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Autorepression of RctB, an Initiator of *Vibrio cholerae* Chromosome II Replication†

Elizabeth S. Egan, Stephane Duigou, and Matthew K. Waldor*

Genetics Program and Department of Molecular Microbiology, Tufts University School of Medicine and Howard Hughes Medical Institute, 136 Harrison Ave., Boston, Massachusetts 02111

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The RctB protein binds to the origin of replication of *Vibrio cholerae* chromosome II (chrII) and is required for $oriCII_{Vc}$-based replication. Here, we found that RctB acts as an autorepressor, inhibiting *rctB* transcription. Integration host factor promotes *rctB* transcription, while Dam and DnaA, factors required for replication of both *V. cholerae* chromosomes, influence RctB autorepression. Thus, RctB appears to regulate chrII replication as both an initiator and a transcription repressor, and its synthesis is modulated by factors that govern replication of both chromosomes.

The genome of *Vibrio cholerae*, the causative agent of the severe diarrheal disease cholera, is divided unequally between two circular chromosomes. Most if not all of the many species that constitute the family *Vibrionaceae* have similarly divided genomes (12, 17–19). As more bacterial genomes have been investigated, it has become clear that multipartite genomes are not uncommon and are found among diverse prokaryotic phyla (2). Almost all studies of bacterial chromosome replication and segregation have utilized organisms with a single chromosome, yet the models derived from these studies may not fully apply to bacteria with multipartite genomes (2).

We previously constructed minichromosomes to identify the minimal replicons of the two *V. cholerae* chromosomes (4). We found that $oriCI_{Vc}$, the origin of replication of the larger chromosome, chromosome I (chrI), is similar in sequence to *oriC*, the well-characterized origin of replication of the *Escherichia coli* chromosome. Like *oriC*, $oriCI_{Vc}$ includes five DnaA boxes (binding sites for the DnaA initiator protein), a putative binding site for integration host factor (IHF, a histone-like protein that bends DNA), several sites for methylation by DNA adenine methyltransferase (Dam, which is involved in regulating timing of replication in *E. coli*), and an AT-rich region (where strand opening is believed to originate).

The origin of replication of chrII, $oriCII_{Vc}$, does not resemble $oriCI_{Vc}$ in terms of sequence identity (4). However, it contains some features common to many bacterial origins of replication, such as one DnaA box, a binding site for IHF, several sites for Dam methylation, and an AT-rich region. In addition to these features, $oriCII_{Vc}$ contains a 12-base-pair repeat that is required for $oriCII_{Vc}$-based replication. While the intergenic region containing $oriCI_{Vc}$ can replicate autonomously in *E. coli*, $oriCII_{Vc}$-based replication requires two novel *V. cholerae* genes which flank $oriCII_{Vc}$ (Fig. 1). One of these genes, *rctB*, encodes an origin-binding protein and is conserved among diverse genera of the family *Vibrionaceae*. The other gene, *rctA*, codes for an untranslated RNA and not a protein.

The distinct replication requirements for the two *V. cholerae* chromosomes suggest that initiation of chrII replication may, at least in part, be controlled independently from chrI. However, our studies of the kinetics of *V. cholerae* chromosome replication revealed that the two chromosomes initiate replication in a coordinated, synchronous manner (3). We found that $oriCI_{Vc}$- and $oriCII_{Vc}$-based replication share requirements for certain factors that have been implicated in the regulation of bacterial chromosome replication in other organisms, such as DnaA and Dam methyltransferase (4). These shared replication factors may help mediate coordinated replication of the chromosomes. However, our understanding of the interplay of the various *V. cholerae* replication factors and how they act to coordinate chromosome replication is limited.

Since the *V. cholerae* RctB protein binds specifically to $oriCII_{Vc}$ and is required only for $oriCII_{Vc}$-based replication, RctB appears to be a chrII-specific replication initiator. Here, we identified the *rctB* promoter and investigated its regulation. We found that RctB is autoregulatory and represses its own transcription. IHF enhances *rctB* transcription, and Dam and DnaA influence RctB-mediated repression of *rctB* transcription. In addition to its own promoter, RctB also regulates the promoter for *rctA*.

To identify the *rctB* transcriptional start site, we isolated RNA from *V. cholerae* strain N16961 and used 5′ rapid amplification of cDNA ends (Invitrogen). This analysis revealed a single putative transcriptional start site 40 bp upstream of the *rctB* start codon (Fig. 1; see also Fig. S1 in the supplemental material). A 209-bp fragment spanning this site could drive transcription from a promoterless *lacZ* gene (see below), suggesting that this region harbors the *rctB* promoter, P_{rctB}. BPROM, a computer program designed to identify sigma 70-dependent promoters for bacterial genes (SoftBerry, Mount Kisco, NY), predicted −10 and −35 sites upstream of the *rctB* transcriptional start site (see Fig. S1 in the supplemental material). Experimental verification that the predicted −10 and −35 sequences are required for activity of P_{rctB} has recently been provided by another lab (14).

* Corresponding author. Mailing address: Genetics Program and Department of Molecular Microbiology, Tufts University School of Medicine and Howard Hughes Medical Institute, 136 Harrison Ave., Boston, MA 02111. Phone: (617) 636-2730. Fax: (617) 636-2723. E-mail: matthew.waldor@tufts.edu.

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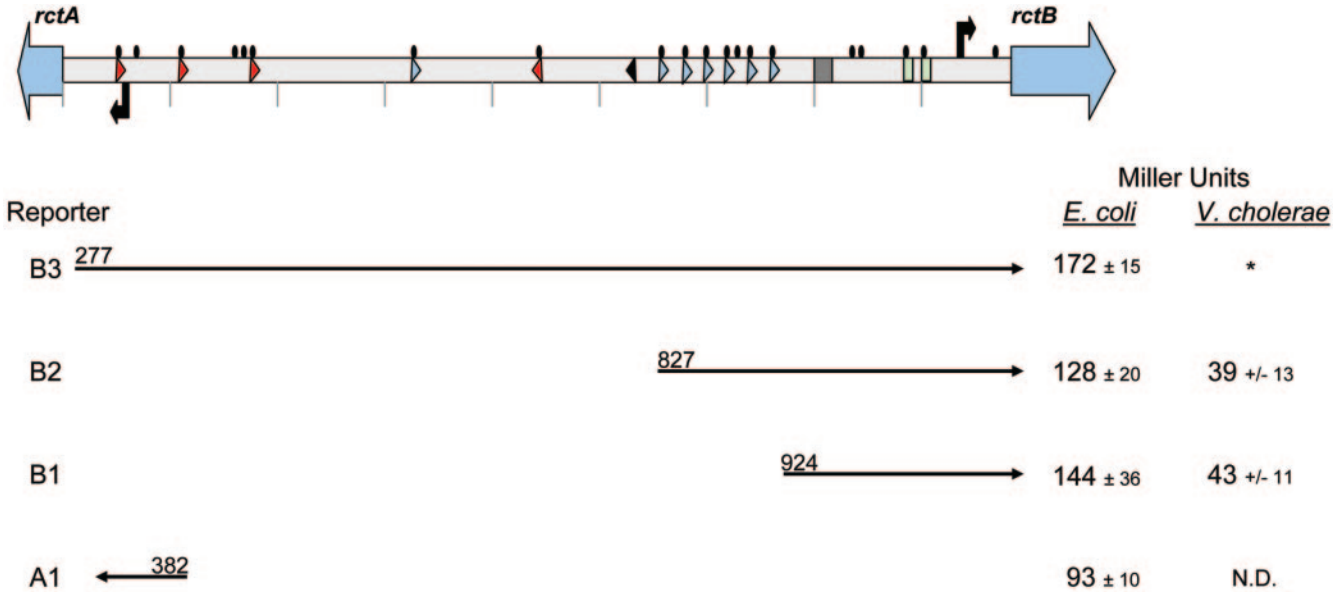

FIG. 1. Reduced β-galactosidase activity of *rctB*::*lacZ* reporters in *V. cholerae* versus *E. coli*. (Top) Schematic representation of the *ig2* region of *V. cholerae* chrII with the following features: the DnaA box is represented as a black triangle, the IHF site as a gray box, AT-rich regions as green boxes, Dam sites (GATC sequences) as black ovals, 12-mer repeats as blue triangles, and 11-mer repeats as red triangles. *rctB* and *rctA* are represented by the blue block arrows. The site of transcription initiation (+1) for *rctB* is represented by the black arrow upstream of *rctB*, and the presumed start site of transcription for *rctA* (based on the location of −10 and −35 sites) is represented by the black arrow upstream of *rctA*. Hatch marks reflect 100-bp intervals. (Bottom) β-Galactosidase activities (Miller units) in *E. coli* MC1061 and *V. cholerae* 2740–80 *lacZ*. The first three reporters are *ig2* fragments B1 (bp 924 to 1265 according to TIGR annotation), B2 (bp 827 to 1265), and B3 (bp 277 to 1265) subcloned into the *lacZ* reporter vector pCB182N. pCB182N is a derivative of pCB182 (16) that contains an rRNA transcription terminator in the SmaI site. Reporter A1 contains a fragment from the left side of *ig2* (bp 382 to 283) upstream of *lacZ* in pCB182. The asterisk indicates that we were unable to obtain a *V. cholerae* strain harboring reporter B3, and N.D. means not done. Each assay was conducted at least three times, and the means and standard deviations are presented.

We explored the influence of sequences upstream of P_{rctB} on *rctB* expression by studying the activity of P_{rctB} using a set of *rctB* transcriptional reporter plasmids. DNA fragments of various lengths from the intergenic region (*ig2*) upstream of the *rctB* open reading frame were introduced upstream of a promoterless *lacZ* gene. The smallest fragment, found in the B1 reporter, started at base pair 924 (numbers are according to the TIGR annotation of the N16961 genome) and included 209 bp upstream of the *rctB* start codon; the largest fragment, in the B3 reporter, included the entire *ig2* intergenic region between *rctB* and the divergently transcribed *rctA*. Each of these reporters exhibited significant β-galactosidase activity in *E. coli*, indicating that there are no *V. cholerae*-specific factors required for P_{rctB} function. Also, the amounts of β-galactosidase activity of all three reporters were very similar (Fig. 1), suggesting that there is only one rightward-facing promoter within *ig2* and that there are no key *cis*-acting sites in *ig2* upstream of base pair 924 that control P_{rctB} expression. In *V. cholerae*, the level of β-galactosidase activity generated by these reporters was ~3-fold lower than in *E. coli*, suggesting that there is a *V. cholerae*-specific factor(s) that represses P_{rctB} activity (Fig. 1). As in *E. coli*, the activities of the B1 and B2 reporters in *V. cholerae* were similar, providing further support for the idea that sequences upstream of base pair 924 do not control P_{rctB} expression. The B3 reporter contains *inc* sequences that negatively regulate chrII replication (4), and as expected, we were unable to introduce this reporter into *V. cholerae*.

We speculated that RctB autorepression might account for the reduced activity of P_{rctB} in *V. cholerae* compared to *E. coli* for two reasons. First, we previously showed that RctB binds to $oriCII_{Vc}$ in the region now known to contain P_{rctB} (4), and second, initiator proteins are commonly autoregulated (1, 10). This hypothesis proved correct; the expression of all three P_{rctB} reporters was repressed by RctB more than fivefold when RctB

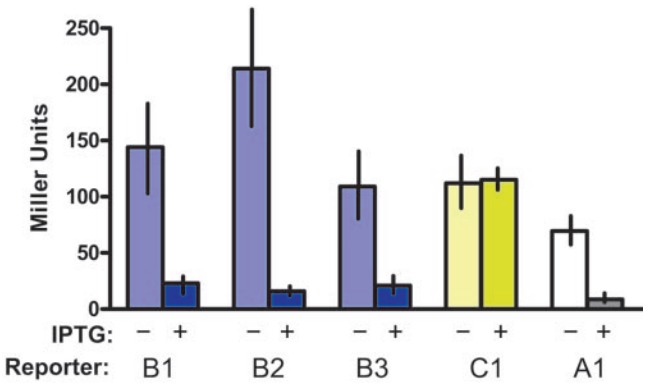

FIG. 2. RctB repression of *rctB* and *rctA* transcription. RctB was produced from pRctB-119, which is a derivative of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression vector pGZ119 (6). C1, a *grpE*::*lacZ* fusion in pCB182N, served as a control. The Miller units presented are the means of at least three independent experiments, and vertical lines represent standard deviations.

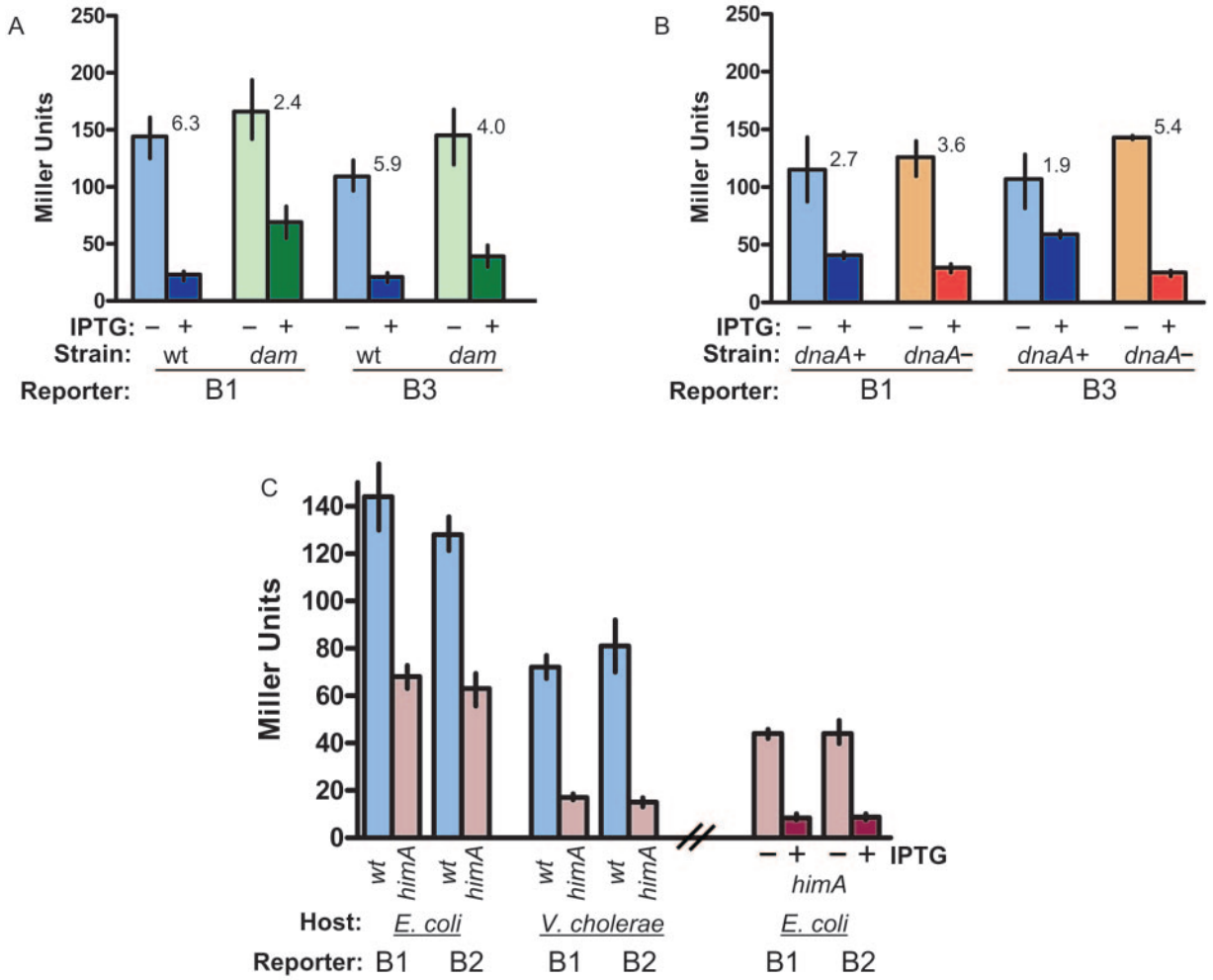

FIG. 3. Host factors influence the activity and regulation of P_{rctB}. In all cases, RctB was produced from the IPTG-inducible expression plasmid pRctB-119, which is a derivative of pGZ119 (6). (A) The wild-type strain was *E. coli* MC1061, and the *dam* strain was *E. coli* KO1607 (MC1061 *dam13*::Tn*9*, gift of A. Wright). (B) The wild-type strain was *E. coli* EH 3896, and the isogenic *dnaA*-independent strain was EH 3894 (CM1565 *zig*::pKN500 Δ*dnaA mad-1 tnaA*::Tn*10* [5]). (C) The wild-type strain was *E. coli* MC1061, and the *himA* strain was an isogenic derivative of MC1061 with a transposon-linked C-terminal deletion of *himA*. This deletion originated from strain RJ3138 (gift of R. Johnson). The *V. cholerae* wild-type strain was 2740-80, and *V. cholerae himA* was an in-frame deletion of *himA* (gift of S. McLeod). All assays were done at least three times, and the means and standard errors of the mean are presented. The repression (*n*-fold) in the presence of overproduced RctB is shown for each reporter.

was overproduced from a plasmid in *E. coli* (Fig. 2). RctB binds to multiple sites in *ig2* (4); however, there was no obvious correlation between the degree of RctB-mediated repression of the three *rctB* reporters and the number of RctB binding sites in the individual reporters, suggesting that potential interactions between different RctB molecules bound throughout *ig2* do not influence P_{rctB} activity. As in *E. coli*, when *rctB* was overexpressed in *V. cholerae*, P_{rctB} activity was also reduced (data not shown). Overexpression of RctB did not repress the activity of a control reporter, indicating that RctB repression of P_{rctB} is specific (Fig. 2). Thus, like both DnaA and Rep proteins of iteron plasmids, RctB appears to be an autoregulatory initiator protein that represses its own synthesis.

To study whether *rctB* transcription is influenced by other factors that are required for chrII replication, such as Dam methyltransferase and DnaA, we measured P_{rctB} activity from the B1 and B3 *rctB* reporters in *E. coli* strains harboring mutations in *dam* or *dnaA*. Dam methylation is known to regulate gene expression (7), and there are several potential sites for methylation by Dam methyltransferase in the vicinity of P_{rctB} (Fig. 1). The β-galactosidase activities of the two *rctB* reporters were similar in wild-type and *dam E. coli* strains, indicating that Dam methylation does not significantly influence P_{rctB} activity (Fig. 3A). However, Dam methylation appears to enhance RctB autorepression. In *dam E. coli*, overexpression of *rctB* resulted in somewhat less repression of the *rctB* reporters than was observed in wild-type *E. coli*; this effect was strongest for reporter B1 (Fig. 3A). These findings may suggest that methylation increases the affinity of RctB for its promoter.

DnaA is known to act as a transcription regulator as well as an initiator of DNA replication (11). Since P_{rctB} is close to the single DnaA box in $oriCII_{Vc}$ (Fig. 1), we tested whether DnaA influences P_{rctB} activity by comparing the levels of *lacZ* expression from the B3 and B1 *rctB* reporters, one of which (B3)

includes the DnaA box and the other of which does not (Fig. 1). The β-galactosidase activities of these two *rctB* reporters were similar (Fig. 1), suggesting that DnaA binding to *ig2* does not influence P_{rctB} promoter activity. Consistent with this idea, we found that the β-galactosidase activities of these two reporters were similar in a *dnaA* deletion strain (with an integrated R1 *ori* [5]) and isogenic wild-type *E. coli* (Fig. 3B). However, the degrees of RctB autorepression were different in these strains (Fig. 3B). Although in both backgrounds RctB repressed its own synthesis, in the absence of DnaA, RctB-mediated repression was enhanced 2.8-fold for reporter B3 and less so for B1. This may suggest that DnaA antagonizes RctB binding to P_{rctB}.

The nucleoid-associated factor IHF is a heterodimer consisting of HimA and HimB and is known to modulate transcription of some bacterial genes (9). We investigated whether IHF influences P_{rctB} activity. $oriCII_{Vc}$ contains a DNA sequence that is similar to the *E. coli* IHF consensus binding site (4), and we have found that the *E. coli* IHF protein binds to the region of $oriCII_{Vc}$ containing this site (our unpublished observations). IHF appears to activate transcription from P_{rctB}, as transcription from P_{rctB} was reduced ~2-fold in an *E. coli himA* mutant strain compared with wild-type *E. coli* (Fig. 3C). The similar reductions in the β-galactosidase activities of the B1 and B2 reporters in *himA* versus wild-type *E. coli* suggest that the activating effect of IHF requires only, at the most, the sequence present in the small B1 reporter. The activity of a control reporter was not reduced in *himA E. coli*, indicating that the effect of the *himA* deletion is not due to a global defect in transcription levels. In *V. cholerae*, IHF activation of transcription from P_{rctB} was even more pronounced, as the β-galactosidase activities of the B1 and B2 reporters were four to five times lower in *himA* than in wild-type *V. cholerae*. These observations suggest that IHF contributes to efficient P_{rctB} activity, as is the case for several other promoters where IHF promotes architectural changes in the DNA. Alternatively, IHF could also activate transcription by directly contacting RNA polymerase (9). IHF does not appear to influence the binding of RctB to P_{rctB}, since the reporters were repressed to similar extents by RctB in *himA* and wild-type *E. coli* (Fig. 3C).

Since RctB binds to the left side of *ig2* just upstream of *rctA* (4), we tested whether RctB regulates *rctA* transcription. Using BPROM, we identified a putative *rctA* promoter (P_{rctA}) approximately 80 bp upstream of the annotated gene (Fig. 1). This region of DNA can drive *lacZ* transcription, since a P_{rctA}::*lacZ* reporter (A1) yielded ~93 Miller units of β-galactosidase activity in *E. coli* (Fig. 1). Introduction of a 3-bp substitution mutation into the putative −10 sequence of P_{rctA} in A1 decreased β-galactosidase activity ~10-fold, indicating that P_{rctA} is indeed a true promoter. RctB overproduction reduced the β-galactosidase activity from A1 more than eightfold, indicating that P_{rctA} is repressed by RctB (Fig. 2). Thus, RctB represses at least two genes, *rctA* and *rctB*, required for chrII replication.

Our findings reveal that RctB is a multifunctional protein. Previously, we found that RctB is required for $oriCII_{Vc}$-based replication and that RctB binds to several sites in *ig2*, suggesting that RctB functions as a chrII-specific replication initiator. This idea is supported by the recent observation that RctB levels determine the copy number of an $oriCII_{Vc}$-based minichromosome in *E. coli* (14). Besides acting as a chrII-specific initiator, RctB also functions as a transcriptional repressor, inhibiting transcription from P_{rctB} and from P_{rctA}. Thus, RctB controls $oriCII_{Vc}$-based replication on at least three levels: as an initiator of replication, as an autorepressor, and as a repressor of *rctA*.

RctB binds in the vicinity of P_{rctB}, and therefore it is likely that RctB directly represses its own promoter. RctB autorepression, which may be a critical determinant of RctB levels and chrII replication, appears to be influenced by DnaA and Dam, two host factors that are essential for replication of both *V. cholerae* chromosomes (4). Dam modestly potentiates RctB autorepression, probably by altering RctB binding to sites that influence P_{rctB} activity. DnaA modestly inhibits RctB autorepression, perhaps by interacting with RctB and inhibiting its binding to P_{rctB}. Interactions between DnaA and plasmid Rep proteins have been described previously (8). Thus, there appears to be a complex interplay of factors essential for chrI and chrII replication that, along with RctB, govern *rctB* transcription. Understanding the molecular details of RctB-DNA and RctB-protein interactions will enhance our understanding of how the two chromosomes initiate replication in synchrony.

IHF promotes transcription from P_{rctB} but does not influence RctB binding to this region. The putative IHF binding site is 129 bp from the start site of *rctB* transcription, and the region upstream of the IHF binding site is relatively AT rich. This arrangement of an AT-rich region and IHF site upstream of a promoter is similar to that of the $ilvP_G$ promoter in *E. coli*, which is also activated by IHF (13). IHF activates $ilvP_G$ by translocating superhelical energy; binding to its cognate site prevents destabilization of the upstream AT-rich region while promoting duplex destabilization of the −10 region of the promoter (15). Destabilization is correlated with open complex formation as well as increased transcriptional activity of $ilvP_G$. It is tempting to speculate that IHF activates P_{rctB} by a similar mechanism.

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