Biochemistry. In the article “Characterization of residual structure in the thermally denatured state of barnase by simulation and experiment: Description of the folding pathway” by Chris J. Bond, Kam-Bo Wong, Jane Clarke, Alan R. Fersht, and Valerie Daggett, which appeared in number 25, December 9, 1997, of Proc. Natl. Acad. Sci. USA (94, 13409–13413), one of the authors regrets that she inadvertently omitted references to the computer program and protein potential function that the authors used for their simulations of barnase cited above. The following sentence should have been the first sentence of the Methods section: Molecular dynamics simulations were performed with the program ENCAD (44) and the potential energy function of Levitt et al. (45).


Biochemistry. In the article “Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin” by Kim C. Quon, Bing Yang, Ibrahim J. Domian, Lucy Shapiro, and Gregory T. Marczyński, which appeared in number 1, January 6, 1998, of Proc. Natl. Acad. Sci. USA (95, 120–125), the authors wish to note that the institutional affiliations in the author line were incorrectly attributed. The correct affiliations are as follows. Bing Yang and Gregory T. Marczyński are at McGill University in Montreal, and Kim C. Quon is now at the Netherlands Cancer Institute.

Biochemistry. In the article “The 30-kDa C-terminal domain of the RecB protein is critical for the nuclease activity, but not the helicase activity, of the RecBCD enzyme from Escherichia coli” by Misook Yu, Jehanne Souaya, and Douglas A. Julin, which appeared in number 3, February 3, 1998, of Proc. Natl. Acad. Sci. USA (95, 981–986), the following correction should be noted. The symbols in the graph (Fig. 3C) were identified incorrectly in the manuscript. The corrected legend and graph with accompanying symbols are printed below.

Biochemistry. In the article “Escherichia coli RNA polymerase terminates transcription efficiently at rho-independent terminators on single-stranded DNA templates” by Susan M. Uptain and Michael J. Chamberlin, which appeared in number 25, December 9, 1997, of Proc. Natl. Acad. Sci. USA (94, 13548–13553), the authors request that the following correction be noted. It is critical that the bands in lanes 6 and 8 of Fig. 3 indicated by the T7Te arrow be visible. The existence of these terminated bands is a major point on which the conclusions of the paper depend. Therefore, to enhance their visibility, Fig. 3 and its accompanying legend are reprinted below with greater contrast.

Cell Biology. In the article “Scavenger receptor class B, type I (SR-BI) is the major route for the delivery of high density lipoprotein cholesterol to the steroidogenic pathway in cultured mouse adrenocortical cells” by Ryan E. Temel, Bernardo Trigatti, Ronald B. DeMattos, Salman Azhar, Monty Krieger, and David L. Williams, which appeared in number 25, December 9, 1997, of Proc. Natl. Acad. Sci. USA (94, 13600–13605), the following correction should be noted. The equation on page 13601 should be as follows:

$$P_{\text{total}} = \frac{[P_{\text{max}}][HDL] + C}{K_{HA} + [HDL]}$$
Cell Biology. In the article “Identification of a family of low-affinity insulin-like growth factor binding proteins (IGFBPs): Characterization of connective tissue growth factor as a member of the IGFBP superfamily” by Ho-Seong Kim, Srinivasa R. Nagalla, Youngman Oh, Elizabeth Wilson, Charles T. Roberts, Jr., and Ron G. Rosenfeld, which appeared in number 24, November 25, 1997, of Proc. Natl. Acad. Sci. USA (94, 12981–12986), the authors request that the following corrections be noted. In Fig. 8, the units on the scale should indicate the number of substitution events, rather than “million years.” The lengths of the branches represent the relative distance between the sequences of mammalian IGFBPs compared in this figure. In the Discussion, the statement “The dendogram depicted in Fig. 8 indicates that, based upon structural similarities, all ten members of the superfamily can be traced back to an ancestor gene 60 million years ago” should read: “The dendogram depicted in Fig. 8 indicates that all ten members of the superfamily share a common ancestral gene based upon their sequence similarities.”

Neurobiology. In the article “Hair cell-specific splicing of mRNA for the α1D subunit of voltage-gated Ca\(^{2+}\) channels in the chicken’s cochlea” by Richard Kollmar, John Fak, Lisa G. Montgomery, and A. J. Hudspeth, which appeared in number 26, December 23, 1997, of Proc. Natl. Acad. Sci. USA (94, 14889–14893), the authors wish to note that the quality of reproduction of Fig. 1 was below standard. In all three panels, the middle parts were affected. Specifically, the reverse (white-on-black) type denoting exons 9a, 22a, and 30a was illegible; parts of the arrows that represented primers such as F9 were missing; and the outlines of several of the boxes that depicted exons such as 9, 10, and 20 were defective. The figure and its legend are reproduced below.

Immunoology. In the article “Parasite-mediated nuclear factor κB regulation in lymphoproliferation caused by Theileria parva infection” by Guy H. Palmer, Joel Machado, Jr., Paula Fernandez, Volker Heussler, Therese Perinat, and Dirk A. E. Dobbelnaere, which appeared in number 23, November 11, 1997, of Proc. Natl. Acad. Sci. USA (94, 12527–12532), the following correction should be noted. The concentration of N-acetylcysteine used in the experiments was 30 mM, not 25 μg/ml as erroneously reported on page 12528, lines 14 and 15 of the paragraph entitled “Cell Lines and Cultures” in the Materials and Methods section.

**FIG. 1.** Alternative splicing of the α1D mRNA in the basilar papilla and the brain. (A) Southern blot of PCR products amplified with primers flanking the insert in the I-II loop (exon 9a). Marker sizes in base pairs are indicated on the left. The diagram below of the putative genomic structure (not drawn to scale) depicts exons as rectangles, introns as horizontal lines, and PCR primers as arrows. To amplify all isoforms together, we used primers F9 and R14. To amplify rare isoforms without interference from more abundant ones, we used exon-specific primers: primer F9a binds across the splice junction of exons 9 and 9a, and primer F10 binds across that of exons 9 and 10. The table at the bottom lists product size and occurrence for each splice variant and primer pair. ++, abundant; +, detectable; (+), barely so; --, not detectable. (B) Same as A, but for the alternative III2 segment (exon 22a). Note the abundance in the basilar papilla of mRNAs with exons for both III2 segments. (C) Same as A, but for the insert in the IVS2–3 loop (exon 30a). Primer F30a binds across the splice junction of exons 30 and 30a, primer F31 binds across that of exons 30 and 31, and primer F31a binds across that of exons 30a and 31a. For the basilar papilla, the lengths of even the minor products were consistent only with splice isoforms containing exon 30a; for the brain, they were consistent only with isoforms lacking exon 30a. Note the abundance in the brain of mRNAs with exons for both IVS3 segments.
Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin

(CtrA response regulator / Caulobacter crescentus / cell cycle control)

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ABSTRACT

Caulobacter crescentus divides asymmetrically generating two distinct cell types at each cell division: a stalked cell competent for DNA replication, and a swarmer cell that is unable to initiate DNA replication until it differentiates into a stalked cell later in the cell cycle. The CtrA protein, a member of the response regulator family of the two-component signal transduction system, controls multiple cell cycle processes in Caulobacter and is present in swarmer cells but absent from stalked cells. We report that CtrA binds five sites within the chromosome replication origin in vitro. These sites overlap an essential DnaA box and a promoter in the origin that is essential for replication initiation. Analysis of mutant alleles of ctrA and point mutations in one of the CtrA binding sites in the origin demonstrate that CtrA represses replication in vivo. CtrA-mediated repression at the origin thus restricts replication to the stalked cell type. Thus, the direct coupling of chromosome replication with the cell cycle is mediated by the ubiquitous two-component signaling proteins.

The coordination of cell cycle progression and cell differentiation with the initiation of DNA replication is a fundamental, yet poorly understood problem in bacteria. The bacterium Caulobacter crescentus provides an amenable system for studying this problem because cell division in this organism is obligatorily asymmetric, and cellular differentiation is an integral part of the cell cycle (1–3). The presdivisional cells exhibit two distinct progenies upon division; the progeny stalked cell immediately initiates DNA replication but the progeny swarmer cell does not replicate its chromosome until shedding its flagellum and differentiating into a stalked cell later in the cell cycle (refs. 4 and 5; Fig. 1). Thus, the chromosomes in the two poles of the presdivisional cell exhibit differential control of replication potential.

To define the molecular basis of the spatial control of replication initiation in the presdivisional cell and the temporal control of replication initiation during the swarmer-to-stalked cell transition, the chromosome replication origin, termed Cori, was cloned and shown to support autonomous plasmid replication (6). Cori plasmid replication, like chromosome replication, initiates specifically in nascent stalked cells, indicating that cell type-specific factors act through regulatory sequences in Cori (6).

The minimal origin region contains many essential regulatory elements, including a strong transcriptional promoter (ref. 7; Fig. 2A), potential binding sites for the DnaA replication initiator (6, 8), and five repeats of a novel GTTAA-N7-TTAA, 9-mer motif (Fig. 2A, sites a–e). Transcription from the strong promoter occurs in stalked cells but not swarmer cells, and two of the five 9-mer sequences overlap the origin promoter (6). In vivo assays of origin promoter activity in synchronized cell populations suggested that expression of the origin promoter is repressed in the swarmer portion of the presdivisional cell (6). Thus, the 9-mer sequences are likely binding sites for a repressor of the origin promoter and, consequently, replication initiation.

We recently demonstrated that several cell cycle-regulated genes have promoters with a similar 9-mer sequence motif that is recognized by the essential cell cycle regulatory protein CtrA (cell cycle transcription regulator) (9). CtrA is a global regulator that controls the transcription of genes encoding flagellar proteins (9), a DNA methyltransferase (9), and cell division proteins (9, 10). Transcription from the origin promoter increased when CtrA levels were reduced in vivo (9), suggesting that CtrA might regulate this essential replication element (7). CtrA is a member of the “response regulator” superfamily of transcription factors (9) activated by aspartyl phosphorylation in the two-component signal transduction pathways (11, 12). Such proteins have been primarily studied in bacteria, but two-component pathways are also present in archea (13) and eukaryotes (14).

In this report, we demonstrate that in vitro CtrA directly binds the five 9-mer sites within the chromosome replication origin. Further, strains with site-directed mutations to one of the 9-mer sites show increased level of replication, as do strains bearing mutant alleles of ctrA. Overexpression of CtrA blocks replication. Thus, CtrA represses the replication origin in vivo. We propose that cell cycle cues are channeled through CtrA to regulate the initiation of chromosome replication.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, Growth Conditions, and Cell Synchrony. The synchronous wild-type strain NA1000 and derivative strains of C. crescentus were grown in PYE complex media or M2G minimal media at 30°C (15). The ctrA401ts strain LS2195 is isogenic to NA1000 (9); the PxyIX:ctrAΔ3 strain has a 1.2-kb (BstBI–EcoRI) deletion of chromosomal ctrA complemented by a derivative of the pJS14 high copy number plasmid, pD42HA, carrying a promoterless ctrAΔ3 allele driven by the xylose-inducible PxyIX promoter (9, 16). CtrAΔ3 lacks three C-terminal amino acids, causing it to be resistant to proteolysis and thus present at all times in the cell cycle (17). Synchronous swarmer cell cultures were obtained by the method of Evinger and Agabian (18).

Fluorescent Cell Cytometry. Cultures of C. crescentus wild-type (ctrA+), ctrA401ts, and PxyIX:ctrAΔ3 were fixed in 70%

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ethanol, and their chromosomal DNA was stained with 10 mg/liter chromomycin A3 (Sigma) and analyzed by a Becton Dickinson FACStar Plus machine as described (20). Data were analyzed by using FACS/DESK software (Stanford University, Stanford, CA).

Protein Abundance and the Percentage of Replicating Chromosomes. Pure populations of swarmer cells were obtained by Ludox density centrifugation (18) and allowed to proceed synchronously through the cell cycle (Fig. 1). Cell samples were analyzed by Western blotting with anti-CtrA antibody as described (17). The relative abundance of CtrA protein was measured by laser densitometry of immunoblots (17). Chromosome replication was measured by the previously described methylation state assay at the dnaA locus approximately 4 kb away from Cori (6). This assay is based on the fact that unreplicated DNA is methylated on both strands, but once replicated, DNA remains hemimethylated until the predivisional cell stage (19). DNA was prepared from aliquots of the same cell samples used for Western blot analysis and was assayed for sensitivity to HindII digestion, based on the fact that one HindII recognition site overlaps the Caulobacter CcrM DNA methylation site at the dnaA gene (19).

Site-Directed DNA Mutagenesis of Cori CtrA Binding Site. To change CtrA binding site d to the Mut-d sequence shown in Fig. 4, the following oligonucleotides were ligated between unique EcoRI and EcoR1 endonuclease sites at Cori base pair positions 430 and 447 (6). The Mut-d top oligonucleotide GCCTTGAACACACAGGTG was annealed to the Mut-d bottom oligonucleotide AATTCACTGTGTGTTCAAGGC, and these oligonucleotides were ligated with double-stranded Cori plasmid pGM1270 (7). As a control, oligonucleotides containing the wild-type sequence were likewise annealed and ligated. Both sequences were confirmed by the Sanger dideoxy-sequencing method by using the T7 sequencing kit (Pharmacia).

CtrA Protein Footprinting Experiments. Dnase I protection footprinting experiments (21) were performed with a purified His6–CtrA fusion protein as described (9). For the experiment described in Fig. 2, the following 32P end-labeled DNA fragments from Cori were employed: first panel, CtrA binding sites a, b, and c were assayed by end-labeling pGM1070 (7) at an artificial HindIII (+221) and by using gel electrophoresis to isolate the short fragment cut at EcoRI (+452); second panel, sites a, b, and c were assayed by end-labeling pGM1070 at EcoRI (+452) and by using gel electrophoresis to isolate the short fragment cut at HindIII; third panel, pGM1070 (7) was 5' end-labeled at the BgII site (+254); fourth panel, pGM1022 (7) was 5' end-labeled at an artificial BamHI linker (+714).

RESULTS

Kinetics of CtrA Disappearance Coincides with the Initiation of DNA Replication in a Synchronized Cell Population. We have shown that a strong promoter within the origin of replication (Fig. 2A) is essential for the temporal control of Cori plasmid replication (7). These studies also suggested that the promoter is bound in vivo by a swarmer cell-specific repressor (7). Consensus binding sites for the CtrA protein overlap the Cori strong promoter (Fig. 2A), and in a strain bearing a ctra ts allele, origin promoter activity is significantly increased (9). Furthermore, we have recently shown that CtrA is present only in the cell type that is unable to initiate DNA replication (17). To assess the temporal relationship of CtrA disappearance and the initiation of chromosome replication during the swarmer-to-stalked cell transition, samples of a synchronized population were assayed by immunoblotting with CtrA antibody and in the same samples, by monitoring the replication state of the chromosome, as described in Materials and Methods (Fig. 1). As swarmer cells differentiated into stalked cells, CtrA protein levels decreased coincident with the
initiation of chromosome replication. Thus, CtrA is likely to be a swarmer cell-specific repressor of chromosome replication.

To directly test this hypothesis, we have assayed both in vitro binding of CtrA to the origin promoter and the in vivo effect on DNA replication of genetically lowering or raising levels of CtrA.

**CtrA Protein Binds to Five Sites within the Caulobacter Replication Origin.** To test for CtrA binding to Cori, we performed DNase I footprinting experiments (21) throughout the Cori region by using purified His6-tagged CtrA protein (Fig. 2B). We observed a CtrA-dependent 24- to 26-bp zone of protection, flanked by hypersensitive sites, centered over each of the five 9-mer motifs (Fig. 2, sites a–e). No additional DNase I protection in the ~1-kb Cori region was seen, demonstrating that CtrA selectively binds the 9-mer motifs.

**Genetic Manipulation of CtrA Demonstrates Repression of Chromosome Replication.** To test whether CtrA represses replication in vivo, we genetically altered CtrA activity and examined chromosome replication. CtrA activity was decreased by shifting the ctrA401 temperature-sensitive (ts) mutant to a restrictive temperature or increased by overexpressing the CtrAΔ3 protein from the xylose-inducible promoter PxyX (17). CtrAΔ3 lacks a C-terminal degradation signal and, unlike wild-type CtrA (Fig. 1), is present in all cell types (17). To monitor the changes in CtrA activity, we measured Cori strong promoter transcription from a lacZ transcription fusion (Fig. 3A). This promoter is coregulated with chromosome replication (7) and is repressed by CtrA in vivo (9). Thus, it provides a physiologically relevant measure of CtrA activity at Cori.

Our genetic experiments demonstrate that CtrA is both necessary and sufficient to repress chromosome replication initiation (Fig. 3B and C). The ctrA401 ts allele decreased CtrA activity, as evidenced by a 4-fold increase in Cori strong promoter transcription (Fig. 3A), and caused ctrA401 cells to progressively accumulate an average of 10 chromosomes per cell within 4 h after the temperature shift (Fig. 3B). Conversely, overexpression of CtrAΔ3 (in a chromosomal CtrA null background) increased CtrA activity, as evidenced by a 3-fold decrease in Cori strong promoter transcription (Fig. 3A), and caused cells to arrest with primarily one or two complete chromosomes (1n or 2n) even after 10 h of induction by xylose. Few cells had DNA contents intermediate between 1n and 2n (Fig. 3C), indicating that they could complete but not initiate chromosome replication (20). Overexpressing wild-type CtrA caused no discernible replication block (data not shown), presumably because it was efficiently removed by proteolysis in stalked cells (ref. 17; Fig. 1).
CtrA represses DNA replication independent of its effects on cell division (9), most likely because of altered transcription of cell division genes (10).

**Direct Repression of Chromosome Replication by Binding CtrA to Origin DNA.** Although direct CtrA binding to Cori in vitro (Fig. 2) implies direct binding in vivo (Fig. 3), CtrA could indirectly repress replication. CtrA regulates the transcription of several genes, and one of these might be a repressor of chromosome replication. To address this issue, we mutated CtrA binding site d as shown in Fig. 4A. This mutation (Mut-d) altered CtrA binding in vitro such that only the unaltered TTAA half site was footprinted (Fig. 4B). To assay the effects of altered CtrA binding to the replication origin, plasmids bearing a wild-type origin or an origin with a Mut-d mutation were moved into a wild-type Caulobacter strain, and plasmid replication activity was monitored (Fig. 4C). Altered binding of CtrA to binding site d increased the in vivo number of Cori plasmids per chromosome approximately 7-fold. These results indicate that wild-type Cori plasmid replication is repressed by CtrA binding in vivo and that the Mut-d Cori base pair changes, shown in Fig. 4A, are by themselves sufficient to relieve this repression.

Our data, in its entirety, therefore demonstrate that CtrA controls chromosome replication during the cell cycle by directly binding and repressing the replication origin in swarmer cells.

**DISCUSSION**

**How Might CtrA Repress Replication?** CtrA could repress replication by binding sites a and b, which completely overlap the strong promoter in the origin of replication (Fig. 3, 2), and blocking RNA polymerase binding. This is a key regulatory step because the Cori strong promoter is essential for replication; it is only transcribed in stalked cells, and its inappropriate transcription abolishes the temporal control of Cori plasmid replication (7). However, the function of the other CtrA binding sites are not explained by this model. Site e lies only 3 bp away from a DnaA box that is essential for autonomous replication (6). CtrA footprinting experiments clearly demonstrate that CtrA overlaps this DnaA box (Fig. 2B). Therefore, CtrA may also act by inhibiting DnaA interaction with the origin. In *Escherichia coli*, chromosome replication is controlled by a balance between positively acting proteins, such as RNA polymerase (22, 23) and DnaA (8, 24), and negatively acting ones such as SeqA (25, 26) and an inhibitor of DnaA (27). In *Caulobacter*, an analogous antagonism may restrict replication to the stalked cells.

At present, the role of CtrA binding site c is unknown, although site d is clearly involved in repression (Fig. 4). How might site d repress replication? The presence of five CtrA binding sites within the minimal Cori sequences suggests that CtrA and Cori form a multimeric nucleoprotein complex. Cooperative protein–protein interactions within such a complex could allow CtrA bound at site d to repress RNA polymerase and/or DnaA at distant sites by a DNA looping mechanism. Such cooperative interactions allow the homologous response regulator OmpR to repress transcription (28, 29), and similar interactions regulate plasmid R6K replication (30).

Negative regulation of chromosome replication seems to be required to prevent premature initiation of chromosome replication. The *Caulobacter* cell cycle provides a striking example of the “once and only once” principle of chromosome replication, a concept normally applied to the eukaryotic S phase, that accounts for exactly one round of chromosome replication per cell cycle. In *E. coli*, negative regulation of chromosome replication is accomplished, at least in part by the SeqA protein binding origin DNA that becomes hemimethylated by new replication through the Dam methylation sites (25, 26). Al...
Interestingly, CtrA may act in two ways to increase the affinity and cooperativity of CtrA binding, as is the case for OmpR (31, 32). Phosphorylation of CtrA is required for selective DNA binding of unphosphorylated CtrA to chromosome replication in Fig. 3 (ref. 17), and overexpression of CtrA probably accounts for the ability of CtrA to repress replication once the wild-type protein is targeted for degradation. This idea is also consistent with our hypothesis that CtrA acts cooperatively at C trI.

**Relevance to Other Chromosome Replication Systems.** Despite much progress in understanding cell cycle control and DNA replication in both eukaryotes and prokaryotes, the signals that couple these two processes remain obscure. For example, in budding yeast, the timed phosphorylation and proteolysis of the Sic1 protein coordinates DNA replication with the cell cycle (33–36), but how Sic1p-mediated signals are ultimately communicated to replication origins is unclear. The identification of CtrA as a global regulator of cell cycle and developmental events (9), as a cell-type-specific protein controlled by proteolysis and phosphorylation (Fig. 1; refs. 9 and 17), and as a repressor of the chromosome replication origin (Figs. 2–4) suggests that CtrA directly communicates cell cycle cues to the Caulobacter replication origin. These observations extend the functions of the versatile two-component regulators to the direct control of DNA replication. Considering the ubiquity and versatility of two-component regulatory systems, it would be surprising if other organisms did not also adapt them to regulate chromosome replication.

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Fig. 4. Directed mutagenesis at CtrA binding site d alters protein binding and increases Cori plasmid copy number in vivo. (A) Wild-type Cori sequence at site d showing the clustered base pair changes in Mut-d. (B) DNase I protection footprinting experiments were performed as in Fig. 2B (third panel). Outside lanes show the 5'-32P end-labeled DNA fragments cut with EcoRI or HpaI. (C) Cori plasmids per chromosome, as determined by Southern blot hybridization. Caulobacter strain CB15N Δbla was electroporated with pBluescriptII plasmids (Stratagene) bearing otherwise identical wild-type (Wt) or mutant (Mut-d) Cori DNA between BamHI sites at −680 and +998 (see Fig. 1A) and analyzed as described (7). Radioactivity was measured with a PhosphorImager (Molecular Dynamics), and the ratio between Cori plasmid and chromosome band intensities is shown.