A developmentally regulated chromosomal origin of replication uses essential transcription elements

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Only one of the two chromosomes in the asymmetric *Caulobacter* predivisional cell initiates replication in the progeny cells. Transcription from a strong promoter within the origin occurs uniquely from the replication-competent chromosome at the stalked pole of the predivisional cell. This regulated promoter has an unusual sequence organization, and transcription from this promoter is essential for regulated (cell type-specific) replication. Our analysis defines a new class of bacterial origins and suggests a coupling between transcription and replication that is consistent with the phylogenetic relationship of *Caulobacter* to the ancestral mitochondrion.

[Key Words: *Caulobacter*; origin of replication; transcription; polar development; *hemE*]

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Caulobacter crescentus offers the opportunity to study the control of DNA replication in the context of a developmental cell cycle. The *C. crescentus* predivisional cell expresses a remarkable asymmetry that polarizes cellular components so that every cell division yields two distinct cell-types: a motile nonreplicative swarmer cell and a sessile replicative stalked cell. The swarmer cell later differentiates into a stalked cell to grow and divide asymmetrically (for recent reviews, see Newton and Ohta 1990; Brun et al. 1994). In addition to distinct surface morphologies, the progeny swarmer and stalked cells receive newly replicated chromosomes that have different sedimentation coefficients (Evinger and Agabian 1979; Swoboda et al. 1982; Gober et al. 1991a), differentially transcribed genes (Gober et al. 1991a,b; Wingrove et al. 1993), as well as different replicative abilities (Marczyński and Shapiro 1992).

The great diversity of bacteria implies that they have evolved sophisticated systems to coordinate DNA replication with their diverse modes of proliferation. Unfortunately, critical information is lacking for most bacterial species regarding the organization of their origins of replication and the coordination of replication with the cell cycle. The *C. crescentus* chromosome, as is the case for *Escherichia coli* and *Bacillus subtilis*, uses one origin of replication (Lott et al. 1987; Dingwall and Shapiro 1989; Marczyński and Shapiro 1992). When placed on a nonreplicating plasmid, the cloned *C. crescentus* origin (*Cori*) supports autonomous plasmid replication (Marczyński and Shapiro 1992). In contrast to other plasmids,

whose replication occurs in both swarmer and stalked cells (Marczyński et al. 1990), "*Cori*-driven" plasmid replication coincides with the initiation of chromosome replication in the stalked cell (Marczyński and Shapiro 1992), indicating that the cloned *Cori* DNA responds to the same regulatory factors as the chromosome. This provides a powerful system in which to study the molecular switch between chromosomes that are active or inactive for replication.

A cell's commitment to replicate its genome is analogous to its commitment to differentiate by transcribing specific genes, and both processes are thought to share similar regulatory principles. Studies in *E. coli* have provided a detailed description of a replication initiation process that requires the ordered assembly of replication proteins (DnaA, helicase, primase, DNA polymerase, and topoisomerase subunits), collectively termed the replisome (Kornberg and Baker 1992), at a unique origin sequence. Targeted replisome assembly is conceptually similar to the action of transcriptional enhancer elements that load and activate RNA polymerase and transcription factors at specific promoters (Mitchell and Tjian 1989; Kustu et al. 1991; Roeder 1991; Heintz et al. 1992). Transcriptional enhancer elements also appear to be auxiliary components of several viral origins of replication (DePamphilis 1988; Guo and DePamphilis 1992; Li and Botchan 1993) and at least one yeast chromosomal origin of replication (Marahrens and Stillman 1992).

We report the DNA sequence requirements and functional organization of the *C. crescentus* chromosomal origin and demonstrate that an origin-internal promoter is important for the regulation of DNA replication. Tran-

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scription from this promoter anticipates chromosome replication in the progeny cells and occurs preferentially at the stalked pole of the predivisional cell. Our results imply that the differential activity of transcription factors at the two poles affects the switch between replicating and nonreplicating chromosomes.

Results

Novel DNA sequence requirements of the *C. crescentus* origin of replication

The cloned *Cori* supports autonomous plasmid replication, and *Cori*-directed plasmid replication coincides with the onset of chromosomal DNA synthesis (Marczynski and Shapiro 1992). These results imply that this DNA region contains cell cycle control elements that restrict chromosome replication to the stalked cells. To define the DNA sequences required for regulated replication in *C. crescentus*, we mutagenized the *Cori* region systematically (Fig. 1A). Randomly generated deletions with *Bam*HI linker insertions (shown as boxes numbered

1–27), as well as site-directed linker insertions and base-pair changes (shown as dagger signs a–h) were created throughout the *Cori* region. These mutated origins were introduced into *C. crescentus* on plasmids that cannot replicate without the functional *Cori* sequence, and replication function was assayed by scoring for *Cori*-plasmid maintenance. As illustrated for six selected *Cori*-plasmids in Figure 1B,C, we observed three classes of *Cori*-plasmid maintenance, designated Rep+, Rep+/-, and Rep-. Mutated *Cori*-plasmids were designated Rep+ when they were indistinguishable from unmutated *Cori*-plasmids by these criteria: high frequency transformation (10^6 – 10^7 colonies/ μ g of DNA), a large colony morphology (Fig. 1B, plates 1,2), and efficient *Cori*-plasmid extraction from liquid cultures (Fig. 1C, lanes 1,2). *Cori*-plasmids designated as Rep+/- gave comparable transformation frequencies, but their colonies were tiny (Fig. 1B, plates 3,4), and their plasmid yields were low from liquid cultures (Fig. 1C, lanes 3,4). The extracted Rep+/- plasmids not visible on ethidium bromide-stained agarose gels, and although Rep+

Figure 1. Mutations defining the *C. crescentus* origin of replication (*Cori*). (A) The region of *C. crescentus* chromosomal DNA that supports cell type-specific autonomous plasmid replication in *C. crescentus* and in vitro-generated mutations aligned with major sequence landmarks. The AT box indicates a 40-bp region of high (85%) AT content. Deletions/insertions (boxes 1–27) and site-directed mutations (daggers a–h) are described precisely in Tables 1 and 2. (■) Mutations that allow DNA replication (Rep+); and open boxes represent mutations that do not allow DNA replication (Rep-) when this cloned region is assayed for autonomous replication, as described below. Likewise, the open daggers represent mutations that impair replication (Rep- or Rep+/-), and the solid dagger represents a Rep+ linker insertion (Table 2). (H) *Hind*III, (P) *Pst*I, (Bg) *Bgl*II, (E) *Eco*RI, and (B) *Bam*HI. (B) The *Cori*-plasmid replication assay as revealed by plating electroporated *C. crescentus* cells on antibiotic selection plates. The following plasmids were tested: (Plate 1) Wild-type (Rep+) pGM946; (plate 2) deletion/insertion 22 (Rep+) pGM1022; (plate 3) targeted 8-mer base pair changes (Rep+/-) pGM1031; (plate 4) targeted 9-mer base pair changes (Rep+/-) pGM1036; (plate 5) targeted DnaA box base pair changes (Rep-) pGM1038; (plate 6) deletion/insertion 8 (Rep-) pGM1008. Each plasmid (~0 ng) was electroporated into *C. crescentus* strain LS108 and plated on PYE medium containing 20 μ g/ml of ampicillin. (C) The *Cori*-plasmid replication assay as demonstrated by Southern blot analysis of plasmids extracted from liquid cultures. Plasmids were extracted from the same strains shown in B, plates 1–6, as described in Materials and methods, and plasmid preparations from equivalent cell numbers were digested with *Eco*RI, subjected to Southern blot analysis, hybridized with 32 P-labeled vector DNA, and exposed to X-ray film for 30 min.

Cori-plasmids were maintained indefinitely by subculturing, Rep+/- *Cori*-plasmids soon lost their autonomous replication state and integrated into the genome (data not shown). *Cori*-plasmids designated as Rep- gave low frequency transformation (<10 colonies/ μ g of DNA) indistinguishable from background, and these few colonies could be accounted for by spontaneous acquisition of antibiotic resistance.

Cori-plasmids containing randomly generated deletions/insertions (Fig. 1A, boxes 1–27) all fall into either the Rep+ or the Rep- classes. These mutations define an essential origin of replication whose left boundary lies between positions +137 and +198, whose right boundary lies between positions +635 and +714, and whose minimal length is between 437 and 577 bp.

DNA sequence motifs that are common to other bacterial origins of replication as well as those that are unique to *Cori* are contained within the essential DNA region defined by these mutations. At the *E. coli* chromosomal origin of replication, the DnaA protein, bound to specific sites (the "DnaA boxes"), promotes the unwinding of an adjacent AT-rich region (Bramhill and Kornberg 1988a; Kowalski and Eddy 1989) and subsequent replisome assembly at this site (Bramhill and Kornberg 1988b). The essential *Cori* region contains two DnaA box homologies that are potential binding sites for the DnaA protein (Yoshikawa and Ogasawara 1991; Zweiger and Shapiro 1994). Site-directed point mutations inside one *Cori* DnaA box (Fig. 1A, dagger h) abolished replication (Marczynski and Shapiro 1992). The other two potential DnaA box homologies fall within a *Cori* region that is not required for replication (deletions/insertions 24–27). Also within the essential origin is an exceptionally AT-rich domain (85% AT between positions +251 and +290) that is present in other bacterial origins of replication (Bramhill and Kornberg 1988b).

The replication region also contains sequence motifs that are unique to *Cori*. Three perfect 9-mer motifs and two 8/9-bp 9-mer matches (spanning the AT-rich region) fall within the essential *Cori* region. Deletions/insertions 11, 29, and 30 (Figs. 1A and 3D; Table 1) demonstrate that the AT-rich region is essential. Targeted mutagenesis experiments demonstrated that all three perfect 9-mer motifs are essential for replication (Fig. 1A, daggers c,d,f,g; Table 2). Adjacent to the AT-rich region is a cluster of four 8-mer motifs, and targeted mutagenesis experiments (Fig. 1A, daggers a,b; Fig. 3C, pGM1031 and pGM1032), as well as deletions/insertions 10 and 28 (Fig. 1A; Table 1) demonstrate that these motifs are essential for replication.

Two promoters overlap the essential *C. crescentus* origin of replication

An open reading frame that overlaps the essential *Cori* sequence was identified by DNA sequence analysis as a homolog of the *E. coli* *hemE* gene (GenBank accession no. U13664). Sequence analysis revealed a 44% amino acid identity and a 61% similarity to human uroporphyr-

Table 1. Deletions and insertions inside the *Cori* region

Mutation	End points (bp) ^a	Mutation	End points (bp) ^a
1	-504/B/-503	16	+385/B/+420
2	-427/B/-426	17	+397/B/+390
3	-337/B/-316	18	+395/B/+454
4	-234/B/-230	19	+511/B/+577
5	-72/B/-53	20	+615/B/+911
6	+8/B/+60	21	+635/B/+975
7	+97/B/+100	22	+714/B/+814
8	+137/B/+198	23	+714/B/+751
9	+185/B/+322	24	+718/B/+836
10	+202/B/+212	25	+764/B/+816
11	+270/B/+300	26	+767/B/+812
12	+308/B/+330	27	+813/B/+885
13	+297/B/+332	28	+212/H/+244
14	+314/B/+345	29	+254/-/+287
15	+326/B/+353	30	+259/-/+274

^aNumbers indicate the wild-type *Cori* base pair positions (Marczynski and Shapiro 1992) present on the left and right sides of each deletion/insertion shown in Figs. 1A and 3C,D. (B/) An intervening *Bam*HI linker, GGGGATCCCC; (H/) an intervening *Hind*III linker, AAGCTTCC; (-/-) no intervening DNA. Mutations 1–30 correspond to the *Cori* plasmid series pGM1001–pGM1030 in Table 3.

inogen decarboxylase (UroD) (EC 4.1.1.37), an intermediate enzyme in heme biosynthesis (Romeo et al. 1986). The *E. coli* *hemE* gene encodes UroD, and we have adopted the bacterial nomenclature by convention (Bachmann 1990). UroD protein homology extends the entire length of the *C. crescentus* *hemE* gene (beginning at position +201), and we have performed protein fusion experiments to confirm that the HemE initiator AUG codon begins at position +201 (data not shown). The mutational analysis shown in Figure 1A indicates that the 5' portion of the *hemE* gene (beginning at position +201) overlaps an essential region of the *C. crescentus* origin suggesting that *hemE* transcription from inside the *Cori* region may influence replication.

To examine the role of *hemE* transcription in the control of replication, we identified the *hemE* promoters and measured promoter activity during the *C. crescentus* cell cycle. The *hemE* promoters were located initially by constructing transcriptional fusions between a promoterless *lacZ* reporter gene and subclones from the *Cori* region (Fig. 2). Constructs 1–3, which retain a large portion of the region 5' to *hemE*, produced between 2500 and 3000 Miller units. Constructs 4–8, which form a progressive deletion series, demonstrated promoter activity directly upstream of the *hemE* protein coding sequence. Construct 8, which deleted all *hemE* 5'-noncoding sequences up to +201, lost all promoter activity. Constructs 9–12, where the *lacZ* gene was inserted just upstream of the *hemE* protein-coding sequence, produced progressively higher levels of *lacZ* expression as rightward deletions removed the 8-mer motifs. This suggests that expression from a strong promoter is impeded

Table 2. Directed mutations inside the *Cori* region

Mutation	Target	Replication	Description
a	8-mer	+/-	(see Fig. 3C, pGM1031)
b	8-mer	+/-	(see Fig. 3C, pGM1032)
c	9-mer	-	[GGGTACCC] insert at +395 (<i>HpaI</i>)
d	9-mer	-	[AATT] insert at +447 (<i>EcoRI</i>)
e	<i>SmaI</i> site	+	[GGGTACCC] insert at +627 (<i>SmaI</i>)
f	9-mer	+/-	GTAA-----TTAA base pair changes at +654–657 to <u>CGCTA</u> -----TTAA
g	9-mer	-	[GGGTACCC] insert at +667 (<i>HpaI</i>)
h	DnaA box	-	TGATCCACA base pair changes at +677 and +678 to TGAG <u>AC</u> ACA

Mutations a–h correspond to dagger symbols in Fig. 1A and plasmid series pGM1031–pGM1038 in Table 3.

by the 8-mer cluster. Construct 12, with *lacZ* inserted at the *Bgl*II site, produced >11,000 Miller units, but construct 13, with only an extra 10-bp rightward deletion, produced only 2100 Miller units. Further rightward deletions past the AT-rich region reduced *lacZ* expression close to background levels (constructs 14–16). These results indicate that a strong promoter lies directly on the right side of the *Bgl*II site inside the AT-rich region, and comparison of *lacZ* expression from constructs 7 and 8 demonstrates a second weaker promoter on the left side of this *Bgl*II site (between positions +201 and +254), because this region by itself produced ~2000 Miller units.

These observations are summarized by the model drawn at the bottom of Figure 2. A weak promoter contributes ~2000 Miller units and an upstream strong promoter contributes ~10,000 Miller units, of which all but ~1000 are impeded by downstream sequences overlapping the 8-mer cluster. A mechanism for impeding strong promoter expression is suggested by potential sec-

ondary structure in the RNA leader sequences, as described below (Fig. 8A).

Primer extension assays were used to confirm this two-promoter model. A primer internal to the *hemE* protein-coding sequence revealed transcripts initiating at positions +201 and +251 (Fig. 3A). A primer at the start of the *hemE* protein-coding sequence revealed a single transcription start site at position +251/252 (Fig. 3B). These transcriptional start sites agree with the *lacZ* reporter experiments shown in Figure 2 and additional *lacZ* reporter experiments described below in Figure 3C,D.

The DNA sequences directly upstream of each *hemE* RNA start site are homologous to established promoters (Fig. 3C,D). Overlapping the 8-mer cluster is a consensus sequence derived from a number of σ^{70} -type *C. crescentus* promoters (B. Ely, pers.). This weak *hemE* promoter has correct spacing between the +1, -10, and -35 regions, and it matches the proposed -10 and -35 consensus in 8/9 positions (Fig. 3C). Its relative transcrip-

Figure 2. *hemE* promoters within the *C. crescentus* origin of replication mapped by *lacZ* transcriptional reporter assays. A partial restriction map of the replication origin (*Cori*) is shown. Subclones of the *Cori* region were ligated into the pRK290 *lacZ* test plasmid (constructs 1–16 corresponding to *lacZ*–plasmid series pGM2001–pGM2016 in Table 3). The lines show the extent of *Cori* DNA present in these constructs, and β -galactosidase (β -gal) activity is shown, in Miller units, to the right of each construct. Translational stop codons in all three reading frames precede the *lacZ* ribosome-binding site and prevent *lacZ*::*hemE* protein fusions. The activities shown are the averages of at least three separate assays with s.d. <10%. The pRK290 *lacZ* test plasmid produced 250 Miller units without any subcloned DNA. Restriction sites and symbols are as presented in Fig. 1. The approximate locations of two promoters are shown at the bottom, along with an impedance region that reduces upstream transcription into *lacZ*.

Developmentally controlled replication origin

Figure 3. Sequences required for transcription and replication within the origin of replication. (A,B) Primer extension analysis of both *hemE* promoters using 32 P-labeled oligonucleotide primers complementary to an internal portion of the HemE-coding sequence (5' position +120 through +137) (A) and the amino terminus of HemE (5' position +181 through +203) (B). Lanes 1–4 (A) and 1–5 (B) present a series of primer extensions carried out at progressively increasing temperature using wild-type *C. crescentus* (NA1000) chromosomal RNA, as described in Materials and methods. Dideoxy-sequencing ladders served as the size standards. Numbered arrows mark the RNA start sites aligned with the *Cori* DNA base pair positions shown in C and D. (C,D) Sequences of two *hemE* promoters and analysis of transcriptional and replicative abilities of mutations in the promoter regions. The bent arrows position the primer extension results from A and B over the *Cori* DNA sequence directly upstream of *hemE*. Boxed sequences in C show the four 8-mer motifs, and the connected ovals in D show the two 9-mer motifs that are present on the wild-type plasmid pGM946. Established promoter sequences and the inferred *hemE* promoter –10 and –35 elements are aligned above the wild-type sequence. Site-directed base pair changes and/or deletions present on mutant *Cori*-plasmids are shown below. Deletions are indicated by open boxes with dots, and base changes are shown with dashes to indicate the unchanged bases. Replication was assayed by electroporating these *Cori*-plasmids (containing the –633 to +998 *Cori* region) into *C. crescentus* strain LS108 and testing plasmid replication, as described in Fig. 1B,C. Transcription was assayed by testing *Cori*-plasmid subclones in the *lacZ* reporter system described in Fig. 2. In C, weak promoter transcription of each mutant origin was assayed by subcloning the *Pst*I–*Bgl*II *Cori* DNA (+1 to +254) into pRK290 *lacZ*; in D, strong promoter transcription was assayed by subcloning the *Afl*III–*Eco*RI *Cori* DNA (+250 to +452) into pRK290 *lacZ*. These *lacZ* reporter plasmids correspond to pGM2021–pGM2028 in Table 3. The transcription assay results, presented in Miller units, are the averages of at least three separate experiments with s.d. <10%.

tion weakness is probably attributable to the one mismatch in the conserved base pair at position 213 (Fig. 3C). The strong *hemE* promoter has a 6/9-bp match to the same consensus, but it retains the most conserved base pairs at positions 261 and 263 in the –10 region, and at positions 285 and 286 in the –35 region (Fig. 3D). The –10 region of this promoter also has a 7/9-bp match to the –10 region of the *hemA* promoters of the closely related bacterium, *Rhizobium meliloti* (Leong et al. 1985).

We performed precise promoter mutation experiments to confirm the presence of the weak promoter within the four 8-mer motifs, and the strong promoter within the two 9-mer motifs. The weak promoter transcriptional start at position +201 (directly over the A of the *hemE*

AUG initiation codon) is unusual but not without precedent (Shean and Gottesman 1992). Only the weak promoter DNA (from *Pst*I at +1 to *Bgl*II at +254) was used in the *lacZ* transcriptional reporter experiments shown in Figure 3C. Although the wild-type weak promoter DNA (from pGM946) produced 1780 Miller units, a deletion of the –35 region (pGM1028) and base pair changes that overlap the –10 region without changing the spacing (pGM1010) produced only 230 and 90 Miller units, respectively (Fig. 3C). Because these mutations were predicted to impair the –35 and –10 promoter elements, respectively, clearly a functional promoter resides directly upstream of the *hemE* protein-coding sequence inside the 8-mer cluster.

In a similar manner, we demonstrated that a strong

promoter overlaps two 9-mer motifs inside the AT-rich region. Only the strong promoter DNA (from *Afl*III at +250 to *Eco*RI at +452) was used in the *lacZ* transcriptional reporter experiments shown in Figure 3D. Although the wild-type strong promoter DNA (from pGM946) produced 12,430 Miller units, the substitution of 3 G's for 3 T's in the conserved -10 region (pGM1303), a deletion of the -10 region (pGM1029), an extensive deletion of both -10 and -35 region (pGM1029) and a deletion and base change in the -35 region (pGM1011) all produced significantly lower Miller units (Fig. 3D). Because all four mutations are predicted to impair the -10 and/or -35 promoter elements, clearly a second promoter resides in the AT-rich region.

Impaired transcription correlates with impaired replication

All mutations that reduced *hemE* transcription, from either the weak promoter (Fig. 3C) or the strong promoter (Fig. 3D), also impaired replication. This suggests that transcription is required for replication. However, this analysis is complicated by the overlap between promoter and replication elements. For the weak promoter, 8/9 of the -10 and -35 promoter base pairs overlap the 8-mer elements, and this precludes a mutational separation between these DNA sequences (Fig. 3C). However, as targeted 8-mer base pair changes (in pGM1031 and pGM1032) impair replication without reducing transcription from the weak promoter (Fig. 3C), we speculate that the four 8-mer motifs and not transcription from the weak promoter are required for *Cori* replication.

For the strong promoter, 6/9 of the *C. crescentus* σ^{70} consensus promoter base pairs overlap the 9-mer replication elements (Fig. 3D). The directed mutation analysis in Figure 1 demonstrated that the consensus 9-mer motifs are essential for autonomous *Cori*-plasmid replication. A comparison between the *C. crescentus* σ^{70} consensus and the *R. meliloti* *hemA* homology suggests that an important portion of the -10 promoter element lies outside the 9-mer elements, implying that a separation between replication and transcription elements is possible. Therefore, we changed the three conserved base pairs (at positions 259–261) from T's to G's (pGM1303). As shown in Figure 3D, comparison of the wild-type promoter in pGM946 with the -10 promoter mutation in pGM1303 revealed that replication is significantly impaired when strong promoter transcription is reduced from 12,430 to 2150 Miller units. Because the pGM1303 point mutations do not affect the 9-mer elements, we infer that transcription from the strong promoter is required for *Cori* replication. Apparently, transcription from the strong promoter contributes a replicative function that is separable from the replicative function contributed by the 9-mer elements.

Cell-type-specific control of transcription within the *C. crescentus* origin of replication

To examine further the function of the two *hemE* promoters in the control of replication, their transcription

was assayed during the developmental cell cycle. Swarmer cells were isolated and allowed to proceed synchronously through the developmental cell cycle, and at the indicated times, cells were removed and assayed for *hemE* transcription (Fig. 4). As described in Materials and methods, this assay measures mRNA transcription through the *lacZ* reporter gene. When positioned downstream of both *hemE* promoters, the *lacZ* gene was transcribed uniformly in all cell types (Fig. 4A). However, when the *lacZ* gene was driven solely by the upstream strong promoter, the *lacZ* reporter exhibited significant cell cycle variation (Fig. 4B). A PhosphorImager quantitation of this experiment is presented in Figure 4C.

Figure 4. Transcription initiated from the two *hemE* promoters during the cell cycle. Samples of synchronized *C. crescentus* cultures bearing *lacZ* transcription fusions to the *hemE* promoters were pulse-labeled with [³⁵S]methionine and immunoprecipitated with anti- β -galactosidase antibody as described in Materials and methods. Autoradiographs of β -galactosidase synthesis driven by both *hemE* promoters (Fig. 2, construct 3) and by the strong promoter alone (Fig. 2, construct 11) are shown in A and B, respectively. (C) The PhosphorImager quantitation of B. The ³⁵S-labeled β -galactosidase (β -gal) units in C are phosphorescence counts per protein band after a 12-hr exposure to the PhosphorImager screen (Molecular Dynamics). Synchronous swarmer cells were released into fresh M2G media at *t* = 0 min and subsequently sampled for analysis of β -galactosidase synthesized from chimeric *lacZ* RNA at the times indicated above each lane. Cell division occurred at *t* = 150 min in both experiments.

scent swarmer cells produced negligible amounts of *lacZ* mRNA, but as the swarmer cells developed into stalked cells, the level of *lacZ* mRNA increased. Peak transcription was observed at ~100 min, during the stalked cell phase. Transcription then decreased in the predivisive cells to ~50% of its peak value. When cell division occurred, at ~150 min, and DNA replication resumed in the progeny stalked cells, transcription again increased from the strong promoter (Fig. 4B,C).

Because the *hemE* strong promoter is virtually inactive in the nascent swarmer cells, but highly active in the nascent stalked cells, we determined the precise time and polar position before cell division that this transcriptional switch occurs. Figure 4C indicates that strong promoter transcription decreased ~50% in the predivisive cells, implying that either transcription decreased equally at both cell poles or that transcription decreased preferentially at the swarmer cell pole. To distinguish these possibilities, predivisive cells (containing the strong promoter *lacZ* transcriptional fusion) were pulse labeled for 5 min with [³⁵S]methionine and chased with unlabeled methionine while they continued to grow and divide (Fig. 5A). The nascent swarmer and

stalked cells were isolated, and the 116-kD β -galactosidase reporter protein was immunoprecipitated from equal amounts of ³⁵S-labeled cell extracts (Fig. 5A). The presence of the ³⁵S-labeled β -galactosidase in the progeny cells reports the subcellular compartment (swarmer pole vs. stalked pole) of the predivisive cell containing the chimeric mRNA at the time of the [³⁵S]methionine pulse. It has been demonstrated previously that the β -galactosidase protein shows no inherent bias toward either pole. However, if a flagellin gene promoter directs *lacZ* transcription, then in this protocol the β -galactosidase protein is recovered preferentially in the progeny swarmer cells (Gober et al. 1991b). As expected, the 25- and 27-kD flagellin proteins are synthesized exclusively at the swarmer pole of these same predivisive cells (Fig. 5A). In contrast, when the strong *hemE* promoter drives *lacZ*, β -galactosidase synthesis occurs primarily at the stalked cell pole before cell division (Fig. 5A). This indicates that the strong promoter is transcribed primarily from the chromosome at the stalked pole before cell division.

Stalked pole-specific transcription of the strong *hemE* promoter might be attributable to selective activation at

Figure 5. Stalked pole-specific transcription of the strong *hemE* promoter. (A) Protocol diagram and example of one assay of pole-specific *hemE/lacZ* transcription. Swarmer cells, bearing the *lacZ* transcription fusion to the strong *hemE* promoter (Fig. 2, construct 11), were allowed to proceed to the predivisive stage. These cells were then pulse labeled with [³⁵S]methionine for 5 min and allowed to divide in the presence of excess unlabeled methionine (chase period). Progeny swarmer (Sw) and stalked (St) cells were isolated, β -galactosidase and, as a control, flagellin proteins were immunoprecipitated from equal amounts of ³⁵S-labeled protein extracts, as detailed in Materials and methods. (B) Time course of swarmer pole and stalked pole transcription from the strong *hemE* promoter before cell division. The protocol described in A was repeated, but the time of the pulse with [³⁵S]methionine was varied from 0 to 40 min before cell division. The relative amount of ³⁵S-labeled β -galactosidase in progeny stalked and swarmer cells was quantified by PhosphorImaging of immunoprecipitated proteins and graphed as shown. The ³⁵S-labeled β -galactosidase units in B and D are phosphorescence counts per protein band after a 12-hr exposure to the PhosphorImager screen (Molecular Dynamics). (C,D) Loss of stalked pole-specific transcription from a mutant strong *hemE* promoter. The pGM1030 *lacZ* transcriptional reporter from Fig. 3D was used, but otherwise these polar transcription assays are identical to those present in A and B.

the stalked pole or selective repression at the swarmer pole. To distinguish between these two mechanisms, we modified the polar transcription protocol shown in Figure 5A, by varying the time of the [35 S]methionine pulse between 0 and 40 min before cell division (Fig. 5B). The progeny swarmer and stalked cells were isolated, and the relative amount of reporter protein was quantitated by immunoprecipitation and 35 S-labeled PhosphorImaging, as described in Materials and methods. Transcription at the stalked pole was found to remain constant, whereas transcription at the swarmer cell pole decreased before cell division (Fig. 5B). One interpretation of these transcription kinetics is that the *hemE* strong promoter is repressed selectively at the swarmer pole of the predivisional cell.

The preferential transcription of the *hemE* strong promoter at the stalked pole (Fig. 5A,B) is probably mediated by the two 9-mer motifs that overlap both the -10 and -35 promoter elements (see Fig. 3D). The deletion in pGM1030 removes sequences from both 9-mer motifs while retaining significant transcription activity (Fig. 3D). If the 9-mer sequences contribute to the differential transcription of the strong promoter within *Cori*, then the mutated 9-mer sequences in pGM1030 should result in a loss of stalked pole transcription preference. Consistent with this hypothesis, the polar transcription experiments in Figure 5, C and D, which parallel those in the preceding panels for the wild-type promoter, demonstrate that this mutant promoter has lost all transcriptional preference for the stalked pole. The mutant promoter possesses a small but consistent transcription bias for the opposite swarmer pole (Fig. 5D). Therefore,

stalked pole-specific regulatory sequences overlap the AT-rich region inside *Cori*. Although the experiment shown in Figure 5B suggests that a swarmer pole-specific repressor binds the 9-mer sequences, we cannot rule out the possibility that transcription is activated preferentially at the stalked pole. The experiment presented in Figure 5D shows that the pGM1030 mutation both decreased basal transcription and yielded equal transcription at both poles.

To confirm that stalked pole-specific transcription is a special property of the *hemE* strong promoter, we repeated the polar transcription protocol for each of four *lacZ* transcription fusions that assayed the combined *hemE* promoters (Fig. 6A), the *hemE* weak promoter alone (Fig. 6B), the wild-type strong promoter alone (Fig. 6C), and the pGM1030 mutant strong promoter alone (Fig. 6D). The ratio of 35 S-labeled protein recovered in stalked versus swarmer cells (St/Sw) as a function of the time of the pulse label before cell division is shown in each case. Most proteins, such as RNA polymerase ($\beta\beta'$) subunits, partition equally to both progeny cells and show a St/Sw ratio of ~ 1.0 , whereas flagellin proteins partition exclusively to the swarmer cells (Gober et al. 1991b) and show a St/Sw ratio of <0.1 (Fig. 6). Transcription from the combined *hemE* promoters, which includes the 8-mer impedance region (Fig. 6A), from the weak *hemE* promoter alone (Fig. 6B) and from the mutant strong promoter alone (Fig. 6D), showed no stalked pole bias. Only when *lacZ* transcription was driven by the *hemE* wild-type strong promoter alone did its mRNA accumulate preferentially at the stalked pole of the predivisional cell (Fig. 6C).

Figure 6. Only the wild-type strong *hemE* promoter exhibits stalked pole-specific transcription. (A–D) Time courses before cell division of the polar localization ratio, defined as the ratio between transcription activity arising from the stalked and swarmer cell poles (St/Sw) of the predivisional cell. Each experiment used the following *lacZ* transcription reporter constructs: (A) the native two-promoter system; (B) the weak promoter alone; (C) the wild-type strong promoter alone; and (D) a mutant strong promoter alone. These experiments were performed as described in Fig. 5 and used *lacZ* transcriptional reporter constructs 3, 7, 11 (Fig. 2), and the *lacZ* construct from pGM1030 (Fig. 3D) shown symbolically in A–D, respectively. Equal amounts of 35 S-labeled protein extracts from progeny stalked cells and swarmer cells were analyzed for each time point. β -Galactosidase and flagellins (25- and 27-kD proteins) were assayed by immunoprecipitation, and the RNA polymerase ($\beta\beta'$ subunits) were observed directly on total cytosolic protein gels. Bands were quantified by PhosphorImaging and plotted as shown.

Abolishing differential transcription from the two poles of the predivisional cell abolishes regulated replication in the progeny cells

The preceding experiments imply that stalked pole-specific transcription from the *hemE* strong promoter is required for stalked cell-specific replication. To test this hypothesis, cell type-specific *Cori*-plasmid replication was assayed using plasmid constructs with either the wild-type (stalked pole-specific, Fig. 5A,B) or the pGM1030 deletion mutant (nonspecific; Fig. 5C,D) *hemE* strong promoters, described above. For these experiments, we used a sensitive replication assay based on the observation that *C. crescentus* DNA is always fully methylated at the A nucleotide of the GANTC sequence on both DNA strands before replication (Zweiger et al. 1994). The GANTC methyltransferase is only present in late predivisional cells; therefore, DNA replication produces two hemimethylated molecules whose hemimethylated state persists until the end of the cell cycle. When the GANTC sequence overlaps a restriction endonuclease site, for example, *Cla*I or *Hinc*II, endonuclease cutting is blocked when both DNA strands are methylated, and the hemimethylated state is revealed by the acquired ability to cut at these sites on one of the two newly replicated DNA molecules. As reported previously, the chromosomal *Hinc*II site upstream of the *dnaA* gene is fully methylated in swarmer cells, and it becomes hemimethylated when swarmer cells differentiate into stalked cells (Zweiger et al. 1994). Because the *C. crescentus* *dnaA* gene is only ~6 kb away from *Cori*, we used this *Hinc*II site to assay the initiation of chromosomal replication. *Cori*-plasmid replication was monitored similarly by inserting an oligonucleotide-bearing overlapping *Cla*I and GANTC methylation sites.

Using this methylation assay, the replication kinetics of wild-type *Cori*-plasmids and mutant pGM1030 *hemE* strong promoter *Cori*-plasmids were compared with that of the chromosome during the swarmer to stalked cell transition period of the cell cycle. Swarmer cells containing these *Cori*-plasmids were prepared and analyzed as they differentiated synchronously into stalked cells. Both plasmid and chromosomal DNA were prepared, the percentage of hemimethylated molecules was determined by Southern blot analysis, and the corresponding percentage of replicated molecules was quantitated as detailed in Materials and methods.

The results presented in Figure 7 demonstrate that the *Cori*-plasmid with the mutant *hemE* strong promoter, unlike the wild-type *Cori*-plasmid, fails to initiate replication synchronously when swarmer cells differentiate into stalked cells. Chromosomal replication served as an internal control, and in both experiments (Fig. 7A,B), the chromosomes initiated replication with identical kinetics. However, although wild-type *Cori*-plasmid replication exhibited a steep sigmoidal curve that closely tracked the sigmoidal curve of the chromosome (Fig. 7A), the mutant *Cori*-plasmid exhibited a linear replication curve distinct from that of the chromosome and indicative of random replication. This linear accumulation of

Figure 7. Wild-type and mutant *Cori*-plasmid replication was measured and compared to chromosomal replication by the methylation state assay reported previously (Zweiger et al. 1994). Wild-type *Cori*-plasmid (pGM946) and mutant *hemE* strong promoter *Cori*-plasmid (pGM1030) were modified so that they contained the RK2 *par* sequences (Roberts et al. 1990) between the *Hind*III and *Kpn*I sites, and the following oligonucleotide sequence 5'-CTCGAGTCAGGCTCATCGATTCGATCGCAGCGGATCC-3', containing the *Cla*I and methylation site overlap, between the *Xho*I and *Bam*HI sites. These are designated as pGM1064 and pGM1065, respectively (Table 3). Swarmer cells bearing these plasmids were isolated and allowed to differentiate into stalked cells by release into PYE medium. At the indicated times, both plasmid and chromosomal DNA were prepared separately and analyzed by Southern blot and PhosphorImaging techniques as described in Materials and methods. Chromosomal DNA was digested with *Hinc*II, and the Southern blots were hybridized with a 420-bp piece of ³²P-labeled DNA spanning the 5' region of the *C. crescentus* *dnaA* gene (Zweiger and Shapiro 1994). Plasmid DNA was linearized with *Pst*I and then redigested with excess *Cla*I. *Cori*-plasmid Southern blots were hybridized with ³⁵P-labeled pGM946 vector DNA between the *Cla*I and *Pst*I sites. (A,B) The time course of the percentage of replicated molecules; chromosomal DNA (○,△) and plasmid DNA (●,▲) were obtained from analyses of images of the corresponding Southern blots. (C,D) Autoradiographs of the corresponding wild-type and mutant *Cori*-plasmid Southern blots. The chromosomal DNA Southern blots are not shown.

replicated plasmids implies that the mutant *Cori*-plasmids replicated at the same rate throughout most of the cell division cycle. In contrast, the sigmoidal curve implies that the wild-type *Cori*-plasmid replicated primarily during the brief 10- to 20-min period spanning the swarmer to stalked cell transition at the start of the

DNA synthesis period. These wild-type *Cori*-plasmid replication results agree with those reported previously using a radioactive pulse-labeling method to assay replication (Marczynski and Shapiro 1992). Because the only difference between the wild-type and mutant *Cori*-plasmids is a 14-bp deletion that abolishes stalked pole-specific transcription in the predivisional cell (Figs. 5C,D and 6D), we conclude that stalked pole-specific transcription from the *hemE* strong promoter is required to regulate replication in the progeny cells.

The stalked pole-specific hemE RNA originating from within the origin of replication is not translated

The 5'-leader sequence of the strong *hemE* promoter mRNA has the potential to form secondary structures that effectively bury the *hemE* AUG initiation codon and block ribosome binding (Fig. 8A). The majority of the base pairs in this RNA structure are formed by the four 8-mer motifs. The 5' end of the downstream weak promoter *hemE* mRNA lacks this structure, and initiates transcription at the start of the AUG for the HemE cod-

ing sequence. Although this *hemE* mRNA lacks a Shine and Dalgarno (S.D.) sequence, it possesses a "downstream box" that is complementary to a region of the *C. crescentus* 16S rRNA (Fig. 8A), as is the case for the λ phage *cl* gene (Shean and Gottesman 1992). The proximity of the *hemE* downstream box to the AUG and the presence in *C. crescentus* 16S rRNA of a domain utilized for these base pairings are directly analogous to several documented *E. coli* downstream boxes (Sprengart et al. 1990; Shean and Gottesman 1992). These considerations suggest that HemE translation might occur primarily from the weak promoter mRNA.

We confirmed this hypothesis by measuring protein synthetic rates from transcripts originating from the two *hemE* promoters. The results in Figure 8B indicate that *hemE* translation derives primarily from the weak promoter mRNA and that most strong promoter mRNA molecules are not translated. This could be attributable to several factors, including premature termination or inability to access the AUG when the transcript initiates at the strong promoter. Comparison of *hemE::lacZ* fusion protein synthesis rates from constructs 1 and 2 re-

Figure 8. Translational efficiency of transcripts originating from the strong and weak *hemE* promoters. (A) Potential RNA structures formed by mRNA initiating from either the *hemE* strong promoter or the downstream weak promoter. Base-pairing across the 8-mer motifs (numbered 1-4) in the leader mRNA originating from the strong promoter might block ribosome access to the AUG start codon. RNA sequences downstream of *hemE* AUG (i.e., the downstream box, *Cori* positions +198 through +386) might align ribosomes on the weak promoter mRNA lacking a S.D. site by base-pairing with *C. crescentus* 16S rRNA from positions 1498 through 1486 (Ely 1992), as is observed for some *E. coli* (Sprengart et al. 1990) and bacteriophage λ (Shean and Gottesman 1992) genes. (B) The mRNA originating from the strong *hemE* promoter is translated inefficiently. *C. crescentus* strains NA1000 containing constructs 1-5, corresponding to plasmids pGM4001-pGM4005 in Table 3, were pulse-labeled with [35 S]methionine, and equal amounts of 35 S-labeled protein extracts were immunoprecipitated with anti- β -galactosidase antibody. To aid the comparison of relative protein synthetic rates, the immunoprecipitated proteins were separated on a single polyacrylamide gel, and the resulting bands were quantitated by PhosphorImaging. The indicated numbers show protein synthesis units expressed as PhosphorImaging counts per band obtained after a 24-hr exposure to the PhosphorImaging screen (Molecular Dynamics). To normalize for transcription termination sequences, we compared constructs with the same lengths of *hemE*-coding sequence, between the *Hind*III site (-633) and the AUG start codon (constructs 1-4). S.D. is the efficient Shine and Dalgarno site directing *lacZ* translation; other symbols are as in Fig. 1A.

veals that most *hemE* translation is directed by the weak promoter, because addition of the strong promoter only increases protein synthesis from 3172 to 4130 Miller units. This is a very small increase, considering the relative strengths of these promoters. When the activities of constructs 1 and 2 are compared with that of constructs 3 and 4, it is evident that *hemE* translation from its native ribosome-binding sites is not very efficient, because a S.D. sequence provided by the *lacZ* reporter gene increased the rate of protein synthesis ~10-fold. The addition of the strong promoter to the weak promoter in constructs 3 and 4 also provided a disproportionately small increase in *lacZ* protein synthesis from 34,783 to 47,438 Miller units. Although the strong promoter's contribution to downstream protein synthesis is relatively minor in constructs 2 and 4, its full protein synthetic potential is revealed in construct 5 where the 8-mer motifs are deleted and an efficient S.D. sequence is provided by the *lacZ* reporter gene. Construct 5 demonstrates that the strong promoter can drive as much as 443,971 units of *lacZ* protein synthesis, but that in its normal context the strong promoter only contributes ~1000 units (cf. constructs 1 and 2). Apparently, most transcripts that initiate from the strong *hemE* promoter do not contribute significantly to *hemE* gene expression. Presumably this RNA serves a replicative function.

Discussion

The *C. crescentus* predivisional cell exhibits a striking asymmetry that yields morphologically distinct progeny whose chromosomes have different replicative fates. Although the swarmer cell delays replication, the stalked cell initiates replication of the entire chromosome from a single origin and replication proceeds bidirectionally at a uniform rate until completion (Lott et al. 1987; Dingwall and Shapiro 1989; Marczyński and Shapiro 1992). Apparently, control mechanisms for replication initiation act asymmetrically in the predivisional cell, and we have focused our studies on the cloned *C. crescentus* origin of replication (*Cori*). Mutation analysis demonstrated that, in addition to anticipated features such as an AT-rich region and DnaA boxes, *Cori* contains novel replication elements such as 8-mer and 9-mer motifs that are also essential for replication (Fig. 1). Interestingly, both 8-mer and 9-mer motifs are clustered in the 5' region of the *hemE* gene, whose unusual transcription and translation properties are summarized in Figure 9A.

From our analysis, we conclude that replication is coupled to transcription from the strong *hemE* promoter originating from inside *Cori*. Seven major experimental results support this conclusion. (1) The 5' region of the *hemE* gene overlaps the essential *Cori* replication region (Fig. 1). (2) Two *hemE* promoters intimately overlap repeated 8-mer and 9-mer motifs that are essential for replication (Figs. 2 and 3). The two 9-mer motifs that overlap the -10 and the -35 regions of the strong promoter are also present at three additional places inside *Cori* where their integrity is essential for replication. (3) Mu-

Figure 9. (A) Organization of the *hemE* 5' region within the *C. crescentus* origin. The symbols are as shown in Fig. 1A. (B) A model indicating selective transcription from the *hemE* strong promoter on the chromosome in the stalked portion of the predivisional cell. We propose that transcripts from the strong promoter potentiate replication in the stalked cell chromosome, perhaps by forming RNA-DNA hybrids inside the *Cori* region.

tations that decrease promoter activity also impair replication (Fig. 3C,D). (4) Targeted mutagenesis that decreases strong promoter transcription (without changing the overlapping 9-mer motifs) impairs replication, implying that transcription is required for replication (Fig. 3D). (5) Translation of the HemE protein is significantly under-represented in comparison to the activity of the strong *hemE* promoter, implying that most transcripts initiating from the strong *hemE* promoter are not translated and presumably serve an alternative replicative function (Fig. 8B). (6) There is preferential transcription from the strong *hemE* promoter in the stalked cell pole of the predivisional cell and in the nascent stalked cell (Figs. 4B,C, 5A,B, and 6C). Therefore, transcription from the strong promoter anticipates chromosome replication. (7) When transcription from this strong promoter is impaired so that it is transcribed comparably at both poles (Figs. 5C,D and 6D), the control of replication is lost in the progeny cells (Fig. 7). We propose that preferential transcription from the strong promoter establishes a polarity in the predivisional cell that generates progeny whose chromosomes have different replicative fates (Fig. 9B).

Polarized transcription in the *C. crescentus* predivisional cell requires a special transcriptional control system. Although most expressed genes are transcribed from both chromosomes in the predivisional cell, four flagellar promoters are known to be transcribed selectively at the swarmer pole (Gober et al. 1991a,b; Gober and Shapiro 1992). We observed that the strong *hemE* promoter decreases transcription at the swarmer pole, whereas stalked pole transcription remains constant during the predivisional cell period (Fig. 5B). This suggests that differential transcription from the strong promoter is attributable either to swarmer pole-directed repression or to stalked pole activation. The promoter deletion in pGM1030 (Fig. 3D), which affects both 9-mers, simultaneously yielded equal transcription at the swarmer and stalked poles (Figs. 5C,D and Fig. 6D) and eliminated normal replication control in the progeny cells (Fig. 7B,D). Therefore, we suggest that a regulatory protein binds differentially to the two 9-mer motifs that overlap the -10 and -35 regions of the strong *hemE* promoters in the predivisional cell. Recent results indicate that a response regulatory element protein that acts at multiple points of the *C. crescentus* cell cycle binds to the 9-mer motifs in the strong *hemE* promoter (G. Marczynski, K. Quon, and L. Shapiro, unpubl.).

The activation of replication by transcription was originally encountered in the *E. coli* λ phage and the *E. coli* chromosomal replication systems (for review, see Baker and Wickner 1992). In both cases, transcription is required for replication, although RNA polymerase probably does not actually synthesize a primer for DNA polymerase (Ogawa et al. 1985; Baker and Kornberg 1988). These systems contrast with plasmid *ColE1* (Masukata and Tomizawa 1990) and mitochondrial DNA replication (de Zamaroczy et al. 1984; Schinkel and Tabak 1989; Clayton 1991), where RNA polymerase synthesizes the rate-limiting RNA primer for DNA polymerase. Plasmid *ColE1* copy number control is dependent on a complex interplay between the secondary structure of the primer RNA and competition between annealing to an antisense RNA repressor or complementary DNA inside the *ColE1* origin (Kues and Stahl 1989). Transcription from the *Cori* strong promoter through the 8-mer cluster blocks HemE translation (Fig. 8B), presumably by forming an RNA secondary structure in the leader sequence that blocks ribosome binding (Fig. 8A). By analogy to *ColE1* plasmids, we speculate that this RNA structure has an additional replicative role inside *Cori*. The majority of the base pairs in this structure form by virtue of the 8-mer motifs, and mutations inside the 8-mer cluster impair replication (Fig. 3C). We speculate that the 8-mer motifs are RNA structural elements, whereas the 9-mer motifs are DNA-binding sites for new replication proteins.

We also speculate that the *C. crescentus* *hemE* promoter/origin overlap represents an ancestral counterpart to the mitochondrial RNA-primed origin. Both *C. crescentus* and the ancestral endosymbiont that gave rise to the modern mitochondrion are members of the α -subdivision of purple bacteria (Yang et al. 1985; Woese 1987;

Schmidt et al. 1991; Stahl et al. 1992). Promoters like the strong *hemE* promoter, which are not coupled to genetic expression, are characteristic of yeast mitochondrial origins of replication (de Zamaroczy et al. 1984; Schmitt and Clayton 1993). The sequences downstream of the strong *hemE* promoter are 83% GC (Fig. 9A), and our mutations define this GC block as absolutely essential for replication (Fig. 3C). As shown in Figure 9B, we propose that this GC block may clamp the 5' end of the *hemE* mRNA preferentially at the stalked pole of the predivisional cell, where we have demonstrated that this promoter is transcribed preferentially before cell division. This clamping mechanism and the proximity of the GC block to the RNA start site are directly analogous to mitochondrial replication origins (Schmitt and Clayton 1993). A persistent *hemE* RNA-DNA hybrid might serve as a heritable replication determinant to preferentially stimulate replication in the progeny stalked cell.

The mechanism for stimulating replication may involve establishing a rate-limiting primer (by RNase H cleavage) or destabilizing the adjacent 85% AT region, as implied in Figure 9B. Short RNA/template hybrids (R-loops) located at or near the *E. coli* origin stimulate replication in vitro, apparently by promoting DNA melting under suboptimal conditions (e.g., low supercoiling, or high HU chromatin protein concentration) that would otherwise inhibit the DnaA-dependent strand-separating reaction at the AT-rich region (Baker and Kornberg 1988; Skarstad et al. 1990). The *E. coli* *mioC* and *gidA* genes flank the origin, but their promoters are separate from the minimal *E. coli* origin (Oka et al. 1980, 1984). However, their transcription can influence replication significantly under special circumstances (Asai et al. 1990; Lobner-Olesen and Boye 1992). Recently, it has been demonstrated that *gidA* and *mioC* transcription is modulated during the *E. coli* cell cycle such that *gidA* transcription increases while *mioC* transcription decreases before the initiation of replication (Theisen et al. 1993; Ogawa and Okazaki 1994). Also, and perhaps more similar to our results, transcription from specific promoters inside the *E. coli* origin may cooperate with the DnaA protein to unwind the AT-rich region (Asai et al. 1992). Our observations on transcription within the *C. crescentus* origin of replication imply that it may use general transcriptional control mechanisms to polarize both replication and morphogenesis in the asymmetric predivisional cell. Although, other mechanisms can be proposed, currently we favor the model presented in Figure 9B because it uses established biochemical properties and it makes experimentally testable predictions.

Materials and methods

Bacterial strains

E. coli strain TG1 was used for routine DNA manipulations. *E. coli* S17-1 (Simon et al. 1983) was used to mobilize pRK290 plasmid derivatives into *C. crescentus* by bacterial conjugation (Ely 1979). *E. coli* strain CJ236 was used to prepare single-strand templates for site directed mutagenesis (Kunkel et al. 1987). *C. crescentus* NA1000 (Evinger and Agabian 1977) was used for

cell synchrony and transcriptional studies, and its ampicillin-sensitive and recombination-defective derivative, LS108 (Table 3) was used for *Cori*-plasmid replication studies.

Cell growth, synchrony, protein labeling, and immunoprecipitation

C. crescentus strains were grown in PYE medium (Poindexter 1964) or in M2G medium (Ely and Johnson 1977). pRK290-derived plasmids and pBluescript II-derived *Cori* plasmids were maintained in *C. crescentus* cultures by supplementing the media with 1 µg/ml of tetracycline or with 20 µg/ml of ampicillin, respectively. Cells were synchronized by collecting the more dense swarmer cells from a Ludox (colloidal silica) gradient and returning them to fresh M2G medium (Evinger and Agabian 1977). Typically, 1 liter of M2G cultures OD₆₆₀ 0.5–1.0 was concentrated into 150 ml by centrifugation before applying the Ludox protocol yielding ~50 ml of OD₆₆₀ = 0.4–0.8 swarmer cells (>95% pure, as judged by light microscopy). Protocols for labeling synchronous *C. crescentus* cells with [³⁵S]methionine and immunoprecipitating radiolabeled proteins have been described (Gomes and Shapiro 1984).

Transcriptional analysis

Transcription initiation at the *hemE* promoters was measured using *lacZ* transcriptional fusions containing translational stop

codons in all three frames preceding the *lacZ* ribosome-binding sites (Yu and Shapiro 1992). This allowed transcription passing through the *lacZ* gene to be measured indirectly by pulse-labeling 1.0 ml of cells (containing the pRK290 *lacZ* constructs) with 0.01 mCi of [³⁵S]methionine (Tran-³⁵S-label, ICN Radiochemicals) for 5 min, and immunoprecipitation with anti-β-galactosidase antibody (Gomes and Shapiro 1984). Polar transcription localization experiments were performed as reported (Gober et al. 1991b), and nascent swarmer cells and stalked cells were isolated by combining the Ludox and differential centrifugation protocols (Gober and Shapiro 1992). β-Galactosidase assays were performed on unsynchronized exponential cultures OD₆₆₀ = 0.2–0.4 of *C. crescentus* strain NA1000 containing the pRK290 *lacZ* reporter plasmids with *Cori* subclones (Fig. 2, constructs 1–16) as described (Miller 1972). For the primer extension analysis, *C. crescentus* RNA was prepared from exponentially growing NA1000 cells in M2G media by the hot phenol method (Gomes et al. 1990) and treated with ribonuclease-free DNase I, reextracted with 50:50 phenol/chloroform, ethanol precipitated, and resuspended in sterile water. Approximately 5 µg of this RNA was annealed with ~100,000 cpm of the specified *hemE* oligonucleotides (Fig. 3A,B), and 5'-end labeled with [γ-³²P]ATP (Amersham) and T4 phage polynucleotide kinase. The primer extension reaction was performed with 5 units of avian myeloblastosis virus reverse transcriptase in the presence of actinomycin C, and the products were analyzed on a 6% polyacrylamide sequencing gel alongside dideoxynucleotide sequencing reactions.

Table 3. Strains and plasmids

Strains/plasmids	Description	Reference/source
<i>E. coli</i>		
TG1	Δ(<i>lac-pro</i>), <i>supE</i> , <i>thi</i> , <i>hsdD5/F'</i> <i>proAB</i> , <i>lacI^q</i> , <i>lacZ</i> ΔM15	T.J. Gibson (unpubl.)
S17-1	<i>E. coli</i> 294::RP4-2(Tc::Mu)(Km::Tn7)	Simon et al. (1983)
CJ236	<i>dut1</i> , <i>ung1</i> , <i>thi</i> , <i>relA</i> /pCJ105	Kunkel et al. (1987)
<i>C. crescentus</i>		
NA1000	synchronizable wild type	Evinger and Agabian (1977)
LS108	NA1000 derivative <i>bla</i> ::Tntet, <i>rec</i> ~Tn5	Marczynski and Shapiro (1992)
Plasmids		
pBluescript II	<i>Amp</i> , <i>ColE1</i> , KS polylinker	Stratagene
pGM946	wild-type <i>Cori</i> -plasmid, 1.7-kb <i>Bam</i> HI– <i>Hind</i> III <i>Cori</i> in pBluescript II	this paper
pGM1000 series		
1001–1030	pGM946 with deletion/insertions	Table 1
1031–1038	pGM946 with targeted mutations	Table 2
1064	pGM946 RK2 <i>par</i> , <i>Clal</i> /CcrM sites	Fig. 7
1065	pGM1030 RK2 <i>par</i> , <i>Clal</i> /CcrM sites	Fig. 7
pRK290 <i>lacZ</i>	derivative of broad host-range pRK290 (Ditta et al. 1980) with <i>lacZ</i> transcriptional reporter and mp18 polylinker	Yu and Shapiro (1992)
pGM2000 series		
2001–2016	pRK290 <i>lacZ</i> containing <i>Cori</i> subclones	Fig. 2
2021–2028	from pGM946 and pGM1000 series	Fig. 3
pJBZ281	<i>lacZ</i> translational fusion vector	Alley et al. (1991)
pGM3901	<i>hemE</i> :: <i>lacZ</i> protein fusion plasmid, <i>Bgl</i> II to <i>Hind</i> III <i>Cori</i> from pGM946 in pJBZ281	this paper
pGM3902	<i>hemE</i> :: <i>lacZ</i> protein fusion plasmid, <i>Eco</i> RI– <i>Hind</i> III <i>Cori</i> from pGM946 in pJBZ281	this paper
pGM4000 series		
4001	pGM3901 in <i>Eco</i> RI site of pRK290	Fig. 8B
4002	pGM3902 in <i>Eco</i> RI site of pRK290	Fig. 8B
4003	<i>Bgl</i> II– <i>Hind</i> III <i>Cori</i> in pRK290 <i>lacZ</i>	Fig. 8B
4004	<i>Eco</i> RI– <i>Hind</i> II <i>Cori</i> in pRK290 <i>lacZ</i>	Fig. 8B
4005	<i>Eco</i> RI– <i>Hind</i> III (+244) <i>Cori</i> from pGM1032 in pRK290 <i>lacZ</i>	Fig. 8B

DNA preparation, sequencing, mutagenesis, and replication analysis

Alkaline lysis plasmid preparations from *E. coli*, digestions by restriction endonucleases, agarose gel electrophoresis, and Southern blot procedures were performed according to established protocols (Sambrook et al. 1989). DNA sequencing was performed by the dideoxynucleotide chain termination method (Sanger et al. 1977) using the Sequenase System II kit (U.S. Biochemical). Single-strand DNA pBluescript II phagemids were prepared as described (Short et al. 1988). The series of *Cori*-plasmid mutations (Tables 1 and 2) were created by combining the methods of Heffron et al. (1978) and Prentki and Krisch (1984). To create specific base pair changes, oligonucleotide-directed site-specific mutagenesis was performed as described (Kunkel et al. 1987). *Cori* DNA that was judged as unmutated on the basis of DNA sequencing analysis was used as a positive control. The ability of these plasmids to replicate autonomously in *C. crescentus* was assayed after electroporation (Fig. 1B; Gilchrist and Smit 1991), by quantitative extraction of plasmid DNA from *C. crescentus* (Fig. 1C; Marczynski et al. 1990) and by *in vivo* ³²P-labeled DNA, as detailed previously (Marczynski and Shapiro 1992).

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