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Mutagenesis of the *Caulobacter crescentus* replication origin: effects on plasmid and chromosome replication

By

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This thesis was submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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Abstract

Caulobacter crescentus has an asymmetric cell division program that yields distinct flagellated swarmer cell and a sessile stalked cell types. Chromosome replication is restricted to the stalked cells where it initiates exactly once per cell cycle. To better understand the DNA sequence requirements for chromosome replication, I systematically altered the cloned chromosome replication origin (*Cori*), and assayed these mutations in a *Cori*-plasmid context and in a whole chromosome context. Previous studies implicated the cell cycle transcription regulator (CtrA) in multiple cell cycle events. CtrA is also proposed to regulate chromosome replication by binding five sites (a-e) inside *Cori*. Altered CtrA binding sites were assayed. CtrA protein failed to bind one mutant (in CtrA site d), and this mutation significantly increased the number of *Cori*-plasmids per cell. Other CtrA binding site mutations decreased plasmid copy number. These results suggest that CtrA plays both positive and negative roles in *Cori* replication. To reveal the essential *Cori* DNA sequences that operate on the chromosome, and not just on a plasmid, 17 mutations throughout the *Cori* region were introduced into the chromosome by homologous recombination. Mutations that could not replace the wild-type chromosome DNA were judged replication defective based on a statistical argument, and effects on replication were separated from effects on adjacent genetic expression by control experiments. 3 mutations allowed replication in the plasmid but not the chromosome context. Surprisingly, 4 out of 17 mutations that blocked *Cori*-plasmid replication allowed replication on the chromosome. Although this deserves further study, only one of

these mutations had a slower growth phenotype, and otherwise cells bearing these altered chromosomes resembled wild-type cells. Presumably, these mutations define a replication system that is essential on the plasmid, but redundant on the chromosome. The 11 exceptional mutations, that block chromosome replication, presumably define the most essential DNA sequences. This is the first systematic mutation analysis of a chromosome replication origin in the natural whole chromosome context. Clearly, both autonomous plasmid and whole chromosome approaches are necessary to define essential replication sequences.

Résumé

La bactérie *Caulobacter crescentus* a une division cellulaire asymétrique donnant naissance à deux types cellulaires distinct. Un possedant un flagelle qui est donc mobile, appelé "swarmer" et l'autre qui est non-motile nommé "stalked' en raison de la présence d'une tige à une extremité. La réplication chromosomique est restreinte aux cellules non-motiles où l'initiation a lieu une seule fois par cycle cellulaire. Afin de mieux comprendre qu'elles sont les séquences d'ADN nécessaires à la réplication, j'ai procédé à la modification systématique de l'origine de réplication clonée (*Cori*) et ces modifications ont été testées dans le contexte d'un plasmide et dans le chromosome. Certaines études faites précédemment suggèrcnt que le régulateur de transcription du cycle cellulaire (CtrA: cell cycle transcription regulator) est impliqué dans plusieurs événements du cycle cellulaire. Il a été proposé que la protéine CtrA régule la réplication chromosomique en se liant à 5 sites (a-e) situés à l'intérieur de *Cori*. Des mutants modifiants ces sites de liaisons ont donc été testés. Un de ces derniers, arborant un mutation au site d, ne pouvais plus être contacté par CtrA, mais il y a toutefois eu augmentation significative du nombre de plasmides *Cori* ayant cette mutation par cellule. D'autres mutations ont fait chuter le nombre de plasmides par cellule. Ces résultats suggèrent à *Cori* une implication tant négative que positive lors de la réplication. Afin de déterminer la séquence essentielle de *Cori* requise non seulement dans un plasmide mais dans le contexte du chromosome, 17 mutations, distribuées dans *Cori* ont été introduites par recombinaison homologue. Les mutations ne pouvant remplacer la séquence chromosomique originale ont été jugées déficientes en ce qui à trait à la réplication en

se basant sur des données statistiquement significatives. Ainsi, les effets sur la réplication ont été départagés d'effets sur l'expression génétique adjacente à làide de contrôles. Trois mutations ont permi la replication dans un contexte de plasmide mais non dans le chromosome. Etonnamment, nous avons eu 4 mutants sur 17 empèchant la réplication d'un plasmide *Cori* mais qui permettait celle du chromosome. Malgré ce fait, seulement un de ces mutant présentait une croissance plus lente comparée à la souche de type sauvage, les autres mutants se comportant comme cette dernière. Nous avons donc présumé que ces mutations définissaient un systéme de réplication essentiel dans un plasmide mais redondant dans le chromosome. Nous croyons que les 11 mutations exceptionnelles empêchant la réplication chromosomique définissent les séquences d'ADN les plus essentielles. Cette étude est la premiere à faire l'analyse systématique de l'origine de réplication chromosomique dans son contexte naturel. II est toutefois evident que les analyses, tant dans le contexte du chromosome que dans celui d'un plamide autonome, sont nécessaires à la définition des sequences de réplication essentielles.

Acknowledgments

I thank my supervisor Gregory Marczynski for his support and patience throughout my study. Dr. Marczynski's lab gave me a great opportunity to study molecular microbiology. Dr. Marczynski's excellent teaching and critical review of my work were invaluable. All that I learned from him will be important for my future research.

I would also like to thank everyone in Dr. Marczynski's lab. These people include Marie-Claude Ouimet, Rania Siam, Karen Brassinga, and Boris Gorbatyuk. Without your help, I would have had a much harder time during my studies. We discussed my project, and cooperated with experiments. I learned techniques and English from you guys. We also shared life experiences and had nice times as friends. Marie-Claude even translated my abstract into French for me, thanks. In a word, I had a great experience here.

Special thanks go to my family, and to my husband Desheng Lu, without your love, support and help, this work would never have been accomplished. I thank my parents for your love, support, and especially for taking care of my two children during my study period.

Preface

The candidate was responsible for all the work presented in this thesis with exception of some plasmid *Cori* mutations which were made by Dr. Marczynski.

Part of the data presented in this thesis are being prepared as the following manuscript for publication:

Yang, B. and Marczynski, G. T. (1999) Mutagenesis of the *Caulobacter crescentus* replication origin: effects on plasmid and chromosome replication.

Part of the data presented in the thesis was published in the following paper:

Quon, K.C., **Yang, B.**, Domian, I.J., Shapiro, L., and Marczynski, G.T. (1998) Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin. Proc. Natl. Acad. Sci. USA 95, 120-125.

Out of 4 figures in this paper, the experiments in Figure 4 containing a *Cori* mutation, , and plasmid copy number experiments were performed by myself, the CtrA protein binding experiment was performed by Dr. Marczynski and myself.

Table of Contents

Abstract.....II

Résumé.....IV

Acknowledgments.....VI

Preface.....VII

Table of Contents.....VIII

List of Figures and Tables.....XI

Introduction.....1

1. Bacterial chromosome replication origins.....2

1.1. Examples of two bacterial chromosome replication origins.....2

1.2. Bacterial replication origins are regulated by many replication proteins.....6

2. The *C. crescentus* chromosome replication origin 7

3. Transcription and replication 10

4. Cell-cycle control of *C. crescentus* chromosome origin 23

5. Chromosome replication assayed by plasmid "minichromosome" replication and in the whole chromosome context.....18

Research Objectives.....25

Methods and Materials.....26

1. Bacterial strains, plasmids, and growth media.....27

2. Plasmid and chromosome DNA preparation.....31

3. Site-directed mutagenesis of *Cori*.....31

4. *Cori*-plasmid "minichromosome" replication assay.....32

5. Mutagenesis of the *C. crescentus* chromosome origin.....32

6. Competitive growth or "Population shift" assay 33

7. Southern blot assay for rare recombination events 36

8. *lacZ/gusA* transcriptional reporter assay.....36

9. Other methods.....36

Results.....39

1. Negative control of minichromosome replication by CtrA binding to site d.....40

2. Mutated CtrA binding sites inside *Cori* can stimulate or repress minichromosome replication.....40

3. CtrA may control replication by regulating transcription inside *Cori*.....44

4. *Cori*-plasmid replication requirements are independent of the plasmid vector 49

5. Systematic mutagenesis of *Cori* suggests that *Cori* behaves differently in plasmid versus a whole chromosome context.....49

6. Control transcription experiments to separate mutant effects on replication from mutant effects on genetic expression.....58

7. Chromosome *Cori* mutants that fail to support plasmid replication, resemble wild-type cells in growth and morphology.....61

Discussion.....69

1. Summary.....70

2. CtrA binding sites influence replication and transcription 72

3. Whole chromosome versus plasmid replication.....74

Conclusions.....78

References.....79

List of Figures and Tables

Figure 1. A comparison of *B. subtilis, E. coli*, and *C. crescentus* chromosome replication origins 3

Figure 2. The *C. crescentus* cell division cycle.....13

Figure 3. CtrA protein binds five sites within the *C. crescentus* chromosome replication origin (*Cori*) 16

Figure 4. Initiation of chromosome replication during the *C. crescentus* cell cycle negatively correlates with CtrA protein abundance 19

Figure 5. Mutations defining the *C. crescentus* replication origin (*Cori*).....23

Table 1. Strains and plasmids.....28

Figure 6. The principle of the chromosomal mutation replacement assay.....34

Figure 7. Directed mutagenesis of CtrA binding site d alters protein binding and increases *Cori* plasmid copy number *in vivo*.....41

Table 2. The effects of *Cori* mutations on plasmid replication.....43

Figure 8. Both higher and lower plasmid replication resulted from altered CtrA binding sites inside *Cori* 45

Figure 9. Transcription correlated with replication and CtrA binding site mutations inside *Cori*.....47

Table 3. Chromosomal replication with *Cori*-mutations.....50

Figure 10. Southern blot analysis to test for the presence or absence of

chromosome mutants following 3 % sucrose counter-selection 52

Figure 11. Schematic representation of plasmid and whole chromosome replication with different *Cori* mutations 55

Table 4. The different effects of chromosomal origin mutations on plasmid and chromosome replication.....57

Figure 12. The effects of *Cori* mutations on transcription inside *Cori* 59

Figure 13. An example of the identification of chromosome mutants 63

Figure 14. Growth curves of chromosome *Cori* mutants and wild-type *C. crescentus*.....65

Figure 15. Competitive growth assays between *Cori* mutants and wild-type *C. crescentus*.....67

Figure 16. Schematic representation of yeast autonomous replication sequences (ARS's), plasmid versus chromosome position effects.....76

Introduction

1. Bacterial chromosome replication origins

Regulated chromosome DNA replication is essential for bacterial growth and survival. Chromosomal replication starts from specific DNA sequences termed the replication origin. These are composed of repetitive sequence elements described below for three representative bacteria. It is not known how the DNA sequence at a bacterial replication origin contributes to the precise regulation of DNA synthesis during the cell cycle. However, significant clues are provided by the literature described below.

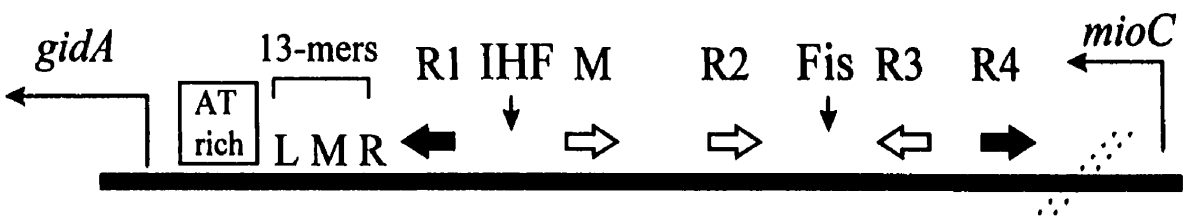
1.1. Examples of two bacterial chromosome replication origins

The *E. coli* replication origin (*oriC*) is the most extensively studied bacterial origin. The minimal *E. coli* replication origin spans 245 bps and assumes a complex DNA-protein structure. The most prominent DNA motifs within *oriC* are the DnaA boxes (TTATCCACA) and the 13-mers (GATC(t/a)nTT(c/a)T(t/a)(t/a)T(t/a)n) present inside the AT rich region (Fig. 1B). Five DnaA boxes have a precise arrangement (R1, R2, R3, R4 and M as shown in Fig.1). These sequences and their spacing are highly conserved among similar Enteric bacteria. The DnaA protein is highly conserved among eubacteria, and it binds all five of the *E. coli* DnaA boxes. A comprehensive set of DnaA box mutations in *oriC* demonstrate that all *E. coli* DnaA boxes have a function in the initiation process (Langer et al. 1996). The three tandem 13-mer repeats are the first places inside the origin where the DNA is unwound with the aid of the DnaA protein. Mutagenesis studies show that non -conserved spacer DNA sequences are also essential for replication. As one

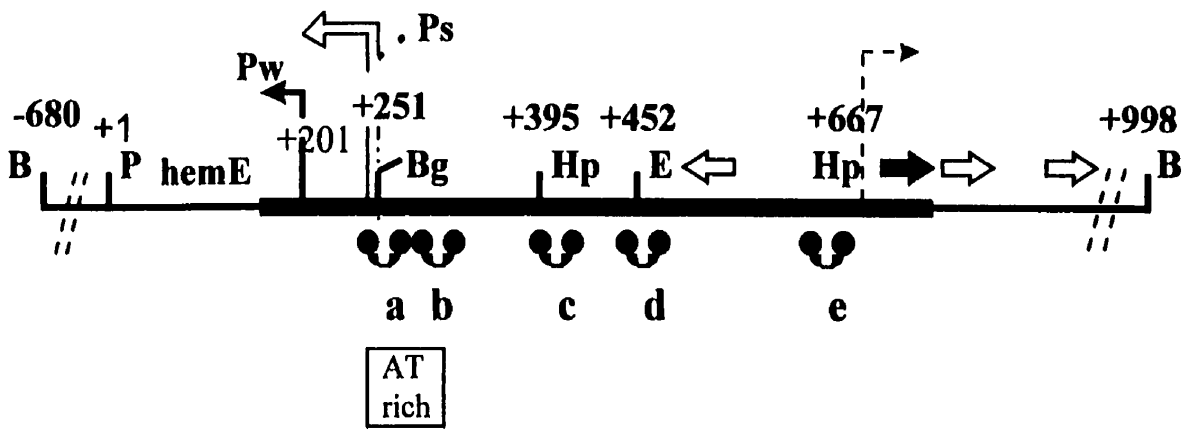
Figure 1. A comparison of *B. subtilis*, *E. coli*, and *C. crescentus* chromosome replication origins. Thick black lines indicate minimal sequences required for autonomous (origin-driven) plasmid replication. The short horizontal arrows mark DnaA boxes. Bent arrows mark the RNA start sites for promoters. The large open box demarcates the protein-coding sequences for the *dnaA* in *B. subtilis* origin. The dumbbell-shaped symbols denote the 9-mer motif GTTAA-N7-TTAA sequences (a-e) in *C. crescentus* replication origin; Figure was modified from a review (Marczynski and Shapiro 1995) and an article (Polaczek et al. 1997).

A. ***B. subtilis origin***

B. ***E.coli origin***



C. ***C. crescentus origin***



example, the addition or subtraction of even a single base pair between the DnaA boxes and the 13-mers drastically reduced *E. coli* chromosome replication both *in vitro* and *in vivo* (Oka et al. 1984). Base pair substitutions between DnaA boxes R3 and R4 did not have an effect on *oriC* plasmid replication. However, a 4 bp insertion or a 5 bp deletion in the same region completely destroyed *oriC* plasmid replication (Oka et al. 1984). Presumably, the spacer DNA in *oriC* as well as the DnaA boxes are important to establish the DNA-protein "replication initiation complex".

Other bacterial replication origins contain both significant differences and similarities with the *E. coli* replication origin. *Bacillus subtilis*, a Gram-positive bacterium, has a spore-forming developmental program (Errington 1993). The *B. subtilis* replication origin consists of two DnaA box regions located upstream and downstream of the *dnaA* gene. Both regions contain multiple repeats of the DnaA box motif (Moriya et al. 1992; 1994). Interestingly, most of these DnaA boxes precisely match the consensus *E. coli* DnaA box (Moriya et al. 1985), and they are also necessary for *oriC* plasmid replication (Moriya et al., 1992; 1994). The left-hand boundary of *B. subtilis* origin also contains an exceptionally AT-rich domain (Fig. 1A). The *in vivo* mapping of the replication origin of *B. subtilis*, made possible by synchronized spore germination, indicates that its replication initiates at the DnaA box region downstream of the *dnaA* gene and proceeds bidirectionally (Moriya and Ogasawara 1996). Therefore, although *B. subtilis* has a conspicuously large origin, it too utilizes the DnaA protein.

1.2. Bacterial replication origins are regulated by many replication proteins

The initiation of bacterial chromosome replication requires the ordered assembly of replication proteins. Protein binding to defined sites within replication origins presumably determines whether an origin is active or inactive. As noted above, the origin recognizing protein is DnaA which binds to DnaA boxes, promotes the separation of the DNA strands in the AT-rich region, and subsequently the initiation process (Fuller et al. 1984; Gill and Messer 1991; Hwang and Kornberg, 1992). Studies in *E. coli oriC* revealed that a number of DnaA proteins interact with *oriC* in an ordered manner (Margulies and Kaguni 1996; Weigel et al. 1997). Although, DnaA binds to most DnaA boxes throughout the cell cycle, and all DnaA boxes in *oriC* are essential for autonomous replication, some studies suggest that DnaA protein binding to R3 (the weakest *E. coli* DnaA box) is the critical rate limiting step in the initiation process, since it occurs only at the time of initiation (Samitt et al. 1989; Asseler et al. 1995). Therefore, DnaA protein is a major regulator and plays a key role in the initiation and regulation of replication.

However, several other proteins also act positively or negatively to regulate replication initiation. Integration host factor (IHF), and factor for inversion stimulation (FIS), are also likely to play important roles in the regulation of *E. coli* replication (Nash 1996; Bramhil and Kornberg 1988). IHF is a DNA-binding and bending protein that is found in a number of Gram-negative bacteria. IHF binds specifically to one site in the *E. coli oriC* and presumably facilitates open complex formation by building a proper nucleoprotein structure (Skarstad et al. 1990; Polaczek et al. 1997). In contrast, FIS remains bound to

oriC between DnaA boxes R2 and R3 (Fig. 1B) through most of cell cycle, but is released at the time of initiation of replication. It prevents formation of an initiation-proficient structure and inhibits the strand separation reaction at *oriC* (Wold et al. 1996).

The interactions of DnaA, IHF and FIS with *oriC* are dynamic and vary during the *E. coli* cell cycle. A FIS-bound nucleoprotein complex is present throughout the majority of the *E. coli* cell cycle. *E. coli oriC* then switches to the IHF-bound form as cells initiate DNA replication. Coincident with binding of IHF, the initiator DnaA binds to its previously unoccupied DnaA box R3. These results indicate that *oriC* exists in at least three different nucleoprotein complexes: *oriC*-FIS exists throughout most of the cell cycle, *oriC*-IHF is present as cells initiate chromosome replication, and *oriC*-DnaA (R3) is formed perhaps exactly when *oriC* initiates replication (Cassler et al. 1995).

The proteins mentioned above are the most extensively studied replication control proteins. Other proteins, such as IciA, and DnaX, may also play important roles in the control of replication initiation (Lee et al. 1997; Levina et al. 1998). There is a vast literature on this subject (Kornberg and Baker 1992). However, there is no consensus view regarding how chromosome replication is coupled to the *E. coli* cell cycle. It is also not known if *E. coli* can serve as a model for all bacterial cells.

2.The *C. crescentus* chromosome replication origin.

As with other bacteria, *C. crescentus* also uses one replication origin (Lott et al. 1987; Marczynski and Shapiro 1992). When placed on a non-replicating plasmid, the cloned *C. crescentus* origin (*Cori*) supports autonomous plasmid replication (Marczynski and Shapiro 1992) Importantly, *Cori*-directed plasmid "minichromosome" replication coincides with the onset of chromosomal DNA synthesis. This suggests that the cloned *Cori* DNA is regulated by the same factors that regulate the entire chromosome. Also, the mechanism of minichromosome replication control is assumed to be similar to that of chromosome. However, some of my results presented below will suggest that this conclusion is too simplistic.

Studies based on autonomous plasmid replication revealed that *Cori* has common and unique properties that distinguish it from other bacterial origins, such as those of *E. coli* and *B. subtilis* (Yasuda and Hirota 1977; Moriya et al. 1985,1992). The minimal *Cori* is between 430 to 720 bp in length and overlaps the *hemE* gene indicated in Fig. 1C. Four potential DnaA boxes were found although none of these motifs is a perfect *E. coli* consensus. At least one DnaA box is essential, the other three DnaA boxes are dispensable for autonomous replication. Mutagenesis of the *dnaA* gene dramatically impaired cell replication and cell division (Gorbatyuk and Marczynski, unpublished data). Therefore, *C. crescentus* DnaA protein is suspected, as in other bacteria, to play a critical role in the initiation of replication by binding to at least one DnaA box inside *Cori*.

A unique feature of the *C. crescentus* replication origin is the presence of five 9 bp motifs GTTAA-N7-TTAA (two motifs are 8/9 bp matches) within the minimal origin

(Marczynski and Shapiro 1992). These five motifs were found to be the binding site (a to e) of cell cycle transcription regulator, CtrA (Marczynski et al. 1995). CtrA is a global regulator for multiple cell cycle events (Quon et al. 1996). This discovery strongly suggested that replication from *Cori* is controlled by CtrA and coordinated with other cell cycle events. Part of my studies, to be described below, revealed that CtrA negatively controls DNA replication by binding to CtrA-binding site d inside *Cori* (Quon et al. 1998). Further studies are in progress to more fully understand the roles of all five CtrA binding sites.

Another important feature of *Cori* is an exceptional AT-rich region, which overlaps the *hemE* gene promoter (the strong promoter) and is spanned by two CtrA binding sites a and b (Marczynski et al. 1995). Deletions of this AT-rich region destroy the ability of *Cori* to replicate autonomously. Adjacent to the AT-rich region is a cluster of four novel 8-mer motifs which span another *hemE* promoter (the weak promoter). Targeted mutagenesis experiments also demonstrated that these 8-mer motifs are essential for replication. Studies of these regions may provide more information about the mechanism of replication.

In summary, *C. crescentus* only has one chromosome origin of replication (*Cori*). It initiates replication when a stalked cell type is generated. It has common bacterial features such as an AT-rich region, and DnaA boxes, as well as unique *C. crescentus* features such as five CtrA binding sites, and strong transcriptional promoters. The CtrA

binding sites overlap promoters and suggest a relationship between transcription and the control of chromosome replication.

3. Transcription and replication

An important relationship between transcription and the initiation of DNA replication has been suggested by a number of studies in both eukaryotes and prokaryotes (DePamphilis 1988; 1993; Marians 1992; Baker and Kornberg 1988; French 1992). DNA replication of eukaryotic viruses, such as during polymavirus-infection, is significantly affected by transcription and by bound transcription factors (Guo and DePamphilis 1992; Murakami et al. 1991). For example, the binding of the transcription factor AP1 to the flanking region of the origin markedly stimulates viral DNA replication *in vivo* (Guo et al. 1992). Colocalization of transcription and DNA replication in nuclei has been shown in permeabilized HeLa cells (Hassan et al. 1994). In higher eukaryotes, early replicating DNA, and presumed replication origins have been identified in the promoters of human *c-myc* (Iguchi-Ariga et al. 1988), human β-globin (Kitsber et al. 1993) and PPV1 human viral genes (Giacca et al. 1994). A correlation between transcription and origin activation was suggested (Leflak et al. 1989; Hatton et al. 1988; Benard et al. 1992). For example, combining a replication initiation zone, upstream of the human *c-myc* gene, and a T7 promoter induced DNA replication, presumably by inducing negative supercoiling (Ohba et al. 1996). In the yeast *S. cerevisiae*, mitochondrial ori/replication sequences contain a transcription promoter upstream of the site of transition from RNA to DNA synthesis, and

mammalian mitochondrial DNA replication is likewise initiated by a similar process where RNA polymerase leaves an RNA primer for initiating DNA replication (Schmitt and Clayton 1993). However, in most cases the molecular mechanisms underlying the relationship between transcription and replication remain unclear.

In *E. coli*, replication and transcription are also interconnected within the chromosome replication origin. Two transcriptional promoters of the *gidA* and *mioC* genes neighbor the *oriC* (Fig. 1B) (Asai et al. 1990; 1992; Lobner-Olesen and Boye 1992; Von Freiesleben 1992). The transcriptional level of *E. coli mioC* and *gidA* genes was found to correlate with the time of initiation of chromosome replication. It is proposed that transcription from the *gidA* gene introduces negative supercoiling which stimulates *oriC* plasmid DNA replication. In contrast, it is proposed that transcription from *mioC* plays a negative role in the initiation of replication by passing through *oriC* (Junker et al. 1986). Furthermore, the origin binding DnaA protein also regulates the transcription of *mioC* and perhaps *gidA* (Ogawa and Okazaki 1994). These authors express an interesting model for replication control through transcription control.

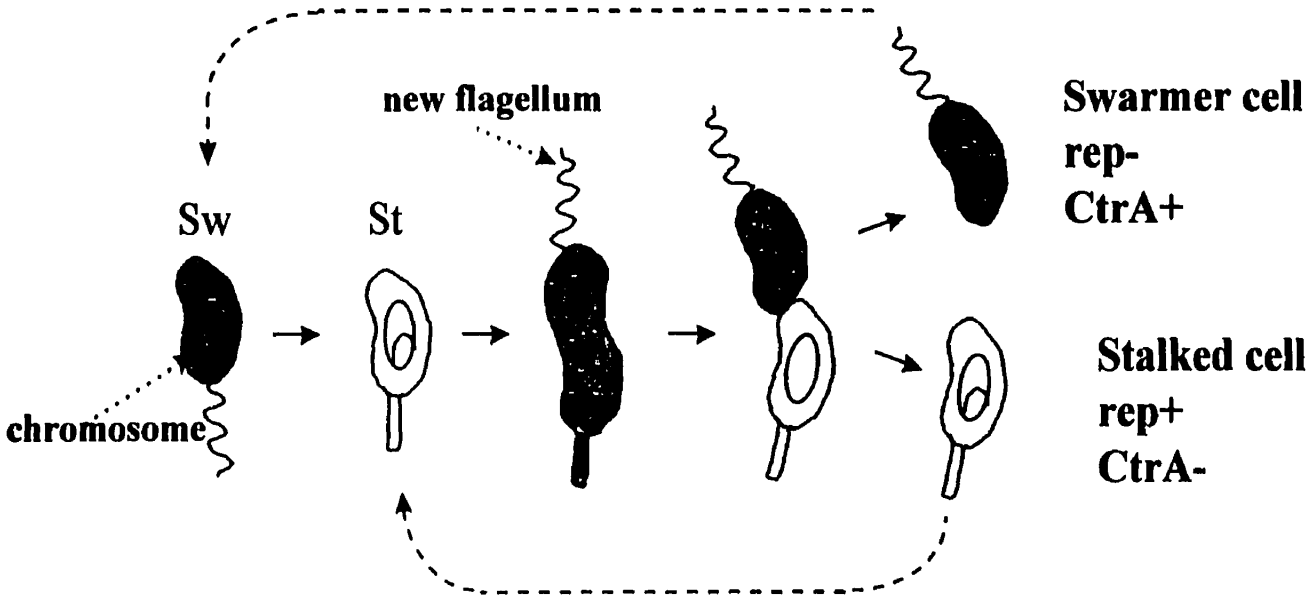
In *C. crescentus*, the minimal DNA sequences required for autonomous *Cori*-plasmid replication overlap with two promoters of a heme biosynthetic gene (*hemE*). One promoter overlaps four 8-mer motifs (AAGCCGG), and the second promoter overlaps two imperfect 9-mer motifs of *Cori*. Both promoters appear to be essential for *Cori*-plasmid replication. The mutations which impair transcription from either promoter also impair minichromosome replication. The unique *hemE* strong promoter, like the *gyrB* promoter

(Rizzo et al. 1993), is selectively transcribed from the stalked cell chromosome and repressed in swarmer cell chromosome. The *hemE* strong promoter -10 and -35 regions overlap with two 9-mer motifs which may be critical regulatory elements that specify *Cori*-plasmid replication in nascent stalked cell (Marczynski et al. 1995). The previous studies revealed that the strong promoter is not coupled to *hemE* gene expression. Primarily, it is the mRNA originating from the downstream weak promoter that directs *hemE* protein synthesis (Marczynski 1995). It is speculated that *C. crescentus hemE* promoter/origin overlap is similar to the mitochondrial RNA primed replication system (Marczynski and Shapiro 1995). These considerations motivated my study of *Cori* mutants and transcription.

4. Cell-cycle control of the *C. crescentus* chromosome replication origin

The cell cycle is an ordered series of events involving chromosomal duplication and segregation, followed by cell division. *Caulobacter crescentus* is a Gram-negative bacterium with a well defined cell cycle, which yields two distinct cell types: a flagellated non-replicative swarmer cell and a non-motile but replicative stalked cell. The swarmer cell later differentiates into a stalked cell to grow and divide asymmetrically as shown in Fig. 2 (Newton 1989; Shapiro and Gober 1989; Brun et al. 1994). Since chromosome replication is restricted to the stalked cell where chromosome replication initiates exactly one per cell cycle (Nathan et al. 1982; Marczynski 1999). Therefore, the *C. crescentus* cell division cycle presents the basic control problems common to all developmentally regulated cells.

Figure 2. The *C. crescentus* cell division cycle. The flagellated swarmer cell (Sw) differentiates into a stalked cell (St) that initiates chromosome replication and asymmetric cell division yielding replicating (rep+) and nonreplicating (rep-) progeny. The shading shows that CtrA is restricted to the swarmer cells and to the swarmer compartment of the predivisional cell .


new flagellum
Sw
St
chromosome
Swarmer cell
rep-
CtrA+
Stalked cell
rep+
CtrA-

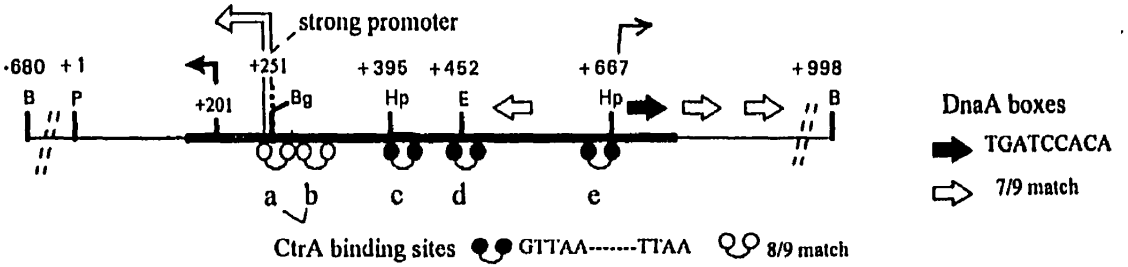
Technical advantages also make *C. crescentus* a good bacterial model system to study the control of DNA replication. Accompanying the distinct morphology, the cells undergo distinct biochemical changes throughout cell cycles. Most importantly, and unlike for most other bacteria, *C. crescentus* cultures are easy to synchronize without metabolic disturbance (Evinger and Agabian 1977).

The cloned chromosome origin (*Cori*) has provided information about the cis-acting elements that mediate the control of DNA replication. Essential regulatory elements were found including a binding site for the initiation protein DnaA, five CtrA binding sites GTTAA-N7-TTAA 9-mer motifs (Figure 3), as well as the transcription promoters described above. Mutagenesis revealed that all these interesting sequences are important for autonomous plasmid replication (Marczynski et al. 1995). Other DNA sequences inside *Cori* also play an important role in replication, such as flanking sequences of the above described motifs, but these are as yet poorly defined.

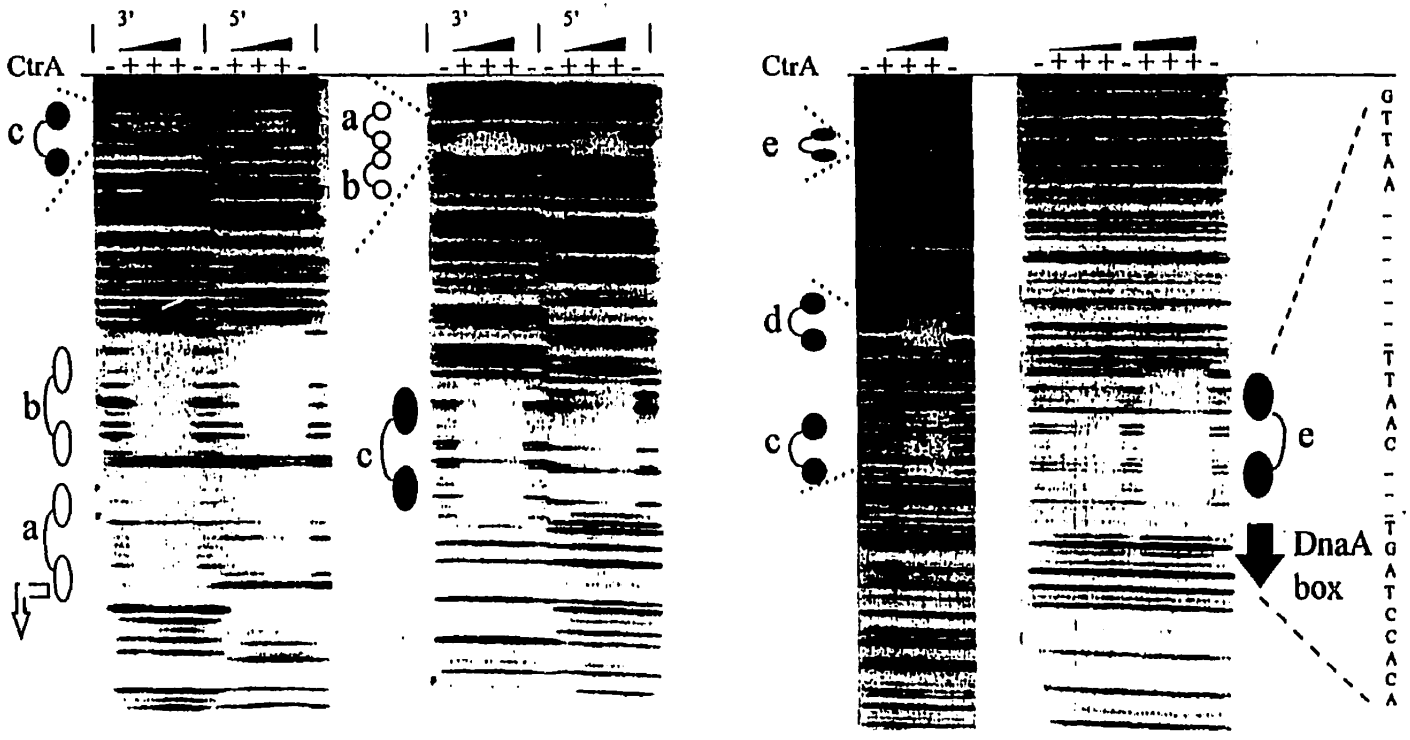
Part of my work deals with the five GTTAA-N7-TTAA, 9-mer motifs that were demonstrated to be the binding sites of an essential cell cycle regulatory protein CtrA (cell cycle transcription regulator) (Quon et al. 1998). CtrA is a two-component signal transduction protein that controls multiple events in the *C. crescentus* cell cycle including cell division, stalk synthesis, and cell cycle-specific transcription (Quon et al. 1996). CtrA is a member of the response regulatory superfamily. As a transcription factor, CtrA regulates the expression of genes for flagellqar biogenesis, DNA methylation, and cell

Figure 3. CtrA protein binds five sites within the *C. crescentus* chromosome replication origin (*Cori*). (A) Schematic of *Cori* as described in figure1. (B) DNase I protection footprinting experiments. + or - indicates that His6-CtrA protein (50 μg/ml maximum) was added or omitted from the footprinting reactions. Figure was taken from (Quon et al. 1998).

A


strong promoter
-680 +1
+251
+395 +452
+667
+998
B P
+201
Bg
Hp E
Hp
B
a b c d e
DnaA boxes
TGATCCACA
7/9 match
CtrA binding sites
GTTAA-------TTAA
8/9 match

B


CtrA
3' 5' 3' 5'
- + + + - - + + + -
c
a
b
b
c
a
CtrA
- + + + -
- + + + - + + + -
e
d
c
e
DnaA box
GTTAA-------TTAACC--TGATCCACA

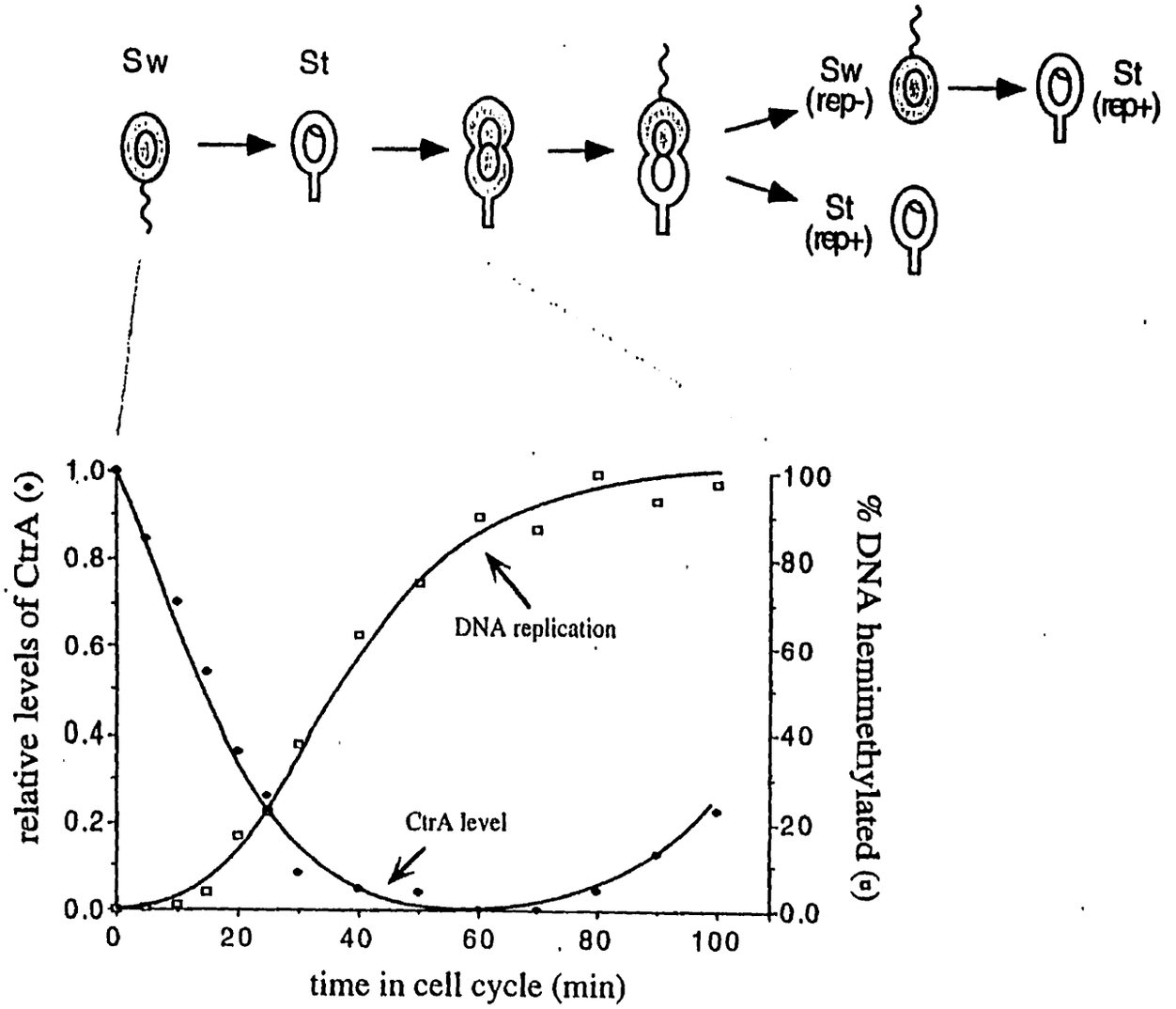
division (*ftsZ*) by binding to their promoters (Brun et al. 1994; Wright et al. 1996; Kelly et al. 1998). CtrA activity is controlled by cell cycle-regulated phosphorylation and proteolysis (Domian et al. 1997). The kinetics of CtrA disappearance coincide with the initiation of DNA replication in a synchronized cell population (Quon et al. 1998) (Figure 4). Its direct binding to *Cori* strongly suggests that CtrA is a swarmer cell-specific repressor of chromosome replication (Quon et al. 1998). However, some of my results argue that CtrA also has a positive role inside *Cori*.

Previous studies demonstrated that DnaA is a replication initiation protein which binds to DnaA boxes inside the replication origins of *E. coli* and *B. subtilis* (Moriya et al. 1992; 1994). Four potential DnaA boxes were found inside *Cori*. One DnaA box is essential for autonomous plasmid replication and lies extremely close to one CtrA binding site (only 3 bp away). Footprinting experiments showed the overlapping binding between these two important proteins (Marczynski, unpublished data). These results suggest that DnaA, as in *E. coli*, may also play a critical role in the initiation of *C. crescentus* chromosomal replication.

5. Chromosome replication assayed by plasmid "minichromosome" replication and in the whole chromosome context

The simplest assay strategy employs autonomously replicating plasmids driven by a cloned replication origin. Several bacterial origins of replication, such as those of *E. coli*, *B. subtilis*, *Pseudomonas putia*, had been isolated and their minimal sequence

Figure 4. Initiation of chromosome replication during the *C. crescentus* cell cycle negatively correlates with CtrA protein abundance. The *C. crescentus* cell division cycle as described in Figure 2. The graphs present the analysis of synchronized cells assayed simultaneously for CtrA protein abundance and the percentage of chromosomes replicated. Figure was taken from (Quon et al. 1998).

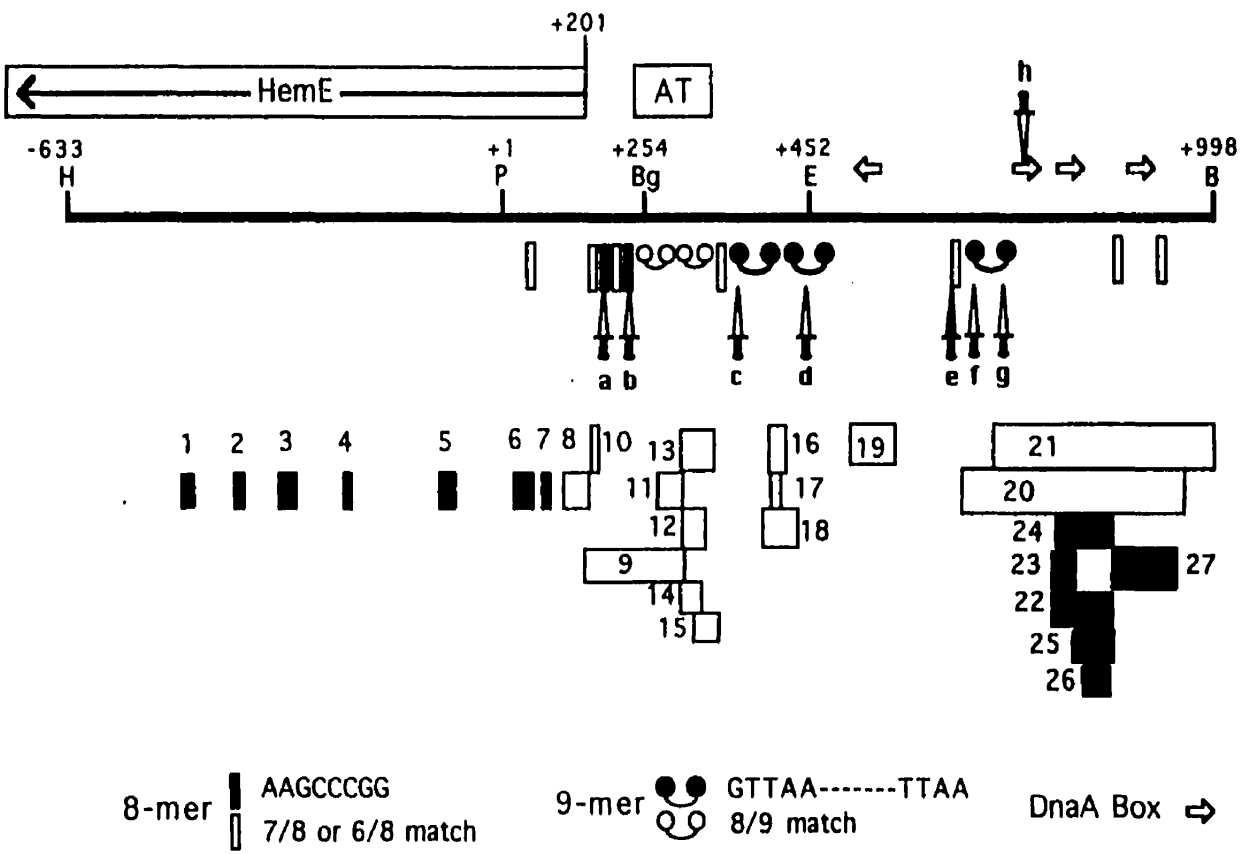

Sw
St
Sw
(rep-)
St
(rep+)
St
(rep+)
relative levels of CtrA (•)
% DNA hemimethylated (▫)
DNA replication
CtrA level
1.0
0.8
0.6
0.4
0.2
0.0
100
80
60
40
20
0.0
0
20
40
60
80
100
time in cell cycle (min)

requirements for replication have been defined by autonomous plasmid replication. *E. coli oriC*-plasmid replication coincides with the initiation of chromosomal DNA synthesis. Likewise, *C. crescentus Cori*-plasmids replicate primarily during the swarmer to stalked cell transition period (Marczynski and Shapiro 1992). Therefore, both of these studies argue that replication control elements are contained within relatively small cloned origin sequences. At present, nearly all of our information about bacterial replication origins is based on plasmid minichromosome assays. However, the minichromosome may not contain all of the cis-acting DNA elements required to precisely regulate chromosome replication. A very limited number of studies have addressed this problem (Asai et al. 1998). A unique example is the mutagenesis of the *E. coli* replication origin. The *E. coli* DnaA box R4 was found to be dispensable on the chromosome although it is clearly essential for plasmid minichromosome replication (Bates et al. 1995). Similar observations have been reported for the *E. coli gidA, mioC* genes and their transcription promoters (discussed above). Although they influence plasmid replication, these genes and their promoters can be deleted from the *E. coli* chromosome without causing a detectable phenotype (Bates et al. 1997).

These considerations motivated my experiments that employed homologous recombination to place *Cori* mutations within the natural context of the entire chromosome. Mutations that defined essential *Cori*-plasmid replication sequences are shown in Fig. 5. I used a subset of these mutations to see if they were also essential in the chromosome context. I will present data that demonstrates that some DNA sequences are

only essential for *Cori*-plasmid replication, while others are absolutely essential for replicating the entire chromosome.

Figure 5. Mutations defining the *C. crescentus* replication origin (*Cori*). 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 in (Marczynski et al. 1995). Closed boxes with numbers indicating a particular deletion or insertion represent 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) *EcoR*I; and (B) *BamH*I. Figure was taken from (Marczynski et al. 1995).


+201
HemE
AT
h
-633
H
+1
P
+254
Bg
+452
E
+998
B
a b
c
d
e f g
1 2 3 4 5 6 7 8 10 13 11 12 9 14 15 16 17 18 19 21 20 24 23 27 22 25 26
8-mer
AAGCCCGG
7/8 or 6/8 match
9-mer
GTTAA-------TTAA
8/9 match
DnaA Box

Research Objectives

Towards defining the DNA sequences required for regulated chromosome replication in *C. crescentus*, my projects focused on testing the consequences of mutated CtrA binding sites inside *Cori* on plasmid replication, and comparing the effects of *Cori*-mutations in both plasmid and whole chromosome contexts.

Methods and Materials

1. Bacterial strains, plasmids and growth media

The strains and plasmids used are listed in Table 1. All *Caulobacter crescentus* strains were derivatives of CB15N. CB15N was used for introducing mutations in chromosome origin to produce a series of chromosome mutants: CM1125 to CM1073. CB15Nbla- was employed in the autonomous plasmid replication assay. *C. crescentus* strains were routinely grown in PYE or M2G at 30°C when cells were synchronized by the Ludox density gradient. PYE contained (per liter) 2 g of Bacto peptone, 1 g of Bacto yeast extract, and 0.2 g of $MgCl_2$, was used with or without agar, and supplemented with appropriate antibiotics: 5 μg/ml ampicillin, 20 μg/ml kanamycin, 1.5 μg/ml tetracycline, 2.5 μg/ml streptomycin.

E. coli strains (DH10B, DH5α and S17-1) were used for construction and transformation and DNA manipulations. *E. coli* strains were grown in LB media at 37°C. LB media contained (per liter) 10 g of Bacto tryptone, 5 g of Bacto yeast extract and 5 g of NaCl used with or without agar, and supplemented with appropriate antibiotics: 100 μg/ml ampicillin, 50 μg/ml kanamycin, 15 μg/ml tetracycline, 2.5 μg/ml streptomycin.

The detailed plasmid description can be found in Table 1. pKS series were used for generating different *Cori*-mutations and studying the autonomously replication in *C. crescentus*. Different *Cori* mutations were also cloned into pACYC177 vector at *BamH*I site or *Spe*I- *Hind*III site. Then *Cori*-driven pACYC177 plasmids were introduced into

Table 1. Strains and plasmids

Strain or plasmid	Description	Source or reference
Strains:		
C. crescentus		
CB15N	wild-type, Amp^R, Kan^S	G.M.*
CB15NΔbla$^-$	wild-type, Amp^S, Kan^R	G.M.
CM1125	CB15NΔAlf in *Cori*	This study
CM1127	CB15NΔAT in *Cori*	This study
CMR4	CB15N changed CtrA binding site d in *Cori*	This study
CM1655	CB15N deleted CtrA binding site c from 386 to 419 in *Cori*	This study
CM1656	CB15N reverse duplication at CtrA binding site c from 397 to 390 in *Cori*	This study
CM1073	CB15N duplication on weaker promoter from 217 to 240 in *Cori*	This study
E. coli		
DH10B	Cloning strain	Stratagene
DH5α	Cloning strain	Stratagene
S17-1	For plasmid mobilization	Simon et al. 1983
LE392	rec+	M. DuBow
Plasmids:		
General purpose vectors		
pBluescript II	Amp, ColE, KS polylinker	Stratagene
pGM946	Wild-type *Cori*-plasmid, 1.7 kb *BamH*I-*Hind*III *Cori* in pBluescript II	G.M. (1995)
pGM1109	kan^R, $SacB^S$, polylinker	G.M.
pGM1112	kan^R, $SacB^S$, *BamH*I site	G.M.
pNTP138S	kan^R, $SacB^S$, polylinker	G.M.
pRSZ6	PRK290 based *gusA*, *lacZ* reporter, Tc	M.R.K. Alley
Subclones of *Cori* region		
pBluescript KS series		
pKS1645	deletion: 9/B/59 , without Ω in *Cori*	G.M.
pKS1647	deletion: 138/B/197, without Ω in *Cori*	G.M.
pKS1648	deletion: 186/B/321, without Ω in *Cori*	G.M.
pKS1649	deletion: 203/B/211, without Ω in *Cori*	G.M.
pKS1650	deletion: 271/B/299, without Ω in *Cori*	G.M.
pKS1652	deletion: 298/B/331, without Ω in *Cori*	G.M.
pKS1654	deletion: 327/B/352, without Ω in *Cori*	G.M.
pKS1655	deletion: 386/B/419, without Ω in *Cori*	G.M.
pKS1656	duplication: 397/B/390, without Ω in *Cori*	G.M.
pKS1658	deletion: 512/B/576, without Ω in *Cori*	G.M.
pKS1662	deletion: 715/B/750, without Ω in *Cori*	G.M.
pKS1667	duplication: 267/B/264, without Ω in *Cori*	G.M.
pKSmut1	Wild-type *Cori*	This study
pKSmutd	4 bp change at left side of CtrA binding site d in *Cori*	This study

----------continued

Strains or plasmids	Description	Source or reference
pKSmut3	10 bp change at left franking sequence of CtrA binding site d in *Cori*	This study
pKSmut4	10 bp change at left franking sequence of CtrA binding site d in *Cori* (R2)	This study
pKSmut5	4 bp change at right side of CtrA binding site d in *Cori* (R4)	This study
pKSmut6	double mutation combining mut3 and 1276	This study
pKSmut7	deletion from 258 to 282 in *Cori*, (MAT1)	This study
pKSmut8	deletion from 203 to 244 in *Cori*, no Pw	This study
pKSmut9	2 bp change at CtrA binding site a and b (MAT2)	This study
pKSmut10	1 bp change at CtrA binding site a (AT1-2)	This study
pKSmut11	1 bp change at CtrA binding site a (AT1-5)	This study
pKSmut12	Δ 2 *Hpa*I site in *Cori*: 393 to 665	This study
pKSmut13	Δ 2 *Nco*I site in *Cori* using pKSmut5	This study
pKS1270	3 bp change from 428 to 430, obtaining *Eco*47 site	G.M.
pKS1276	4 bp change at left CtrA binding site e	G.M.
pKS1073	duplication: 217/H/240	G.M.
pKS1076	deletion: 217/H/240	G.M.
pACYC177 series		
pGM1125	deletion: 265 to 286	G.M.
pGM1127	deletion: 269 to 272	G.M.
pACYC177-1645	deletion: 9/B/59	This study
pACYC177-1647	deletion: 138/B/197	This study
pACYC177-1648	deletion: 186/B/321	This study
pACYC177-1649	deletion: 203/B/211	This study
pACYC177-1650	deletion: 271/B/299	This study
pACYC177-1652	deletion: 298/B/33	This study
pACYC177-1654	deletion: 327/B/352	This study
pACYC177-1655	deletion: 386/B/419	This study
pACYC177-1656	duplication: 397/B/390	This study
pACYC177-1658	deletion: 512/B/576	This study
pACYC177-1662	deletion: 715/B/750	This study
pACYC177-1667	duplication: 267/B/264	This study
pACYC177-mut5	4 bp change at right side of CtrA binding site d in *Cori* (R4)	This study
pACYC177-1276	4 bp change at left of CtrA binding site e	This study
pACYC177-1073	duplication: 217/H/240	This study
pACYC177-1076	deletion: 217/H/240	This study
pNTPS138 series		
pNTPS138-1645	deletion: 9/B/59	This study
pNTPS138-1647	deletion: 138/B/197	This study
pNTPS138-1648	deletion: 186/B/321	This study
pNTPS138-1649	deletion: 203/B/211	This study
pNTPS138-1650	deletion: 271/B/299	This study
pNTPS138-1652	deletion: 298/B/33	This study
pNTPS138-1654	deletion: 327/B/352	This study

----------continued

strains or plasmids	Description	Source or reference
pNTPS138-1655	deletion: 386/B/419	This study
pNTPS138-1656	duplication: 397/B/390	This study
pNTPS138-1658	deletion: 512/B/576	This study
pNTPS138-1662	deletion: 715/B/750	This study
pNTPS138-1667	duplication: 267/B/264	This study
pNTPS138-mut5	4 bp change at right side of CtrA binding site d in *Cori* (R4)	This study
pNTPS138-1276	4 bp change at left of CtrA binding site e	This study
Transcriptional fusion		
pRSZ6*lacZ*/mutated *Cori*/*guA*:		
pRSZ6-mut1	constructed from pKSmut1	This study
pRSZ6-mutd	constructed from pKSmut2	This study
pRSZ6-mut3	constructed from pKSmut3	This study
pRSZ6-mut4	constructed from pKSmut4	This study
pRSZ6-mut5	constructed from pKSmut5	This study
pRSZ6-mut6	constructed from pKSmut6	This study
pRSZ6-mut7	constructed from pKSmut7	This study
p RSZ6-mut8	constructed from pKSmut8	This study
pRSZ6-mut9	constructed from pKSmut9	This study
pRSZ6-1276	constructed from pKS1276	This study
pRSZ6-1645	constructed from pKS1645	This study
pRSZ6-1647	constructed from pKS1647	This study
pRSZ6-1648	constructed from pKS1648	This study
pRSZ6-1649	constructed from pKS1649	This study
pRSZ6-1650	constructed from pKS1650	This study
pRSZ6-1652	constructed from pKS1652	This study
pRSZ6-1654	constructed from pKS1654	This study
pRSZ6-1655	constructed from pKS1655	This study
pRSZ6-1656	constructed from pKS1656	This study
pRSZ6-1658	constructed from pKS1658	This study
pRSZ6-1662	constructed from pKS1662	This study
pRSZ6-1667	constructed from pKS1667	This study
pRSZ6-1073	constructed from pKS1073	This study
pRSZ6-1076	constructed from PKS1076	This study

B: *BamH*I; H: *Hind*III * Marczynski'lab strain collection

C. crescentus by electroporation. Consequently, mutations were mobilized onto the chromosome by the homologous recombination strategy described below. pGM964, pNTP138S were used to provide convenient restriction enzyme sites. Wild-type and mutated *Cori* pRSZ6*lacZ*/*gusA* plasmid series were constructed by homologous recombination with plasmid pRSZ6-s*ac*B-*Kan* in *E. coli* LE392.

2. Plasmid and chromosome DNA preparation

Plasmid DNA was extracted by the standard alkaline lysis method. To prepare total cellular DNA, 2 ml of overnight PYE broth culture was collected. Total DNA (chromosome and plasmid) was prepared by treatment of cells with 50 mM Tris-HCl, 50 mM EDTA, 0.1% Triton X-100, pH 8.0. Then proteinase K 0.5 mg/ml 55°C overnight incubation, followed by two phenol and one chloroform extraction and ethanol precipitation.

3. Site-directed mutagenesis of *Cori*

The oligonucleiotides used for mutagenesis were designed to introduce a variety of base pair changes to the interesting regions of *Cori*. For cloning purposes, the convenient restriction sites were also designed into the oligonucleotides. These oligonucleotides were phosphorylated by kinase and annealed. DNA polymerization, restriction enzymes digestion and ligation were carried out concomitantly in the presence of all four

deoxynucleosides triphosphates (dNTPs), T4 DNA polymerase, restriction enzymes and T4 DNA ligase. After the DNA fragments containing the wild-type and mutated *Cori* region were inserted into pBluescrip II derivative pGM1270, the resulting plasmids were transformed into *E. coli* strain DH10B and the mutated clones were identified by DNA sequence analysis.

Convenient restriction enzyme sites inside *Cori* were also used to delete the targeted regions. *Cori*-plasmid fragments were isolated by low melting temperature agarose gels, followed by ligation, transformation and DNA sequencing.

4. *Cori*-plasmid "minichromosome" replication assay

The autonomous *Cori*-plasmids containing wt or mutated *Cori* were introduced into CB15NΔbla cell by electroporation. Total DNA (chromosome and plasmid) were prepared from the culture of transformed clones selected on PYE Ampicillin plates, and then total DNA were digested by appropriated restriction. Following Southern blot analysis, the ability of these plasmids to replicate in *C. crescentus* was determined by quantitation of plasmid DNA contents; the relative plasmid copy numbers were estimated by calculating the ratio of plasmid and chromosome band densities.

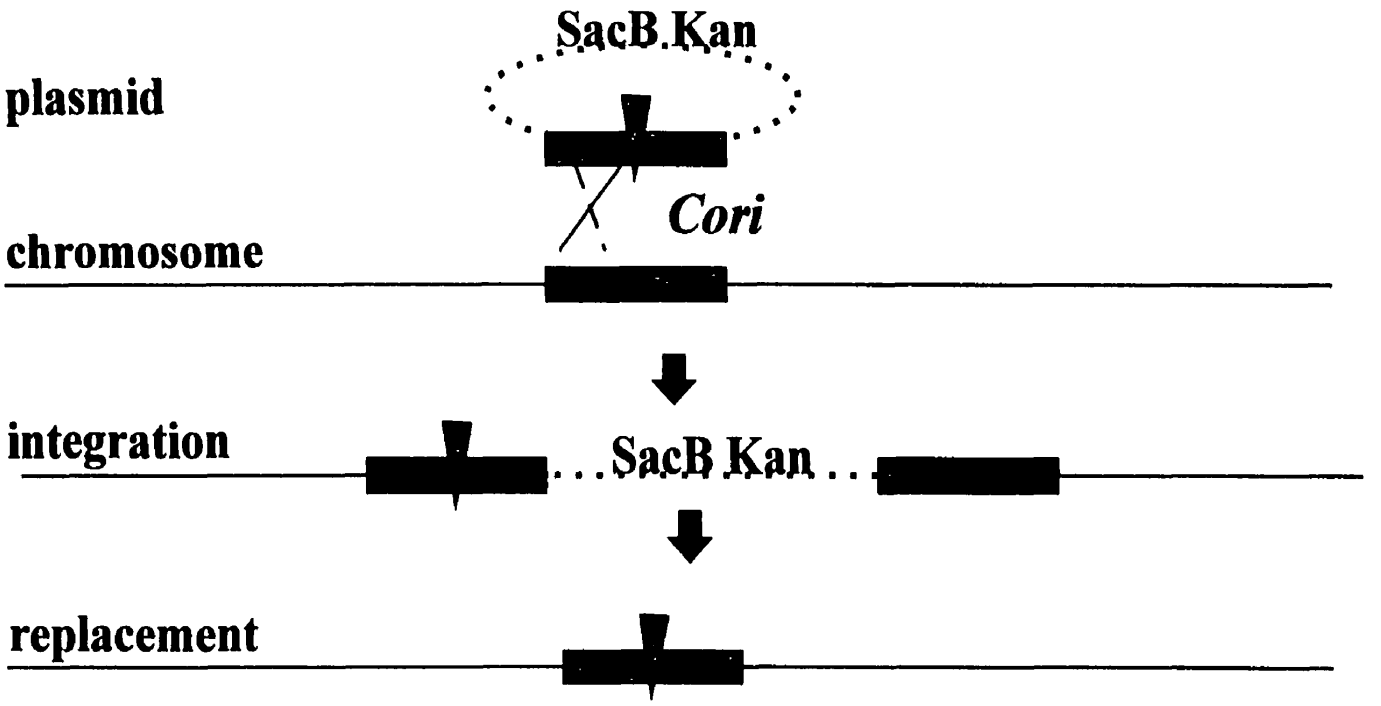
5. Mutagenesis of the *C. crescentus* chromosomal origin

The principle was shown on Fig. 6. A series of mutated-*Cori* were cloned into the *C. crescentuse*-non-replicating plasmid pACYC177-*sac*B, and introduced to CB15N competent cells by electroporation. The integrated clones were selected (and confirmed by Southern blot) on PYE Kan plates and cultured in PYE broth at 30°C overnight. The cultures were diluted 10^{-3} times and spread on 3% sucrose PYE, 3% sucrose hemin PYE or 3% sucrose hemin Tc PYE, as necessary. The pools of all clones on 3% sucrose plates were washed and examined by Southern blot to detect the possible mutants or to get statistically significant negative results. Between 5-15 individual clones on 3% sucrose plates were later individually examined to identify a chromosomal mutant, if the mutant was detectable by the Southern blot analysis of pooled colonies.

6. Competitive growth or "population shift" assay

Wild-type *C. crescentus* and chromosomal origin mutants were cultured separately in PYE broth at 30°C overnight. After mixing equal amounts of wild-type and *Cori* mutant cultures, 20 μl was taken and subcultured in 2 ml PYE broth at 30°C overnight. During the 4 times of repeated subcultures , the cells were collected for total DNA preparation. The Southern blot assays and quantitation of band densities were performed. The competitive ability of mutants to wild-type *C. crescentus* was determined by comparing the ratio of mutant to wild-type DNA.

Figure 6. The principle of the chromosomal mutation replacement assay. The thick black lines indicate the *Cori* homologous DNA. The black triangles indicate the location of mutations inside *Cori*. The pACYC177 plasmids harboring mutated *Cori* were introduced to wild-type *Caulobacter* by electroporation. The transformed clones and potential integrated clones were selected on PYE Kan plates. The clones with the second recombination (replacement) were selected on 3 % sucrose plates.


SacB.Kan
plasmid
Cori
chromosome
integration
...SacB.Kan...
replacement

7. Southern blot assay for rare recombination events

Total DNA was digested with the appropriate restriction enzymes as specified (New England Biolabs or Biotech) 37°C overnight. The fragments were separated on 0.8% agarose gel, and transferred to Hybond H+ nylon membrane (Amersham) under alkaline conditions, then UV cross-linked, and probed with the *Cori* containing 680 bp *BamH*I-*Pst*I fragment that had been labeled with α-^{32}P-dCTP by random priming. After hybridization, the membrane was washed at 65°C with 2XSSC (0.15 M NaCl plus 0.015 M Sodium Citrate, 0.2 % SDS), 1XSSC and 0.1XSSC. Then the membrane was covered with Saran wrap and exposed to Kodak film or exposed to an imaging plate and the intensities of bands were quantified by a PhosphorImager (Molecular Dynamics).

8. *LacZ/gusA* transcriptional reporter assay

Wild-type or mutated *Cori* DNA were constructed into the pRSZ6*lacZ/gusA* test plasmid by homologous recombination using *E. coli* strain harboring pRSZ6*lacZ/gusA* test plasmid. The *Cori* DNA sequence is located between the two promoterless reporter genes. Then, the constructed pRSZ6 test plasmids were introduced into the wild-type *C. crescentus* strain by bacterial conjugation. The *lacZ* and *gus*A activity (millar units of each culture) were measured as described (Marcyznski et al. 1995).

9. Other methods

• Electroporation

8 μl filtered plasmid DNA was added to 0.1 cm cuvette containing 30 μl *C. crescentus* competent cells, mixed well. The cuvette was then inserted in the Electroporator II (Invitrogen, San Diego). Electroporation was performed at 25 mA , 1500 V and 25 watts, the cells were then transformed to 1 ml PYE broth culture for 1.5 hr at 30°C, and spread on PYE Kan plate.

• Sequencing

DNA sequencing was performed by Sanger dideoxynucleotide chain termination method using the T7 sequencing kit (Pharmacia).

• Synchronization of *C. crescentus*

The culture for synchronization was grown in PYE medium, and swarmer cells were isolated by Ludox density gradient centrifugation. Swarmers were released into M2G medium at 30°C. Progression through the cell cycle was monitored microscopically. The cells were collected at each time point. Total DNA were isolated as previously described.

• CtrA protein footprinting

DNase I protection footprinting experiments were performed with a purified His6-CtrA fusion protein as described (Quon et al. 1998).

RESULTS

1. Negative control of minichromosome replication by CtrA binding to site d

To test the effects of CtrA binding on replication, CtrA binding sites were mutated, and the wild-type and mutated *Cori*-plasmids were electroporated into a wild-type *C. crescentus* strain in which *Cori*-plasmid copy number was measured by Southern blot analysis. CtrA binding site d was mutated as shown in Fig. 7A. The 5 base pair change of site d clearly blocks CtrA binding as only the unaltered half site was footprinted (Fig. 7B). Due to this mutation, the plasmid copy number significantly increased about 7 fold (Fig. 7C). In additional, but similar copy number experiments, the copy number increase was approximately 2 to 3-fold (Fig. 8, and other data not shown). Thus, impaired CtrA binding resulted in increased *Cori*-plasmid replication. This suggests that CtrA maintains a repressed state by directly binding to site d.

2. Mutated CtrA binding sites inside *Cori* can stimulate or repress minichromosome replication

If CtrA served only to repress replication, then mutations that weaken binding to all five sites should increase *Cori*-plasmid copy number, as observed for site d in Figure 7. However, data in Figure 8 argues that site d is unique, and suggests that CtrA binding to sites a, b, and e is required to support *Cori*-plasmid replication. As observed before, mutations (base pair changes shown in Table 2) in either the left (L) or right (R) sides of site d elevate *Cori*-plasmid copy number. Interestingly, randomized mutations flanking

Figure 7. 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 described in Materials and Methods. Outside lanes show the same 5'-^{32}P end-labeled DNA fragments cut with *EcoR*I or *Hpa*I. (C) *Cori* plasmids per chromosome, as determined by Southern blot hybridization. *C. crescentus* strain CB15N Δbla was electroplated with pBluescriptII plasmids (Stratagene) bearing otherwise identical wild-type (WT) or mutant (Mut-d) *Cori* DNA between *BamH*I sites at -680 and +998 and analyzed as described in Methods and Materials. Radioactivity was measured with a PhosphorImager (Molecular Dynamics), and the ratio between *Cori* plasmid and chromosome band intensities is shown. Figure was taken from (Quon et al. 1998).

A

ACAGG Mut-d

| | | | | *EcoRI*

...CTTGAACACCTTAATGAATTCTTAACGTCCT...

d

B

Mut-d Wt

-+++++- -+++++- CtrA

EcoRI

d

c

HpaI

C

Wt Mut-d

Cori-plasmid

Chromosome

Ratio 1.9 13.0 6.8x

Table 2. The effect of *Cori*-mutation on plasmid replication

	Mutants	*Cori* position	Ratio of P/C	Rep.
1.	mut-d	d-9mer (L) CTTAA→ACAGG	3.63	↑
2.	mut-3	Flanking sequence of d-9mer GCCTTGAACAC→CGATACTGCCA	0.72	↓
3.	R2	Flanking sequence of d-9mer GCCTTGAACAC→CTTAgCCTGTA	3.4	↑
4.	R4	d-9mer (r). NcoI. TTAAC→CATGG	3	↑
5.	AT1-2	a-9mer	< 1	↓
6.	AT1-5	a-9mer	0.5	↓
7.	MAT1	258-282 deletion 1/2a.b-9mer	-	-
8.	MAT2	1 bp change in a-9mer and b-9mer	0.16	↓
9.	1270 HpaI	deletion between two HpaI sites	-	-
10.	R4NcoI	deletion between two NcoI sites	-	-

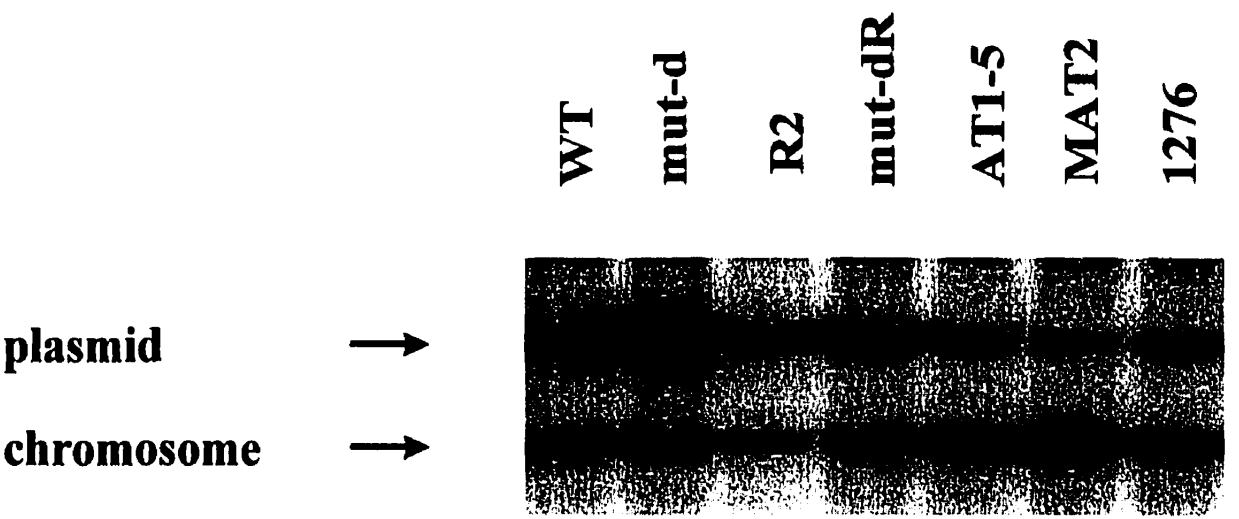
the consensus sequence to site-d (R2) also increased *Cori*-plasmid copy number. Whether R2 affects CtrA binding directly or through an unknown helper protein remains to be determined. However, base pair changes directed to the a, b, and e CtrA binding sites lowered *Cori*-plasmid copy number (Figure 8). Interestingly, mutations causing the lowest *Cori*-plasmid copy numbers, MAT2 and 1276 also block transcription from *Cori*.

3. CtrA may control replication by regulating transcription inside *Cori*

The *C. crescentus* replication origin (*Cori*) is characterized by overlapping transcription elements which harbor CtrA binding sites. For example, CtrA binding sites a and b overlap the *hemE* gene strong promoter implicated in cell cycle replication control (Marczynski et al. 1995). To further examine the role of transcription in chromosome replication, the wild-type *Cori*-plasmid and mutated *Cori*-plasmids harboring various deletions and base pair changes at the CtrA binding sites, as well as other sequences inside *Cori*, were constructed between two opposite and promoterless reporter genes: *lacZ* and *gusA* in pRSZ6 plasmids, as described in Methods and Materials. Transcription from *Cori* was monitored by measuring the expression of *lacZ* and *gusA* reporter genes. The results are shown in Fig. 9.

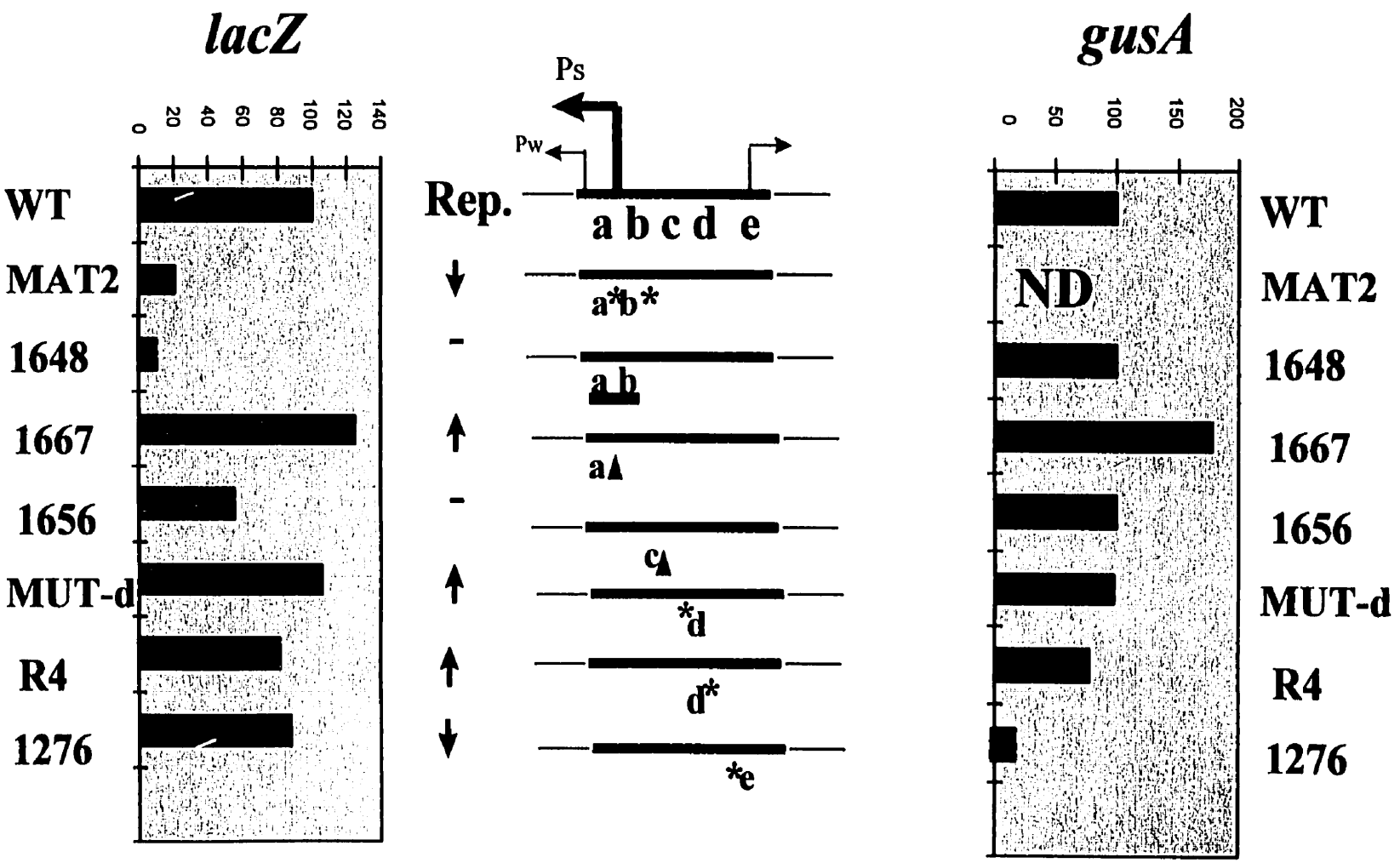
Nearly all leftward transcription can be accounted by mutations MAT2 and 1648 affecting CtrA sites a and b over the strong promoter. Likewise nearly all rightward transcription can be accounted by mutation 1276 that changes CtrA site e. Therefore, nearly all transcription from *Cori* results from promoters regulated by CtrA. Point mutations that

Figure 8. Both higher and lower plasmid replication resulted from altered CtrA binding sites inside *Cori*. Copy number, expressed as *Cori*-plasmids per chromosome, was determined as described in Figure 7C. These mutations are all base pair changes described in Table 2.



	WT	mut-d	R2	mut-dR	AT1-5	MAT2	1276
plasmid / chromosome	1.7	2.8	3.0	2.8	0.9	0.1	0.3
Ratio changed:	—	↑	↑	↑	↓	↓	↓
CtrA binding sites :		d (L)		d (R)	a	a b	e

Figure 9. Transcription correlated with replication and CtrA binding site mutations inside *Cori*. Wild-type *Cori* and *Cori* bearing altered CtrA binding sites were constructed between two opposite *lacZ* and *gusA* reporter genes in the pRSZ6*lacZ*/*gusA* test plasmid as described in Methods and Materials. These plasmids were introduced into wild-type *C. crescentus* by bacterial conjungation , *LacZ* and *gusA* activity was measured and presented here as % of the wt levels. Plasmid replication assay as described in Figure 7 and 8.


lacZ
0
20
40
60
80
100
120
140
WT
MAT2
1648
1667
1656
MUT-d
R4
1276
Rep.
Ps
Pw
a b c d e
a*b*
a b
a
c
*d
d*
*e
gusA
0
50
100
150
200
ND
WT
MAT2
1648
1667
1656
MUT-d
R4
1276

lower transcription, MAT2 1276, also lower *Cori*-plasmid copy number. This contrasts with, for example mutations in site d, Mut-d and R4, that do not affect transcription, but increase the copy number.

4. *Cori*-plasmid replication requirements are independent of the plasmid vector

To control for the effects of vector sequences on *Cori*-plasmid replication, all 17 *Cori*-mutations described in Table 1 and Table 3 were constructed in three different plasmid vectors: pKS, pNTPS138 and pACYC177-*sac*B. These plasmids were electroporated into wild-type *C. crescentus* strain CB15N, and replication was scored by colony formation on selective PYE plates. All *Cori* mutaions scored as Rep (+) produced vigorous growth on PYE Amp (20) for pKS vector, or on PYE Km (50) for the pNTPS138 and pACYC177-*sac*B vectors. Likewise, all *Cori* mutations scored as Rep (-) produced no growth, with the exception of a few (approximately one in 10^7) large colonies due to *Cori*-plasmid integration into the chromosome (see below). *Cori* mutations that produced a lower than wt copy number (e.g. Figure 8) showed reduced growth and colony size compared to the wild-type *Cori*-plasmid control. Likewise, *Cori*-plasmid mut-d constructs, produced more vigorous growth and larger colonies indicative of their higher copy numbers.

5. Systematic mutagenesis of *Cori* suggests that *Cori* behaves differently in a plasmid versus whole chromosome context

To reveal the full requirements of *Cori* on the entire chromosome, 17 mutations throughout the *Cori* region (described in Table 3) were introduced into the chromosome as

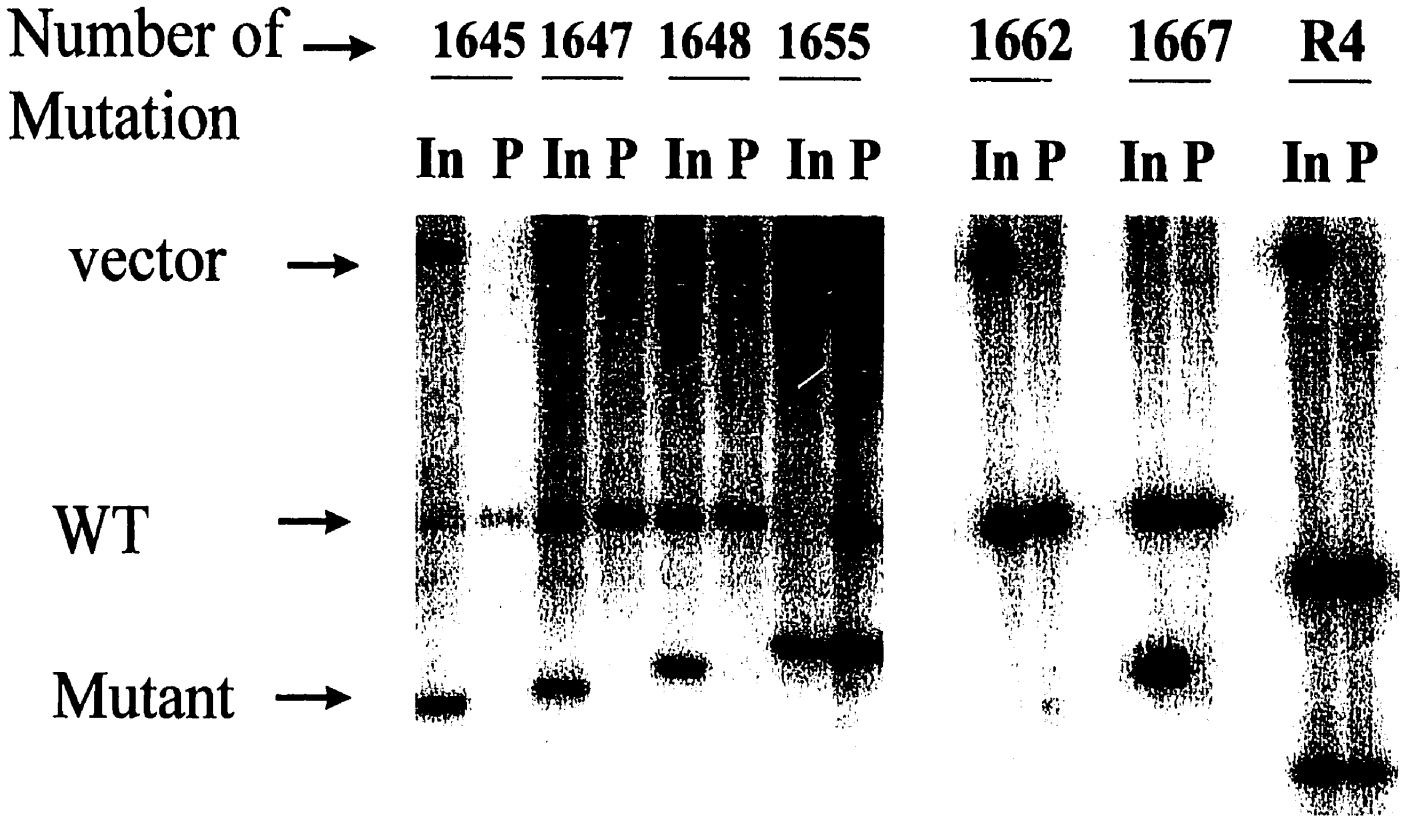
Table 3. Chromosome replication with Cori-mutations

mutation		*Cori* position	Plasmid replication	Chromosome replication
1	1073	240/H/217 duplication	-	+
2	1076	217/H/240 deletion	-	-
3.	1125	a, b-9mer, ▲ Alf II	-	+
4.	1127	a, b-9mer, ▲ AT	+	+
5.	1645	9/B/59	+	-
6.	1647	138/B/197	-	-
7.	1648	186/B/321	-	-
8.	1649	203/B/211	-	-
9.	1650	271/B/299	-	-
10.	1652	298/B/331	-	-
11.	1654	327/B/352	-	-
12.	1655	386/B/419	-	+
13.	1656	397/B/390	-	+
14.	1658	512/B/576	-	-
15.	1662	715/B/750	+	-
16.	1667	267/B/264duplication	+	-
17.	R4	d-9mer (right),NcoI site	+	+

described in Methods and Materials. Our strategy for homologous recombination and allelic replacement is summarized in Figure 6. The first step is a homologous recombination between plasmid and chromosome *Cori* DNA. This produced a stable duplication of the wt *Cori* and the mutated *Cori* with intervening plasmid vector sequences (Figure 6) for each of the 17 mutations tested. Examples of integrated *Cori*-plasmids are shown by the Southern-blot analysis in Figure 10. Subsequent counter selection on sucrose plates kills these cells unless they remove the vector DNA by homologous recombination. Only between one in 10^3 to 10^4 cells survive this counter-selection step. A successful allelic replacement, i.e. transfer of the *Cori* mutation to the chromosome, is scored by Southern-blot and revealed by lose of vector DNA and the gaining of a novel restriction site (*BamH*I, *Hind*III or *Nco*I, see Table 3) inside *Cori*.

Since chromosome mutations are potentially lethal, we adopted a strategy that screens many (at least 100) separate recombination events. Following sucrose counter-selection, at least 100 isolated colonies were pooled, and their DNA prepared and subjected to Southern-blot analysis. Figure 10 compares integrated (In) versus pooled (P) DNA prepared from selected *Cori* mutations. For example, mutations 1645, 1647, 1648 are present as integrated DNA, but completely absent in the pooled DNA (lower bands in Figure 10). Note that the vector DNA is also absent in the pooled DNA, indicating a successful second homologous recombination. Since we are confident that we can detect mutant DNA present at a frequency of about 1 %, the absence of mutant DNA in our pools argues that these deletions are lethal on the chromosome. This contrasts with for example

Figure 10. Southern blot analysis to test the presence or absence of chromosome mutants following 3% sucrose counter-selection. The indicated *Cori* mutants are described in Table 3. "In", represents total chromosome DNA containing the integrated *Cori*-plasmid before sucrose counter-selection. "P", represents DNA from pooled colonies following sucrose counter-selection. Its Total DNA was prepared, digested with appropriate restriction enzymes and subjected to Southern-blot analysis as described in Material and Methods. The 6 kb vector band and the 1.6 kb wt *Cori* are shown. The bands lower than 1.6 kb represent mutated *Cori* marked with novel restriction sites.


Number of Mutation
1645
1647
1648
1655
1662
1667
R4
In P In P In P In P In P In P In P
vector
WT
Mutant

mutations 1655 and R4 in Figure 10, where both mutant and wt *Cori* DNA is readily detected in the pooled colony DNA preparations.

We score a mutation as chromosome rep (+) if it is readily detected in a pooled DNA preparation, and if we can obtain cloned cell cultures that only contain mutant *Cori* DNA (for example, see Figure 13 below). We score a mutation as chromosome rep (-) if it is not detected in pooled DNA preparation and if we control for effects on the expression of flanking genes. This was done by genetic complementation with a cosmid vector containing approximately 12 kb of *Cori* flanking genomic DNA (pGM2466). To further control for the lose of *hemE* and other heme biosynthetic genes, hemin was added to the sucrose selection plates.

Comparing plasmid and chromosome replication, four classes of expected and unexpected mutations were observed: (i) 2 mutations that allowed replication in both plasmid and chromosome contexts (as expected); (ii) 8 mutations that block replication in both plasmid and chromosome contexts (as expected); (iii) Surprisingly, 4 mutations that block replication in plasmid but not the chromosome context. These mutations define a replication system that is essential only on the plasmid; (iv) 3 mutations that allowed replication in the plasmid but not the chromosome context. Therefore, 7 mutations were found to have different effects on plasmid and chromosome replication. Most importantly, 11 mutations were found that block chromosome replication, and these presumably define the most essential replication sequences. These results are summarized in Table 3, Table 4 and Figure 11.

Figure 11. Schematic representation of plasmid and whole chromosome replication with different *Cori* mutations. Top line shows *Cori* and its three promoters, part of *hemE* gene and five CtrA binding sites. Below: the approximate location of each mutation inside *Cori*. Top box of each pair represents plasmid replication, bottom box represents chromosome replication, black box represents replication while blank box represents abolished replication.

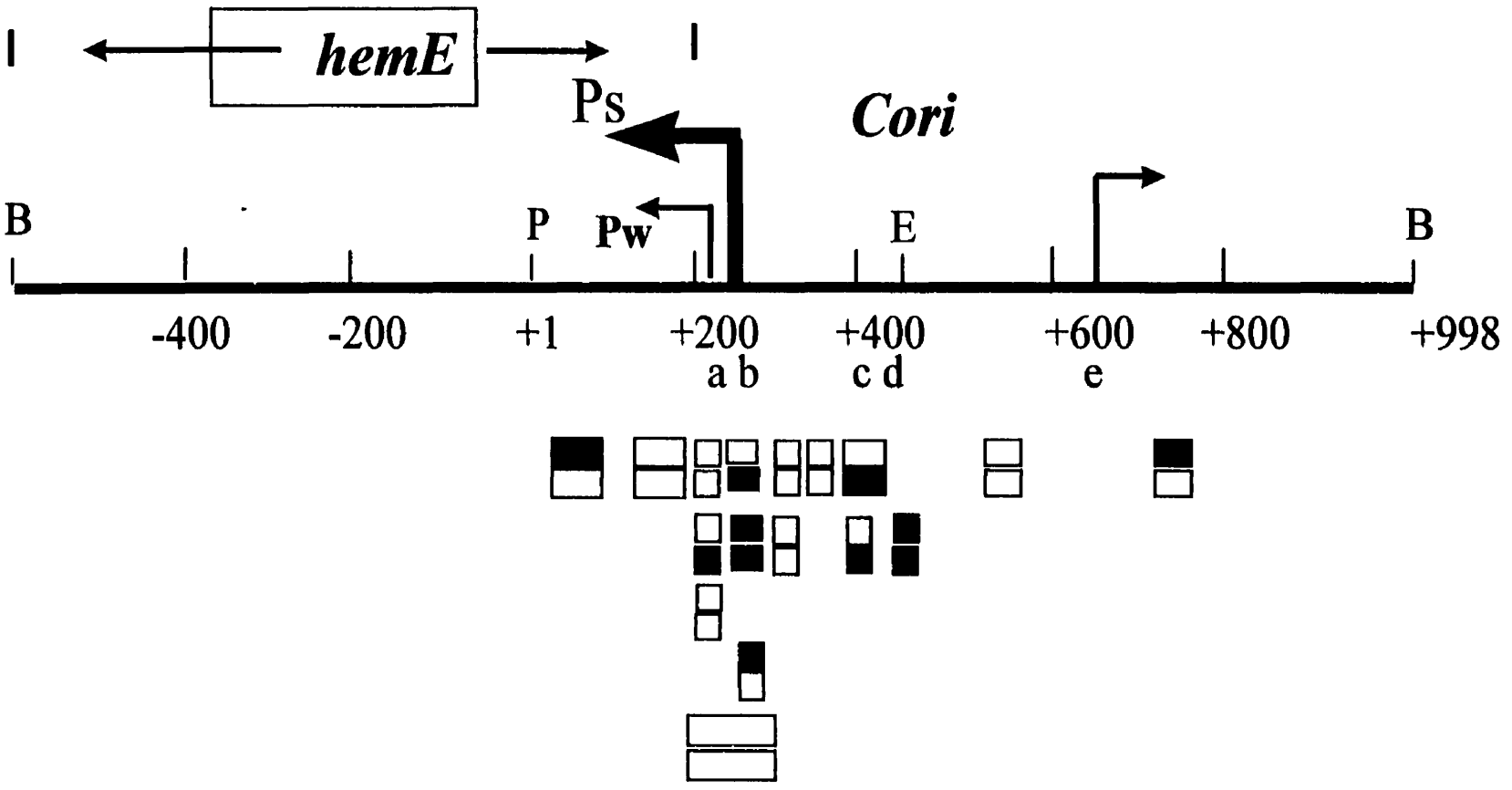

hemE
Ps
Cori
B
P
Pw
E
B
-400
-200
+1
+200
+400
+600
+800
+998
a b
c d
e

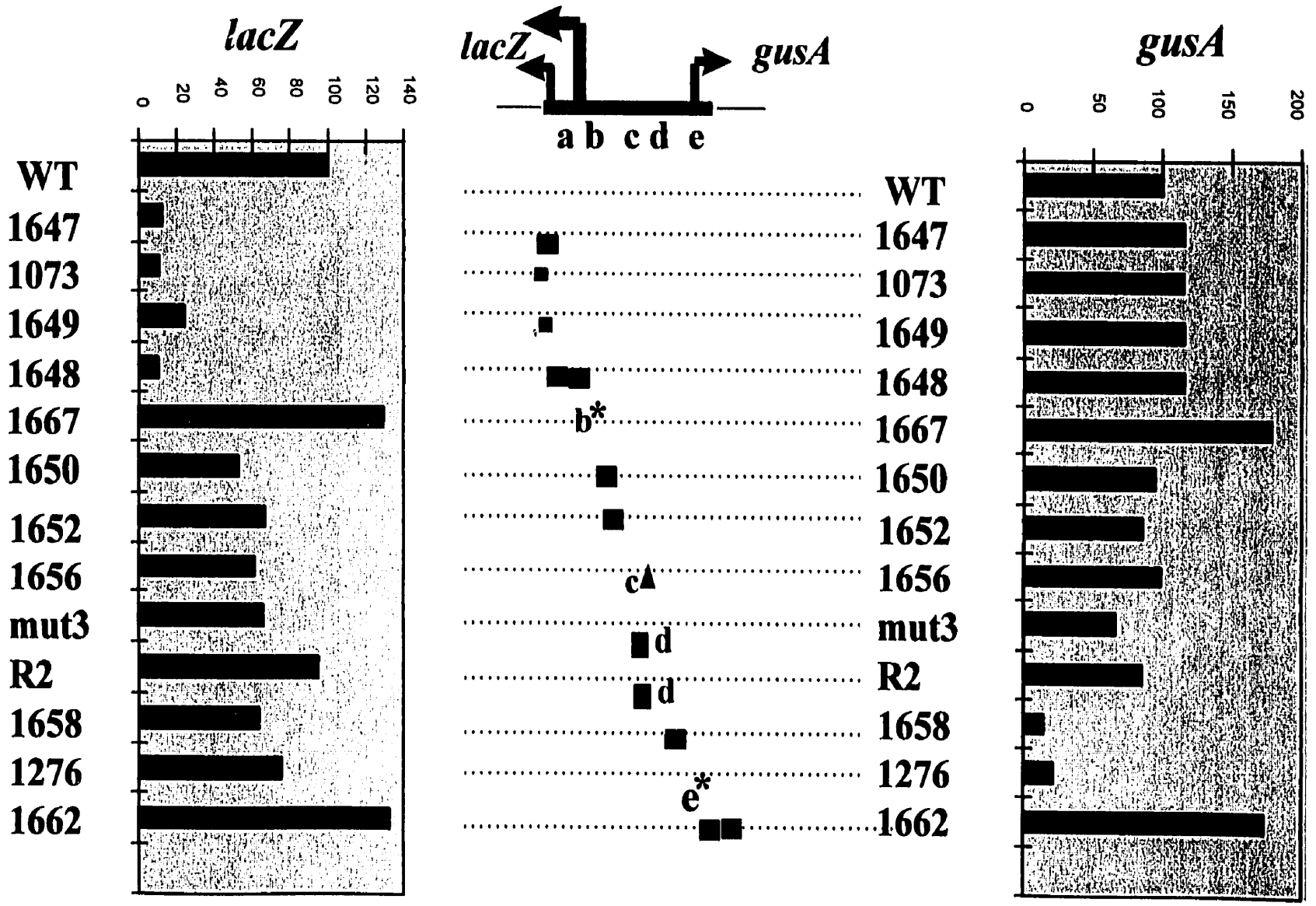
Table 4: The different effects on plasmid and chromosome replication

Class	plasmid replication	+	+	-	-	
	chrom. replication	+	-	-	+	
						total
Number		2	3	8	4	17

6. Control transcription experiments to separate mutant effects on replication from mutant effects on genetic expression

As was argued above, a mutation in the chromosome may be lethal either because it abolishes replication or because it blocks the expression of an essential gene. To help rule out genetic lethality, we expanded the transcription studies described in Figure 9 to include all of the chromosome rep (-) mutants that we discovered by our integration replacement analysis. Figure 12 presents this transcription data, and argues that for most chromosomal rep (-) mutations, transcription from *Cori* into flanking genomic DNA is not significantly effected. The major exceptions are mutations 1073, 1647, 1648, and 1649, that delete inside the *hemE* 5' coding and the *hemE* weak promoter. Although these transcription levels are lower than wild-type, they are nonetheless, significant, and should support cell viability even if the downstream genes encode essential biosynthetic genes. The inclusion of hemin in our selection protocal (discussed above) would also eliminate the requirement for transcribing the *hemE*, and other heme biosynthetic genes. Deletion mutation 1658 also significantly decreases rightward transcription, as does the base pair change mutation 1276 (Figure 12). However, while 1658 is lethal on the chromosome, and both plasmid and chromosome replication is blocked (Table 3), mutation 1276 allows both plasmid and chromosome replication (Marczynski., unpublished), although the plasmid copy number is significantly lower than wild-type (Figure 8). Therefore low rightward transcription is not in itself lethal. Also, as noted above, essential rightward genes would be complemented by plasmid pGM2466 containing 12 kb of rightward genomic DNA.

Figure 12. The effects of *Cori* mutations on transcription inside *Cori*. The wild type and a series of mutated *Cori* were constructed between the *lacZ*/*gusA* transcription reporter genes as described in Materials and Methods. See also Figure 9 legend.


lacZ
0
20
40
60
80
100
120
140
WT
1647
1073
1649
1648
1667
1650
1652
1656
mut3
R2
1658
1276
1662
lacZ
gusA
a b c d e
b*
c
d
d
e*
WT
1647
1073
1649
1648
1667
1650
1652
1656
mut3
R2
1658
1276
1662
gusA
0
50
100
150
200

7. Chromosome *Cori* mutants that fail to support plasmid replication, resemble wild type cells in growth and morphology

We detected four *Cori* mutations that, although they blocked autonomous plasmid replication, could nonetheless be placed on the chromosome by homologous recombination (Table 3 and Figure 11 presented summaries). These mutations, 1125, 1655, 1656, and 1073 were first identified in pooled DNA preparations, and examples of this analysis were shown in Figure 11. To confirm these mutations, and to prepare them for a more detailed analysis, we isolated clones from the pool, and screened isolated colonies by Southern-blot analysis. A representative example is shown in Figure 13, where mutant and wild-type *Cori* clones were clearly resolved (compare with Figure 10). *C. crescentus* strains containing *Cori* mutations resembled the wild-type strain. All four *Cori* mutants, 1125, 1655. 1656 and 1073 produce normal looking swarmers and stalked cells, as judged by light microscopy. However, *Cori* 1125, a deletion in the strong promoter, tends to produce some more filamentous cells, especially at room temperature, but this phenotype is relatively subtle. The growth curves in Figure 14 demonstrate that chromosome *Cori* mutants grow very well, at maximal rates indistinguishable from wild-type cells.

To detect subtle growth differences, we performed competitive growth assays between chromosome *Cori* mutants and wild-type *Cori* cells. Both mutant and wild-type cells were mixed and cultured together. Upon successive subculturings, DNA was prepared and analyzed by Southern-blot. The results in Figure 15 demonstrate that only *Cori*

mutant 1125 has a subtle growth disadvantage, since only this mutation is slowly outgrown by the wild-type cells (Figure 15).

Figure 13 An example of the identification of chromosome mutants. Total DNA of isolated clones from each chromosome replication positive pool were prepared and digested with *BamH*I, Southern blots experiment were performed using the 0.7 kb *Pst*I-*BamH*I *Cori* fragment as probe. The 1.6 kb band represents the wild-type *Cori*, and the bands between 1.6 kb and 0.5 kb represent the mutated *Cori* DNA marked with a *BamH*I linker.

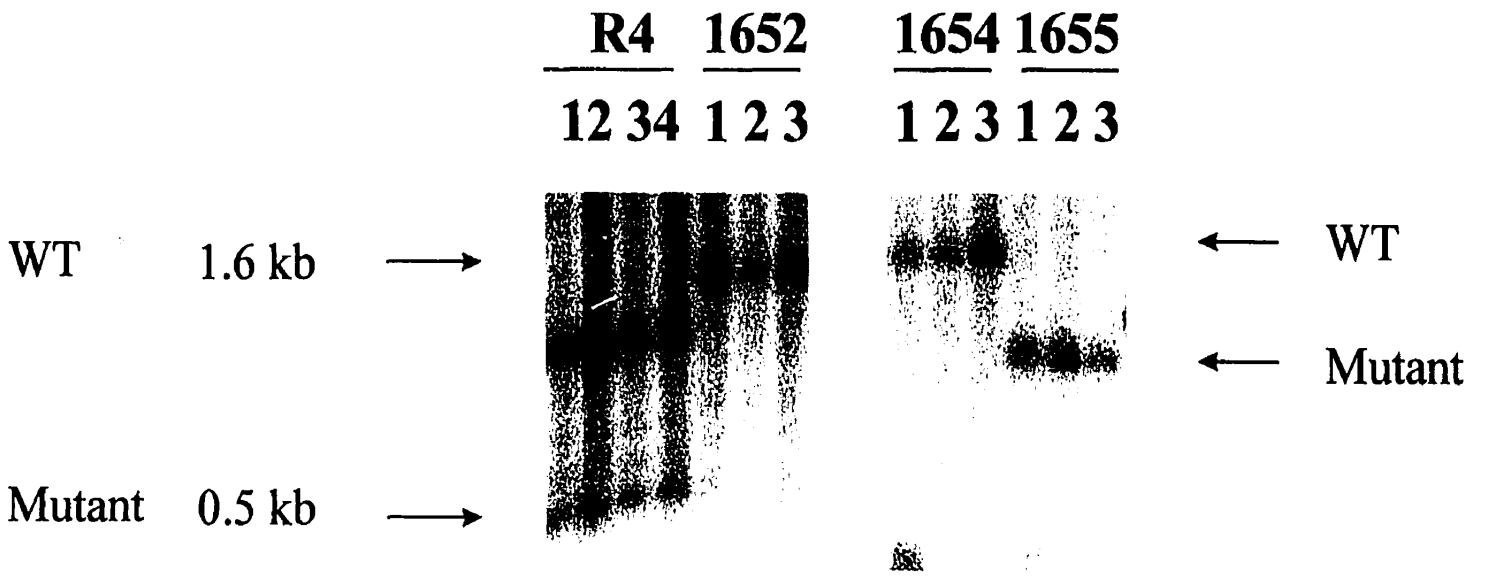
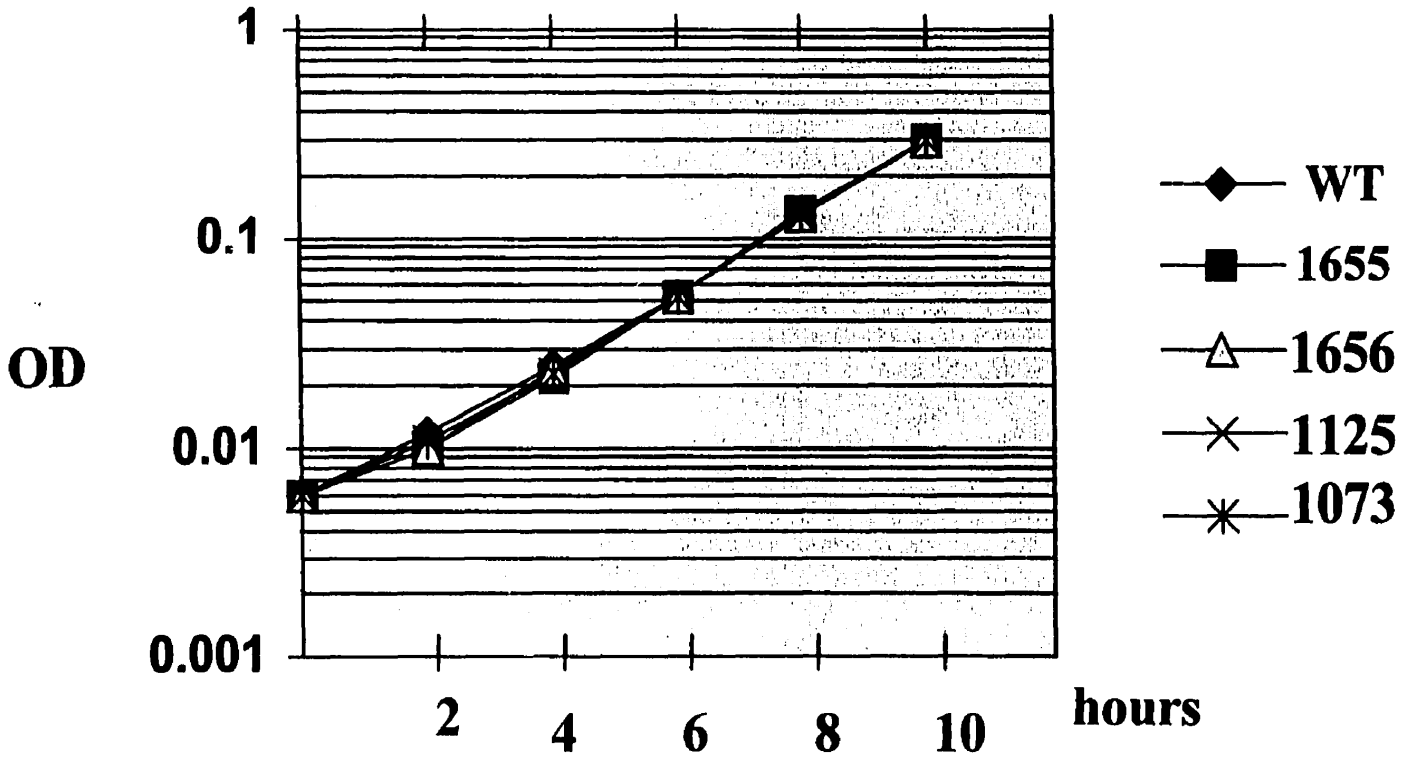
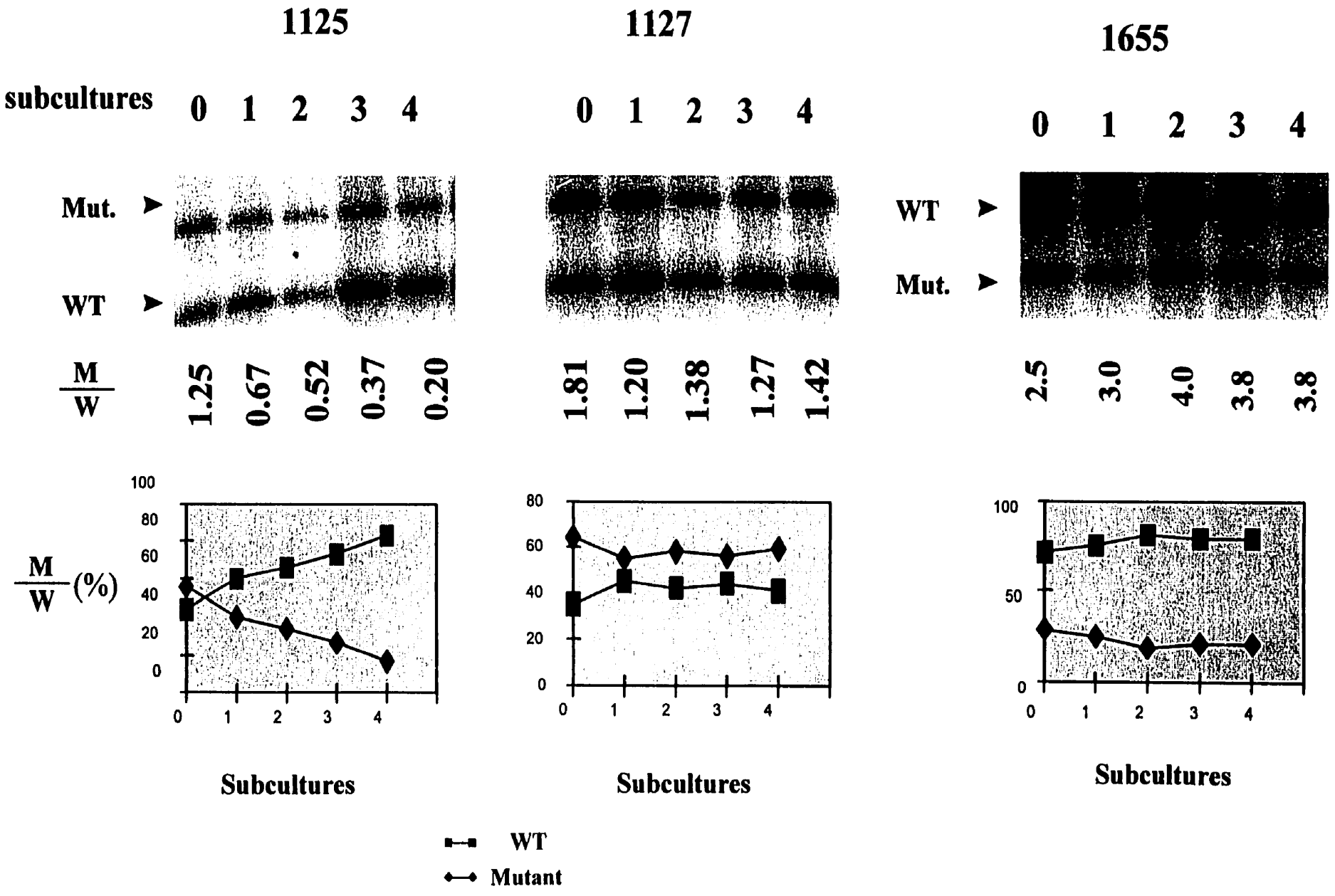

R4
1652
1654
1655
12 34 1 2 3
1 2 3 1 2 3
WT
1.6 kb
Mutant
0.5 kb
WT
Mutant

Figure 14. Growth curves of *Cori* mutants and wild-type *C. crescentus*. Wild-type and mutants were cultured in 1xPYE broth, 30°C air shaker. All samples were taken during log-phase. The doubling time (80 min) of all mutants and wild-type were calculated using log-phase data. Optical density (OD) was measured at 660 nm.



DT = 80 min

Figure 15. Competitive growth assays between *Cori* mutants and wild-type *C. crescentus*. A. A mixture of mutant and wild type cells were subcultured 4 times. 0, 1, 2, 3, and 4 successive subcultures were examined by Southern blotting as described above. A "population shift" is determined by measuring the ratio of mutant (M) to wild-type (WT) chromosome DNA. *Cori* mutations 1125, 11127, and 1655 are described in the text and Table 3.


1125
1127
1655
subcultures
0 1 2 3 4
0 1 2 3 4
0 1 2 3 4
Mut.
WT
WT
Mut.
M/W
1.25 0.67 0.52 0.37 0.20
1.81 1.20 1.38 1.27 1.42
2.5 3.0 4.0 3.8 3.8
M/W (%)
100 80 60 40 20 0
80 60 40 20 0
100 50 0
0 1 2 3 4
Subcultures
Subcultures
Subcultures
WT
Mutant

Discussion

1. Summary

Previous studies have demonstrated that *C. crescentus* uses one replication origin to initiate chromosome replication, and that the cloned *C. crescentus* origin (*Cori*) supports autonomous plasmid replication (Marcynzski and Shapiro 1992). Studies based on autonomous plasmid replication revealed that *Cori* has significant similarities and difference with other bacterial origins. The objective of this study was to determine DNA sequence requirements for developmentally regulated chromosome replication in *C. crescentus*. To test the effect of cell cycle transcription regulator (CtrA) on replication, site-directed mutagenesis was employed to mutate the CtrA binding sites inside *Cori*, and the *Cori*-plasmid "minichromosome" replication assay was used to determine the copy number of altered *Cori*-plasmids. These experiments demonstrated the negative control of minichromosome replication by CtrA binding to site d. 5 bp changes at site d increase the *Cori*-plasmid copy number. However, mutated CtrA binding sites inside *Cori* can also impair minichromosome replication. Altered CtrA binding site a, b and e inside *Cori* caused much lower *Cori*-plasmid copy numbers. These results suggest that CtrA plays both positive and negative roles in minichromosome replication. Interestingly, CtrA binding site mutations, which lower *Cori*-plasmid number, also blocked transcription from *Cori*. This strongly suggests that CtrA may control replication through transcription regulation inside *Cori*. *Cori*-plasmid replication requirements are independent of plasmid vector, since similar replication abilities were shown when *Cori*-mutations were constructed in three different plasmid vectors: pKS, pNTPS138 and pACYC177-*sac*B.

Since minichromosomes may not contain all of the cis-acting DNA elements required to precisely regulate chromosome replication, I designed and executed experiments to reveal the full requirements of *Cori* on the entire chromosome. 17 mutations throughout the *Cori* region were placed within the natural context of the entire chromosome by homologous recombination. Control transcription experiments were used to separate mutant effects on replication from mutant effects on genetic expression. Comparing plasmid and chromosome replication, four classes of expected and unexpected mutations were observed: (i) 2 mutations that allowed replication in both plasmid and chromosome contexts (as expected); (ii) 8 mutations that block replication in both plasmid and chromosome contexts (as expected); (iii) Surprisingly, 4 mutations that block replication in plasmid but not the chromosome context. These mutations define a replication system that is only essential on the plasmid. Further studies revealed that these chromosome mutants resemble wild-type cells in growth and morphology, and only one mutant had a slightly slower growth phenotype; (iv) 3 mutations that allowed replication in the plasmid but not the chromosome context. A total of 7 mutations were found to have different effects on plasmid and chromosome replication. The 11 mutations that block chromosome replication presumably define the most essential DNA sequences. These should be the subject of further detailed studies to understand the control of whole chromosome replication.

This is the first systematic mutation analysis of a chromosome origin in the whole chromosome context. The data presented in this thesis demonstrate that it is necessary to

employ both autonomous plasmid and whole chromosome approaches to define essential replication sequences.

2. CtrA binding sites influence replication and transcription

CtrA is a global transcription regulator. Among its many cell cycle roles, CtrA controls the transcription from the strong promoter by binding site a and b which overlap this promoter in the *C. crescentus* replication origin. This regulations coincides with cell cycle-regulated chromosome replication, since transcription only occurs in stalked cells. Also, mutations of CtrA binding sites a or b impair transcription and replication. For example, a deletion of 14 bp across sites a and b (1125) impairs transcription and cell cycle *Cori*-plasmid replication. A base pair duplication (1667) increases transcription, but blocks chromosome replication, although interestingly not *Cori*-plasmid replication (Table 3, Figure 12). This suggests a complex relationship between CtrA binding, transcription, and the regulation of replication. Increasing the spacing between site a and site b reduces cooperative binding interactions between CtrA proteins (Siam and Marczynski, unpublished results). It does not seem likely that CtrA only functions as a repressor of the *Cori* replication. Point mutations in site d elevated *Cori*-plasmid copy number, but point mutations in sites a, b, and e clearly decrease *Cori*-plasmid copy number, presumably by impairing replication (Figure 8). It is possible that binding of CtrA to site b and e activates transcription by cooperating with transcription factors or RNA polymerase.

CtrA binding to site e is especially interesting, because CtrA foodprinting experiments clearly demonstrated that CtrA binding overlaps an essential DnaA box (Fig. 3). Point mutations in site e, and the proposed interactions between CtrA and DnaA imply that both proteins may play a positive role in the initiation of chromosome replication. A 4 base pair change to site e, adjacent to the essential DnaA box, completely abolishes the rightward transcription, and significantly lowers *Cori*-plasmid copy number (1276, see Figure 8 and Figure 12). In *E. coli*, transcription from *gidA* and *mioC* is involved in the regulation of initiation of replication. DnaA activates transcription from *gidA* which has a positive role in initiating replication, and DnaA represses the transcription from *mioC*, a negative factor of initiation. In *C. crescentus*, the rightward transcription from *Cori* may be a positive factor for the initiation of replication.

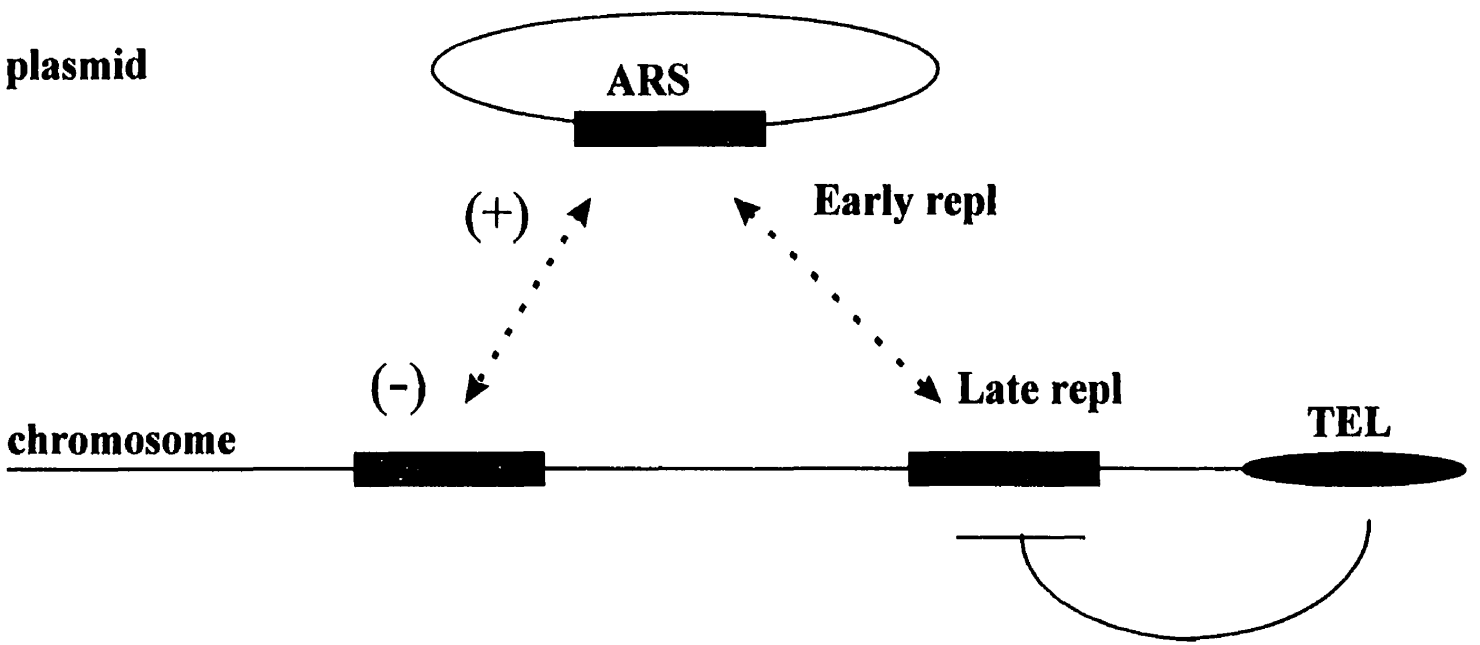
Although site d is clearly involved in repression of replication, there is little evidence for the role of CtrA binding site c. A deletion across site c abolished *Cori*-plasmid replication, but not that of the whole chromosome (1655, 1656, see Table 3). In fact, these cells were not distinguishable from wild-type with respect to growth and morphology. Site c was recently found to overlap a strong IHF binding site (Siam and Marczynski, unpublished data). In *E. coli*, IHF is a replication protein which bends the DNA into a proper structure (Nash, 1996; Polaczek, 1990). Does this imply that CtrA unbends *Cori* by competitive binding with IHF at site c? Certainly, more experiments are needed before we can make firm conclusions.

3. Whole chromosome versus plasmid replication

The cloned *C. crescentus* replication origin (*Cori*) can support autonomous plasmid replication, and these studies demonstrated many important features which suggest potential control mechanisms of replication. However, *Cori* mutagenesis on the whole chromosome has revealed some unexpected results. Comparing mutations on *Cori*-plasmids versus the whole chromosome produced four classes of mutation (Table 4). Some mutations, such as deletions in the *hemE* gene start codon (1645, see Table 3) and DNA sequences upstream of the essential DnaA box (1662, see Table 3), have no effect on autonomous *Cori*-plasmid replication, but are nonetheless essential for chromosome replication. On the other hand, other groups of mutations which are essential for autonomous *Cori*-plasmid replication, such as a deletion of CtrA binding sites a, b and c (1125, 1127, 1655 and 1656, see Table 3), are nonetheless dispensable for chromosome replication. Plasmid versus chromosome position effects have also been observed for yeast origins (ARS elements). Figure 16 summarizes data from the Fangman and Brewer labs that show ARS elements can be activated and/or their timing can be advanced by plasmid context (Brewer and Fangman 1993). What is the function of the cloned replication origin on the *C. crescentus* chromosome? 2-D agarose gels indicate that *Cori* may initiate chromosome replication by an alternative mechanism which is significantly different from that of *E. coli* chromosome. The detected X-shaped replication intermediates suggest that replication doesn't initiate from the center of *Cori*, but instead chromosome replication appears to initiate from *Cori* flanking sequences (Brassinga and Marczynski, unpublished data). One possible explanation is that the *Cori* stimulates replication of flanking DNA which may include more than one origin. The cell cycle

regulators, such as CtrA, bind to *Cori* to stimulate and/or repress the initiation of replication from flanking sequences during the cell cycle. *Cori* may function as a regulator or "enhancer" and/or "silencer" rather than a simple replication starting site. Similar mechanisms have been proposed in eukaryotes (Heintz et al. 1992; Newton 1997) and in *E. coli* plasmid R6K (Mukherjee et al. 1988; Doran et al. 1998).

Figure 16. Schematic representation of yeast autonomous replication sequence (ARS's) that also demonstrate plasmid versus chromosome position effects. An ARS that does not initiate on the chromosome (-) does in fact initiate replication (+) and support plasmid replication. Also an ARS linked to a telomere (TEL) will replicate late in S-phase, but relatively early in S-phase in the plasmid context.


plasmid
ARS
(+)
Early repl
(-)
Late repl
chromosome
TEL

Conclusions

These studies addressed a number of critical questions regarding the regulation of *C. crescentus* chromosome replication. First, CtrA plays both positive and negative roles on *Cori*-plasmids. Second, systematic mutagenesis demonstrated that *Cori* behaves differently in plasmid vesus chromosome contexts. Mutations that abolish replication in both plasmid and chromosome contexts presumably define the most critical elements. Third, the correlation between replication and transcription inside *Cori* implies a mechanism that links both processes. Most replication studies assay plasmid replication, and relatively few studies have targeted the entire chromosome. This thesis presents the most intensive whole chromosome mutation study to date of a bacterial chromosome replication origin.

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