Conserved Promoter Motif Is Required for Cell Cycle Timing of \( \text{dnaX} \) Transcription in \text{Caulobacter}

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Cells use highly regulated transcriptional networks to control temporally regulated events. In the bacterium \text{Caulobacter crescentus}, many cellular processes are temporally regulated with respect to the cell cycle, and the genes required for these processes are expressed immediately before the products are needed. Genes encoding factors required for DNA replication, including \text{dnaX}, \text{dnaA}, \text{dnaN}, \text{gyrB}, and \text{dnaK}, are induced at the G1/S-phase transition. By analyzing mutations in the \text{dnaX} promoter, we identified a motif between the −10 and −35 regions that is required for proper timing of gene expression. This motif, named RRF (for repression of replication factors), is conserved in the promoters of other coordinately induced replication factors. Because mutations in the RRF motif result in constitutive gene expression throughout the cell cycle, this sequence is likely to be the binding site for a cell cycle-regulated transcriptional repressor. Consistent with this hypothesis, \text{Caulobacter} extracts contain an activity that binds specifically to the RRF in vitro.

The timing of DNA replication is tightly controlled in \text{Caulobacter}. Each cell division in \text{Caulobacter} produces two different cell types, but in each cell, DNA replication is initiated only once per cell cycle (3, 9, 10). The swarmer cell enters the cell cycle in G1 phase and initiates DNA replication at a discrete time during the swarmer-to-stalked cell transition, whereas the stalked cell immediately initiates replication and enters the cell cycle in S phase (Fig. 1). Before initiation of replication, transcription of genes encoding many replication factors is induced, suggesting that these genes are regulated by a shared repressor or activator (Fig. 1) (6, 12, 14). The promoter region has been mapped for genes that encode five replication factors: \text{dnaN}, encoding the β subunit of DNA polymerase III (12); \text{dnaX}, encoding the γ and τ subunits of DNA polymerase III (14); \text{gyrB}, encoding the β subunit of DNA gyrase (12); \text{dnaA}, encoding a replication initiation factor (15); and \text{dnaK}, encoding a chaperone required for initiation of replication (5). For each of these genes, the primary promoter has canonical −10 and −35 sequences for σ73, the major sigma factor in \text{Caulobacter} (7). \text{dnaA}, \text{dnaX}, and \text{gyrB} each have a single σ73 promoter; \text{dnaK} has a σ73 promoter and a heat shock promoter; and \text{dnaN} has a σ73 promoter, a heat shock promoter, and an additional promoter. The transcription of each of these genes is induced before DNA replication is initiated, although there is some divergence in the expression patterns (Fig. 1). Transcription from the \text{dnaA} promoter is induced first, followed by coordinated induction of \text{dnaN}, \text{dnaX}, \text{gyrB}, and \text{dnaK}. These promoters do not contain CtrA binding sites and are not directly controlled by CtrA (6), so understanding how they are coordinately induced requires identification of novel cell cycle regulatory elements.

Alignments of the promoter sequences for these five genes have suggested that there are two conserved sequence motifs, called the “8-mer” and the “13-mer” (the number of bases in the 13-mer motif varies among promoters) (12, 14), which may be binding sites for transcription factors. The 13-mer lies in the region between the −10 and −35 sequences, and two conflicting consensus sequences have been proposed for this element (12, 14). In order to characterize this regulatory element, we systematically mutagenized each position in this region of the \text{dnaX} promoter and examined the effects on the level and timing of transcription. Our results show that there is a regulatory element present between the −35 and −10 sequences in the \text{dnaX} promoter and that this element is required for cell cycle regulation of transcription. This element is required for the repression of transcription during periods of the cell cycle when the replication factor genes are not expressed, so we have named it RRF (for repression of replication factors). Furthermore, we demonstrate that \text{Caulobacter} extracts contain an activity that binds stably to the RRF of the \text{dnaX} promoter and to the analogous regions of the \text{dnaA}, \text{dnaN}, \text{gyrB}, and \text{dnaK} promoters.

MATERIALS AND METHODS

Strains and plasmids. The wild-type \text{Caulobacter crescentus} strain used in these studies is CB15N (4). \text{Caulobacter} cultures were grown at 30°C in PYE.
DNA binding assays. DNA probes were constructed by end labeling oligonucleotides corresponding to the following sequences with T4 polynucleotide kinase (13) and annealing them to complementary oligonucleotides: complete dnaX; GTTGCGGCGGAGCTTTCGTCGGCCCATACACTCCGCGCC; dnaX. TTTGCGGCGGCTTCGCGCCCATACACTCCGCGCC; dnaA. TTTGCGGCGGCTTCGCGCCCATACACTCCGCGCC; gsrβ. GGCGGCGGAATCCGCGCC; gsrβ. GGCGGCGGAATCCGCGCC.

gel mobility shift assays. Typically, a 300-ml log-phase culture of Caulobacter was harvested by centrifugation and resuspended in 30 ml of buffer B (10 nM Tris-HCl [pH 8.0], 100 mM NaCl, 50 mM MgCl₂, 1 mM dithiothreitol). The cells were lysed by the addition of lysozyme to a final concentration of 10 µg/ml and then sonicated until the optical density at 600 nm was reduced to less than 10% of the initial level. The insoluble material was removed by centrifugation for 30 min at 27,000 × g. Alternatively, extracts were made by extraction with B-PER detergent following the manufacturer’s protocol (Pierce). The concentration of total protein in the extracts was 1 to 3 mg/ml and 1 to 5 µl of extract was used for a 25-µl DNA-binding reaction. For gel-shift assays, extract was incubated for 2 h with 200 µM 32P-end-labeled DNA probe in buffer B containing 10 µg of bovine serum albumin per ml and 50 µg of sheared calf thymus DNA per ml as a nonspecific competitor, separated by electrophoresis on native 10% polyacrylamide gels in 0.5× Tris-borate-EDTA buffer, and visualized by autoradiography (1). Ideally identical results were obtained under the range of conditions described above.

RESULTS

Mutagenesis of the dnaX promoter. Previous mutagenic studies with the dnaX promoter have defined the −10 and −35 sequences required for transcription and have suggested that there is a negative regulatory element in this region (14). In order to define this regulatory element more precisely, we performed a thorough mutational analysis of the promoter. Each of the 14 bases in this region was individually changed to the other 3 possible bases, and transcriptional activity was measured by β-galactosidase assays. Figure 2 shows the activity of each mutant promoter relative to the wild-type promoter in unsynchronized log-phase culture. Mutations at seven positions resulted in a change in transcription of at least 50%. In all cases in which a sizable change in transcription was observed, the promoter activity increased, suggesting that these mutations disrupted a negative regulatory element. Overall, these mutations define a redundant sequence preference extending over 12 bases, starting at position −29 and continuing to position −18. There is a lesser preference at positions −17 and −16. Sequences upstream of −29 and downstream of −16 may also be required for the RRF, but since these bases are also part of the RNA polymerase binding site (14) (Fig. 2A), mutations at these positions cannot be interpreted.

Four positions, −29, −28, −26, and −21, are crucial for regulation, because mutations at these positions result in an increase in promoter activity of twofold or more (Fig. 2). There is a strong preference for a G at −29 and a C at −26, because no other bases at these positions produce wild-type promoter activity. At −28, a pyrimidine is preferred. The greatest change in activity was observed for the C(−21)T substitution, which increased activity by 3.4-fold, but other substitutions at this position had only a mild effect. The individual mutations studied here provide a redundant sequence preference for the RRF: GYRCnmmmCNsYM (Y = C or T; R = A or G; S = G or C; M = A or C; and n = any base).

Cell-cycle regulation of a mutant promoter. A priori, mutations in the RRF could increase the observed promoter activity
by increasing transcription throughout the cell cycle while retaining the normal regulatory pattern, by increasing the peak level of transcription, or by relieving repression so that peak-level transcription occurs throughout the cell cycle. To determine how mutations in the RRF affect the cell cycle regulation of the dnaX promoter, we isolated swarmer cells from Caulobacter bearing a lacZ reporter driven by either the wild-type dnaX promoter or the C(21)T variant. We then allowed the cells to pass synchronously through the cell cycle and assessed transcription at different times by using a pulse-label immunoprecipitation assay (Fig. 3). The major effect of the C(21)T mutation is loss of repression of the dnaX promoter in the swarmer and late predivisional cells. Whereas the wild-type promoter is induced 10-fold during the swarmer-to-stalked cell transition, expression from the C(21)T variant changes by only 1.5-fold and exhibits expression levels throughout the cell cycle that are near the wild-type maximum. In fact, the minimal level of expression from the C(21)T variant is 7.5-fold higher than that in the wild type. There is also a small change in the peak expression from the C(21)T variant: maximal expression is approximately 15% higher than in the wild type and occurs earlier in the swarmer-to-stalked cell transition. It is possible that this increase in peak expression level is due to a positive regulatory factor that prefers the C(21)T variant.

However, the increased expression levels in the swarmer and predisional cells are more significant than the small increase during the swarmer-to-stalked cell transition. Therefore, the increased amount of gene expression in unsynchronized cultures bearing the C(21)T variant appears to be due primarily to a loss of cell cycle-regulated repression.

DNA-binding activity for the RRF. The most straightforward explanation for the activity of the RRF is that a repressor binds to this sequence and inhibits transcription. Mutations that disrupt this binding site would then decrease repression and lead to a higher level of transcription. To investigate whether such a repressor exists, we assayed Caulobacter extracts for a DNA-binding activity specific for the dnaX RRF. Binding site probes were constructed that correspond to the dnaX promoter RRF (positions -25 to -10) or to the RRF flanked by shorter DNA sequences to control for binding of RNA polymerase σ factors to the -35 and -10 regions. We found that Caulobacter extracts contain an activity that binds to the dnaX promoter RRF, but does not bind to a similar DNA probe in which the RRF has been replaced by an arbitrary sequence (Fig. 4). A dnaX promoter probe containing the C(21)T mutation was also shifted (data not shown), indicating that this sequence retains enough of the protein-DNA contacts to bind under these assay conditions.

We tested these extracts for binding activity to the compa-
rable region of the dnaA, dnaN, dnaK, and gyrB promoters and found that these probes are also shifted (Fig. 4). Since the different promoter probes are shifted to the same mobility, it is likely that they are bound by the same protein or protein complex. These results are consistent with the model that a transcriptional repressor binds to this region in all of the promoters.

Identification of RRFs in other promoters. Based on the redundant RRF sequence from the dnaX promoter, we identified putative RRF sequences in the promoters of dnaA, dnaN, dnaK, and gyrB (Fig. 5). Although these promoters are induced in a similar fashion and are all proposed to have s73 binding sites, none of the −10 or −35 sequences is the same, and none exactly matches the s73 consensus sequence (8). Similarly, none of the RRF sequences is an exact match with the preferences defined for the dnaX promoter. It is possible that bases in the −10 or −35 regions also influence the RRF, so that each RRF sequence is optimized to the surrounding DNA. Another possible reason for variation in the RRF sequences is that changes in the RRF among the promoters alter the affinity of the repressor and thereby account for the differences in cell cycle regulation observed for the different promoters. For instance, the dnaA RRF is the most divergent, so it would be predicted to bind the repressor with altered affinity. In fact, the dnaA gene is induced earlier in the cell cycle than the other replication genes, consistent with weaker binding of the repressor.

**DISCUSSION**

Control of the cell cycle in Caulobacter requires coordinated expression of functionally related groups of genes. The genes for DNA replication factors are coordinately induced, but the
The mechanism of this regulation is not known. To gain a better understanding of how these genes are controlled, we performed a thorough mutational analysis of the region between the −35 and −10 sequences of the dnaX promoter. This study identified a promoter element, the RRF, which is required for cell cycle-regulated expression from the dnaX promoter and is present in all of the coordinately regulated genes of replication factors. Our results show that the RRF is a negative regulatory element essential for the correct timing of gene expression and suggest that it is the binding site for a repressor protein.

The analysis of single-base substitutions in the dnaX promoter has defined the RRF preference as GYRCnnnnC and looking for a consensus sequence. However, two different consensus sequences, but since it is based on mutational data, it is likely to be more functionally relevant than those based wholly or largely on manual sequence alignment. This consensus sequence has been thoroughly tested and refined for the dnaX promoter.

It is not surprising that the other replication factor RRFs differ from the dnaX RRF preference, because even in the context of the dnaX promoter, the dnaX RRF is not the optimal repressor binding site. Some mutations resulted in lower promoter activity, by as much as 30%, consistent with increased binding of a repressor to the RRF. There may be physiological reasons why an optimal repressor binding site is not used. For example, if the RRF repressor is bound too tightly, it could not be removed when the gene needs to be expressed.

Caulobacter extracts contain an activity that binds to the RRF, and although the repressor has not yet been identified, the dnaX promoter mutants predict some characteristics of the repressor-DNA interaction. The bases that are important for the RRF, −29 to −26 and −21 to −18, are predicted to be on the same face of B-form DNA, so a repressor (or repressor complex) could bind without wrapping around the DNA helix. Because G(−29) and C(−26) cannot be functionally replaced by any other bases, it is likely that these residues are directly recognized by the repressor. The discrimination against T at position −21 suggests an interaction between a protein and the major groove of the RRF at this position, since T has a bulky methyl group protruding into the major groove, which can cause steric hindrance.

Although a probe with the C(−21)T mutation is shifted by cell extracts, this mutation clearly affects promoter activity. It is possible that the C(−21)T mutant binds to the repressor with lower affinity than the wild-type sequence, such that in vivo the wild-type promoter is bound and the C(−21)T promoter is not. Such a difference in affinity would not be detected in our assays if the concentration of repressor is above the dissociation constant for the C(−21)T promoter. Alternatively, it is possible that the C(−21)T mutant binds to the repressor with comparable affinity to the wild type, but in a conformation that does not result in transcriptional repression. Identification of the repressor that binds to the RRF and characterization of its interactions with wild-type and mutant replication factor promoters are essential to distinguish between these possibilities and to understand how the RRF mediates its effects on transcription.

**TABLE 1. Relative activity of doubly and singly substituted variants of the dnaX promoter**

<table>
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<tr>
<th>dnaX promoter variant</th>
<th>RRF sequencea</th>
<th>Fold induction (repression)</th>
<th>Reference</th>
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<tr>
<td>Wild type</td>
<td>GGCCTTCCGCCC</td>
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<tr>
<td>C(−26)A/C(−25)T</td>
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<tr>
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<tr>
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<td>14</td>
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<tr>
<td>C(−19)A</td>
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<tr>
<td>T(−23)G</td>
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a Substitutions are given in boldface.
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REFERENCES