JOURNAL OF BACTERIOLOGY, June 1997, p. 3981–3988 Vol. 179, No. 12
0021-9193/97/$04.00+0

Translation of the Leaderless *Caulobacter dnaX* mRNA

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Received 8 January 1997/Accepted 8 April 1997

The expression of the *Caulobacter crescentus* homolog of *dnaX*, which in *Escherichia coli* encodes both the γ and τ subunits of the DNA polymerase III holoenzyme, is subject to cell cycle control. We present evidence that the first amino acid in the predicted DnaX protein corresponds to the first codon in the mRNA transcribed from the *dnaX* promoter; thus, the ribosome must recognize the mRNA at a site downstream of the start codon in an unusual but not unprecedented fashion. Inserting four bases in front of the AUG at the 5′ end of *dnaX* mRNA abolishes translation in the correct frame. The sequence upstream of the translational start site shows little homology to the canonical Shine-Dalgarno ribosome recognition sequence, but the region downstream of the start codon is complementary to a region of 16S rRNA implicated in downstream box recognition. The region downstream of the *dnaX* AUG, which is important for efficient translation, exhibits homology with the corresponding region from the *Caulobacter hemE* gene adjacent to the replication origin. The *hemE* gene also appears to be translated from a leaderless mRNA. Additionally, as was found for *hemE*, an upstream untranslated mRNA also extends into the *dnaX* coding sequence. We propose that translation of leaderless mRNAs may provide a mechanism by which the ribosome can distinguish between productive and nonproductive templates.

The prokaryotic 30S ribosomal subunit recognizes mRNA at a region in the untranslated leader region approximately 10 bp upstream of the start codon (24). In *Escherichia coli*, this region, referred to as a Shine-Dalgarno sequence, is almost indispensable for translation. In a few notable cases, proteins, primarily from bacteriophage and *Streptomyces* species, are known to be translated from mRNAs that lack any leader sequence, implying that ribosomal recognition can occur in the absence of a Shine-Dalgarno sequence (1, 10, 11, 13, 23). We have found that the DnaX homolog from *Caulobacter crescentus* is translated from a leaderless mRNA; the start codon of the *Caulobacter dnaX* gene, which in *E. coli* encodes the γ and τ subunits of the DNA polymerase III, appears to be coincident with the first codon of the mRNA expressed from the *dnaX* promoter.

During the *Caulobacter* cell cycle, many genes are expressed only at the time in the cell cycle when their products are required (2). For example, several genes required for DNA replication are activated in the stalked cell, coincident with the period of DNA replication (9, 18, 20, 27). We have recently shown that the promoter for the *dnaX* gene is expressed preferentially in the stalked cell portion of the cell cycle (28). The *dnaX* gene, however, is located downstream of the constitutively expressed *ffs* gene, encoding the small stable untranslated 4.5S RNA, and transcripts originating at the *ffs* promoter extend into the *dnaX* gene but terminate before reaching the end of *dnaX* (27). We show here that only the transcripts originating at the *dnaX* promoter are translated. It may be that the longer transcript is not translated because a Shine-Dalgarno sequence is not found upstream of the AUG, and the shorter transcript is translated because the AUG is placed at the 5′ end of the transcript, which compensates for the lack of a Shine-Dalgarno sequence. In vitro mutagenesis indicates that placement of the AUG at the 5′ end is essential for ribosomal recognition in the absence of a Shine-Dalgarno sequence. The paradigm of an untranslated upstream mRNA followed by a translated leaderless mRNA is also found for the *Caulobacter hemE* gene, located adjacent to the origin of DNA replication (14).

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MATERIALS AND METHODS

Growth of bacterial strains. Strains and plasmids are described in Table 1. *C. crescentus* strains were routinely grown at 32°C in peptone yeast extract (PYE) (19) or M2 minimal glucose medium (M2G) (5), tetracycline (1 μg/ml), kanamycin (10 μg/ml), or ampicillin (2.5 μg/ml) when appropriate. *E. coli* strains were grown at 37°C in LB medium or Superbroth (21), supplemented with ampicillin (100 μg/ml), tetracycline (10 μg/ml), or kanamycin (50 μg/ml) as necessary. For assays of β-galactosidase activity (15), *C. crescentus* strains were grown in PYE to stationary phase; cells were then diluted 1:20 into fresh PYE and allowed to grow to an optical density at 600 nm (OD_{660}) of 0.2 to 0.4 at 32°C. Plasmids were introduced into *C. crescentus* strains by electroporation or by mating as previously described (5).

Sequence analysis. Codon preference and *Caulobacter* rare codon usage for the *dnaX* open reading frame were determined by using the codon preference module in the Genetics Computer Group computer software package (3).

Assays of synchronized cultures. Cultures of *C. crescentus* LS107 were grown in M2G to an OD_{660} of 0.8 and then centrifuged in a colloidal silica (Ludox) gradient as previously described (6). Pure populations of swarmer cells were suspended in fresh M2G at an OD_{660} of 0.4 and allowed to proceed synchronously through the cell cycle at 32°C. Samples (1 ml) were removed periodically, labeled with 1 μl of [^{35}S]methionine (1,280 Ci/mmol, 11.21 μCi/μl; ICN) for 5 min at 32°C, and chased for 1 min with 20 μM unlabeled methionine–20 μM cysteine–0.2% tryptone. Immunoprecipitations were performed with antibodies to β-galactosidase (5 Prime 3 Prime, Boulder, Colo.) and to the flagellins as previously described (28). Immune complexes were resuspended in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer (125 mM Tris-HCl [pH 7.0] 0.4% SDS, 20% glycerol, 10% β-mercaptoethanol) and separated by electrophoresis through an SDS–8% polyacrylamide gel.

Immunoblots. *C. crescentus* LS107 (1 ml) containing the appropriate plasmid was grown to mid-log phase (OD_{660} = 0.4) in PYE and collected by centrifugation. Cells were resuspended in SDS-PAGE loading buffer and lysed. Proteins were separated by electrophoresis through an SDS–8% polyacrylamide gel. Proteins were transferred to Immobilon P membranes by electroblotting, and membranes were probed with a 1:1,000 dilution of anti-β-galactosidase antibody (5 Prime 3 Prime). Antibody binding was visualized by probing with a 1:10,000 dilution of goat anti-rabbit antibody conjugated to horseradish peroxidase (Boehringer Mannheim) followed by washing with Renaissance chemiluminescence detection solution (NEN).

Nuclease S1 analysis. For nuclease S1 protection assays, 10 μg of probe derived from plasmid pEW81.30 containing 5×10^5 cpm was hybridized to 20 μg of *Caulobacter* RNA prepared from strain NA1000 or 10 μg of yeast tRNA (Sigma) as previously described (21). Nuclease S1 (Boehringer Mannheim) was prepared at a concentration of 250 or 1,000 U/ml. The probe was hybridized to the RNA for 12 h at 58°C. DNA-RNA hybrids were digested for 1 h at 37°C.

TABLE 1. Strains and plasmids used

Strain or plasmid	Genotype or method of construction	Reference or source
E. coli		
S17-1	*E. coli* 294::RP4-2 (Tc::Mu)(Km::Tn*7*), for plasmid mobilization	25
DH10B	*mcrA* Δ(*mrr-hsd* RMS-*mcr* BC)	Gibco BRL
Caulobacter		
LS101	NA1000 wild type (synchronizable)	6
LS107	NA1000 Δ*bla*	1
LS2383	LS107 containing pEW86.280 integrated into the chromosome at the *dnaX-ffs* locus	This study
LS2384	LS107 containing pEW89.282 integrated into the chromosome at the *dnaX-ffs* locus	This study
LS2385	LS107 containing pEW118.280 integrated into the chromosome at the *dnaX-ffs* locus	This study
LS2386	LS107 containing pEW87.281 integrated into the chromosome at the *dnaX-ffs* locus	This study
LS2387	LS107 containing pEW88.282 integrated into the chromosome at the *dnaX-ffs* locus	This study
LS2388	LS107 containing pEW121 integrated into the chromosome at the *dnaX-ffs* locus	This study
LS2389	LS107 containing pEW124 integrated into the chromosome at the *dnaX-ffs* locus	This study
LS2761	LS107 containing pEW140.E integrated into the chromosome at the *dnaX-ffs* locus	This study
Plasmids		
pEW111.U	A plasmid containing a transcriptional fusion of P_{dnaX} to *lacZ*	28
pEW118.280	An integrative plasmid encoding a protein fusion of the DnaX N terminus (at base 391 [Fig. 1]) to β-galactosidase. To construct the plasmid, the 0.8-kb *Bam*HI-to-*Hin*dIII fragment from pRTW1 was ligated into the same sites in pJBZ280.	This study
pEW121	An integrative plasmid encoding a protein fusion of the DnaX N terminus to β-galactosidase. pEW39 was cut with *Stu*I and *Eco*RI. A 0.8-kb band was gel purified and ligated to an 8-kb fragment derived from cutting pEW118.280 with *Eco*RI and *Stu*I.	This study
pEW141	pEW118.280 was cut with *Bam*HI and *Hin*dIII. A 0.7-kb fragment was gel purified and cloned into the *Bam*HI and *Hin*dIII sites of placZ290.	This study
pEW142	pEW124 was cut with *Bam*HI and *Hin*dIII. A 0.7-kb fragment was gel purified and cloned into the *Bam*HI and *Hin*dIII sites of placZ290.	This study
pEW135.E	A plasmid containing a transcriptional fusion of β-galactosidase to a mutant *dnaX* promoter	28
pEW140.E	The 150-bp *Bsa*I-to-*Nco*I fragment from pEW135.E was gel purified and cloned into pEW118.280 from which the *Bsa*I-to-*Nco*I fragment had been removed.	This study
pEW124	Integrative plasmid encoding a protein fusion of the DnaX N terminus to β-galactosidase. To make this plasmid, pEW118.280 was cut with *Nco*I. The ends were filled in with Klenow enzyme, and the plasmid was self-ligated, generating a duplication of the CATG in the *Nco*I site.	This study
pEW39	A plasmid containing 7 kb of the *dnaX* region in pDELTA (Gibco BRL)	27
pEW81.30	A 0.5-kb *Apo*I-to-*Sal*I fragment containing the 5′ end of *dnaX* was ligated into the *Eco*RI and *Sal*I sites of pJAMY30.	This study
pEW86.280, pEW87.281, pEW88.282, pEW89.282	Integrative plasmids encoding protein fusions of the DnaX N terminus to β-galactosidase. To make the plasmids, pEW39 (27) was cut with *Sma*I (pEW87.281) or *Ecl*136 i (pEW88.282), with *Nco*I and blunted (pEW89.282), or with *Apo*I and blunted (pEW86.280) and then cut with *Pst*I. A fragment of 0.8, 1.0, 0.6, or 0.5 kb, respectively, was gel purified and ligated into a pJBZ vector, which had been cut with *Hin*dIII, blunt ended, and then cut with *Pst*I.	This study
pJAMY30	An integrative plasmid for generation of translational fusions to β-lactamase	M. R. K. Alley
pJBZ280, pJBZ281, pJBZ282	Integrative plasmids for generation of translational fusions in frame 1, frame 2, and frame 3, respectively, to *lacZ*	M. R. K. Alley
placZ290	A *lacZ* transcriptional fusion vector, Tet^r, IncP-1 replicon, mob^+; used for *lacZ* fusions; replicates in *C. crescentus* at 2–5 copies/cell	8
pRTW1	A pBluescript KS− derivative containing a 0.8-kb *Asc*I-to-*Bam*HI fragment encoding the *ffs* gene and the N terminus of *dnaX*	Rob Wheeler

Nucleic acids were precipitated and electrophoresed on an 8% polyacrylamide gel. ^{35}S-labeled size standards were prepared by performing a dideoxy sequencing reaction on control template M13 single-stranded DNA, using the −40 primer provided in the Sequenase kit (United States Biochemical).

Nucleotide sequence accession numbers. The complete *ffs* and *dnaX* gene sequences can be obtained from GenBank under accession no. U22036 and U49738, respectively.

RESULTS

Translational initiation of *dnaX*. Examination of the sequence in the 5′ region of *dnaX* revealed three possible translation start sites: S1 (AUG), S2 (GUG), and S3 (AUG) (Fig. 1). Although the S1 start site is preceded by a possible Shine-Dalgarno sequence, comparison to other *dnaX* homologs revealed that the AUG in S1 would be within the predicted *dnaX* open reading frame (28). Furthermore, initiation of translation at S1 would produce a protein shorter than the one predicted from rare codon usage and third-position G or C bias (data not shown). An alternative start site, S2, is located near the start of the homology to the predicted *E. coli* DnaX, but the spacing between a possible upstream Shine-Dalgarno sequence is longer than usual (nine bases) and S2 would utilize the less common start codon GUG instead of AUG. As with S1, third-position G or C bias and rare codon usage indicated a potential start even further upstream than this position. A third possible start site, S3, was correctly placed with regard to third-position G or C bias and rare codon usage but had nothing resembling a Shine-Dalgarno sequence either upstream or downstream of the AUG. Furthermore, nuclease S1 analysis, primer extension analysis, site-directed mutagenesis, and construction of transcriptional fusions had established that the A of the AUG is

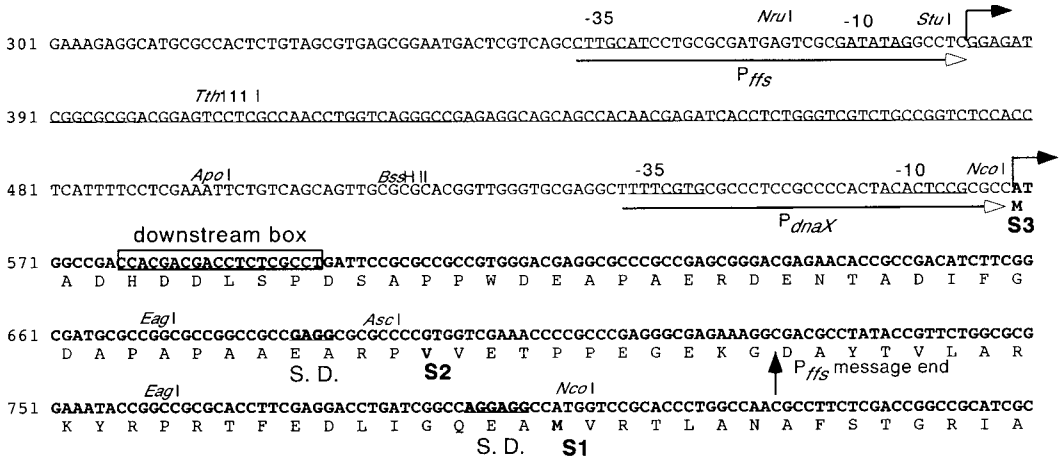

FIG. 1. Nucleotide sequence of the *ffs-dnaX* intergenic region showing potential translational start sites, S1, S2, and S3. The mature 4.5S RNA sequence is underlined, and the predicted DnaX coding sequence is shown in boldface. Promoter sequences are indicated by open-headed arrows below sequences, and transcription start sites are shown by bent arrows (27, 28). Potential Shine-Dalgarno sequences are underlined and indicated by S. D. The putative downstream box involved in mRNA recognition by the ribosome is boxed.

the first base in the mRNA transcribed from P_{dnaX} (28). Utilization of S3 on the mRNA derived from P_{dnaX} would require that the ribosome recognize the mRNA based solely on the AUG and sequences downstream, a mechanism not without precedent in *Caulobacter* (14) but highly unusual in *E. coli*. To differentiate among these potential start sites, translational fusions were constructed (Fig. 2A). In these fusions, a *lacZ* gene lacking its native translational start site and promoter was fused to the *dnaX* gene at several different locations (Fig. 2A). The plasmids were then integrated into the chromosome by homologous recombination to generate strains containing both the wild-type *dnaX* gene and the translational fusion of *dnaX* to *lacZ*. Fusions were constructed, in all cases, for all three reading frames, although only those in the predicted frame are shown in Fig. 2A. Assays of β-galactosidase activity revealed that only three strains, LS2385, LS2386, and LS2387, showed activity above background. These three contained fusions to the predicted reading frame shown below the nucleotide sequence in Fig. 1 (fusions in the incorrect frames showed only background levels of activity). Based on this finding, S1 and S2 could be eliminated as possible translation start sites because LS2385 had activity comparable to that of the wild type (LS2387) but did not contain either S1 or S2. (While the putative S2 Shine-Dalgarno region remained intact in LS2385, the novel junction was sequenced and no possible start codons were observed in any frame.) As a control, a translational fusion to a region 5′ to the S3 site, in LS2383, had, as expected, no activity.

To further confirm the use of S3 as the translational start site and to determine if only a single start site was being used,

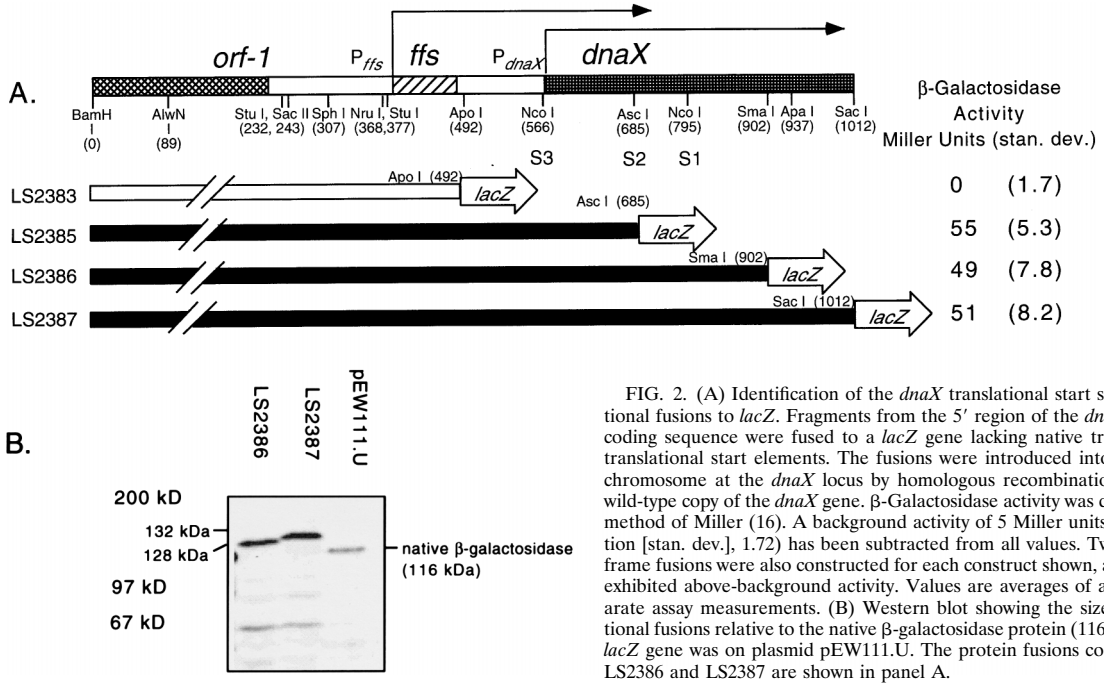

FIG. 2. (A) Identification of the *dnaX* translational start site, using translational fusions to *lacZ*. Fragments from the 5′ region of the *dnaX* promoter and coding sequence were fused to a *lacZ* gene lacking native transcriptional and translational start elements. The fusions were introduced into the *Caulobacter* chromosome at the *dnaX* locus by homologous recombination, maintaining a wild-type copy of the *dnaX* gene. β-Galactosidase activity was determined by the method of Miller (16). A background activity of 5 Miller units (standard deviation [stan. dev.], 1.72) has been subtracted from all values. Two similar out-of-frame fusions were also constructed for each construct shown, and none of these exhibited above-background activity. Values are averages of at least three separate assay measurements. (B) Western blot showing the sizes of two translational fusions relative to the native β-galactosidase protein (116 kDa). The native *lacZ* gene was on plasmid pEW111.U. The protein fusions contained in strains LS2386 and LS2387 are shown in panel A.

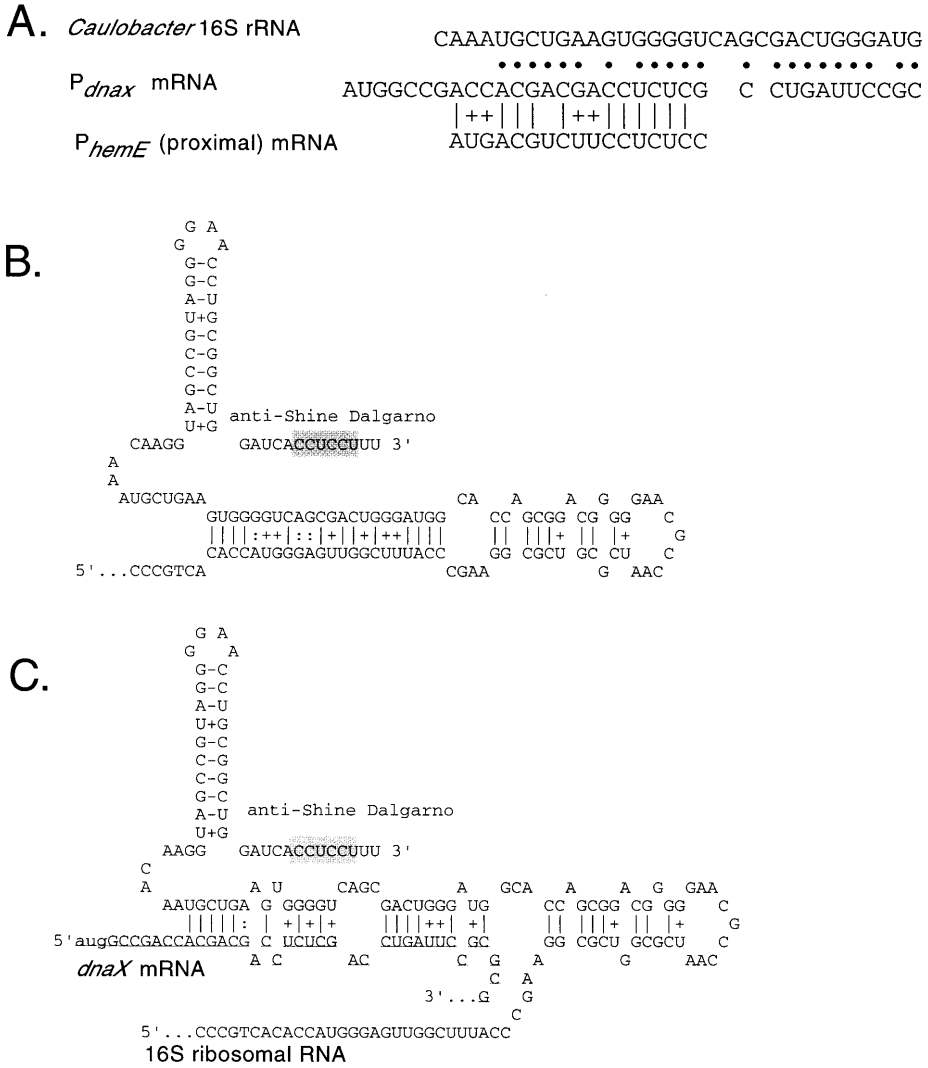

FIG. 3. (A) Potential base pairing, indicated by • between the 5′ end of the mRNA produced from P_{dnaX} and the region on the 16S rRNA (4) implicated in downstream box recognition (26). Below, the *dnaX* 5′ mRNA sequence is compared to 5′ end of the mRNA produced from the weak proximal *hemE* promoter (14). |, identity with the *dnaX* sequence; +, a base that is not identical to that contained in *dnaX* but still able to pair with the 16S RNA. (B and C) Potential secondary structure of the *Caulobacter* 16S RNA in the absence (B) and presence (C) of the *dnaX* mRNA (underlined). The anti-Shine-Dalgarno sequence is shown in boldface. The *dnaX* mRNA start codon is shown in lowercase. +, G-to-U base pairing; :, A-to-G base pairing.

immunoblot analysis using an anti-β-galactosidase antibody was performed on extracts of the protein fusion strains LS2386 and LS2387, as well as on a strain containing a plasmid-based transcriptional fusion (pEW111.U) of the *dnaX* promoter driving the native *lacZ* gene. Protein fusions, larger than the native β-galactosidase protein (encoded in pEW111.U), were observed for the extracts derived from strains LS2386 and LS2387 (Fig. 2B). The smaller bands are cross-reacting products that were also present in control extracts lacking β-galactosidase. The migration distances of the LS2386 and LS2387 bands yielded apparent molecular masses consistent with the predicted molecular masses of 128 kDa (LS2386) and 132 kDa (LS2387), expected if the S3 site was employed. Thus, it appears that only the S3 translation start site is used.

It was previously reported that the *Caulobacter* HemE protein also appears to be translated from a leaderless transcript (14). The region downstream of the *hemE* translational start site was found to be similar to a region implicated as a downstream ribosomal recognition site in certain highly expressed *E. coli* genes (7, 17, 25). This region, which in *E. coli* has been referred to as a downstream box, is capable of forming base pairs with a region of the 16S rRNA separate from that involved in Shine-Dalgarno sequence recognition. The region downstream of the *hemE* mRNA AUG, likewise, has the capacity to base pair with a similar region of the *Caulobacter* 16S rRNA (14). As shown in Fig. 3A, the region within the 5′ portion of the *dnaX* mRNA aligns with a sequence in the 5′ region of the *hemE* mRNA that is complementary to the region in the 16S rRNA (4) implicated in downstream box recognition. As expected, the region downstream of the start codon in the *dnaX* mRNA was also partially complementary to this region of the *Caulobacter* 16S rRNA (4) (Fig. 3A). Internal alignments of a portion of the *Caulobacter* 16S rRNA in the absence and presence of the *dnaX* transcript, indicating the locations of the potential interactions between the rRNA and the box downstream of the S3 translational start site, are shown in Fig. 3B and C, respectively. To test the importance of this region, we constructed the translational fusion contained in

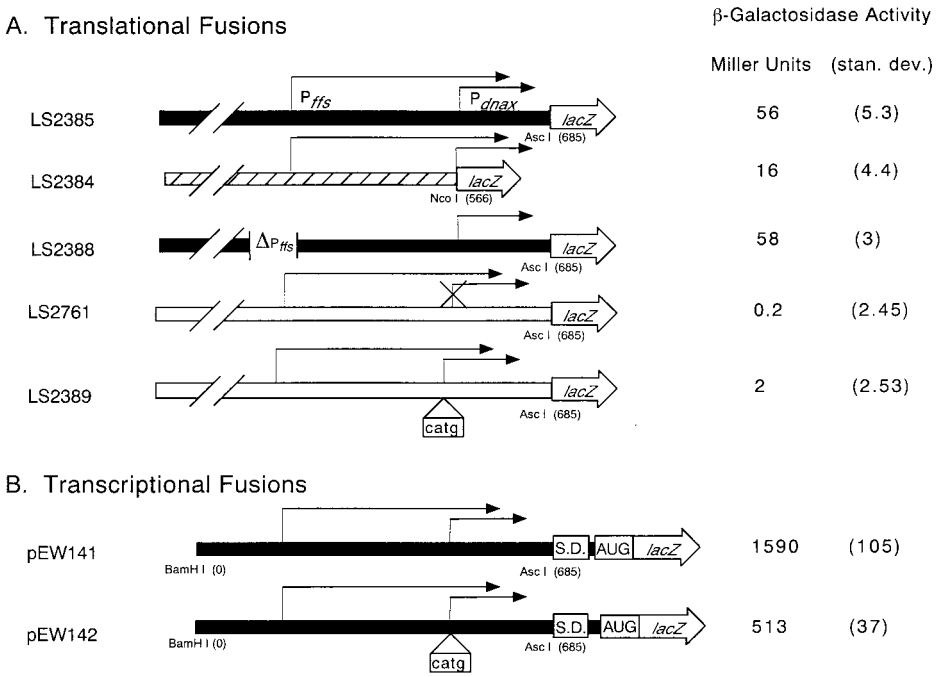

FIG. 4. (A) Translational fusions of mutant 5′ regions of *dnaX* to *lacZ*. Fragments from the *dnaX* 5′ region were fused to a *lacZ* gene lacking native transcriptional and translational start elements. The fusions were introduced into the *Caulobacter* chromosome at the *dnaX* locus by homologous recombination, maintaining a wild-type copy of the *dnaX* gene. The correct orientation was determined by Southern blot analysis. β-Galactosidase activity was determined by the method of Miller (16). To construct the fusion contained in strain LS2389, the plasmid (pEW118.280) used to make LS2385 was cut with *Nco*I (which cuts the plasmid pEW118.280 once at S3), filled in, and self-ligated, creating plasmid pEW124. This plasmid was integrated into the chromosome to make strain LS2389. (B) Transcriptional fusions of P_{ffs} and P_{dnaX} to a promoterless *lacZ* gene containing a native Shine-Dalgarno signal (S.D.) and an AUG. The fusions were constructed in the vector pLACZ290, which has a background activity of approximately 100 Miller units. Background activity of 5 Miller units for translational fusions or 100 Miller units for transcriptional fusions has been subtracted from shown values, which are averages of at least three separate assay measurements. stan. dev., standard deviation.

strain LS2384, in which the region downstream of the AUG was deleted but all upstream sequences remained intact (Fig. 4A). LS2384 had very little β-galactosidase activity, despite containing the AUG in S3 and all upstream sequences, suggesting that the region downstream of the AUG does function as a translational enhancer.

Transcripts originating at P_{ffs} extend into *dnaX*. Previous work suggested that transcripts originating at the *ffs* gene upstream of the *dnaX* promoter extend partially into the *dnaX* open reading frame (29). Although transcripts originating at P_{ffs} terminate before reaching the end of the *dnaX* gene, when a promoterless *lacZ* gene is fused to the *dnaX* promoter at a site just downstream of the promoter, reporter activity is decreased by 40% when P_{ffs} is deleted (29). To determine the fraction of transcripts originating upstream of P_{dnaX} and extending to the *Asc*I site within the *dnaX* gene, nuclease S1 analysis was carried out (Fig. 5). Previous work, including the construction of promoter fusions, site-directed mutagenesis, nuclease S1 analysis, and primer extension, had shown that no other promoters exist between P_{ffs} and P_{dnaX} (Fig. 1) (28, 29). The S1 probe (Fig. 5A) was designed to be complementary to the region in between *dnaX* and *ffs* but not to be complementary to the mature form of the 4.5S RNA, which is stable and abundant and could compete the probe away from the longer transcripts. The probe was labeled at the 5′ end and hybridized to *Caulobacter* RNA. The results show two major protected products, with sizes of about 195 and 122 bases, as would be expected if transcripts originating at P_{ffs} extended into *dnaX* (Fig. 5B). Densitometry of the bands indicated that the longer product contributed to about 35% of the total protected DNA.

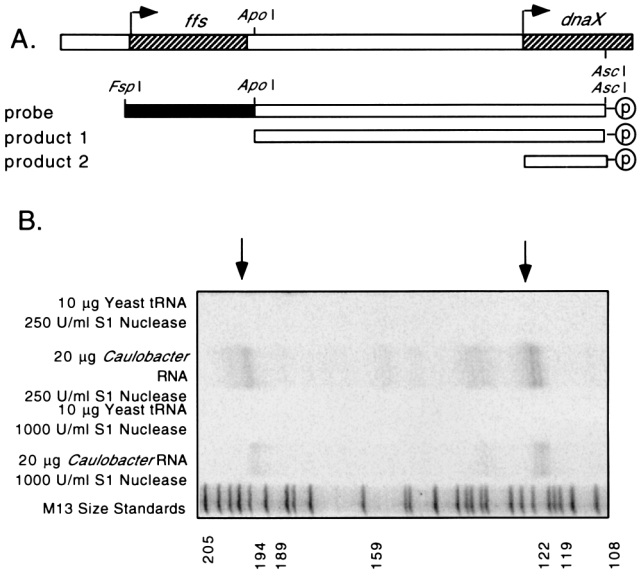

FIG. 5. Nuclease S1 protection assay of the *ffs-dnaX* intergenic region. (A) Diagram showing the locations of the probe and two expected products in the nuclease S1 assay. The probe was prepared by cutting plasmid pEW81.280 with *Asc*I, end labeling the fragment, and then incubating it with *Fsp*I, which cuts in the vector portion of the plasmid. Nonhomologous vector sequence is shown as a solid bar. (B) Autoradiogram of nuclease S1 analysis using the probe shown above and *Caulobacter* or yeast RNA. As a size standard, M13 single-stranded DNA was sequenced by using the −40 primer. Only the T reaction is shown.

Only the mRNA originating at P_{dnaX} is translated. In an attempt to determine the contribution of P_{ffs} to *dnaX* translatable mRNA, we constructed a strain (LS2388) that contained the same translational fusion to β-galactosidase as in strain LS2385 but in which all of P_{ffs} had been deleted (Fig. 4A). β-Galactosidase activity was measured in LS2388 (ΔP_{ffs}) and compared to that of the parent strain, LS2385, containing intact P_{ffs}. The relative amount of β-galactosidase activity in strain LS2388 was the same as that of LS2385, indicating that deletion of P_{ffs} had little effect on the level of activity of the translational fusion. While it is possible that the loss of P_{ffs} might be compensated for by an increase in activity resulting from some other factor such as RNA stability, it seems most likely that the longer mRNA is not translated. This result was confirmed by constructing a translational fusion of *lacZ* to *dnaX* in which P_{dnaX} but not P_{ffs} had been inactivated by a point mutation. Previous work has shown that transcriptional fusions to an isolated P_{dnaX} carrying this mutation have no activity (28). When this mutant promoter was placed in front of the β-galactosidase::DnaX protein fusion (LS2761), no β-galactosidase activity was detected, indicating that the longer transcript is not translated.

The AUG must be placed at the end of the message for recognition by the ribosome. There are several possible reasons why the mRNA originating at P_{ffs} might not be translated. One is that the positioning of the AUG at the 5′ end of the mRNA might be important for recognition by the ribosome in the absence of a Shine-Dalgarno sequence. In the longer transcript originating at P_{ffs}, the AUG would be well buried within the transcript instead of at the 5′ end, as in the case of the shorter transcript. To test if a 5′-terminal AUG is required for translation, we made a derivative of strain LS2385 in which four base pairs, CATG, were inserted 5′ to the CATG of S3 (Fig. 4A). The resulting strain containing this insertion, LS2389, maintained the integrity of P_{dnaX} with respect to the first base in the transcript and maintained the integrity of spacing between the AUG and the downstream box. The amount of activity of LS2389 was statistically indistinguishable from background. The loss of activity could be due to the original AUG becoming nonfunctional when it is moved away from the end of the mRNA or remaining functional but not being utilized when a new out-of-frame AUG, which competes with the original AUG for ribosome binding, is found at the end. In either case, the conclusion that the AUG functions best at the end for optimal ribosome binding is supported. We also tested whether this observed difference could have resulted from accidently destroying the *dnaX* promoter by constructing a fusion of the mutant *dnaX* to *lacZ* containing a native translational start signal (plasmid pEW142 in Fig. 4B). Substantial promoter activity was still present, though activity levels were reduced (compare to pEW141). The observed decrease in β-galactosidase activity is most likely due to the polar effect of the insertional mutation on translation of the *lacZ* mRNA. This experiment suggests that the placement of the AUG at the very beginning of the mRNA is critical for ribosomal recognition and provides a likely rationale for why the longer mRNA is not translated. An additional reason why the mRNA originating at the *ffs* promoter might not be translated is that the RNA from the intergenic region between the end of the 4.5S RNA and the beginning of *dnaX* has the potential to form a strong hairpin structure with a free energy value of −43 kcal/mol. In this case, the AUG and downstream box might be base paired within the mRNA structure and thus be unavailable for interaction with the 16S rRNA.

Temporal expression of *dnaX* is not modulated by translational control. We have shown that the *dnaX* promoter is more active in the stalked cell portion of the cell cycle, coincident with the time period of DNA replication (28). Because translation of the *dnaX* mRNA is unusual, we compared temporal expression pattern of a transcriptional fusion of P_{dnaX} to a promoterless *lacZ* gene containing its own Shine-Dalgarno sequence to that of a translational fusion of P_{ffs} and P_{dnaX} to a *lacZ* gene, lacking both a promoter and a Shine-Dalgarno sequence. Cultures of strain LS107 containing a *dnaX*::*lacZ* transcriptional fusion on plasmid pEW111.U or strain LS2387, containing a chromosomal copy of the *dnaX*::*lacZ* translational fusion, were synchronized and allowed to progress through the cell cycle. Aliquots were removed at times indicated in Fig. 6 and pulse-labeled with [^{35}S]methionine. Newly synthesized β-galactosidase was immunoprecipitated from these extracts and visualized following separation by SDS-PAGE. Autoradiography (Fig. 6A) and densitometry (Fig. 6B) of these results indicate that the temporal expression of the fusion protein is virtually identical to expression of the native β-galactosidase containing its own Shine-Dalgarno sequence. Therefore, we conclude that the unusual translation mechanism of *dnaX* does not contribute to the cell cycle expression pattern of this gene.

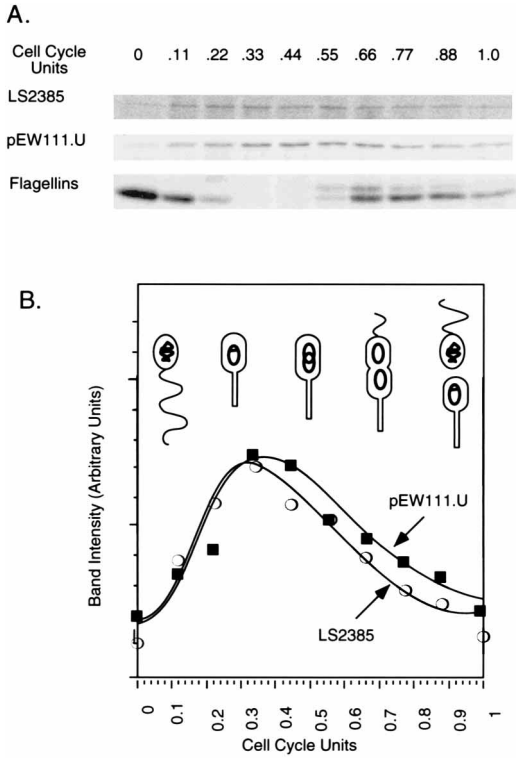

FIG. 6. Cell cycle-dependent expression of *dnaX*. (A) Immunoprecipitation of a β-galactosidase from a *lacZ* reporter gene in cell cycle extracts of strain LS107 containing a transcriptional fusion of *lacZ* to P_{dnaX} alone (pEW111.U) and LS2385 containing a chromosomal translational fusion of *lacZ* to P_{ffs} and P_{dnaX}. Immunoprecipitation of flagellin protein is shown as an internal control of cell synchrony. Isolation of synchronous swarmer cells and immunoprecipitations were performed as described in Materials and Methods. (B) Schematic diagram of the *Caulobacter* cell cycle relative to a graph showing quantitation of bands shown in panel A. Values were determined by integrating bands on a Molecular Dynamics PhosphorImager. For purposes of comparison, values were normalized and are intended to reflect patterns of temporal activity, not absolute levels of activity.

DISCUSSION

Almost all translated mRNAs in *E. coli* carry a 5′ untranslated leader sequence which includes a Shine-Dalgarno se-

quence 5 to 15 bp upstream of the initiation codon. Shine-Dalgarno sequences are complementary to the 3′ end of 16S rRNA in the 30S ribosomal subunit and thus are thought to facilitate recognition of the mRNA by the ribosome (24). The region downstream of the mRNA start site is also thought to play some role in allowing the ribosome to recognize the mRNA. In general, genes that are very efficiently translated have a less than random distribution of bases in the region 5 to 20 bp downstream of the initiation codon (22). This region, referred to as the downstream box, has been shown to be important for the efficient translation of some prokaryotic mRNAs (7, 17, 25). In most cases, the downstream box functions to enhance translation but not to support it entirely. In a few cases, however, mRNAs lacking a leader sequence are thought to be recognized by the ribosome solely through interactions with the AUG and downstream sequences (11, 13, 23). In these cases, the initiation codon is the first three bases in the mRNAs. In the case of the *aph* mRNA from *Streptomyces griseus*, as with the *Caulobacter* P_{dnax} mRNA, moving the position of the AUG four bases away from the transcriptional start site stops translation initiation in that frame (11). These data imply that in the absence of a Shine-Dalgarno sequence, the presence of a leader sequence interferes with recognition at the downstream box. This conclusion was also reached in studies of translation of the λ *c*I repressor leaderless-mRNA produced from the Prm promoter (23). It was shown that mutant ribosomes with decreased amounts of S2 protein were more capable of recognizing leaderless transcripts than were wild-type ribosomes. The presence of a leader sequence actually inhibited the ability of these mutant ribosomes to recognize the mRNA. We have found that deleting the *dnaX* downstream box lowers translational efficiency but does not appear to entirely abolish translation. However, moving the AUG away from the beginning of the transcript does abolish all translation, supporting the idea that position is more important than the downstream sequence. For translation of leaderless mRNAs from *Streptomyces*, sequences downstream of the AUG are also of lesser importance than the positioning of the AUG at the beginning of the transcript (12).

The translation of a leaderless mRNA has been observed for another *C. crescentus* gene, *hemE*, which encodes an enzyme involved in heme biosynthesis (14). *hemE* is of particular importance because it is found adjacent to the minimal *Caulobacter* origin of replication, and the promoter region has been implicated in the initiation of DNA replication. *hemE* also has a region downstream of the AUG that has the potential to base pair with the 16S rRNA. As for *dnaX*, expression of *hemE* mRNA is produced from two promoters, a strong distal promoter and a weak proximal promoter. Like P_{dnax}, the transcript produced by the weak *hemE* promoter starts with an AUG, which is also the first codon of the protein. The *hemE* strong promoter is cell cycle regulated and is about fivefold more active in the stalked cell portion of the cell cycle. This promoter is one of the strongest *Caulobacter* promoters known, but evidence indicates that most of the mRNA originating at this promoter, like the mRNAs originating at the strong P_{ffs}, are not translated (14). Instead, the RNA originating at the *hemE* strong promoter has been proposed to play a critical role in initiation of DNA replication either by priming the DNA at the origin of replication or by facilitating the formation of the replisome.

Why should the *Caulobacter* cell utilize an alternative mechanism for the initiation of translation? The most probable reason may be that a gene producing a stable RNA is located upstream of *dnaX*, and on the basis of nuclease S1 analysis, about 35% of transcripts reaching the *Asc*I site of the *dnaX* open reading frame originate at P_{ffs}. Because the transcripts originating at P_{ffs} do not extend the length of the *dnaX* gene (29), it would be inefficient for the cell to produce the truncated proteins which would result from translation of mRNAs originating at the *ffs* promoter. While the 3′ end of the *hemE* strong promoter mRNA has not been mapped, the data support the idea that transcript originating at the strong promoter also does not extend completely through the *hemE* gene and terminates prematurely (14), analogous to the way the P_{ffs} message terminates within *dnaX*. Because, in these cases, efficient translation occurs only when the AUG is located at the end of the mRNA, the ribosome appears to be able to distinguish between what would be productive and nonproductive translation events.

We propose that translation of leaderless messages may be more common in *Caulobacter* than in *E. coli*. One reason for this may be that *Caulobacter* is adapted to living in low-nutrient conditions, and thus the organism may need to be more parsimonious with its resources than *E. coli*. Steps toward economy might include placing promoters under cell cycle control so that gene products are made only when they are required. Another step might include only translating mRNAs that encode full-length proteins. By demanding that the AUG be placed at the end of the mRNA, the cell is able to prevent translation of truncated messages or message fragments and to prevent translation of messages that might originate at an upstream constitutively active promoter. The translation of leaderless messages is also more commonly observed in two other classes of organisms: *Streptomyces* species (1, 10, 11) and bacteriophage (23). The value of leaderless transcripts to bacteriophage can be rationalized by the selective pressure to maintain as small a genome as possible. In the case of *Streptomyces* species, which, like *Caulobacter*, are adapted to living in low-nutrient conditions and have complex developmental patterns, translation of leaderless messages might provide a means by which the organism can differentiate between transcripts originating at proximal developmentally regulated promoters and distal, constitutively active promoters.

ACKNOWLEDGMENTS

We thank members of the Shapiro group for careful reading of the manuscript.

This work was supported by NIH grants GM51426 and GM32506/512OMZ. E.A.W. was supported by NIH training grant 2T32GM07790.

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