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## Identification of a weak promoter for the *dam* gene of *Escherichia coli*

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We have used a combination of techniques to identify a weak promoter located about 70 nucleotides before the start site of translation of the *Escherichia coli dam* gene which encodes a DNA methyltransferase. The promoter activity was identified by the use of *lacZ* fusions to fragments containing different lengths of upstream DNA. In vitro run-off transcription and primer extension determinations revealed transcription initiation sites at either 69 or 73 nucleotides prior to the ATG of the *dam* coding sequence. No ribosome binding sequence was present close to the ATG codon suggesting that the transcript may be inefficiently translated.

### Introduction

The *dam* gene of *Escherichia coli* is located at min 74 on the genetic map [1,2]. This gene encodes a DNA methyltransferase which modifies adenines in -GATC- sequences in double-stranded DNA to produce 6-methyladenine. The substrate -GATC- sequences are located on the newly synthesized DNA chain and there is a lag period between synthesis and subsequent modification. This delay in modification is sequence dependent and can vary from less than 1.5 min to 9.5 min [3].

The hemi-methylated DNA immediately following the replication fork is different from bulk DNA which is fully methylated. This difference is exploited by various systems in the cell including repair of replication errors, alteration of gene expression as well as initiation of chromosome replication and segregation (reviewed in Refs. 4–9). The efficiency of these processes is dependent on the level of Dam methyltransferase in the cell; high levels of methyltransferase can interfere with the above processes. For example, overproduction of Dam methyltransferase increases spontaneous mutability due to decreased repair [10,11] and causes asynchronous initiation of chromosome replication [12].

Conversely, cells with reduced amounts of Dam

methyltransferase exhibit increased spontaneous mutagenesis and asynchronous initiation of chromosome replication [12,13]. In addition, an increased induction of lysogenic phages, increased breaks in DNA, increased transposition of Tn5 and Tn10, increased expression of certain genes and lethality in cells deficient in DNA repair are observed [6]. The level of Dam methyltransferase in the cell is thus critical for a variety of processes.

The level of Dam methyltransferase can be varied by the use of multicopy plasmids which generally increase enzyme activity due to increased plasmid copy number. An exception is *pdam118* which was used to establish the nucleotide sequence of the *dam* gene [14]. Although this plasmid is a pBR322 derivative, the level of Dam methyltransferase in the cell is only twice as high as that of a wild type cell suggesting that only a weak promoter is present in the 119 nucleotides of chromosomal DNA contained on plasmid *pdam118* and preceding the *dam* gene [15]. In this communication, we characterize this promoter.

### Materials and Methods

#### Bacterial strains

Strain NK5031 (del *lacM5265* SuIII<sup>+</sup> Nal<sup>R</sup>) was obtained from N. Kleckner (Harvard University). GM4707 is a *pcnB* derivative of NK5031 prepared by P1 transduction of the closely linked Tn10 from strain MRi93 [16]. Transductants were screened for the *pcnB* marker as described [16]. Other *E. coli* strains used

were AB1157 [17], GM3819 (del *dam-16*, [17]); and LJ24 [18], a gift of L.J. Rasmussen. Strain ALO454 is a *dnaA46 tna::Tn10* derivative of LJ24 and was obtained from A. Lobner-Olesen. Plasmid pMQ184 was constructed by inserting a *PstI-SalI* fragment containing the *dam* gene into the same sites of pEMBL8(-) [19].

### Methods

**Construction of *lacZYA* fusions.** Operon fusions were constructed as described by Simons et al. [20]. DNA fragments from pMQ133 [15] were isolated from polyacrylamide gels [21] and subcloned into pRS415 [20]. The derivatives containing the *PvuII-EcoRV*, *PvuII-NlaIII*, *PvuII-BstBI* and *BstBI-EcoRV* fragments (Fig. 1) were designated pMQ235, pMQ290, pMQ277 and pMQ276, respectively. Strains NK5031 and GM4707 were transformed by these plasmids [22] to yield high copy number and low copy number plasmid derivative strains, respectively. The strain bearing pMQ235 was infected with  $\lambda$  RS45 [20] and  $\lambda$  *lacZ*-fusion derivatives were identified and characterized [20]. Eight lysogens were assayed for  $\beta$ -galactosidase activity [23] to ensure that only a monolysogen was selected. The phage containing the promoter fusion was designated  $\lambda$  GM104.

**RNA polymerase gel retardation assays.** Various amounts of RNA polymerase (0.5–2.0 units; New England Biolabs) were mixed with 0.5  $\mu$ g of plasmid DNA in binding buffer (50 mM KCl, 10 mM MgCl<sub>2</sub>, 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol (DTT), 4% glycerol) for 10 min at 37°C. Enzymes *PstI* and *EcoRV* (1.5 units of each; New England Biolabs) were added and incubation continued for 30 min at 37°C before addition of stop mix (0.1% bromophenol blue, 50% glycerol, 100 mM EDTA) to terminate digestion. The fragments were separated on a 1.5% agarose gel in TGE buffer (25 mM Tris, 190 mM glycine, 1 mM EDTA). The gel was stained with ethidium bromide and photographed under short wavelength ultraviolet light using Polaroid 55 positive/negative film.

**In vitro transcription.** Restriction fragments (*PstI-BamHI*; *PstI-EcoRV*; *Fnu4HI*) were obtained after digestion of pMQ184 and purified from polyacrylamide gels [21]. These were used as templates for in vitro transcription [24] and transcripts were labelled with either [ $\alpha$ -<sup>32</sup>P]CTP or [ $\gamma$ -<sup>32</sup>P]GTP (New England Nuclear) and separated on 8% polyacrylamide/7 M urea gels and visualized by autoradiography. The length of the transcripts was estimated by comparing them to size markers run on the same gel.

**Initiation of transcription with dinucleotides.** The 5'-terminal dinucleotides of the in vitro transcripts were determined essentially as described [25]. The purified *Fnu4HI* fragment from pMQ184 was incubated with RNA polymerase (2 units; New England Biolabs) and 500  $\mu$ M priming dinucleotide (GpU, GpA, GpC or ApG; Sigma) for 5 min in transcription buffer [24]. The

four rNTPs, including [ $\alpha$ -<sup>32</sup>P]CTP, were added to 50  $\mu$ M and incubated for a further 3 min. Rifampicin (30  $\mu$ g/ml) and rNTPs (500  $\mu$ M) were added to extend existing transcripts and to prevent initiation of new ones. The reaction was terminated after 30 min and the products resolved on 8% acrylamide/7 M urea gels as described above.

**Primer extension assay.** Two different 20-mer DNA primers were used. These were designated primers P1 (5'-GCACCTACAAAAGGCTCAAC-3') and P2 (5'-ATAGAGACTGATCAGGTCGC-3'; see map in Fig. 6). RNA was extracted from *E. coli* [26] and 50  $\mu$ g of RNA was mixed with either 2.5 or 0.25 pmol primer, as indicated, in 5  $\mu$ l of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, before denaturation at 70°C for 5 min and annealing at 42°C for 2 h. RNasin (15 units; Promega) was present during annealing. 9  $\mu$ l of extension mix was then added which contained 30  $\mu$ M each of dATP, dGTP and dTTP, 10 mM DTT, 15 units of RNasin, 10  $\mu$ Ci (3.3 pmol) of [<sup>32</sup>P]dCTP and 2.5 units of AMV Reverse Transcriptase (Boehringer-Mannheim), all in 1.5  $\times$  annealing buffer. Primer extension was allowed to proceed for 10 min at 42°C before addition of all four dNTPs each at 2.5  $\mu$ M. Incubation at 42°C was continued for 60 min. This pulse-chase regimen increases the radioactive labelling and allows the detection of weak promoters. Primer extension was terminated by transfer to 70°C for 3 min and onto ice for 5 min. RNase A (1  $\mu$ g in 1  $\mu$ l) was added and the digestion terminated after 5 min by addition of 11  $\mu$ l of 99% formamide. Extension products were resolved on 6.6% polyacrylamide/7 M urea gels and visualized by autoradiography. Product sizing was performed by comparison with a dideoxy sequencing reaction [27] run in parallel on the same gel.

### Results

#### Promoter activity in *lacZ* fusion vectors

A 506 bp *PvuII-EcoRV* chromosomal DNA fragment containing 380 bp upstream of the *dam* gene (Fig. 1) was tested for promoter activity in *lacZYA* operon fusion vectors with different plasmid copy num-

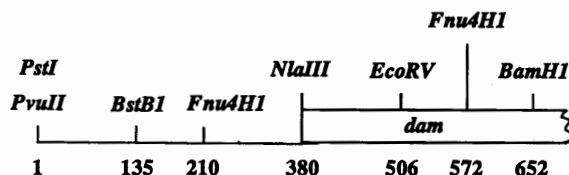


Fig. 1. Restriction map of the chromosomal DNA preceding and including the proximal part of the *dam* gene of *E. coli*. Restriction endonuclease sites are indicated and numbered from the *PstI* site. The sequence information was obtained from the GenBank Ecodam file (V00272).

TABLE I

*β*-galactosidase activity from various plasmid constructs containing chromosomal DNA

| Fragment                      | Size (bp) | <i>β</i> -Galactosidase (units)       |                                      |   |
|-------------------------------|-----------|---------------------------------------|--------------------------------------|---|
|                               |           | high copy number plasmid <sup>a</sup> | low copy number plasmid <sup>b</sup> | single copy number plasmid <sup>c</sup> |
| None <sup>d</sup>             | 0         | 6                                     | 4                                    | 1                                       |
| <i>Pvu</i> II- <i>Eco</i> RV  | 506       | 378                                   | 42                                   | 12                                      |
| <i>Pvu</i> II- <i>Nla</i> III | 380       | 147                                   | 13                                   | –                                       |
| <i>Pvu</i> II- <i>Bst</i> B1  | 135       | 18                                    | 4                                    | –                                       |
| <i>Bst</i> B1- <i>Eco</i> RV  | 371       | 330                                   | 46                                   | –                                       |

<sup>a</sup> High copy number plasmids are derivatives of plasmid pBR322.

<sup>b</sup> Low copy number plasmid activity was determined in a *pcnB* derivative of the strain used to assay high copy plasmid activity.

<sup>c</sup> Single copy plasmids were phage  $\lambda$  lysogens.

<sup>d</sup> Activity from the vector plasmids without insert DNA.

bers. As shown in Table I, a pBR322 derivative produces 378 units of *β*-galactosidase in a wild type strain but only 42 units in a *pcnB* derivative in which copy number is reduced to 3–4 per chromosome [16]. In a single copy  $\lambda$  vector, the 506 bp chromosomal fragment produces 12 units of activity compared to 1–2 units for the control  $\lambda$  vector lacking any chromosomal DNA (Table I).

To determine more precisely the location of the promoter region, we measured *β*-galactosidase activity in three further subclones. The results in Table I indicate that promoter activity was found in the *Bst*B1-*Eco*RV and *Pvu*II-*Nla*III fragments but not in the *Pvu*II-*Bst*B1 fragment. This places the promoter activity between the *Bst*B1 site and beginning of the gene (Fig. 1).

The promoter activity from the *Pvu*II-*Nla*III fragment was consistently lower, in both high and low copy number plasmids, than that for the other fragments containing promoter activity. (Table I). At present the significance of this reduction is not known. It is possible that some feature of the DNA sequence in the first part of the *dam* gene aids transcription initiation.

#### RNA polymerase gel retardation assays

Plasmid pMQ184 is a derivative of pEMBL8(–) which contains the *dam* gene on a 1923 bp *Pst*I-*Sal*I fragment. This plasmid and pBR322, which served as the control, were each incubated with various amounts of RNA polymerase after which the plasmids were digested with various restriction endonucleases to identify RNA polymerase binding sites. A 376 bp fragment

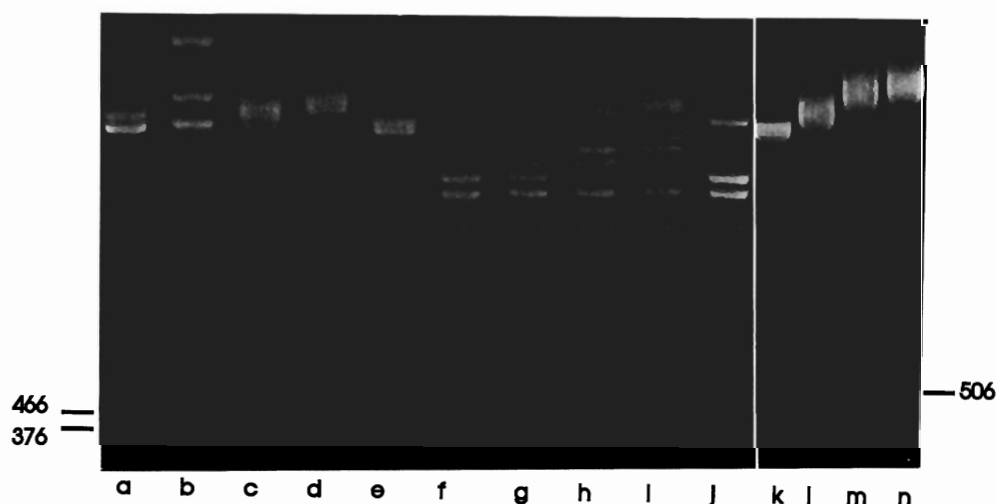


Fig. 2. RNA polymerase binding as measured by gel retardation. Plasmid DNA (pBR322, lanes a–j; pMQ184, lanes k–n) was incubated with RNA polymerase, 0.5 unit (lanes a, f, l), 1.0 unit (lanes b, g, m), 2.0 units (lanes c, h, n) and 0 units (lanes e, j, k) for 10 min (pMQ184) or 30 min (pBR322) at 37°C. The DNA was then restricted with *Eco*RI and *Ssp*I (lanes a–e), *Eco*RI and *Acc*I (lanes f–j), *Pst*I and *Eco*RV (lanes k–n). The fragments were separated on a 1.5% agarose gel. The numbers to the left of the figure indicate the 376 and 466 bp pBR322 fragments and the number to the right of the figure indicates the 506 bp fragment from pMQ184.

of pBR322 known to bear the *bla* and *tet* promoters (*Ssp*I-*Eco*RV) was retarded after incubation with the enzyme (Fig. 2, lanes a-e), whereas a 466 bp fragment (*Eco*RV-*Acc*I) devoid of promoter activity showed no measurable retardation (Fig. 2, lanes f-j). The 506 bp *Pst*I-*Eco*RV fragment (Fig. 1) containing the putative *dam* gene promoter (Table I) was also retarded (Fig. 2, lanes k-n) confirming the presence of an RNA polymerase binding site.

#### *In vitro* transcription

Run-off transcripts were obtained from various restriction fragments after incubation with RNA polymerase, to identify the location of the promoter (Fig. 3). The *Pst*I-*Bam*HI, *Pst*I-*Eco*RV, and *Fnu*4HI fragments (Fig. 1) yielded transcripts of 390, 240 and 270 nucleotides, respectively (Fig. 3A, lanes 1-3) indicating

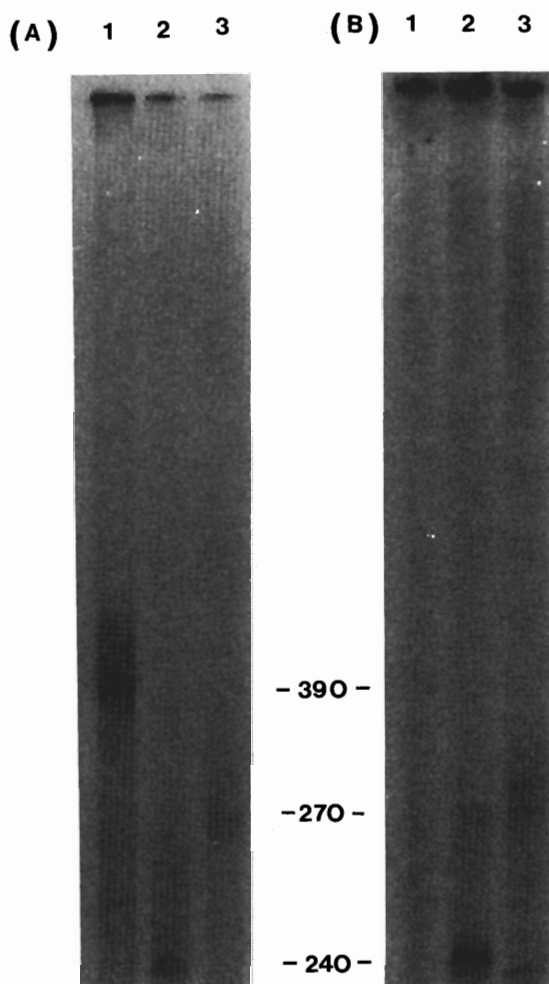


Fig. 3. Run-off transcripts from fragments containing the promoter. (A) Fragments *Pst*I-*Bam*HI (lane 1), *Pst*I-*Eco*RV (lane 2), *Fnu*4HI (lane 3) were incubated with RNA polymerase in the presence of NTPs (including  $[\alpha\text{-}^{32}\text{P}]\text{CTP}$ ) and the products separated on a 8% polyacrylamide/7 M urea gel. (B) Same as (A) but the transcripts were labelled with  $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ . The numbers at the side of the gel indicate the length of the transcripts.

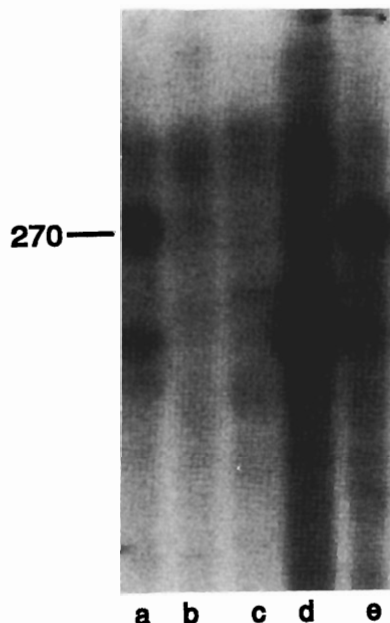


Fig. 4. Initiation of transcription with dinucleotides. The *Fnu*4HI fragment was incubated with RNA polymerase in the presence of 500  $\mu\text{M}$  priming dinucleotide for 5 min after which dNTPs at 50  $\mu\text{M}$  (including  $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ ) were added. 3 min later, rifampicin and 500  $\mu\text{M}$  dNTPs were added and the reaction allowed to continue for 30 min. Lane a, GpU; lane b, GpA; lane c, GpC; lane d, ApG; lane e, no dinucleotide. The number to the left of the gel indicates the size of the transcript.

that the promoter was located between the *Fnu*4HI site and the beginning of the *dam* gene. The data in Fig. 3B show that the transcript begins with a guanosine residue since it was labelled with  $[\gamma\text{-}^{32}\text{P}]\text{GTP}$  but not  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . When various dinucleotides were used to label the 5' end of the message, a strong band was detected with the GpU dinucleotide (Fig. 4, lane a) at the same position as the control transcript (Fig. 4, lane e). A weak band was also seen with the ApG dinucleotide (Fig. 4, lane d) but since the transcript begins with a guanosine (Fig. 3) this band must be non-specific. That the transcript begins with GpU together with the length of the run-off transcripts (Fig. 3) allowed us to assign the transcript start to 70 nucleotides upstream of the ATG codon as shown in Fig. 6.

#### Primer extension experiments

Extension of primer P1 resulted in several products (Fig. 5, lane A). The addition of lower amounts of primer, however, showed that only a 187 nucleotide extension product appeared to be primer specific (Fig. 5, lane B). Similarly, primer P2 gave several products and dilution of the primer showed that a 263 nucleotide extension product was specific (Fig. 5, lanes C and D). Primer P2 did not hybridize to RNA from strain GM3819 which contains a deletion of the *dam* gene and therefore no sequence complementary to it

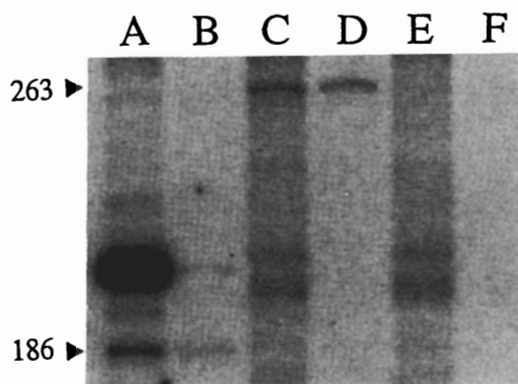


Fig. 5. Primer extension of RNA isolated from *dam*<sup>+</sup> and *dam*<sup>-</sup> cells. Primer extension products were electrophoresed on a polyacrylamide gel and subjected to autoradiography. 60  $\mu$ g of RNA isolated from strains AB1157 (lanes A through D) or GM3819 (lanes E and F) was hybridized with primer 1 (lanes A and B) or primer 2 (lanes C through F). The amount of primer used was 2.5 pmol (lanes A, C, E) or 0.25 pmol (lanes B, D, F) and the primers were extended by 2.5 U of AMV Reverse Transcriptase. A DNA sequencing reaction was run in parallel (not shown) allowing sizing of the extension products (indicated on the left side, in base pairs). The intense blob in lane A is not relevant.

(Fig. 5, lanes E and F). The results with both primers indicate a start site for transcription *in vivo* at the nucleotide with an exclamation mark in Fig. 6.

#### Transcription in a *dnaA46* strain

It has been reported that the *dam* gene is negatively regulated by the DnaA protein [28] presumably through DNA binding of the protein at a putative DnaA box located about 30 nucleotides upstream of the ATG codon (Fig. 6). We tested the effect of the *dnaA46* allele on production of  $\beta$ -galactosidase from a single copy  $\lambda$  prophage *lacZ* fusion containing the 506 bp chromosomal DNA fragment containing promoter ac-

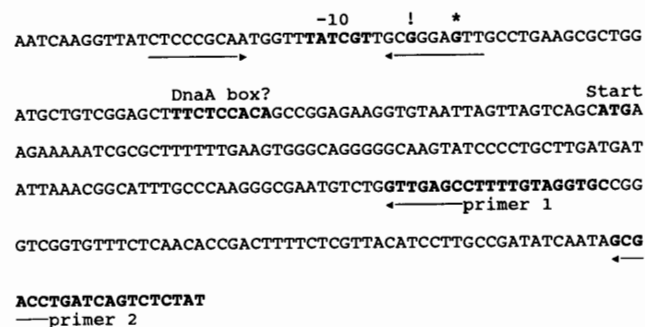


Fig. 6. Landmarks in the nucleotide sequence. The mRNA start site determined *in vivo* (!) and *in vitro* (\*) are indicated together with a putative -10 sigma 70 recognition sequence. The arrows under the first line indicate inverted repeats. On the second line are the possible DnaA box and the translation initiation codon. Sequences complementary to those of primers 1 and 2 used in the primer extension experiments are also indicated. All these landmarks are shown in bold type.

TABLE II

*$\beta$ -galactosidase activity from single-copy plasmids in wild type (LJ24) and *dnaA46* (ALO454) strains*

Each strain was lysogenized with a  $\lambda$  phage containing the 506 bp *PvuII-EcoRV* fragment fused to the *lacZYA* genes. Cells were incubated for 60 min at 42°C before sampling for enzyme assays.  $\beta$ -Galactosidase values did not deviate by more than 10%.

| Strain        | $\beta$ -Galactosidase activity |
|---------------|---------------------------------|
| LJ24 (30 C)   | 12                              |
| LJ24 (42 C)   | 12                              |
| ALO454 (30 C) | 129                             |
| ALO454 (42 C) | 103                             |

tivity. The *dnaA46* mutation is conditionally lethal and produces a protein which is only partially active at the permissive temperature [29]. The data in Table II indicate that there was a significant difference in  $\beta$ -galactosidase production between the *dnaA46* mutant lysogenic strain and its wild type parent at both permissive and non-permissive temperatures. The *tna::Tn10* mutation in the *dnaA46* strain was not responsible for this effect (data not shown).

#### Discussion

Our results indicate that a weak promoter is present about 70 nucleotides upstream of the *dam* gene. Since a fully induced *lac* promoter would be expected to yield about 1000  $\beta$ -galactosidase units, the upstream *dam* promoter has only about 1% the strength of the *lac* promoter. When present on a multi-copy plasmid such as pBR322 derivatives, the *dam* structural gene transcribed from only this promoter is sufficient to provide wild type levels of Dam methyltransferase [30]. In single copy, however, it is probably too weak to provide the necessary level of enzyme during logarithmic growth. This indicates that the *E. coli* chromosome must contain additional promoters upstream of the sequence we have examined. Recent results (Ref. 28; A. Lobner-Olesen, E. Boye and M.G. Marinus, unpublished data) indicate that this is indeed the case.

The *in vitro* transcription and primer extension experiments indicated two start sites for RNA transcription which are separated by three bases (Fig. 6). The *in vitro* start site is preceded by a reasonable -10 region (TATCGT) similar to that for  $\sigma$ -70 RNA polymerase promoters (TATAAT) but does not appear to have a discernable -35 region. This may be the basis for the observed weak promoter strength. The start site determined by primer extension, in contrast, has no discernable -10 region but a weak -35 region (TAATCA) compared to consensus (TTGACA). We have not found any significant homology to other *E. coli* sigma factor binding sites in this region. The sequence containing

both the transcriptional start sites is part of an inverted repeat (Fig. 6). At present we do not know if this feature has any significance for transcription initiation.

The closest ribosome-binding-sequence appears as a polypurine sequence 17 nucleotides prior to the start of the *dam* gene (Fig. 6). This distance is probably too great to allow ribosomes to load onto the message suggesting that the transcripts from this weak promoter will be inefficiently translated.

In agreement with the results obtained by Jonczyk et al. [28], an increase in expression of the promoter region was observed in a *dnaA46* strain. It has been reported that the DnaA protein can modulate gene expression by blocking transcribing RNA polymerase at *dnaA* boxes [31]. A problem with our results, however, is that the same amount of  $\beta$ -galactosidase was produced at both 30°C and 42°C in the *dnaA46* strain. We expected to see a larger difference in activity at the two temperatures if the only effect of DnaA was to act as a repressor. This may mean that some feature other than DNA binding is involved. We are currently altering the DNA sequence of the *dnaA* box by site-directed mutagenesis in order to evaluate further the effect of DnaA protein on gene expression.

The weak promoter described here may have a physiological function in cells that are growing slowly or which are entering stationary phase. Under these conditions, a small amount of enzyme may be enough to satisfy physiological requirements. To evaluate the physiological role for this promoter, however, it will be necessary to place a transcriptional terminator between it and the *dam* gene on the chromosome. Such experiments are currently in progress.

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