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# Identification and characterization of a second, inducible promoter of *relA* in *Escherichia coli*

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The alarmone ppGpp is an important signal molecule for the stringent response. *Escherichia coli relA* encodes a ppGpp synthetase, and although the regulation of RelA protein activity has been studied extensively, the regulation of *relA* transcription remains unclear. Here, we describe a novel *relA* promoter, *relA*P2. According to quantitative measurement of mRNA by primer extension analysis, the previously reported promoter *relA*P1 is constitutively active throughout growth, while *relA*P2 is induced temporarily at the transition state between the exponential growth and stationary phases. A chromosomal transcriptional *lacZ* fusion (*relA*P2-*lacZ*) showed that *relA*P2 is positively regulated by H-NS and CRP. Furthermore, the reduced activity of *relA*P2-*lacZ* in an *hns* mutant could be rescued by an *rpoS* mutation, which is sufficient to derepress the *relA*P2-*lacZ* activity. These data suggest that transient expression from the *relA*P2 promoter is controlled by several global regulators. This may account for the complex regulation of *relA* expression in *Escherichia coli*.

**Key words:** CRP, growth-dependent promoter, H-NS, *relA*, RpoS

## INTRODUCTION

As it enters stationary phase, *Escherichia coli* must regulate precisely the expression of many genes to adapt to environmental changes: genes involved in replication are repressed, while those required for stress responses are induced. Changes in expression patterns are observed at the transcriptional level in the transition state between logarithmic growth phase and entry into stationary phase (Hengge-Aronis, 1996). Global regulators, including histone-like nucleoid structuring protein (H-NS), factor for inversion stimulation (Fis), leucine-responsive regulatory protein (LRP), integration host factor (IHF), cAMP receptor protein (CRP; also known as CAP), RpoS, and guanosine tetraphosphate (ppGpp), are responsible for this transcriptional reprogramming. For example, the *rrn* P1 promoter (the major promoter of *rrn* operon encoding rRNA) is activated by Fis and repressed by H-NS and ppGpp (Schneider et al., 2003). During log phase, the *rrn* P1 promoter is strongly activated by Fis. Fis protein levels decrease during the transition to stationary phase (Nilsson et al., 1992), whereas H-NS increases at the transition state (Ali Azam et al., 1999). In addition, ppGpp accumulates during the transition to stationary phase (Cashel et al., 1996). These factors reduce *rrn* P1 promoter activity at the transition state. Thus, the expression from the *rrn* P1 promoter is fine-tuned by multiple global regulators at the transition state. Although some aspects of regulation by multiple global regulators have been studied (e.g. Browning et al., 2004; Germer et al., 2001; Johansson et al., 1998; Schneider et al., 2003; Soutourina et al., 1999), our current understanding of the complex mechanism of transcriptional reprogramming at the transition state is incomplete.

The cAMP-CRP complex regulates the expression of more than 200 genes in response to carbon source condition. At the molecular level, CRP is activated to bind to target sites on DNA (consensus: TGTGA-6 bp-TCACA; Ebright et al., 1989) by cAMP binding, enhancing RNA polymerase (RNAP) recruitment to the promoter and initiation of transcription.

H-NS can bind directly to DNA, preferentially to curved sequence, making the topology of DNA supercoiled and acting mainly as a repressor (Dorman, 2004; Tupper et al., 1994). H-NS represses various kinds of genes, including those related to environmental changes such as cold shock or osmotic stress (Dersch et al., 1994; Hommais et al., 2001; Vrentas et al., 2005), by altering DNA topology (Shin et al., 2005) and/or occupying their pro-

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Table 1. Bacterial strains and plasmids

| Name | Description | Reference |
|---|---|---|
| ***Escherichia coli* K-12 strains** | | |
| MC4100 | *Δ(argF-lac)U169 rpsL150 relA1 araD139 flbB5301 deoC1 ptsF25* | Casadaban 1976 |
| AN627 | MC4100 Φ(8relAupM-*lacZ*) | This study |
| AN623 | MC4100 Φ(5relAupM-*lacZ*) | This study |
| AN506 | MC4100 Φ(P1-*lacZ*) | This study |
| AN624 | MC4100 Φ(1relAupM-*lacZ*) | This study |
| AN526 | MC4100 Φ(P2-*lacZ*) | This study |
| AN507 | MC4100 Φ(P1m10-*lacZ*) | This study |
| AN508 | MC4100 Φ(P1m35-*lacZ*) | This study |
| AN616 | MC4100 Φ(P2m10-*lacZ*) | This study |
| AN731 | MC4100 Φ(P2m35-*lacZ*) | This study |
| AN634 | MC4100 Φ(CRPdown-*lacZ*) | This study |
| AN635 | MC4100 Φ(CRPup-*lacZ*) | This study |
| AN529 | AN526 *hns-205* | [a]Yamashino et al., 1995 |
| AN543 | AN526 *crp*::Km$^r$ | [a]Baba et al., 2006 |
| AN557 | AN526 *rpoS*::Tet$^r$ | [a]Yamada et al., 1991 |
| AN558 | AN529 *rpoS*::Tet$^r$ | [a]Yamada et al., 1991 |
| W3110 | Wild-type (lineage A) | Jishage and Ishihama, 1997 |
| AN582 | W3110 transformed with p8M | This study |
| AN682 | W3110 *rumA*::Km$^r$ | [a]Baba et al., 2006 |
| AN718 | W3110 with -10 box mutation of *relA*P2 | This study |
| AN766 | W3110 with -10 box mutation of *relA*P1 | This study |
| AN767 | W3110 with -10 box mutation of both *relA*P1 and *relA*P2 | This study |
| BL21(DE3)pLysS | F$^-$ *ompT hsdS*$_B$(r$_B^-$ m$_B^-$) *gal dcm* pLysS | Novagen |
| MAN001 | BL21(DE3)pLysS transformed with pCRP-His | This study |
| **Plasmids** | | |
| pMKt1403 | *lacZ* operon fusion plasmid | M. Kawano |
| p8M | pMKt1403 containing 8relAupM-*lacZ* | This study |
| pET29b | expression vector | Novagen |
| pCRP-His | pET29b containing *crp* open reading frame | This study |
| pSTV29 | cloning vector | Takara |
| pUC19-*hns* | pUC19 containing *hns* open reading frame and the promoter | T. Mizuno |
| pHNS | pSTV29 containing *hns* open reading frame and the promoter | This study |
| pKO3 | mutation introducing vector | Link et al., 1997 |
| prelAP2m10HR | pKO3 containing the *relA* promoter region including P2m10 mutation | This study |
| prelAP1m10P2m10 HR | pKO3 containing the *relA* promoter region including P1m10 and P2m10 mutations | This study |

[a] The sources describe the origins of the strains from which new mutations were constructed by P1 transduction.

moter regions (Dorman, 2004). H-NS accumulates at the transition state (Ali Azam et al., 1999), and regulates growth phase-dependent promoters (e.g. Afflerbach and Schroder, 1998; Chiuchiolo et al., 2001). Thus it is conceivable that H-NS is involved in transcriptional reprogramming at transition state via remodeling of nucleoid organization.

RpoS is an RNAP (RNA polymerase) σ factor that activates numerous genes under various stress conditions and during the transition state to the stationary phase (Tanaka et al., 1993). Although transcription of *rpoS* mRNA is observed during log phase, RpoS protein is stabilized and accumulates only after the transition state (Lange and Hengge-Aronis, 1994). While RpoS recog-

nizes stationary phase-inducible promoters, some genes regulated by house-keeping sigma factor $\sigma^{70}$ are negatively controlled by RpoS (Jishage et al., 2002; Patten et al., 2004), perhaps as a result of RpoS binding competitively to the RNAP core (Jishage et al., 2002).

*relA* encodes a ppGpp synthetase, RelA, whose intrinsic ppGpp synthetic activity is weak but increases greatly upon binding to stalled ribosomes which contain non-aminoacylated tRNA (Cashel et al., 1996). ppGpp binds to RNAP via DksA and/or RpoZ (the ω subunit of RNAP), and affects its transcriptional activity (Chatterji et al., 1998; Gourse et al., 1998; Vrentas et al., 2005). Amino acid starvation, in particular, causes the ppGpp accumulation by RelA. Transcriptional regulation by ppGpp has been studied extensively in relation to the *rrn* P1 promoter (Schneider et al., 2003). Because a very large amount of rRNA is transcribed during log phase, a large number of RNAPs are used for the transcription from the *rrn* P1 promoter. Under conditions of nutrient depletion, such as amino acid starvation, glucose starvation or during stationary phase, ppGpp represses *rrn* P1 promoter activity by destabilizing the open complex between RNAP and the *rrn* P1 promoter (Barker et al., 2001a), and RNAP relieved from *rrn* transcription can instead transcribe other genes (Barker et al., 2001b). ppGpp thus affects genome-wide transcription not only through its direct binding to RNAP but also, by an indirect effect, via *rrn* regulation.

Although the precise position of one *relA* promoter has been determined (Metzger et al., 1988), the transcriptional regulation of *relA* is poorly understood and it has been suggested that additional promoters may occur upstream of *relA* (Cashel et al., 1996). We demonstrate that *relA*P2, a newly identified promoter of the *relA* gene, may provide a new model to study transcriptional reprogramming by multiple global regulators. *relA*P2 is regulated by CRP, H-NS and RpoS. Our observation that *relA*P2 is affected by multiple global regulators raises the possibility that synthesis of ppGpp, an important molecule for cell physiology, is finely regulated at the transcriptional level.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions** The *E. coli* strains used in this study are listed in Table 1. Each mutant allele was introduced into various strains from its original strain by P1 transduction (Miller, 1972). Bacterial cultures were grown at 37°C in LB medium under aeration.

**Construction of strains containing a *lacZ* transcriptional fusion gene** To construct a *lacZ* transcriptional fusion gene, we used pMKt1403 (unpublished, laboratory stock) transcription fusion vector derived from pMC1403 (Casadaban, 1976). pMKt1403 contains a *rrnB*T1T2 terminator in the upstream of the cloning site, and the downstream sequence is replaced with a *trp'BA'-lacZ'* operon fusion fragment from pMS434 (Hirano et al., 1987). *relA* promoter regions were amplified by PCR with appropriate primers. Primers are listed in Table 2. For site-directed mutation alleles, fragments were constructed by a previously described cross-over PCR method (Chen and Przybyla, 1994). Each PCR fragment was cloned into the *Sma*I site of pMKt1403, and transcriptionally fused to the *lacZ* gene. This plasmid was recombined with λ-RZ5 phage in vivo, followed by lysogenization into *att* site of MC4100. Single-copy insertion of the fusion gene was verified using a PCR method (Powell et al, 1994).

Table 2. Primers

| Name | Sequence |
|---|---|
| pr58 | TCGACGTCAAACAATGCCC |
| pr55 | ATGTGCAACCTGAAGATCGCG |
| pr53 | GCCGTGGGCGAAAAACG |
| pr51 | GGATATGTTCCCACACACGG |
| pr3M | CATCGTCCTCTCCTTTAGGG |
| pr35 | CCATACCGCAGAACAGATCC |
| pr5P1m10 | CTATTCGTATAGTTTATG***CCCGGG***GTAACCCTGCAA[a] |
| pr3P1m10 | TTGCAGGGTTAC***CCCGGG***CATAAACTATAC[a] |
| pr5P1m35 | AAATTATAAAAC***CCCGGG***CTATTCGTATAG[a] |
| pr3P1m35 | CATAAACTATACGAATAG***CCCGGG***GTTTTATAATTT[a] |
| pr5P1mAT | GCAGCAAATTAT***CCCC***CTGGAACCTATTCG[a] |
| pr3P1mAT | CGAATAGGTTCCAG***GGGG***ATAATTTGCTGC[a] |
| pr5P2m10 | CCCCGATAGTGAG***GAT***C***C***CGAAACCGTCTC[a] |
| pr3P2m10 | GAGACGGTTTCG***G***G***ATC***CTCACTATCGGGG[a] |
| pr5P2m35 | GAAGGCCTGGAT***GGATCC***CTCGCCCCCGATAG[a] |
| pr3P2m35 | CTATCGGGGGCGAG***GGATCC***ATCCGGCCTTC[a] |
| pr5P2mCD | ATCGCGAAAAAC***GGATC***CGCTTTTTCGCATTC[a] |
| pr3P2mCD | GAATGCGAAAAGCG***GATCC***GTTTTTCGCGAT[a] |
| pr5P2mCU | CGAAAAACTG***TG***ACGCTTTTC***A***CATTCTGAAGG[a] |
| pr3P2mCU | CCTTCAGAATG***T***GAAAAGCGT***CA***CAGTTTTTCG[a] |
| pr5P2-120 | GATGATTTTGCGCCATACC |
| pr3P2-90 | GGTTCACACCCGCATTGACC |
| pr5relAP1HR | TAAGCGGCCGCCGGCAAAACGCTATTTATCC[b] |
| pr3relAP1HR | TAAGCGGCCGCATAGCAATCCTGTAAACG[b] |
| pr5relAP2HR | TAAGCGGCCGCTCATCATAAACCGAAATCG[b] |
| pr3relAP2HR | TAAGCGGCCGCGGTTAACGACCGACTTACC[b] |
| pr5CRP | GGCCATGGTGCTTGGCAAACCGC[b] |
| pr3CRP | CCCTCGAGACGAGTGCCGTAAACGACGATG[b] |

[a] Bold and italic letters indicate the mutated bases.
[b] Underlined letters indicate bases added for restriction.

**Construction of -10 box mutants of *relA*P1 and *relA*P2** First, we constructed a -10 box mutant of

*rel*AP2 with the pKO3 system (Link et al., 1997). pKO3 system is plasmid-based gene knockout system using temperature-sensitive origin, *sacB* suicide gene, and antibiotics resistance. A DNA fragment containing the P2m10 mutation (Table 3) was amplified by PCR with the primer sets pr5relAP2HR-pr3P2m10 and pr5P2m10-pr3relAP2HR (Table 2), and cloned into the *Not*I site of pKO3 to generate prelAP2m10HR. The sequence of this construct was confirmed. W3110 (wild-type strain) was transformed with prelAP2m10HR. A single-crossed strain was selected by chloramphenicol resistance at 42°C because the origin of pKO3 is temperature-sensitive, and a saccharose-resistant strain, which does not have *sacB* gene, was chosen as the double-crossed strain as described by Link et al. (Link et al., 1997). The -10 box of the *rel*AP2-mutated strain (AN718) was confirmed to be plasmid-free by PCR and *Bam*HI digestion (data not shown). Next, to construct single *rel*AP1 and *rel*AP1-*rel*AP2 double mutants (Table 3, same mutation as shown in Fig. 4A), PCR was carried out using strain AN718 as a template and primer sets pr5relAP1HR-pr3P1m10 and pr5P1m10-pr5relAP1HR. The resulting a *rel*AP1-*rel*AP2 double-mutated DNA fragment was cloned into the *Not*I site of pKO3, yielding prelAP1m10P2m10HR, and its sequence was confirmed. W3110 and AN718 were transformed with prelAP1m10P2m10HR. The double-crossed strain was selected as described by Link et al. (Link et al., 1997), and the mutations were confirmed by *Bam*HI and/or *Sma*I digestion of the appropriate PCR fragments (data not shown).

Table 3. Promoter fragments

| Name | Primer set | Location[a] |
|---|---|---|
| 8relAupM | pr58-pr3M | +3 to –880 |
| 5relAupM | pr55-pr3M | +3 to –502 |
| P1 | pr53-pr3M | +3 to –300 |
| 1relAupM | pr51-pr3M | +3 to –108 |
| P2 | pr58-pr35 | –457 to –880 |
| P1m10 | pr53-pr3P1m10, pr5P1m10-pr3M | +3 to –300 |
| P1m35 | pr53-pr3P1m35, pr5P1m35-pr3M | +3 to –300 |
| P1mAT | pr53-pr3P1mAT, pr5P1mAT-pr3M | +3 to –300 |
| P2m10 | pr58-pr3P2m10, pr5P2m10-pr35 | –457 to –880 |
| P2m35 | pr58-pr3P2m35, pr5P2m35-pr35 | –457 to –880 |
| CRPdown | pr58-pr3P2mCD, pr5P2mCD-pr35 | –457 to –880 |
| CRPup | pr58-pr3P2mCU, pr5P2mCU-pr35 | -457 to –880 |

[a] The numbers indicate the position relative to the initiation codon of RelA (A of ATG designated as +1).

**Construction of H-NS-expressing plasmid** The *Bam*HI-*Hin*dIII fragment of pUC19-*hns* which contains *hns* structural gene with an intact promoter (a gift of Dr. Takeshi Mizuno) was cloned into the same sites of pSTV29 (Takara).

**β-galactosidase assays** Cultured cells were harvested at various times after inoculation and diluted up to 0.4 ml (1- to 10-fold dilution) with LB medium, and mixed with 0.6 ml Z-buffer (Miller, 1972) in an ice bath. Cells were then disrupted with SDS and chloroform. β-galactosidase activity was measured with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as described by Miller (Miller, 1972).

**RNA extraction and primer extension** RNA extraction was carried out by a modified hot phenol method (Conway et al., 1991). A volume of hot phenol equivalent to that of the culture was added to each sample immediately after mixing the cells with 1/10 volume 10X solution A (10% SDS, 200 mM sodium acetate, 10 mM EDTA, pH 5.5). After ethanol precipitation, the pellet was dissolved in water and the solution was treated with an RNeasy Mini Kit (Qiagen). With this method, genomic DNA contaminated the sample; each sample was therefore treated with DNaseI (Takara). Total RNA was then extracted with phenol-chloroform and precipitated with ethanol, and the amount of RNA in the re-dissolved pellet was measured spectrophotometrically. Primer extension experiments were carried out with AMV reverse transcriptase (Life Science, Inc.) (Ausubel et al., 1987). Ten (Fig. 1B) or 70 μg (Fig. 2B) total RNA and $4 \times 10^4$ cpm [γ-$^{32}$P]ATP-end-labeled primers (pr3M and pr35 for *rel*AP1 and *rel*AP2), respectively were used.

**Detection of RelA protein by Western blotting** Cultured cells were harvested by centrifugation. The pellet of cells was resuspended in one-tenth the culture volume of TES buffer (50 mM Tris-HCl pH 7.9, 0.3 M NaCl, and 0.1 mM EDTA). Cells were sonicated and subjected to SDS-PAGE and the proteins were electroblotted on to an Immobilon-P PVDF membrane (Millipore). RelA was detected with polyclonal mouse serum raised against RelA protein (a gift of Dr. Hirofumi Aiba). Signal intensity was quantitated by the Dolphin-1D image analyzer (Wealtec).

**Purification of CRP-His protein** To construct a plasmid expressing C-terminally $His_6$-tagged CRP protein (CRP-His), the PCR product generated from purified genomic DNA using pr5CRP and pr3CRP (Table 2) was cloned into the *Nco*I and *Xho*I sites of pET29b (Novagen). The sequence and expression level were confirmed by sequencing and SDS-PAGE, respectively (data not shown). CRP-His was expressed in *E. coli* BL21 (DE3 pLysS) (Novagen). The cells were harvested by centrifugation from an overnight culture in LB medium containing 25 μg/ml kanamycin and 1 mM IPTG. The pellet of cells was resuspended in one-tenth the culture

volume of TES. After lysis by sonication, samples were centrifuged for 30 minutes at 10,000 *g* at 4°C and the supernatant was loaded on to a Ni-NTA (Qiagen) column equilibrated with TES buffer. The column was washed six times with TES buffer containing 50 mM imidazole and eluted with TES buffer containing 250 mM imidazole. Protein concentration was measured by the Bradford assay (Bio-Rad) using bovine serum albumin as

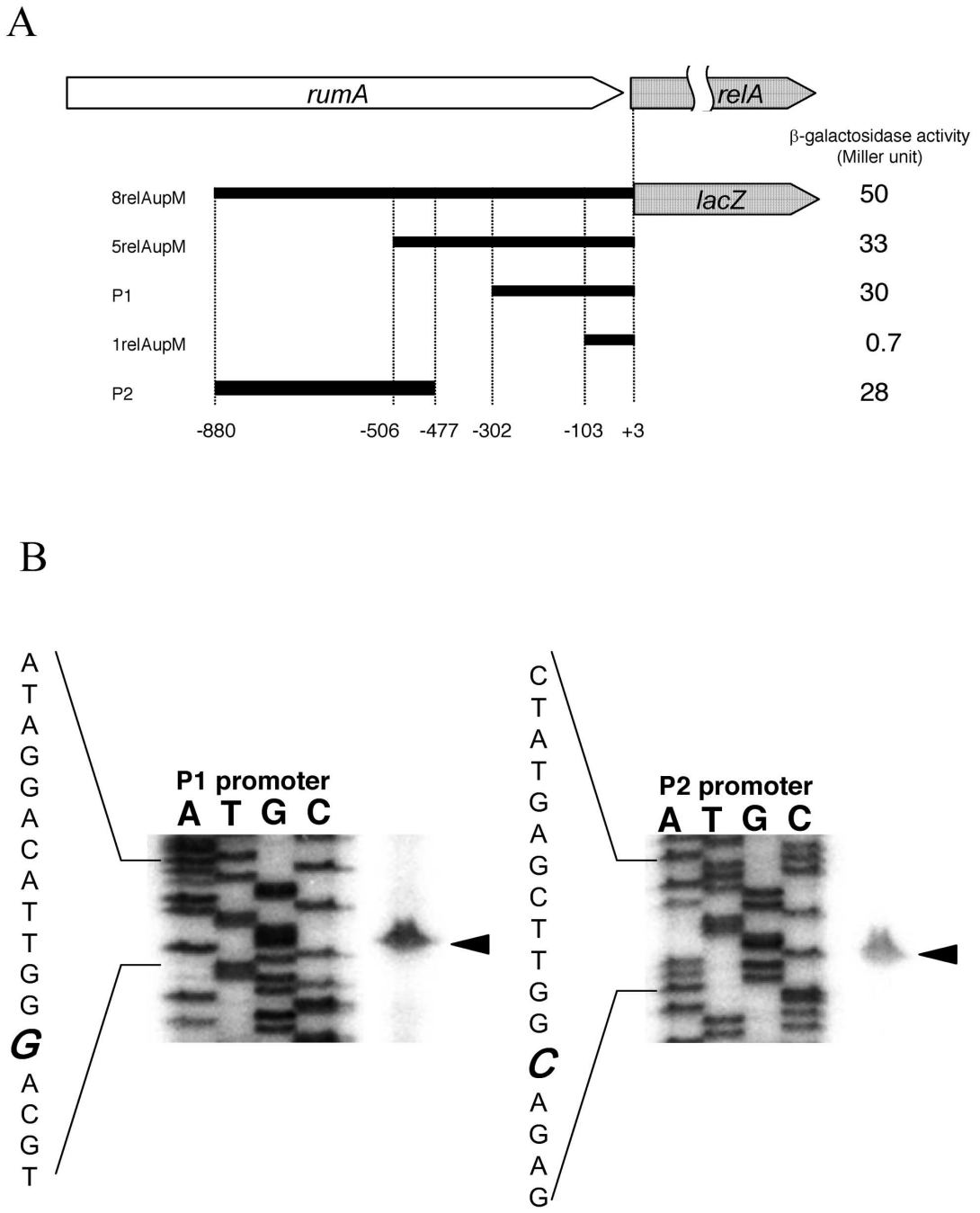


Fig. 1. Determination of *relA* gene promoter region and transcriptional start sites. A) Schematic representation of *relA* upstream region deletions. The *relA* promoters locate in the *rumA* gene encoding 23 S ribosomal RNA m5U1939 methyltransferase (Agarwalla et al., 2002). Distances in bp from the initiation codon of RelA (A of ATG designated as +1) are indicated at the bottom, and promoter names and values of β-galactosidase activity are shown on the left and on the right, respectively. Cells were grown on LB medium for 15 h and harvested. B) Primer extension analysis. The proximal promoter (*relA*P1) is on the left and the distal promoter (*relA*P2) on the right. Total RNA was harvested from strain AN582 at $OD_{600}$ = 1.5. Arrowheads indicate primer extension products. The sequences around transcriptional start sites are shown to the left and the base at the start site is indicated by a bold and italicized letter, –178 bp (*relA*P1) and –626 bp (*relA*P2) upstream from the initiation codon of RelA.

a standard.

**Electrophoresis mobility shift assay** The 209-bp *relA*P2 region was generated by PCR from plasmids containing 8relAupM, CRPdown or CRPup (Table 3) with primers pr5P2-120 and pr3P2-90 (Table 2). DNA fragments were gel-purified and labeled with [$\gamma$-$^{32}$P]ATP by T4 polynucleotide kinase (Takara). Labeled DNA ($8 \times 10^4$ cpm) was incubated for 30 min at 37°C with various concentrations of CRP-His in a final volume of 10 µl containing 50 mM Tris-HCl pH7.9, 100 mM NaCl, 3 mM $MgSO_4$, 0.1 mM cAMP (Sigma), 0.1 mM DTT, 0.1 mM EDTA, and 200 ng poly(dI-dC)-(dI-dC) (Pharmacia). Samples were resolved on a 5% polyacrylamide gel containing 0.1 mM cAMP at 17 V/cm. After electrophoresis, the gel was dried and analyzed by Fujix bioimaging analyser (BAS2500, Fuji).

## RESULTS

**Detection of *relA* promoters** To seek additional promoters in the region upstream of *relA*, various deletions were transcriptionally fused to the *lacZ* gene and integrated into the *E. coli* chromosome with λ phage (Fig. 1A; see Materials and Methods). After 15 h cultivation of strains, β-galactosidase assays were performed. The activity of the 880-bp region upstream from the *relA* initiation codon was about 50 Miller units (Fig. 1A, 8relAupM). When sequences between –880 and –502 were deleted from 8relAupM, the activity decreased to about 30 Miller units (Fig. 1A, 5relAupM), suggesting that the region between –880 bp and –502 has promoter activity. In support of this suggestion, the region between –880 and –457 alone had significant activity (Fig. 1A, P2). In contrast, the activity of P1 was almost identical to that of 5relAupM, suggesting that the region between –502 and –300 has no promoter activity. Finally, 1relAupM lacked significant activity. Collectively, these data indicate that at least two regions within 880 bp upstream of *relA* are responsible for *relA* expression: one is located between –880 and –502, and the other between –300 and –108. The latter region includes the promoter identified by Metzger et al. (Metzger et al., 1988). The activities of these two regions are almost identical (Fig. 1A).

To determine the precise positions of transcriptional initiation sites, primer extension analysis was carried out using AN582, W3110 harboring a multicopy plasmid containing the 880-bp region upstream of *relA* (Fig. 1B). One site is located 178 bp upstream of the RelA translational start site, and the other is located 626 bp upstream. The proximal site is the same as that identified by Metzger et al. (Metzger et al., 1988), and this promoter is referred to as *relA*P1. The distal promoter is newly identified and referred to as *relA*P2.

***relA* transcription and effect of each promoter on RelA protein** To investigate whether *relA* transcription is dependent on growth phase, promoter activities in the wild-type strain W3110 were determined quantita-

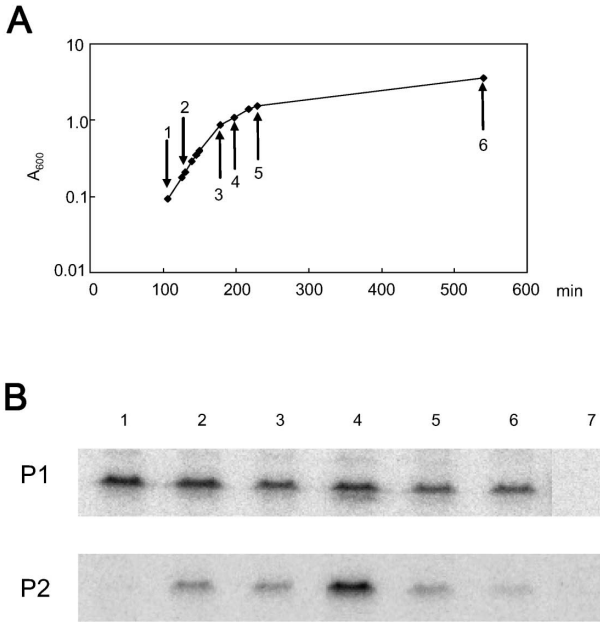


Fig. 2. Growth dependent regulation of *relA* mRNA. An overnight culture of W3110 was diluted 500-fold into LB medium. Total RNA was extracted at various cell densities and primer extension was carried out as described in Materials and Methods. Growth curves (A) and the results of primer extension analysis (B) are shown. A) Arrows indicate the harvesting time points and numbers correspond to lane numbers in B). B) Total RNA was extracted at $OD_{600}$ = 0.2 (lane 1), 0.4 (lane 2), 0.8 (lane 1), 1.0 (lane 4), 1.5 (lane 5), and 3.5 (lane 6), and from a *rumA* deletion mutant at $OD_{600}$ = 1.5 (lane 7). The position of each transcriptional start site was confirmed by comparison with sequence ladders (data not shown). Each sample was normalized for the amount of total RNA (70 µg).

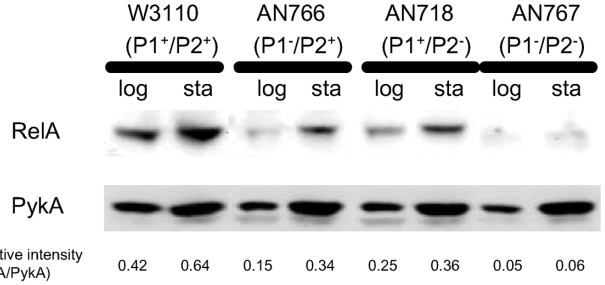


Fig. 3. Contribution of each promoter to RelA protein production. The amount of RelA protein was assessed by Western blot analysis with anti-RelA antiserum in a wild-type strain (W3110), *relA*P1 mutant (AN766), *relA*P2 mutant (AN718) and *relA*P1-*relA*P2 double mutant (AN767) during log phase (log) and stationary phase (sta). Cells were harvested at $OD_{600}$ = 0.4 in log phase and $OD_{600}$ = 2.5 in stationary phase. Each sample contained an equivalent number of cells, normalized according to $OD_{600}$. The relative intensities were obtained by dividing the signal intensity of RelA by that of PykA.

tively by primer extension analysis with promoter-specific primers (prP1 and pr3relAup5 for *relA*P1 and *relA*P2, respectively) at six growth points (Fig. 2A). The band intensities for *relA*P1 are almost the same, indicating that *relA*P1 is constitutively active during all growth phases (Fig. 2B, upper). In contrast to *relA*P1, the *relA*P2 signal at $OD_{600}$ = 1.0 is stronger than the others, indicating that the expression from *relA*P2 is transiently induced at the transition state between the exponential growth phase and the stationary phase (Fig. 2B, lower panel). Thus, the two promoters have different properties. We did not observe any reverse transcripts in either primer extension experiment for a *rumA* deletion mutant (AN682, Table 1) lacking both *relA*P1 and *relA*P2 (Fig. 2B, lanes 7, see also Fig. 1A), confirming that the observed bands for the wild-type strain are specific for *relA* transcripts.

To investigate how this difference between *relA*P1 and *relA*P2 contributes to RelA protein synthesis, RelA protein was detected by Western blotting using anti-RelA antiserum in strains W3110 (*relA*P1$^+$, *relA*P2$^+$), AN766 (*relA*P1$^-$, *relA*P2$^+$), AN718 (*relA*P1$^+$, *relA*P2$^-$) and AN767 (*relA*P1$^-$, *relA*P2$^-$) (Fig. 3). Each -10 box promoter mutation (Fig. 4A) was introduced into the genomic *relA* promoter locus of W3110 by the pKO3 system (Link et al., 1997; see Materials and Methods). Promoter activities were almost abolished by these mutations (Fig. 4, described below). To check no significant difference of

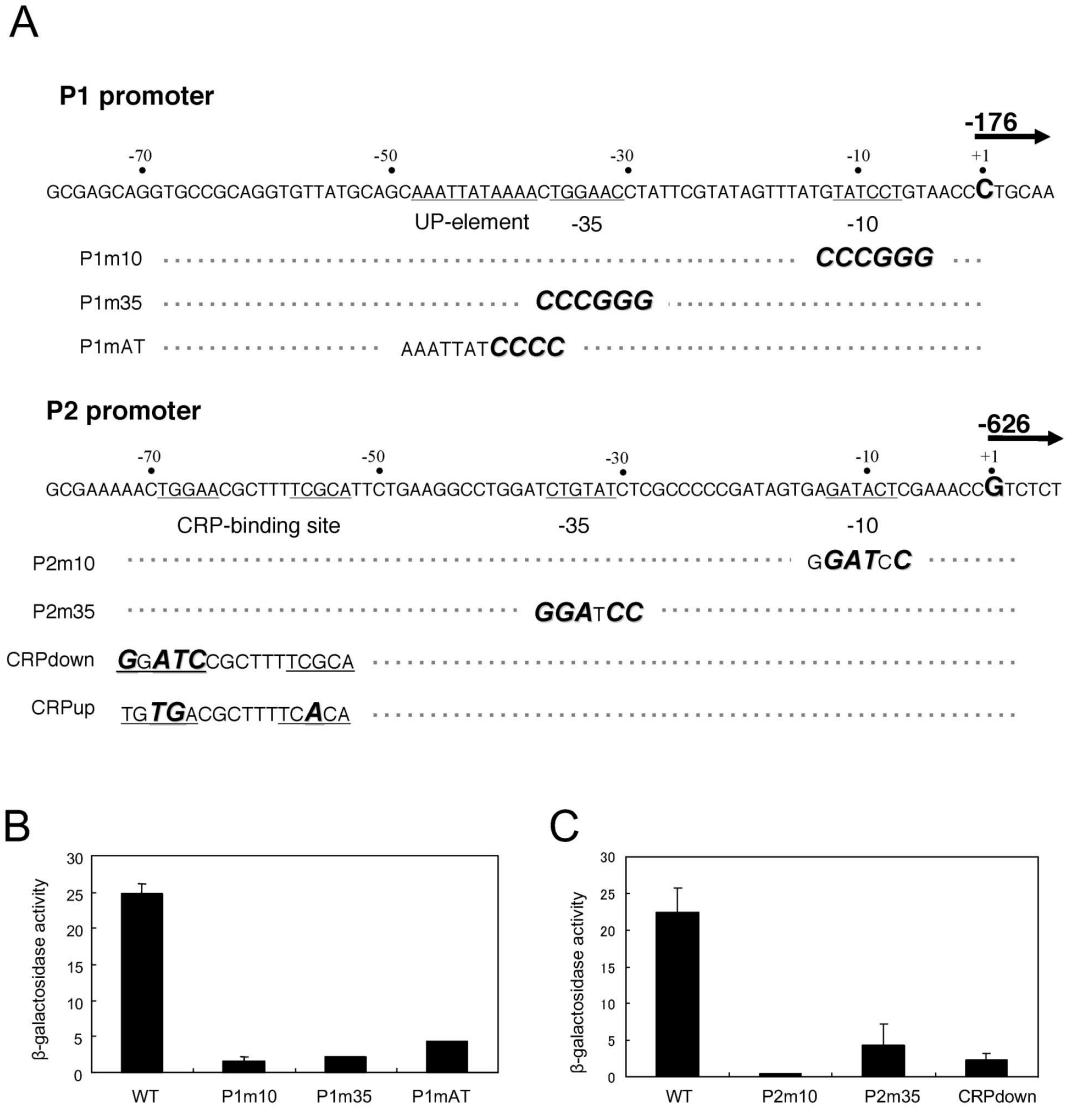


Fig. 4. Effect of putative regulatory element mutation on *relA* promoter activity. Each mutated promoter was fused transcriptionally to *lacZ*. A) Mutation alleles in *relA*P1 and *relA*P2. Bold and italic letters indicate mutated sequences. Except for the mutation site, all mutated promoters have the same sequence as the wild-type allele. The lengths of P1 and P2 are 303 bp (–300 to +3) and 424 bp (–880 to –457), respectively (Table 3). B), C) β-galactosidase activities of mutation series in *relA*P1 (B) and *relA*P2 (C) are shown. Error-bar indicates the standard deviation from two independent experiments.

applying protein amounts between the strains, Western blot analysis of PykA protein was also carried out using the same membrane. Although PykA was accumulated in stationary phase, there is no significant difference between tested strains in the accumulation pattern (Fig. 3, lower panel). In W3110 and single-promoter mutants AN766 and AN718, RelA protein accumulated to stationary phase; however, it was almost abolished in the double promoter mutant AN767 (Fig. 3, upper panel). In addition, both in single promoter mutants, the RelA accumulation in stationary phase was decreased to about 50% of wild-type. In AN766, RelA synthesis in log phase was decreased by 40% compared to AN718 at log phase, whereas almost the same levels were synthesized at stationary phase. These observations, together with the transcription data (Fig. 2B), suggest that *relA*P1 is mainly responsible for RelA production during log phase, and that both promoters are required for maximal accumulation of RelA during stationary phase.

**Mutation analysis of putative regulatory elements in each promoter** There are candidate $\sigma^{70}$ -10 boxes (consensus: TATAAT; Dombroski et al., 1992) in both promoters (Fig. 4A). To determine whether these -10 regions are functional or not, we constructed mutation alleles fused to *lacZ* gene and measured with β-galactosidase activity. Each putative -10 box was replaced by a sequence recognized by a restriction enzyme (Fig. 4A; P1m10, P2m10). The mutation in each -10 region had no significant promoter activity (Fig. 4B and 4C; P1m10 and P2m10). Although the regions around -35 in both promoters have very low similarities to the -35 box consensus (TTGACA; Dombroski et al., 1992), putative -35 box mutants were also constructed. The β-galactosidase activities of both mutants were inactivated (P1m35 and P2m35 in Fig. 4), suggesting that -35 boxes of both promoters might be functional. Taken together, these observations indicate that *relA* has two transcriptional initiation sites, both of which are probably regulated by $\sigma^{70}$.

Immediately upstream of the -35 box of the *relA*P1 promoter is an UP-element-like sequence (Fig. 4A). An UP-element is an AT-rich sequence that enhances promoter recognition by RNAP (Ross et al., 1998). To investigate the contribution of this sequence to *relA*P1 promoter activity, a mutant of the putative UP-element was constructed (Fig. 4A, P1mAT). This mutation drastically decreased β-galactosidase activity compared with the wild-type promoter (Fig. 4B), indicating that the UP-element-like sequence of *relA*P1 is essential for promoter activity.

We also found a putative CRP-binding site (consensus: TGTGA-6 bp-TCACA; Ebright et al., 1989) around the *relA*P2 promoter region, centered 60.5 bp upstream from the transcriptional start site. This distance is appropriate for Class I-type activation by CRP (Ebright, 1993), implying that CRP may directly activate *relA*P2. To test the importance of this element in transcription from *relA*P2, the activity of a promoter containing a mutation in the putative CRP-binding site (CRPdown, Fig. 4A) was measured by β-galactosidase assay. As expected, CRP-down had no significant activity (Fig. 4C). These data suggest that *relA*P2 is directly activated by CRP.

**CRP- dependent regulation of *relA*P2** For further analysis of the regulation of *relA*P2 by CRP, a β-galactosidase assay was performed with a *crp* mutant (AN543, Table 1). *relA*P2 activity in the *crp* mutant was almost abolished in all growth phases (Fig. 5), but was restored by CRP expressed from a plasmid (data not shown), indicating that *relA*P2 is activated by CRP.

The results of the *relA*P2 reporter assay with the *crp* mutant (Fig. 5B) and site-directed mutagenesis (Fig. 4C) strongly suggested that *relA*P2 is directly activated by CRP. To confirm this, an electrophoresis mobility shift assay (EMSA) was performed with a DNA fragment including the region upstream of *relA*P2. Three DNA probes were prepared: a wild-type fragment around *relA*P2 (from –91 to +26 with respect to the transcription start site), a fragment carrying a putative CRP-binding

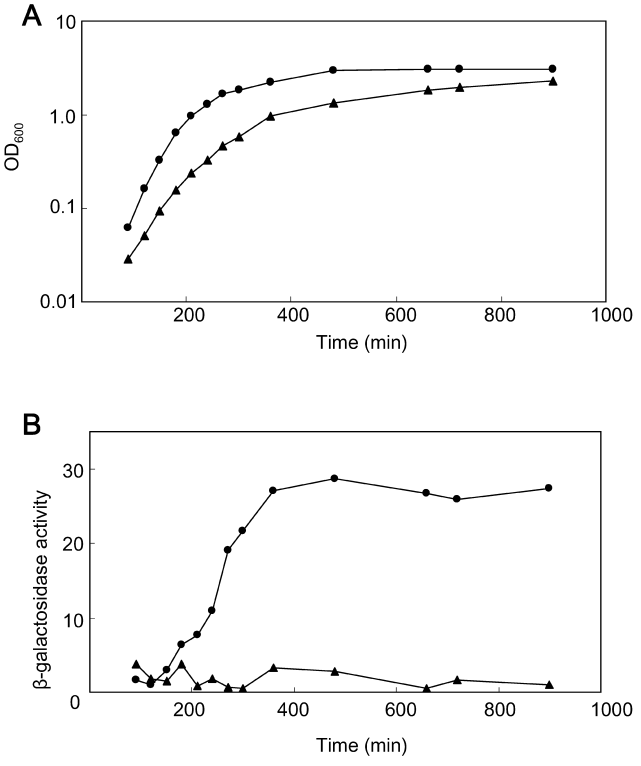


Fig. 5. Effect of *crp* mutation on *relA*P2 activity. Overnight cultures were diluted 500-fold into LB medium. Cultures were harvested at various times during growth. $OD_{600}$ (A) and β-galactosidase activities (B) of indicated strains were monitored. Circles: AN526 (*hns*$^+$, *crp*$^+$), triangles: AN543 (*hns*$^+$, *crp*$^-$).

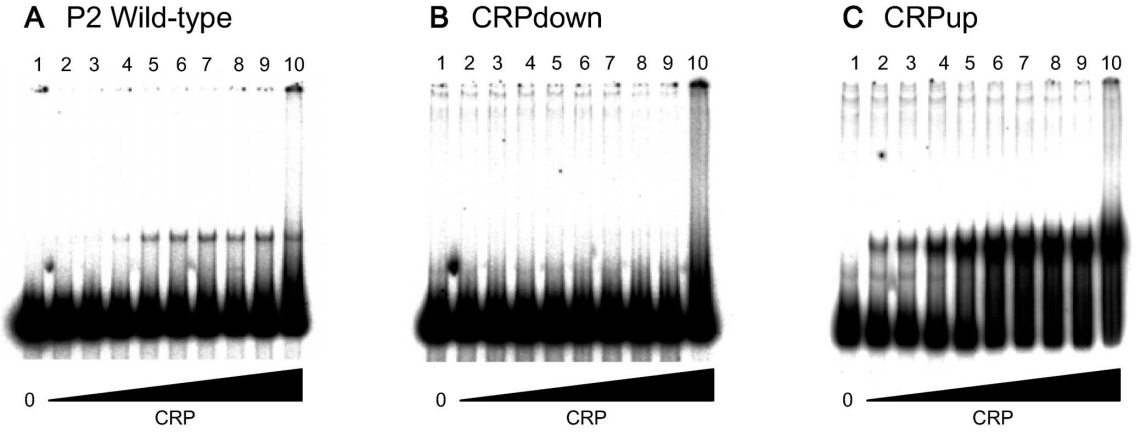


Fig. 6. Specific binding of CRP-His to putative CRP-binding site in *relA*P2. Labeled DNA probe (80,000 cpm) either with or without a CRP-binding site mutation, was mixed with various amounts of purified CRP-His protein and incubated for 30 min at 37°C. Results are shown for the wild-type probe (A), the CRPdown probe (B; same mutation as in Fig. 4A) and the CRPup probe (C; same mutation as in Fig. 4A). CRP-His was added to samples in lanes 1-10 to final amounts of 0, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200, and 400 ng, respectively. 400 ng of CRP-His indicates about 2300 times the amount of probe DNA molecules.

half-site converted to a *Bam*HI recognition site (referred to as CRPdown in Fig. 4A), and a fragment with a perfect CRP-binding consensus sequence instead of the putative CRP-binding site (referred to as CRPup in Fig. 4A). Each fragment was incubated with various amounts of purified C-terminally His-tagged CRP protein (CRP-His) and run on a 5% polyacrylamide gel containing 0.1 mM cAMP. As shown in Fig. 6A, in the case of the wild-type fragment, a specific shifted band could be observed with 3.125 ng (about 18 times the amount of probe DNA molecules) or more of CRP-His (Fig. 6A, lanes 4–10). With the CRP-down fragment, CRP-His did not form a significant amount of complex with the DNA fragment even with 400 ng. CRP-His in the reaction (Fig. 6B). These results indicate that CRP-His binds specifically to the putative CRP-binding site of *relA*P2. CRPup was recognized more efficiently by CRP-His (Fig. 6C) than was the wild-type fragment (Fig. 6A), implying that the affinity of wild-type *relA*P2 is much lower than that of CRPup.

**Effect of *hns* mutation on *relA*P2** It was conceivable that global regulators other than CRP, acting as growth-dependent genes, could regulate *relA*P2-directed expression. To investigate this, β-galactosidase assays of *relA*P2-*lacZ* were carried out for several global regulator mutants. Among these analyses, *relA*P2 activity was drastically reduced in an *hns* mutant (AN529, Table1) throughout growth (Fig. 7B), suggesting that *relA*P2 is positively regulated by H-NS. In support of this suggestion, supply of H-NS from a multicopy plasmid could complement the deactivation of *relA*P2 in the *hns* mutant (Table 4). Furthermore, in contrast to the *hns* mutant, exogenous supply of H-NS could not complement the *crp* mutation (Table 4). These observations suggest that H-NS cannot activate *relA*P2 by itself but can positively regulate *relA*P2 activity together with CRP.

Table 4. Complementation analysis for supply of H-NS from a multicopy plasmid[a]

| Host genotype | Plasmid | |
|---|---|---|
| | pSTV29 | pHNS |
| *hns*$^+$ *crp*$^+$ | 23.4 ± 0.28 | 34.1 ± 2.50 |
| *hns*$^-$ *crp*$^+$ | 4.39 ± 0.50 | 32.8 ± 1.10 |
| *hns*$^+$ *crp*$^-$ | 0.60 ± 1.21 | 0.88 ± 0.85 |

[a] β-galactosidase activity during stationary phase (after 15 hr cultivation), shown in Miller units.

**Effect of *rpoS* mutation on *relA*P2** *rpoS* encodes $\sigma^{38}$, one of the alternative sigma factors of RNAP, which controls a regulon of more than 30 genes expressed during the transition to stationary phase (Hengge-Aronis, 1996). Furthermore some $\sigma^{70}$-dependent promoters are recognized by $\sigma^{38}$. The fact that *relA*P2 is transiently expressed during the transition state (Fig. 2B, P2) prompted us to investigate the involvement of RpoS in *relA*P2 activity. An *rpoS* mutation allele was transduced into AN526, and we measured β-galactosidase activity (Fig. 7). The activity of *relA*P2-*lacZ* in the *rpoS* mutant was almost identical to that of the wild-type strain until mid-log phase; however, during the rest of growth, a higher level of β-galactosidase activity was observed, and the eventual activity in the *rpoS* mutant was 2-fold higher than that in wild-type cells (Fig. 7B). These data suggest that *relA*P2 is partially repressed by RpoS after mid-log phase.

Some genes are regulated cooperatively by RpoS and H-NS (Ohta et al., 1999). To assess cooperativity between RpoS and H-NS for the *relA*P2 promoter, we introduced an *rpoS* mutation allele into an *hns* mutant (AN529) and measured β-galactosidase activity (Fig. 7). Surprisingly, the additional mutation of *rpoS* could rescue the decreased *relA*P2 activity in the *hns* mutant after mid-log

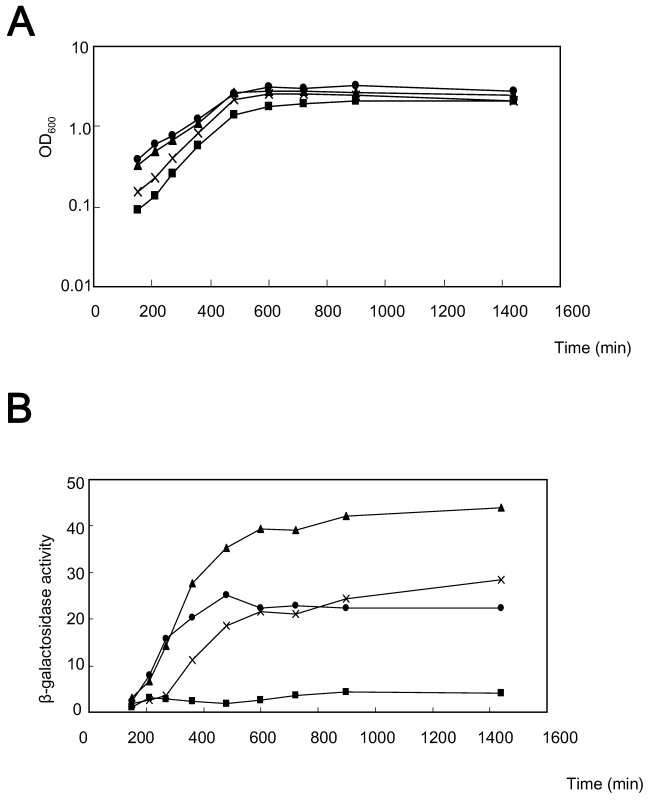


Fig. 7. Effects of *hns* and *rpoS* mutations on *rel*AP2 activity. Overnight cultures of indicated strains were diluted 500-fold into LB medium, and $OD_{600}$ and β-galactosidase activities were monitored during growth. $OD_{600}$ (A) and β-galactosidase activities of *rel*AP2-*lacZ* (B) in wild-type (AN526, circles), *hns* mutant (AN529, squares), *rpoS* mutant (AN557, triangles) and *hns-rpoS* double mutant (AN558, crosses) cells are shown.

Table 5. Complementation analysis of *rpoS* mutant for supply of H-NS from a multicopy plasmid[a]

| Host genotype | Plasmid | |
|---|---|---|
| | pSTV29 | pHNS |
| *hns*$^+$ *rpoS*$^+$ | 19.9 ± 0.40 | 33.5 ± 1.89 |
| *hns*$^-$ *rpoS*$^+$ | 3.08 ± 2.62 | 23.9 ± 1.41 |
| *hns*$^+$ *rpoS*$^-$ | 28.5 ± 0.63 | 45.4 ± 0.48 |
| *hns*$^-$ *rpoS*$^-$ | 16.1 ± 2.11 | 51.3 ± 1.37 |

[a] β-galactosidase activity during stationary phase (after 15 hr cultivation), shown in Miller units.

phase (Fig. 7B), and the maximum activity in the *hns-rpoS* double mutant was almost identical to that in wild-type. These results suggest that increased *rel*AP2 activity in the *rpoS* mutant is independent of H-NS. Because *rpoS* is repressed by H-NS at the posttranscriptional level (Brescia et al., 2004), we hypothesized that the positive effect of H-NS on *rel*AP2 might be mediated by H-NS repression of *rpoS*. To test this, we measured β-galactosidase activity when H-NS was supplied from a multicopy plasmid in an *rpoS* mutant and an *hns-rpoS* double mutant (Table 5). pHNS could increase the activity of *rel*AP2-*lacZ* even in the *rpoS* mutant (Table 5). In the *hns-rpoS* double mutant, the activity of *rel*AP2-*lacZ* was increased 3-fold by exogenous supply of H-NS, almost the same level as in the *rpoS* single mutant (Table 5). These results suggest, contradictory to our hypothesis, that H-NS can regulate *rel*AP2 without RpoS, and therefore that positive regulation by H-NS is not mediated by *rpoS* repression.

## DISCUSSION

In this work, we report three observations regarding *relA* transcriptional regulation. 1) The *relA* gene has two promoters. 2) *rel*AP1 is a constitutive promoter and *rel*AP2 is an inducible promoter. 3) *rel*AP2 is regulated by multiple global regulators, including at least CRP, H-NS and RpoS.

Systematic deletion analysis of the region upstream of *relA* suggested that *relA* is transcribed from two $\sigma^{70}$-dependent promoters (Fig. 1B, Fig. 4). One promoter, *rel*AP1, has been identified by Metzger et al. (Metzger et al., 1988) and the other, *rel*AP2, is newly identified in this study (Fig. 1B). Although the transcriptional start site for *rel*AP2 is far upstream (626 bp) of the RelA initiation codon, *rel*AP2 contributes to the accumulation of RelA protein (Fig. 3). RelA accumulation is drastically reduced in a *rel*AP1-*rel*AP2 double mutant (Fig. 3, AN767), indicating that *relA* expression is regulated almost exclusively by *rel*AP1 and *rel*AP2 on LB medium.

Transcription from *rel*AP1 is constitutive throughout growth (Fig. 2B) and depends on an UP-element located about 40 bp upstream of the transcription start site (Fig. 4B) like some stable RNA (rRNA and tRNA) promoters (Ross et al., 1998). In contrast, we found that transcriptional control of *rel*AP2 is strongly dependent upon CRP (Fig. 5 and Fig. 6), which is activated by cAMP accumulated during glucose starvation (Notley-McRobb et al., 1997). In addition, the ppGpp synthetic activity of RelA is responsible for ppGpp accumulation in the early stage of glucose starvation (Chaloner-Larsson et al., 1978; Murray et al., 2003), and we therefore speculated that the accumulation of ppGpp might be stimulated by CRP activation of *rel*AP2. Although we monitored the amount of ppGpp in W3110 and AN718 during glucose starvation, we could not obtain definitive evidence that CRP regulation contributes to ppGpp accumulation (data not shown). The CRP dependency of *rel*AP2 may thus have some other biological function.

We observed that the unique transcription pattern of *rel*AP2 which is transiently activated at the transition state (Fig. 2B). Since H-NS accumulates at the transition state (Ali Azam et al., 1999), the activity of *rel*AP2 seems to be correlated with the amount of H-NS; however, there is a possibility that the activation of CRP by cAMP accu-

mulation due to glucose starvation is also involved in the transient activation of *relA*P2. Although *relA*P2-*lacZ* has almost no activity in an *hns* mutant throughout growth (Fig. 7), H-NS cannot activate *relA*P2 without CRP (Table 4). CRP binding to the *relA*P2 promoter sequence was relatively weak in vitro. H-NS binding induces DNA bending and looping in vivo, thus some conformational change of the *relA*P2 promoter sequence by H-NS would facilitate the CRP binding.

In addition to H-NS dependent activation of *relA*P2, H-NS independent transcription also occurred at *relA*P2 after mid-log phase, although repressed by RpoS, because significant *relA*P2 activity was observed in *hns-rpoS* double mutant (Fig. 7B). Since *relA*P2 had no significant activity in a *crp-rpoS* double mutant (data not shown), we conclude that this H-NS independent activation also depends on CRP. Although the mechanism of the H-NS independent activation is unknown at moment, other nucleoid binding protein induced after mid-log phase might facilitate CRP binding to *relA*P2, instead of H-NS. The negative effect of RpoS on the H-NS independent *relA*P2 might be due to RNAP competition, as occurred with the *uspB* gene regulated by $\sigma^{70}$ (Jishage et al., 2002), and this repression will be responsible for the shut off of the *relA*P2 expression in stationary phase where H-NS level is reduced.

To ascertain the biological meaning of *relA*P2 regulation by multiple global regulators, it will be necessary to investigate the relationship between transcriptional induction from *relA*P2 and regulation of the ppGpp pool in the cell. In this study, we did not succeed in finding the biological meaning of the transient induction of *relA*P2 (data not shown). However, we have determined that *relA*P2 regulation is fine-tuned by at least three global regulators, CRP, H-NS and RpoS. Given the known importance of ppGpp, these observations may provide biological clues about how *E. coli* survives in extremely complex environment.

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