

Negative Effect of Glucose on *ompA* mRNA Stability: a Potential Role of Cyclic AMP in the Repression of *hfq* in *Escherichia coli*^{∇†}

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Glucose is a carbon source that is capable of modulating the level of cyclic AMP (cAMP)-regulated genes. In the present study, we found that the stability of *ompA* mRNA was reduced in *Escherichia coli* when glucose (40 mM) was present in Luria-Bertani (LB) medium. This effect was associated with a low level of cAMP induced by the glucose. The results were confirmed with an adenylyl cyclase mutant with low levels of cAMP that are not modulated by glucose. Northern blot and Western blot analyses revealed that the host factor I (Hfq) (both mRNA and protein) levels were downregulated in the presence of cAMP. Furthermore, we showed that a complex of cAMP receptor protein (CRP) and cAMP binds to a specific P_{3_{hfq}} promoter region of *hfq* and regulates *hfq* expression. The regulation of the *hfq* gene was confirmed *in vivo* using an *hfq*-deficient mutant transformed with an exogenous *hfq* gene containing the promoter. These results demonstrated that expression of *hfq* was repressed by the CRP-cAMP complex. The presence of glucose resulted in increased Hfq protein levels, which decreased *ompA* mRNA stability. An additional experiment showed that cAMP also increased the stability of *fur* mRNA. Taken together, these results suggested that the repression of Hfq by cAMP may contribute to the stability of other mRNA in *E. coli*.

The degradation of mRNA appears to be a major player in controlling the level of gene expression, in addition to the regulation of transcription and translation. The stability of mRNA from a single gene can be modulated in response to growth conditions, environmental signals, and translational efficiency. Therefore, the regulation of mRNA stability can provide a means to actively adapt the cellular translation to the changes in growth conditions.

The degradation of the outer membrane protein A (OmpA) mRNA is used as a model to study the regulation of mRNA decay in *Escherichia coli*. OmpA is abundant in the outer membrane, with a copy number of ~100,000 per cell, and plays important roles in a variety of functions (30). Previous publications have shown that the stability of *ompA* mRNA increased as the cell growth rate increased due to the medium composition (25). In other words, the stability of mRNA in bacteria can be adjusted in response to growth conditions. The 5' untranslated region (5' UTR) of the *ompA* transcript is important for regulating mRNA stability in response to changing growth rates (8, 11). For example, the binding of host factor I (Hfq), an RNA chaperon protein, to the 5' UTR of the *ompA* transcript negatively regulates growth rate-dependent degradation of *ompA* mRNA (36). Hfq stimulates *ompA* mRNA decay by binding to an A/U-rich sequence of the *ompA* 5' UTR region which overlaps an endoribonuclease RNase E (*rne*) cleavage site and thus appears to interfere with ribosome binding, by which Hfq stimulates *ompA* mRNA decay (1, 22, 37).

Glucose is a major source of carbon for the growth of *E. coli*. For glucose metabolism, *E. coli* has developed pleiotropic regulators for the transcriptional regulation, such as cyclic AMP (cAMP) and its receptor protein (CRP) complex. It is known that cAMP forms a complex with CRP (cAMP-CRP complex) that binds to a specific promoter region of genes to either activate or inhibit transcription depending upon the position bound by the complex relative to the promoter (3). As a result, the cAMP-CRP complex regulates the expression of hundreds of genes (10, 38). However, little is known about the relationship between glucose regulation and *hfq* expression in post-transcriptional regulation. In this study, we found that glucose significantly enhanced the decay of *ompA* mRNA with a half-life of ~11.5 to ~8.1 min. This change in the decay rate was associated with a decrease in cAMP levels. Given the fact that Hfq is involved in the destabilization of *ompA* mRNA, we tested whether there is a relationship between the cAMP levels and the expression of *hfq*. Interestingly, we found that cAMP-CRP was capable of binding to a specific region of the P_{3_{hfq}} promoter and subsequently repressed *hfq* expression. The finding was further substantiated by expressing an exogenous *hfq* gene using *hfq* and *lacZ* promoters in an *hfq*-deficient mutant. Overall, we provide the first evidence and mechanism(s) for the regulation of mRNA stability by glucose. In the absence of glucose, cAMP can repress *hfq* expression and thereby stabilizes *ompA* mRNA. In contrast, enhanced *hfq* expression in the presence of glucose destabilizes *ompA* mRNA.

MATERIALS AND METHODS

Bacterial strains. The strains and plasmids used in this work are listed in Table 1. All primers are shown in Table S1 in the supplemental material. *E. coli* strain BW25113 (wild type) and strains JW3778 (cAMP-deficient or *cya* mutant) and JW4130 (*hfq*-deficient mutant), knocked out by the Keio collection system (2), were kindly provided by the National Institute of Genetics of Japan. An *Hfq* and *cya* double mutant (CPT021) was produced in our laboratory by transforming

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference ^a
Strains		
BW25113	<i>lacI^h rrmB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1</i>	NIG
JW3778	BW25113 <i>Δcya::kan</i>	NIG
JW4130	BW25113 <i>Δhfq::kan</i>	NIG
CPT021	JW4130 <i>Δhfq Φ(Δcya::kan)</i>	This study
Plasmids		
pRW50	Vector for creating <i>hfq</i> promoter- <i>lacZ</i> reporter fusions; Tet ^r	28
pRW50-P _{3_{hfq}} - <i>lacZ</i>	pRW50 cut with EcoRI and HindIII containing 275-bp P _{3_{hfq}} promoter region; Tet ^r	This study
pCP20	Plasmid for removal of the <i>kan</i> marker from JW4130; Amp ^r	NIG
pSP73	Vector for <i>hfq</i> gene expression; Amp ^r	Promega
pSP73-P _{hfq} - <i>hfq</i>	pSP73 cut with HpaI and BamHI containing 275-bp <i>hfq</i> promoter region and 334-bp <i>hfq</i> gene; Amp ^r	This study
pSP73-P _{NCH} - <i>hfq</i>	Non-CRP binding site was corrected with mutagenic primers from pSP73-P _{hfq} - <i>hfq</i> ; Amp ^r	This study
pSP73-P _{lacZ} - <i>hfq</i>	pSP73 cut with HpaI and BamHI containing 264-bp <i>lacZ</i> promoter region and 334-bp <i>hfq</i> gene; Amp ^r	This study
pQE30	Vector for CRP protein expression; Amp ^r	Laboratory stock
pQE30- <i>crp</i>	pQE30 cut with BamHI and KpnI containing 633-bp <i>crp</i> gene; Amp ^r	This study

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plasmid pCP20 (6) with the kanamycin marker removed into JW4130. A P1 phage containing a *Δcya::kan* fusion from JW3778 was used to construct a CPT021 double deletion using a standard protocol of OpenWetWare from Robert Sauer's Laboratory at the Massachusetts Institute of Technology (http://openwetware.org/wiki/Sauer:P1vir_phage_transduction).

Plasmid construction. To construct the P_{3_{hfq}}-*lacZ* fusion, the P_{3_{hfq}} promoter region (containing the 275 bp upstream of the *hfq* start codon) was inserted into pRW50 (kindly provided by S. J. Busby, University of Birmingham [19, 28]) to get plasmid pRW50-P_{3_{hfq}}-*lacZ*. The *Hfq* expression plasmid (pSP73-P_{hfq}-*hfq*) containing the same P_{3_{hfq}} promoter region, and a putative CRP binding sequence, was constructed by insertion of the *hfq* gene (including a 309-bp coding region and 25-bp terminator from the genomic DNA of *E. coli* wild-type BW25113) into a pSP73 vector, in which the T7 promoter was deleted by HpaI and BamHI. A pSP73-P_{NCH}-*hfq* plasmid was similarly constructed, except that the putative CRP binding region at TGGGA AGGGGT TCACT was replaced by a non-CRP binding region or ACGCT AGGGGT AGAGT (namely, NCH). This replacement was done with a complementary sequence described by Hollands et al. (13) using a KOD-Plus mutagenesis kit (Toyobo, Osaka, Japan). To construct the pSP73-P_{lacZ}-*hfq* plasmid, the promoter region (total of 275 bp) of pSP73-P_{hfq}-*hfq* was replaced with a *lacZ* promoter (containing the 264 bp upstream of the start codon) using PCR-driven overlap extension (12). For expression and purification of CRP protein, the 633-bp *crp* gene was amplified in our laboratory using primers *crpF* and *crpR* by PCR. The PCR products were inserted into the BamHI and KpnI sites of the pQE30 vector to obtain pQE30-*crp*.

Media and growth conditions. Cells were grown in Luria-Bertani (LB) medium with or without 40 mM glucose at 37°C until reaching the exponential phase with an optical density at 600 nm (OD₆₀₀) of 0.3 to 0.5. Tetracycline, ampicillin, or kanamycin was added as needed to the medium at a concentration of 50 μg/ml. Initiation of mRNA transcription was inhibited by the addition of rifampin to a final concentration of 500 μg/ml before harvesting the total cellular RNA. After adding rifampin, cultured cells were harvested at different time points and chilled in a dry ice/ethanol bath for 1 min prior to RNA extraction.

RNA preparation. RNA isolation was performed according to the procedures of the TRI reagent-RNA kit (Molecular Research Center, Cincinnati, OH). Cells collected by centrifugation (8,000 × g for 10 min) at 4°C were resuspended in 1.5 ml TRI reagent. The homogenate was incubated at 65°C for 10 min, 0.3 ml chloroform was added, and the mixture was vortexed vigorously for 30 s and incubated at room temperature for 5 min. The mixture was then centrifuged at 12,000 × g for 15 min. The supernatant was mixed with 0.75 ml isopropanol, and the RNA was precipitated at -20°C for 1 h before centrifugation at 12,000 × g for 10 min. The pellet was washed twice with cold 75% ethanol and dissolved in 50 μl of deionized water pretreated with 0.1% diethyl pyrocarbonate (DEPC).

Northern blot analyses. Equal amounts of total RNA (determined by measuring its absorbance at the OD₂₆₀) were loaded and separated on a 1.2% or 2% agarose-formaldehyde gel for the analysis of *ompA*, *hfq*, or *fur*. The integrity and quality of RNA samples were confirmed by the presence of 23S and 16S rRNA. Following electrophoresis, gels containing RNA were transferred to a positively charged nylon membrane (Millipore, Bedford, MA) using a 10× saline sodium

citrate (SSC) buffer solution (1× SSC is 0.15 M NaCl and 15 mM sodium citrate, pH 7.0), and the membrane was cross-linked with UV light. The membrane was equilibrated in a hybridization buffer containing 5× Denhardt's solution (1× Denhardt's solution contains 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 5× SSC, 1% SDS, 5% dextran sulfate, and 50% formamide and hybridized with digoxigenin (DIG)-labeled DNA probes for at least 12 h at 42°C. DNA probes specific to *E. coli ompA*, *hfq*, and *fur* were amplified with dNTPs and a DIG-11-dUTP mixture (Roche Applied Science, Mannheim, Germany) as previously described (18). Following hybridization, membranes were washed five times (twice with 2× SSC and 0.1% SDS at 25°C for 5 min and three times with 0.1× SSC and 0.1% SDS at 37°C for 15 min). After washes, the membranes were incubated with anti-DIG antibody conjugated with alkaline phosphatase and detected by adding disodium 3-(4-methoxy-psiropi{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) pheryl phosphate (CSPD) chemiluminescent substrate (Roche Applied Science, Indianapolis, IN). The blots were then developed by exposure to Kodak films. The half-life of mRNAs was represented as the mean ± standard deviation (SD) of the band intensity analyzed by Northern blotting in triplicates, while the band intensity was determined by the Zero-Dscan image analysis system (Scanalytics, Billerica, MA).

Real-time PCR analyses. Cells were grown to reach to an exponential phase. Total RNA was isolated as described above and treated with DNase I (Promega, Madison, WI). Briefly, cDNA was synthesized from 5 μg of total RNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA), with the corresponding primers designed for *hfq* or *me* by the use of Primer Express software (Applied Biosystems; Foster City, CA). Real-time PCR using SYBR Premix ExTag (Takara, Tokyo, Japan) was executed with an ABI PRISM 7000 (Applied Biosystems) according to the standard operation procedures. The relative level of 16S rRNA was used as an internal control to normalize *hfq* and *me* expression. The relative expression of *hfq* and *me* was calculated by the comparative threshold cycle ($\Delta\Delta C_T$) method according to the manufacturer's recommendation. SD was calculated using Microsoft Excel (Redmond, WA), and Student's *t* tests were used for statistical analysis. Differences were considered statistically significant if *P* values were <0.05.

β-Galactosidase assay. Plasmid pRW50-P_{hfq}-*lacZ* was transformed into BW25113 and JW3778. Cells were cultured as indicated above. The activity of β-galactosidase was measured as described previously (21). The β-galactosidase values were represented as the average of results from at least three independent experiments, with a variation of no more than 5% from the mean.

EMSA. Electrophoretic mobility shift assays (EMSA) were carried out with modifications as described previously (26). In addition to *hfq* and NCH fragments which were commercially synthesized, the rest of the fragments were amplified by PCR. All DNA fragments used in the assays were labeled with [γ -³²P]dATP and purified over G25 columns (GE-Healthcare, Uppsala, Sweden). The pQE30-*crp* plasmid was transformed into *E. coli* BL21(DE3), and the CRP recombinant protein was isolated using a His tag column system purchased from Qiagen (Hilden, Germany) (26). The binding reactions (20 μl) were performed by incubating 10 ng of labeled DNA fragments with various amounts

of purified CRP (0 to 200 nM) and cAMP (0 to 200 μ M) in a binding buffer [2 μ g/ml of sonicated herring sperm DNA, 4 mM Tris-HCl (pH 7.0), 5 mM sodium chloride, 2 mM magnesium chloride, 2 mM dithiothreitol, 50 mg/ml bovine serum albumin (BSA), and 15 μ g/ml poly(deoxyinosinic-deoxycytidylic acid)]. After incubation at 37°C for 30 min, samples were electrophoresed in a native polyacrylamide gel (6%) in 0.5 \times Tris-borate (TBE) buffer (1 \times TBE contains 0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA, pH 7.0). After electrophoresis, the gel was dried on a 3M paper for 80 min and autoradiographed on Kodak film.

DNase I footprinting. A DNase I footprinting assay was performed as described previously (5, 16, 39). The DNA fragment from nucleotide position -251 to -32 (as indicated in Fig. 3) was generated by PCR using the 5' 6-carboxy-fluorescein (FAM)-labeled primers. Binding of the cAMP-CRP complex to the labeled DNA fragment was carried out as described for EMSA. Then, the binding mixtures were partially digested with 0.5 units DNase I containing 40 mM Tris-HCl (pH 8.0), 10 mM MgSO₄, and 1 mM CaCl₂. After incubation for 1 min at 37°C, the reaction was stopped by heating at 65°C for 10 min in the presence of 20 mM EGTA, pH 8.0. The digested DNA fragments were purified using the MinElute PCR purification kit from Qiagen. The purified DNA fragments were detected with a 3730 DNA analyzer (Applied Biosystems). The protected regions were then analyzed with GeneMarker (SoftGenetics, State College, PA) and then aligned to the sequences generated using the same primers.

Western blot analysis. The Western blot analysis used for Hfq protein expression was essentially similar to that described previously (34). In brief, cells were harvested at an OD₆₀₀ of 0.3 to 0.5 and resuspended in 100 mM potassium phosphate buffer (pH 7.6), and sonication for 5 min and centrifugation at 12,000 \times g for 15 min at 4°C followed. A total of 8 μ g of extract protein per lane was electrophoresed on 12% SDS-polyacrylamide gel for 2 h at 100 V. After transferring to nitrocellulose paper and washing, the protein was detected using an antibody specific against Hfq (kindly provided by U. Bläsi, University of Vienna) and developed by an ECL detection system (Amersham, Arlington Heights, IL).

RESULTS

Effects of glucose and cAMP on the stability of *ompA* mRNA.

ompA is abundantly expressed in *E. coli* and is often used to investigate mRNA stability. In the present study, we found that glucose had a negative effect on the stability of *ompA* mRNA when comparing *E. coli* grown in LB medium supplemented with or without glucose. Figure 1A reveals that *ompA* mRNA was degraded faster when *E. coli* was grown in the presence of glucose. Since it is well established that cAMP levels are low when growing in the presence of glucose (23), we further tested whether the addition of exogenous cAMP could stabilize the *ompA* mRNA. Figure 1B shows that the addition of cAMP restored the stability of the *ompA* mRNA, suggesting that cAMP played a role in stabilizing the *ompA* mRNA. To substantiate this hypothesis, we showed that an *E. coli* mutant lacking cAMP (JW3778 or the Δ *cya* mutant) caused even faster decay of *ompA* mRNA (Fig. 1C, top) compared to the wild type (BW25113) (Fig. 1A, top). The addition of glucose to this mutant did not further destabilize the mRNA (Fig. 1C, bottom), indicating that such a negative effect of glucose on *ompA* mRNA stability in wild-type *E. coli* is cAMP dependent. Taken together, our data suggest that glucose destabilization of the *ompA* mRNA is attributable to a decrease in cAMP levels.

***hfq* expression in cAMP-deficient mutant.** One study reported that Hfq facilitates the degradation of *ompA* mRNA (36). We attempted to determine whether there is a linkage between cAMP and the expression of *hfq*. We examined the effect of cAMP on *hfq* gene expression in a cAMP-deficient mutant. This mutant expressed significantly higher *hfq* mRNA levels ($P < 0.05$) than wild-type *E. coli* as determined by a real-time PCR assay (Fig. 2A). On the other hand, the expres-

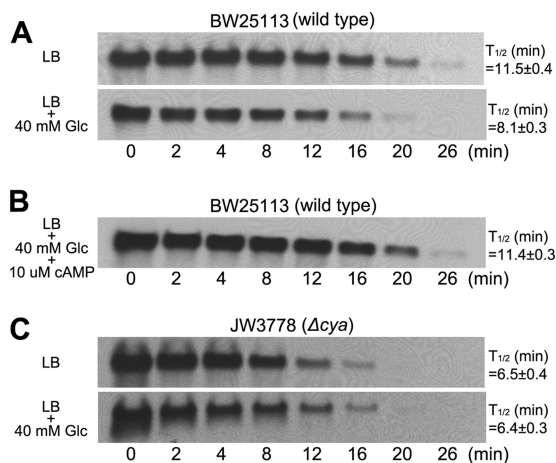


FIG. 1. Effects of glucose and cAMP on the stability of *E. coli ompA* mRNA as determined by Northern blot analysis. (A) Wild-type cells (BW25113) were grown in LB medium supplemented with and without 40 mM glucose (Glc). (B) Wild-type cells (BW25113) were grown in LB with 40 mM glucose and 10 μ M cAMP. (C) cAMP-deficient cells (JW3778) were grown in LB with and without 40 mM glucose. After exponential phase was reached (OD₆₀₀ = 0.3 to 0.5), rifampin was added to the cultured cells to inhibit new synthesis of mRNA, and the cells were harvested at different time points from 0 to 26 min. Analysis of the *ompA* mRNA levels was performed by fractionation of total RNAs on 1.2% agarose-formaldehyde gel and transfer of the RNA onto a nylon membrane. The membrane was then hybridized with DIG-labeled *ompA* DNA. Each experiment was repeated three times and subjected to statistical analysis.

sion of *me*, known to participate in *ompA* mRNA degradation, was not affected in the cAMP-deficient mutant (Fig. 2A, right). Previous studies have shown that *hfq* can be transcribed from three different promoters, P1_{*hfq*}, P2_{*hfq*}, or P3_{*hfq*} (32). In order to determine which promoter is regulated by cAMP, we examined the *hfq* mRNA level transcribed from each of the promoters. The levels of *hfq* mRNA transcribed from the P1_{*hfq*} and P2_{*hfq*} promoter did not markedly change (data not shown) in response to glucose. In contrast, significant changes were seen in the level of *hfq* transcribed from the P3_{*hfq*} promoter in this study. We showed that growing wild-type *E. coli* in glucose-supplemented medium, a culture condition that reduces cAMP levels, upregulated the *hfq* mRNA in the wild type. A similar increase in *hfq* mRNA was seen in the cAMP-deficient mutant (Fig. 2B). To determine whether the P3_{*hfq*} promoter is regulated by cAMP, we examined the expression of the P3_{*hfq*}-*lacZ* construct consisting of 275 bp of the P3_{*hfq*} promoter cloned into a *lacZ* expression vector, pRW50. The β -galactosidase levels were about 2-fold higher in LB medium supplemented with glucose than in LB medium. There was a >2-fold increase in P3_{*hfq*}-*lacZ* expression by the cAMP-deficient mutant (Fig. 2C). Increases in Hfq protein expression was confirmed using Western blotting. Our results show that a glucose-induced reduction in cAMP levels in the wild type or cAMP-deficient mutants resulted in increased Hfq protein expression (Fig. 2D). These data support the theory that cAMP can repress the expression of *hfq* from the P3_{*hfq*} promoter, leading to a reduced Hfq level.

Interaction between cAMP-CRP and P3_{*hfq*} promoter. We tested the hypothesis that the cAMP-CRP complex binds to

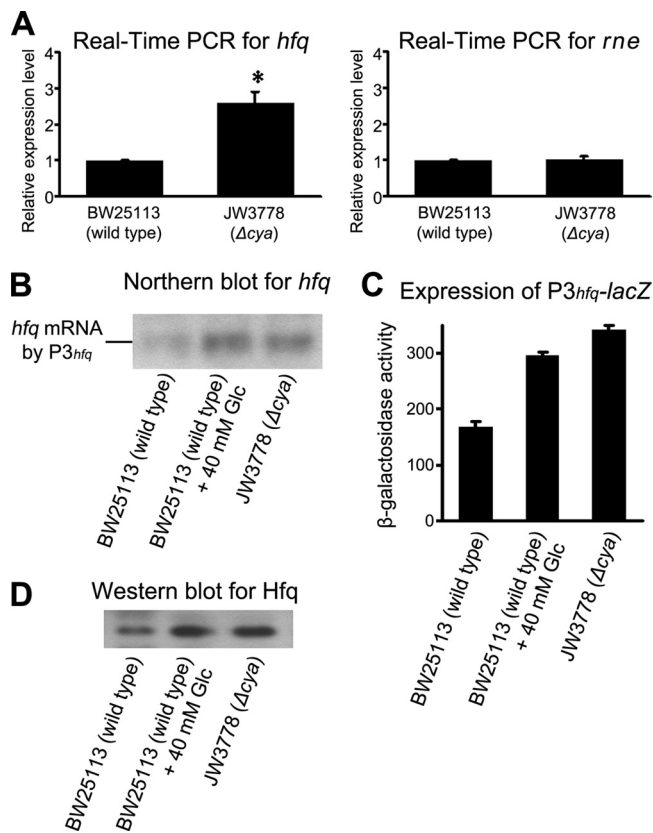


FIG. 2. Increases in *hfq* expression of cAMP-deficient mutant. (A) *E. coli* was grown in LB medium. Relative expression of *hfq* and *rne* mRNA in wild type (BW25113) and cAMP-deficient mutant (JW3778) measured by a real-time PCR (in triplicate). *, $P < 0.05$. (B) Expression of *hfq* mRNA measured by Northern blot analysis. BW25113 was grown in LB supplemented with or without 40 mM glucose (Glc). JW3778 was grown in LB. (C) Activity of β -galactosidase was measured for BW25113 and JW3778 transformed with pRW50-P3_{*hfq*}-*lacZ*. The cells were grown as described above for panel B. (D) Hfq protein expression was confirmed using Western blotting.

the P3_{*hfq*} promoter region of the *hfq* gene and acts as a transcriptional factor, by the use of an EMSA. First, we prepared a full-length recombinant CRP and incubated it with cAMP to form the cAMP-CRP complex. We then examined the binding of the cAMP-CRP complex with four sequential fragments spanning the P2_{*hfq*} and P3_{*hfq*} promoter region (sequence -429 to 68) (Fig. 3A, top) and found that the sequence from -151 to -32 was sufficient for the binding (Fig. 3A, bottom). Second, we used the MATCH program prediction software to delineate a potential binding region over the P3_{*hfq*} promoter (-151 to -32). The MATCH program is a weight matrix-based tool for searching the putative binding region for a transcription factor in DNA sequences (20). The cAMP-CRP complex binding region was putatively identified as being located between -104 and -89 relative to the P3_{*hfq*} promoter. The binding profile of CRP was constructed based on similarity to the consensus sequences inferred from known CRP binding sites using RegulonDB, version 6.4. A fragment (-115 to -78) was synthesized within the proposed sequence (-104 to -89) for EMSA to test the binding of the cAMP-CRP complex. Figure 3B depicts that the cAMP-CRP complex bound to this

synthetic fragment in a CRP dose-dependent manner (0 to 200 nM), whereas cAMP or CRP alone (Fig. 3B, bottom) failed to bind to the proposed region. Finally, the specificity of the binding region was confirmed because the cAMP-CRP complex could not bind to the other synthetic fragment containing the mutated sequences, ACGCTAGGGGTAGAGT (Fig. 3B, top). The EMSA results demonstrated that the cAMP-CRP complex directly interacts with the *hfq* promoter region (-104 to -89) in a dose-dependent manner. Next, we used a DNase I footprinting assay to verify the cAMP-CRP complex binding site. A DNA fragment from positions -251 to -32 was subjected to DNase I footprinting in the absence or presence of CRP (400 nM). The digested fragments were separated by capillary electrophoresis and peak heights on the chromatograms. We detected only one CRP protected region located from -112 to -87 (including a hypersensitive region) (Fig. 3C, bottom) when comparing sequence patterns in the absence or presence of CRP (Fig. 3C, top and middle). Therefore, the CRP protected region verified by the DNase I footprinting assay is similar to the binding site detected by EMSA. The results shown in Fig. 3 suggest that a binding region of the cAMP-CRP complex is located on the -429-to-68 fragment.

Stability of *ompA* mRNA following the *hfq* knockout in cAMP-deficient mutant. Our data suggested that elevated expression levels of *hfq* in response to low levels of cAMP were associated with the increased degradation of *ompA* mRNA. To further test this hypothesis, we used a cAMP-deficient mutant, which already displayed a high degradation level of *ompA* mRNA (Fig. 1A and C, top), to create a *cya hfq* double mutant using a phage P1-generalized transduction method. The *cya hfq* double mutant should have low levels of both cAMP and Hfq. Figure 4 shows, interestingly, that once the *hfq* gene was deleted, *ompA* mRNA became markedly more stable. The increases in stability were independent of the cAMP levels because both the *hfq* mutant and *cya hfq* double mutant exhibited similar increases in the stability of *ompA* mRNA. These findings confirm that high levels of *hfq* expression reduce the stability of *ompA* mRNA.

Roles of transformed *hfq* in *ompA* mRNA stability of *hfq* knockout mutant in the presence of glucose. In addition, we used a *hfq* knockout mutant to assess the role of *hfq* in the presence of glucose by transforming the *hfq* knockout mutant with a plasmid containing the P3_{*hfq*} promoter and wild-type *hfq* gene. The half-life of *ompA* mRNA was more stable in the *hfq* knockout mutant than in the wild-type strain (Fig. 4, bottom). When *hfq* levels were restored by transforming the mutant with a plasmid containing the wild-type *hfq* operon, the *ompA* mRNA was destabilized (Fig. 5A, top). However, the stability of the *ompA* mRNA was further reduced by the addition of glucose to the growth medium (Fig. 5A, bottom). This suggests that the presence of the *hfq* gene is necessary for the negative effects of glucose on *ompA* mRNA stability in response to the reduced cAMP levels. On the other hand, glucose did not have a negative effect when the P3_{*hfq*} promoter region was mutated (namely, P_{NCH}, with a mutation of the binding region for the cAMP-CRP complex) (Fig. 5B). This finding reveals that, first, the predicted cAMP-CRP binding region of the P3_{*hfq*} promoter is essential for glucose response, and second, the underlying mechanisms involved in the negative regulation by glucose of *ompA* mRNA stability is associated with the activation

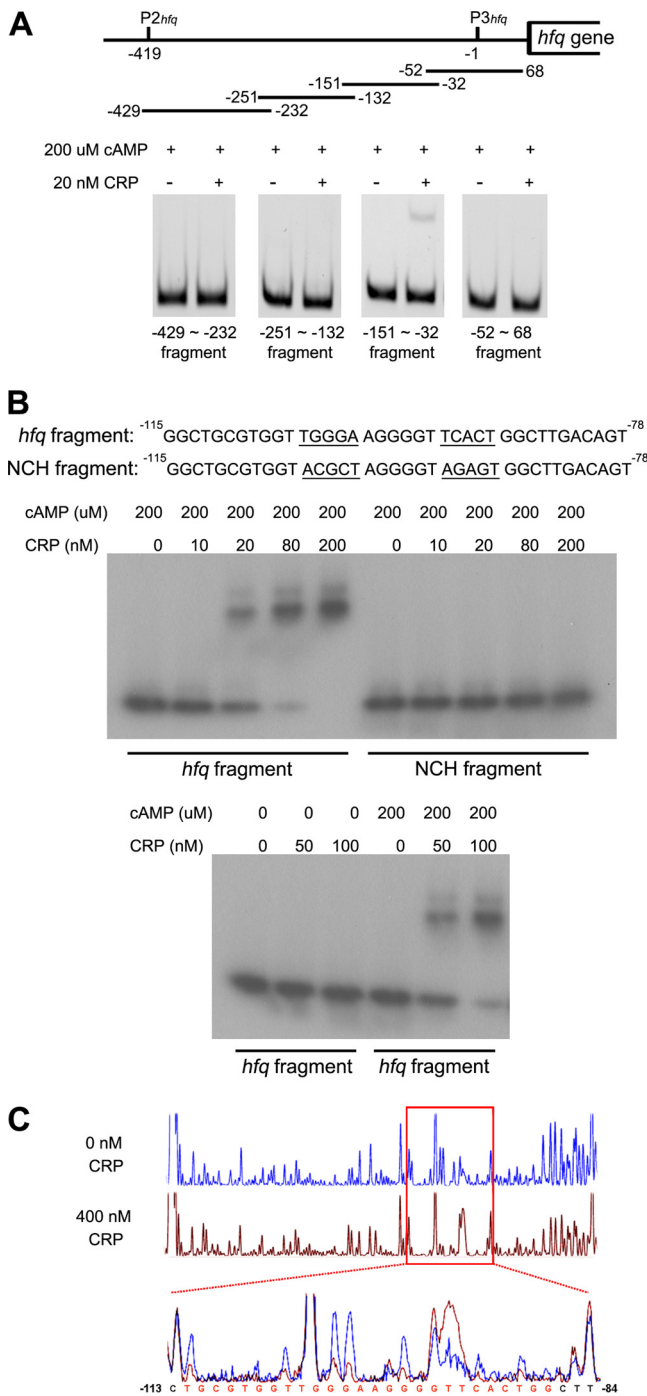


FIG. 3. Delineation of *hfq* promoter region interacted with cAMP-CRP complex and its specificity. (A) (Top) Four DNA fragments of the P3_{hfq} promoter region ranging from sequence position -429 to 68 were used to narrow down the potential binding region of the cAMP-CRP complex. The numbers indicate the DNA sequences counted from the P3_{hfq} promoter. (Bottom) The band shift resulted from the binding of cAMP-CRP complex by EMSA. (B) The MATCH program predicted and screened the putative binding region (*hfq* fragment). The *hfq* and NCH fragments were synthesized and used for EMSA. NCH indicates non-CRP binding region mutant. (Top) EMSA results as shown by ³²P-labeled *hfq* or NCH fragment in the presence of 200 uM cAMP and different concentrations of CRP as indicated. (Bottom) EMSA results as shown by ³²P-labeled *hfq* fragment. The concentrations of cAMP and CRP are indicated above the panels. (C) Full-

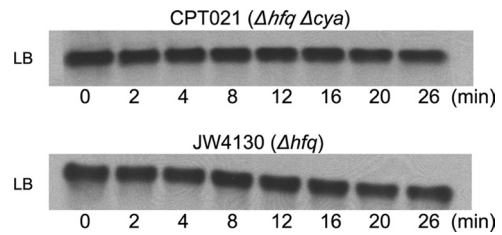


FIG. 4. Stability of *ompA* mRNA following the *hfq* knockout in cAMP-deficient mutant. Northern blot analysis was used to examine the effect of *hfq* deletion on the stability of *ompA* mRNA in two *E. coli* strains. CPT021 was a double mutant with both *hfq* and cAMP deficiency. JW4130 was the mutant with *hfq* deficiency.

of the *hfq* gene caused by the loss of the repressive effects of the decreased cAMP level (Fig. 1B).

In contrast to the repressive effects of the cAMP-CRP complex on the P3_{hfq} promoter, it is well known that the complex can positively activate the *lacZ* promoter. Next we replaced the P3_{hfq} promoter with the *lacZ* promoter and used it to address whether the negative effects of glucose on *ompA* mRNA stability could be reversed by reducing *hfq* expression at low cAMP levels. We found that using the *lacZ* promoter resulted in a stabilization of *ompA* mRNA after adding glucose (Fig. 5C). This indicates that the decreased level of cAMP induced by glucose failed to activate the *lacZ* promoter, resulting in lower expression of *hfq*. Under such conditions, the transformed cells mimicked those for the *hfq* knockout mutant (Fig. 4, bottom). Western blot analysis also indicated that the stability of *ompA* mRNA correlates with Hfq levels (Fig. 5D).

Positive effects of cAMP and negative effects of Hfq on *fur* mRNA stability. The relationship between cAMP and Hfq on mRNA stability was examined using another mRNA (*fur*) with a degradation rate that is regulated by the Hfq protein in the *E. coli* wild type (35). Compared to the wild type (BW25113) (Fig. 6A), *fur* mRNA was less stable in cAMP-deficient mutants (Fig. 6B). However, knockout of the *hfq* gene in the *cya* and *hfq* double mutant (CPT021) markedly stabilized the *fur* mRNA (Fig. 6C). These results were closely similar to those described in the legend to Fig. 4 for the stability of *ompA* mRNA.

DISCUSSION

While the decay rate of *ompA* mRNA in *E. coli* has been reported (29), our finding that glucose negatively regulates the stability of *ompA* mRNA has not been reported previously according to the best of our knowledge. The growth rate-dependent stability of *ompA* mRNA is probably due to the fact that glucose is a preferred carbon source for *E. coli* for most of

length electropherograms of DNase I footprinting assays showing the protected pattern of the -251-to-32 fragment in the absence (blue, top) or presence (red, middle) of CRP. The red square shows a significant difference in the peak pattern. The square was expanded to demonstrate where the region was protected by aligning the results for 0 nM and 400 nM CRP (bottom). The red sequence indicates the protected region.

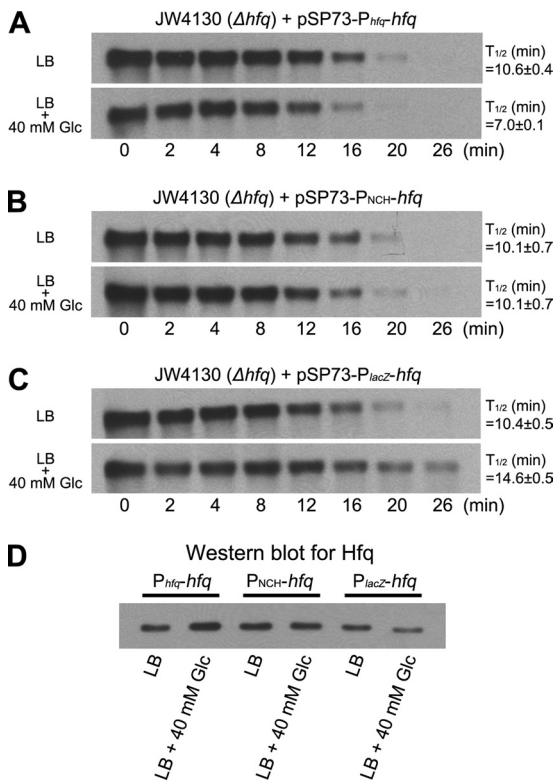


FIG. 5. Roles of transformed *hfq* in *ompA* mRNA stability. (A) *OmpA* mRNA stability was measured in *hfq*-deficient cells (JW4130) transformed with pSP73-*P_{hfq}-hfq* plasmid when growing in LB medium supplemented without (top) or with (bottom) 40 mM glucose (Glc). (B) *OmpA* mRNA stability was measured in *hfq*-deficient cells transformed with a negative-control pSP73-*P_{NCH}-hfq* plasmid. (C) *OmpA* mRNA stability was measured in *hfq*-deficient cells transformed with a hybrid plasmid containing *lacZ* promoter and *hfq* gene (pSP73-*P_{lacZ}-hfq*). All the experiments were conducted by Northern blotting in triplicate. (D) Hfq protein expression in response to glucose supplement was analyzed by Western blotting.

its nutrients, based on carbon catabolite repression (9). It is therefore expected that the uptake of glucose results in the enhanced growth rate of the cells, and in turn, the increased growth rate results in the increase in the stability of *ompA* mRNA (25, 36). However, our study has shown that the glu-

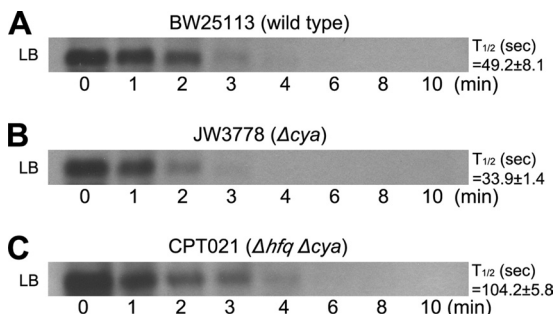


FIG. 6. Stability of *fur* mRNA following the *hfq* knockout in cAMP-deficient mutant. Northern blot analysis was used to examine the effects of *hfq* deletion on the stability of *fur* mRNA in the three *E. coli* strains. (A) Wild type. (B) Mutant with cAMP deficiency. (C) Double mutant with both *cya* and *hfq* deficiency.

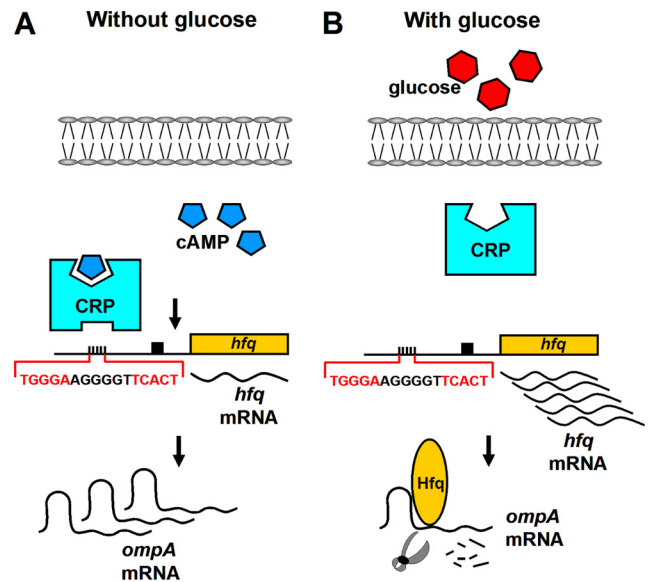


FIG. 7. Schematic drawing for the cascade effect of cAMP and Hfq on *ompA* mRNA stability. (A) In LB medium without the addition of glucose, cAMP forms a complex with CRP and specifically binds to the *hfq* promoter to lower the transcription of *hfq* gene. The reduced Hfq protein attenuates the *ompA* mRNA degradation. (B) In contrast, after the addition of glucose the cAMP levels are reduced, preventing the cAMP-CRP complex from binding to the *hfq* promoter. This results in an increase in *hfq* transcription, and then Hfq protein promotes *ompA* mRNA degradation.

ucose supplement did not make a significant difference in the growth rates when *E. coli* was growing in LB medium. These findings are consistent with those of the previous study showing that the growth rate of *E. coli* is barely affected when glucose is added to LB media (31). In this study, the doubling times were similar: 41 min when wild-type *E. coli* was grown in LB and 37 min when it was grown in LB supplemented with glucose (40 mM). Therefore, the data presented reflect the effects of glucose on degradation of *ompA* mRNA that is independent of the growth rate. The half-life (~11.5 min) for *ompA* mRNA in the wild type (Fig. 1) is in close agreement with those found in previous studies (25, 36).

It is well known that cAMP levels are low in *E. coli* growing in the presence of glucose (23). We have shown that changing cAMP levels in *E. coli* regulates *ompA* mRNA stability. Increasing cAMP levels by the addition of exogenous cAMP to LB increased the stability of *ompA* mRNA (Fig. 1B). Subsequently, we showed that the half-life of *ompA* mRNA was significantly attenuated in the cAMP-deficient mutant, suggesting that the regulation of *ompA* mRNA stability was cAMP dependent (Fig. 1C).

The mechanism(s) involved in the negative effects of glucose on *ompA* mRNA in the present study seems to be complicated. A schematic drawing to simplify our working hypothesis is depicted in Fig. 7. There are several lines of evidence in the present study, which have not been reported previously, suggesting that cAMP is associated with the expression of *hfq*. First, *hfq* was derepressed in the cAMP-deficient mutant when assessed by real-time PCR, Northern blot analysis, and Western blot analysis (Fig. 2). Second, cells grown in the presence

of glucose (a condition that lowers the cellular cAMP levels) caused an increase in *hfq* expression (Fig. 2B and D). Third, only the cAMP-CRP complex, but not cAMP or CRP alone, could directly bind to the promoter of the *hfq* gene (Fig. 3B). The identified bound region (sequence -104 to -89) for the cAMP-CRP complex appears to be specific and essential, as mutation of this region abolishes the binding of the complex in an EMSA and DNase I footprinting assay (Fig. 3B and C). In many cases, the cAMP-CRP complex binding site is positioned at a relatively long distance from the transcription start site (38). Downregulation of the *guaB* promoter by the cAMP-CRP complex has been reported at position -117.5 (14). In this study, the results shown in Fig. 3 show that only one binding site for the cAMP-CRP complex existed in the -429-to-68 fragment. Fourth, *ompA* mRNA was destabilized in cAMP-deficient mutants, but the mRNA stability was markedly restored by deletion of the *hfq* gene in the *cya hfq* double mutant (Fig. 4). Our results suggest that the cAMP-CRP complex acts as a transcriptional factor to repress the expression of *hfq*. Fifth, subsequent transformation of the wild-type *hfq* operon back to the *hfq* mutant substantially destabilized *ompA* mRNA. Then, the regulation of the wild-type *hfq* operon for *ompA* mRNA stability in the presence of glucose was the same as that of the wild-type strain (BW25113) (Fig. 1A and 5A). In contrast, substituting the P_{3_{hfq}} promoter with a *lacZ* promoter (that is positively regulated by cAMP-CRP) increased the stability of the *ompA* mRNA in the presence of glucose. This is because a glucose-induced reduction in cAMP levels only minimally activates P_{lacZ-hfq}, and *hfq* expression was not sufficient to promote the *ompA* mRNA degradation (Fig. 5C).

Hfq has been proposed to control posttranscriptional or translational regulation by targeting small RNAs and mRNAs (4). In addition to forming base pairs of small RNA and mRNA, Hfq associates with the C-terminal scaffold region of RNase E to activate *ptsG* mRNA degradation (17, 24). Recently, Hfq is regarded as a limiting factor *in vivo* for small RNA signaling (15). The base-pairing of MicA small RNA (also named SraD) to the *ompA* mRNA in the vicinity of the ribosome-binding site is enhanced by the presence of Hfq, leading to mRNA decay and subsequent translational inhibition. MicA participates in the downregulation of *ompA* mRNA in the stationary phase (OD₆₀₀ values of 1.5 to 2.0), and the MicA mutant does not affect the stability of *ompA* mRNA during exponential growth (OD₆₀₀ = 0.4) or the conditions used in our experiments (data not shown) (27, 33). Furthermore, CyaR (also named RyeE, the CRP-activated small RNA) negatively regulated the expression of *ompX* but had no effects on *ompA* mRNA (7). Therefore, the small RNA, which is already known to affect CRP or *ompA* mRNA stability, would not play a role for glucose regulation of *ompA* stability in this study.

Taken together, our data reveal that glucose can destabilize the *ompA* mRNA by reducing the cAMP-CRP complex-mediated activation of *hfq* gene expression. We also identified a binding site for the cAMP-CRP complex in the P_{3_{hfq}} promoter, which may be responsible for the repression. Our findings provide new insights into the transcriptional regulation of *hfq* and posttranscriptional regulation of *ompA* by glucose. Since Hfq is an important RNA binding protein, the changes in cAMP levels induced by glucose appear to indirectly modulate

the stability of mRNA, such as *ompA* and *fur*, by an Hfq-dependent mechanism. This mechanism may play an important role in regulating the stability of other mRNA in *E. coli*.

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