Participation of Regulator AscG of the β -Glucoside Utilization Operon in Regulation of the Propionate Catabolism Operon[▽]

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Received 21 May 2009/Accepted 17 July 2009

The *asc* operon of *Escherichia coli* is one of the cryptic genetic systems for β -D-galactoside utilization as a carbon source. The *ascFB* genes for β -D-galactoside transport and catabolism are repressed by the AscG regulator. After genomic SELEX screening, AscG was found to recognize and bind the consensus palindromic sequence TGAAACC-GGTTTCA. AscG binding was detected at two sites upstream of the *ascFB* promoter and at three sites upstream of the *prpBC* operon for propionate catabolism. In an *ascG*-disrupted mutant, transcription of *ascFB* was enhanced, in agreement with the repressor model of AscG. This repression was indicated to be due to interference of binding of cyclic AMP-CRP to the CRP box, which overlaps with the AscG-binding site 1, as well as binding of RNA polymerase to the promoter. Under conditions of steady-state *E. coli* growth in a rich medium, the intracellular level of AscG stayed constant at a level supposedly leading to tight repression of the *ascFB* operon. The level of *prpR*, encoding the activator of *prpBCDE*, was also increased in the absence of AscG, indicating the involvement of AscG in repression of *prpR*. Taken together, these data suggest a metabolic link through interplay between the *asc* and *prp* operons.

Both laboratory strains and natural isolates of *Escherichia coli* possess a number of cryptic genes for β -glucoside utilization (5). The most well-characterized *bgl* operon is the major genetic system for utilization of β -glucoside sugars such as arbutin and salicin. The *bglGFB* operon includes the genes encoding the enzymes for uptake of β -glucoside sugars and for hydrolysis of the phosphorylated substrates (15, 26, 33, 34). BglG is a positive regulator of this *bgl* operon (26, 33) which prevents termination of transcription in the presence of β -glucosides. The *bgl* operon is, however, cryptic and uninducible in most *E. coli* strains under laboratory culture conditions (3, 23), but it is expressed and functional in some *E. coli* strains (5, 26, 31). The cryptic *chbBCARF* (or *celABCDF*) operon also includes the genes for transport and phosphorylation of cellobiose, arbutin, and salicin and for hydrolysis of phosphorylated substrates (11, 12, 24, 28). The *chbR* (*celD*) gene, encoding a repressor-activator dual regulator, is organized within this operon (11, 12, 25). A set of pathogenic *E. coli* strains carry an additional cryptic *bgc* operon encoding the enzymes for transport and hydrolysis of β -glucoside sugars (19). The divergently transcribed *bgcR* gene encodes a GntR-type transcription factor for positive control of the *bgc* operon. The *asc* operon (arbutin-salicin-cellobiose) was discovered by selecting for cellobiose utilization in a *bgl chb* (or *bgl cel*) double mutant (4, 5, 23). The cryptic *asc* operon encodes a repressor (*ascG*), a phosphotransferase system enzyme II (*ascF*) for the transport of arbutin, salicin, and cellobiose, and a phospho- β -glucosidase (*ascB*) that hydrolyzes the sugars which are phosphorylated during transport. The *ascG* and *ascFB* operons are transcribed from divergent promoters. The *ascFB* genes are paralogous to the cryptic *bglGFB* genes, and the *ascG* gene is paralogous to *galR*.

Various kinds of β -D-glucosides are released from plants, and thus the expression of a genetic system for utilization of β -glucosides is beneficial for bacterial survival in nature. Genes for utilization of β -D-glucosides are considered to be gained by horizontal transfer from gram-positive bacteria, and in most laboratory *E. coli* strains, they are retained silently by tight repression by genome silencing factors such as the nucleoid protein H-NS (31). For utilization of β -D-glucosides such as arbutin and salicin as a sole carbon source by laboratory *E. coli* strains, at least one of the cryptic operons must be activated, enhancing the adaptive potential of the strains. The cryptic operons for β -glucoside utilization can be activated by gaining mutations in the signals involved in transcription initiation or in the regulatory genes. In the case of the *bglGFB* operon, both the *cis*-acting regulatory sequence and the *trans*-acting regulatory proteins that bind to the regulatory sequences respond to silencing of the *bgl* promoter (18, 29, 32, 35). The major class of activating mutations are effective in *cis* and have been characterized as insertions of insertion elements or point mutations within the regulatory sequences. Mutations in the cryptic *chb* operon also allow constitutive utilization of β -D-glucosides such as arbutin, salicin, and cellobiose (25).

In an attempt to gain insight into the regulation of the cryptic genes for β -D-glucoside utilization, we performed detailed analyses of the relatively ill-characterized cryptic *asc* operon, focusing on the function of the regulatory gene *ascG*. After a systematic search for the regulation targets of AscG, we determined that in addition to the *asc* operon, the *prp* operon for catabolism of propionate is under the control of AscG. The physiological relevance of this interplay between the *asc* and *prp* operons is discussed.

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[▽] Published ahead of print on 24 July 2009.

MATERIALS AND METHODS

Bacterial strains. *E. coli* KP7600 (W3110 *lacI^q lacZΔM15 galK2 galT22*) and JD27135 (with a transposon insertion within the *ascG* coding sequence of KP7600 in the opposite direction from that of *ascG* [a gift from T. Miki, Fukuoka

TABLE 1. Primers used in this study

Primer group and name	Length (nucleotides)	Sequence
Primers for amplification of SELEX fragment DNA		
EcoRV-F	20	CTTGTTATGCCCGGTACTGC
EcoRV-R	20	GCGATGCTGTCGGAATGGAC
Primers for sequencing of SELEX fragment DNA		
T7-F primer	20	TAATACGACTCACTATAGGG
T7-R primer	21	GGTTTTCCAGTCACACGACG
Primer for gel shift and DNase footprinting analyses		
FITC-T7 primer	20	TAATACGATCACTATAGGG
Primers for Northern blot analysis		
ascG-F	25	CAGTTTAAACGCCGTGGCAGAGTTG
ascG-R	22	AAGGAGCAATGAGTGAGTCGCG
ascF-F	23	ATGCACCGCGTCTTTACGCCAAC
ascF-R	25	AAGACTTACTTCTTTGGCGACGGTC
ascB-F	23	TTTCAGCTATTACGCCTCGCGCT
ascB-R	29	TAAATCTTCCCCATTACTGGCAATCACTT
prpR-F	23	GGCATCAGCCTGTCCGGTAGAT
prpR-R	21	TTTTCAGCCGCCGCCAGAACG
prpB-F	25	GTCATCGTCCGAATAAAGCGATCGT
prpB-R	22	ACGGGCAAACAGGTTGTCGAGC
prpE-F	24	GTGTATTACAGACCATCTGGGGCGA
prpE-R	22	TGGCGGATCTGATCCAACGACG
Primers for primer extension		
FITC anti-GFP	19	AGGGTCAGCTTGCCGTAGG
FITC ascF	28	CCATCGGATTTATCCTGTTATCAGTAG

Dental College]) were used in various experiments for the analysis of the regulatory roles of AscG, including primer extension assays, promoter assays, and Northern and Western blot analyses. *E. coli* BL21(DE3) [F^- *ompT* *hsdS_B*(r_B^- m_B^-) *dcm* *gal* λ (DE3)] was used for expression and purification of AscG. Cells were cultured in LB medium or M9-0.4% glucose medium at 37°C. When necessary, 100 μ g/ml ampicillin was added to the medium.

Expression and purification of His-tagged AscG protein. For construction of plasmid pAscG for AscG expression, a DNA fragment corresponding to the AscG coding sequence was amplified by PCR, using *E. coli* W3110 genomic DNA as a template and a pair of primers designed so as to hybridize upstream or downstream of the AscG coding sequence. After digestion with NdeI and NotI (note that the restriction enzyme sites were included within the primer sequences), PCR products were cloned into pET21a(+) (Novagen) between NdeI and NotI sites. The plasmid construct was confirmed by DNA sequencing. For protein expression, pAscG plasmid was transformed into *E. coli* BL21(DE3). Transformants were grown in 200 ml of LB medium, and at a cell optical density at 600 nm (OD_{600}) of 0.6, isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After 3 h of incubation, cells were harvested by centrifugation, washed with a lysis buffer (50 mM Tris-HCl, pH 8.0, at 4°C, with 100 mM NaCl), and then stored at -80°C until use.

For protein purification, frozen cells were suspended in 3 ml of lysis buffer containing 100 mM phenylmethylsulfonyl fluoride. Cells were treated with lysozyme and then subjected to sonication for cell disruption. After centrifugation at 15,000 rpm for 20 min at 4°C, the resulting supernatant was mixed with 2 ml of 50% Ni-nitrilotriacetic acid agarose solution (Qiagen) and loaded onto a column. After being washed with 10 ml of the lysis buffer, the column was washed with 10 ml of washing buffer (50 mM Tris-HCl, pH 8.0, at 4°C, with 100 mM NaCl) and then with 10 ml of washing buffer containing 10 mM imidazole. Proteins were eluted with 2 ml of an elution buffer (lysis buffer plus 200 mM imidazole), and AscG peak fractions were pooled, dialyzed against a storage buffer (50 mM Tris-HCl, pH 7.6, at 4°C, 200 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, and 50% glycerol), and stored at -80°C until use. Protein purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE).

Genomic SELEX of AscG-binding sequences. The genomic SELEX system was used as described previously (36). Genomic DNA of *E. coli* W3110 was sonicated to generate fragments of 150 to 300 bp in length. The *E. coli* DNA library was constructed after cloning of these 150- to 300-bp DNA fragments into plasmid pBR322 at the EcoRV site. A collection of 150- to 300-bp DNA fragments could be regenerated by PCR amplification, using an *E. coli* DNA plasmid library as the template and a set of primers, EcoRV-F and EcoRV-R, which

hybridize to the pBR322 vector at EcoRV junctions (for the primer sequences, see Table 1). PCR products thus generated were purified by 5% PAGE. For genomic SELEX screening of AscG-binding sequences, 5 pmol of DNA fragments and 20 pmol of His-tagged AscG were mixed in a binding buffer (10 mM Tris-HCl, pH 7.8, at 4°C, 3 mM magnesium acetate, 150 mM NaCl, 1.25 μ g/ml bovine serum albumin) and incubated for 30 min at 37°C. The mixture was applied to a Ni-nitrilotriacetic acid column, and after washing of unbound DNA with binding buffer containing 10 mM imidazole, DNA-AscG complexes were eluted with an elution buffer containing 200 mM imidazole. When necessary, this SELEX cycle was repeated several times. For sequencing of AscG-bound DNA fragments, DNA was isolated from DNA-AscG complexes by PAGE and then PCR amplified. PCR products were cloned into pT7 Blue-T vector (Novagen), using a blunt-end cloning kit (Takara), and transformed into *E. coli* DH5 α . Sequencing analysis was carried out using a T7 primer (Table 1) and an ABI model 3130xl DNA sequencer.

SELEX chip analysis of AscG-binding sequences. Mapping of SELEX fragments along the *E. coli* genome was also performed by using a 22,000-feature DNA microarray (Oxford Gene Technology, Oxford, United Kingdom) (38). One genomic SELEX sample obtained with AscG was labeled with Cy3, while another SELEX sample obtained in the absence of AscG or with a different transcription factor was labeled with Cy5. After hybridization of samples to the tiled DNA array, the Cy5/Cy3 ratio was measured and the peaks of scanned patterns were plotted against the positions of DNA probes along the *E. coli* BW25113 chromosome.

Gel mobility shift assay. Gel mobility shift assays were performed as described previously (20, 21). In brief, probes were generated by PCR amplification of AscG-binding sequences in the SELEX analysis by use of a pair of primers, 5'-fluorescein-isothiocyanate (FITC)-labeled T7-F and T7-R (Table 1), the genomic SELEX plasmids containing the respective AscG recognition sequences as templates, and Ex *Taq* DNA polymerase (Takara). PCR products with FITC at their termini were purified by PAGE. For gel shift assays, 1.0 pmol each of the FITC-labeled probes was incubated at 37°C for 30 min with various amounts of AscG in 12 μ l of gel shift buffer consisting of 10 mM Tris-HCl, pH 7.8, at 4°C, 150 mM NaCl, and 3 mM magnesium acetate. After the addition of a DNA dye solution, the mixture was directly subjected to 6% PAGE. Fluorescently labeled DNA in gels was detected using a Phorox FX Plus system (Bio-Rad).

DNase I footprinting assay. DNase I footprinting assays were carried out using FITC-labeled DNA fragments as described previously (20, 21). Each 1.0 pmol of FITC-labeled probes was incubated at 37°C for 30 min with various amounts of AscG in DNase I footprinting buffer consisting of 25 μ l of 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 3 mM magnesium acetate, 5 mM $CaCl_2$, and 25 μ g/ml bovine

serum albumin. After incubation for 30 min, DNA digestion was initiated by the addition of 12.5 ng of DNase I (Takara). After digestion for 30 s at 25°C, the reaction was terminated by the addition of 25 µl of phenol. Digested products were precipitated with ethanol, dissolved in formamide-dye solution, and analyzed by electrophoresis on a 6% polyacrylamide gel containing 7 M urea.

Measurement of promoter activity. Promoter strength was determined as described previously (16). In brief, green fluorescent protein (GFP) was expressed under the control of a test promoter, while red fluorescent protein (RFP) was expressed under the control of a reference promoter. The promoter assay vector pGRP carries two types of fluorescent protein genes, one for RFP under the control of reference promoter *lacUV5* and the other for GFP under the control of a test promoter. The sequences upstream from the initiation codons of *ascF* and *ascG* were PCR amplified by use of their respective primers and inserted into the pGRP vector. The primers contained the recognition sequences for EcoT22I, BglII, or BamHI, which could be used for promoter cloning. The PCR products were digested with the respective restriction enzymes and then ligated into pGRP at EcoT22I and BglII sites. The insertions in the promoter assay plasmids thus constructed were confirmed by sequencing. The plasmids used were pGRK259 (pGRP*ascF*), pGRK559 (pGRP*ascG*), and pGRN024 (pGRP*prpR*).

For measurement of the fluorescence intensity of RFP or GFP expressed in *E. coli* transformants, each carrying one promoter assay vector (16), cells were grown in LB medium or M9–0.4% glucose medium to an OD₆₀₀ of 0.3 to 4.0, harvested by centrifugation, and resuspended in phosphate-buffered saline [PBS(–)]. The cell suspensions were diluted with PBS(–) to obtain approximately the same cell density (OD₆₀₀ of 0.6) for all samples. For the measurement of bulk fluorescence, aliquots of a 0.2-ml cell suspension were added to 0.4-ml flat-bottomed 96-well plates, and the fluorescence was measured with a Wallac 1420 ARVOsx instrument (Perkin-Elmer Life Sciences), where GFP was measured using a 485-nm excitation wavelength and a 535-nm emission wavelength and RFP was measured using a 544-nm excitation wavelength and a 590-nm emission wavelength. The fluorescence intensity of GFP expressed from the test promoter was normalized using the equation $(X/Y)/(A/B)$, in which *X* and *Y* indicate the fluorescence intensities of GFP (test promoter) and RFP (*lacUV5* promoter), respectively, while *A* and *B* represent the fluorescence intensities of GFP (*lacUV5* promoter) and RFP (*lacUV5* promoter), respectively (16).

Northern blot analysis. Northern blot analysis of *ascF*, *ascG*, *prpR*, and *prpB* RNAs was performed essentially as described previously (20, 21). In brief, total RNAs were prepared by the hot phenol method from both *E. coli* wild-type strain KP7600 and the *ascG*-disrupted mutant JD27135, separated by agarose gel electrophoresis in the presence of formamide, and transferred to a positively charged nylon membrane (Roche). Digoxigenin (DIG)-labeled probes were prepared by use of a PCR DIG probe synthesis kit (Roche) and a pair of oligonucleotide primers, each complementary to the 5'-proximal or 3'-proximal sequence of test mRNA (see Table 1 for the primer sequences). Hybridization was carried out at 50°C for 16 h in DIG Easy Hyb (Roche) as recommended by the supplier. Hybridized probe on the membrane was detected by CDP-Star ready-to-use reagent (Roche).

Quantitative Western blot analysis. Antibodies against AscG, cyclic AMP receptor protein (CRP), and PrpR were raised in rabbits by injection of the respective purified proteins. A quantitative Western blot analysis was carried out by a standard method as described previously (9). In brief, *E. coli* cells grown in 10 ml of either LB or M9–0.4% glucose medium were harvested by centrifugation and resuspended in 0.3 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% glycerol, and 1 mM dithiothreitol), and then lysozyme was added to a final concentration of 20 µg/ml. Total proteins were subjected to 12% sodium dodecyl sulfate-PAGE and blotted onto polyvinylidene difluoride membranes by use of a semidry transfer apparatus. Membranes were first immunodetected with anti-AscG, anti-CPA, or anti-PrpR, treated with fluorescently labeled secondary antibodies, and then developed with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). Images were analyzed with an LAS-1000 Plus Lumino image analyzer and Image Gauge (Fuji Film).

RESULTS AND DISCUSSION

AscG is a GalR-type transcription factor consisting of 337 amino acid residues with a molecular mass of 36,944 Da. The gene encoding AscG is transcribed divergently from the *ascFB* operon and has been proposed to be involved in repression of the cryptic *ascFB* operon, which is involved in transport and

TABLE 2. SELEX search for AscG-binding sites^a

Left gene	SELEX fragment (size [bp]) ^b	Right gene	No. of fragments
<i>ascG</i> (←)	S (244)	<i>ascF</i> (→)	36
<i>prpR</i> (←)	S (204)	<i>prpB</i> (→)	22
<i>recR</i> (←)	S (187) (<i>htpG</i>)	<i>adk</i> (→)	1
<i>ybiS</i> (←)	S (211) (<i>ybiT</i>)	<i>ybiU</i> (←)	1
	Others		19
<i>cspF</i> (→)	AscG box*	<i>quuQ</i> (→)	
<i>fimA</i> (→)	AscG box**	<i>fimC</i> (→)	

^a Genomic SELEX analysis for a search of AscG-binding sequences was performed using purified His-tagged AscG alone in the absence of effectors. The SELEX fragments were also analyzed by a SELEX chip system. A total of 79 DNA SELEX fragments were isolated, among which 36 fragments contained a 244-bp sequence within spacer regions between the *ascG* and *ascF* coding frames and 22 fragments contained a 204-bp sequence between the *prpR* and *prpB* coding sequences. Each of the other 21 SELEX fragments contained a different sequence originating from a different region of the *E. coli* genome, among which AscG binding within the coding regions of *htpG* and *ybiT* was also detected by the SELEX chip system. After sequence analysis of the entire *E. coli* genome, an AscG box-like sequence was detected in the *cspF-quuQ* and *fimA-fimC* spacer regions. The arrows indicate the direction of transcription of the neighboring genes, and the genes shown in bold represent possible target genes under the control of AscG.

^b *, TAAACCGTTTC; **, GAACCGTTTC.

utilization of the β-glucoside sugars arbutin, salicin, and cellobiose (4).

Screening of AscG-binding sequences by genomic SELEX. To search for AscG-regulated targets within the *E. coli* genome, attempts were made to identify DNA sequences that are recognized by AscG by use of the genomic SELEX system (36), in which a complete library of *E. coli* genome DNA fragments is used as substrates instead of synthetic oligonucleotides, with all possible sequences used in the original SELEX method (2, 41, 43). This genetic SELEX screening has been employed successfully for identification of the whole set of regulation targets for transcription factors including AilR (7), CitB (47), Cra (36), LeuO (39), NemR (44), PdhR (21), RstA (20), and RutR (37). A fourfold molar excess of the purified His-tagged AscG protein was added to a mixture of *E. coli* genomic DNA fragments of 150 to 300 bp in length, and the AscG-DNA complexes were affinity purified. In the early stage of this genomic SELEX cycle, the AscG-bound DNA fragments gave smear bands on PAGE gels, as did the original genome fragment mixture. After two and three SELEX cycles, several discrete bands were identified, indicating that some DNA fragments with high affinity to AscG were enriched. These DNA fragments were recovered from the gel and cloned into pT7 Blue-T plasmid (Novagen) for sequencing.

Among a total of 79 independently isolated clones, two major species were isolated from two different regions, including one spacer region between the *ascG* and *ascF* genes and another between *prpR* and *prpB* in the *E. coli* genome (Table 2). Since regulatory proteins affecting transcription initiation generally bind upstream of the respective target genes, the major regulation targets of AscG were indicated to be *ascG* and/or *ascF* and *prpR* and/or *prpB*. This finding agrees well with the prediction that AscG regulates the promoter for the *ascFB* operon. However, experimental evidence indicating the involvement of AscG in the regulation of *prpR* and/or *prpB* has not been reported. The number of SELEX isolates correlates

FIG. 1. Gel mobility shift assay of AscG-target DNA complex formation. Fluorescently labeled DNA probes (1.0 pmol each) containing a SELEX segment from the *ascG-ascF* spacer (A) or the *prpR-prpB* spacer (B) were incubated at 37°C for 30 min in the absence (lane 1) or presence of increasing concentrations of AscG (lane 2, 2.5 pmol; lane 3, 5.0 pmol; and lane 4, 10 pmol). The reaction mixtures were directly subjected to PAGE. P, probe DNA; C1 to C3, AscG-probe DNA complexes.

with the binding affinity of the test transcription factor to the substrate DNA (36, 44). If this is the case with AscG, then the *prp* operon must be a major regulation target of AscG, in addition to the *asc* operon.

The binding of AscG to the *ascG-ascF* and *prpR-prpB* intergenic spaces was confirmed by analysis of the SELEX fragments by use of a tiled array (OGT) (38). In addition to these two major sites, binding of AscG was identified on *htpG*, encoding the HSP90 chaperone, and *ybiT*, encoding an ABC-type transporter, by both genomic SELEX and SELEX chip analyses (Table 1).

Identification of AscG-binding DNA sequences. The binding of AscG protein to the two major sequences identified by the SELEX screening was examined by gel shift assay. As shown in Fig. 1, both SELEX DNA fragments formed AscG complexes in a protein dose-dependent manner. One unexpected finding is that both probes contained multiple sites for AscG binding. Upon increases in the AscG concentration, the AscG complexes with both *ascG-ascF* (Fig. 1A) and *prpR-prpB* (Fig. 1B) probes were supershifted, altogether forming two complex bands in the case of *ascG-ascF* (Fig. 1A) and three complex bands with the *prpR-prpB* probe (Fig. 1B). This finding indicates that at least two and three binding sites exist in the *ascG-ascF* and *prpR-prpB* probes, respectively.

To identify the recognition sequence on each of the AscG-binding SELEX fragments, we next performed DNase I footprinting assays, using both SELEX fragments as probes. After formation of complexes in the presence of a fixed amount of the fluorescently labeled DNA probe and increasing amounts of AscG, DNase I treatment was carried out for a short period, and the partially digested DNA products were analyzed by PAGE (Fig. 2). In the case of the *ascG-ascF* probe, two protected regions were detected, one between positions –84 and –59 with respect to the *ascF* promoter and another between positions –8 and +11 (Fig. 3A), in good agreement with the formation of two bands in gel shift assays (Fig. 1A). On the other hand, in the case of the *prpR-prpB* probe, clear protection by AscG was observed at a single long region consisting of a 54-bp sequence between *prpR* positions –11 and –64 (Fig. 3A), indicating that three AscG protomers bind in tandem within this sequence.

FIG. 2. Identification of AscG box sequence. A fluorescently labeled SELEX segment (1.0 pmol) from the *ascG-ascF* spacer (A) or the *prpR-prpB* spacer (B) was incubated in the absence or presence of increasing concentrations of purified AscG (2.5, 5.0, 10, and 20 pmol, from right to left) or CRP (0.3, 0.6, 1.25, and 2.5 pmol, from right to left) and then subjected to DNase I footprinting assays. Lanes A, T, G, and C represent the respective sequence ladders.

Prediction of the AscG recognition sequence. After alignment of the two DNase I protection patterns obtained by the use of two different probes, we could identify the 14-bp-long palindromic sequence TGAAACC-GGTTTCA as the AscG recognition sequence, and it is hereafter referred to as the AscG box (Fig. 4A). The AscG-protected site 1 on the *ascF* promoter includes this AscG box between positions –80 and

FIG. 3. Locations of promoters and AscG- and CRP-binding sites. (A) The transcription initiation sites of *ascG* and *ascF* and the binding sites for AscG and CRP are shown along the 256-bp-long *ascG-ascF* spacer. The transcription initiation sites were determined by primer extension assay (data not shown), while the binding sites for AscG and cAMP-CRP were determined by DNase I footprinting assay as described in the legend to Fig. 2. The numbers in parentheses represent distances from the transcription initiation site of *ascF*, which is under the control of AscG. Open boxes at the termini of the DNA bar represent the initiation codons of *ascG* and *ascF*. (B) The transcription initiation sites of *prpR* and *prpB* and the binding sites for AscG and CRP are shown along the 200-bp-long *prpR-prpB* spacer. The binding sites for AscG and cAMP-CRP were determined by DNase I footprinting assay as described in the legend to Fig. 2. The numbers in parentheses represent distances from the transcription initiation site of *prpR*, which is under the control of AscG. Boxes at the termini of the DNA bar represent the initiation codons of *prpR* and *prpB*.

FIG. 4. Consensus sequence of AscG box and its location within the *asc* and *prp* operons. (A) The sequences represent those protected by AscG within the *ascG-ascF* and *prpR-prpB* spacer regions (see Fig. 2 and 3). The consensus sequence of the AscG box was identified after comparison of these AscG-binding sequences. The numbers in parentheses represent distances (bp) from the transcription initiation site of *ascF* or *prpR*. The underlined nucleotides represent those identical with the consensus AscG box. (B) The sequences represent those protected by cAMP-CRP within the *ascG-ascF* and *prpR-prpB* spacer regions (see Fig. 2 and 3). The numbers in parentheses represent distances (bp) from the transcription initiation site of *ascF* or *prpR*.

–67 with respect to the transcription initiation site of *ascF* (Fig. 3A). On the other hand, the AscG-protected site 2 includes the complete 14-bp-long palindromic AscG box between positions –4 and +9 (Fig. 4A). Since AscG site 2 overlaps with promoter position –10 and the transcription initiation site of *ascF*, AscG bound at site 2 may play a major role in repression of *ascFB* transcription. Since the AscG box sequence in *ascF* site 2 is identical with the consensus AscG box, the binding affinity of AscG should be strong, presumably leading to strong repression (see below for promoter assay and mRNA analysis). After sequence analysis of the entire *E. coli* genome, the AscG box-like sequence was identified in the spacer regions of both *cspF-quuQ* and *fimA-fimC* (Table 1), raising the possibility that both *quuQ* (Qin antiterminator) and *fimC* (pilus chaperone) are under the control of AscG.

The cluster of genes required for propionate catabolism was first identified in *Salmonella enterica* serovar Typhimurium (6, 8), and a closely related cluster was found in *E. coli* after genome sequencing (30). These genes constitute a locus composed of two divergently transcribed units. One unit is the single gene *prpR*, which encodes a regulatory protein, PrpR, a member of the RpoN-dependent activator family (22, 40). The second transcriptional unit contains the *prpBCDE* operon, which encodes the enzymes for propionate catabolism, allowing growth on propionate as a sole carbon and energy source (8). After DNase I footprinting assay of the 200-bp spacer between *prpR* and *prpB* (Fig. 2), one 54-bp-long region protected by AscG was detected between positions –64 and –11 with respect to the *prpR* promoter (Fig. 3). Within this 54-bp sequence, two copies of the left half of the AscG box (L1 and L2) (Fig. 4A) and three copies of its right half (R1, R2, and R3) (Fig. 4A) were identified. Thus, the formation of three complex bands upon gel shift assay (Fig. 1) indicates sequential binding of three AscG protomers within this modified AscG

box. Since this AscG-binding sequence overlaps the *prpR* promoter, AscG should repress transcription initiation of the *prpR* gene. This AscG-binding site also overlaps the predicted PrpR-binding site for activation of the *prpBCDE* operon (13), so the binding of AscG may also interfere with the activation of the *prpBCDE* operon by PrpR.

Identification of CRP-binding DNA sites. In *E. coli*, glucose controls the utilization of alternative carbon sources by regulating transcription of the genes involved in their catabolism in response to glucose depletion (1, 10, 14). Since the genes for β -D-glucoside utilization were predicted to be expressed in the absence of glucose and since the AscG box sequence is similar to the CRP box sequence (Fig. 4), we analyzed binding in vitro of cyclic AMP (cAMP)-CRP to the *ascF* promoter. By gel shift and DNase I footprinting assays, we indeed identified binding of cAMP-CRP between positions –83 and –59 (Fig. 4B), completely overlapping with AscG-binding site 1 (between positions –84 and –59) (Fig. 4A). From the location of the CRP-binding site, CRP was indicated to be involved in activation of the *ascFB* operon.

Lee et al. (13) proposed the involvement of cAMP-CRP in transcription activation of both the divergently transcribed *prpR* gene and the *prpBCDE* operon. We then analyzed the binding site of cAMP-CRP within the *ascG-ascF* spacer region. After DNase I footprinting assay, one cAMP-CRP-binding site was identified, including a 29-bp sequence between positions –82 and –54 of the *prpR* promoter or between positions –119 and –92 of the *prpBCDE* promoter (Fig. 3). A CRP box-like sequence was identified within this sequence (Fig. 4), which overlaps partially with the AscG-binding site (Fig. 3).

Influence of AscG on in vivo promoter activity. The possible influence of AscG on transcription initiation in vivo from the *ascFB*, *prpR*, and *prpBCDE* promoters was then examined using a two-fluorescent-protein (TFP) promoter assay system. For this purpose, we first identified the hitherto unidentified promoter for the *ascFB* operon. Using a primer extension assay in the absence of the AscG repressor, a transcription initiation site of *ascF* was identified at position –95 relative to the initiation codon (data not shown). We then cloned sequences of about 500 bp in length upstream from the initiation codons of the *ascF*, *prpR*, and *prpB* genes (see Fig. 3 and 8 for the gene organization), each including transcription initiation sites and recognition sequences for a transcription factor, i.e., AscG (*ascF*), CRP (*ascF* and *prpB*), or PrpR (*prpB*), and inserted them into pGRP to construct the promoter assay vectors pGRK259 (pGRP*ascF*), pGRK559 (pGRP*ascG*), and pGRN024 (pGRP*prpR*). The expression of GFP was under the control of each test promoter, while RFP expression was directed by the reference promoter *lacUV5* on the same vector (see Materials and Methods).

Promoter activity was detected for transformants with pGRK259 (pGRP*ascF*) in both wild-type *E. coli* KP7600 and the mutant JD27135 with a disrupted *ascG* gene. The promoter activity in wild-type *E. coli* increased concomitantly with the advance of cell growth but was always two- to threefold higher for the mutant without AscG protein (Fig. 5A). This finding supports the prediction that AscG represses the expression of the *ascFB* operon under ordinary culture conditions. Using transformants with pGRN024 (pGRP*prpR*), the expression of GFP under the control of the *prpR* promoter was examined

FIG. 5. Assay of the *ascF* and *prpR* promoters in wild-type and *ascG* mutant *E. coli*. The *ascF* and *prpR* promoter fragments were inserted into the TFP promoter assay vector pGRP, and the resulting promoter plasmids, pGRP*ascF* (A) and pGRP*prpR* (B), were transformed into wild-type KP7600 and its *ascG*-disrupted mutant, JD27135. The promoter activities were determined by measuring the GFP/RFP ratio. Closed bars represent the promoter activities in wild-type KP7600, while open bars represent the promoter activities in the *ascG* mutant JD27135.

(Fig. 5B). Even though the promoter activity of *prpR* was lower than that of *ascF*, it was significantly higher in the mutant lacking AscG (Fig. 5B), supporting the prediction that AscG is involved in repression of the *prpR* promoter. To confirm the results of the promoter assay using the TFP vector, we next performed direct measurement of mRNAs.

Regulation in vivo of the *ascFB* and *prpBCDE* promoters by AscG. Total RNAs were isolated from both wild-type KP7600 and *ascG*-disrupted JD27135 and subjected to Northern blot analysis using a fluorescently labeled probe for detection of the 5'-proximal region of *ascF* mRNA. A single hybridized band was detected (Fig. 6), and its band intensity increased markedly in the *ascG* mutant, in particular when grown in LB medium (Fig. 6, lanes 1 and 2). When cells were grown in M9-glucose medium, the level of *ascF* transcription was very low because of the lack of cAMP in the presence of glucose (Fig. 6, lanes 3 and 4). Based on this finding, we concluded that AscG is the repressor of transcription initiation from the promoter of the *ascFB* operon.

By Northern blot analysis, high-level expression of the *prpR* gene was detected in wild-type *E. coli* grown in LB medium (Fig. 6). The level of *prpR* mRNA increased slightly in the *ascG* mutant. Taking these observations together with the promoter assay results (Fig. 5), we concluded that AscG plays a repressive role, albeit at a low level, for the *prpR* gene encoding the activator of the *prpBCDE* operon. In agreement with the slight increase in *prpR* expression in the *ascG* mutant, the mRNA level of *prpB*, the activation target of PrpR, significantly increased (Fig. 6). The level of *prpB* mRNA in M9-glucose culture (Fig. 6, lanes 3 and 4) was lower than that in LB culture (Fig. 6, lanes 1 and 2), in agreement with the involvement of CRP in activation of the *prpBCDE* operon (13).

FIG. 6. Northern blot analysis of *asc* and *prp* operon RNAs. Overnight cultures of wild-type *E. coli* KP7600 (wt) and its *ascG*-disrupted mutant JD27135 ($\Delta ascG$) in either LB or M9-glucose medium were transferred into the respective fresh medium. After shaking culture at 37°C, cells were harvested in the middle of log phase (OD_{600} , 0.6). Total RNA was isolated as described in Materials and Methods and fractionated by PAGE in the presence of urea. After blotting of RNA onto filters, *ascF*, *ascB*, *prpR*, and *prpB* RNAs were detected with fluorescently labeled probes. The probes used were prepared by PCR, using the primers described in Table 1. The total amounts of RNA samples were adjusted based on the staining intensity of rRNA with ethidium bromide.

Competition between AscG and CRP. The AscG-binding sites identified herein are similar to the CRP box sequence (Fig. 3). We next analyzed binding in vitro of cAMP-CRP to the *ascG-ascFB* spacer region. By gel shift and DNase I footprinting assays, we indeed identified binding of cAMP-CRP upstream of the *ascFB* promoter (Fig. 2). Within the protected regions, a sequence similar to the consensus palindromic CRP box sequence, TGTGA-TCACA, was identified (Fig. 3). The binding site (between positions -83 and -59) of cAMP-CRP for activation of the *ascFB* operon completely overlaps that (between positions -84 and -59) of repressor AscG-binding site 1 (Fig. 3). This immediately suggests that one of the possible mechanisms of *ascFB* repression by AscG is competition between two regulators, AscG and CRP, for binding to their respective target sites. This possibility was examined by competitive gel shift assay, as employed for AscG binding to the targets identified after genomic SELEX screening (Fig. 1). Protein species bound to the shifted DNA complexes were determined by immunoblot analysis, using specific antibodies against AscG and CRP. Probe DNA-bound CRP was decreased by adding increasing amounts of AscG (Table 3). Likewise, the amount of DNA-bound AscG decreased with an increase in cAMP-CRP. This finding supports the prediction that AscG represses *ascFB* transcription not only through interference of RNA polymerase binding to the promoter (at AscG site 2) but also through interference of binding of the activator cAMP-CRP (at AscG site1).

In the case of the *prpB* promoter, the cAMP-CRP-binding site does not overlap with the AscG-binding site on the *prpR* promoter (Fig. 3). Thus, the repression of *prpR* and *prpBCDE* transcription by AscG might not be due to interference of the action of cAMP-CRP.

Search for effectors influencing expression of the *ascFB* operons. To identify the inducer(s) that enhances or reduces the induction of the *ascFB* operon, we performed a phenotype microarray assay using the whole set of 20 standard phenotype

TABLE 3. Competition between AscG and CRP in binding to the *ascF* promoter^a

AscG level (pmol)	Amt (pmol) of added cAMP-CRP	% DNA-bound protein	
		AscG	CRP
2.5	0	100	0
5.0	0	100	0
5.0	0.3	70	30
5.0	0.6	50	30
0	0.3	0	100
0	0.6	0	100
2.5	0.6	80	70
5.0	0.6	70	50

^a A fluorescently labeled *ascG-ascF* spacer DNA probe (1.0 pmol) was incubated with the indicated amounts of AscG and/or CRP at 37°C for 30 min in the presence of 0.05 mM cAMP. The reaction mixtures were directly subjected to PAGE. Proteins were blotted onto polyvinylidene difluoride membranes by use of a semidry transfer apparatus. Membranes were first immunodetected with anti-AscG or anti-CRP, treated with fluorescently labeled secondary antibodies, and then developed with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). The intensities of stained bands were measured with an LAS-1000 Plus Lumino image analyzer and Image Gauge (Fuji Film). The approximate amounts of DNA-bound proteins were estimated from the band intensities.

microarray plates. Significant differences in cell growth between wild-type KP7600 and the isogenic *ascG* mutant JD27135 were identified in several wells. In the presence of arbutin or salicin as a sole carbon source, cell growth was observed only with the *ascR* mutant lacking AscG (data not shown). This finding supports the prediction that in wild-type *E. coli*, AscG represses the genes needed for utilization of arbutin and salicin, but in the absence of AscG, the *ascFB* genes for the catabolism of these compounds as carbon sources are induced.

Intracellular levels of AscG. mRNA analysis indicated that in wild-type *E. coli*, the *ascFB* genes, encoding the enzymes for utilization of arbutin and salicin, are strictly repressed (Fig. 6). We then determined the intracellular level of AscG in wild-type *E. coli* grown in LB medium by using quantitative Western blot analysis (9). From exponential growth phase to stationary phase, the intracellular concentration of AscG stayed almost constant, at a level of about 500 to 700 molecules per genome equivalent of DNA (Fig. 7). This finding indicates that a high level of AscG is always present in wild-type *E. coli* cells and represses transcription of the *ascFB* operon.

Using the same cell lysates, we also determined the intracellular concentrations of CRP and PrpR (Fig. 7). Surprisingly, the level of PrpR (1,500 to 1,700 molecules per genome equivalent of DNA) was as high as that of the most abundant global regulator, CRP, even in the absence of propionate. At present, it is not clear why such a high level of PrpR is present under normal growth conditions in the absence of propionate. One possibility is that PrpR has an as yet unidentified physiological role, including the regulation of other genes besides the only known target, the *prpBCDE* operon.

Physiological roles of AscG. At least four operons for β -D-glucoside utilization have been identified in *E. coli*, but most of these operons are preserved as cryptic states in most laboratory *E. coli* strains. Each of these operons is associated with a specific regulatory gene for the control of gene expression. For instance, the genes for the activators BglG and ChbR (CelR) are organized within the respective target operons *bglGFB* (15,

FIG. 7. Determination of intracellular concentrations of AscG, CRP, and PrpR. Wild-type *E. coli* KP7600 was grown in LB medium at 37°C for various times. Cells were harvested at turbidities of 0.3, 0.6, 0.9, and 1.5, and whole-cell lysates were prepared as described in Materials and Methods. Quantitative Western blot analysis was performed by a standard procedure (11), using anti-AscG, anti-CRP, and anti-PrpR antibodies. The intensities of immunoblot bands were measured with an LAS-1000 Plus Lumino image analyzer and Image Gauge (Fuji Film).

26, 33, 34) and *chbBCARF* (or *celABCDF*) (12, 24, 25, 28). On the other hand, the genes for the regulators AscG and BgcR form single-gene operons, which are located next to the divergently transcribed target operons, namely, *ascFB* (11, 12) and *bglEFIHA* (19), respectively. The modes of action are also different for these regulators. BglG activates the *bglGFB* operon by preventing transcription termination in the presence of β -glucosides (33), while ChbR (or CelD) is a positive factor for transcription initiation (25). In contrast, AscG is the repressor for keeping the *ascFB* operon silent under ordinary culture conditions (12; this report). In fact, the level of AscG is maintained at a high level to keep the *ascFB* genes silent under steady-state cell growth (Fig. 7). In the presence of β -D-glucosides but the absence of useful carbon sources such as glucose in natural environments, however, the expression of these cryptic genes may provide an advantage for growth. These cryptic genes for β -D-glucoside utilization are preserved in a silent state in most wild-type *E. coli* strains. Silencing of these cryptic operons for β -D-glucoside utilization may be needed to prevent the utilization of toxic aryl- β -D-glucoside substrates (31).

The direct action of AscG as a repressor of the *prpR* gene could provide a regulatory link of utilization between β -D-glucosides and propionate (Fig. 8). In *E. coli*, propionate arises

FIG. 8. Model of the regulatory roles of AscG. AscG was found to regulate not only the *ascFB* operon but also the *prpR* gene, which encodes a positive regulator of the *prpBC* operon for propionate catabolism. Two AscG-binding sites were identified in the *ascF* promoter, while three sites were identified in the *prpR* promoter.

as the terminal three-carbon fragment in the oxidation of odd-chain-length fatty acids (45, 46). Propionate is catabolized to pyruvate through α -oxidation. The 200-bp-long intergenic region between the *prpR* and *prpBCDE* operons contains an RpoD-dependent promoter for *prpR* and an RpoN-dependent promoter for *prpBCDE*. In addition, the regulatory factors PrpR, cAMP-CRP, and IHF have been suggested to participate in the transcriptional activation of the *prpBCDE* operon (8, 14, 22, 42). Expression of *prpBCDE* is dependent on the RpoN RNA polymerase holoenzyme and PrpR (8, 22, 42). PrpR is an NtrC family activator which interacts directly with RpoN RNA polymerase (17). NtrC family activators are generally involved in nitrogen regulation in *E. coli*, since nitrogen assimilation consumes energy and intermediates of central metabolism (27). Involvement of CRP and AscG indicates another link between carbon and nitrogen metabolism. Taking these observations together, we propose that the catabolism of propionate is under the control of several regulators of the genes for both carbon and nitrogen metabolism.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid (17076016, 18310133, and 21241047) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the Nano-Biology Project of the Micro-Nano Technology Research Center of Hosei University.

We thank T. Miki for the JD27135 mutant strain and T. Shimada, H. Ogasawara, and J. Teramoto (Hosei University) for helpful discussions.

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