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Analysis of Promoter Targets for *Escherichia coli* Transcription Elongation Factor GreA In Vivo and In Vitro▽†

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Transcription elongation factor GreA induces nucleolytic activity of bacterial RNA polymerase (RNAP). In vitro, transcript cleavage by GreA contributes to transcription efficiency by (i) suppressing pauses and arrests, (ii) stimulating RNAP promoter escape, and (iii) enhancing transcription fidelity. However, it is unclear which of these functions is (are) most relevant in vivo. By comparing global gene expression profiles of *Escherichia coli* strains lacking Gre factors and strains expressing either the wild type (wt) or a functionally inactive GreA mutant, we identified genes that are potential targets of GreA action. Data analysis revealed that in the presence of chromosomally expressed GreA, 19 genes are upregulated; an additional 105 genes are activated upon overexpression of the wt but not the mutant GreA. Primer extension reactions with selected transcription units confirmed the gene array data. The most prominent stimulatory effect (threefold to about sixfold) of GreA was observed for genes of ribosomal protein operons and the *tna* operon, suggesting that transcript cleavage by GreA contributes to optimal expression levels of these genes in vivo. In vitro transcription assays indicated that the stimulatory effect of GreA upon the transcription of these genes is mostly due to increased RNAP recycling due to facilitated promoter escape. We propose that transcript cleavage during early stages of initiation is thus the main in vivo function of GreA. Surprisingly, the presence of the wt GreA also led to the decreased transcription of many genes. The mechanism of this effect is unknown and may be indirect.

In bacteria, the transcription process is initiated when the RNA polymerase (RNAP) holoenzyme binds to a promoter DNA sequence and forms an open promoter complex (RPo). In the presence of nucleoside triphosphates (NTPs), RNAP in the RPo synthesizes short (typically 2 to 9 nucleotides [nt] long) transcripts that rapidly dissociate from the complex. However, some of these "abortive" transcripts are extended beyond a threshold of 9 to 12 nt, which allows RNAP to start transcript elongation (36, 30). RNAP in the elongation complex (EC) can transcribe over long distances until it reaches a terminator, where the EC dissociates into individual components (the DNA template, the RNA product, and RNAP, which can reinitiate transcription). Every stage of the transcription cycle can be limiting to the overall process and subject to regulation.

Transcription elongation can be slowed or even blocked at certain points of the template, with the resultant formation of paused or arrested complexes, respectively. In these complexes, RNAP shifts along the DNA template in the direction opposite to that of transcription. As a result of such backtracking (18, 24) the 3′ end of RNA disengages from the RNAP catalytic center, making further elongation impossible. An arrested complex can resume transcript elongation only following endonucleolytic cleavage of the nascent RNA that generates a new 3′ end of the transcript in the RNAP catalytic center. The endonucleolytic reaction performed by the RNAP catalytic center is slow but greatly stimulated by transcript cleavage factors GreA and GreB. The products of GreA-induced cleavage are di- and trinucleotides, whereas the products of GreB-induced cleavage are RNA fragments 2 to 18 nt long (see references 7 and 13 and references therein).

Gre factors use their C-terminal domain to bind near the opening of the RNAP secondary channel. This channel is thought to serve as a port of entry for NTPs during transcription elongation, as an exit path for the RNA 3′ terminus during backtracking (3, 38, 40), and likely as an exit route for abortive transcripts. The N-terminal coiled-coil domain of Gre factors reaches the RNAP catalytic center through the secondary channel and directly stimulates the transcript cleavage reaction (21, 25, 34). Factor-stimulated endonucleolytic cleavage of RNA is followed by the dissociation of the 3′-proximal fragment from the EC; the 5′-proximal fragment remains bound and can be further extended, giving RNAP another opportunity to transcribe through a problematic sequence. Both GreA and GreB can prevent elongating RNAP from falling into an arrested conformation (7, 8) and thus stimulate the overall rate and efficiency of transcription elongation. Backtracking is also observed when nucleotide analogs that disrupt RNA-DNA hybrid are incorporated into RNA in in vitro transcription assays (24, 32). By activating the cleavage reaction, Gre factors remove misincorporated nucleotides and thus may contribute to transcription proofreading and fidelity (13, 39). Another in

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TABLE 1. Strains and plasmids

E. coli K-12 strain	Genotype	Source or reference
BW28357	*rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568*	41
BW32611	BW28357 Δ*greA*::*kan*	Kan^r by Red-mediated recombination
BW32613	BW28357 Δ*greB*::*kan*	Kan^r by Red-mediated recombination
BW32645	BW32611 Δ*greA*; BW32670 Δ*greB*	Kan^s with pCP20, Kan^s with pCP20
pET19b[NPH]greA	pET19b; NPH-greA	21
pET19b[NPH]greA-D41E	pET19b; NPH-greA-D41E	21

vitro activity of Gre factors, facilitation of transition from abortive initiation to productive elongation, also appears to involve the stimulation of the transcript cleavage reaction (14, 15, 16). However, which stage of transcription is (are) most dependent on Gre-induced cleavage in vivo is yet unclear.

There are several lines of evidence that emphasize the biological importance of Gre factors in nature. Genes coding for Gre proteins and their homologs have been found in the genomes of most bacteria. Moreover, the *greA* gene is among ~200 genes that are essential for the viability of *Mycoplasma pneumonia*, an organism with one of the smallest known genomes (17). In several bacterial species, the deletion of *greA* leads to hypersensitivity toward environmental assaults, such as ionic detergents, elevated temperatures, and osmotic shock (9, 23, 26, 37). A recent report identified *greA* as a member of the *sigE* regulon in *E. coli*, further underscoring the potential role of GreA in cellular stress response (31). GreA is also found to be one of only few proteins that are upregulated during low-pH growth of *Streptococcus mutans* (22) and one of only nine proteins that are upregulated in *Staphylococcus aureus* in response to a challenge by cell wall-active antibiotic (33). In *Escherichia coli*, the overexpression of GreA confers resistance to toxic levels of divalent metal ions, such as Zn^{2+} and Mn^{2+} (35). Collectively, these observations strongly implicate Gre factors in the survival of microorganisms in harsh or restrictive environments. However, the relationship, if any, between the known biochemical functions of Gre proteins and their physiological roles has not yet been established. To find such a link, in this work, we performed an in vivo transcription profiling of *E. coli* cells lacking GreA and/or overproducing wild-type (wt) GreA, or inactive GreA mutant that binds RNAP but is unable to promote transcript cleavage. Follow-up in vitro studies of several randomly chosen genes whose expression depends on the presence of wt GreA indicate, unexpectedly, that stimulation of promoter escape may be the main function of this transcription elongation factor.

MATERIALS AND METHODS

Construction of *E. coli gre* deletion strains. Deletions of *greA* and *greB* genes were made in the *E. coli* K-12 derivative BW28357 (41) (Table 1) by using Red-mediated recombination as described previously (10). The *greA* deletion was constructed by using a PCR product generated on pKD13 as the template with primers *gre*A#1 (5′-AATATTCAAGAGGTATAACAAATGCAAGCTATTCC GTGTAGGCTGGAGCTGCTTCGAAG-3′) and *gre*A#2 (5′-TTACAATACA TCAACATCTTGAGTATTGGGTAATTCATTCCGGGGATCCGTCGACC TG-3′). The *greB* deletion was similarly made, except with primers *gre*B#1 (5′-TATTGATTCTGTTGATATGATCACGTTATACCCAATTGTAGGCTG GAGCTGCTTCGAAG-3′) and *gre*B#2 (5′-AAATGCCAGCCATCGGCAGGA GGTTAAGACTCTTCCGATCCGTCGACCTGCAGTTC-3′). Mutations were confirmed by PCR as described previously (10). The kanamycin resistance gene was eliminated by using pCP20 helper plasmid. P1*kc* transduction was carried out as described elsewhere.

Culture conditions and RNA isolation. All growths of liquid culture were carried out at 30°C. Overnight cultures prepared from frozen stocks of individual strains were diluted 200-fold into fresh Luria-Bertani broth (LB; Difco), supplemented with antibiotics and 50 μM IPTG (isopropyl-β-D-thiogalactopyranoside) where necessary, and allowed to grow to mid-exponential phase (an optical density at 600 nm [OD_{600}] of 0.4) under vigorous aeration. Growth was stopped by quick cooling in an ice water bath, and cells were harvested by centrifugation at 2°C. Cell pellets were flash frozen in an ethanol-dry ice bath and stored at −84°C or were processed immediately for RNA isolation. RNA was isolated from frozen cell pellets by using QIAGEN RNeasy mini kit. On-column DNase I digestion (QIAGEN) was used to maximally eliminate the contaminating genomic DNA. RNA concentrations were determined by absorbance at 260 nm on a spectrophotometer, and the quality of RNA preparation was analyzed by agarose gel electrophoresis.

***E. coli* genome arrays.** The GeneChip *E. coli* Genome 2.0 arrays were purchased from Affymetrix. An array contains approximately 10,000 probe sets for 20,366 genes present in four (K-12 plus three pathogenic) strains of *E. coli*. The probe sets encompass the entire complement of annotated open reading frames and over 700 intergenic regions of K-12 *E.coli* genome.

Microarray analyses. cDNA synthesis, fragmentation, 3′-terminal biotin labeling, array hybridization, and computational array data analyses were performed by Cognition Therapeutics LLC. (Rockville, MD) as detailed in Affymetrix protocols (2). After the hybridization of labeled cDNA to Affymetrix *E. coli* Genome 2.0 array chips, the arrays were washed, stained with R-phycoerythrin streptavidin (Molecular Probes) using the Affymetrix Fluidic Station 400, and scanned by a GeneChip Scanner 3000 (Affymetrix). The scanned probe array images were analyzed and sorted using Affymetrix GeneChip operating software (2). Datasets from three independent experiments were combined and filtered to select for genes consistently showing a change in expression of 1.5-fold or more.

Primer extension. For total RNA purification, *E. coli* cells were grown under the same conditions as those for gene array analysis and total RNA was purified as described above. RNA concentrations were estimated from the absorbance at 260 nm, and verified by denaturing agarose gel electrophoresis, followed by ethidium bromide staining. For each primer extension reaction, 10 μg of total RNA was used. Primers (30 pmol) were radiolabeled by 10 U of T4 polynucleotide kinase (NEB) using 9 pmol of [γ-^{32}P]ATP (4,500 Ci/mmol). Three picomoles of ^{32}P-labeled, gene-specific primers (0.45 μCi/pmol) (see Table S1 in the supplemental material) was annealed to RNA under identical conditions, using a temperature gradient from 65°C to 56°C for 20 min. The primer extension reaction was carried out by using 200 U of SuperScript III reverse transcriptase (Invitrogen) for 50 min at 55°C in the presence of deoxynucleoside triphosphates (1 mM each) in 20 μl. The reaction was stopped by the addition of 20 μl of formamide sample buffer, and reverse transcripts were separated in 8 M urea-10% polyacrylamide gel along with ^{32}P-labeled ϕX174 DNA/Hinf I markers (Promega). Reverse transcripts were identified by using standard size markers and by DNA sequencing and quantified by a Typhoon PhosphorImager (GE Healthcare) by using ImageQuant (Molecular Dynamics).

Immunoblotting. To estimate the expression level of chromosomally encoded wt GreA and plasmid-encoded wt and mutant GreA in vivo, we used quantitative immunoblotting analysis (see Fig. S1 in the supplemental material). Two ml of *E. coli* cell cultures was grown as described above to an OD_{600} of 0.6 and harvested by centrifugation, and the pellets were resuspended in 300 μl of lysis buffer containing 40 mM Tris HCl, pH 7.9, 300 mM NaCl, 1 mM EDTA, 5% glycerol, 5 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride. Cells were lysed by sonication and centrifuged to remove debris. Total protein concentration in clear lysates was determined with Bradford assay reagent (Bio-Rad). Indicated amounts of total soluble protein were resolved by Tris-glycine sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (PAGE) and electroblotted onto nitrocellulose membranes (Advantec MFC, Inc.) Blots were probed

TABLE 2. Genes that are activated by wt GreA under both native and overexpressed conditions

Name[a]	Alias	Effect of wt GreA (fold)		Gene description or function
		Chromosomal	Plasmid encoded	
cydA	b0733	+1.7	+1.6	Cytochrome *d* terminal oxidase, polypeptide subunit I
cydB	b0734	+1.5	+1.6	Cytochrome *d* terminal oxidase polypeptide subunit II
ndh	b1109	+1.5	+2.6	Respiratory NADH dehydrogenase/cupric reductase
emrY[b]	b2367	+2.4	+1.6	Predicted multidrug resistance protein Y (multidrug efflux system)
yfiD	b2579	+2.4	+2.5	Formate acetyltransferase; stress-induced alternate pyruvate formate-lyase subunit
ypjL[c]	b2639	+1.9	+2.2	Putative pump protein, CP4–57 prophage; predicted inner membrane protein
dctR[c]	b3507	+1.9	+1.6	Protein involved in metabolism of C4-dicarboxylates; involved in acid resistance (*yhiF*)
gadW	b3515	+1.7	+1.7	AraC-type transcriptional repressor; controls several genes of acid resistance system (*yhiW*)
yibH[c]	b3597	+2.1	+5.7	Putative membrane protein
nlpA[c]	b3661	+1.8	+1.6	Cytoplasmic membrane lipoprotein 28
dgoT[c]	b3691	+1.7	+1.6	D-Galactonate transport
tnaL	b3707	+3.4	+4.2	Tryptophanase leader peptide
tnaA	b3708	+2.2	+1.5	Tryptophanase
tnaB	b3709	+1.7	+2	Low-affinity tryptophan permease
yidZ[c]	b3711	+1.8	+1.7	LysR-type putative transcriptional regulator; involved in protection against NO
yieH[c]	b3715	+1.6	+1.7	6-Phosphogluconate phosphatase
cbrB[c]	b3716	+2.1	+1.8	Predicted inner membrane protein (*yieI*)
yijF[c]	b3944	+1.9	+1.6	Conserved protein
yjfZ[c]	b4204	+1.8	+1.9	ORF, hypothetical protein

[a] Genes comprising polycistronic operons are grouped together. All unannotated genes are monocistrons.
[b] Genes from polycistronic operons where expression of other genes were not affected by GreA.
[c] Genes with unknown operon structure.

with rabbit polyclonal anti-GreA antibodies (a generous gift from R. Landick) and developed by a standard protocol using goat anti-rabbit horseradish peroxidase conjugate antibodies (Sigma) with the ECL$^+$ reagent kit (GE Healthcare). Developed immunoblots were scanned by PhosphorImager and quantified by ImageQuant (GE Healthcare).

In vitro transcription assay. The *E. coli* RNAP was purified from *greA greB* mutant *E. coli* strain as described previously (5, 26). *E. coli* wt GreA, GreA-D41E mutant, and *Thermus thermophilus* GreA proteins were overexpressed and purified as described previously (6, 21).

Transcription assays were performed using PCR-amplified promoter DNA fragments that include about 150 bp both upstream and downstream of the transcription start site. For a list of the primers used for PCRs, see Table S1 in the supplemental material. Because RNAP may become arrested at the end of the DNA fragment during transcription on some templates it may impede the multiround transcription. Therefore, to avoid this problem and to analyze and compare transcription efficiency of RNAP in the absence and in the presence of GreA on different promoters in the multiround runoff transcription assay, we included terminator in all our templates. Thus, all templates contained an additional 115 bp, including tR2 terminator (68 bp), downstream from the initial transcribed region of a promoter, followed by an additional 47 bp. Reactions were carried out in standard transcription buffer (40 mM Tris-HCl, pH 7.9, 70 mM KCl, 0.1 mM dithiothreitol, 10 mM $MgCl_2$) by using 0.06 μM RNAP holoenzyme, 0.1 mM NTP, 5 μCi [α-^{32}P]CTP (3,000 Ci/mmol), 0.02 μM template DNA, and 4.5 μM Gre factors in a total volume of 10 μl. DNA, RNAP, Gre proteins, and NTPs were premixed on ice, and the samples were incubated for 5 or 15 min at 37°C. We found that the efficiency of transcription termination was not affected by GreA factors (data not shown). To observe the accumulation of abortive products during the multiround runoff assay, transcription reactions were carried out as above by using 30 μM NTP and the incubation time was increased to 30 or 60 min. All reactions were stopped with formamide sample buffer, and the RNA products were separated by 8 M urea-10% or 23% PAGE. Runoff and termination RNA products were identified by using ^{32}P-labeled ϕX174 DNA/HinfI markers (Promega) and quantified by the Typhoon PhosphorImager (GE Healthcare) by using ImageQuant software (Molecular Dynamics). The identity of short abortive RNAs was verified by RNA sequencing by using γ-^{32}P-radiolabeled initiating NTP and terminating 3′ deoxy NTPs as described previously (15).

RESULTS AND DISCUSSION

Of the two Gre factors encoded in the *E. coli* genome, GreA appears to be more important based on its higher abundance and stronger evolutionary conservation. Moreover, previous analysis showed that while cells lacking GreB are virtually indistinguishable from wt cells, *greA* mutants exhibit several growth defects, including sensitivity to salt and divalent metal ions (data not shown) (35). For this work, we constructed isogenic single (*greA*$^+$ *greB* mutant) and double (*greA greB* mutant) *E. coli* mutants and used them, together with appropriate analysis methods, to compare the global expression profiles of the two strains (see Materials and Methods). We reasoned that the comparison of transcriptomes from these two

TABLE 3. Genes that are down-regulated by wt GreA under both native and overexpressed conditions

Name[a]	Alias	Effect of wt GreA (fold)		Gene description or function
		Chrom	Plasm	
talB	b0008	−1.9	−2.0	Transaldolase
dnaK	b0014	−3.5	−3.1	Hsp70 chaperone; DNA biosynthesis; autoregulated heat shock proteins
dnaJ	b0015	−2.4	−2.3	Heat shock protein; chaperone with DnaK
acnB	b0118	−1.9	−1.7	Aconitate hydrase B (TCA cycle)
betA	b0311	−1.7	−2.6	Choline dehydrogenase, a flavoprotein
betB	b0312	−2.5	−3.2	NAD^{+}-dependent betaine aldehyde dehydrogenase
betI	b0313	−4.4	−4.3	Probably transcriptional repressor of bet genes
bolA	b0435	−2.8	−2.8	Transcription regulator of penicillin binding proteins, β-lactamase, and possibly, of murein genes; osmotic stress-induced morphogen
clpP	b0437	−2.0	−2.3	ATP-dependent proteolytic subunit of clpA-clpP and clpX-clpP serine protease, heat shock protein
clpX	b0438	−2.4	−2.1	ATPase and specificity subunit of ClpX-ClpP ATP-dependent serine protease
lon	b0439	−2.1	−2.8	DNA-binding, ATP-dependent protease La; heat shock K protein
	b0725	−4.5	−2.6	ORF, hypothetical protein in *sdh-suc* operon
sucA	b0726	−4.1	−3.7	2-Oxoglutarate dehydrogenase (decarboxylase component)
sucB	b0727	−2.9	−4.1	2-Oxoglutarate dehydrogenase (dihydrolipoyltranssuccinase E2 component)
IG		−3.5	−4.0	Intergenic region between *sucB* and *sucC*
sucC	b0728	−3.1	−4.4	Succinyl-CoA synthetase, beta subunit
sucD	b0729	−2.4	−3.7	Succinyl-CoA synthetase, alpha subunit
gpmA	b0755	−1.9	−3.2	Phosphoglyceromutase 1
galM	b0756	−2.0	−1.9	Galactose-1-epimerase (mutarotase)
galK	b0757	−1.9	−2.9	Galactokinase
galT	b0758	−1.9	−2.8	Galactose-1-phosphate uridylyltransferase
galE	b0759	−1.7	−2.6	UDP-galactose-4-epimerase
glnH[b]	b0811	−1.8	−2.3	Periplasmic glutamine-binding protein; permease
cspD	b0880	−1.8	−1.9	Cold shock protein homolog; stress induced DNA replication inhibitor
clpA	b0882	−2	−2.8	ATP-binding component of serine protease, chaperone activity
lrp	b0889	−3.3	−2.2	Regulator for leucine (or *lrp*) regulon and high-affinity branched-chain amino acid transport system
lolA[c]	b0891	−2.1	−1.9	Chaperone for lipoproteins; periplasmic protein effects translocation of lipoproteins from inner membrane to outer membrane
ompF	b0929	−1.7	−2.6	Outer membrane protein 1a (Ia;b;F)
pepN[c]	b0932	−1.8	−2.5	Aminopeptidase N
icdA	b1136	−2.6	−2.5	Isocitrate dehydrogenase, specific for NADP+ (TCA cycle)
oppA	b1243	−2.8	−2.4	Oligopeptide transport; periplasmic binding protein
oppB	b1244	−2.9	−2.5	Oligopeptide transport permease protein
trpA	b1260	−1.8	−3.5	Tryptophan synthase, alpha protein
trpB	b1261	−1.8	−2.5	Tryptophan synthase, beta protein
aldA	b1415	−4.5	−5.1	Aldehyde dehydrogenase A, NAD-linked
maeA	b1479	−2.1	−2.6	NAD-linked malate dehydrogenase (decarboxylating, NAD-requiring) (malic enzyme) (TCA cycle)
rpsV	b1480	−2.7	−3.2	30S ribosomal subunit protein S22; stationary phase-induced ribosome-associated protein
fumA	b1612	−3.4	−2.6	Fumarase A fumarate hydratase class I; aerobic isozyme
mglC	b2148	−3.0	−2.6	Methyl-galactoside transport and galactose taxis
mglA	b2149	−3.2	−2.8	ATP-binding component of methyl-galactoside transport and galactose taxis
mglB	b2150	−4.1	−4.6	Galactose-binding transport protein; receptor for galactose taxis
IG		−5.0	−4.3	Intergenic region between *mglB* and *galS*
galS	b2151	−1.5	−1.9	*mgl* repressor, galactose operon inducer
glpQ	b2239	−2.2	−3.2	Glycerophosphodiester phosphodiesterase, periplasmic

Continued on following page

TABLE 3—*Continued*

Name[a]	Alias	Effect of wt GreA (fold)		Gene description or function
		Chrom	Plasm	
glpT	b2240	−2.9	−3.3	sn-Glycerol-3-phosphate permease
maeB[c]	b2463	−1.8	−2.6	Fused malic enzyme predicted oxidoreductase/predicted phosphotransacetylase
clpB	b2592	−2	−3.7	Heat shock protein
raiA[c]	b2597	−1.5	−2.3	Putative *yhbH* sigma 54 modulator; cold shock protein associated with 30S ribosomal subunit (also known as *yfiA*)
rpoS	b2741	−2.3	−2.6	RNA polymerase, sigma S (sigma38) factor; synthesis of many growth phase related proteins
nlpD	b2742	−2.1	−2.1	Lipoprotein
yqjC[c]	b3097	−1.7	−2.8	Conserved protein
yqjD[c]	b3098	−1.5	−2.3	Conserved protein
yhcH	c3975	−2.6	−2.6	(CFT073) hypothetical protein
nanK	b3222	−2.4	−1.8	Predicted *N*-acetylmannosamine kinase
nanE	b3223	−2.8	−3.0	Predicted *N*-acetylmannosamine-6-P epimerase
nanA	b3225	−3.6	−5.4	*N*-Acetylneuraminate lyase (aldolase); catabolism of sialic acid; not K-12?
mdh	b3236	−2.5	−4.1	Malate dehydrogenase (TCA cycle)
hslR[c]	b3400	−1.8	−1.9	Ribosome-associated heat shock protein Hsp15
malQ	b3416	−1.8	−2.8	4-Alpha-glucanotransferase (amylomaltase)
malP	c4194	−2.0	−3.5	(CFT073) Maltodextrin phosphorylase
glpD	b3426	−2.1	−4.9	sn-Glycerol-3-phosphate dehydrogenase (aerobic) (divergent from glpEGR operon)
dppA[c]	b3544	−2.5	−2.7	Dipeptide transport protein
lldP	b3603	−2	−2.6	L-Lactate permease
lldR	b3604	−2.5	−3.5	Transcriptional regulator
lldD	b3605	−3.1	−2	L-Lactate dehydrogenase
glpK	b3926	−2.3	−4.4	Glycerol kinase
glpF	b3927	−2.9	−4.9	Facilitated diffusion of glycerol
hslU	b3931	−2.2	−2.6	Heat shock protein hslVU, ATPase subunit, homologous to chaperones
hslV	b3932	−3.2	−2.8	Heat shock protein hslVU, proteasome-related peptidase subunit
ftsN[c]	b3933	−1.6	−1.6	Essential cell division protein
aceB	b4014	−2.3	−4.3	Malate synthase A (glyoxylate cycle)
aceA	b4015	−2.2	−4.1	Isocitrate lyase (glyoxylate cycle)
malG	b4032	−2.1	−2.8	Part of maltose permease, inner membrane
malF	b4033	−3.2	−3.7	Part of maltose permease, periplasmic
malE	b4034	−1.9	−5.8	Periplasmic maltose-binding protein; substrate recognition for transport and chemotaxis
malK	b4035	−4.2	−7.5	ATP-binding component of transport system for maltose
lamB	b4036	−3.1	−7.1	Phage lambda receptor protein; maltose high-affinity receptor
malM	b4037	−4.5	−6.5	Periplasmic protein of mal regulon
groS	b4142	−2.7	−3.5	GroES, 10-kDa chaperone binds to Hsp60 in presence of Mg-ATP, suppressing its ATPase activity
groL	b4143	−3.1	−3.2	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shock protein
yjiA	b4352	−3.4	−2.2	Predicted GTPase
yjiX	b4353	−3.9	−3.7	Conserved protein
yjiY	b4354	−5.1	−7.1	Putative carbon starvation protein; predicted inner membrane protein
osmY	b4376	−2.5	−2.8	Hyperosmotically inducible periplasmic protein

[a] Names of the genes comprising polycistronic operons are grouped. All unannotated genes are monocistrons.
[b] Genes from polycistronic operons where expression of other genes were not affected by GreA.
[c] Genes with unknown operon structure.

TABLE 4. Genes that are activated by wt GreA only under overexpressed conditions

Name[a]	Alias	Effect of wt GreA (fold)	Gene description or function
rpsT	b0023	+2	30S ribosomal subunit protein S20
aceE	b0114	+4.9	Pyruvate dehydrogenase (decarboxylase component)
aceF	Z0125	+4.9	(EDL933) pyruvate dehydrogenase (dihydrolipoyltransacetylase component)
t44	b4414	+3.3	Small RNA of unknown function (*tff*)
rpsB	b0169	+2.3	30S ribosomal subunit protein S2
IG		+2.5	Intergenic region between *rpsB* and *tsf*
tsf	b0170	+2.0	Protein chain elongation factor EF-Ts
cyoD	b0429	+3.5	Cytochrome *o* ubiquinol oxidase subunit IV
cyoC	b0430	+3	Cytochrome *o* ubiquinol oxidase subunit III
cyoB	b0431	+2.3	Cytochrome *o* ubiquinol oxidase subunit I
cyoA	b0432	+4.4	Cytochrome *o* ubiquinol oxidase subunit II
ylaC[c]	b0458	+1.7	Predicted inner membrane protein
cusF[b]	b0573	+2.5	Periplasmic copper-binding protein (*ylcC*)
IG		+2.1	Intergenic region upstream of *ompX*
ompX	b0814	+2.3	Outer membrane protein X
rpsA[b]	b0911	+4.0	30S ribosomal subunit protein S1
flgE[b]	b1076	+2.0	Flagellar biosynthesis, hook protein
sraB[c]	b4418	+3.2	Small RNA
yceD	b1088	+3.1	Conserved protein
rpmF	b1089	+4.1	50S ribosomal subunit protein L32
ptsG	b1101	+2.5	PTS system, glucose-specific IIBC component
lpp	b1677	+1.8	Murein lipoprotein
rplT	b1716	+1.7	50S ribosomal subunit protein L20 and regulator
rpmI	b1717	+1.8	50S ribosomal subunit protein A
infC	Z2747	+2.0	(EDL933) initiation factor IF-3
rplY[c]	b2185	+2.5	50S ribosomal subunit protein L25
IG		+5.1	Intergenic region upstream of ackA
ackA	b2296	+3.9	Acetate kinase
pta	b2297	+3.4	Phosphotransacetylase
yfdH[c]	b2351	+2.5	Putative glycan biosynthesis enzyme CPS-53 (KpLE1) prophage; bactoprenol glucosyl transferase
rplS	b2606	+2.2	50S ribosomal subunit protein L19
trmD	b2607	+2.0	tRNA methyltransferase; tRNA (guanine-7-)-methyltransferase
rimM	b2608	+2.1	16S rRNA processing protein; ribosome maturation protein
rpsP	b2609	+2.3	30S ribosomal subunit protein S16
deaD[c]	b3162	+1.9	Inducible ATP-independent RNA helicase
nlpI[c]	b3163	+2.2	Conserved proteins, lipoprotein involved in cell division
rpsO	b3165	+2.5	30S ribosomal subunit protein S15
rbfA	b3167	+1.9	Ribosome-binding factor A
yhbC	b3170	+1.8	Conserved protein
yhbY[c]	b3180	+1.6	Predicted RNA-binding protein containing KH domain; possibly a ribosomal protein
yhbE[c]	b3184	+2.3	Conserved inner membrane protein

Continued on following page

TABLE 4—*Continued*

Name[a]	Alias	Effect of wt GreA (fold)	Gene description or function
rplU	b3186	+2.2	50S ribosomal subunit protein L21
rpsI	b3230	+2.6	30S ribosomal subunit protein S9
rplM	b3231	+2.7	50S ribosomal subunit protein L13
accB	b3255	+1.9	Acetyl-CoA carboxylase, BCCP (biotin carboxyl carrier protein) subunit; carrier of biotin
accC	b3256	+1.8	Acetyl-CoA carboxylase, biotin carboxylase subunit
dusB	b3260	+2.2	tRNA-dihydrouridine synthase B (putative dehydrogenase, *aka yhdG*)
fis	b3261	+3.8	Site-specific DNA inversion stimulation factor; DNA-binding protein; a transactivator for transcription
rplQ	b3294	+2.8	50S ribosomal subunit protein L17
rpoA	b3295	+2.3	RNA polymerase, alpha subunit
rpsD	b3296	+2.5	30S ribosomal subunit protein S4
rpsK	b3297	+2.6	30S ribosomal subunit protein S11
rpsM	b3298	+2.3	30S ribosomal subunit protein S13
rpmJ	b3299	+2.7	50S ribosomal subunit protein L36
prlA	b3300	+4.3	Putative ATPase subunit of translocase
rplO	b3301	+3.1	50S ribosomal subunit protein L15
rpmD	b3302	+3.7	50S ribosomal subunit protein L30
rpsE	b3303	+3.7	30S ribosomal subunit protein S5
rplR	b3304	+3.1	50S ribosomal subunit protein L18
rplF	b3305	+4.1	50S ribosomal subunit protein L6
rpsH	b3306	+3.2	30S ribosomal subunit protein S8, and regulator
rpsN	b3307	+3.2	30S ribosomal subunit protein S14
rplE	b3308	+2.7	50S ribosomal subunit protein L5
rplX	b3309	+3.2	50S ribosomal subunit protein L24
rplN	b3310	+3.3	50S ribosomal subunit protein L14
rpsQ	b3311	+4.2	30S ribosomal subunit protein S17
rpmC	b3312	+3.1	50S ribosomal subunit protein L29
rplP	b3313	+3.5	50S ribosomal subunit protein L16
rpsC	b3314	+3.2	30S ribosomal subunit protein S3
rplV	b3315	+3.3	50S ribosomal subunit protein L22
rpsS	b3316	+3.1	30S ribosomal subunit protein S19
rplB	b3317	+3.1	50S ribosomal subunit protein L2
rplW	b3318	+2.9	50S ribosomal subunit protein L23
rplD	b3319	+3.0	50S ribosomal subunit protein L4, regulator of *rpsJ* operon
rplC	b3320	+2.3	50S ribosomal subunit protein L3
rpsJ	b3321	+2.9	30S ribosomal subunit protein S10
fusA	b3340	+2.0	GTP-binding protein chain elongation factor EF-G
rpsG	b3341	+2.0	30S ribosomal subunit protein S7, initiates assembly
rpsL	b3342	+2.5	30S ribosomal subunit protein S12
htrL[c]	b3618	+1.6	Involved in lipopolysaccharide biosynthesis
rpmG	b3636	+2.4	50S ribosomal subunit protein L33
rpmB	b3637	+2.6	50S ribosomal subunit protein L28
rpmH	b3703	+2.4	50S ribosomal subunit protein L34
rnpA	b3704	+2.6	RNase P, protein component; protein C5
atpD	b3732	+1.9	Membrane-bound ATP synthase, F1 sector, beta-subunit
atpG	b3733	+1.7	Membrane-bound ATP synthase, F1 sector, gamma-subunit
atpA	b3734	+2.2	Membrane-bound ATP synthase, F1 sector, alpha-subunit
atpH	b3735	+1.7	Membrane-bound ATP synthase, F1 sector, delta-subunit
atpE	b3737	+2.4	Membrane-bound ATP synthase, F0 sector, subunit c
atpB	b3738	+1.6	Membrane-bound ATP synthase, F0 sector, subunit a
rbsD[b]	b3748	+1.7	D-Ribose high-affinity transport system; membrane-associated protein

Continued on following page

TABLE 4—*Continued*

Name[a]	Alias	Effect of wt GreA (fold)	Gene description or function
trxA[c]	b3781	+1.6	Thioredoxin 1
rhoL	b3782	+2.3	*rho* operon leader peptide
rho	b3783	+1.9	Transcription termination factor Rho; polarity suppressor
typA[c]	b3871	+1.8	GTP-binding protein; predicted membrane GTPase involved in stress response
rpmE	b3936	+3.4	50S ribosomal subunit protein L31
rplK	b3983	+3.2	50S ribosomal subunit protein L11
rplA	b3984	+2.5	50S ribosomal subunit protein L1, regulates synthesis of L1 and L11
rplJ	b3985	+2	50S ribosomal subunit protein L10
rplL	b3986	+1.9	50S ribosomal subunit protein L7L12
rpoB	b3987	+1.9	RNA polymerase, beta subunit
rpoC	b3988	+2	RNA polymerase, beta prime subunit
rpsF	b4200	+4.5	30S ribosomal subunit protein S6
priB	b4201	+5.2	Primosomal replication protein N
rpsR	b4202	+4.2	30S ribosomal subunit protein S18
rplI	b4203	+2.1	50S ribosomal subunit protein L9
hsdS[c]	b4348	+1.9	Specificity determinant for *hsdM* and *hsdR*

[a] Genes comprising polycistronic operons are grouped together. All unannotated genes are monocistrons.
[b] Genes from polycistronic operons where expression of other genes were not affected by GreA.
[c] Genes with unknown operon structure.

strains would allow us to identify those *E. coli* genes whose expression depends, directly or indirectly, on chromosomally encoded GreA.

In vivo gene expression analysis. The presence of chromosomally expressed GreA affected the transcription of only a few genes, a finding which is consistent with results obtained by others (35). Nineteen genes were upregulated in the *greA*$^+$ *greB* mutant relative to the *greA greB* mutant (Table 2), indicating that GreA is required for their optimal expression. The most prominent effect was observed for the *tna* genes of the tryptophanase operon. At the same time, the expression of 82 genes was downregulated in the *greA*$^+$ *greB* mutant compared to the *greA greB* mutant (Table 3), suggesting that chromosomally encoded GreA normally suppresses the expression of these genes.

That expression levels of only few genes are affected by GreA under the conditions used for microarray experiments may be due to the fact that, normally, *greA* expression is relatively low but is upregulated under certain stress conditions, such as heat shock, acid shock, or antibiotic treatment (22, 33). Recently it was shown that in *E. coli greA* expression is under the control of a σ^E-dependent promoter (31). Indeed, the intracellular levels of GreA were observed to increase about six- to eightfold upon the overexpression of SigE (31), a condition designed to mimic the activation of σ^E in response to heat shock, hyperosmotic stress, divalent metal ion exposure, etc. (1, 4, 12, 11). The relatively modest effects caused by the absence of GreA may also be due to the fact that under normal growth conditions, GreA is present in a conformation that prevents its interaction with RNAP (20). Finally, Gre activity may be obscured by antagonists, such as DksA, an abundant protein that shares a common RNAP binding site with GreA and conceivably may outcompete GreA for binding to RNAP (27, 28, 29).

The aforementioned stress conditions that activate the SigE regulon, and that in turn may induce GreA expression, have a complex effect on cellular physiology that is unrelated to GreA activity. Therefore, to identify all or at least the majority of *E. coli* genes that are specifically responsive to GreA, we compared the expression profile of *greA greB* mutant cells harboring a pTRC99A vector plasmid with that of an isogenic strain harboring a pTRC99A plasmid carrying wt *greA* gene under control of an IPTG-inducible *tac* promoter. Upon induction with 0.05 mM IPTG, the level of plasmid-encoded GreA increases about 10-fold compared to that of chromosomally expressed GreA at our standard growth conditions (based on the results of immunoblotting with anti-GreA antibodies [see Materials and Methods]). Thus, the amount of GreA overproduced from a plasmid is comparable to the amount of chromosomally encoded GreA present in the cell upon derepression of the SigE regulon (31). Since we are specifically interested in genes whose expression is affected by the transcript cleavage activity of GreA, we also performed transcription profiling of similarly induced *greA greB* mutant cells harboring a pTRC99A plasmid containing a *greA* gene with a point mutation that substitutes Asp^{41} for Glu (GreA-D41E). Previously, we showed that such a substitution severely impairs the transcript cleavage activity of GreA without affecting its ability to interact with *E. coli* RNAP or EC (21). Western blot analysis revealed that upon IPTG induction, the expression levels of GreA-D41E were identical to those of the wt GreA (see Materials and Methods).

The induction of the wt but not the mutant GreA led to changes in the expression levels of 189 genes (Tables 2 to 5). The set of genes downregulated by the wt GreA overexpression was almost the same as that of genes downregulated by chromosomally encoded GreA. These "GreA-repressed" genes are listed in Table 3. Twelve additional genes whose expression is downregulated only by plasmid-encoded wt GreA are presented in Table 5. In most cases, the magnitude of the

TABLE 5. Genes that are downregulated by wt GreA only under overexpressed conditions

Name[a]	Alias	Effect of wt GreA (fold)	Gene description or function
ybeL[b]	b0643	−3.2	Conserved protein; putative alpha helical protein
gatD	b2091	−3.2	Galactitol-1-phosphate dehydrogenase
gatC	b2092	−5.6	PTS system galactitol-specific enzyme IIC
gatB	b2093	−3.4	Galactitol-specific enzyme IIB of phosphotransferase system
gatA	b2094	−2.9	Galactitol-specific enzyme IIA of phosphotransferase system
gatZ	b2095	−2.6	Putative tagatose 6-phosphate kinase 1
gatY	b2096	−2.0	Tagatose-bisphosphate aldolase 1
glpB	b2242	−2.7	sn-Glycerol-3-phosphate dehydrogenase (anaerobic), membrane anchor subunit
glpC	b2243	−3.5	sn-Glycerol-3-phosphate dehydrogenase (anaerobic), K-small subunit
elaB[b]	b2266	−2.5	Conserved protein
talA	b2464	−2.7	Transaldolase A
gcvH[b]	b2904	−2.7	In glycine cleavage complex, carrier of aminomethyl moiety via covalently bound lipoyl cofactor
srlE_b[b]	Z4007	−3.3	(EDL933) PTS system, glucitolsorbitol-specific IIB component and second of two IIC components; frag
srlE_2[b]	Z4009	−2.8	(EDL933) PTS system, glucitolsorbitol-specific IIB component and second of two IIC components; frag

[a] Genes comprising polycistronic operons are grouped together. All unannotated genes are monocistrons.
[b] Genes with unknown operon structure.

inhibitory effect was more pronounced at conditions of GreA overexpression, suggesting that GreA-mediated downregulation of transcript levels is concentration dependent. Follow-up primer extension analysis confirmed that several genes selected from Table 3 are indeed downregulated in the presence of overexpressed wt GreA but not the mutant protein (Fig. 1). However, GreA failed to inhibit transcription from the corresponding promoters in vitro (data not shown). Therefore, the

GENE	Chromosomal expression of wt GreA			Plasmid expression of wt GreA			Plasmid expression of GreA-D41E		
	gene array (fold)	primer extension (fold)	ΔgreAΔgreB ΔgreB	gene array (fold)	primer extension (fold)	pTrc99A pGreA	gene array (fold)	primer extension (fold)	pTrc99A pGreAD41E
ompX	NC/SD	SI		+2.3	+2.5		—1.5	±1.5*	
rplN	NC/SD	+1.5		+3.3	+2.3		NC	—1.5	
rpsB	NC/SD	SI		+2.3	+1.7		NC/SD	NC	
rpsJ	NC/SD	NC/SI		+2.9	+2.6		NC/SD	SD	
tnaL	+3.4	+ >10		+4.2	+ >10		NC	NC	
rpsP	NC/SD	NC/SI		+2.3	+1.9		NC	NC/SD	
rplY	NC/SD	NC/SI		+2.5	+1.6		NC	NC	
lpp	NC	+1.5		+1.8	+1.6		NC	NC	
aceE	NC	NC/SD		+4.9	+3.5		NC	NC/SD	
atpB	—1.7	NC		+1.6	+3.0		NC	NC	
betI	—4.4	—3.7		—2.8	—2.5		NC	NC	
gatY	—1.5	NC		—2.0	—2.5		NC	NC	
glpD	—2.1	NC/SI		—4.9	—3.5		NC	NC	
malE	—1.9	NC		—5.8	—4.7		NC	NC	
ompA	NC/SD	NC		NC	NC		NC	NC	
ppa	NC/SD	NC		NC	NC		NC	NC	

FIG. 1. Comparison of the results of microarray and primer extension analyses of the effects of chromosome-encoded or plasmid-encoded wt GreA or of plasmid-encoded mutant GreA-D41E on the cellular RNA levels of selected transcript units. The gene array and primer extension columns of each panel show the average *n*-fold difference in the RNA level of an indicated gene quantified from the results of at least three independent experiments of gene array and primer extension analysis between the control strain lacking *gre* genes and experimental strain expressing *gre*. Positive and negative values indicate, respectively, an increase or decrease in the RNA level in *gre*-expressing strain relative to control. NC, no change; SD, small decrease; SI, small increase. The third column of each panel shows an autoradiogram of denaturing PAGE of a typical result of a primer extension reaction. Each band represents a cDNA product obtained using specific primer complementary to a region located at about 100 to 150 nt downstream from the transcription start site (see Materials and Methods).

TABLE 6. Operons that are upregulated by wt GreA

Transcript unit	Description	σ	Activators	Repressors
Translation apparatus				
rpsT	Ribosome biosynthesis			
t44-rpsB-tsf[a]	Ribosome biosynthesis; translation; sRNA			
PrpsA-himD	Ribosome biosynthesis			
PyceD-rpmF	Ribosome biosynthesis			
PinfC-rpmI-rplT	Ribosome biosynthesis; translation	σ^{70}		FNR
PrpsP-rimM-trmD-rplS	Ribosome biosynthesis; tRNA modification	σ^{70}		
rplU[a]	Ribosome biosynthesis			FNR
PrplM-rpsI	Ribosome biosynthesis			AccB
PrpsMKD-rpoA-rplQ	Ribosome biosynthesis	σ^{70}		
PrplNXE-rpsNH-rplFR-rpsE-rpmD-rplO-prlA-rpmJ	Ribosome biosynthesis			FNR
PrpsJ-rplCDWB-rpsS-rplV-rpsC-rplP-rpmC-rpsQ	Ribosome biosynthesis	σ^{70}	ArcA	
PrpsLG-fusA-tufA	Ribosome biosynthesis; translation			
PrpmBG	Ribosome biosynthesis			
PrpmH-rnpA	Ribosome biosynthesis			
PrplKAJL-rpmE-rpoBC	Ribosome biosynthesis; transcription; transcription			
rpsF-priB-rpsR-rplI[a]	Ribosome biosynthesis; replication			
P1dusB-fis	tRNA modification; transcriptional regulator	σ^{70}	CRP, IHF	
Cellular respiration/energy metabolism				
PaceEF	Anaerobic respiration; transcriptional regulator	σ^{70}	FNR, ArcA	FNR, ArcA
PcyoABCDE	Aerobic respiration	σ^{70}	CRP, GadE	ArcA, Fur, FNR
PcydAB	Microaerobic respiration	σ^{70}	ArcA, FruR	FNR, H-NS
Pndh	Aerobic respiration	σ^{70}	ArcA, Fis	Fis, FNR, IHF
yfiD	Anaerobic respiration, stress response		ArcA, CRP, FNR	FNR
PatpIBEFHAGDC	ATP biosynthesis under aerobic cell growth	σ^{70}		
PackA-pta[a]	Acetate and pyruvate catabolism		ArcA, FNR	PdhR, FNR
PaccBC	Fatty acid biosynthesis	σ^{70}		CRP, Fis
PptsG	Glucose uptake/phosphorylation, component of PTS system		CRP, Fis	DgsA, ArcA, Fis
PtnaLAB	Trytophanase operon, carbon utilization; tryptophan catabolism	σ^{70}	TorR, CRP	FNR
Cell structure				
ompX	Outer membrane protein	σ^{70}		FNR
lpp	Peptidoglycan biosynthesis	σ^{70}/σ^{24}		
Transcription regulator				
gadW[a]	Transcriptional regulator of *gad* operons, acid resistance			
PrhoL-rho	Transcription, termination factor ρ			

[a] Putative operons.

in vivo inhibitory effect of GreA may depend on additional factors (repressors or activators) that are present in the cell. Elucidation of the mechanism of GreA-mediated inhibition will have to await the identification of these factors and is the subject of our ongoing research.

Genes whose expression increased either in the presence of chromosomally encoded GreA or upon the overexpression of plasmid-encoded wt GreA are presented in Table 2. Genes whose transcript levels increased only under conditions of GreA overexpression are listed in Table 4. Of the 105 genes listed in Table 4, 52 are ribosomal protein genes, 7 are translation apparatus genes, 1 is a replication gene, and 3 are genes encoding RNAP subunits α, β, and β′, all of which belong to ribosomal protein operons. The remaining 42 genes code for proteins involved in carbohydrate catabolism, respiration, and proton transport and proteins of unknown function. The overexpression of GreA-D41E did not lead to any gene activation and caused only mild inhibitory effects on a subset of genes whose expression was affected by the wt GreA (data not shown). We therefore conclude that most (or perhaps all) changes in gene expression levels listed in Tables 2 to 5 are caused, directly or indirectly, by the cleavage activity of the wt GreA.

Next we analyzed the operon structure of GreA-responsive

TABLE 7. Operons that are downregulated by wt GreA[a]

Transcript unit	Description	σ	Activators	Repressors
Metabolism				
talB[b]	Central intermediary metabolism, pentose-phosphate shunt, non-oxidative branch			
PtalA	Central intermediary metabolism, pentose-phosphate shunt, non-oxidative branch	σ^{38}		
PgpmA	Central intermediary metabolism, gluconeogenesis/glycolysis	σ^{70}		Fur
PaldA	Central intermediary metabolism, carbohydrate catabolism (glycolate)	σ^{70}	CRP	ArcA, DnaA
PlldPRD	Carbohydrate catabolism (lactate); transcriptional repressor		PdhR	ArcA, LldR
PgalS	Carbon utilization (galactose), transcriptional repressor	σ^{70}	CRP	GalS/R
PgalETKM	Carbohydrate catabolism (galactose)	σ^{38}/σ^{70}	CRP GalS/R	GalS/R, HU, CRP, HNS
PmglBAC	Carbohydrate catabolism (methyl-galactose), β-D-galactoside transport	σ^{38}/σ^{70}	CRP	GalS/R, FlhDC, Fis
PgatYZABCD	Carbohydrate catabolism, galactitol uptake/degradation, component of PTS system	σ^{70}	CRP	GatR, ArcA
PmalPQ	Carbohydrate catabolism, glycogen degradation, maltose catabolism	σ^{70}	CRP, MalT	
PmalEFG	Carbohydrate catabolism, maltose/maltodextrin transport system	σ^{70}	CRP, MalT	
PmalK-lamB-malM	Carbohydrate catabolism, maltose/maltodextrin transport system	σ^{70}	CRP, MalT	
PacnB	Aerobic respiration, TCA cycle	σ^{70}	CRP	ArcA, FruR, Fis
PsucABCD	Aerobic respiration, TCA cycle	σ^{70}		ArcA, FNR, IHF
PicdA	Anaerobic respiration, TCA cycle	σ^{70}	FruR	ArcA
PfumA	Anaerobic respiration, TCA cycle	σ^{70}	CRP	ArcA, FNR
Pmdh	Carbohydrate catabolism, anaerobic respiration, glyoxylate bypass, TCA cycle	σ^{70}	CRP	FlhDC, ArcA
PaceBAK	Central intermediary metabolism, glyoxylate bypass	σ^{70}	IHF, FruR	ArcA, IclR, CRP
PglpABC	Anaerobic respiration, glycerol degradation	σ^{70}	CRP, FNR, FlhDC	GlpR, ArcA
glpD[b]	Aerobic respiration, glycerol metabolism	σ^{70}	CRP	GlpR
PglpTQ	Glycerol metabolism, glycerol-3-phosphate uptake	σ^{70}	CRP, FNR	GlpR, IHF
PglpFKX	Glycerol metabolism/degradation	σ^{70}	CRP	GlpR
Plrp	Amino acid (leucine, valine) biosynthesis, transcriptional regulation	σ^{70}	GadE	Lrp
PtrpCBA	Amino acid (tryptophane) biosynthesis	σ^{70}		
PnanATEK-yhcH	Amino sugar biosynthesis; yhcH, conserved ORF	σ^{70}	CRP	NanR
Stress response				
PdnaKJ	Heat shock/stress response, protein folding, chaperoning, repair (refolding)	σ^{32}		
PclpPX	Heat shock/stress response, protein degradation	σ^{32}		
Plon	Heat shock/stress response, protein degradation	σ^{32}	GadX	
PclpB	Heat shock response, protein folding, chaperoning	σ^{32}		
PhslVU	Heat shock response, protein folding, chaperoning	σ^{32}		
groSL[b]	Heat shock response, protein folding, chaperoning, repair (refolding)	σ^{32}		
PclpA	Protein chaperone/degradation	σ^{70}		
PcspD	Cold shock/stress response	σ^{70}	HNS	
PbetIBA	Response to osmotic stress, betaine biosynthesis; transcriptional regulator	σ^{70}		ArcA, BetI
PbolA	Response to osmotic stress, transcriptional regulator	σ^{38}/σ^{70}		OmpR, HNS
PrpoS	Transcription, response to oxidative/osmotic stress	σ^{70}	GadX, CRP	ArcA, CRP
PnlpD-rpoS	Unknown function; transcription, response to oxidative/osmotic stress			
osmY[b]	Response to osmotic stress, periplasmic protein	σ^{70}/σ^{38}		Lrp, CRP, IHF
PrpsV	Ribosome biosynthesis (stationary phase-induced), unknown function	σ^{38}	IHF, Fis	OmpR
yjiYXA[b]	Unknown function; putative carbon starvation protein; P-loop guanosine triphosphatase			
Cell structure				
PompF	Outer membrane porin	σ^{70}	CRP, IHF, Fur, Lrp, OmpR, EnvY	OmpR, CpxR, IHF
PoppABCDF	Peptidoglycan biosynthesis, oligopeptide transport	σ^{70}		ModE, Lrp, ArcA

[a] Operons of related functions are grouped together.
[b] Putative operons.

genes. A list of operons that respond positively and negatively to the presence of the wt GreA is given in Tables 6 and 7, respectively. Promoters of operons upregulated by GreA contain two major groups: (i) operons of translational apparatus/ribosome biosynthesis (most do not require activators), and (ii) operons involved in cellular respiration/energy metabolism, all of which are tightly regulated by activators and repressors. Two additional small groups of promoters include four monocistronic operons involved in cell structure and transcriptional regulation (Table 6). Almost all upregulated promoters are transcribed by the σ^{70}-RNAP holoenzyme. Among upregulated operons, three were involved in stress response (acid/alkaline resistance): *yfiD*, *gadW*, and *tna*. Promoters of operons downregulated by GreA can be divided into two large groups and one small group (Table 7). The first large group is involved in cell metabolism (central intermediary metabolism, carbohydrate catabolism, aerobic and anaerobic respiration, glycerol metabolism, trichloroacetic acid cycle, etc.), and most of operons are positively and negatively regulated; the second large group is involved in stress response, such as heat and cold shock, oxidative and osmotic stress, protein folding and degradation, etc., with some of the operons regulated by activators and repressors. Most of these operons are under control of alternative σ factors, such as heat shock σ^{32} and stationary phase σ^{38}. Finally, a small group of promoters includes two operons involved in cell structure (Table 7).

Detailed analysis of Tables 6 and 7 and raw microarray data reveal that if a gene in an operon was affected by GreA, so were the other genes in the same operon, though the magnitude of the effect varied and was sometimes below the arbitrarily chosen cutoff level of 1.5 that was used to select individual genes listed in Tables 2 to 5. Importantly, in all cases, all the genes in an operon responded to GreA in the same (positive or negative) way. Together, these results suggest that GreA affects transcription initiation (see below).

The results of global transcription profiling were further confirmed by primer extension analysis using primers specific to several randomly chosen promoter-proximal genes in GreA-responsive operons identified in microarray experiments. The results presented in Fig. 1 show that there is a good correlation between the microarray and primer extension data and further support the notion that GreA regulates gene expression at the level of transcription initiation and/or promoter escape.

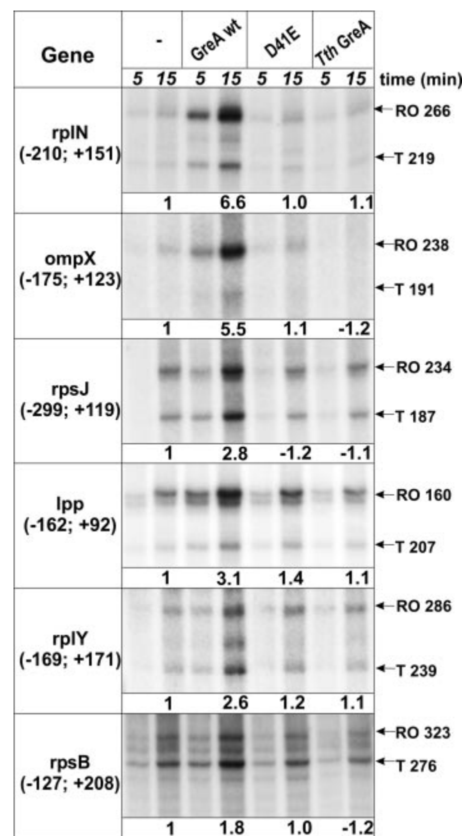

FIG. 2. Effect of GreA factors on multiround runoff transcription from promoters of selected genes. For each DNA fragment used, the terminal nucleotide positions relative to transcription start site are indicated in the left column. Each panel is an autoradiogram of the denaturing 10% PAGE of radiolabeled RNA products. Transcription reactions were conducted at 37°C for 5 and 15 min under standard reaction conditions (see Materials and Methods) using 0.5 μM RNAP, 0.15 μM DNA, 100 μM NTPs, and 0.2 μCi [α-^{32}P]CTP in 10 μl of reaction volume, in the absence or presence of 4 μM GreA factor. Positions of the runoff products (RO) and terminated transcripts (T) are indicated on the right. The average *n*-fold difference between the amount of total RNA product (terminated transcript plus runoff) synthesized after 15 min in the absence of GreA factors and in the presence of wt GreA, mutant GreA-D41E, or *T. thermophilus* GreA is shown below each panel. Positive and negative values indicate, respectively, stimulatory and inhibitory effect of GreA factors on transcription relative to control. The average values were calculated from at least four independent experiments. Standard deviation from experiment to experiment was typically less than 20%.

In vitro transcription analysis of GreA-responsive genes. To determine whether the stimulatory effect of GreA can be demonstrated in vitro, steady-state runoff transcription from six DNA fragments containing promoters activated by GreA in vivo was carried out in the absence or in the presence of wt or mutant D41E GreA. As a control, we used *T. thermophilus* GreA. This protein does not bind to *E. coli* RNAP or its ECs (21, 19). The amount of the runoff product from each of the promoter DNA fragments tested was increased in the presence of the wt GreA (Fig. 2, compare lanes 3 and 4 with lanes 1 and 2, respectively). The stimulatory effect was promoter dependent and under our assay conditions varied from 1.5- to 6-fold. Very little if any stimulation was observed in the presence of GreA-D41E (lanes 5 and 6) and no stimulation was evident in the presence of *T. thermophilus* GreA (Fig. 2, lanes 7 and 8).

GreA-dependent increase in steady-state levels of runoff transcripts could, in principle, be due to the stimulation of (i) promoter binding by RNAP, (ii) promoter melting, (ii) promoter clearance, (iv) transcription elongation, (v) RNAP recycling. To determine which of these possibilities are realized, we performed a series of discriminative assays. Each aimed to reveal the effect of GreA on a particular step of the transcription cycle. We observed no difference in the ability of RNAP to form closed (i) or open (ii) promoter complexes in the presence or the absence of GreA on any of the promoter DNA fragments tested (by using specific DNA band-shift assays and by $KMnO_4$ and DNase footprinting assays) (data not shown). Furthermore, GreA had no visible effect on the rate of transcription elongation on the templates used (data not shown). However, an analysis of abortive transcription reactions (Fig.

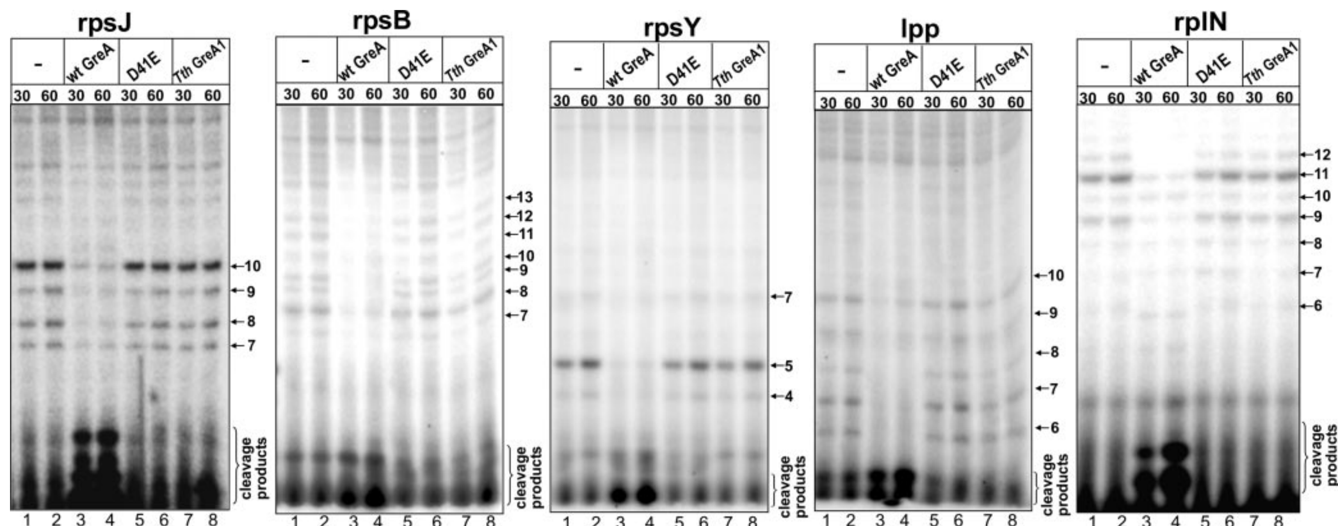

FIG. 3. Effect of GreA factors on the pattern of abortive transcripts generated during multiround transcription reactions from promoters of selected genes. Each panel is an autoradiogram of the denaturing 23% PAGE separating short radiolabeled RNA products. Transcription reactions were carried out as described in the legend for Fig. 2, except that 30 μM NTPs were used and reactions were incubated for 30 and 60 min. Positions of abortive RNAs and products of GreA-induced cleavage are indicated on the right.

3) revealed that for each promoter tested, the addition of wt but not the mutant or *T. thermophilus* GreA decreased the amount of abortive synthesis relative to the amount of the runoff product (compare Fig. 2 and 3). The exception was the *ompX* gene promoter where no abortive products were observed in the presence or the absence of GreA (data not shown). In all cases, the decrease of abortive transcripts was accompanied by accumulation of shorter radiolabeled products (di- and trinucleotides) visible on denaturing PAGE. The mobility of these products decreased upon treatment with alkaline phosphatase (data not shown), indicating that they contain 5′ phosphate and thus represent 3′ end-proximal products of GreA-induced cleavage. Together, these data suggest that in most cases, (i) the stimulatory effect of GreA is exerted during the early stages of transcription initiation, and (ii) this stimulation requires the presence of the transcript cleavage activity of GreA. This finding is an unexpected one, as heretofore GreA was mostly thought to be a bona fide "elongation" factor influencing transcription pausing and arrest (8, 7, 13). Recently it has been proposed that GreA may increase the rate of promoter escape by affecting an equilibrium between productive and the so-called "moribund" promoter complexes that are unable to leave the promoter (35). While the structural characteristics of the moribund complexes are unknown, the effect of GreA was not associated with the transcript cleavage activity but was rather thought to be due to a conformational change in RNAP that occurs upon GreA binding (35). Clearly, the stimulatory effect on GreA-responsive genes studied here is different as it is dependent on the transcript cleavage activity of the factor. For example, transcript cleavage by GreA may be necessary to relieve early transcriptional arrest during promoter clearance (14, 16). Even if only a small fraction of RNAP molecules becomes arrested, in a multiround transcription, this can lead to a significant decrease in the overall number of runoff product. Thus, GreA-induced cleavage of such arrested complexes would enhance promoter clearance and the overall efficiency of productive initiation. Alternatively, GreA may increase the efficiency of promoter escape directly by promoting the retention (and therefore elongation) of short transcripts by RNAP. The latter scenario is possible if the 5′ end-proximal cleavage products are more stably associated with RNAP than are the transcripts GreA acts upon. Availability of GreA-responsive promoters uncovered in this work will allow us to determine which of these (or other) possibilities is realized.

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