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Antisense Downregulation of σ^{32} as a Transient Metabolic Controller in *Escherichia coli*: Effects on Yield of Active Organophosphorus Hydrolase

RANJAN SRIVASTAVA,[1,2] HYUNG JOON CHA,[1,2]† MARVIN S. PETERSON,[3]‡ AND WILLIAM E. BENTLEY[1,2]*

Department of Chemical Engineering, University of Maryland,[1] and Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute,[2] College Park, Maryland 20742, and Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington 98121[3]

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Plasmids containing an antisense fragment of the σ^{32} gene were constructed and introduced into *Escherichia coli* cells. Downregulation of the σ^{32}-mediated stress response was evaluated under heat shock and ethanol stress and during the production of organophosphorus hydrolase (OPH). Northern blot analyses revealed that σ^{32} sense mRNA was virtually undetected in antisense-producing cultures from 5 to 20 min after antisense induction. However, lower-molecular-weight bands were found, presumably due to partial degradation of σ^{32} mRNA. While a >10-fold increase in σ^{32} protein level was found under ethanol stress in the control cultures, antisense producing cultures resulted in a <3-fold increase, indicating downregulation of σ^{32}. Correspondingly, antisense synthesis resulted in a decreased level of a σ^{32} regulated chaperone (GroEL) for the first 2 h after induction relative to control cultures without σ^{32} antisense mRNA. The total yield of OPH in the presence of σ^{32} antisense was, on average, 62% of the yield without antisense. However, during σ^{32} antisense production, a sixfold-higher specific OPH activity was observed compared to non-antisense-producing cultures.

The σ^{32}-mediated stress response in *Escherichia coli* is induced by a variety of factors, including ethanol and heat shock, as well as the overexpression of recombinant protein (16, 17, 21, 22, 25, 27). The hallmark of this response is a rapid increase in the concentration of the σ^{32} sigma factor (3, 16, 17, 21, 26, 27, 30). For both heat shock and ethanol stress, σ^{32} accumulation is mediated through control of transcription and translation, as well as σ^{32} protein stabilization (3, 7, 8, 14, 26, 30, 31). Conversely, the σ^{32} accumulation following production of recombinant protein is due to stabilization (16, 21). When bound to RNA polymerase, forming the holoenzyme Eσ^{32}, σ^{32} directs the production of a number of chaperone proteins (e.g., GroEL, GroES, DnaK, DnaJ, and GrpE) and proteases (e.g., Lon, ClpB, and FtsH) (7, 8, 12–15, 19, 21, 22, 26, 31, 32). Chaperones often help to fold proteins into their proper configuration, while they and other proteins with unfoldase activity also facilitate the degradation of proteins by folding them into protease-susceptible configurations. The stress proteases then degrade the targeted proteins.

Under ethanol stress or heat shock conditions, it is well known that the synthesis of σ^{32} increases (16, 17, 21, 22, 25, 27). Additionally, the σ^{32} protein that is already present in the cytoplasm is stabilized (3, 16, 17, 21, 26, 27, 30). Under nonstress conditions, σ^{32} has a high turnover rate with a half-life on the order of 1 min (21, 26, 27). Under stress conditions, the half-life of σ^{32} protein has been reported to increase by as much as a factor of 10 (27). FtsH degrades σ^{32} only after σ^{32} has bound to DnaK, DnaJ, and GrpE, creating a multiprotein complex (7, 8). All of these proteins are heat shock chaperone proteins except for FtsH, which is a heat shock protease (31). Under stress conditions, the chaperones bind to misfolded proteins that arise due to the imposed stress (7, 8, 26, 29). The result is a sequestering of the σ^{32} binding these proteases and chaperones and increased stability of σ^{32}. This, in turn, further increases production of stress proteins. Then, as chaperone proteins accumulate, σ^{32} is degraded more swiftly.

To facilitate the expression of recombinant proteins in *E. coli*, it may be convenient at times to downregulate the activity and/or concentration of chaperone proteins and/or proteases (29). This is especially true since increased proteolytic activity accompanying the overexpression of recombinant proteins in *E. coli* can be detrimental to product yield (15). One strategy to overcome proteolytic degradation has been to use knockout mutations (18). However, multiple knockouts can be detrimental to cell growth, and, additionally, some mutations are lethal (14, 31, 32). Hypothetically, in the event that a global regulator such as the σ^{32} sigma factor was downregulated, the level of all σ^{32} activated proteases, including those not currently characterized, could be simultaneously reduced. Since σ^{32} mutations are lethal at temperatures greater than 20°C (21, 32), a method that transiently downregulates the σ^{32} stress response in vivo could be advantageous.

Recently, antisense RNA was introduced as a mechanism for manipulating biosynthesis pathways in prokaryotes for the synthesis of commercially relevant products, specifically acetone and butanol (6). However, there have been no reports demonstrating antisense RNA as a transient and potentially tunable mechanism for enhancing production of such biologicals, including proteins. Moreover, there have been no reports demonstrating control of a regulatory network using antisense RNA. Both naturally occurring and artificial antisense transcripts accomplish downregulation by either blocking ribosome binding or reducing mRNA stability (2, 5, 6, 10, 20). In the present work, an antisense sequence targeting a 284-bp segment of σ^{32}, including the ribosomal binding site, was cloned into a plasmid under the control of the *trc* promoter as shown in Fig. 1A. This vector and a subsequent vector for coexpres-

* Corresponding author. Mailing address: University of Maryland, 5115 Plant Sciences Bldg. #36, College Park, MD 20742. Phone: (301) 405-4321. Fax: (301) 314-9075. E-mail: bentley@eng.umd.edu.

† Present address: Department of Chemical Engineering, Pohang University of Science and Technology, Pohang 790-784, Korea.

‡ Present address: Signal Pharmaceuticals Inc., San Diego, CA 91212.

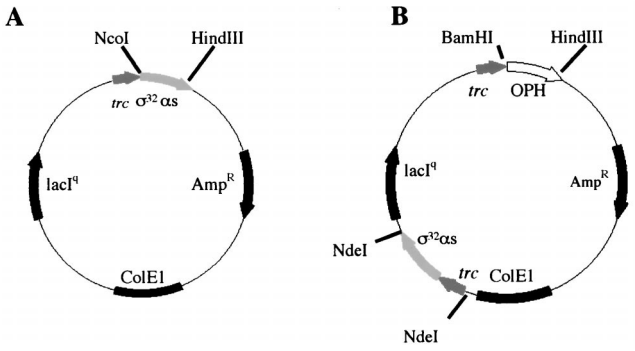


FIG. 1. Maps of σ^{32} antisense expression plasmid pSE420αs (A) and OPH-σ^{32} antisense expression plasmid pTOas (B). Antisense was inserted into pSE420αs between *Nco*I and *Hin*dIII restriction sites under control of the *trc* promoter. For the pTOas plasmid, *trc*-σ^{32} antisense fragment from pSE420αs was engineered with *Nde*I restriction enzyme sites and inserted into a plasmid containing the OPH gene (pTO), also under *trc* control.

sion of organophosphorus hydrolase (OPH) were evaluated to examine whether plasmid-encoded σ^{32} antisense RNA could influence the levels of σ^{32} sense RNA, σ^{32} protein, and GroEL (normally upregulated by σ^{32} under stress) and both the level and activity of OPH.

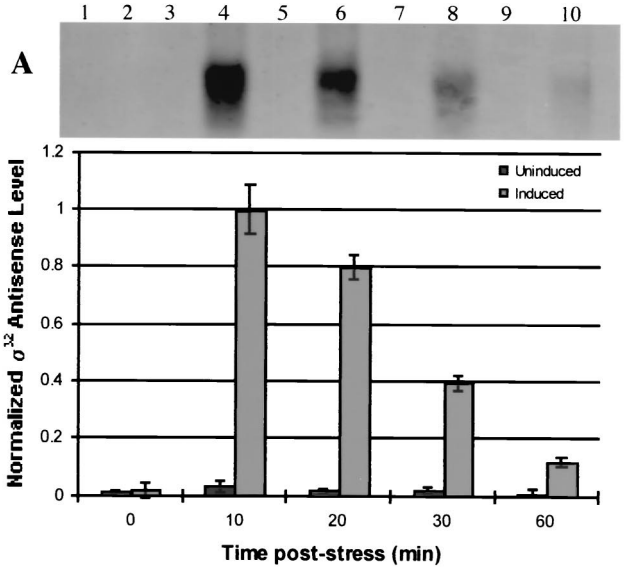
MATERIALS AND METHODS

Bacterial strains. *E. coli* TOP10 [F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ*80lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*) *7697 galU galK rpsL* (Str^r) *endA1 nupG*] (Invitrogen, Carlsbad, Calif.) was used for plasmid construction. *E. coli* strain JM105 [*supE endA sbcB15 hsdR4 rpsL thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] was used for expression (ATCC).

Construction of antisense plasmid. Primers were designed to amplify a 284-bp segment of the σ^{32} gene from the *E. coli* K-12 genome using PCR. A naturally occurring *Hin*dIII site was found at the 5′ end of the segment of interest, while a naturally occurring *Nco*I site was found ~284 bp downstream. This enabled flanking restriction sites to be incorporated into the homologous sequence of interest by appropriate primer design. The 5′ primer sequence used was 5′-CCG AAG CTT GCA TTG AAC TTG TGG-3′, while the 3′ primer was 5′-GCT GCT TCC AGA TCG CCA TGG-3′. After PCR the isolated segment (purified via a Bio-Rad Prep-A-Gene kit) was inserted in the antisense orientation between the *Nco*I site and the *Hin*dIII site of the pSE420 plasmid (Invitrogen) as shown in Fig. 1A. PCR was further used to amplify the promoter, antisense, and termination sequence from pSE420αs and also incorporate *Nde*I restriction enzyme sites at either end of the fragment. The resulting PCR-amplified (5′ primer, 5′-TTC ATT CAT ATG CGA CAT CAT AAC GGT TCT GGC AAA TATTC-3′; 3′ primer, 5′-TAA GCT CAT ATG GCG GAT TTG TCC TAC TCA AGG AGA GCG-3′) and purified fragment was then inserted into a unique *Nde*I site in the pTO vector as shown in Fig. 1B. The resulting vector coexpresses σ^{32} antisense RNA and OPH upon IPTG (isopropyl-β-D-thiogalactopyranoside) addition.

Media, chemicals, and culture conditions. Experiments were performed in which cell cultures were exposed to either heat stress or ethanol stress and induced to produce σ^{32} antisense RNA. The effects of the antisense σ^{32} mRNA on sense σ^{32} mRNA, σ^{32} protein, and GroEL protein (as a σ^{32}-regulated model) were subsequently monitored. That is, GroEL was monitored to examine whether σ^{32} antisense could influence the level of a protein normally upregulated by σ^{32} under stress conditions. OPH was coexpressed to see whether the σ^{32} antisense RNA could influence both the level and activity of a model recombinant protein product. Minimal M9 medium supplemented with thiamine (0.17 μg/ml) and ampicillin (50 μg/ml) was used for all experiments (28). One vial (1.0 ml) of −80°C *E. coli* freezer stock was grown overnight at 30°C in 50 ml of medium in 250-ml Erlenmeyer flasks in an air incubator-shaker. The shaker speed was set at 250 rpm. Fresh culture was inoculated with 5% (vol/vol) overnight culture for a final working volume of 210 ml. Nonstressed, ethanol shocked, and OPH producing cultures were all grown at 37°C. Cultures were grown to an optical density at 600 nm (OD_{600}) of 0.3; at this point, they were induced (1 mM IPTG) and/or stressed (4% [vol/vol] ethanol). Cultures that were heat shocked and their controls were grown at 30°C using a 100-ml working volume in 250-ml Erlenmeyer flasks. For heat shock, the cultures were moved to a 42°C incubator-shaker water bath at an OD_{600} of 0.3, where the elevated temperature (42°C) was reached in less than 3 min (determined experimentally). The temperature was maintained at 42°C for the duration of the experiment.

RNA extraction, detection, and analysis. Twenty-five-milliliter samples from each culture were harvested periodically as noted. Total RNA was isolated using an RNAqueous kit (Ambion, Austin, Tex.). Total RNA concentration was determined using the OD_{260} method (23, 24). Expression of sense and antisense σ^{32} mRNA was analyzed via Northern analysis. RNA (10 μg/well) was run on a formaldehyde denaturing gel with 1% agarose (wt/vol). Total RNA per sample was analyzed using ethidium bromide to ensure legitimate comparison between lanes. The gel was blotted to a nylon membrane (Boehringer-Mannheim, Indianapolis, Ind.) using the capillary action method (24). The membrane was probed for either sense or antisense σ^{32} mRNA. Several probes ranging in size from 20 to 60 bp were tested for their specificity and binding sensitivity; ultimately 40-bp



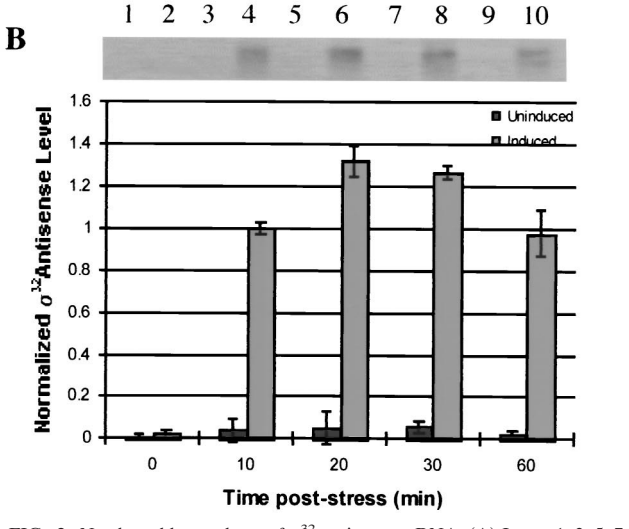


FIG. 2. Northern blot analyses of σ^{32} antisense mRNA. (A) Lanes 1, 3, 5, 7, and 9 are time course samples from unstressed cultures to which no IPTG has been added (no induction of σ^{32} antisense mRNA). Lanes 2, 4, 6, 8, and 10 are time course samples from unstressed cultures that were induced for σ^{32} antisense mRNA synthesis by addition of 1 mM IPTG at 0 min. All quantified values were normalized to the value of lane 4 (10 min). (B) Lanes 1, 3, 5, 7, and 9 are time course samples from cultures that were ethanol stressed (4%, vol/vol) at 0 min but to which no IPTG has been added (Uninduced). Lanes 2, 4, 6, 8, and 10 are time course samples from cultures that were both ethanol stressed and induced for antisense synthesis (Induced). All quantified values were normalized to the value of lane 4. Error bars in both panels represent standard error from multiple blots from multiple experiments.

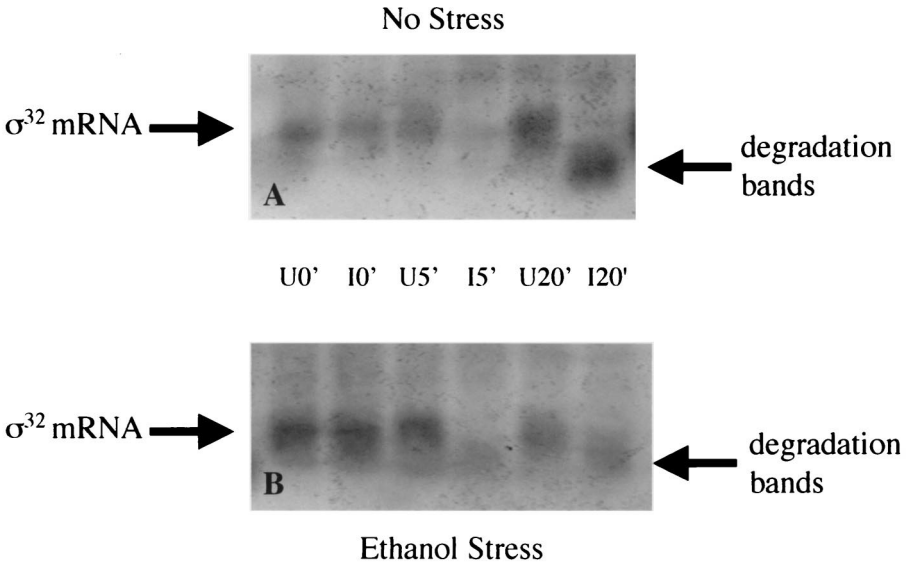


FIG. 3. Northern analyses of σ^{32} sense mRNA. All samples were taken at 0, 5, and 20 min postinduction. (A) Northern blot of nonstressed cell culture over a 20-min time course. Uninduced 0-, 5-, and 20-min samples (U0′, U5′, and U20′, respectively) were taken from a culture that was not induced; induced 0-, 5-, and 20-min samples (I0′, I5′, and I20′, respectively) were taken from a culture that had 1 mM IPTG added at 0 min. (B) Northern blot of cell cultures stressed with ethanol (4%, vol/vol) at 0 min. Lanes are as described for panel A.

probes were selected (sense probe, 5′-CAGACCATGGTAATGCAGCTTTTC AGCCAGCGCCCGCTTT-3′; antisense probe, 5′-CGACTCTAGATCGATTG AGAGGATTTGAATGACTGACAAA-3′). All probes were labeled at the 3′ end with digoxigenin (Boehringer-Mannheim) for fluorescence detection. Membranes were developed using a wash and blocking kit (Boehringer-Mannheim). For σ^{32} sense mRNA detection, 5 μl of the chemiluminescence solution (Boehringer-Mannheim) was diluted in 495 μl of 1× detection buffer (Boehringer-Mannheim). The solution was applied to the membrane, and the membrane was incubated at 37°C for 1 h. The membrane was exposed to Fuji X-ray film for 10 min, after which the film was developed. For the σ^{32} antisense mRNA detection, 300 μl of Vistra ECF substrate solution (Amersham Life Sciences, Princeton, N.J.) was applied in place of the chemiluminescent/detection substrate. The membrane was then scanned using a STORM860 fluorescence imaging system (Molecular Dynamics, Sunnyvale, Calif.) and quantified using ImageQuant software (Molecular Dynamics).

Protein extraction, detection, and analysis. Culture volumes equivalent to 1 ml at an OD_{600} of 2 were withdrawn. The samples were centrifuged at 7,500 × *g* for 5 min at 4°C and decanted, and the pellets were stored at −80°C until needed. The pellets were then resuspended in gel running buffer (0.5 M Tris-HCl [pH 6.8], 10% glycerol, 5% sodium dodecyl sulfate, 5% β-mercaptoethanol, 0.25% bromophenol blue), heated to 100°C for 5 min, and vortexed again. The samples were loaded onto a sodium dodecyl sulfate–12.5% polyacrylamide gel for electrophoresis. The gels were blotted onto supported nitrocellulose membranes (Bio-Rad) using a mini-trans blot cell (Bio-Rad) and Bjerrum and Schafer-Nielsen transfer buffer (48 mM Tris, 29 mM glycine, 20% methanol) for 20 min at 10 V and another 20 min at 20 V. Anti-GroEL mouse monoclonal antibody (StressGen, Vancouver, Canada) was diluted 1:2,000 in antibody buffer (0.5% Tween 20 [vol/vol], Tris-buffered saline with 1% [wt/vol] nonfat dry milk) and used to probe for GroEL. Anti-σ^{32} mouse monoclonal antibody, kindly shared by the laboratory of Richard Burgess (University of Wisconsin), was diluted 1:1,000 in antibody buffer and used to probe for σ^{32}. Antihistidine mouse monoclonal antibody was diluted 1:3,000 in antibody buffer and used to probe for the N-terminal hexahistidine tag on OPH (Sigma, St. Louis, Mo.). The membranes were then transferred to a 1:4,000 diluted goat anti-mouse antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) solution. The membranes were washed and developed colorometrically with 5-bromo-4-chloro-indolyl phosphate–nitro blue tetrazolium tablets (Sigma). The membranes were then scanned and their images were analyzed using NIH Image software. Images depicted in figures are from representative experiments. Data depicted in bar charts are averaged from duplicate or triplicate assays for each. Thus, there might not be a direct visual correspondence between the depicted image and bar chart in all cases.

OPH activity assays. Two milliliters of cell culture was collected, frozen with liquid nitrogen, and stored at −80°C until needed. Thawed samples were centrifuged at 7,500 × *g* for 5 min at 4°C. The samples were then decanted and suspended in 2 ml of phosphate-buffered saline (20 mM sodium phosphate and 500 mM sodium chloride) at a pH of 8.5. They were then sonicated for 1 min using a 0.5-s pulse with a Fisher Scientific 550 Sonic Dismembrator. After sonication, the samples were spun down as before, and the supernatant was collected. After the supernatant had come to room temperature, 75-μl were added to 900 μl of phosphate-buffered saline and to 25 μl of 1 mM paraoxon. The absorbance of each sample was measured at 400 nm, for which the extinction coefficient is 17,000 M^{-1} cm^{-1}. Activities were expressed in micromoles of paraoxon hydrolyzed per minute per OD_{600} of whole cells.

RESULTS

Plasmid-mediated expression of antisense. *E. coli* JM105 (pSE420αs) was induced and probed for antisense σ^{32} mRNA under nonstressed (Fig. 2A) and ethanol-stressed (Fig. 2B) conditions. In all cases preinduction samples had virtually no detectable antisense σ^{32} mRNA; the antisense σ^{32} mRNA was, however, detected in the samples from 10 min postinduction. Subsequent time points were thus normalized to the 10-min level. Antisense σ^{32} mRNA cultures that were induced but not stressed had maximal antisense RNA 10 min following induction. Cultures that were stressed showed an increase in antisense σ^{32} mRNA levels up to 20 min. After 20 min, the level decreased steadily. There was no detectable σ^{32} mRNA in cultures that were not induced. There was also no noticeable change in cell growth rate after induction (data not shown).

Antisense effect on sense σ^{32} mRNA. To evaluate regulation of σ^{32} mRNA, σ^{32} sense mRNA was probed using Northern blotting analysis. Cultures were examined under ethanol shock and nonstress conditions (as described in Materials and Methods). As seen in Fig. 3, σ^{32} mRNA was present in each culture before induction of the antisense as expected. In all antisense-induced cultures, σ^{32} mRNA decreased within 5 min and remained low up to 20 min after induction (Fig. 3). Interestingly our 5′-targeted probe detected putative σ^{32} mRNA degradation bands at lower molecular weights in these same cultures. Also, there was no rapid accumulation of σ^{32} mRNA in uninduced but ethanol-stressed cultures (Fig. 3B), which is consistent with σ^{32} protein stabilization as a principal means of σ^{32} upregulation under stress (16, 21).

Antisense effect on σ^{32} protein levels. σ^{32} protein levels were monitored by Western blotting. The cells without σ^{32} antisense mRNA showed a 10-fold increase in σ^{32} after ethanol stress, which was sustained for over 30 min. In the cultures producing antisense, σ^{32} increased threefold initially and then dropped to

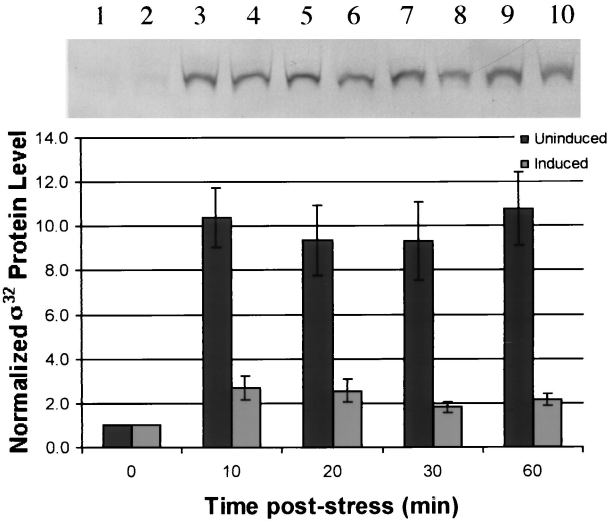
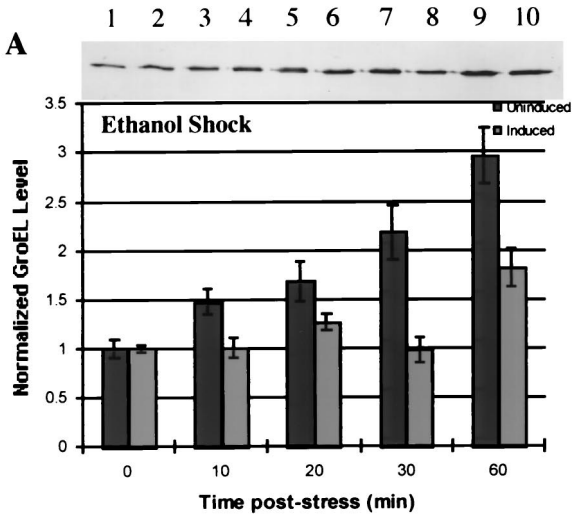


FIG. 4. Time course analysis of σ^{32} determined from Western blot quantification. Cultures were stressed at 0 min with ethanol (4%, vol/vol). Uninduced cultures had no IPTG added. Induced cultures had 1 mM IPTG added at 0 min. Samples were taken at 0, 10, 20, 30, and 60 min. Uninduced samples were normalized to the 0-min uninduced sample value, and induced samples were normalized to the 0-min induced sample value. Error bars represent standard error from duplicate experiments.



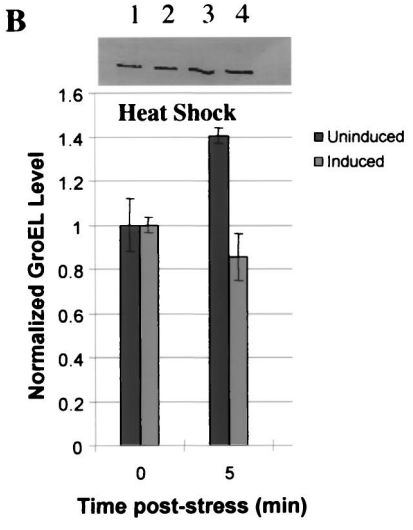


FIG. 5. Time course analysis of GroEL determined from Western blot quantification. (A) Cultures were stressed at 0 min with ethanol (4%, vol/vol). Uninduced cultures had no IPTG added. Induced cultures had 1 mM IPTG added at 0 min. Uninduced samples were normalized to the 0-min uninduced sample value, and induced samples were normalized to the 0-min induced sample value. Samples were taken at 0, 10, 20, 30, and 60 min. (B) Analysis of GroEL level after heat shock via Western quantification. Samples were normalized to their pre-heat shock values. Uninduced cultures had no IPTG added. Induced cultures had 1 mM IPTG added at 0 min. Samples were taken at 0 min (30°C) and again after cultures had been at 42°C for 5 min. Error bars on both panels represent standard error from multiple blots from multiple experiments.

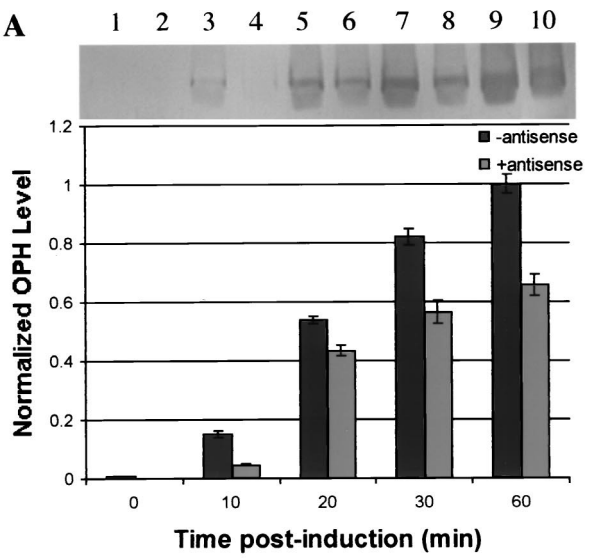
twice the initial value after 30 min and remained there for the duration of the experiment.

Antisense effect on GroEL. GroEL was used as a model target protein to evaluate σ^{32} antisense downregulation of σ^{32}-regulated proteins (cascade effect). For the first hour after stressing the cultures with ethanol, σ^{32} antisense mRNA-producing cultures showed significantly lower levels of GroEL than those cultures not producing antisense (Fig. 5A). In samples taken beyond 2 h, the levels of GroEL in both cultures were comparable (data not shown). In cultures that were not stressed and not induced, GroEL concentration was constant throughout (also not shown). Also, in unstressed but induced cultures, there was a 33% reduction in GroEL during the first 10 min postinduction. In the remaining 50 min, the GroEL level was typically one-half to two-thirds that in control cultures without σ^{32} antisense. Similarly, the effect of antisense on GroEL was also evaluated under heat shock conditions (Fig. 5B), where there was a 30% decrease in GroEL in the antisense-producing cultures after 5 min.

Antisense effect on OPH levels and activity. The effect of the coexpression of σ^{32} antisense on OPH production was investigated by comparing the induction of pTO to the induction of pTOas (Fig. 6). There was no OPH detected from pTO prior to induction, and there was a very low level of OPH detected for pTOas at the zero time point; hence, values were normalized to the final nonantisense OPH-producing culture samples. Throughout the postinduction period, the pTOas culture produced less OPH than the pTO cultures and the absolute difference increased monotonically. Ultimately, at 60 min, the σ^{32} antisense-producing cultures yielded roughly two-thirds the OPH yielded by the controls.

OPH activity results are depicted in Fig. 6B. In this case, results were not normalized to a specific time point although they were indicated on a per cell or OD basis. After induction, the specific activity of the OPH produced by the pTO was maximal (0.02 U) after 20 min and then dropped to a final specific activity of 0.01 U at the end of 1 h. The specific activity of OPH in cultures making σ^{32} antisense mRNA was over 0.06 U within the first 10 min and then dropped monotonically for the remaining 50 min. At the end of the hour, the antisense-containing cultures exhibited a threefold-greater activity than cultures not producing antisense.

Finally, GroEL levels were determined in these experiments (Fig. 6C), and they generally decreased over time in the σ^{32} antisense RNA producing cultures. Moreover, they generally decreased relative to the nonantisense controls, which is consistent with the previous experiments with no OPH synthesis.



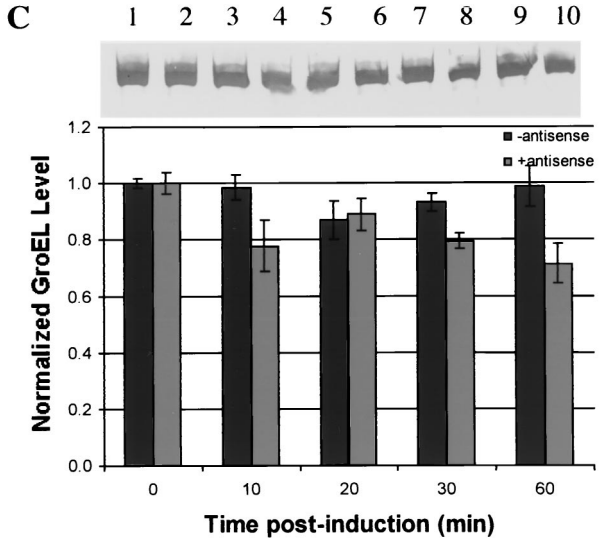
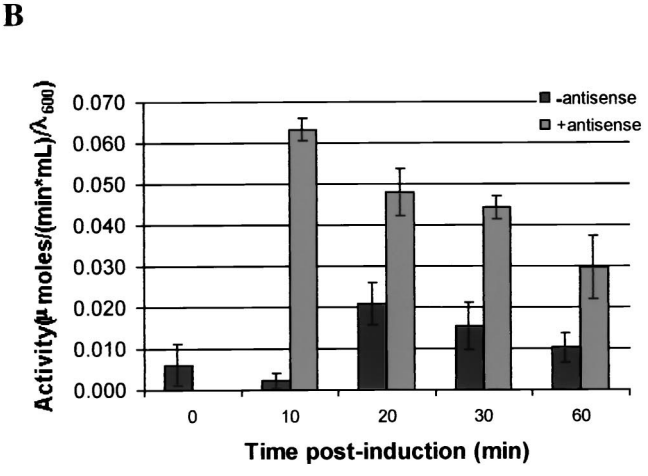


FIG. 6. (A) Time course analysis of OPH determined from Western blot quantification. Cultures were induced at 0 min with 1 mM IPTG. Cultures contained either the pTO plasmid (lanes 1, 3, 5, 7, and 9) and produced only OPH, or contained pTOαs plasmid (2, 4, 6, 8, and 10) and produced OPH and σ^{32} antisense RNA. (B) OPH activity assay results for non-antisense- and for antisense-producing cells. (C) Time course analysis of GroEL determined from Western blot quantification during OPH production. Cultures were induced as described for panel A. Error bars shown represent multiple assays from one experiment, which was duplicated with the same result.



DISCUSSION

Importantly, we demonstrated that pSE420αs produced σ^{32} mRNA in antisense configuration and that this rapidly accumulated within 10 min and was still prevalent at 60 min postinduction. We also found that sense σ^{32} mRNA was lost within the first 5 to 20 min following stress and σ^{32} antisense production. Significantly less σ^{32} protein in antisense-producing cultures was found (Fig. 4), demonstrating a correlation between σ^{32} mRNA and σ^{32} protein levels. The σ^{32} protein in the antisense-induced and ethanol-stressed cultures increased by a factor of 3 within 10 min. This is substantially less than the 10-fold increase seen in the non-antisense-producing cultures. Therefore, in σ^{32} antisense mRNA-producing cells, the capacity for the synthesis of σ^{32} protein was reduced.

Importantly, we found the amount of OPH produced in the presence of σ^{32} antisense mRNA was roughly two-thirds that produced without antisense. This could in part be rationalized by a transcriptional limitiation. Specifically, there is one *trc* promoter on the pTO vector and two *trc* promoters on the pTOas plasmid (Fig. 1); hence, for the antisense producing cultures there are two sites enabled for RNA transcription, with only one leading to OPH protein. The resulting competition for RNA polymerase could therefore be the reason that less OPH was found in the antisense-producing cultures. An additional and consistent observation was made in that the cells containing the antisense vector produced no detectable OPH prior to induction, while the other vector (pTO) resulted in low but detectable OPH. This makes the pTOas vector potentially useful for the production of toxic proteins in *E. coli.* Most importantly, however, the pTOas vector was found to enable a threefold increase in biologically active OPH. It was also interesting that the level of σ^{32} antisense mRNA tracked almost exactly the level of active OPH (highest 10 min after induction followed by a gradual decrease until 60 min), suggesting a temporal relationship between cause and effect. We do not know the specific molecular mechanisms for both the reduced OPH level and increased OPH activity, however. There are many σ^{32}-regulated proteins in *E. coli*, each of which may have contributed to these phenomena. Perhaps OPH undergoes a chaperone-assisted proteolytic degradation (e.g., DnaK-, DnaJ-, or GrpE-mediated FtsH degradation of σ^{32}), and while the antisense acts to suppress the chaperone level (which would otherwise increase), the available chaperones are sequestered away from an OPH degradation pathway. This would be consistent with the increased loss of OPH activity and decreased accumulation rate observed as the antisense RNA disappeared (compare Fig. 6B and 2A). We are presently evaluating the transcriptional response to σ^{32} antisense production on a global basis using reverse transcription-PCR to begin to elucidate these mechanisms (10).

While the use of antisense RNA has been shown to increase the yield of biologicals by manipulating pathway enzymes in prokaryotes, this work is the first to demonstrate an effect on heterologous protein. Importantly, as a temporal metabolic

control mechanism, the potential for affecting a downstream product, GroEL, through downregulation of a global regulatory protein, σ^{32}, was demonstrated here. Results indicated that GroEL production was significantly reduced compared to cells not producing σ^{32} antisense when exposed to either ethanol or heat shock. It was interesting that for the OPH-producing cultures, GroEL levels were roughly constant in cells not producing antisense. However, GroEL was found to decrease in the presence of antisense (Fig. 6C), though not as dramatically as in the ethanol stress or heat shock cases. This may be due to the differences in the mechanisms by which σ^{32} is accumulated in the various cases. During recombinant protein expression, σ^{32} protein is stabilized. For heat shock and ethanol stress, not only is σ^{32} stabilized, but the synthesis of σ^{32} is increased. Since our σ^{32} antisense mRNA targets the synthesis mechanism, it may have had more of an impact in heat and ethanol shock where σ^{32} synthesis normally increases.

In summary an in vivo antisense system for effective delivery and downregulation of σ^{32} was clearly demonstrated. Additionally, downregulation of a σ^{32}-regulated protein, GroEL, was shown. One unique attribute of our result was to demonstrate that antisense RNA could be used to downregulate expression of a protein that, if unavailable or nonfunctional, would be lethal (σ^{32} knockouts are lethal above 20°C). We have also shown that by downregulating a sigma factor, we were able to influence the level of a downstream gene product for which the sigma factor is responsible under stress, GroEL. Through manipulation of a global regulatory unit, the ability to potentially affect the expression of an entire regulatory system can potentially be achieved. The transient nature of the process provides a further advantage by not causing any permanent change to the cell system being employed. Finally, we were able to show that by using antisense we were able to increase specific activity of OPH.

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