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Heat Shock-Dependent Transcriptional Activation of the *metA* Gene of *Escherichia coli*

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In *Escherichia coli*, the growth rate at elevated temperatures is controlled by the availability of endogenous methionine, which is limited because of the temperature sensitivity of the *metA* gene product, homoserine transsuccinylase (HTS). In order to determine the relationship between this control mechanism and the heat shock response, we estimated the cellular levels of HTS during heat shock by Western (immunoblot) analysis and found an increase following induction by temperature shift and by addition of ethanol or cadmium ions. The elevated level of HTS was a result of transcriptional activation of the *metA* gene. This activation was heat shock dependent, as it did not take place in *rpoH* mutants, and probably specific to the *metA* gene, as another gene of the methionine regulon (*metE*) was not activated. These results suggest a metabolic link between the two systems that control the response of *E. coli* to elevated temperatures: the *metA* gene, which codes for the enzyme responsible for regulating cell growth as a function of temperature elevation (HTS), is transcriptionally activated by the heat shock response.

Homoserine transsuccinylase (HTS) (EC 2.3.1.46) is the first enzyme of methionine biosynthesis, catalyzing the synthesis of succinyl-homoserine from homoserine and succinyl-coenzyme A (for a review, see reference 27). In *Escherichia coli*, its activity is reduced at temperatures higher than 33°C, resulting in a limitation of methionine (18). The change in enzyme activity is immediate; therefore, soon after a shift to an elevated temperature, cultures assume a new growth rate that is dependent on the activity of HTS and the resulting availability of methionine (18, 19). At temperatures above 44°C, the activity of HTS is so low that *E. coli* cultures do not grow in minimal medium without the addition of exogenous methionine. The control of growth rate by the activity of HTS is not unique to *E. coli* and has been demonstrated for other enteric bacteria (16) and *Bacillus polymyxa* (28). Expression of the gene that codes for HTS, *metA* (5), is controlled by the repressor of the methionine regulon, MetJ (15). This protein functions as a dimer and binds to an 8-bp sequence (met box) present in multiple successive copies in the promoter region of genes involved in methionine biosynthesis (15, 20). Several met box sequences are also present in the regulatory region of the *metA* gene. This region contains two transcription start points, as shown by S1 analysis. The major *metA* promoter is under negative control by methionine, and the other promoter is very weak and constitutive with respect to methionine concentration (10).

Another temperature-dependent growth control is the heat shock response, which is a rapid response to shifts to higher temperatures (13). This response is characterized by a rapid, transient increase in the rate of synthesis of the heat shock proteins within seconds of the shift (1b, 13). In *E. coli*, at least 17 heat shock genes are known; these are also induced by other stress conditions, such as exposure to ethanol or heavy metals (25). The transcriptional induction of heat shock proteins is mediated by the σ^{32} subunit of RNA polymerase, encoded by the *rpoH* (*htpR*) gene, which constitutes the positive regulator of the heat shock response (7). This sigma factor recognizes a specific consensus sequence upstream of the heat shock genes (2, 23) which is different from the consensus sequence in promoters recognized by the vegetative sigma factor, σ^{70} (8).

In *E. coli*, the need to synthesize a large number of new proteins following a shift to high temperatures could present a problem because under these conditions, cells are severely limited for methionine, which is required for protein synthesis. Previous studies suggested that the total cellular activity of HTS increased when the heat shock response was induced (17), therefore making methionine more available under these conditions.

To further understand the interaction of these two control systems in the adaptation of *E. coli* to elevated temperatures, we analyzed the expression of the *metA* gene during heat shock. Using antibodies specific to HTS, we found an increase in the concentration of this enzyme under conditions of heat shock, and mRNA analysis indicated that this increase was due to transcriptional activation of the gene.

MATERIALS AND METHODS

Bacterial strains and plasmids. All of the strains used in this study were the following *E. coli* K-12 strains: GW1000 [*recA441 sulA11* Δ(*lacU*) *169 thr-1 leu-6 argE3 his-4 ilv*(Ts) *galK2 rplsL31*] and its isogenic *htpR* (*rpoH*)(Am) *malPQ*::Tn*5* derivative, GW4701 (6), kindly provided by G. C. Walker; AB1932 (*argH metA thi lacZ xyl galK tsk*), kindly provided by B. Bachmann at the Coli Genetic Stock Center; and 71-18 [F′ *lacI*^{q} Δ(*lacZ*) M15 *galK strA*] (29).

The plasmids used were pMA-6 (11) and pME-2 (constructed by S. Michaeli in our laboratory), which are pBR322 containing the *E. coli metA* and *metE* genes, respectively, and pGS-04, which is pUC18 containing the *E. coli rpoD* gene (constructed by G. Segal in our laboratory).

Growth conditions. Cultures were grown at 32°C in minimal medium A of Davis and Mingioli (4) or in MOPS (morpholinepropanesulfonic acid) medium (12) (when treated with cadmium). Media were supplemented with glucose (0.2%), required amino acids (50 μg/ml), and thiamine (1 μg/ml). Ampicillin was added, when required, at a final concentration of 50 μg/ml. For induction of the heat shock response, exponentially growing cultures (about 5×10^7 bacteria per ml) were transferred to 42°C or treated with 4% ethanol or 600 μM cadmium. Treatments were stopped by harvesting on ice.

Western blot (immunoblot) analysis. One-milliliter aliquots of control and treated cultures were centrifuged at 10,000 × *g* for 10 min and resuspended in 50 μl of sample buffer, and 10 μl was loaded and analyzed on a sodium dodecyl sulfate (SDS)–10% polyacrylamide slab gel according to the method of Laemmli

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(9). Proteins were electroblotted to nitrocellulose membrane filters (Schleicher and Schuell), and HTS protein was detected by using rabbit anti-HTS serum at a dilution of 1:5,000. Antibodies were obtained by vaccination with a synthetic peptide that consisted of the first 20 amino acids of HTS protein. Nitrocellulose membranes were processed by using the Renaissance kit (DuPont).

Preparation of RNA. Ten-milliliter culture aliquots were centrifuged at 10,000 × *g* for 10 min and suspended in 3 ml of STE solution (1 mM EDTA, 100 mM NaCl, 10 mM Tris [pH 7]), and then an equal volume of hot aqueous 90% phenol was added. The mixture was shaken at 65°C for 10 min and centrifuged for 5 min at 4,000 × *g*. The aqueous phase was removed, and the phenol phase was reextracted with 3 ml of STE solution as described above. After centrifugation, the aqueous phases were mixed and the solution was extracted twice with phenol, once with phenol-chloroform, and twice with ether. RNA was precipitated by the addition of 2 volumes of ethanol and incubated at −20°C overnight. RNA concentrations were estimated by A_{260} (1 A_{260} unit = 40 μg of RNA per ml).

RNA dot blot analysis. RNA samples were suspended in 30 μl of a solution that contained TE buffer (1 mM EDTA, 10 mM Tris [pH 7.5]), 20× SSC (3 M NaCl, 0.30 M sodium citrate), and 37% formaldehyde (5:3:2, respectively). The mixture was incubated for 15 min at 60°C, and 2 μg of RNA was spotted on nitrocellulose membranes. The following DNA probes were used: (i) the 1,010-bp *Bam*HI-*Pvu*II restriction fragment that contains part of the *metA* gene, (ii) the 1,500-bp *Eco*RV-*Eco*RV restriction fragment that contains part of the *metE* gene, and (iii) the 220-bp *Bam*HI-*Eco*RV restriction fragment that contains part of the *rpoD* gene. DNA fragments were ^{32}P labelled by the random prime labelling kit (Boehringer Mannheim Biochemica), denatured, and used for hybridization. Prehybridization was carried out at 65°C for 4 h in a solution that contained 5× SSPE (0.9 M NaCl, 50 mM NaH_2PO_4 [pH 7.7], 5 mM EDTA), 5× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.3% SDS, and 150 μg of denatured herring sperm DNA per ml. Labelled probes were then added to the prehybridization solution, and incubation continued overnight. Membranes were washed at 65°C for 20 min in 2× SSPE–0.1% SDS, 20 min in 1× SSPE–0.1% SDS, and 20 min in 0.1× SSPE–0.1% SDS. Membranes were then exposed to Fuji RX X-ray film at −70°C for autoradiography.

Densitometric analysis. Densitometric analysis was performed with an Ultrascan XL enhanced laser densitometer (LKB).

Northern (RNA) hybridization. RNA samples (10 μg) were electrophoresed on 1.5% formaldehyde agarose gels and transferred to nitrocellulose membranes by capillary transfer in 10× SSC. The labelling of the *metA* probe, prehybridization, and hybridization were performed as described above for RNA dot blot analysis.

Primer extension of mRNA transcripts. A synthetic 17-mer oligonucleotide (5′-GTTGTCATCACAAAGAC-3′) complementary to the nucleotides at positions 104 to 120 of the *metA* gene (5, 10) was used for primer extension and sequencing. ^{32}P-end-labelled primer (0.5 pmol) and 10 μg of RNA were suspended to a 6-μl final volume in RT buffer (120 mM Tris [pH 8.3], 19.2 mM $MgCl_2$, 96 mM KCl, 2.4 mM dithiothreitol) that contained dA, dC, dG, and dT (2.5 mM each), RNasin (10 U), and 5 U of avian myeloblastosis virus reverse transcriptase (Molecular Biology Research, Inc.). The reaction mixture was incubated for 5 min at 45°C and then at 37°C for 30 min. Four microliters of a dye solution was added, and the samples were heated for 5 min at 90°C and then electrophoresed on a 6% polyacrylamide sequencing gel. A dideoxy sequencing reaction carried out with the same primer was electrophoresed in parallel.

RESULTS

Effect of the heat shock response on HTS level. The level of HTS was determined following three treatments known to be inducers of the heat shock response. These were a temperature shift from 32 to 42°C, addition of 4% ethanol, and treatment with 600 μM of cadmium chloride (25). HTS level was determined by Western blotting of cellular proteins analyzed by SDS-polyacrylamide gel electrophoresis and detection by HTS-specific antibodies. The results presented in Fig. 1 indicate that all these treatments resulted in substantial increases in the level of HTS.

Activation of the *metA* gene during heat shock is at the transcriptional level. Cultures growing at 32°C were divided into two parts, one of which was shifted to 42°C for 5 min. RNA from each culture was analyzed after gel electrophoresis by Northern hybridization with a *metA* probe. The results presented in Fig. 2A show a significant increase in the level of *metA*-specific mRNA. In a similar experiment, the treatment was the addition of 600 μM of cadmium chloride, also known to be an inducer of the heat shock response. This treatment also brought about an increase in *metA* mRNA (Fig. 2B).

The results presented in Fig. 3 show the kinetics of transcriptional activation of the *metA* gene during heat shock, as determined by Northern hybridization. These data indicate that the rate of transcription of the *metA* gene reaches its highest level within 5 min of a shift from 32 to 42°C and then starts to decrease. These kinetics are characteristic of transcriptional activation of heat shock genes, with a very rapid increase (within less than 5 min of induction) in the rate of synthesis of mRNA followed by a slow decrease (1b, 13).

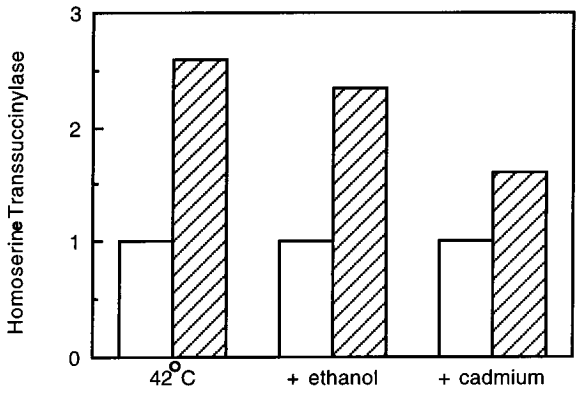

FIG. 1. Effects of heat shock on the concentration of HTS. Heat shock was induced as described in Materials and Methods with AB1932(pMA-6). Samples were removed before (32°C) and 30 min after induction of the heat shock response by a temperature shift from 32 to 42°C, the addition of 4% ethanol, or treatment with 600 μm of cadmium chloride. The level of HTS protein was determined by using anti-HTS antibodies. Sampling, treatment of samples, and Western analysis were performed as described in Materials and Methods. The results of densitometric analysis of the blot are presented and have been normalized by setting the amount found in cells at 32°C before induction equal to 1.

Transcriptional activation of the *metA* gene during heat shock depends on activity of σ^{32}. In order to find out whether the activity of σ^{32} is required for transcriptional activation of the *metA* gene, RNA was prepared from cells of strain GW1000 and its mutant, GW4701, that carries an amber mutation in the *rpoH* (*htpR*) gene that codes for σ^{32}, as well as an unidentified amber suppressor. As the cellular demand for σ^{32} activity increases following a shift to a higher temperature, the mutant expresses a phenotype of temperature sensitivity for growth. The results presented in Fig. 4 show that increased transcription of the *metA* gene following a 10-min treatment at 42°C occurred only in the wild-type strain. For comparison, the levels of transcription of the *rpoD* and *metE* genes were also determined in the same experiment. The *rpoD* gene codes for the vegetative sigma factor (σ^{70}) and has previously been

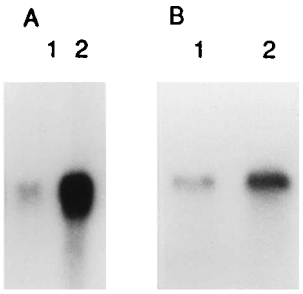

FIG. 2. Northern analysis of *metA* mRNA following heat shock induction. Total RNA of strain 71-18 was isolated before (32°C) and 5 min after induction of the heat shock response. Each lane contained 10 μg of RNA and was probed with the ^{32}P-labelled *Pvu*II-*Bam*HI fragment (1,010 bp) of the *metA* gene. (A) Lane 1, 32°C; lane 2, 5 min after heat shock at 42°C. (B) Lane 1, 32°C; lane 2, 5 min after being treated with 600 μM of cadmium chloride.

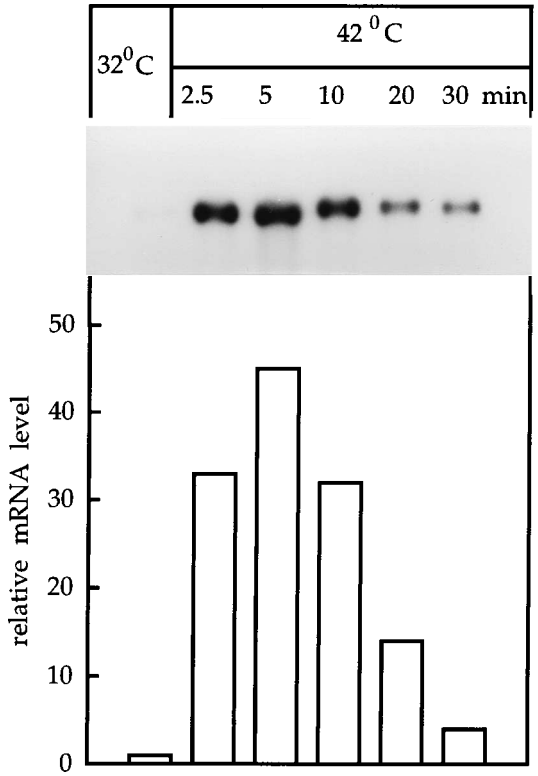

FIG. 3. Northern hybridization of *metA* mRNA during heat shock. Total RNA of strain 71-18 was isolated before (32°C) and at different times (2.5, 5, 10, 20, and 30 min) after heat shock (42°C). Each lane contained 10 μg of RNA and was probed with the ^{32}P-labelled *Pvu*II-*Bam*HI fragment (1,010 bp) of the *metA* gene. The results of densitometric analysis of the blot are presented in the lower part of the figure and have been normalized by setting the amount found in cells at 32°C before induction equal to 1.

shown to be a heat shock gene (13), and the *metE* gene codes for one of the biosynthetic enzymes in the methionine pathway (5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase) (27) and represents the methionine regulon. The

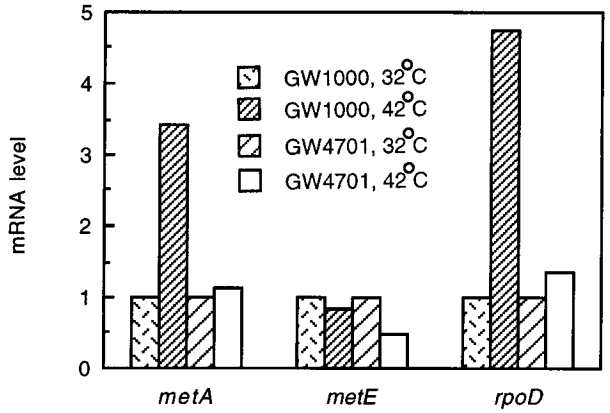

FIG. 4. Densitometric analysis of RNA dot blots. Total RNA was isolated from strain GW1000 (with a wild-type allele of the *rpoH* gene) and strain GW4701, an *rpoH* mutant, before (32°C) and 10 min after heat shock (42°C). RNA (2.5 μg) was dot blotted and probed with one of the following ^{32}P-labelled fragments: 1,010-bp *Pvu*II-*Bam*HI fragment of the *metA* gene, 1,500-bp *Eco*RV-*Eco*RV fragment of the *metE* gene, or 220-bp *Eco*RI-*Bam*HI fragment of the *rpoD* gene. The results were normalized by setting the amount found in cells at 32°C before induction equal to 1.

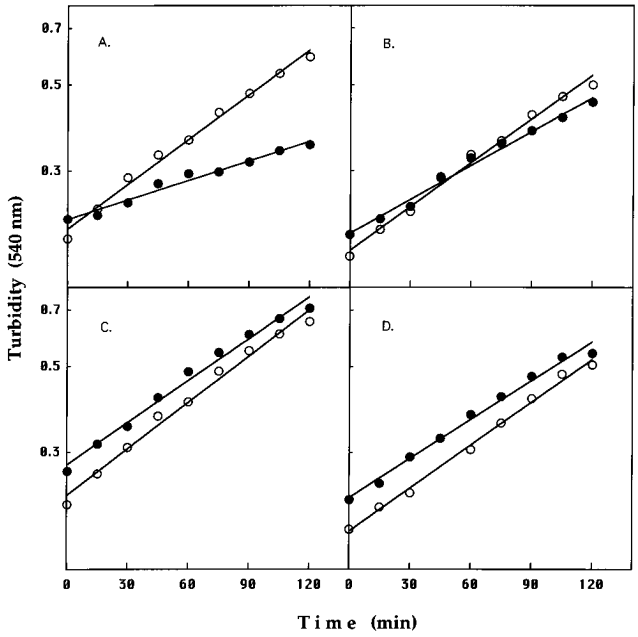

FIG. 5. Effects of elevated temperatures and *metA* activity on growth rate of an *rpoH* mutant. Cultures of strain GW1000 (C) and its *rpoH* mutant, GW4701 (A), were grown at 32°C to about 2×10^7 cells per ml in minimal medium A (open circles) and then divided to two parts, one of which was transferred to 42°C (solid circles). A parallel experiment was performed with these two strains after transformation with pMA-6, which contains the *metA* gene (B and D). Growth was determined by turbidity.

levels of transcription of the *metA* gene were similar to those of the *rpoD* gene, showing activation in the wild type but not in the *rpoH* mutant. In contrast, the *metE* gene did not show transcriptional activation during heat shock.

Since the activation of *metA* during heat shock is dependent on the availability of functional σ^{32} and the *metA* gene product (HTS) is a growth limiting factor at elevated temperatures, we examined the possibility that *rpoH* mutants require methionine upon a shift to higher temperatures. Determinations of the growth rates of such mutants at 32 and 42°C indicated that this is indeed the case. As shown in Fig. 5, the growth rate of strain GW4701 is reduced by about 80% at 42°C (Fig. 5A) and is restored by the presence of a multicopy plasmid that carries the *metA* gene (pMA-6) (Fig. 5B) or by the addition of methionine (data not shown). Isogenic strain GW1000, which carries the wild-type allele of *rpoH*, does not require additional copies of *metA* or exogenous methionine at the higher temperature.

Transcription start point of the *metA* gene during the heat shock response. The 5′ end of *metA* mRNA was determined by primer extension analysis to locate the promoter activated during heat shock. Heat shock was induced by a shift to a higher temperature or the addition of cadmium. In both cases (Fig. 6), the notable increase in transcription of the *metA* gene was from the major promoter. We did not observe a new start point or change in the position of the 5′ end as a consequence of the heat shock response. There was no change in the transcription level from the weak, upstream promoter of *metA* in response to heat shock (data not shown).

As the major *metA* promoter is controlled by methionine via the MetJ repressor, we examined the effect of this repressor on heat shock-dependent transcriptional activation. The results presented in Fig. 7 indicate that the MetJ repressor can reduce heat shock-dependent activation of the *metA* gene, as this activation was decreased in the presence of methionine.

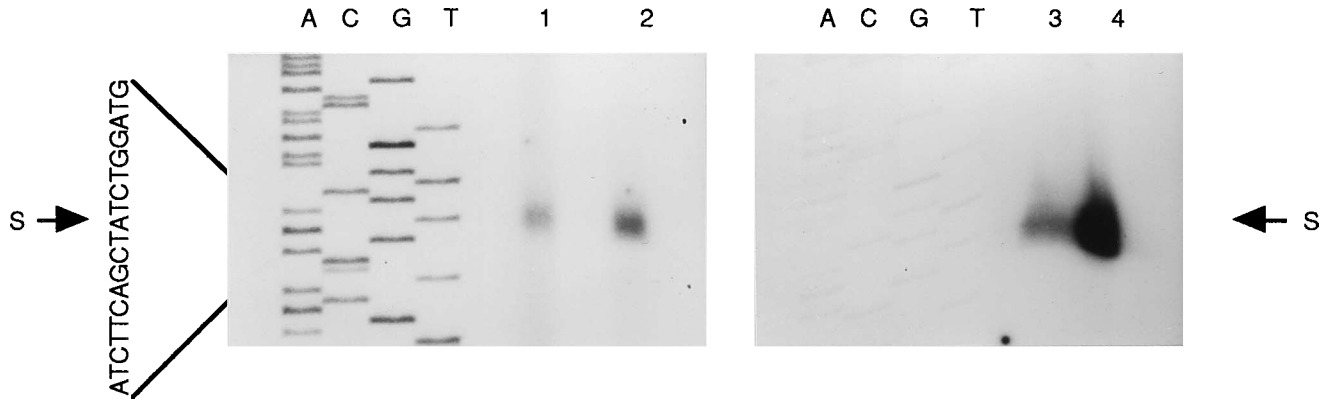

FIG. 6. Mapping of the 5′ end of *metA* mRNA during the heat shock response by primer extension. A primer complementary to the 5′ end of *metA* was hybridized to total RNA (10 μg) isolated from strain 71-18 before (lanes 1 and 3) and 5 min after (lanes 2 and 4) induction of the heat shock response. Lanes A, C, G, and T, products of sequencing reactions obtained with the same primer. The sequence of the sense strand is presented. The arrows at S indicate the base that represents the 5′ end of mRNA. Heat shock response induction by 600 μM of cadmium chloride (left panel) and by temperature shift from 32 to 42°C (right panel).

DISCUSSION

The results presented in this paper indicate that the *metA* gene, which codes for the first enzyme in methionine biosynthesis, is activated during heat shock at the level of transcription. This transcriptional activation results in an elevated concentration of the gene product HTS, as was shown by the use of specific antibodies (Fig. 1). It should be noted that the increase in HTS protein (usually two- to fivefold) is lower than expected from the increase in specific *metA* mRNA level (up to 50-fold [see Fig. 3 for quantitation]) and may result from the relative instability of HTS (1a).

Activation of the *metA* gene was observed upon a shift to a higher temperature of growth (from 32 to 42°C) or following treatment with ethanol or cadmium ions. The kinetics of changes in the level of *metA* mRNA were also characteristic of the heat shock response, with a rapid increase followed by a decrease.

The increase in transcription of the *metA* gene during heat shock appears to be specific for this gene, as there was no stimulation of transcription of the *metE* gene (Fig. 4) and no increase in activity of the *metC* gene product (1). Therefore, it is clear that the response to heat shock inducers is not a common characteristic of all the genes in the methionine pathway. This result, together with previous findings that HTS is the only enzyme in the methionine biosynthetic pathway involved in regulating growth rate as a function of temperature (16, 18, 19), points out the unique role of this enzyme in adaptation to elevated temperatures.

Although transcriptional activation of the *metA* gene by heat shock was not coupled with activation of other genes in the regulon, it was still repressible by the MetJ protein, as it was significantly reduced in the presence of methionine (Fig. 7). This finding suggests that transcriptional activation during heat shock reflects MetJ derepression of the gene at higher temperatures when methionine becomes limited because of the low activity of this enzyme. However, the following findings indicate that heat shock-dependent activation cannot be explained by MetJ derepression: (i) expression of two other genes in the regulon (*metE* and *metC*) is unchanged during heat shock, (ii) the *metA* gene is also activated by ethanol and cadmium at 32°C, when the availability of methionine is not limiting, and (iii) activation of the *metA* gene does not occur in *rpoH* mutants at 42°C. Moreover, *rpoH* mutants, which have a wild-type *metJ* control system but an impaired heat shock response, require methionine at elevated temperatures, presumably because they are incapable of activating the *metA* gene.

Heat shock-dependent transcriptional activation of heat shock genes is due to increased activity of the heat shock

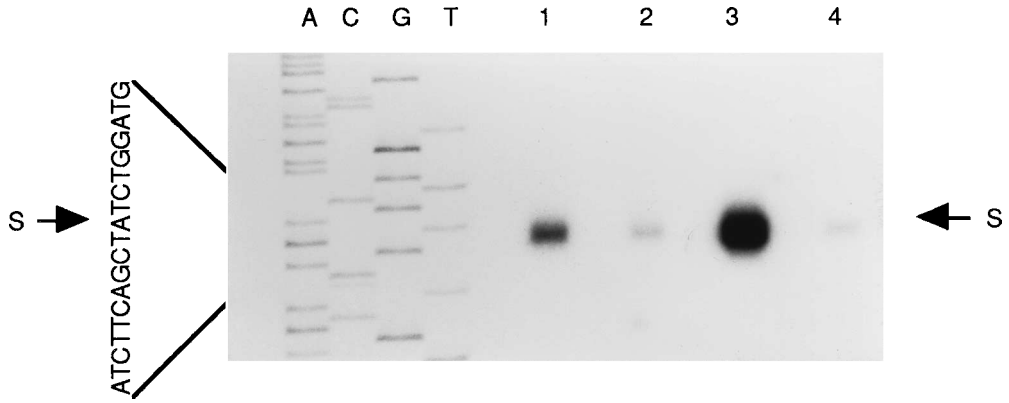

FIG. 7. Effects of methionine on transcriptional initiation of the *metA* gene during heat shock. The experiment was performed as described in the legend to Fig. 6 except that parts of cultures were grown with 200 μg of methionine per ml. Cells grown with (lanes 2 and 4) and without (lanes 1 and 3) methionine before (lanes 1 and 2) and 5 min after (lanes 3 and 4) heat shock (42°C).

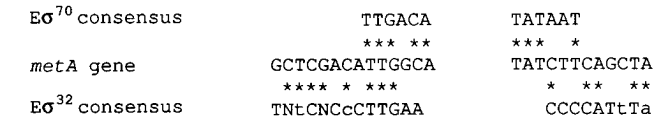

FIG. 8. Sequence analysis of the *metA* major promoter region. Comparison of the major promoter region sequence with the consensus sequence recognized by σ^{70} and that recognized by σ^{32}. Asterisks represent identity. E, *E. coli.*

response activator σ^{32} factor (7). Under conditions of heat shock, this sigma factor associates with the RNA polymerase core enzyme and recognizes specific heat shock promoters. When the heat shock response is induced, a rapid rise in the level of σ^{32} is achieved by an increase in σ^{32} synthesis and stability (21, 24). The promoters recognized by σ^{32} are unique and have a consensus sequence that differs from that of the vegetative σ^{70} promoters (2). Examination of known heat shock genes shows that several of them have two separable promoters, one recognized by σ^{70} and another recognized by σ^{32} (2, 12, 31).

Several lines of evidence suggest that heat shock-dependent transcriptional activation of the *metA* gene may be mediated by σ^{32}. These include the findings that activation is brought about by several different inducers of the heat shock response (temperature, ethanol, and cadmium) and that it does not occur in *rpoH* mutants defective in σ^{32}. However, we found no change in the transcription start point during heat shock; rather, it appears that stimulated transcription is from the major promoter of the *metA* gene.

As transcription is from the same promoter during heat shock and vegetative growth, it is possible that the observed transcriptional activation of the *metA* gene is an indirect result of the heat shock response. For example, other heat shock genes could be involved in the induction of transcription. The final test would involve in vitro experiments, which should indicate whether σ^{32} is directly involved in *metA* transcription. However, although we transcribed this gene in a transcription-translation system with S-30 crude extract, we did not succeed with a fractionated system dependent on RNA polymerase with either σ^{70} or σ^{32} (30). There are probably additional activators (so far unidentified) that are required for in vitro transcription of this gene. These factors are probably also necessary for the binding of RNA polymerase holoenzyme, as deduced from our inability to show binding of purified polymerase to either of the sigma factors by footprinting (3).

If transcription during heat shock is mediated by σ^{32}, the heat shock promoter must overlap the σ^{70} promoter. The possible existence of a σ^{32} promoter that overlaps the major σ^{70} promoter is supported by sequence analysis of the *metA* regulatory region (Fig. 8). Although the situation of overlapping vegetative and heat shock promoters is unusual in *E. coli*, it has already been demonstrated for the *rrnB* gene, for which it was shown in vitro that σ^{32} and σ^{70} bind to the same promoter (14). Additional examples are found in *B. subtilis*, in which there are genes that are activated by more than one RNA polymerase holoenzyme and their promoter regions contain overlapping consensus sequences recognized by two different sigma factors (22, 26).

Methionine biosynthesis is involved in controlling a large number of cellular activities, protein synthesis, DNA and RNA syntheses, synthesis of polyamines, and the transfer of C1 fragments (for the synthesis of purines, pyrimidines, and various methylation reactions) (18). Therefore, regulation of its biosynthesis is expected to be tied in with the global regulatory systems designed to control both growth rate and survival. The role of HTS in this global regulation may be even more central; as HTS is the most temperature-sensitive biosynthetic enzyme, its denaturation may well be one of the signals for the induction of the heat shock response.

ACKNOWLEDGMENTS

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