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Transcription of the *mutL* Repair, *miaA* tRNA Modification, *hfq* Pleiotropic Regulator, and *hflA* Region Protease Genes of *Escherichia coli* K-12 from Clustered $E\sigma^{32}$-Specific Promoters during Heat Shock

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The *amiB-mutL-miaA-hfq-hflX-hflK-hflC* superoperon of *Escherichia coli* contains genes that are important for diverse cellular functions, including DNA mismatch repair (*mutL*), tRNA modification (*miaA*), pleiotropic regulation (*hfq*), and proteolysis (*hflX-hflK-hflC*). We show that this superoperon contains three $E\sigma^{32}$-dependent heat shock promoters, P_{mutL}HS, P_{miaA}HS, and $P1_{hfq}$HS, in addition to four $E\sigma^{70}$-dependent promoters, P_{mutL}, P_{miaA}, $P2_{hfq}$, and $P3_{hfq}$. Transcripts from P_{mutL}HS and P_{miaA}HS were most prominent in vivo during extreme heat shock (50°C), whereas $P1_{hfq}$HS transcripts were detectable under nonshock conditions and increased significantly after heat shock at 50°C. The P_{mutL}HS, P_{miaA}HS, and $P1_{hfq}$HS transcripts were not detected in an *rpoH* null mutant. All three promoters were transcribed by $E\sigma^{32}$ in vitro at 37°C and contain −35 and −10 regions that resemble the $E\sigma^{32}$ consensus. In experiments to assess the possible physiological relevance of the P_{mutL}HS and P_{miaA}HS promoters, we found that *E. coli* prototrophic strain MG1655 increased in cell mass and remained nearly 100% viable for several hours at 50°C in enriched media. In these cells, a significant fraction of *mutL* and *hfq-hflA* region transcripts were from P_{mutL}HS and $P1_{hfq}$HS, respectively, and the amounts of the *miaA*, *hfq*, *hflX*, *hflK*, and *hflC* transcripts increased in comparison with those in nonstressed cells. The cellular amounts of MutL and the *hfq* gene product (HF-I protein) were maintained during heat shock at 44 or 50°C. Consistent with their expression patterns, *miaA* and *hfq* were essential for growth and viability, respectively, at temperatures of 45°C and above. Together, these results suggest that there is a class of $E\sigma^{32}$ promoters that functions mainly at high temperatures to ensure *E. coli* function and survival.

The *amiB-mutL-miaA-hfq-hflX-hflK-hflC* superoperon of *Escherichia coli* K-12 contains a complicated arrangement of genes that mediate several important cellular processes (see Fig. 1). *amiB* encodes *N*-acetylmuramoyl-L-alanine amidase II, involved in cell wall hydrolysis (71). *mutL* encodes a highly conserved protein involved in at least three major DNA repair pathways, methyl-directed mismatch repair (reviewed in reference 50), very-short-patch (VSP) repair (43), and transcription-coupled nucleotide excision repair (47). *E. coli mutL* mutants exhibit a 100- to 1,000-fold increase in spontaneous mutability (49), and *mutL* homologs play major roles in the maintenance of chromosomal stability in eukaryotes, including humans (50). *miaA* encodes the tRNA modification enzyme tRNA dimethylallyl diphosphate transferase (7, 12). *miaA* mutants lacking the ms^2i^6A-37 and i^6A-37 tRNA modifications exhibit pleiotropic phenotypes (17, 26), including mutator and antimutator effects on spontaneous mutation frequencies (13, 40).

hfq encodes HF-I, a host factor required for the initiation of plus-strand synthesis by the replicase of the Qβ RNA bacteriophage (31, 32). The exact role of HF-I, which is heat stable and has a high affinity for single-stranded RNA (22), in uninfected bacteria is unknown, but *hfq* mutants show a variety of pronounced pleiotropic phenotypes, including altered growth properties, a large decrease in the negative supercoiling of plasmid DNA in stationary-phase cells, and alterations and multicopy suppression of *hns* phenotypes (61, 66). Recently, HF-I was shown to be required for translation of the *rpoS*-encoded σ^{38} subunit (6a, 50a), which mediates expression of a regulon when cells enter stationary phase or are osmotically stressed (45). A homolog of HF-I is required for *nifA* expression in *Azorhizobium caulinodans* (33) and enterotoxin production in *Yersinia enterocolitica* (51). *hflX* encodes a putative GTP-binding protein (HflX) (54), and *hflK* and *hflC* encode a membrane-localized protease (54) that mediates bacteriophage lambda lysogeny (10) and the expression levels of some *E. coli* proteins (9).

Previously, we demonstrated that all adjacent genes in this superoperon were cotranscribed and that transcription initiated from at least five promoters (P_{mutL}, P_{miaA}, $P1_{hfq}$, $P2_{hfq}$, and $P3_{hfq}$) during exponential growth in Luria-Bertani (LB) medium under nonstress conditions (66, 68, 69, 71). However, expression of the superoperon in response to other growth or stress conditions was not analyzed. In a recent study of global control of *E. coli* transcription it was found that the *hflA* region in this complex superoperon may be induced by heat shock from an $E\sigma^{32}$-specific promoter (11). Our earlier results indicated that transcription of the *hflA* region is completely dependent on promoters located upstream of *hfq* (66, 70). Therefore, we wanted to locate the putative promoter responsible for *hflA* region heat shock expression. In addition, we wanted to test whether expression of the *mutL* repair gene was affected by heat shock since high temperatures significantly increase spontaneous mutation rates (60, 62) and certain forms of DNA

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TABLE 1. *E. coli* K-12 strains and plasmids

Strain or plasmid	Genotype[a] or description	Source (reference)
Strains		
CAG9301	MG1655 *rpoH120*::*kan zhg-21*::Tn*10*	C. Gross (76)
DEV15 *miaA*	DEV15 *miaA* (*trpX*) (ochre)	D. Elseviers (55)
JC7623	*recB21 rec22 sbc-15 ara arg his leu pro thr*	J. Clark (37)
MG1655	*E. coli* K-12 prototroph	C. Gross (3)
NU426	W3110 *sup*(Am) prototroph (probably W1485E)	C. Yanofsky (see reference 1)
NU816	W3110 Δ*lacU169 tna-2 sup*^{0}	C. Yanofsky
TX2542	JC7623 *mutL*::Ω(Km^r; *Bsa*AI)	Laboratory stock (71)
TX2545	JC7623 *miaA*::Ω(Km^r; *Nru*I)	JC7623 × linear pTX345
TX2554	MG1655 *miaA*::Ω(Km^r; *Nru*I)	MG1655 × P1*vir*(TX2545)
TX2652	CC106 *mutL*::Ω(Km^r; *Bsa*AI)	Laboratory stock (71)
TX2724	JC7623 *amiB*::Ω(Km^r; *Eco*RV)	Laboratory stock (71)
TX2758	JC7623 *hfq2*::Ω(Km^r; *Kpn*I)	Laboratory stock (66)
TX2808	JC7623 *hfq1*::Ω(Km^r; *Bcl*I)	Laboratory stock (66)
TX2816	MG1655 *amiB*::Ω(Km^r; *Eco*RV)	MG1655 × P1*vir*(TX2724)
TX2817	MG1655 *hfq2*::Ω(Km^r; *Kpn*I)	MG1655 × P1*vir*(TX2758)
TX2821	MG1655 *hfq1*::Ω(Km^r; *Bcl*I)	MG1655 × P1*vir*(TX2808)
TX3641	MG1655 *amiB*::Ω(Km^r; *Eco*RV) *miaA*(*trpX*) (ochre)	MG1655 × P1*vir*(TX3681)
TX3660	MG1655 *mutL*::Ω(Km^r; *Bsa*AI)	MG1655 × P1*vir*(TX2542)
TX3681	DEV15 *amiB*::Ω(Km^r; *Eco*RV) *miaA* (*trpX*) (ochre)	DEV15*miaA* × P1*vir*(TX2724)
Plasmids		
pNU127	pBR325 containing *Kpn*I(1)-*Kpn*I(6045) *E. coli* K-12 fragment; *urf1′-urf2*$^+$*-amiB*$^+$*-mutL*$^+$*-miaA*$^+$*-hfq′*; Cm^r Tc^r	Laboratory stock (12)
pTX312	pUC19 cut with *Sal*I and *Kpn*I containing *Sal*I(2667)-*Kpn*I(6045) fragment of pNU127; *amiB′-mutL*$^+$*-miaA*$^+$*-hfq′*; Ap^r	Laboratory stock (71)
pTX315	pGEM3Z cut with *Bam*HI and *Sal*I containing 2.4-kb *Sal*I(2667)-*Bam*HI(5075) fragment of pNU127; *amiB′-mutL*$^+$*-miaA′*; source of RNA probes 3 and 4; Ap^r	Laboratory stock (69)
pTX317	pGEM3Z cut with *Bam*HI and *Kpn*I containing 1.0-kb *Bam*HI(5075)-*Kpn*I(6046) fragment of pNU127; *miaA′-hfq′*; source of RNA probes 5 and 6; Ap^r	This work
pTX338	pGEM3Z cut with *Hin*cII containing 1.2-kb *Bss*HII(2131)-*Bgl*I(3293) fragment of pNU127; *amiB′-mutL′*; source of RNA probes 1 and 2; Ap^r	Laboratory stock (71)
pTX345	pTX312 *miaA*::Ω(Km^r; *Nru*I); by blunt-end ligations; Km^r Ap^r	This work
pTX348	pBR322 cut with *Sal*I containing *Sal*I(2667)-*Sal*I(9085) fragment from Kohara phage 3A1; *amiB′-mutL*$^+$*-miaA*$^+$*-hfq*$^+$*-hflX*$^+$*-hflK*$^+$*-hflC′*; Ap^r	Laboratory stock (66)
pTX368	pGEM3Z cut with *Hin*cII containing 0.7-kb *Xmn*I(5865)-*Pvu*II(6577) fragment of pTX348; *hfq′-hflX′*; source of RNA probe 7; Ap^r	Laboratory stock (66)
pTX405	pGEM3Z cut with *Kpn*I and *Hin*cII containing 1.6-kb *Kpn*I(6045)-*Eco*RV(7687) fragment of pTX348; *hfq′-hflX*$^+$*-hflK′*; source of RNA probe 8; Ap^r	This work
pTX406	pGEM3Z cut with *Kpn*I and *Hin*dIII containing 5.0-kb *Kpn*I(6045)-*Hin*dIII(10998) fragment from Kohara phage 3A1; *hfq′-hflX*$^+$*-hflK*$^+$*-hflC*$^+$*-purA′*; Ap^r	Kohara et al. (34); this work
pTX407	pGEM3Z containing *Bss*HII(9567)-*Hin*dIII(10998) fragment of pTX406; source of RNA probe 9; *hflC′-purA′*; Ap^r	This work

[a] Positions of omega (Ω) cassette insertions are depicted in Fig. 1. Insertion designations indicate the resistance conferred by the Ω cassette and the restriction site in the bacterial chromosome into which it was inserted. Numbers following restriction sites in plasmid constructs refer to coordinates starting with the *Kpn*I (position 1) site of the sequence with GenBank no. L19346 (71) and melding in succession as follows: Z11831 (67), M63655 (13), D00743 (31), and U0005 (54). Ap^r, Km^r, and Cm^r indicate resistance to ampicillin, kanamycin, and chloramphenicol, respectively.

damage such as deamination (23). *mutL* is thought to regulate or stimulate VSP repair, which corrects G · T mismatches that result from deamination in certain contexts (41, 43). The results reported here show that *mutL*, *miaA*, *hfq*, and the *hflA* region are transcribed from clustered Eσ^{32}-specific promoters in vivo and in vitro. Our results suggest that this heat shock transcription plays a physiologically important role, because *miaA* and *hfq* gene functions are required for growth and viability, respectively, of *E. coli* K-12 cells at temperatures of 45 to 50°C.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. Strain CAG9301 was grown at 23°C. pGEM3Z, pBR322, pUC19, and pACYC184 vectors were obtained from Promega Corp. and New England Biolabs Inc. Cloning and genetic manipulations were carried out by standard methods (2). Genetic markers were moved between bacterial strains by generalized transduction with bacteriophage P1*vir* (48). LB rich broth and agar containing 10 g of NaCl per liter (59) were prepared with capsules purchased from Bio 101, Inc. Enriched minimal salts-glucose medium (EMMG) contained Vogel-Bonner (1× E) salts (16), 0.01 mM $FeSO_4$, 0.4% (wt/vol) glucose, and 0.5% (wt/vol) vitamin assay Casamino Acids (Difco Laboratories). Minimal salts-glucose medium (MMG) was the same as EMMG, except that it lacked Casamino Acids. Antibiotics were added at the following concentrations to rich media: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 25 μg/ml.

Heat shock conditions and determination of cell viability after heat shock at 50°C. Cells from a single colony of each bacterial strain were added to 5 ml of the medium used in each experiment, and the cultures were grown overnight with shaking at 37°C. Fresh medium (15 to 30 ml) in a 250-ml Nephelo or Erlenmeyer flask was inoculated with a 0.5 to 2% volume of overnight culture. Exponential cultures were grown with shaking to turbidities of 40 to 50 Klett (660 nm) units (≈4×10^8 CFU/ml) at 23 to 30°C before being rapidly shifted to 42, 44, or 50°C (±1°C).

For determinations of cell viability, strains MG1655 and NU816 and isogenic mutants of MG1655 (Table 1) were inoculated from overnight cultures into 20 to 30 ml of EMMG or LB in 250-ml Erlenmeyer flasks (starting turbidities ≈0.06 optical density at 600 nm [OD_{600}] units). The cultures were grown with shaking at 30°C to exponential phase (turbidities ≈0.4 OD_{600} units [≈2.3×10^8 CFU/ml]) and then shifted to 50°C. Alternatively, 20 ml was inoculated with 1 μl of overnight cultures and grown at 30°C to a turbidity of ≈0.1 OD_{600} unit before

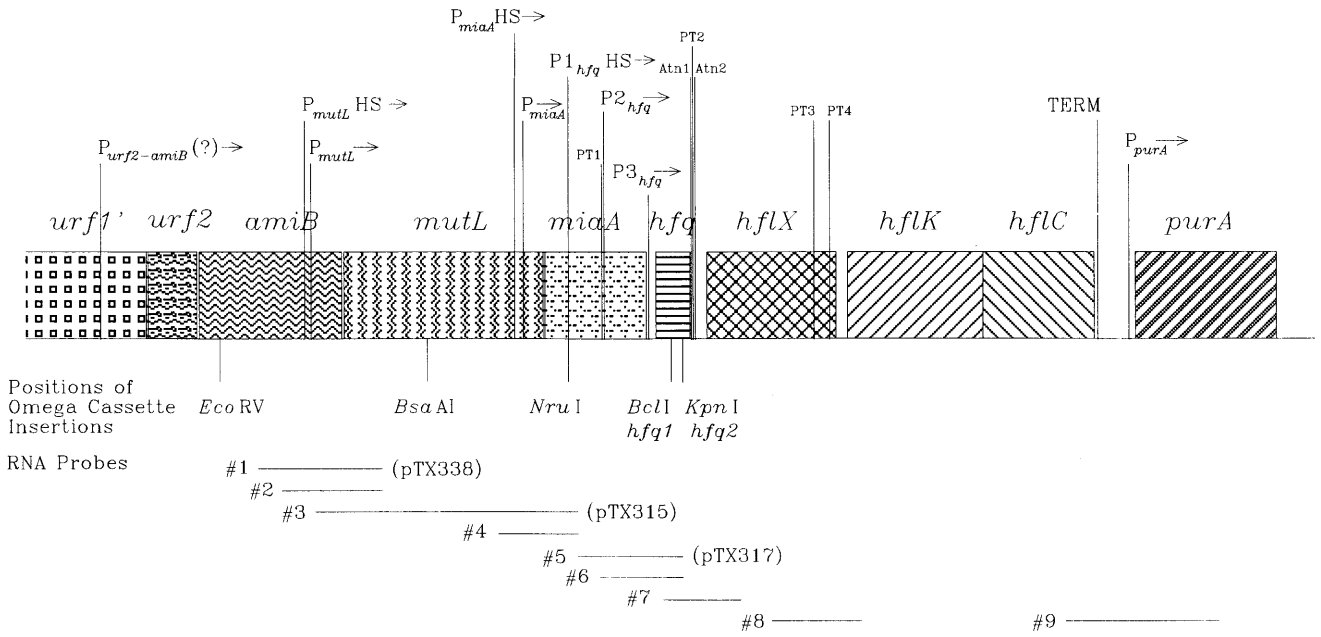

FIG. 1. Structure and transcription of the complex *amiB-mutL-miaA-hfq-hflX-hflK-hflC* superoperon at 94.78 min in the *E. coli* K-12 chromosome (Kroeger-Wahl map [35]). The figure, which is drawn to scale, is based on the sequences whose GenBank accession numbers are listed in the footnote to Table 1. The following sites are indicated: putative $P_{urf2\text{-}amiB}$ promoter; confirmed P_{mutL}HS, P_{miaA}HS, and $P1_{hfq}$HS $E\sigma^{32}$ promoters (see Results); confirmed P_{mutL}, P_{miaA}, $P2_{hfq}$, and $P3_{hfq}$ $E\sigma^{70}$ promoters (see Results) (69–71); Atn1 and Atn2 attenuators (see Results); PT1, PT2, PT3, and PT4 transcript processing sites (see Results) (66, 70); and terminator (TERM) at the end of the superoperon (see Results). Also indicated are sites in the bacterial chromosome containing polar omega cassettes, inserts in plasmids (pTX338, pTX315, and pTX317) used as templates in in vitro transcription reactions, and RNA probes 1 to 9 used in RNase T_2 protection assays (see Materials and Methods and Results).

being shifted to 50°C. Samples (0.1 ml) were withdrawn at various times before and after the upshifts and serially diluted in 0.9 ml of 1× E salts. Dilutions were spread in triplicate onto LB plates, which were incubated at 33°C for up to 3 days until the number of CFU per plate was constant.

RNA isolation, RNase T_2 protection assays, and in vitro capping assays. RNA was purified from cells grown exponentially at 23, 30, or 37°C and from cells shifted to 42 or 50°C for 7 min. RNA was prepared by adding portions of bacterial cultures directly to lysis solutions without intervening steps as described previously (68). RNA preparations were analyzed by agarose gel electrophoresis to ensure that similar amounts of rRNA were used in hybridization reactions, especially when cells were grown in LB media (68). RNase T_2 protection assays of mRNA transcribed from the bacterial chromosome were performed as described before (68). RNA probes (Fig. 1) were synthesized by using the following phage RNA polymerases (RNAP) and linearized plasmid templates: probe 1, T7, pTX338 with *Hin*dIII; probe 2, T7, pTX338 with *Bsa*A1; probe 3, T7, pTX315 with *Hin*dIII; probe 4, T7, pTX315 with *Bsa*HI; probe 5, T7, pTX317 with *Hin*dIII; probe 6, T7, pTX317 with *Sma*I; probe 7, SP6, pTX368 with *Eco*RI; probe 8, SP6, pTX405 with *Acc*I; probe 9, SP6, pTX407 with *Bss*HII. A series of labeled, undigested probes of known length were used as size standards to determine the lengths of the protected probes. Each hybridization reaction mixture contained 5 to 25 μg of total RNA from cells grown in EMMG or 50 μg of A_{260}-absorbing material from cells grown in LB medium. The radioactivity in gel bands was quantitated by using a betascope (Betagen Corp.). An area equal to that of the largest band was counted for each protected species, and a background band of the same area was determined from a region of the gel lacking radioactivity. Relative molar yields were calculated by correcting for the number of ^{32}P-labeled C residues in segments of protected probes.

For capping assays of $P3_{hfq}$ transcripts, total RNA from strain NU426 or NU426(pNU127) was end labeled in vitro in reaction mixtures containing [α-^{32}P]GTP and the vaccinia virus guanylyltransferase capping enzyme (BRL Life Technologies, Inc.) as described previously (68). Hybridization reaction mixtures contained 50 μg of total capped RNA and 50 μl of unlabeled probe 5 (Fig. 1). Products of RNase T_2 protection assays of capped hybrids and of hybrids formed between ^{32}P-labeled RNA probe 5 and the unlabeled total RNA preparation that was used in the capping reactions were loaded in adjacent lanes (68).

In vitro transcription. In vitro transcription reaction mixtures contained *E. coli* $E\sigma^{70}$ RNAP holoenzyme ($E\sigma^{70}$) (Epicentre Technologies, Madison, Wis.) or $E\sigma^{32}$ RNAP holoenzyme, which was purified as described elsewhere (14) and generously provided by Alicia Dombroski from the Department of Microbiology and Molecular Genetics, University of Texas—Houston Medical School. Plasmid DNA templates were purified by the Wizard miniprep kit (Promega, Inc.). Reaction mixtures (50 μl), which contained 0.3 to 0.5 μg of pTX315, pTX317, or pTX338 plasmid DNA; 40 mM Tris-HCl (pH 7.9); 50 mM (for $E\sigma^{70}$) or 200 mM (for $E\sigma^{32}$) KCl; 10 mM $MgCl_2$; 0.1 mM EDTA; 0.1 mM dithiothreitol; 0.1 mg of bovine serum albumin (BSA) per ml; 0.5 μl of RNasin (25 U/μl; Promega Corp.); and 2 U of $E\sigma^{70}$ or 0.35 μg of $E\sigma^{32}$ RNAP holoenzyme, were incubated at 37°C for 10 min. ATP, CTP, GTP, and UTP were added to a final concentration of 0.2 mM each. Reaction mixtures were incubated for 20 min, and reactions were stopped by addition of 150 μl of 0.3 M sodium acetate, 10 μg of phenol-extracted carrier tRNA, and 160 μl of phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous phase was further extracted once with 160 μl of chloroform-isoamyl alcohol (24:1) and twice with diethyl ether (68). RNA was precipitated with 2 volumes of ethanol, and dried RNA pellets were suspended in 40 μl of water that had been treated with diethyl pyrocarbonate (68). A 5-μl volume of resuspended RNA was used in each primer extension reaction (see below).

Primer extension. Six 27- to 30-mer synthetic oligonucleotides were chosen as primers specific for regions 80 to 120 nucleotides (nt) downstream from putative transcript 5′ ends. Primers and their coordinates in the published complementary coding sequence (sequence with GenBank accession number L19356 [71] melded in succession with Z11831 [67] and M63655 [13]) were as follows: $PriP_{mutL}$HS/P_{mutL} for identification of transcript starts at P_{mutL} HS and P_{mutL} positions 2720 to 2749; $PriP_{miaA}$HS, positions 4559 to 4588; $PriP_{miaA}$, positions 4689 to 4717; $PriP1_{hfq}$HS, positions 5001 to 5027; $PriP2_{hfq}$, positions 5410 to 5439; and $PriP3_{hfq}$, positions 5821 to 5848. Primer extension was carried out as previously described (68) with the following modification to increase sensitivity. (i) Kinase reaction mixtures (20 μl) contained 0.03 μg of oligonucleotide primer, 1× polynucleotide kinase buffer (Promega Corp.), 16.0 μl of a solution of 10 mCi of [γ-^{32}P]ATP per ml (3,000 Ci/mmol; Amersham Corp), and 1.0 μl (8 U/μl) of T4 polynucleotide kinase (Promega Corp). After purification with a Nuctrap push column, 100 μl of 5′-end-labeled primer (30,000 to 60,000 cpm [Cerenkov]/μl) was obtained. (ii) Hybridization reaction mixtures contained 200,000 cpm of 5′-end-labeled primer and 25.0 μg of total RNA from cells grown in EMMG, 100 to 200 μg of A_{260}-absorbing material from cells grown in LB medium, or 5.0 μl of product from in vitro transcription reactions (see above). (iii) Following the reverse transcriptase reaction, 1.0 μl of 0.5 M Na_2EDTA and 0.75 μl of a solution of 0.5 mg of RNase per ml (Boehringer Mannheim Corp., Indianapolis, Ind.) were added to each reaction mixture. Primer extension products were electrophoresed on the same gel with products of dideoxy sequencing reactions carried out with the same primer and plasmid pTX338, pTX315, or pTX317 as the template.

Western blotting (immunoblotting). Protein samples were prepared from cultures of strains MG1655, TX2652 (CC106 *mutL*::Ω), and TX2821 (MG1655 *hfq1*::Ω) grown exponentially in EMMG at 23 or 30°C and shifted to 44°C for 7 and 30 min or 50°C for 1, 2, and 6 h, respectively. Cultures (4.5 ml) were divided

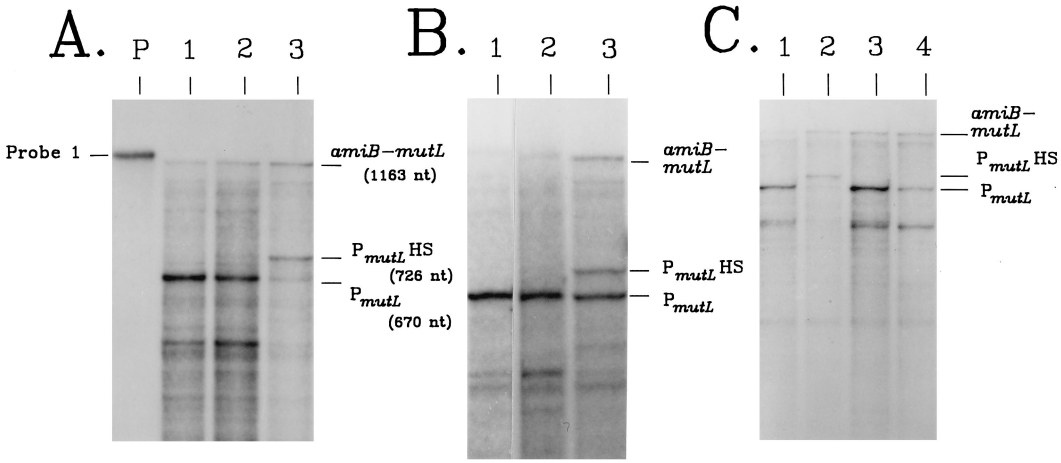

FIG. 2. Identification of the $E\sigma^{32}$-specific P_{mutL}HS promoter by RNase T_2 protection assays. Total RNA isolated from nonshocked or heat-shocked cells was hybridized with RNA probe 1 (Fig. 1) (see Materials and Methods). Bands corresponding to the *amiB-mutL* cotranscript and transcription initiation from the P_{mutL} and P_{mutL}HS promoters are indicated. RNA was prepared from cultures 7 min after shifts to the higher temperatures. (A) Lane P, RNA probe 1; lane 1, MG1655 grown exponentially in EMMG at 30°C; lane 2, MG1655 shifted from 30 to 42°C; lane 3, MG1655 shifted from 30 to 50°C. A 25-μg amount of total RNA was used in each hybridization. (B) Lane 1, NU426 grown exponentially in LB medium at 30°C; lane 2, NU426 shifted from 30 to 42°C; lane 3, NU426 shifted from 30 to 50°C. A 50-μg amount of A_{260}-absorbing materials was used in each hybridization. (C) Lane 1, MG1655 grown exponentially in EMMG at 23°C to a density of 60 Klett (660 nm) units; lane 2, MG1655 shifted from 23 to 50°C; lane 3, CAG9301 (*rpoH* null mutant originally isogenic with MG1655) grown exponentially in EMMG at 23°C to a density of 60 Klett (660 nm) units; lane 4, CAG9301 shifted from 23 to 50°C. A 15-μg amount of total RNA was used in each hybridization.

into three microcentrifuge tubes which were centrifuged at 16,000 × *g* for 20 s at room temperature. Cells in each tube were rapidly suspended in 15 μl of 2% (wt/vol) sodium dodecyl sulfate (SDS) warmed to 95°C and lysed by boiling for 5 min. The lysates were combined, 10 μl was diluted 20-fold with 0.5% (wt/vol) SDS, and protein concentrations were determined by using the D_c protein assay kit (Bio-Rad, Inc.). The remaining amount of lysate was diluted twofold with 2× Laemmli dissociation solution (38) lacking SDS and boiled for 5 min. Lysate volumes containing 125 or 20 μg of total protein were loaded in each lane on 11 or 15% polyacrylamide (5.6% stacking) gels containing SDS (38) for MutL or HF-I detection, respectively. Proteins were electrotransferred to polyvinylidene difluoride membranes and incubated with antisera as described previously (75). His_6-tagged MutL from *E. coli* was purified as described previously (20). Anti-MutL and anti-HF-I antibodies were generous gifts from P. Modrich (Duke University) and A. Ishihama (National Institute of Genetics, Mishima, Shizuoka, Japan), respectively.

Pulse-labeling and immunoprecipitation. MG1655 and TX2821 (MG1655 *hfq1*::Ω) were grown with shaking to a turbidity of 50 Klett (660 nm) units in 1× E salts containing 0.4% (wt/vol) glucose and 19 amino acids (no Met) or 18 amino acids (no Met or Cys) when labeling was carried out with L-[^{35}S]Met (1,000 Ci/mmol; Amersham Corp.) or tran^{35}S-label mixture (L-[^{35}S]Met and L-[^{35}S]Cys; 1,146 Ci/mmol; ICN Pharmaceuticals, Inc.), respectively. Immediately before heat shock and 6 min after cultures were shifted to 44 or 50°C, 1 ml of culture was transferred to plastic test tubes (17 by 100 mm) containing 70 μCi of L-[^{35}S]methionine or tran^{35}S-label mixture and incubated at the appropriate temperature for 1 min. A 0.11-ml volume of 50% trichloroacetic acid was added to stop the pulse-labeling, and the tubes were kept on ice for 30 to 60 min. Pellets were collected in microcentrifuge tubes by centrifugation at 16,000 × *g* at 4°C for 10 min. Pellets were washed twice with cold 80% acetone, vacuum dried, and suspended in 40 μl of 2% (wt/vol) SDS. The resulting solutions were boiled for 10 min and diluted with 760 μl of phosphate-buffered saline (PBS) (27)–2% (wt/vol) BSA. Immunoprecipitation was carried out by using 1.5×10^6 to 10×10^6 cpm of labeled protein, anti-HF-I antibody, and PANSORBIN cells (Calbiochem, Inc.) or Dynabeads M-280 sheep anti-rabbit immunoglobulin G (Dynal, Inc.) according to the manufacturer's protocols. Incubations with antibody and washes were carried out in PBS–0.1% (wt/vol) SDS–2% (wt/vol) BSA. Immunoprecipitated proteins were fractionated by SDS–15% polyacrylamide gel electrophoresis (PAGE) (38). Gels were fixed in 30% methanol for 20 min, soaked in fluorography solution (138.1 g of salicylic acid per liter) for 30 min, vacuum dried, and exposed to X-ray film against an intensifying screen at −70°C.

RESULTS

Identification of the P_{mutL}HS promoter. *mutL* transcript levels are comparatively low in vivo, and their detection is difficult by Northern (RNA) blotting (68, 71). Consequently, we performed RNase T_2 protection assays with several RNA probes of transcripts synthesized from the chromosomal *mutL* region during heat shock (Fig. 1 and 2). MG1655 or NU426 (W3110) bacteria grown exponentially in EMMG or LB medium at 30°C produced two identifiable bands corresponding to the *amiB-mutL* cotranscript and transcripts initiated from the previously identified P_{mutL} promoter within *amiB* (Fig. 2A and B, lanes 1) (71). The relatively high-level background is characteristic of detection of the low-abundance, intact *amiB-mutL* and P_{mutL} transcripts and does not reflect unusually high degrees of RNA degradation in these preparations (e.g., see Fig. 5). A shift of the strains from 30 to 42°C for 7 min did not significantly change the transcription pattern (Fig. 2A and B, lanes 2). However, a new band appeared when the strains were shifted from 30 to 50°C for 7 min (P_{mutL}HS, Fig. 2A and B, lanes 3). There were differences in the relative amounts of P_{mutL}HS and P_{mutL} transcripts in the MG1655 strain in EMMG and the NU426 (W3110) strain in LB at 50°C. The amount of P_{mutL} transcript decreased significantly in MG1655 in EMMG at 50°C (Fig. 2A, lane 3; Table 2), whereas it did not change very much in the NU426 (W3110) strain in LB medium (Fig. 2B, lane 3). Nonetheless, the basic transcription patterns were similar in the two different genetic backgrounds in the two growth media.

Several criteria were used to demonstrate that the P_{mutL}HS band corresponded to an $E\sigma^{32}$ promoter and not a processing site or an $E\sigma^{70}$ promoter. Insertion of a polar omega (Ω) cassette into the *Eco*RV site upstream of P_{mutL} and P_{mutL}HS (Fig. 1) caused the disappearance of the *amiB-mutL* cotranscript but not the P_{mutL}HS and P_{mutL} transcripts from NU426 (W3110) cells shifted from 30 to 50°C in LB medium (data not shown). The same 726-nt P_{mutL}HS and 670-nt P_{mutL} bands were detected when RNA from the *amiB*::Ω(*Eco*RV) mutant was hybridized to probe 1 or probe 2, which have a common distal end (Fig. 1; also data not shown). This result confirmed the placement of P_{mutL}HS just upstream of P_{mutL} in the orientation shown in Fig. 1. Results from protection assays of RNA from an *rpoH* null mutant following extreme heat shock from 23 to 50°C were consistent with P_{mutL}HS being an $E\sigma^{32}$-specific promoter. In MG1655, the P_{mutL}HS band was the major transcript detected following the shift from 23 to 50°C in

TABLE 2. Relative molar amounts of mRNA segments in the *amiB-mutL-miaA-hfq-hflX-hflK-hflC* superoperon under nonshock and heat shock conditions[a]

RNA probe[b]	Transcript	Relative molar amt of transcript[c] at:		Fold change by heat shock[d]
		30°C	50°C for 7 min	
1	*amiB-mutL*	≡1.0	1.3	1.3
	P_{mutL}HS	ND[e]	3.4	
	P_{mutL}	7.1	3.6	0.5
4	*mutL-miaA*	4.5	6.4	1.4
	P_{miaA}HS	ND	7.9	
	P_{miaA}	22	71	3.2
	$P1_{hfq}$HS	64	1,395	22
5	*miaA-hfq*	36	750	21
	$P2_{hfq}$	103	ND	
	$P3_{hfq}$	95	ND	
7	*hfq-hflX*	85	260	3.0
	hflX at PT2	87	292	3.4
	hfq at Atn2	331	1,503	4.5
	hfq at Atn1	416	1,679	4.0
8	*hflX-hflK*	37	198	5.4
	hflK at PT3	63	90	1.4
	hflK at PT4	63	123	2.0
9	P_{purA}	249	27	0.11
	hflC at terminator	178	307	1.7

[a] *E. coli* MG1655 was grown to mid-exponential phase in EMMG at 30°C, and total RNA was isolated from cells before and after incubation with shaking at 50°C for 7 min as described in Materials and Methods. Cellular RNA was hybridized with RNA probes in RNase T_2 protection assays.

[b] Positions of RNA probes are depicted in Fig. 1. Autoradiograms of RNA species hybridized with probes 1, 4, 5, 7, 8, and 9 are shown in Fig. 2A, 5A, 7, and 8A, B, and C, respectively.

[c] Relative molar amounts of transcripts were determined as described in Materials and Methods.

[d] Fold changes by heat shock were not calculated for RNA species that could not be detected under one of the two experimental conditions.

[e] ND, not detected, no distinct band was detected under condition tested.

EMMG (Fig. 2C, lane 2). However, the P_{mutL}HS band did not appear in the MG1655 *rpoH* null mutant following the same temperature upshift (Fig. 2C, lane 4; see also below).

The same P_{mutL}HS transcript band was detected in primer extension assays done with RNA from NU426 (W3110) cells shifted from 30 to 50°C in LB medium (Fig. 3, lane 2) or RNA synthesized in vitro from supercoiled templates by purified Eσ[32] RNAP (lane 4). The P_{mutL} transcript was synthesized by purified Eσ[70], but not by Eσ[32], RNAP (Fig. 3, lanes 3 and 4). No band corresponding to P_{mutL}HS was detected for the in vitro Eσ[70] reaction, despite a relatively large number of spurious background bands (Fig. 3, lane 3). The apparent difference in the relative amounts of the in vivo P_{mutL}HS and P_{mutL} transcripts detected in RNase T_2 protection (Fig. 2B, lane 3) and primer extension (Fig. 3, lane 2) assays may reflect an inability of reverse transcriptase to extend synthesis to the end of the longer P_{mutL}HS transcript. The primer extension assays (Fig. 3) pinpointed the end of the P_{mutL}HS transcript to a site 54 nt upstream of the start of the P_{mutL} transcript (Fig. 4A). Analysis of the region upstream from the 5′ end of the P_{mutL}HS transcript resembled the consensus for Eσ[32] promoters (Fig. 4A). Taken together, these results establish P_{mutL}HS as an Eσ[32]-specific promoter.

Identification of P_{miaA}HS and $P1_{hfq}$HS as Eσ[32]-specific promoters. RNase T_2 protection assays were extended to the region spanning *mutL* and *miaA* (Fig. 1 and 5). These experiments revealed two more Eσ[32]-specific promoters, P_{miaA}HS and $P1_{hfq}$HS. RNA from strain MG1655 in EMMG or NU426 (W3110) in LB medium at 30°C or shifted from 30 to 42°C gave three major protected bands (Fig. 5A, lanes 3 and 4, and 5B, lanes 1 and 2) corresponding to the *mutL-miaA* cotranscript and transcripts from the previously identified P_{miaA} and $P1_{hfq}$HS (formerly $P1_{hfq}$) promoters within the *mutL* and *miaA* coding sequences, respectively (Fig. 1) (69). Shifting either strain from 30 to 50°C for 7 min resulted in three main changes in the transcription pattern: the apparent amount of intact P_{miaA} transcript increased in cells in EMMG, a new P_{miaA}HS band appeared, and the amount of $P1_{hfq}$HS transcript increased dramatically (>20-fold) (Fig. 5A, lane 5, and 5B, lane 3; Table 2). MG1655 and NU426 (W3110) gave similar results in EMMG following a shift from 30 to 50°C (Fig. 5A, lanes 5 and 6).

Experiments analogous to those described above were performed to establish P_{miaA}HS and $P1_{hfq}$HS as Eσ[32] promoters and P_{miaA} as a likely Eσ[70] promoter. The positions of the promoters were established by hybridization of RNA from heat-shocked cells with either probe 3 (Fig. 5A, lane 1) or probe 4 (Fig. 5A, lane 6), which have a common distal end (Fig. 1). MG1655 shifted from 23 to 50°C in EMMG showed the appearance of P_{miaA}HS, increased amounts of P_{miaA} and $P1_{hfq}$HS, and decreased amounts of *mutL-miaA* cotranscript in comparison with what was observed for growth at 23°C (Fig. 5C, lanes 1 and 2). The *rpoH* null mutation completely abolished the $P1_{hfq}$HS transcript at 23°C, as well as the $P1_{hfq}$HS and P_{miaA}HS transcripts after a shift from 23 to 50°C (Fig. 5C, lanes 3 and 4). Primer extension assays of RNA synthesized in vivo after a shift from 30 to 50°C or in vitro in reactions done with purified Eσ[32] or Eσ[70] RNAP gave results consistent with the interpretation that P_{miaA}HS (Fig. 6A) and $P1_{hfq}$HS (Fig. 6C and D) were Eσ[32] promoters and P_{miaA} was an Eσ[70] promoter (Fig. 6B). Finally, alignment of regions upstream from the transcription start sites of P_{miaA}HS, $P1_{hfq}$HS, and P_{mutL}HS showed that all three promoters match the Eσ[32] promoter

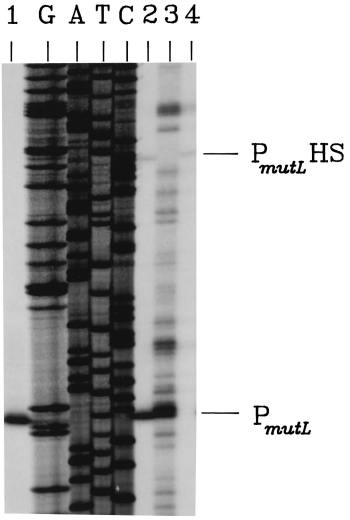

FIG. 3. Primer extension analysis to locate the P_{mutL} and P_{mutL}HS transcription start sites by using mRNA synthesized in vivo and in vitro. Primer extension assays were performed as described in Materials and Methods with the PriP_{mutL}HS/P_{mutL} primer and the RNA preparations indicated below. G, A, T, and C marker bands were from dideoxy sequencing reactions done with the PriP_{mutL}HS/P_{mutL} primer and plasmid pTX338 template (Fig. 1). Lane 1, total RNA (100 μg of A_{260}-absorbing material) isolated from NU426 grown exponentially in LB at 30°C; lane 2, NU426 total RNA (100 μg of A_{260}-absorbing material) isolated 7 min after a shift from 30 to 50°C; lane 3, RNA synthesized in vitro from pTX338 by Eσ[70] RNAP; lane 4, RNA synthesized in vitro from pTX338 by Eσ[32] RNAP.

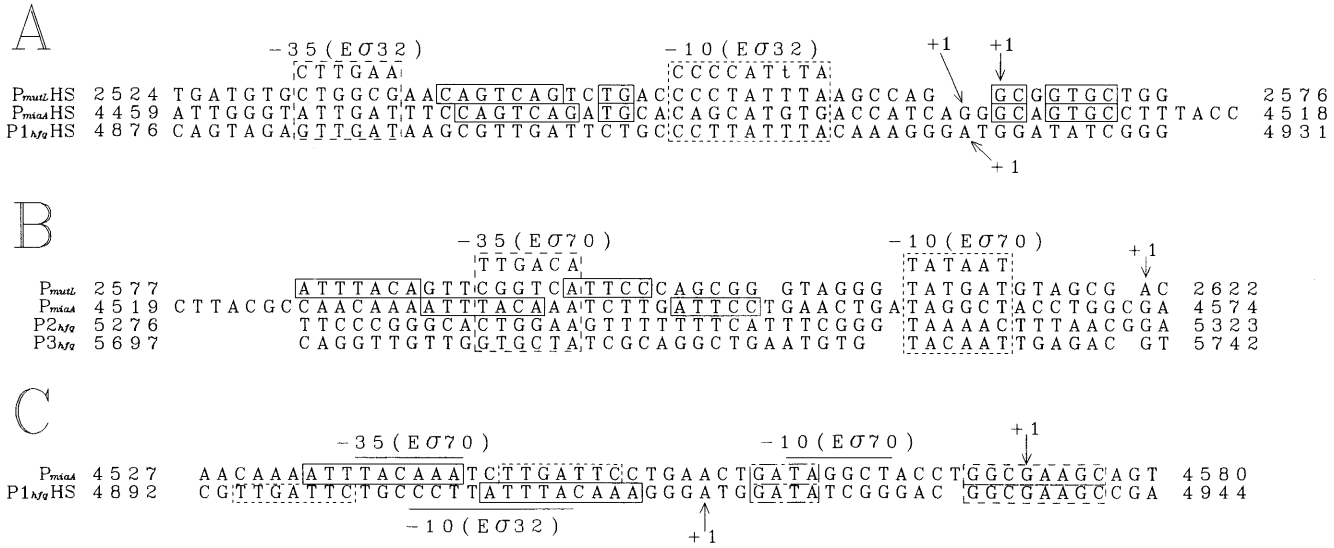

FIG. 4. Comparisons of the P_{mutL}HS, P_{mutL}, P_{miaA}HS, P_{miaA}, and $P1_{hfq}$HS promoters. (A) Alignment of P_{mutL}HS, P_{miaA}HS, and $P1_{hfq}$HS with the −35 and −10 regions of the $E\sigma^{32}$ consensus sequence (25). CAGTCAG(1 or 2 N)TG and GCNGTGC (boxed) are present in the spacer region between the −10 and −35 regions and near the transcription start sites, respectively, of P_{mutL}HS and P_{miaA}HS, but not $P1_{hfq}$HS. (B) Alignment of P_{mutL} and P_{miaA} with the −35 and −10 regions of the $E\sigma^{70}$ consensus sequence (44). ATTTACA(7 or 8 N)ATTCC (boxed) is present near the −35 regions of both promoters. (C) Alignment of P_{miaA} and $P1_{hfq}$HS showing the conserved ATTTACAAA, TTGATTC, and GATA(7 or 8 N)GGCGAAGC sequences. The P_{miaA} and $P1_{hfq}$HS sequences are aligned with the GATA(7 or 8 N)GGCGAAGC conserved sequence.

consensus sequence reasonably well (Fig. 4A). Unusual structural features of these three $E\sigma^{32}$ promoters are described in Discussion.

Heat shock control of *hfq* and the *hflA* region originates from P_{miaA}HS and $P1_{hfq}$HS. We next determined whether the increased transcription from the $E\sigma^{32}$-specific $P1_{hfq}$HS promoter led to heat shock control of *hfq* and the *hflA* region. In extending the RNase T_2 protection analyses to the *miaA-hfq* junction, we mapped and analyzed two additional $E\sigma^{70}$-specific promoters, one within *miaA* ($P2_{hfq}$) and the other in the intercistronic region between *miaA* and *hfq* ($P3_{hfq}$) (Fig. 1; Fig. 7, lane 2). RNA isolated from MG1655 grown in EMMG at 30°C and hybridized to RNA probe 5 (Fig. 1) gave six major protected bands, corresponding to the $P2_{hfq}$ and $P3_{hfq}$ transcripts, the *miaA-hfq* cotranscript, two processed segments of the *miaA-hfq* cotranscript (PT1-5′ and PT1-3′), and an as-yet-unidentified transcript (Fig. 7, lane 2). PT1-5′, PT1-3′, and the other transcripts were positioned in this region by using overlapping probes 5 and 6, which have a common distal end (Fig. 1; see also above and data not shown). The 728-nt $P2_{hfq}$ transcript band corresponded to the longest RNA that hybridized to probe 5 from a *miaA*::Ω(*Nru*I) mutant (data not shown). The location of the $P2_{hfq}$ promoter was pinpointed in primer extension assays of in vivo RNA and in vitro RNA synthesized by $E\sigma^{70}$ RNAP (Fig. 6E). The upstream polar *miaA*::Ω(*Nru*I) insertion eliminated the 975-nt *miaA-hfq* spanner band as expected, in addition to the 215-nt PT1-5′ and 740-nt PT1-3′ bands (data not shown). This result and the heat shock pattern described below indicated that PT1-5′ and PT1-3′ species arose by endonucleolytic cleavage of the *miaA-hfq* cotranscript. The $P3_{hfq}$ promoter was identified by in vitro capping of in vivo RNA (see Materials and Methods) and by primer extension analyses of in vivo RNA and in vitro RNA synthesized by $E\sigma^{70}$ RNAP (Fig. 6F).

When MG1655 was shifted in EMMG from 30 to 42°C for 7 min, there was an increase in the amount of *miaA-hfq* spanner transcript (Fig. 7, lanes 3 and 6) but otherwise the transcription pattern was not markedly changed from that of cells maintained at 30°C (Fig. 7, lanes 2 and 5). A shift from 30 to 50°C resulted in large increases in the amounts of the *miaA-hfq* cotranscript (≈20-fold [Table 2]), the PT1-3′ transcript (≈7-fold), and the PT1-5′ transcript (Fig. 7, lanes 4 and 7). In contrast, the amounts of the $P2_{hfq}$ and $P3_{hfq}$ transcripts sharply decreased so that no corresponding bands were detected above background (Fig. 7, lanes 4 and 7; Table 2). Thus, the increase in the transcript amounts in the *hfq* and *hflA* region during heat shock (see below) originated mainly from $P1_{hfq}$HS.

Finally, we examined whether this heat shock transcription extended into the *hflA* region. The transcription pattern downstream of $P3_{hfq}$ is briefly described here, but the evidence for these features is beyond the scope of this paper and will be reported elsewhere. We did not detect an independent promoter for the *hflA* region in nonstress conditions, and transcription of *hflX*, *hflK*, and *hflC* seems to depend completely on the upstream P_{miaA}, $P1_{hfq}$HS, $P2_{hfq}$, and $P3_{hfq}$ promoters (66, 70). However, the *hfq-hflX* intercistronic region contains a *rho*-independent attenuator (Atn1), a *rho*-dependent attenuator (Atn2), and an RNA processing site (PT2) (70). On the basis of steady-state transcript amounts, but not rate-of-transcription experiments, as much as 80% of transcripts from the *hfq* region may be terminated at Atn1 (45%) and Atn2 (32%) in unstressed cells (70).

The amounts of *hfq-hflX* cotranscript, processed *hflX* (PT2), and *hfq* transcripts terminated at Atn1 and Atn2 increased three- to fivefold in MG1655 cells in EMMG shifted from 30 to 50°C (Fig. 8A, lane 4; Table 2) relative to amounts in cells maintained at 30°C (Fig. 8A, lane 2) or shifted to 42°C (Fig. 8A, lane 3). Likewise, the amount of the contiguous *hflX-hflK* spanner transcript increased about fivefold in cells shifted to 50°C (Fig. 8B, lane 4) in comparison with the amount in cells maintained at 30°C (Fig. 8B, lane 2; Table 2) or shifted to 42°C (Fig. 8B, lane 3). Finally, the amount of transcript terminated at the end of *hflC* in bacteria shifted to 50°C increased about twofold (Fig. 8C, lane 4; Table 2) relative to the amount in

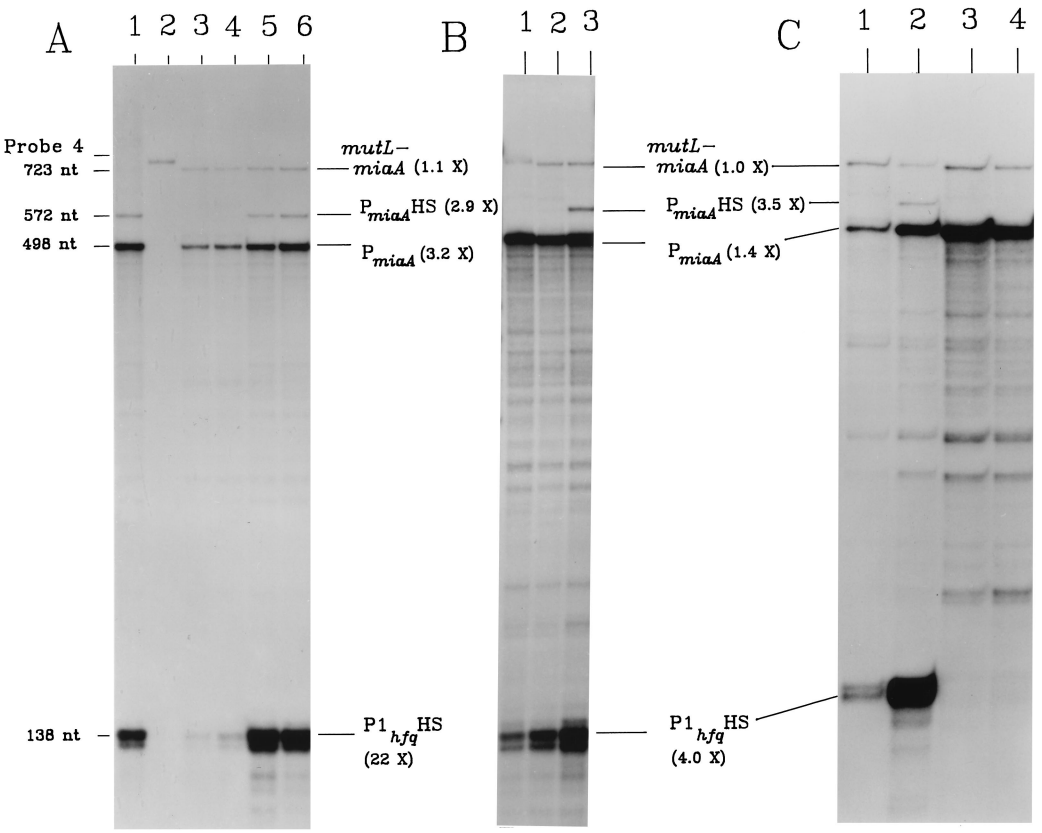

FIG. 5. Identification of the Eσ[32]-specific P_{miaA}HS and $P1_{hfq}$HS promoters by RNase T_2 protection assays. Total RNA isolated from nonshocked or heat-shocked cells was hybridized with RNA probe 3 (lane 1 in panel A) or 4 (all other lanes) (Fig. 1; also see Materials and Methods). Bands corresponding to the *mutL-miaA* spanner transcript and transcription initiations from the P_{miaA}HS, P_{miaA}, and $P1_{hfq}$HS promoters are indicated. RNA was prepared from cultures 7 min after shifts to higher temperatures. (A) Lane 1, NU426 shifted from 30 to 50°C in EMMG (probe 3 was so long that no *mutL-miaA* cotranscript was detected); lane 2, RNA probe 4; lane 3, MG1655 grown exponentially in EMMG at 30°C; lane 4, MG1655 shifted from 30 to 42°C; lane 5, MG1655 shifted from 30 to 50°C; lane 6, NU426 shifted from 30 to 50°C in EMMG. A 25-μg amount of total RNA was used in each hybridization. (B) Lane 1, NU426 grown exponentially in LB at 30°C; lane 2, NU426 shifted from 30 to 42°C; lane 3, NU426 shifted from 30 to 50°C for 7 min. A 50-μg amount of A_{260}-absorbing material was used in each hybridization. (C) Lane 1, MG1655 grown in EMMG at 23°C to a density of 60 Klett (660 nm) units; lane 2, MG1655 shifted from 23 to 50°C; lane 3, CAG9301 (*rpoH* null mutant originally isogenic with MG1655) grown in EMMG at 23°C to a density of 60 Klett (660 nm) units; lane 4, CAG9301 shifted from 23 to 50°C. A 15-μg amount of total RNA was used in each hybridization. Numbers in parentheses refer to panels A and B and are the ratios of transcript amounts in cells shifted to 50°C compared with amounts in exponentially growing cells.

those maintained at 30°C (Fig. 8C, lane 2) or shifted to 42°C (Fig. 8C, lane 3). In contrast, the amount of *purA* transcript decreased dramatically (≈10-fold; Table 2) at 50°C in comparison with its levels under the other conditions (Fig. 8C). This finding, together with a lack of a detectable *hflC-purA* cotranscript, indicated that the superoperon ends at the terminator after *hflC*.

Cellular levels of MutL and HF-I and synthesis of HF-I during heat shock. To determine whether the cellular amounts of MutL and HF-I proteins increased during heat shock, we performed Western immunoblotting with anti-MutL and anti-HF-I antibodies and protein samples obtained from MG1655 grown in EMMG (see Materials and Methods). Cellular levels of MutL remained constant 7 and 30 min after upshift to 44°C (Fig. 9A, lanes 6 to 8) and 20 min and 1, 2, and 6 h after upshift to 50°C (data not shown). Similarly, HF-I protein amounts remained constant after 7 and 30 min at 44°C (Fig. 9B, lanes 2 to 4) and after 7 min and 1, 2, and 6 h at 50°C (data not shown).

To determine whether the rate of HF-I synthesis increased during heat shock, we performed radioimmunoprecipitation experiments using extracts of cells that were pulse-labeled before or 6 min after heat shock at 44°C (see Materials and Methods). Preliminary results showed that an 11-kDa polypeptide, which had the same molecular mass as HF-I, was present in the heat-shocked hfq^+ strain but not in the unshocked hfq^+ control. This 11-kDa polypeptide was absent in an unshocked or heat-shocked *hfq1*::Ω mutant and thus likely was the HF-I polypeptide (data not shown). So far, we have been unable to detect MutL protein by immunoprecipitation procedures.

Growth and viability of hfq^+ and *hfq* strains at 50°C. Previous studies of viability of *E. coli* at 50°C were mostly performed with strain W3110 grown in morpholinepropanesulfonic acid (MOPS) medium supplemented with glucose and thiamine or with glucose, 20 amino acids, and 5 vitamins (72). These studies showed that viability dropped to about 1% when cells were incubated at 50°C for 1 h. Since the transcript amounts from P_{mutL}HS, P_{miaA}HS, and $P1_{hfq}$HS were most abundant in cells grown in EMMG at 50°C, we reexamined the growth and viability of our commonly used MG1655 and W3110 strains under these conditions. We found that the turbidity of MG1655 cultures in EMMG continued to increase when cultures were shifted from 28 to 50°C (Fig. 10A). A large increase in OD_{600} occurred in the first 10 min after the temperature upshift, and the OD_{600} continued to increase gradually for at least 6.5 h. The numbers of viable cells at 1, 2.2, 3.5, and 6.5 h after temperature upshift remained about 100% of that before

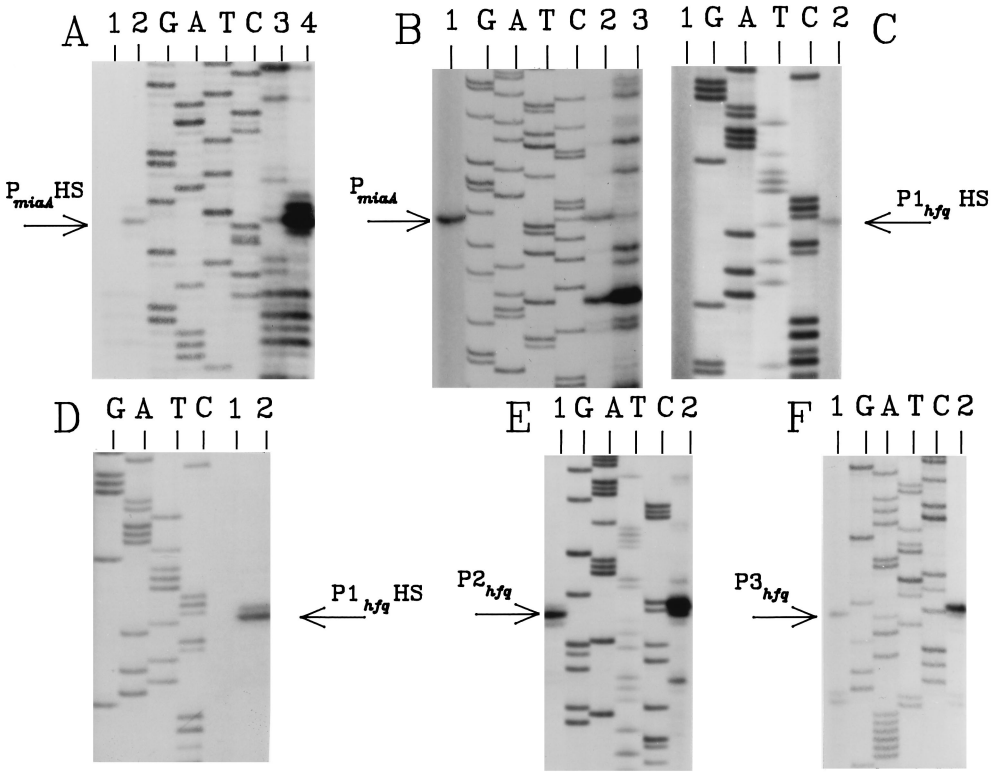

FIG. 6. Primer extension analyses to locate P_{miaA}HS, P_{miaA}, $P1_{hfq}$HS, $P2_{hfq}$, and $P3_{hfq}$ transcription start sites by using RNA synthesized in vivo and in vitro. Primer extension assays were performed by using the primers listed below and described in Materials and Methods. G, A, T, and C marker bands were from dideoxy sequencing reaction mixtures containing the primer used in primer extension analyses and plasmid pTX315 or pTX317 (Fig. 1) as a template. (A) $PriP_{miaA}$HS primer. Lane 1, 25 μg of total RNA from MG1655 grown exponentially in EMMG at 30°C; lane 2, 25 μg of total MG1655 RNA isolated 7 min after a shift from 30 to 50°C; lane 3, RNA synthesized in vitro from pTX315 by $E\sigma^{70}$ RNAP; lane 4, RNA synthesized in vitro from pTX315 by $E\sigma^{32}$ RNAP. (B) $PriP_{miaA}$ primer. Lane 1, 25 μg of total RNA isolated from MG1655 grown exponentially in EMMG at 30°C and shifted to 50°C for 7 min; lane 2, RNA synthesized in vitro from pTX315 by $E\sigma^{70}$ RNAP; lane 3, RNA synthesized in vitro from pTX315 by $E\sigma^{32}$ RNAP. (C) $PriP1_{hfq}$HS primer. Lane 1, total RNA (200 μg of A_{260}-absorbing material) from MG1655 grown exponentially in LB at 23°C; lane 2, total MG1655 RNA (200 μg of A_{260}-absorbing material) isolated 7 min after a shift from 23 to 50°C. (D) $PriP1_{hfq}$HS primer. Lane 1, RNA synthesized in vitro from pTX315 by $E\sigma^{70}$ RNAP; lane 2, RNA synthesized in vitro from pTX315 by $E\sigma^{32}$ RNAP. (E) $PriP2_{hfq}$. Lane 1, total RNA (100 μg of A_{260}-absorbing material) from NU426 grown exponentially in LB at 37°C; lane 2, RNA synthesized in vitro from pTX317 by $E\sigma^{70}$ RNAP. (F) $PriP3_{hfq}$. Same as panel E except for the primer.

the temperature upshift (0 h) (Fig. 10B). Consistent with the viable-cell counts, microscopic examination of the cells with the Live/Dead BacLight bacterium viability kit (Molecular Probes, Inc.) showed fewer than 1% dead cells before temperature upshift and only about 15% dead cells after 6 h at 50°C (data not shown). Strain NU816 (W3110 prototroph) grown in EMMG and strains MG1655 and NU816 grown in LB also maintained viability for several hours at 50°C (data not shown).

We tested whether *hfq* function was required for viability during extreme heat shock by comparing the survival rates of *hfq1*::Ω and *hfq2*::Ω mutants. *hfq1*::Ω lacks HF-I function and is strongly pleiotropic, whereas *hfq2*::Ω has phenotypic properties similar to those of the hfq^+ parent; both *hfq1*::Ω and *hfq2*::Ω are completely polar on transcription of the downstream *hflA* genes (Fig. 1) (66, 70). Cultures of the *hfq1*::Ω mutant abruptly stopped growing during the first 15 min at 50°C but then gradually increased in turbidity, similar to the behavior of the hfq^+ parent strain (Fig. 10A). On the other hand, the viability of the *hfq1*::Ω mutant decreased with time at 50°C to ≈20% at 6.5 h (Fig. 10B). The *hfq2*::Ω mutant behaved like its hfq^+ MG1655 parent (data not shown), indicating that the loss of viability by the *hfq1*::Ω mutant was caused by lack of *hfq* function and not by polarity on the *hflA* region. Likewise, *amiB*::Ω, *mutL*::Ω, *miaA*::Ω, and *amiB*::Ω *miaA* (*trpX*) (ochre) mutants had the same growth and viability as the MG1655 parent when shifted from 30 to 50°C in EMMG (data not shown).

Finally, the loss of viability by MG1655 and its isogenic mutant derivatives was much more pronounced at 50°C in MMG, which lacked Casamino Acids, than in EMMG. After 2 h at 50°C, the numbers of viable MG1655, *amiB*::Ω, *mutL*::Ω, *miaA*::Ω, *amiB*::Ω *miaA* (*trpX*) (ochre), and *hfq2*::Ω cells ranged from about 2 to 12% of numbers before the temperature upshift (data not shown). Nonetheless, this observation suggests that a sizable population of *E. coli* K-12 cells survived prolonged exposure at 50°C, even in MMG. As expected from the results with EMMG (above), the number of viable *hfq1*::Ω mutant cells dropped to only 0.015% of the preshift value, which was about 400-fold less than the value for the hfq^+ and *hfq2*::Ω strains.

***miaA* mutants are defective in colony formation at 45°C.** Since many genes of the $E\sigma^{32}$ transcription regulon are essential for growth at high temperatures (57, 58), we tested whether mutations in the *amiB-mutL-miaA-hfq-hflX-hflK-hflC* superoperon caused defects in colony formation at 45°C. MG1655 and its isogenic *amiB*::Ω, *mutL*::Ω, *miaA*::Ω, *amiB*::Ω *miaA*, *hfq1*::Ω, and *hfq2*::Ω mutants were grown in LB medium at 37°C to stationary phase, and triplicates of serially diluted cultures were spread on LB plates and incubated at 30 or 45°C for 24 h. For MG1655 and its isogenic *amiB*::Ω, *mutL*::Ω,

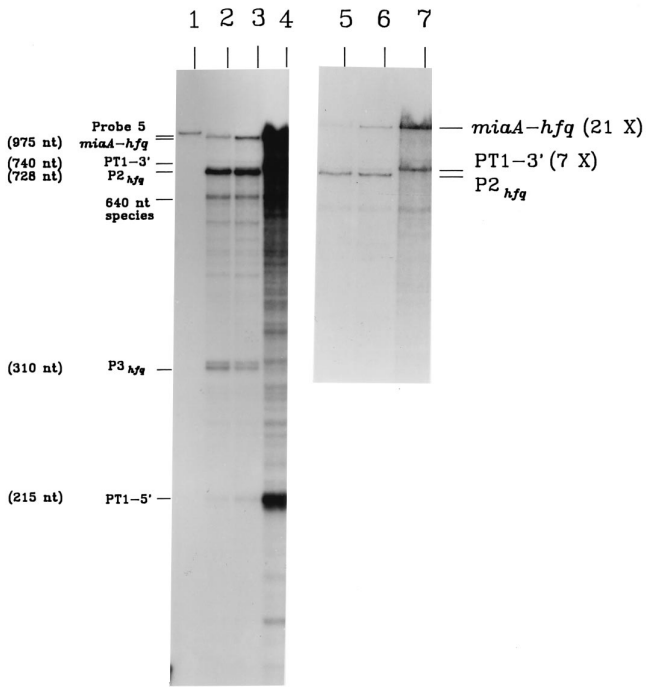

FIG. 7. Identification of the Eσ[70]-specific $P2_{hfq}$ and $P3_{hfq}$ promoters. RNase T_2 protection assays were performed with RNA probe 5 as described in Materials and Methods. The bands indicated correspond to the *miaA-hfq* spanner transcript, the PT1-3′ and PT1-5′ processed transcripts (see Results), and transcription initiations from the $P2_{hfq}$ and $P3_{hfq}$ promoters. The identities of the 640-nt band and other faint degradation bands are unknown. Lane 1, RNA probe 5; lanes 2 and 5, total RNA isolated from MG1655 grown exponentially in EMMG at 30°C; lanes 3 and 6, total MG1655 RNA isolated 7 min after a shift from 30 to 42°C; lanes 4 and 7, total MG1655 RNA isolated 7 min after a shift from 30 to 50°C. Lanes 5 to 7 are shorter exposures and higher magnifications of lanes 2 to 4. Numbers in parentheses are the ratios of transcript amounts in cells shifted to 50°C compared with amounts in exponentially growing cells.

hfq1::Ω, and *hfq2*::Ω mutants, the sizes of the colonies formed at the two temperatures were similar for each strain, and the number of colonies formed at 45°C was around 100% of that at 30°C (data not shown). In contrast, the numbers of colonies formed by *miaA*::Ω and *amiB*::Ω *miaA* (*trpX*) (ochre) mutants at 45°C were 0.16 and 0.4%, respectively, of those formed at 30°C, and the sizes of colonies formed by the *miaA* mutants at 45°C were much smaller and less uniform than those formed at 30°C. Similar results were obtained when MG1655 and MG1655 *miaA*::Ω cells growing exponentially in LB medium were sampled, diluted, and spread onto plates that were incubated at the two temperatures. The fact that strains with *miaA* insertion and point mutations but not downstream *hfq* insertions showed this plating defect supports the conclusion that an intact *miaA* gene is required for colony formation at 45°C.

DISCUSSION

We show in this paper that the *E. coli* K-12 *amiB-mutL-miaA-hfq-hflX-hflK-hflC* superoperon contains a complicated arrangement of alternating Eσ[32] promoters (P_{miaA}HS, P_{mutL}HS, and $P1_{hfq}$HS) and Eσ[70] promoters (P_{miaA}, P_{mutL}, $P2_{hfq}$, and $P3_{hfq}$) (Fig. 1, 2, 5, and 7). The relative molar amounts of mRNA originating from each promoter and the sums of transcripts for each gene are shown in Tables 2 and 3, respectively. During heat shock at 50°C, the amount of *mutL* transcript was maintained and the amounts of the *miaA*, *hfq*, *hflX*, *hflK*, and *hflC* transcripts increased two- to fivefold (Table 3). These results add the genes for *mutL* repair, *miaA* tRNA modification, *hfq* pleiotropic regulator, and the *hflA* region protease to the Eσ[32] RNAP transcription regulon. In addition, the transcript amounts for the individual genes could also be regulated by other features in the region, including Rho-dependent and -independent transcription attenuation at two sites (Atn1 and Atn2 [Fig. 1]), RNA processing at four sites (PT1, PT2, PT3, and PT4 [Fig. 1]) (Fig. 7) (66, 70), and possibly differential transcript destabilization by RNase E (69). This exceedingly complicated arrangement of regulatory sites provides considerable versatility for the control of cellular amounts of MiaA, HF-I, and the HflA protease, which in turn regulate other important cellular processes, including translational fidelity (17), spontaneous mutation rate (13, 40), *nif* gene expression (33), *yst* virulence (51), RpoS amount (6a, 50a), DNA topology (66), and phage lysogeny (10). Finally, results presented here show that the superoperon extends to a terminator beyond *hflC* and that expression of *purA*, which is in the same orientation as the *hflA* region, is independent of the superoperon (Fig. 1 and 8).

Without additional data on transcript stability, rates of mRNA synthesis at low and high temperatures, and the amounts of transcripts present as a function of time after temperature upshift, we cannot accurately compare the relative in vivo strengths of the seven promoters in the superoperon. Nonetheless, several regulatory patterns emerge from the present study. P_{mutL}HS functioned mainly at the high physiological temperature of 50°C, so that the total *mutL* transcript amount was maintained at the same level (Table 3). In contrast, although P_{miaA}HS was also functional only at 50°C, its contribution to *miaA* transcript amounts at high temperatures was minor because of the relatively high rate of transcription from P_{miaA} at both low and high temperatures (Table 2). $P1_{hfq}$HS was functional at temperatures ranging from 23 to 50°C (Fig. 2 and 5). In this regard, $P1_{hfq}$HS was similar to the Eσ[32]-specific promoters that drive the *dnaK*, *dnaJ*, and *htpG* genes (76). Transcription from $P1_{hfq}$HS increased strongly at 50°C (22-fold [Fig. 5; Table 2]) and was the main source of increased transcription of the *hfq*, *hflX*, *hflK*, and *hflC* genes (Fig. 7 and 8; Table 3). For the Eσ[70]-specific promoters, the amount of transcripts from P_{miaA} increased after heat shock from 30 to 50°C (Fig. 5), whereas amounts of intact transcripts from P_{mutL}, $P2_{hfq}$, $P3_{hfq}$, and P_{purA} decreased (Fig. 2, 7, and 8).

The promoters of the *amiB-mutL-miaA-hfq-hflX-hflK-hflC* superoperon show several unusual structural features (Fig. 1 and 4). First, the promoters precede the genes they transcribe by a considerable distance and are present in the coding regions of known upstream genes. This gene arrangement means that the *mutL*, *miaA*, and *hfq* primary transcripts contain extraordinarily long leader regions (i.e., 366 nt for P_{mutL}HS, 312 nt for P_{mutL}, 271 nt for P_{miaA}HS, 201 nt for P_{miaA}, 891 nt for $P1_{hfq}$HS, and 488 nt for $P2_{hfq}$). Long leaders in other bacterial genes often have been found to play regulatory roles (e.g., see references 4 and 74). The presence of promoters within coding regions also raises the possibility that cotranscriptional interference may occur (73), especially for transcription of *miaA* (Fig. 1). Second, P_{mutL}HS and P_{miaA}HS contain the sequence CAGTCAG(1 or 2 N)TG between the −35 and −10 regions and the sequence GCNGTGC near the transcription start sites (Fig. 4A). These two sequences are absent from all published Eσ[32] promoter sequences, and conservation of promoter spacer regions is not normally observed (25). Third, the P_{mutL} and P_{miaA} promoter regions contain multiple potential Fis protein-binding sites (69, 71) and another conserved sequence, ATTTACA(7 or 8 N)ATTCC, near their −35 regions (Fig. 4B). Last, three sequences, ATTTACAAA, TTGATTC, and

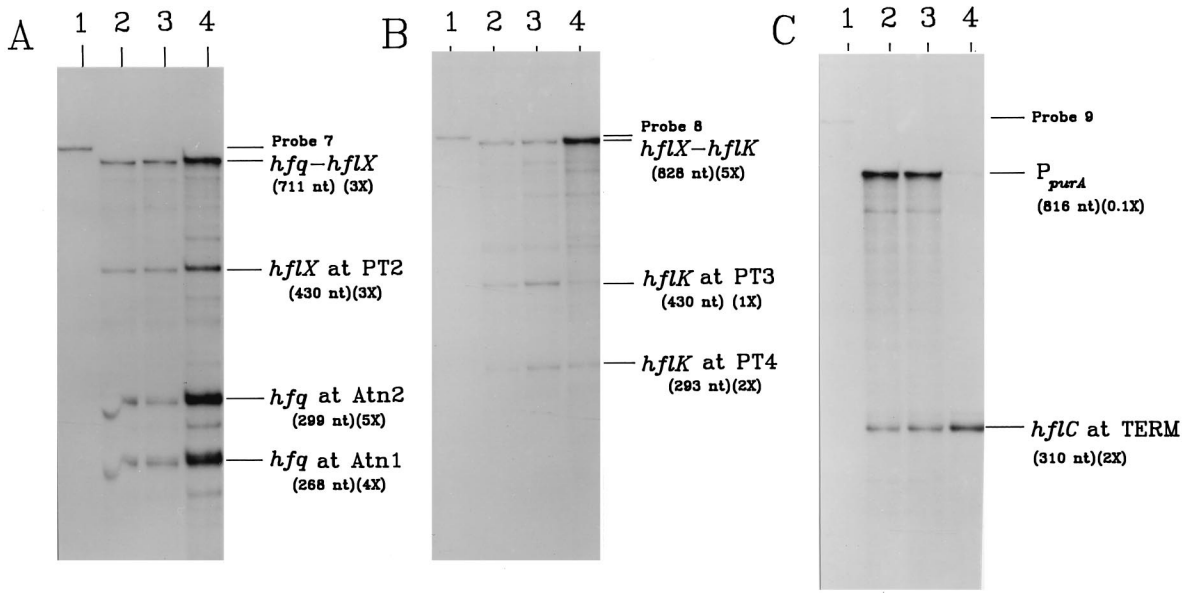

FIG. 8. Increased amounts of transcripts from *hfq* and the *hflA* region after heat shock. RNase T_2 protection assays (see Materials and Methods) were performed with hybrids formed between 25 μg of total RNA isolated from nonshocked or heat-shocked cells and RNA probe 7 (A), probe 8 (B), and probe 9 (C). The bands indicated correspond to the *hfq-hflX* and *hflX-hflK* spanner transcripts, the processed *hflX* transcript at a site designated PT2, the terminated *hfq* transcripts at the Atn1 (Rho factor independent) and Atn2 (Rho factor dependent) attenuators, the processed *hflK* transcripts at sites designated PT3 and PT4, the terminated *hflC* transcript at the end of the superoperon, and the independent *purA* transcript, which is synthesized in the same direction as the superoperon (Fig. 1; see also Results). Lanes (all three panels): 1, RNA probe; 2, 25 μg of total RNA from MG1655 grown exponentially in EMMG at 30°C; 3, 25 μg of total MG1655 RNA isolated 7 min after a shift from 30 to 42°C; 4, 25 μg of total MG1655 RNA isolated 7 min after a shift from 30 to 50°C. Numbers in parentheses are the ratios of transcript amounts in cells shifted to 50°C compared with amounts in exponentially growing cells. TERM, terminator.

GATA(7 or 8 N)GGCGAAGC, are present in the −40-to-+5 region of the $E\sigma^{70}$-specific P_{miaA} promoter and the −26-to-+22 region of the $E\sigma^{32}$-specific $P1_{hfq}$HS promoter (Fig. 4C). Although the functions, if any, of these sequences are yet to be determined, their conservation is suggestive of shared regulation.

We showed in this study that prototrophic *E. coli* MG1655 and W3110 maintained nearly 100% viability in enriched growth media, such as EMMG and LB, after an upshift to 50°C (Fig. 10) (see Results), which has generally been considered a lethal temperature for *E. coli* (63, 72). This observation lends significance to the fact that $E\sigma^{32}$ promoters (11), including P_{mutL}HS, P_{miaA}HS, and $P1_{hfq}$HS, are functional at high levels in vivo at 50°C. In addition, the $E\sigma^{32}$ activity level remains high at 50°C in vitro (5). Consistent with our results, mutants of various *E. coli* strains that not only remain viable but also grow at 48 and 54°C have been isolated (17a).

Transcription from P_{mutL}HS and $P1_{hfq}$HS at a high temperature likely contributed to the maintenance of the total cellular amounts of the MutL and HF-I proteins, which remained constant (Fig. 9) at low and high temperatures. Many members of the $E\sigma^{32}$ transcription regulon are dually transcribed from $E\sigma^{70}$ promoters (8, 39, 65, 76), as is the case for the genes in the *amiB-mutL-miaA-hfq-hflX-hflK-hflC* superoperon (Fig. 1). In some cases (e.g., *groEL*), the rate of synthesis and the total amount of protein increase after heat shock (52). However, in other cases (e.g., *topA*), the total protein amount remains constant after heat shock (39), as it did for MutL and HF-I (Fig. 9). In this latter class of genes, transcription from $E\sigma^{32}$-specific promoters may act to maintain transcript levels, since transcript amounts from many $E\sigma^{70}$ promoters decrease at higher temperatures (5, 18).

Transcription of the *mutL* repair gene by $E\sigma^{32}$ RNAP might fulfill several physiological roles. The MutL protein is not particularly abundant under nonstress conditions (19), and MutL seems to be limiting under some conditions of increased mutagenesis (15, 60a). Maintenance of the MutL amount may be necessary to repair any DNA replication errors that occur under heat shock conditions and after temperature downshift and resumption of growth. In addition, a constant MutL amount may ensure efficient mismatch repair at high temper-

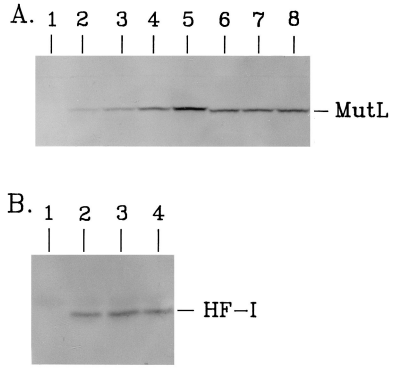

FIG. 9. Cellular amounts of MutL and HF-I before and after heat shock. (A) Western blot of MutL. Lanes 1 to 5, quantitation standards containing 125 μg of total protein from TX2652 (CC106 *mutL*::Ω) grown exponentially at 37°C in EMMG to which 0, 3, 6, 12, and 24 ng of purified His_6-MutL (20) was added, respectively; lanes 6 to 8, MutL in 125 μg of total protein from MG1655 grown exponentially at 23°C and shifted to 44°C for 7 and 30 min, respectively. (B) Western blot of HF-I. Lane 1, 20 μg of total protein from TX2821 (MG1655 *hfq1*::Ω); lanes 2 to 4, HF-I in 20 μg of total protein from MG1655 grown exponentially at 23°C and shifted to 44°C for 7 and 30 min, respectively. The intensity of the HF-I bands increased about twofold when 40 instead of 20 μg of total protein was loaded per lane (data not shown), indicating that detection was not saturated at 20 μg of total protein.

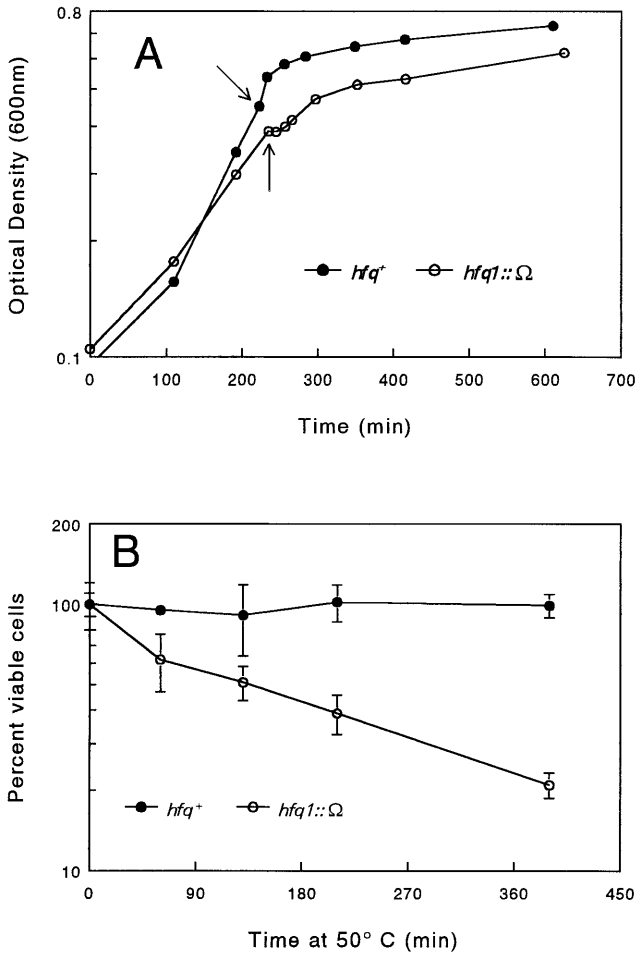

FIG. 10. Growth curves and viabilities of MG1655 *hfq*$^+$ and isogenic *hfq1*::Ω mutant TX2821 before and after a temperature upshift from 30 to 50°C. Cultures were grown with shaking at 30°C to exponential phase and shifted to 50°C (arrows) as described in Materials and Methods. (A) Culture turbidities; (B) percent viable cells determined by plating onto LB medium (see Materials and Methods). *hfq2*::Ω mutant TX2817 grew and survived like MG1655 (data not shown). Viable-cell counts were confirmed by using fluorescent staining (see Materials and Methods).

atures by other pathways, such as by VSP repair (43). Deamination of 5-methylcytosine to T, which leads to the kind of G · T mismatch corrected by the VSP repair pathway (42), occurs in the DNA of nonreplicating and nonrecombining cells and increases significantly in frequency at high temperature (23). Finally, the well-documented overlap of the Eσ[32] heat shock regulon with other stress regulons (52) raises the possibility that *mutL* transcription, as well as that of *miaA*, *hfq*, and the *hflA* region, may be induced by other stress conditions.

This study is the first to show that a tRNA modification gene is transcribed from both Eσ[32]- and Eσ[70]-specific promoters and that mutations in a tRNA modification gene can lead to defective growth at high temperatures (see Results). It is not known whether cellular amounts of tRNA isoaccepting species increase at higher temperatures, but, suggestively, the *rrnB* P1 promoter, which drives the formation of rRNA and tRNA molecules, is transcribed by Eσ[32] RNAP in vitro (53). Transcription from the P_{miaA} promoter, which was classified as Eσ[70] specific on the basis of in vitro transcription experiments (Fig. 6B), was unusual. P_{miaA} transcript amounts increased during heat shock (Fig. 5), whereas levels of most Eσ[70]-specific transcripts decreased (Results) (18). In addition, transcription from P_{miaA} was maintained at a relatively high level in stationary-phase cells, whereas many Eσ[70]-specific transcripts decreased to undetectable levels (19). On the basis of these properties, it is possible that P_{miaA} may be transcribed at some level by Eσ[38] (RpoS) in vivo (45, 64). Consistent with this idea, the −10 region of P_{miaA} (TAGGCT) matches both the Eσ[38] (TATACT) and Eσ[70] (TATAAT) consensus sequences (30). Transcription of the superoperon by RNAP molecules other than Eσ[70] and Eσ[32] and in response to global regulatory factors is currently being investigated. Finally, we do not yet know whether the requirement of an intact *miaA* gene to form colonies at 45°C reflects the need for MiaA-mediated tRNA modification or a second gene product translated from the extraordinarily long $P1_{hfq}$HS or $P2_{hfq}$ leader sequence (Fig. 1; also see above).

TABLE 3. Relative molar transcript amounts of each gene in the *amiB-mutL-miaA-hfq-hflX-hflK-hflC* superoperon and the downstream gene *purA* under nonshock and heat shock conditions[a]

Transcript	Relative molar amt of transcript[b] at:		Fold change by heat shock
	30°C	50°C for 7 min	
amiB	≡1.0	1.3	1.3
mutL	8.1	8.3	1
miaA	27	85	3.1
hfq	832	3,442	4.1
hflX	37	198	5.4
hflK	163	411	2.5
hflC	178	307	1.7
purA	249	27	0.11

[a] *E. coli* MG1655 was grown to mid-exponential phase in EMMG at 30°C, and total RNA was isolated from cells before and after incubation with shaking at 50°C for 7 min as described in Materials and Methods. Cellular RNA was hybridized with RNA probes in RNase T_2 protection assays.

[b] Relative molar amounts of transcripts for each gene were determined from individual or sums of mRNA segments shown in Table 2: *amiB*, *amiB-mutL*; *mutL*, *amiB-mutL* plus P_{mutL}HS plus P_{mutL}; *miaA*, *mutL-miaA* plus P_{miaA}HS plus P_{miaA}; *hfq*, *hfq-hflX* plus *hfq* at Atn2 plus *hfq* at Atn1; *hflX*, *hflX-hflK*; *hflK*, *hflX-hflK* plus *hflK* at PT3 plus *hflK* at PT4; *hflC*, *hflC* at the terminator; and *purA*, P_{purA}.

Our findings that synthesis of *hfq*-encoded HF-I increased (data not shown) and the cellular level of HF-I was maintained at a high temperature (Fig. 9) are consistent with those of Chuang and Blattner (11), who showed that a low-molecular-weight protein similar in size to HF-I was induced from the *hflA* region during heat shock. Our results also show that *hfq* function is required for survival at high temperatures (Fig. 10) (Results). Previously, we showed that HF-I acts as a pleiotropic regulator in *E. coli* at all temperatures (66). The exact function of HF-I, which is itself heat stable, is not known. The *Y. enterocolitica* homolog of HF-I Yrp is required for the expression of a heat-stable enterotoxin virulence factor (Y-ST) (51), and the production of Y-ST (6) and the expression of other virulence functions (46) are temperature regulated. *miaA* and *hfq* are juxtaposed in *Y. enterocolitica* as they are in *E. coli* (51), suggesting that *miaA* acts as a gene and the promoter or regulatory region of *hfq* in both organisms. However, *hfq* is at a chromosomal location separate from that of *amiB-mutL-miaA* in *Haemophilus influenzae* (21) and may be regulated differently in this organism compared with *E. coli* and *Y. enterocolitica.*

The *A. caulinodans* HF-I homolog NrfA was required for the expression of the *nifA* gene (33). In *Y. enterocolitica* and *A.*

caulinodans, lack of the HF-I homologs impaired transcription of the *yst* and *nifA* genes, respectively (33, 51). In contrast, HF-I function was required for translation of the *rpoS*-encoded σ^{38} subunit of *E. coli* (6a, 50a). This finding may explain some, but not all, of the phenotypes of the *hfq1*::Ω null mutant (50a, 66), including the loss of viability at high temperatures reported here (30a, 45). It has not yet been established whether the reported effects of HF-I on transcript amounts or translation efficiency are direct or indirect. Since HF-I binds to Qβ RNA and other RNAs directly, but apparently not to DNA (22), and is found largely in association with ribosomes (32), it seems likely that HF-I may play some role in presentation of mRNA to ribosomes (33). Increased translation mediated by HF-I may result in mRNA stabilization (70), which could account for the apparent effects of HF-I on transcript amounts (33, 51). On the other hand, HF-I, which is subject to positive growth rate control (32) in addition to the regulation discussed here, may play multiple regulatory roles.

Our demonstration that the *hflA* region is in the $E\sigma^{32}$ heat shock regulon confirms the previous conclusion of Chuang and Blattner (11). They identified the HflX putative GTP-binding protein and the HflK and HflC protease subunits as heat shock proteins. We show here that heat shock transcription from the *hflA* region originates mainly way upstream at $P1_{hfq}$HS near the start of the *miaA* coding sequence and that *hfq* and the *hflA* region are cotranscribed during heat shock. Several other protease genes were previously shown to be members of the $E\sigma^{32}$ heat shock regulon (24). Among the heat shock genes involved in proteolysis (*hflB* [29], *clpP* [36], *clpB* [63], *lon* [56], and *htrC* [57]), *hflB* is essential at all temperatures (28), *clpB* is necessary for optimal growth and survival at high temperatures (63), and *htrC* is essential for viability at high temperatures (57). In contrast, *hfq2*::Ω mutants, which completely lacked *hflA* region transcription (66), were not defective in growth at 45°C or viability at 50°C (see Results). This general lack of phenotype of *hflA* mutants (66) may be due to functional substitution by the HflB protease under the conditions tested so far (28, 29).

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