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Mutations in a Gene Encoding a New Hsp70 Suppress Rapid DNA Inversion and *bgl* Activation, but Not *proU* Derepression, in *hns-1* Mutant *Escherichia coli*

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Mutations in *hns*, the gene encoding the nucleoid-associated protein H-NS, affect both the expression of many specific unlinked genes and the inversion rate of the DNA segment containing the *pilA* promoter in *Escherichia coli*. A second-site mutation, termed *hscA1*, compensated for the effect of an *hns-1* mutant allele on the *pilA* promoter inversion rate and on activation of the *bgl* operon. The *proU* operon, induced in an *hns-1* background, remained derepressed in an *hns-1 hscA1* strain and was induced at an intermediate level in an *hns hscA1* strain. An insertion mutant allele, *hscA2-cat*, conferred the same partial *hns-1* compensatory phenotype as the *hscA1* allele. The *hscA* gene encoded a 66-kDa protein product that is a member of the Hsp70 protein class. The gene encoding this product is part of a bicistronic operon that is preceded by a possible σ^{32} promoter and also encodes a 21-kDa protein with significant homology to the DnaJ protein family. The mutation defining the *hscA1* allele resulted in a phenylalanine substituting a conserved serine residue located in the ATP-binding region of other Hsp70 proteins.

H-NS (H1) is an approximately 16-kDa neutral protein that is a major nucleoid component in *Escherichia coli* (10, 35). Homodimers of this protein bind tightly to double-stranded DNA, conferring increased thermal stability to the double-stranded DNA substrate (10, 13, 35). Although H-NS does not appear to bind DNA in a sequence-dependent fashion (32), this protein has a higher affinity for curved DNA than for DNA substrates with similar sequences that do not possess intrinsic curvature (30, 40). H-NS is expressed at high levels in *E. coli*, as each cell contains approximately 20,000 copies of this protein (35). However, H-NS expression is further induced at least threefold following cold shock (22). The H-NS protein has the additional property of acting to inhibit the expression of a subset of specific genes that are not linked on the *E. coli* chromosome (3, 17, 42). Consequently, a number of biological properties can be attributed to this one small protein: it is a part of the prokaryotic nucleoid, it is a stress response protein, and it has an important role in modulating gene expression.

Mutations in *hns*, the gene encoding H-NS, affect gene expression and site-specific DNA inversion in *E. coli* (7, 12, 24, 36, 42), *Salmonella* strains (15), and *Shigella* strains (9, 23). In *E. coli hns* mutants, the inversion rate of the DNA segment containing the *pilA* promoter is increased over 100-fold (18), the normally cryptic *bgl* operon is activated (7, 15), the *proU* operon (normally expressed only under conditions of high osmolarity) is derepressed (15, 24), and Pap pilus expression is also derepressed in strains containing an intact *pap* locus (12). Different *hns* mutant alleles display these same phenotypes, but the specific mechanism by which these phenotypes are induced has been the subject of much debate. A number of studies with *Salmonella* strains have shown that *hns* mutations result in an increased level of negative supercoiling in reporter plasmids and that this increased negative supercoiling is responsible for derepressing *proU* expression in the absence of osmotic shock (15, 17, 30). However, H-NS also specifically inhibits initiation of *proU* transcription in vitro by directly binding the *proU* promoter (38). Furthermore, different *hns* alleles confer the same mutant phenotypes without altering the superhelical density of reporter plasmids (18, 41). Clearly, under some conditions, a subset of *hns* mutant alleles have unpredictable effects on DNA supercoiling levels. It is also evident that these changes in DNA topology may not account for all of the phenotypes associated with *hns* mutations. This possibility is particularly compelling for the effect of *hns* mutations on the DNA inversion rate in *E. coli*. Two different mutant *hns* alleles, *hns-1* and *hns-2-tetR* (formerly termed *pilG1* and *pilG2-tetR* respectively [18]), cause a 100-fold-increased inversion rate for the 314-bp invertible DNA segment containing the promoter of the *pilA* gene in *E. coli*. These two mutant alleles have no discernible effect on reporter plasmid superhelical density (18). It is reasonable to conclude that H-NS may affect gene expression and DNA inversion by a variety of mechanisms.

We have begun to dissect the mechanism by which H-NS is involved in modulating gene expression and DNA inversion by isolating second-site mutations that compensate for *hns-1* mutant phenotypes in *E. coli*. Specifically, we have looked for mutations that restore wild-type, or nearly wild-type, *pilA* promoter inversion frequency in an *hns-1* mutant background and then assessed the effect of these second-site compensatory mutations on other phenotypes associated with *hns* mutations. In this study we describe one such mutation defining an allele termed *hscA1* that compensated for promoter inversion frequency and *bgl* activation, but not *proU* derepression, in an *hns-1* strain. It has been presumed for some time that DnaK is the only member of the Hsp70 family of shock response proteins expressed in *E. coli*. However, the lesion conferring the *hscA1* phenotype was in a gene encoding a second Hsp70 protein that has yet to be described in *E. coli*.

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TABLE 1. Bacteria, bacteriophages, and plasmids

Strain, phage, or plasmid	Description	Source or reference
Bacteria		
DH5α	F⁻ ϕ80 *lacZ*ΔM15 Δ(*argF-lac*)U169 *endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	Laboratory collection
CAG18469	MG1665 *gua-26*::Tn*10*	34
CAG18481	MG1655 *zff-208*::Tn*10*	34
CAG18480	MG1655 *nadB51*::Tn*10*	34
GM50	MC4100 Φ(*proU'-lacZYA-kan*)	25
ORN105	*thr leu proA2* Δ(*argF-lac*)U169 *galK his argE rpsL supE mtl xyl recBC sbcB* (has Tn*10* between *hsd* and *serB*)	18
ORN116	*thr-1 leuB thi-1* Δ(*argF-lac*)U169 *malA1 xyl-7 ara-13 mtl-2 gal-6 rpsL fhuA2 supE44 hns-1* Φ(*pilA'-lacZYA-kan*)	29
ORN132	*thr-1 leuB thi-1* Δ(*argF-lac*)U169 *malA1 xyl-7 ara-13 mtl-2 gal-6 rpsL fhuA2 trpA62 supE44 hns-1* Φ(*pilA'-lacZYA-kan*)*zcg*::Tn*10*	36
THK31	Same as ORN116, except *tetR* adjacent to *hns*⁺ allele	18
THK32	Same as ORN116, except *tetR* adjacent to *hns-1* allele	18
THK35	Same as ORN132, except *hscA1*	UV mutagenesis of ORN132
THK38	Same as ORN115, except *tetR* adjacent to *hns*⁺ allele	P1 transduction from THK31
THK46	Same as THK35, except is Tet^S	Bochner selection of THK35 (4)
THK47	Same as THK46, except *mal*⁺	Selected on MinA maltose
THK51	Same as ORN116, except *hscA1 zfg*::Tn*10*	P1 transduction from pooled tetracycline-resistant THK46 resulting from λ::Tn*10* infection
THK55	Same as THK46, except *pilA*⁺ and has Tn*10* between *fimB* and *fimE*	P1 transduction from AF249 (P. Orndorff)
THK60	Same as THK38, except Φ(*proU'-lacZYA-kan*)	P1 transduction from GM50
THK63	Same as ORN115, except *tetR* adjacent to *hns-1*	P1 transduction from THK32
THK64	Same as THK63, except Φ(*proU'-lacZYA-kan*)	P1 transduction from GM50
THK65	Same as THK55, except Φ(*proU'-lacZYA kan*)	P1 transduction from GM50
THK68	Same as THK55, except is Tet^S	Bochner selection of THK55 (4)
THK72	Same as THK68, except *tetR* adjacent to *hns*⁺ allele	P1 transduction from THK31
THK73	Same as THK68, except *tetR* adjacent to *hns-1* allele	P1 transduction from THK32
THK74	Same as THK72, except Φ(*proU'-lacZYA-kan*)	P1 transduction from GM50
THK75	Same as THK73, except Φ(*proU'-lacZYA-kan*)	P1 transduction from GM50
THK80	ORN105 *hscA2-cat*	Linear transformation using plasmid pTHK300 (Fig. 3)
Phages		
P1	*vir*	Laboratory collection
λ	Kohara phage 430	20
Plasmids		
pUC19	Ap^r ColE1	
pTHK200	pUC19 with 5.4-kb *Eco*RI fragment from Kohara phage 430; *hscA*⁺	This study (Fig. 3)
pTHK201	pUC19 with 3.35-kb partial *Hin*dIII-*Eco*RI fragment from pTHK200 insert; *hscA*⁺	This study (Fig. 3)
pTHK203	pUC19 with 1.85-kb *Hpa*I-*Eco*RI fragment from pTHK200; *hscA* mutant	This study (Fig. 3)
pTHK204	pUC19 with 1.5-kb partial *Hin*dIII-*Hpa*I fragment from pTHK200; *hscA* mutant	This study (Fig. 3)
pTHK205	pUC19 with 3.0-kb *Stu*I-*Eco*RI fragment from pTHK200; *hscA*⁺	This study (Fig. 3)
pTHK300	pTHK200 with *cat* gene inserted into the unique *Hpa*I site; *hscA2-cat*	This study (Fig. 3)
pJM41	pBR322 *dnaKJ*	G. Walker

MATERIALS AND METHODS

Bacterial strains, plasmids, and genetic techniques. The bacterial strains, bacteriophage strains, and plasmids used in this study are listed in Table 1. Generalized transductions using P1 *vir* were carried out as described previously (27). Mutant alleles constructed in vitro were introduced into the *E. coli* chromosome via electroporation with linear DNA into strain ORN154 (*recBC sbcB*) as described previously (18). UV mutagenesis of *E. coli* ORN132 was performed as described elsewhere (27). Briefly, cells from 5 ml of a late-log-phase culture were pelleted and resuspended in 2.5 ml of 10 mM $MgSO_4$. The cell suspension was subjected to 1,000 ergs of UV irradiation. Irradiated cells were plated onto lactose MacConkey plates and incubated at 30°C. Survivors were assessed for phenotype with respect to the *pilA* promoter inversion rate. Mutations that conferred reduced promoter inversion frequency in the *hns-1* background were ultimately transduced into nonmutagenized strains to ensure that resulting phenotypes were not due to multiple lesions. The *pilA* promoter inversion frequencies were calculated as described previously (29).

Mapping the *hscA* allele. The *hscA* allele was located to approximately 54.5 min on the *E. coli* chromosome map. A tetracycline-sensitive, *mal*⁺ THK35 derivative (*hscA1*), THK47, was infected with λ::Tn*10*. A P1 lysate was made with the pooled resulting tetracycline-resistant colonies. This lysate was used to transduce strain ORN116 (*hns-1 pilA'-lacZYA-kan*), selecting for tetracycline resistance and scoring for *hscA1*

phenotype at 30°C on lactose MacConkey agar. Tetracycline-resistant recipients showing reduced *pilA* promoter inversion frequency were picked to determine linkage between the Tn*10* insertion and the *hscA1* allele. One such strain, THK51, had Tn*10* 20% linked to *hscA1* as determined by P1 cotransduction analysis. This result also confirmed that a single mutation conferred the *hscA1* phenotype. Chromosomal DNA was prepared from strain THK51, cut with *Bam*HI restriction endonuclease, electrophoresed on agarose, transferred to nylon, and probed with purified DNA containing a *tetR* gene. Restriction endonuclease *Bam*HI cuts Tn*10* such that the intact *tetR* gene was excised along with chromosomal DNA flanking a Tn*10* insert. An approximately 7-kb *Bam*HI fragment from THK51 DNA that hybridized to the *tetR* gene was identified. Size-fractionated, *Bam*HI-digested THK51 DNA was ligated into pUC19. Ligation products were transformed into *E. coli* DH5α, selecting for tetracycline resistance. One such transformant contained pUC19 with a 7.0-kb insert that hybridized to the *tetR* gene. This cloned fragment containing approximately 1 kb of chromosomal DNA flanking the original Tn*10* insertion was radiolabeled and used to probe the entire Kohara clone miniset (20) (Takara Biomedicals). The cloned DNA hybridized to Kohara phage clones 432 and 433, corresponding to approximately 55 min on the *E. coli* chromosome (20).

Media, enzymes, and chemicals. Media consisted of L agar, L broth, and MacConkey agar (Difco Laboratories, Detroit, Mich.) and minimal broth (27). When used, antibiotics were added to final concentrations of 100 μg (ampicillin), 20 μg (tetracycline), or 40 μg (kanamycin) per ml of medium. Chemicals and antibiotics were purchased from Sigma Chemicals (St. Louis, Mo.). Restriction and other DNA-modifying enzymes were used according to the manufacturer's recommendations (GIBCO-BRL, Gaithersburg, Md.). β-Galactosidase assays were performed essentially as described elsewhere (27) on cultures grown in minimal media to an A_{600} of ca. 0.1 to 0.3. The in vitro transcription-translation reactions using plasmid DNA templates were performed with *E. coli* S-30 extracts (Promega) under the conditions recommended by the manufacturer.

DNA sequencing and analysis. Plasmid clones containing the *hscA* allele were sequenced with synthetic oligonucleotides as primers for double-stranded dideoxy sequencing by using Sequenase as described by the manufacturer (United States Biochemical, Cleveland, Ohio). Synthetic oligonucleotides corresponding to the wild-type *hscA* sequence were used as primers to amplify 400- to 500-bp regions from strain THK47 (*hscA1*). Overlapping fragments that collectively encompassed the entire *hscA* locus were generated. These amplified segments were cloned into pUC19, and their sequences were determined as described above. Multiple templates were used for each amplified fragment to ensure that the *hscA1* mutation was assigned properly. Sequences were analyzed by using the Genetics Computer Group DNA and protein sequence analysis programs (8). Homology between the *hscA* gene product and the Hsp70 signature sequences was found by using the motif program (Genetics Computer Group) for searching the Prosite data base.

Nucleotide sequence accession number. The DNA sequence of *hscA* and 769 flanking bases has been deposited in the GenBank data base under accession number V01827. The small open reading frame (ORF) preceding the *hscA* gene is identified in the data base submission and has been designated *yfhE* by National Center for Biotechnology Information, GenBank.

TABLE 2. Effect of *hscA1* and *hns-1* on *pilA* promoter inversion frequency

Strain	Variation rate[a]	
	On → off	Off → on
ORN131 (*hns*$^+$)	4.0×10^{-4}	1.4×10^{-3}
ORN132 (*hns-1*)[b]	1.5×10^{-2}	3.2×10^{-2}
THK46 (*hns-1 hscA1*)	4.0×10^{-3}	6.5×10^{-3}

[a] Variation rates were calculated as described in Materials and Methods and are expressed as switches per cell per generation.
[b] The inversion frequency for ORN132 was calculated previously (18).

RESULTS

A second-site compensatory mutation, *hscA1*, confers reduced *pilA* promoter inversion frequency in an *hns-1* mutant background. Type 1 pilus expression is subject to reversible on-to-off phase variation. This phase variation is mediated predominantly by the orientation of a 314-bp DNA segment containing the promoter for the *pilA* gene (1, 19). The *pilA* gene encodes pilin, the major protein subunit of the type 1 pilus. Using strains containing a *pilA'-lacZYA-kan* transcriptional fusion allows us to assay *pilA* promoter orientations by colony phenotype on lactose MacConkey agar plates. Cells with the promoter in the on orientation form red colonies, and cells with the promoter in the off orientation form white colonies (29). *E. coli pilA'-lacZYA-kan* strains containing mutant *hns* alleles form pink colonies on lactose MacConkey plates. In these strains the *pilA* promoter inversion rate is so rapid that their colonies are composed of an approximately equal mixture of cells with the *pilA* promoter in each orientation (18). Thus, every colony generated by these strains contains a mixed population of Lac$^+$ and Lac$^-$ cells. The *hns-1* allele confers the rapid inversion phenotype at 30 but not 42°C because of a mutation in the *hns* promoter sequence (18, 36). Consequently, all assays were performed at 30°C. *E. coli* ORN132 (*pilA'-lacZYA-kan hns-1*) was subjected to UV mutagenesis and then screened for reduced *pilA* promoter inversion frequency phenotype by looking for red or white colonies on lactose MacConkey plates at 30°C. We isolated one such mutant, termed THK35, with reduced *pilA* promoter inversion frequency at 30°C (Table 2). Using P1 *vir* cotransduction analysis, we found that the lesion in this mutated strain did not map to either the *hns* or the *pilA* region of the chromosome. The allele defining the *hns-1* compensatory phenotype was called *hscA1* since this gene encodes a heat shock cognate (see results below). We found that *hscA1* did not suppress the rapid *pilA* promoter inversion conferred by the *hns* insertion mutant allele *hns-2-tetR* (data not shown). Also, the *hscA1* mutation did not affect promoter inversion in a wild-type *hns* strain (data not shown).

The *hscA1* allele suppresses the effect of *hns-1* on *bglGFB* but not *proU* expression. The *bglGFB* operon, which encodes products necessary for metabolizing salacin, is normally cryptic in *E. coli*. Mutations in *hns* result in activation of *bglGFB* expression (7). Strains containing an activated *bgl* operon were detected phenotypically on L agar containing the colorimetric indicator 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (X-Gluc). Under these conditions, strains with activated *bgl* will hydrolyze the X-Gluc and form blue colonies, whereas nonactivated strains form white colonies. As reported previously, *hns-1* mutant strains demonstrated activated *bgl* (blue colonies on X-Gluc) at 30 but not 42°C (white colonies) (18). Strains containing both the *hns-1* and *hscA1* alleles together or the *hscA1* allele alone had repressed or deactivated *bgl* expression.

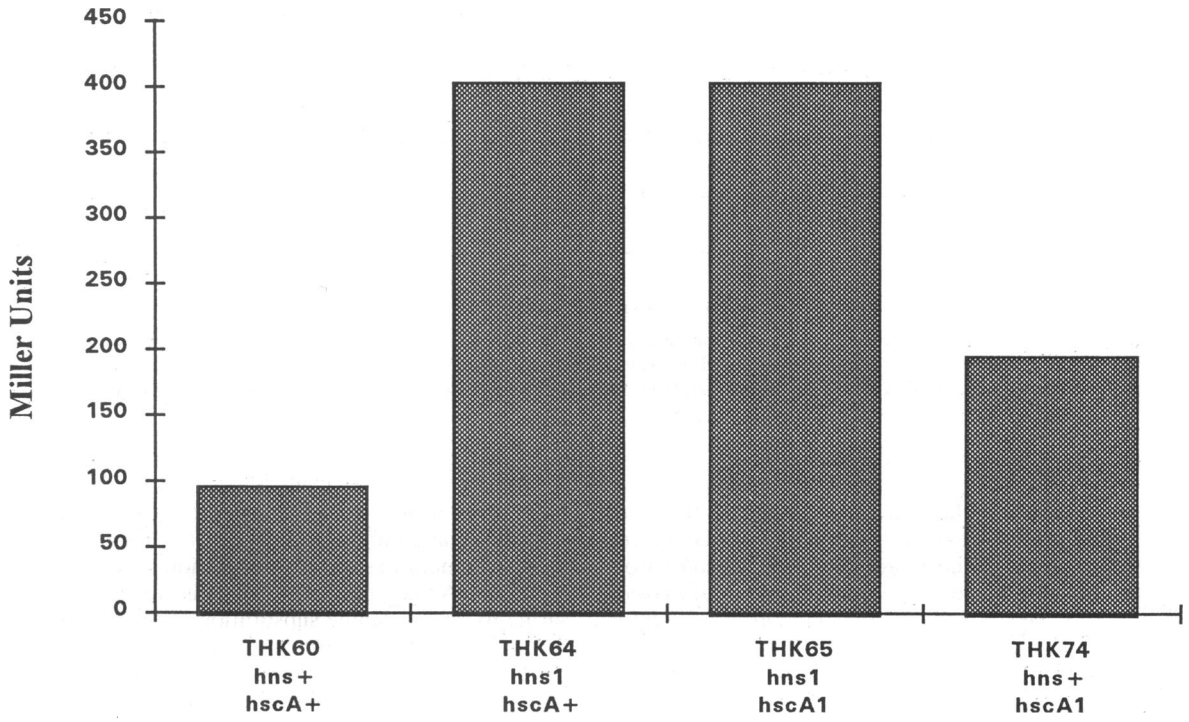

FIG. 1. Effect of *hscA* mutant alleles on *proU* expression. Strains with the indicated relevant genotypes and containing a *proU-lacZ* transcriptional fusion were grown to mid-log phase at 30°C and then assayed for levels of β-galactosidase as described previously (27). β-Galactosidase levels are expressed as Miller units. The data shown represent the average of results from three independent experiments.

Therefore, *hscA1* compensated for the *hns-1* mutant phenotype with regard to *bgl* activation.

The *proU* operon is normally repressed under conditions of low salt but is induced under conditions of increased osmolarity (25). Various mutant *hns* alleles result in *proU* expression even under low-salt conditions (15, 16). Expression of *proU* was measured by using a *proU'-lacZYA-kan* transcriptional fusion (25). This fusion was moved via P1 transduction into strains with different *hns* and *hscA* alleles (Table 1). As expected, the *hns-1* allele derepressed *proU* expression (Fig. 1), which was measured by β-galactosidase assays performed during mid-log growth at 30°C in each of strains tested. In the absence of osmotic induction, *proU* expression was minimal in the wild-type *hns* strain and derepressed in both the *hns-1* and *hns-1 hscA1* strains. Consequently, *hscA1* did not compensate for *proU* derepression in *hns-1* mutants. Interestingly, *proU* was moderately derepressed in the *hns hscA1* strain. This increase, though small, was consistent and reproducible.

Mapping and cloning the *hscA* allele. To map *hscA*, we first isolated a strain with Tn*10* 20% linked to the *hscA1* allele (THK51) as determined by P1 cotransduction frequencies (see Materials and Methods). Cloned DNA containing the intact *tetR* gene plus flanking chromosomal DNA from this strain was used to probe the Kohara clone miniset (20) (Takara Biomedicals), which represents the entire *E. coli* chromosome. The cloned DNA hybridized to Kohara λ phage clones 432 and 433, corresponding to approximately 55 min on the *E. coli* chromosome (21). We then used the Tn*10* location in THK51 to more accurately map *sinA*. Strains containing Tn*10* at 54, 54.75, and 55.75 min (Table 1) were used as donors to cross the transposons into the tetracycline-sensitive *hscA1* mutant strain THK47 via P1 transduction. The cotransduction frequencies for *hscA1* and the introduced transposons were determined (Fig. 2) by transducing a *pilA'-lacZYA-kan hns-1* recipient, selecting for tetracycline resistance, and scoring for a reduced *pilA* promoter inversion rate. This linkage assessment allowed us to position the *hscA* allele between *gua-26*::Tn*10* and *zff-208*::Tn*10*. Thus, *hscA* was at approximately 54.5 min (2) on the *E. coli* chromosome.

We obtained a set of Kohara λ phage clones encompassing the *E. coli* chromosome region determined to contain *hscA* (clones 427 to 433 [20]). Each of these Kohara phages was used to infect strain THK47 (*pilA'-lacZYA-kan hns-1 hscA1*). Infected cells were assessed for conversion from the mutant to the wild-type *hscA* phenotype on lactose MacConkey plates. Kohara clone 430 converted the *hscA1* allele to wild type in strain THK47, presumably by allelic exchange. Thus, we surmised that *hscA* was contained on the phage 430 insert. A 5.4-kb *Eco*RI fragment from this phage 430 insert cloned into pUC19 (pTHK200, Fig. 3) suppressed the mutant *hscA1* phenotype in *trans*. Further subcloning and deletion analysis of pTHK200 allowed us to isolate a 3.4-kb region (pTHK201) that suppressed the mutant *hscA1* phenotype (Fig. 3). We identified two ORFs in this DNA segment which were arranged in tandem and separated by 16 bases (Fig. 4). There were no identifiable promoter sequences preceding the second ORF. The first ORF was preceded by a promoter sequence with significant homology to *E. coli* promoters requiring the alternate σ^{32} RNA polymerase transcription factor (6) (Fig. 4). Consequently, it is likely that these two genes are part of a single operon that may be induced in response to stress. Deleting the 5′ end of the first ORF had no effect on the ability of the remaining cloned DNA, pTHK205, to suppress *hscA1*. Also, a pTHK200 derivative, pTHK300 (Fig. 2), containing a chloramphenicol resistance gene (*cat*) inserted into a unique *Hpa*I site in the second ORF (*hscA2-cat*) did not suppress the

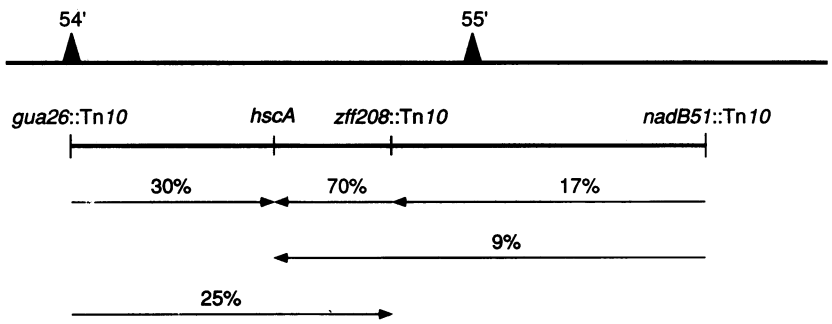

FIG. 2. Chromosomal location of the *hscA* locus as determined by P1 cotransduction. Cotransduction frequencies are expressed as percentages above the arrows. The arrows point towards the unselected marker. Cotransduction frequencies between two designated Tn*10* insertions were determined previously where the unselected Tn*10* marker had been replaced by Tn*10-kan* (34). The chromosome map positions are indicated in minutes on the line above the cotransduction frequencies.

mutant *hscA1* phenotype. Therefore, we concluded that the second ORF represented the *hscA* allele. When the *hscA2-cat* insertion mutant allele was introduced into the *E. coli* chromosome (Fig. 3), it conferred the same phenotypes described for the *hscA1* allele. That is, *hscA2-cat* resulted in reduced *pilA* promoter inversion frequency in an *hns-1* background, yet *proU* expression was derepressed independent of the *hns* genotype.

The *hscA* allele encodes an Hsp70 protein. A number of interesting features were revealed by analyzing the two ORFs, and the predicted gene products, present on pTHK201. The first ORF, which was not required to suppress *hscA1*, was predicted to encode a 21-kDa protein product. By data base screening, this product showed limited similarity to several DnaJ-related proteins. Multiple alignment analysis (33) revealed that the protein product contained the most highly conserved tetrapeptide Y-H-P-D within a 40-amino-acid-residue block conserved in the DnaJ superfamily (21a, 43).

The larger ORF, which was located distal to the first ORF and required to suppress *hscA1*, was predicted to encode an approximately 66-kDa protein product. Plasmid pTHK205 (Fig. 3), which suppressed the *hscA1* phenotype and contained only the larger ORF, expressed this 66-kDa protein in a coupled in vitro transcription-translation assay (Fig. 5). This product was not made by the pTHK300 plasmid containing the *hscA2-cat* insertion mutation (data not shown). The *hscA* predicted product amino acid sequence had 42% identity and 62% similarity to that of the well-described DnaK Hsp70 protein of *E. coli*, and it contained perfect matches to two amino acid sequences that are conserved among all of the known Hsp70 proteins (Prosite data base). These sequences are the octapeptides I-D-L-G-T-T-N-S, beginning at amino acid position 29, and D-L-G-G-G-T-F-D, beginning at position 212 of the *hscA* gene product (Fig. 4). The relative positions of these two sequences in the *hscA* product are also consistent with the positioning of these sequences in the characterized Hsp70 proteins. The second of these octapeptides is thought to form a part of the ATP-binding domain of Hsp70 proteins (11). In the *E. coli* Hsp70 protein DnaK, the threonine located in this octapeptide (position 199 in DnaK; position 217 in the *hscA* product) can be phosphorylated (26). It has been believed for some time that DnaK was the only Hsp70 protein expressed in *E. coli*. However, our evidence indicates that *hscA* encodes an additional Hsp70 in this organism.

Repeated attempts to clone the intact *hscA1* allele failed. Consequently, to determine the nature of the *hscA1* mutation, we sequenced PCR-amplified DNA corresponding to the *hscA* allele from a mutant *hscA1* strain (see Materials and Methods). The only difference between the wild-type and mutant *hscA* sequences was a C-to-T transition at position 1290 in the *hscA1* DNA sequence (Fig. 4). This transition resulted in a serine-to-phenylalanine substitution in the *hscA* product. This serine is conserved in Hsp70 proteins and is located 2 amino acids away from the ATP-binding octapeptide described above. Although we do not have direct experimental evidence that the *hscA* product binds ATP, a phenylalanine substitution so near or within an ATP-binding domain might change the conformation of this region of the protein, thereby dramatically altering its ability to bind or hydrolyze ATP.

DISCUSSION

We have identified a gene, *hscA*, encoding a new Hsp70 in *E. coli*. Both *hscA* mutant alleles that we studied (*hscA1* and *hscA2-cat*) will compensate for a subset of phenotypes associated with *hns* mutations in *E. coli*. The *hscA* allele was initially identified by a mutation conferring reduced *pilA* promoter inversion frequency in an *hns-1* mutant. This mutation also suppressed the effect of *hns-1* on *bgl* activation. Further analysis revealed that this second-site mutation did not suppress the effect of *hns-1* on *proU* expression. Paradoxically, mutations in *hscA* moderately derepressed *proU* expression in wild-type *hns* strains.

We identified the location of a Tn*10* insertion linked to *hscA1* by hybridizing DNA flanking the transposon to the Kohara phage miniset. We then mapped *hscA* more accurately using a series of Tn*10* insertions surrounding the region identified by the Southern hybridizations and used this information to select a minimal number of Kohara phage clones to test for the presence of the wild-type *hscA* allele. We converted the mutant *hscA1* allele to wild type using Kohara phage 430. An approximately 3-kb DNA segment cloned from this phage, pTHK201, suppressed the *hscA1* phenotype in *trans*. The DNA insert on pTHK201 contained two ORFs arranged in tandem and preceded by a potential σ^{32} promoter. Both ORFs were expressed in an in vitro coupled transcription-translation assay. We could not identify a good σ^{70} promoter within 300 bases proximal to the first ORF on the pTHK200 insert. However, there is a poor potential σ^{70} promoter located immediately 5′ to the identified σ^{32} promoter sequences. It is likely that the cloned genes were expressed in vitro from this poor promoter, a plasmid promoter, or there was enough σ^{32} present in the S-30 extract to allow expression from the σ^{32} promoter. Expression of the two ORFs should be increased under

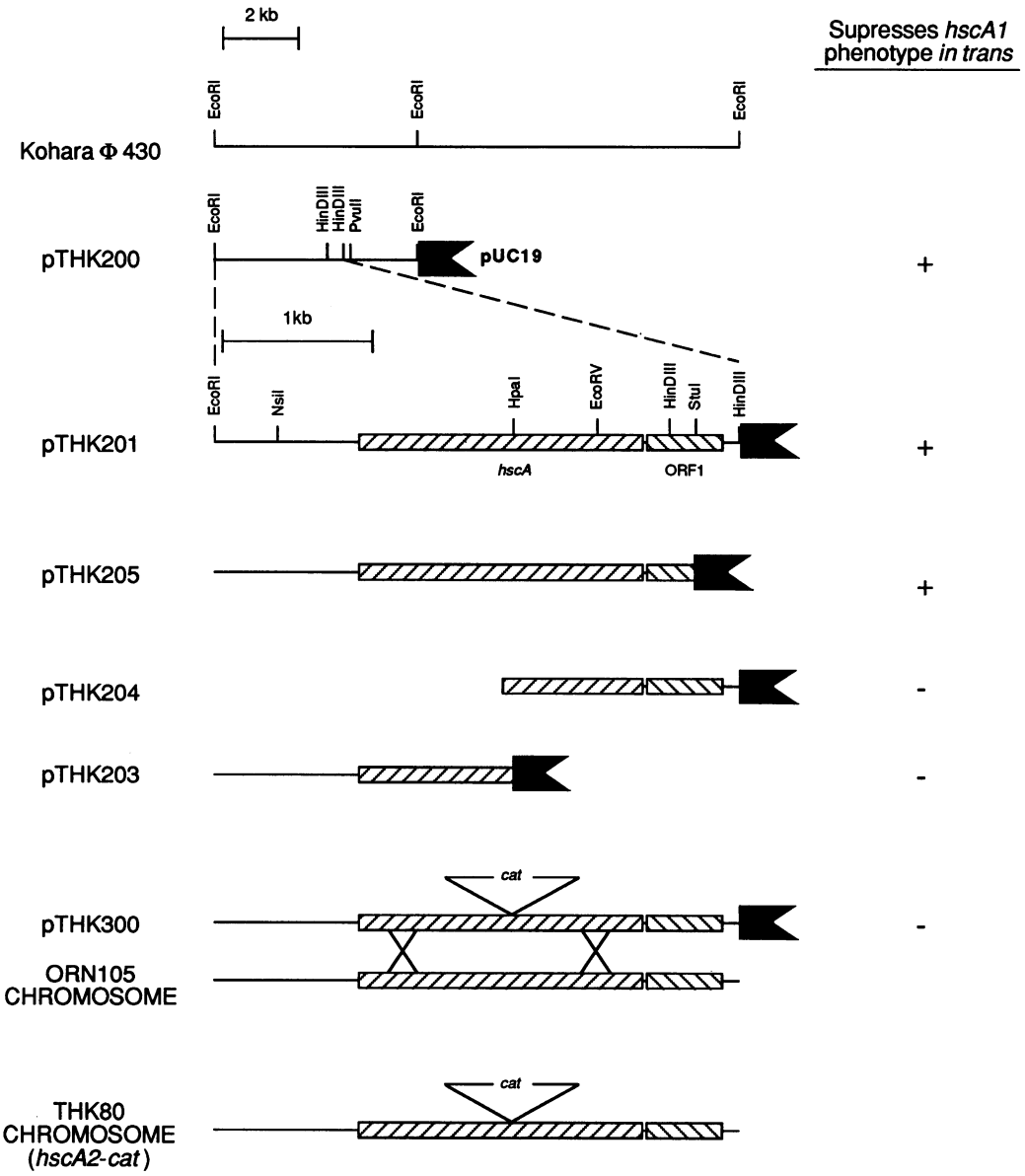

FIG. 3. Cloning the *hscA* allele and construction of the chromosomal *hscA2-cat* allele. The Kohara phage 430 insert is oriented clockwise with respect to the *E. coli* chromosome map. The plasmid clone pTHK200 contains a 5.4-kb DNA insert from Kohara phage 430 which suppressed the *hscA1* phenotype. Phage, plasmid, and strain designations are given in the left column. The phenotype of each construct with respect to the ability to suppress *hscA1* in *trans* is indicated in the column on the right. Vector sequences are designated by solid boxes, and the ORFs determined from the DNA sequence are designated by the boxes containing slanted lines. The transcription direction of ORF1 and *hscA* is from right to left, which is counterclockwise with respect to the *E. coli* chromosome map. A *cat* gene (encoding chloramphenicol acetyltransferase) was inserted in a unique *Hpa*I site located 857 bases downstream from the *hscA* initiation codon. The *hscA2-cat* insertion mutation was introduced into the *E. coli* chromosome via linear transformation into strain ORN105 (*recBC sbcB*), generating strain THK80.

conditions in which σ^{32} is more available in the cell, e.g., heat shock. However, we have not yet been able to detect *hscA* transcripts in mRNA prepared from cells prior to or following heat shock. The genetic evidence indicating that *hscA* is expressed in vivo is compelling: (i) two different mutations in the *hscA* gene had direct phenotypic effects on cells grown at 30°C, and (ii) a cloned wild-type *hscA* gene suppressed the mutant *hscA1* phenotype in *trans*. Our inability to detect *hscA* mRNA in *E. coli* may simply reflect that *hscA* is expressed poorly under both normal and heat shock conditions.

The second of the two ORFs identified on pTHK201 encoded a 66-kDa protein that was sufficient to suppress the *hscA1* phenotype in *trans*. The amino acid sequence of this protein has significant homology to the strongly conserved Hsp70 protein family. This homology includes perfect alignment to the two identified signature octapeptides found in all Hsp70s to date. These data suggest that *hscA* encodes an Hsp70 protein and is a part of a bicistronic stress response operon in which the *hscA* gene is preceded by another ORF. The predicted product of this proximal ORF contains a block of amino acids with significant homology to the DnaJ family of stress response proteins. The gene encoding the other known

```
   1  AAGCTTCCACGTTTGATGCGCATACCGATAACCCCCACCGTGGTCGCCTGCGCGTGGGGTTTGTTTTACCTGATTCGCCGTTATCGCGGCGGATCGCAGCC

 101  CTGAGAATGTTATGGATTACTTCACCCTCTTTGGCTTGCCTGCCCGCTATCAACTCGATACCCAGGCGCTGAGCCTGCGTTTTCAGGATCTACAACGTCA
                M  D  Y  F  T  L  F  G  L  P  A  R  Y  Q  L  D  T  Q  A  L  S  L  R  F  Q  D  L  Q  R  Q

 201  GTATCATCCTGATAAATTCGCCAGCGGAAGCCAGGCGGAACAACTCGCCGCCGTACAGCAATCTGCAACCATTAACCAGGCCTGGCAAACGCTGCGTCAT
       Y  H  P  D  K  F  A  S  G  S  Q  A  E  Q  L  A  A  V  Q  Q  S  A  T  I  N  Q  A  W  Q  T  L  R  H

 301  CCGTTAATGCGCGCGGAATATTTGCTTTCTTTGCACGGCTTTGATCTCGCCAGCGAGCAGCATACTGTGCGCGACACCGCGTTCCTGATGGAACAGTTGG
       P  L  M  R  A  E  Y  L  L  S  L  H  G  F  D  L  A  S  E  Q  H  T  V  R  D  T  A  F  L  M  E  Q  L  E

 401  AGCTGCGCGAAGAGCTGGACGAGATCGAACAGGCGAAAGATGAAGCGCGGCTGGAAAGCTTTATCAAACGTGTGAAAAAGATGTTTGATACCCGCCATCA
        L  R  E  E  L  D  E  I  E  Q  A  K  D  E  A  R  L  E  S  F  I  K  R  V  K  K  M  F  D  T  R  H  Q

 501  GTTGATGGTTGAACAGTTAGACAACGAGACGTGGGACGCGGCGGCGGATACCGTGCGTAAGCTGCGTTTTCTCGATAAACTGCGAAGCAGTGCCGAACAA
       L  M  V  E  Q  L  D  N  E  T  W  D  A  A  A  D  T  V  R  K  L  R  F  L  D  K  L  R  S  S  A  E  Q

 601  CTCGAAGAAAAACTGCTCGATTTTTAATTTCTGGAAGCTAAACATGGCCTTATTACAAATTAGTGAACCTGGTTTGAGTGCTGCGCCGCATCAGCGTCGT
       L  E  E  K  L  L  D  F  *                    M  A  L  L  Q  I  S  E  P  G  L  S  A  A  P  H  Q  R  R

 701  CTGGCGGCCGGTATTGACCTGGGCACAACCAACTCGCTGGTGGCGACAGTGCGCAGCGGTCAGGCCGAAACGTTAGCCGATCATGAAGGCCGTCACCTGC
       L  A  A  G  [I  D  L  G  T  T  N  S]  L  V  A  T  V  R  S  G  Q  A  E  T  L  A  D  H  E  G  R  H  L  L

 801  TGCCATCTGTTGTTCACTATCAACAGCAAGGGCATTCGGTGGGTTATGACGCGCGTACTAATGCAGCGCTCGATACCGCCAACACAATTAGTTCTGTTAA
       P  S  V  V  H  Y  Q  Q  Q  G  H  S  V  G  Y  D  A  R  T  N  A  A  L  D  T  A  N  T  I  S  S  V  K

 901  ACGCCTGATGGGACGCTCGCTGGCTGATATCCAGCAACGCTATCCGCATCTGCCTTATCAATTCCAGGCCAGCGAAAACGGCCTGCCGATGATTGAAACG
       R  L  M  G  R  S  L  A  D  I  Q  Q  R  Y  P  H  L  P  Y  Q  F  Q  A  S  E  N  G  L  P  M  I  E  T

1001  GCGGCGGGGCTGCTGAACCCGGTGCGCGTTTCTGCGGACATCCTCAAAGCACTGGCGGCGCGGGCAACTGAAGCCCTGGCAGGCGAGCTGGATGGTGTAG
       A  A  G  L  L  N  P  V  R  V  S  A  D  I  L  K  A  L  A  A  R  A  T  E  A  L  A  G  E  L  D  G  V  V

1101  TTATCACCGTTCCGGCGTACTTTGACGATGCCCAGCGTCAGGGCACCAAAGACGCGGCGCGTCTGGCGGGGCCTTCACGTCCTGCGCTTACTTAACGAACC
        I  T  V  P  A  Y  F  D  D  A  Q  R  Q  G  T  K  D  A  A  R  L  A  G  L  H  V  L  R  L  L  N  E  P
                                                                                                  <T>
1201  GACCGCTGCGGCTATCGCCTACGGGCTGGATTCCGGTCAGGAAGGCGTGATCGCCGTTTATGACCTCGGTGGCGGGACGTTTGATATTTCCATTCTGCGC
       T  A  A  A  I  A  Y  G  L  D  S  G  Q  E  G  V  I  A  V  Y  [D  L  G  G  G  T  F  D]  I  (S)  I  L  R

1301  TTAAGTCGCGGCGTGTTTGAAGTGCTGGCAACCGGCGGTGATTCCGCGCTCGGCGGCGATGATTTCGACCATCTGCTGGCGGATTACATTCGCGAGCAGG
       L  S  R  G  V  F  E  V  L  A  T  G  G  D  S  A  L  G  G  D  D  F  D  H  L  L  A  D  Y  I  R  E  Q  A

1401  CGGGCATTCCTGATCGTAGCGATAACCGCGTTCAGCGTGAACTGCTGGATGCCGCCATTGCAGCCAAAATCGCGCTGAGCGATGCGGACTCCGTGACCGT
        G  I  P  D  R  S  D  N  R  V  Q  R  E  L  L  D  A  A  I  A  A  K  I  A  L  S  D  A  D  S  V  T  V

1501  TAACGTTGCGGGCTGGCAGGGCGAAATCAGCCGTGAACAATTCAATGAACTGATCGCGCCACTGGTAAAACGAACCTTACTGGCTTGTCGTCGCGCGCTG
       N  V  A  G  W  Q  G  E  I  S  R  E  Q  F  N  E  L  I  A  P  L  V  K  R  T  L  L  A  C  R  R  A  L

1601  AAAGACGCGGGTGTAGAAGCTGATGAAGTGCTGGAAGTGGTGATGGTGGGCGGTTCTACTCGCGTGCCGCTGGTGCGTGAACGGGTAGGCGAATTTTTCG
       K  D  A  G  V  E  A  D  E  V  L  E  V  V  M  V  G  G  S  T  R  V  P  L  V  R  E  R  V  G  E  F  F  G

1701  GTCGTCCACCGCTGACTTCCATCGACCCGGATAAAGTCGTCGCTATTGGCGCGGCGATTCAGGCGGATATTCTGGTGGGTAACAAGCCAGACAGCGAAAT
        R  P  P  L  T  S  I  D  P  D  K  V  V  A  I  G  A  A  I  Q  A  D  I  L  V  G  N  K  P  D  S  E  M

1801  GCTGTTGCTTGATGTGATCCCACTGTCGCTGGGCCTCGAAACGATGGGCGGCCTGGTGGAGAAAGTGATTCCGCGTAATACCACTATTCCGGTGGCCCGC
       L  L  L  D  V  I  P  L  S  L  G  L  E  T  M  G  G  L  V  E  K  V  I  P  R  N  T  T  I  P  V  A  R

1901  GCTCAGGATTTCACCACCTTTAAAGATGGTCAGACGGCGATGTCTATCCATGTAATGCAGGGTGAGCGCGAACTGGTGCAGGACTGCCGCTCACTGGCGC
       A  Q  D  F  T  T  F  K  D  G  Q  T  A  M  S  I  H  V  M  Q  G  E  R  E  L  V  Q  D  C  R  S  L  A  R

2001  GTTTTGCGTGCGTGGTATTCCGGCGCTACCGGCTGGCCGGTGCGCATATTCGCGTGACGTTCCAGGTCGATGCCGACGGTCTTTTGAGCGTGACGGCGAT
        F  A  C  V  V  F  R  R  Y  R  L  A  G  A  H  I  R  V  T  F  Q  V  D  A  D  G  L  L  S  V  T  A  M

2101  GGAGAAATCCACCGGCGTTGAGGCGTCTATTCAGGTCAAACCGTCTTACGGTCTGACCGATAGCGAAATCGCTTCGATGATCAAAGACACAATGAGCTAT
       E  K  S  T  G  V  E  A  S  I  Q  V  K  P  S  Y  G  L  T  D  S  E  I  A  S  M  I  K  D  T  M  S  Y

2201  GCCGAGCAGGACGTAAAAGCCCGAATGCTGGCAGAACAAAAAGTAGAAGCGGCGCGTGTGCTGGAAAGTCTGCACGGCGCGCTGGCTGCTGATGCCGCGC
       A  E  Q  D  V  K  A  R  M  L  A  E  Q  K  V  E  A  A  R  V  L  E  S  L  H  G  A  L  A  A  D  A  A  L

2301  TGTTAAGCGCAGAACGTCAGGTCATTGACGATGCTGCCGCTCACCTGAGTGAAGTGGCGCAGGGCGATGATGTTGACGCCATCGAACAAGCGATTAAAAA
        L  S  A  E  R  Q  V  I  D  D  A  A  A  H  L  S  E  V  A  Q  G  D  D  V  D  A  I  E  Q  A  I  K  N

2401  CGTAGACAAACAAACCCAGGATTTCGCCGCTCGCCGCATGGACCAGTCGGTTCGTCGTGCGCTGAAAGGCCATTCCGTGGACGAGGTTTAATATGCCAAA
       V  D  K  Q  T  Q  D  F  A  A  R  R  M  D  Q  S  V  R  R  A  L  K  G  H  S  V  D  E  V  *

2501  GATTGTTATTTTGCCTCATCAGGATCTCTGCCCTGATGGCGCTGTTCTGGAAGCTAATAGCGGTGAAACCATTCTCGACGCAGCTCTGCGTAACGGTATC

2601  GAGATTGAACACGCCTGTGAAAAATCCTGTGCTTGCACCACCTGCCACTGCATCGTTCGTGAAGGTTTTGACTCACTGCAGGAAAGCTCAGAGCACCAAG

2701  ACGACATGCTGAACAAAGCCAGGGGACTGGAGCCGGAAAGCCGTTTAAGCTGCCAGGCGCGCAGTTACCACGAAGATTTAGTAGTCGAAATCCCGCGTTA

2801  CACTATCAACCATGCGCGTGAGCATTAACAGAGGTTAGTATGGGACTTAAGTGGACCGATAGCCGCGAAATTGGCGAAGCACTGTACGATGCGTATCCCG

2901  ATCTTGATCCGAAAACGGTTCGATTCACCGATATGCATCAGTGGATTTGCGATCTGGAAGATTTCGACCGGACCGACCCAGGCATCCCAACGAAGAAAAT
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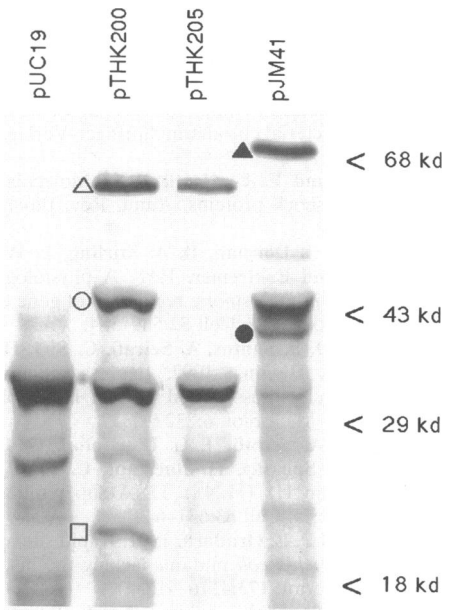

FIG. 5. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis of translation products from *hscA*-containing clones. The plasmids designated above each lane were used as DNA templates in coupled in vitro transcription-translation reactions (see Materials and Methods) containing [^{35}S]methionine to radiolabel protein products. Translation products were electrophoresed on an SDS–12.5% polyacrylamide gel which was subsequently dried and subjected to autoradiography. Plasmid pTHK200 expressed three proteins of ca. 21 (open square), 45 (open circle), and 66 (open triangle) kDa not present in the pUC19 vector control reaction. Plasmid pTHK205, which was sufficient to suppress *hscA1* in *trans*, expressed only the 66-kDa protein. The 21-kDa product expressed by pTHK200 was encoded by the ORF immediately preceding the gene encoding the 66-kDa protein. The additional 45-kDa protein expressed by pTHK200 was encoded by the 2.0-kb DNA segment of this clone that we did not sequence. Plasmid pJM41 containing the *dnaKJ* operon expressed the 69-kDa DnaK (closed triangle) and 41-kDa DnaJ (closed circle) proteins.

Hsp70 in *E. coli* (DnaK) is also part of a bicistronic stress response operon which contains the *dnaJ* gene encoding DnaJ (28). However, in this operon the *dnaJ* gene is located distal to *dnaK*.

The 3′ end of the *hscA* gene was previously sequenced by Ta and Vickery as DNA flanking the *fdx* gene in *E. coli* (37). Since writing this report we have learned that the *hscA* gene has subsequently been sequenced, expressed, and identified as *hsc* by this same group (33a).

The *hscA1* allele, which suppressed rapid *pilA* promoter inversion in *hns-1 E. coli*, contained a C-to-T transition in a serine codon that is conserved in Hsp70 proteins (our comparison of Hsp70 amino acid sequences), resulting in a phenylalanine in place of the conserved serine. The location is as informative as the nature of the mutation defining the *hscA1* allele. The serine-to-phenylalanine conversion occurred only 2 amino acids from one of the conserved octapeptides that is in the region known to be involved in ATP binding by Hsp70 proteins (11, 39) and 4 amino acids C terminal to the threonine that can be phosphorylated in *E. coli* DnaK (26). We have yet to show experimentally that the *hscA* product binds ATP. However, we hypothesize that, by analogy to other Hsp70 proteins such as DnaK, the serine-to-phenylalanine exchange could be blocking either ATP binding, hydrolysis, or both by the *hscA1* product. While it is clear that Hsp70 proteins bind and hydrolyze ATP, the functional ramifications of this particular activity on the biological properties of Hsp70s are not totally understood. Mutants deficient in DnaK ATP binding have phenotypes that closely approximate those associated with *dnaK* null mutations (39). Strains containing deletion and insertion mutations in *dnaK* are deficient in cell division and fail to grow at 42°C (5, 31). Strains containing a *hscA2-cat* insertion mutant allele on the chromosome grew more slowly than wild-type *hscA* strains but were morphologically normal and viable at 42 as well as 30°C. Consequently, the effect of this mutation was not nearly as severe as *dnaK* insertion or deletion mutations are on cell morphology and growth. It is interesting to note that we were able to maintain a cloned *hscA2-cat* insertion mutation on a high-copy-number vector but have been unable to clone the *hscA1* mutant allele. It is possible that the *hscA1* mutation confers a dominant lethal phenotype when present at a high copy number, even in the presence of a chromosomally located wild-type allele. There is precedence for this type of mutation in which a dominant lethal phenotype was conferred by a mutation in the region of the *dnaK* gene encoding the ATP-binding domain of this protein (39).

The question of how mutations in *hscA* compensate for a subset of *hns-1* mutant phenotypes remains. The mutation defining the *hns-1* allele is located in the −30 region of the *hns* promoter (18). This mutation likely results in below-normal levels of H-NS expression, thus conferring the mutant phenotypes. It is conceivable that *hscA* mutations compensate for *hns-1* by effectively raising the amount of available H-NS. This hypothesis is consistent with our observation that *hscA* mutations do not compensate for an *hns* insertion mutation, *hns-2-tetR*. However, this possible mechanism does not adequately explain the fact that *hscA* mutations not only fail to suppress the effect of *hns-1* on *proU* derepression but also effectively derepress *proU* expression in wild-type *hns* strains. Since the *hscA* product is an Hsp70 protein, models explaining the activity of *hscA* must account for both our observed mutant phenotypes and the biological properties of Hsp70 proteins. These proteins act as chaperones, binding nascent polypeptides and aggregated proteins to promote or restore proper protein folding (reviewed in reference 14). It has been demonstrated that H-NS may be present in the cell in at least two different isoforms (35). It is possible that these two H-NS forms have different biological activities. Perhaps one form is active in inhibiting *pilA* promoter inversion, while the other may function to inhibit *proU* expression. The *hscA* product could function to influence the final conformation of H-NS towards that inhibiting *proU* expression. In this case, the lack of the *hscA* product in the cell would effectively increase the amount of the H-NS form active in inhibiting *pilA* promoter

FIG. 4. Nucleotide sequence of the *hscA* allele and flanking DNA. The *hscA* sequence was determined by the dideoxynucleotide method as described in Materials and Methods. Only the sense strand is shown along with the single-letter amino acid designations for the two ORFs. The possible σ^{32}-dependent promoter sequence preceding the two ORFs is underlined. The two octapeptide amino acid sequences corresponding to the Hsp70 signature motifs are bracketed in the translated second ORF (*hscA*). The mutation creating the *hscA1* allele is indicated by the <T> placed above the C that it replaced at position 1290. The resulting phenylalanine-for-serine substitution is designated by the (S) corresponding to the mutated codon.

inversion while decreasing the amount available to inhibit *proU* expression. We are currently testing this hypothesis by assessing the different H-NS forms found in *hscA* mutant strains.

H-NS has many intriguing properties, such as its role in nucleoid structure, its effects on DNA topology and gene expression, and its possible activity as a stress response protein. Mutations in the gene encoding H-NS result in constitutive expression or activation of a number of genes typically subject to some form of environmental regulation. Our isolation of a second-site mutation that compensates for only a subset of *hns* mutant phenotypes provides the first genetic evidence that the biological activities of H-NS may be modulated by other cellular components. This compensatory mutation is located in a gene encoding a new Hsp70, suggesting a dynamic interaction between H-NS and a possible global stress response regulon component, thus providing a mechanism for directing the biological activities of this multifunctional protein.

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