Isolation, Characterization, and Sequence of an *Escherichia coli* Heat Shock Gene, *htpX*

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Received 30 October 1990/Accepted 27 February 1991

We isolated and characterized a new *Escherichia coli* gene, *htpX*. The *htpX* gene has been localized at min 40.3 on the chromosome. We determined its transcription and translation start site. *htpX* expresses a 32-kDa protein from a monocistronic transcript; expression of this protein is induced by temperature upshift. *htpX* is expressed from a σ23-dependent promoter and is thus part of the heat shock regulon. Cells carrying a *htpX* gene disruption grow well at all temperatures and under all conditions tested and have no apparent phenotype. However, cells which overexpress a truncated form of the protein display a higher rate of degradation of puromycyl peptides.

The heat shock response consists in a transient increase, after temperature upshift, in the synthesis of a small subset of proteins, the heat shock proteins (hsp). This response has been observed in all cells so far examined (reviewed in reference 31). In *Escherichia coli*, temperature upshift, as well as other treatments such as UV irradiation, bacteriophage infection, and overproduction of abnormal proteins, leads to the preferential synthesis of at least 17 hsp (reviewed in reference 38). The central regulator of the heat shock response in *E. coli* is the heat shock gene-specific RNA polymerase subunit, σ23 (22, 23).

Some hsp have been highly conserved throughout evolution. Three *E. coli* hps, GroEL, DnaK, and C62.5, are homologous to the mitochondrial HSP70 protein and to the eukaryotic HSP70 and HSP90 protein families, respectively (5, 6, 35). In contrast, the small hsp, although often conserved within the same organism, display little similarity between different organisms (32).

GroE is involved in the assembly of multiprotein complexes (15, 20), and DnaK, DnaJ, and GrpE have been shown to participate in the refolding of denatured proteins (14). In addition, strains carrying mutations in these genes are defective in the proteolysis of abnormal proteins (48). The Lop protease, which degrades abnormal proteins in the cell (21, 34), is a heat shock protein as well (16). A common denominator of most conditions that induce the synthesis of the hsp is thought to be the appearance of unfolded proteins in the cell (4, 17, 41). It is possible that the primary role of the heat shock response is to remove unfolded proteins from the cell, either by refolding or by degrading them.

The lysogenization process of bacteriophage λ involves several phage proteins, such as the CI transcriptional activator, which is required for the initiation of transcription of the repressor and integrase genes (45), and the CI protein, whose role is to stabilize CI (3, 24). Several host genes, e.g., mrc (2) and hsf (7), are involved in this process as well. We were interested in isolating additional host genes participating in the regulation of phage lysogenization.

A screening procedure aimed at isolating such genes from an *E. coli* phage library yielded several clones; one of these clones was characterized further and found to carry a new *E. coli* heat shock gene, designated *htpX*. Overexpression of a truncated form of the HtpX protein does lead to an increase in the degradation of abnormal proteins. However, disruption of the chromosomal copy of this gene does not affect cell viability, nor does it affect the plaque morphology of an infecting phage. The function of HtpX in the cell therefore remains unknown.

MATERIALS AND METHODS

**Strains.** The *E. coli* and phage strains used are described in Table 1.

**Media.** Bacteria were propagated in LB medium containing 40 μg of ampicillin per ml. LBMM is LB plus 0.2% (wt/vol) maltose and 10 mM MgSO₄. TM is 10 mM Tris (pH 7.4) plus 10 mM MgSO₄. Phages were plated on TB plates. For protein labeling, the cells were grown in M56 minimal medium supplemented with 0.5% (vol/vol) glycerol and 20 μg of ampicillin per ml.

**Plasmid and phage constructions.** Standard cloning procedures were as described previously (33). λPR6 was isolated from an *E. coli* library, constructed by introducing fragments from a partial *Sac*III digest of the *E. coli* genome into the BamHI site of the λDE9 vector (37). pPR6L contains the 3.5-kb *Bgl*II-*Hind*III fragment of the λPR6 insert cloned in *Hind*III-BamHI-digested pGEM-3 (Promega Biotech); pPR6M contains the 1.5-kb *Acc*II-*Hind*III fragment (after end filled in with Klenow DNA polymerase) of the λPR6 insert cloned in pGEM-3 digested with *Hind*III and *Sal*I; Pr6S contains the 1-kb *Hind*III-*Eco*RI fragment of the λPR6 insert cloned in pGEM-3 digested with the same enzymes. Plasmid pPR6S' was built by introducing the *Bam*HI-*Eco*RI fragment of the λPR6 insert into pGEM-3 digested with the same enzymes; this construct was used to sequence the 70 bp between the *Bam*HI site and the *Hind*III site upstream of the gene. pPR6Cm was built by introducing the cat gene carried on a *Pst*I fragment into the unique *Pst*I site of pPR6S. The disrupted *htpX* gene was transferred to phage λPR6 by recombination in the following way. λPR6 was grown on A5039 cells carrying pPR6Cm. The resulting lysate was used to lysogenize a strain carrying pINT57 (12), which expresses the λ *int* gene (this is necessary since the bacterial insert of λPR6 disrupts the phage *int* gene), and the lysogens were...
selected for chloramphenicol resistance. These lysogens were then pooled and induced with mitomycin C. Phages were screened for chloramphenicol resistance and ampicillin sensitivity. The DNA of one Cm⁺ Ap⁺ phage, APR6Cm, was further analyzed with a set of restriction enzymes to confirm that it carries the cat fragment within the hpx coding sequence.

**Strain construction.** The conjugation and P1 transduction procedures were performed as described by Miller (36).

(i) Transfer of the disrupted hpx gene to the bacterial chromosome. In a first stage, the immunity region of APR6Cm (imm²) was exchanged with the λcI857 immunity region by a regular phage cross (36). W3350 cells were coinfected by both λcI857 and APR6Cm and UV irradiated for 20 sec. The resulting lysate was then propagated on a strain lysogenic for imm² to select against the APR6Cm phage. This lysate was used to lysogenize strain A5039/ pmapC57 in order to select for λcI857 phages carrying the chloramphenicol resistance marker; chloramphenicol-resistant lysogens were heat induced at 42°C to yield a lysate of the recombinant phage, λcI857PR6Cm. In the second stage, this phage was used to lysogenize strain W3350. In the absence of functional int gene product, the phage is unable to integrate in the specific λ attachment site within the bacterial chromosome; stable lysogens are obtained only by homologous recombination between the phage insert and the bacterial chromosome. These stable lysogens were selected by virtue of the chloramphenicol resistance conferred by the phage. Such cells carry two copies of the hpx gene region, one with the original bacterial hpx gene and one with the phage-carried disrupted gene, and the phage genome inserted between these two copies. The lysogens were grown at 42°C to select for loss of the λcI857 phage, which carries a thermolabile repressor. Cells able to grow at 42°C would have lost, by homologous recombination, one of the hpx copies along with the phage genome. Some 10% of these cells were Km⁺, indicating that they were left with the disrupted hpx copy in the cell. This was confirmed by Southern blot analysis (see Fig. 9). The resulting strain, W3350 hpxCmc, was designated A6006.

(ii) Construction of A6007 and A6512. The hpxCmc gene disruption was transferred to A5039 and SG20322, respectively, by P1 transduction from A6006, using the chloramphenicol resistance marker.

(iii) Construction of A5039 and A6007 derivatives. A6515 and A6518 were constructed by transduction of clpA82::ATnJO from SG12049; A6516 and A6519 were constructed by transduction of lon-146::ΔTn10 (34) from SG20322; A6517 and A6520 were constructed by transduction of slp-1::ΔTn10 from JK20000. A6533 and A6536 were constructed by transduction of hflAI::Tn10 from A1964, using the tetracycline resistance marker of Tn10. The strains were tested for transduction of the hflAI mutation linked to the Tn10 marker by observing the plaque morphology of a λ c⁺ phage.

**Analysis of labeled proteins.** (i) In vivo. Cells were grown in supplemented M56 minimal medium at 30°C to an optical density of 0.4 at 600 nm. At the indicated times after the temperature shift to 42°C, 0.2-ml aliquots were pulse-labeled for 1 min with 10 μCi of [35S]methionine, precipitated with 10% (wt/vol) trichloroacetic acid (TCA), and subjected to gel electrophoresis on a 10 to 26% polyacrylamide gradient gel. The amount of protein synthesis was measured by scanning densitometry of the autoradiogram using a Molecular Dynamics 300A densitometer.

(ii) In vitro. DNA of plasmid pPR6L was added to an S30 protein synthesis extract supplied by Amersham as specified by the manufacturer.

**DNA sequencing.** Exonuclease III in conjunction with mung bean nuclease (Stratagene) and restriction enzymes were used to generate a set of nested deletions in the insert of plasmids pPR6S and pPR6M. Primers complementary to the vector were initially used, followed by a set of hpx-specific primers to complete the sequence of both strands. DNA sequence analysis was performed by the dideoxynucleotide chain termination method (44) on supercoiled plasmid DNA prepared as described by Chen and Seeberg (8).

**mRNA analyses.** Total cellular RNA was extracted as described by Sälsér et al. (43). This method yields DNA-free RNA.

(i) Dot blot. The total RNA concentration was determined by measuring the optical density at 260 nm, and decreasing amounts of RNA were bound to nitrocellulose filters (50). As a control, some samples were treated with DNase-free RNase before being loaded on the filters. The filters were probed with the labeled antisense hpx mRNA synthesized from the T7 promoter of HindIII-digested plasmid pPR6S.

(ii) Northern (RNA) blot. The RNA was extracted as described above, glyoxyl treated, electrophoresed, and transferred to nitrocellulose as described by Maniatis et al. (33). The antisense probe described above was used.
(iii) Primer elongation. An end-labeled oligonucleotide primer complementary to nucleotides 81 to 98 of the htxX sequence (see Fig. 3) was annealed to 5 μg of the RNA isolated as described above and then elongated with avian myeloblastosis virus reverse transcriptase (Promega Biotec) as described before (1). The extension products were analyzed on a sequencing gel.

Extension inhibition by the 30S ribosomal subunit. The toeprint assay was done as described previously (1). Briefly, annealing mixtures contained RNA synthesized in vitro from plasmid pPR6S by SP6 RNA polymerase and an oligonucleotide complementary to nucleotides 81 to 98 of the htxX coding sequence. After annealing the 32P-end-labeled oligonucleotide to the RNA, 1 to 2 μM purified E. coli 30S ribosomal subunits (kindly provided by R. Traut) was added to the mixture, followed by fMet-tRNA and 0.5 U of avian myeloblastosis virus reverse transcriptase. The reaction was terminated by the addition of loading dye; the reaction products were analyzed by electrophoresis on a sequencing gel.

In vivo protein degradation experiments. The assay was performed as described previously (19). Cells were grown in M56 minimal medium-0.5% glycerol-0.05% yeast extract at 37°C to an optical density at 600 nm of 0.3 to 0.4. Puromycin (Sigma, St. Louis, Mo.) was added to 80 μg/ml; after 10 min, 3[H]leucine (Amersham) was added to 0.5 μCi/ml. After an additional 10 min, the cells were collected by filtration on a GF/C filter and washed twice with and resuspended in chase medium (growth medium plus penicillin [75 μg/ml]). At the indicated times, 0.5-ml aliquots were removed; bovine serum albumin was added to 0.4 mg/ml, and TCA was added to 5% (wt/vol). The aliquots were left on ice overnight and then centrifuged. A 0.25-ml sample of the supernatant was added to 5 ml of Insta-Gel scintillation liquid (Packard) and counted. The amount of radioactivity at time zero was regarded as background and subtracted from all other values. The total incorporation of [3H]leucine was measured by counting 0.25 ml of the culture before TCA precipitation.

Sequence analyses. The protein hydrophathy profile, database searches, and sequence comparisons were performed by using the University of Wisconsin sequence analysis software package (11).

Nucleotide sequence accession number. The GenBank accession number for the htxX sequence is M58470.

RESULTS

Isolation of the E. coli DNA fragment carrying htxX. To identify bacterial genes involved in the regulation of the lysogenization process of bacteriophage λ, E. coli DNA fragments were cloned into the phage vector λΔ69 (37). The rationale was that a host gene carried by the phage would be overexpressed upon infection, both because of the higher copy number and because of the fact that a gene cloned in the BamHI site of λΔ69 would be transcribed from the strong pL promoter. Thus, a gene interfering with the lysis/lysogenicity decision, if located on the phage, could change the plaque morphology due to its overexpression.

The E. coli λΔ69 library was grown on the bacterial strain W3350, and a number of phages forming clear plaques were isolated. One of these isolates, λPR6, was investigated further by subcloning several overlapping fragments of its bacterial insert into a plasmid vector (pGEM-3) to yield plasmids pPR6L, pPR6M, and pPR6S (Fig. 1b to d).

htxX is a heat shock gene. We found that cells carrying these plasmids form normal-sized colonies at 30 and 37°C. At 42°C, however, cells carrying pPR6S formed very small colonies, and no colonies were visible with cells carrying pPR6M or pPR6L. Since at least some of the heat shock genes are required for cell survival at high temperatures (40, 42, 55), we tested whether the heat shock response, i.e., the pattern of synthesis of hsps upon temperature upshift (38), was affected in cells carrying pPR6S or pPR6L. The results of this analysis (Fig. 2) showed that whereas the pattern of synthesis of the major hsps remains essentially unchanged, two new protein bands appear: a 22 kDa band in cells carrying pPR6S, and a 33-kDa band in cells carrying pPR6L. Each of these bands became the major synthesized protein after heat shock: over 30% of the methionine is incorporated in the 33-kDa band at 42°C. It is possible that the deregulated high expression of these proteins is responsible for the loss of cell viability at high temperatures.

To test whether the 33-kDa protein band represented a plasmid-encoded protein, an in vitro protein synthesis assay was performed. pPR6L DNA yielded a protein which migrated with the 33-kDa protein band obtained in vivo, supporting this hypothesis (not shown).

The insert of pPR6L includes that of pPR6S (Fig. 1); however, pPR6L does not direct the synthesis of the 22-kDa protein synthesized in cells carrying pPR6S. We inferred that this protein is a truncated form of the 33-kDa protein and that only part of the 33-kDa coding sequence is present in pPR6S. We called the protein HtxP and the gene encoding it htxX (for high-temperature production X).

Sequence analysis of the htxX gene. The insert of plasmid pPR6M is smaller than that of plasmid pPR6L (Fig. 1), but it directs the synthesis of the same 33-kDa protein (not shown). We sequenced both strands of the insert of pPR6M. The DNA sequence (Fig. 3) reveals only one open reading frame (ORF) long enough to encode a 33-kDa protein. The predicted molecular weight of the derived protein sequence is 31,923, in good agreement with the molecular weight.
estimated from gel electrophoresis (Fig. 2). Approximately 60 nucleotides upstream to the ORF, there is a sequence similar to the ρ-35-specific promoters consensus sequence (38; Table 2): CTTGAA at −35 (identical to the consensus sequence) and ACCCAT at −10 (versus CCCCAT for the consensus sequence). The spacing between these two elements in the htpX promoter, 12 nucleotides, is slightly shorter than the spacing in the other heat shock promoters (which ranges from 13 to 15 nucleotides [Table 2]), whereas the distance between the −10 region and the transcription start site of htpX is 1 to 2 nucleotides longer than this distance in the other heat shock promoters.

The DNA sequence suggests that HtpX is expressed from a monocistronic transcript. Two inverted repeats followed by a row of thymines are present, one before and one after the htpX ORF. These sequences could represent rho-independent transcription terminators, the first one of the gene preceding htpX and the second one of htpX itself.

A weak ribosome-binding site consensus sequence (AAG) is present before the ORF. A primer extension inhibition (toeprint) experiment demonstrated that this sequence effectively binds ribosomes. In this experiment, purified 30S ribosomal subunit, bound in vitro to the mRNA, inhibits primer elongation with reverse transcriptase; the primer elongation is usually found to terminate 15 nucleotides downstream of the initiation codon (1, 53). 30S ribosomes were found to bind effectively to in vitro-synthesized htpX mRNA, as indicated by the ribosome-induced transcription termination (Fig. 4). The site of termination is in agreement with the assignment of the ribosome-binding sequence (Fig. 3) and allows us to determine that the first of the two consecutive AUG codons at the beginning of the htpX ORF serves as the initiation codon (Fig. 4).

In plasmid pPR6S the ORF is truncated at the EcoRI site, yielding an ORF of 236 amino acids (223 from the insert and 13 from the vector sequence). The predicted molecular weight of the resulting protein is 25,656, which accounts for the 22-kDa protein band visible in cells carrying this plasmid (Fig. 2).

htpX mRNA size analysis. The distance between the putative promoter and terminator sequences is about 970 nucleotides. The mRNA from cells carrying the vector plasmid, pPR6S or pPR6L, was electrophoresed on an agarose gel and transferred to a nitrocellulose filter. The htpX mRNA was then visualized with an RNA antisense probe (Fig. 5). Cells carrying the control plasmid (lane 1) or pPR6L (lane 3) display a single band of about 1 kb, i.e., in good agreement with the predicted size of 970 nucleotides. As expected, the amount of htpX mRNA is much lower in cells carrying only the chromosomal copy of the gene (lane 1) than in cells carrying the gene on a multicopy plasmid (lane 3). In cells carrying pPR6S, a set of bands of 1 kb and higher are visible (lane 2). This is probably due to the fact that pPR6S lacks the natural terminator of htpX; instead, the transcription appears to terminate at various, probably weaker, transcription terminators within the vector.

htpX mRNA synthesis is enhanced after heat shock induc-
TABLE 2. Comparison of the htpX promoter region sequence with sequences of other known heat shock promoters*

<table>
<thead>
<tr>
<th>Gene</th>
<th>-35 region</th>
<th>13- to 15-bp region</th>
<th>-10 region</th>
<th>+1</th>
</tr>
</thead>
<tbody>
<tr>
<td>groE</td>
<td>TTTCCCCCTTGAA</td>
<td>GGGGGGAGGCCGTGACT</td>
<td>CCCCATTTCTCTGGTCAC</td>
<td></td>
</tr>
<tr>
<td>dnaKp1</td>
<td>TCTCCCCCTTGAT</td>
<td>GACGTTTCTACGA</td>
<td>CCCATTAGTAGTCAA</td>
<td></td>
</tr>
<tr>
<td>dnaKp2</td>
<td>TTGGGACTTGAA</td>
<td>ACCGAGCTTCG</td>
<td>CCCCATTACAGCTCAC</td>
<td></td>
</tr>
<tr>
<td>htpGp1</td>
<td>GCTTCTGTCTGAA</td>
<td>ATATTCTCCCTGTG</td>
<td>CCCCATCTCTGCCACAT</td>
<td></td>
</tr>
<tr>
<td>rpoDpBb</td>
<td>GCTCCACCTTGAA</td>
<td>AAACGATGATGG</td>
<td>GAGCTATAGCAGATAA</td>
<td></td>
</tr>
<tr>
<td>lon*</td>
<td>TCTGGGCTCTTGA</td>
<td>TGTGGGGGTACATG</td>
<td>CCCCATTACAGCTCAC</td>
<td></td>
</tr>
<tr>
<td>htpX</td>
<td>ATCCAGACTTGAA</td>
<td>ATAGCTCGTAT</td>
<td>ACCCATACATGCTGTAT</td>
<td></td>
</tr>
<tr>
<td>Consensus</td>
<td>T tC CoCTTGAA</td>
<td></td>
<td>CCCAATT1TA</td>
<td></td>
</tr>
</tbody>
</table>

* The promoter sequences and the derived consensus sequence were taken from Neidhardt and VanBogelen (38) except for the groE sequence, which was taken from Cowing and Gross (10), and the htpX sequence (this work).


b The lon transcription start site has not yet been determined.

The expression of the heat shock genes is only transiently induced after a temperature shift; in E. coli, heat shock mRNA synthesis reaches a peak approximately 5 min after the temperature shift and decreases gradually afterwards until it again reaches pre-heat shock levels or a new plateau specific for the new growth temperature (reviewed in reference 38). The observation that the level of HtpX synthesis does not decrease after the initial rise subsequent to the temperature shift (Fig. 2) prompted us to assay the mRNA levels at several times after the temperature shift. The htpX mRNA was quantitated by dot blot analysis and scintillation counting of the filter. The results (Fig. 6A) show that the chromosomal copy of the gene, after an initial 10-fold induction, undergoes a shuts off and reaches a level of only 1.5 times the pre-heat shock level within 30 min. In contrast, when the gene is on a multicopy plasmid, pPR6L (Fig. 6B), the mRNA level barely decreases after the initial rise, and even 30 min after the temperature shift it is still sixfold higher than the pre-heat shock level. This observation is consistent with the prolonged high level of HtpX protein synthesis observed in Fig. 2. We do not know whether this phenomenon is general to plasmid-encoded heat shock genes or whether it is particular to htpX.

htpX is σ32 regulated. The gel depicted in Fig. 2 demonstrated that the expression of htpX is heat induced. Since, besides σ32, another heat-shock-specific σ factor has recently been isolated in E. coli (σ34; 13, 52), we set out to determine whether htpX is under the control of σ32. We used a strain which carries an amber mutation within the gene encoding σ32, htpR165(Am), and a temperature-sensitive amber suppressor, supF(Ts) (54). In this strain, the expression of σ32-regulated genes is expected to be about normal at 30°C but strongly reduced at 42°C. The total cellular RNA from this strain and from the isogenic htpR+ strain was isolated before and 6 min after temperature upshift, and the htpX-specific mRNA was quantitated by dot blot analysis.
(Fig. 7) and by Northern blot analysis (not shown). The dot blot experiment shows that heat shock induction of the mRNA is strongly reduced in the htpR165(Am) supF(Ts) strain (2-fold induction versus 15-fold in the control strain), as would be expected from an HtpR-regulated gene. The residual induction observed in the mutant strain could be due to stabilization of σ^{32} present in the cells before the heat induction; indeed, the stabilization of the very labile σ^{32} protein, and not necessarily its de novo synthesis, is thought to be a major mechanism of the induction of the heat shock proteins (47, 51). In summary, this experiment demonstrates that htpX expression is induced by the σ^{32} heat shock transcription factor.

We assigned the position of the htpX promoter on the basis of the similarity to known σ^{32}-regulated promoters (Fig. 3; Table 2). To confirm this assignment, we determined the position of the 5' end of the htpX mRNA by a primer extension experiment. Total cellular RNA isolated from pPR6L-carrying htpR^+ and htpR165(Am) supF(Ts) cells, before and after heat shock induction, was used as a template for primer elongation with reverse transcriptase, using an htpX-specific primer. The results (Fig. 8) show that the position of the 5' end of the mRNA is as expected from the position of the consensus σ^{32} promoter sequence. This 5' end therefore probably represents the site of transcription initiation. The relative band intensities before and after heat induction in the wild-type and mutant cells were as observed with the mRNA from the chromosomal copy of the gene (Fig. 7). No other bands, which could have resulted from the existence of additional promoters or from processing of the mRNA, could be detected on the gel.

**Disruption of the chromosomal htpX gene.** We wanted to (i) determine whether htpX is responsible for the clear-plaque phenotype of λPR6 and (ii) gain insight into the function of the HtpX protein in the cell. Therefore, the coding region of htpX was disrupted by a DNA fragment carrying the chloramphenicol acetyltransferase gene, cat. A PstI fragment carrying the cat gene was first inserted into the PstI site of plasmid pPR6S, yielding pPR6Cm (Fig. 1a). This gene disruption was transferred to the original phage clone (λPR6) by phagemid recombination (Materials and Methods) to yield λPR6Cm (Fig. 1b). Phage λPR6Cm, carrying the disrupted copy of htpX, forms clear plaques, indicating that the phenotype of λPR6 is due not to the htpX gene but probably is due to the presence of another gene on the 8-kb-long insert.

The gene disruption was then transferred from the phage to the bacterial chromosome by recombination to yield strain htpXVCm as described in Materials and Methods. The structure of the disrupted gene was confirmed by Southern blot analysis. Using the insert of plasmid pPR6S as a probe, a change in the restriction pattern of strain htpXVCm (Fig. 9, lanes b) in comparison with the parental strain (lanes a) could be observed. An increase of about 2 kb was observed in the BamH1 and BglII restriction fragment size, as expected from the size of the cat fragment. Digestion with EcoRI yields two fragments in the htpXVCm DNA (R+H and R,
lanes b), as expected from the fact that the cat sequence carries an EcoRI recognition site.

The htpXVCm strain, carrying the disrupted htpX, has no observable phenotype. It grows as well as the parental strain at all temperatures (i.e., up to 47°C); it grows on minimal medium; no restrictive carbon source could be found; it is not UV hypersensitive; lambdoid phages grow well on htpXVCm and lysogenize it efficiently (data not shown).

Mapping of htpX on the E. coli chromosome. We used the Kohara sequential phage library (29) to determine the location of htpX, using a probe made from the insert of plasmid pPR6S. We found a single clone, clone 335 of the miniset, that hybridized to our probe. This clone corresponds to the region around nucleotide 1,930,000 of the E. coli map published by Kohara et al. (29), or min 40.3 on the genetic map. The restriction map derived from the analysis of the insert of pPR6 (Fig. 1) and the restriction map derived from the Southern blot analysis (Fig. 9) are in good agreement with this part of the map of Kohara et al.

We also determined the map position of htpX by means of Hfr recombination and P1 transduction, using the htpXVCm strain and a sequentially ordered antibiotic resistance marker strain library (46). Hfr mapping confirmed the location of htpX between min 36 and 45 of the genetic map. We then transduced htpXVCm to strain CAG12122 (zea-3125::Tn10Kan; 46) using phage P1, thus obtaining a strain (CG12122 htpXVCm) which carries a Tn10Kan marker at min 40.25 and a chloramphenicol resistance marker within the htpX gene. A P1 lysate was grown on this strain and used to transduce a kanamycin-sensitive, chloramphenicol-sensitive strain. The transductants were selected for kanamycin resistance and then screened for chloramphenicol resistance. All of the seven transductants carried both resistances, demonstrating that the two markers are closely linked. From all of these experiments, we concluded that htpX maps close to min 40.25 on the E. coli genetic map.

Effect of the truncated HtpX protein on the rate of lysogenization of λ. We found that λ forms clear plaques on a strain carrying pPR6S but not on a strain carrying the larger insert, pPR6L. We quantitated the effect of pPR6S on phage growth by measuring the frequency of lysogenization of cells carrying this plasmid. The cells were infected with a wild-type phage, a cII-overexpressing mutant (tor862), and a mutant defective in cII (cll611). The frequency of lysogenization of all three phages was much lower in cells carrying pPR6S than in the control strain carrying the vector plasmid pGEM-3 (Table 3), indicating that the presence of pPR6S in the cells interferes with the lysogenization process. We screened additional known phage mutants and found that the plaque morphology of a cII can-1 phage is unchanged in cells carrying pPR6S. cII can-1 carries a mutation that stabilizes the CI protein from proteolytic decay (25), suggesting that the presence of pPR6S in the cells may affect lysogenization through an effect on the stability of the CI protein.

Presence of the truncated HtpX protein in the cell enhances proteolysis. The fact that the truncated htpX present on pPR6S reduced the rate of lysogenization of several phages
TABLE 3. Frequency of lysogenization of λII wild-type and mutants on W350 cells carrying pPR6S

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Phage</th>
<th>% Survivors</th>
<th>% Nonimmune</th>
<th>% Lysogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM-3</td>
<td>λc1857</td>
<td>59</td>
<td>3.5</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>λc1857::862</td>
<td>84</td>
<td>1.7</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>λc1857::916111</td>
<td>22</td>
<td>0.5</td>
<td>22</td>
</tr>
<tr>
<td>pPR6S</td>
<td>λc1857</td>
<td>13</td>
<td>3.9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>λc1857::862</td>
<td>15</td>
<td>3.5</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>λc1857::916111</td>
<td>12</td>
<td>6.5</td>
<td>6</td>
</tr>
</tbody>
</table>

* The cells were grown in LBM medium at 30°C at an optical density of 0.3 at 600 nm, centrifuged, resuspended in TM, and infected at a multiplicity of infection of 3.

but did not affect mutant can-1, which expresses a more stable CII protein, led us to test whether the cloned htpX would affect the rate of general proteolysis. We compared the rate of proteolysis of puromycinyl peptides (18) in cells carrying either pPR6S or pPR6L with the rate in cells carrying the vector plasmid pGEM-3. The antibiotic puromycin is incorporated into the growing polypeptide chain and induces its premature release from the ribosome, yielding short, unstable polypeptides. To measure the rate of puromycinyl peptide degradation, cells were exposed to puromycin and [3H]leucine; the label was then chased by washing the cells and resuspending them in medium containing unlabeled leucine (Materials and Methods). The results of these experiments (Fig. 10A) show that the rate of proteolysis is indeed strongly enhanced in cells carrying pPR6S, which express the truncated form of HtpX: 24% of the labeled amino acid was incorporated into the proteins in the presence of puromycin became TCA soluble after 90 min, versus only 14% in the control cells. The presence of pPR6L, directing the synthesis of the full-length HtpX in the cells, increased proteolysis only slightly, to 16% TCA-soluble radioactivity after 90 min.

In the same set of experiments, we tested whether cells carrying the htpX<sup>VCm</sup> gene disruption display reduced proteolysis. As can be seen in Fig. 10, the htpX<sup>VCm</sup> strain displays the same kinetics of puromycinyl peptide degradation as the parental strain, indicating that the absence of HtpX did not affect the rate of general proteolysis.

We further asked whether the enhanced proteolysis induced by pPR6S is limited to the puromycinyl peptides or whether normal proteins are also more rapidly degraded in these cells. The same experiment was performed as before, but puromycin was omitted from the medium. The results (Fig. 10B) show that the rate of degradation is similar in cells carrying pPR6S and in cells carrying the control plasmid, pGEM-3. Therefore, the proteolytic activity associated with the truncated form of HtpX seems to be specific for abnormal proteins.

**DISCUSSION**

We have isolated and characterized an *E. coli* heat shock gene, htpX. This gene directs the synthesis of a 32-kDa protein, HtpX; the synthesis of the protein is greatly enhanced upon shift to a higher temperature. The size of the mRNA as determined by Northern blot indicates that HtpX is expressed from a monocistronic transcript. By measuring the htpX mRNA levels in an htpR amber mutant, we determined that htpX is under the regulation of the σ<sup>32</sup> factor. The site of transcription initiation was determined by primer extension; the htpX promoter shows high similarity to known heat shock promoters. A strain in which the htpX coding sequence was disrupted is viable under all conditions tested, including high temperature; htpX therefore belongs to the group of the nonessential *E. coli* heat shock genes, such as *lon* (34).

Comparison of the htpX DNA and derived amino acid sequence with a sequence data base revealed no significant homologies. According to its size, HtpX is part of the small hsp subgroup, of which the eukaryotic representatives show very little conservation (32). Determination of the hydropathy profile, which reveals some similarities among the eukaryotic small hsps, allowing them to be classified into one superfamily (32, 49), failed to uncover similarities with HtpX. Analysis of the hydropathy profile of the HtpX sequence according to Kyte and Doolittle (30), however, suggests that it is an integral membrane protein with four transmembrane segments, including a putative signal sequence at the amino terminus. Indeed, in this analysis, when a window of 20 residues is used, a segment is predicted to cross the membrane whenever the hydrophobicity value reaches 1.6 or higher (28, 30); four segments of HtpX, around residues 13, 43, 167, and 205, fulfill this condition. It should further be noted that the amino end of the protein resembles the signal peptide consensus sequence, with a basic residue at the amino end (Arg-3) and a hydrophobic core of 15 residues, followed by the sequence Val-17-X-gly-19; if indeed this sequence functions as a signal peptide, the cleavage by the signal peptidase would be expected to occur after this last residue (39). In fact, upon HtpX overexpression, a protein band does appear which, according to its relative migration in the gel, may represent the processed form of the protein (Fig. 2, pPR6L). Further experiments will be needed to prove this hypothesis.

Overexpression of the truncated HtpX enhances the rate of degradation of puromycinyl peptides. However, neither overexpression of the full-length HtpX nor insertional inactivation of htpX was found to have a measurable effect on the rate of puromycinyl peptide degradation. In addition, we found that the viability of cells carrying an insertional inactivation of htpX is unaffected even when the htpX<sup>VCm</sup> allele is combined with the mutant allele of a set of known protease genes: *lon* (34), *cplA* (27), *slp* (26a), or hflA (7, 9) (not shown). Since the truncated HtpX is extremely unstable, with a half-life of less than 1 min (not shown), the
simplest hypothesis is that the mere overproduction of this rapidly degraded protein results in the induction of cellular proteolytic systems and that this effect is unrelated to the normal function of HtpX.

Although htpX is part of the heat shock regulon, cells in which the chromosomal copy of htpX was inactivated by a gene insertion do not display any particular phenotype, even at an elevated temperature. One possible explanation for this lack of phenotype is that the effects of the inactivation of HtpX are too subtle to be detected in our experiments. Another possibility is that HtpX is required for cell viability, but only under a particular set of conditions, yet to be discovered. Indeed, a bacterial cell can be exposed in nature to a wide range of stress conditions, only part of which are usually being reproduced in laboratory experiments.

ACKNOWLEDGMENTS

We thank Hilla Giladi, Max Gottesman, and Ariella Oppenheim for critical reading of the manuscript, R. Traut for supplying purified 30S ribosomal subunits, Susan Gottesman for sending bacterial strains, Carol Gross for sending the strain library, and Y. Kohara for sending the sequential phase library.

This work was supported by a grant from the National Council for Research and Development, Israel, the Gesellschaft fü r Biotechnologie e.V., Funschung, mbH, Braunschweig, Germany, and grant GM38694 from the National Institutes of Health. Part of this work was performed in the Irene and Davide Sala Laboratory for Molecular Genetics.

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ary conserved mitochondrial protein is structurally related to

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