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Structure and Organization of *hip*, an Operon That Affects Lethality Due to Inhibition of Peptidoglycan or DNA Synthesis

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High-frequency persistence to the lethal effects of inhibition of either DNA or peptidoglycan synthesis, the Hip phenotype, results from mutations at the *hip* locus of *Escherichia coli* K-12. The nucleotide sequence of DNA fragments which complement these mutations revealed an operon consisting of a possible regulatory region, including sequences with modest homology to an *E. coli* promoter, and two open reading frames which are translated both in vitro and in vivo. The stop codon of a 264-bp open reading frame, *hipB*, and the start codon of a 1,320-bp open reading frame, *hipA*, share an adenine residue. Assays of promoter strength, the location of the probable promoter with respect to the start of transcription, and codon usage all indicate that *hipB* and *hipA* are weakly expressed genes. The activity of the promoter is impaired by an adjacent downstream sequence which includes the coding region of *hipB*. The impairment is partially relieved by insertion of a premature translation termination signal within the coding region of *hipB*, suggesting involvement of the HipB protein in the regulation of this promoter. The arrangement of *hipB* and *hipA* within the operon and the toxicity of *hipA* for strains defective in or lacking *hipB* suggest an important interaction between the products of these genes.

Small fractions of *Escherichia coli* K-12 populations, 10^{-6} to 10^{-5}, remain viable after prolonged inhibition of either peptidoglycan or DNA synthesis. These survivors are not mutant organisms. Subcultures of the survivors have the same frequency of persistence upon subsequent inhibition. They retain full sensitivity to the wide variety of chemical and genetic blockages of peptidoglycan or DNA synthesis used in these studies. Thus, this phenomenon, persistence, involves divergent responses by genetically homogeneous populations rather than selection of mutants from among genetically heterogeneous populations (31, 38). Shortly after the introduction of penicillin, persistence was discovered as the result of an inquiry into the occasional failure of penicillin to eradicate infections caused by penicillin-sensitive staphylococci (4). It was subsequently observed among other bacteria susceptible to β-lactam antibiotics (26). The original description of persistence almost 50 years ago suggested that persisters might be in a nondividing phase and thereby insusceptible to the bactericidal action of penicillin (4). Later elaborations of this suggestion linked persistence to the division cycle on the basis of the periodicity of potentially related phenomena: the bactericidal effects of β-lactam antibiotics (16) and the synthesis (6) and hydrolysis (3, 12) of peptidoglycan. Other explanations (10) attributed persistence to selection of mutants for reduced susceptibility to the bactericidal action of penicillin as a result of defects in the activity or expression of peptidoglycan hydrolases or of the ability to form stable spheroplasts. However, explanations proposing selection of mutants or assigning a special role to peptidoglycan metabolism are not tenable in view of the evidence that genetic heterogeneity does not account for persistence (31) and the fact that the persistent response can also be initiated by inhibition of DNA synthesis (38).

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Persistence and its possible relationship to cell division have been studied by genetic analysis of the Hip phenotype, which is characterized by high frequency of persistence to inhibition of peptidoglycan (31) or DNA (38) synthesis. In Hip strains, persisters occur at a frequency of 10^{-2} to 10^{-1}. Mutations responsible for the Hip phenotype map to 33.8 min on the *E. coli* chromosome (31). This locus, designated *hip*, is in the terminus region (15) close to *terC3*, a site of termination of the clockwise replication fork (14). The original Hip mutants (31), in addition to having a vast increase in the frequency of persistence, have a cold-sensitive block in cell division (38). Plasmids containing the *hip* region restore the frequency of persistence of Hip strains to that of the parental strain (32, 38) and relieve the cold sensitivity of the Hip strains (38). One of the products of *hip* is a weakly expressed protein of about 50,000 Da, which may be toxic when overexpressed (32). This report is part of an effort to understand the physiological role of *hip* and its products. It describes the nucleotide sequence, transcription, and gene organization of the *hip* operon, as well as demonstrating an additional, *trans*-acting protein encoded by *hip*.

MATERIALS AND METHODS

Bacterial strains and media. An additional Hip strain, HM1000, was isolated by using the strategy for isolating the earlier Hip mutants (31) but with two potentially important modifications. First, cycles of inhibition of peptidoglycan synthesis were imposed gradually by starvation of a *dapE6* strain, AT984, for diaminopimelic acid rather than abruptly by addition of antibiotics. Second, strains with partial impairment of growth were examined for the Hip phenotype, whereas such strains had been excluded in previous searches (31). The earlier Hip mutants failed to grow at 20°C but had unimpaired growth rates at 37°C. In contrast, HM1000 has slightly reduced rates at both temperatures. The mutation responsible for the Hip phenotype of HM1000 was shown to be in or close to the *hip* locus by P1 transduction using the

TABLE 1. *E. coli* K-12 strains used in this study

Strain	Relevant genotype	Source or reference
AT984	*dapE6*	5
C600K	*galK*	1
HM21	*dapE6 zde-264*::Tn*10*	31
HM25	*zdd-262*::IS*10* Cm^r IS*10* *zdd-263*::Tn*5*	32
HM721	*dapE6 hipA7 recA56 srlC300*::Tn*10*	32
HM1000	*dapE6 hipB2*	See Materials and Methods
HM1002	*dapE6 hipB2 zdd-263*::Tn*5*	P1 · HM25 × HM1000 → Km^r
HM1025	*dapE6 hipB2 zdd-263*::Tn*5*	P1 · HM1002 × AT984 → Km^r
HM1026	*dapE6 hipB2 zdd-263*::Tn*5* *recA56 srlC300*::Tn*10*	P1 · JC10240 × HM1025 → Tc^r
JC10240	*recA56 srlC300*::Tn*10*	7

selective markers previously shown to be closely linked to the mutations *hipA7* and *hipA9* of the original Hip strains (38). The mutant allele of HM1000 was designated *hipB2*. The construction or sources of the other strains used in this work are listed in Table 1.

The culture media were LB broth (30); TYE agar, which contains 10 g of tryptone, 5 g of yeast extract, 8 g of NaCl, and 15 g of agar per liter; and Mueller-Hinton agar supplemented with 0.1 U of thymidine phosphorylase per ml to ensure thymidine deficiency in this otherwise rich medium (9). For growth of *dapE6* strains, 75 mg of diaminopimelic acid per liter was added to these media. When used, antibacterial agents were added in the following amounts per liter: ampicillin, 100 mg; chloramphenicol, 25 mg; kanamycin · SO_4, 75 mg; tetracycline · HCl, 20 mg; and trimethoprim, 2 mg.

Determination of frequency of persistence. The parental frequency of persistence to inhibition of peptidoglycan synthesis, approximately 10^{-6}, was distinguished from the high-frequency persistence of Hip mutants, approximately 10^{-2}, by methods previously described in detail (31, 32). Small streaks from fresh colonies are made in a grid pattern on agar media in which peptidoglycan synthesis is selectively blocked. After 16 to 20 h the block is reversed; at the end of another 16 h the streaks from parental organisms contain zero to three colonies and the streaks from Hip organisms are confluent. For the most part, this is done by using *dapE6* strains which are transiently deprived of diaminopimelic acid. In the case of ampicillin-sensitive strains, 16- to 20-h exposures to the drug are terminated with penicillinase. These methods have proven satisfactory not only for screening purposes, but also for most other determinations, including complementation analyses. A quantitative version of the method (32) is used when small changes in the frequency of persistence might be significant.

The routine method for quantitating persistence to selective inhibition of DNA synthesis is based on transient deprivation of thymidine. This is accomplished by 24 h of inhibition by trimethoprim in Mueller-Hinton agar supplemented with thymidine phosphorylase; inhibition is terminated by spraying plates with approximately 0.3 ml of a sterile solution containing 10 mg of thymidine per ml; persistent cells form visible colonies within 8 to 12 h of further incubation. The parental and Hip frequencies of persistence by this method of inhibition of DNA synthesis, 10^{-5} and 10^{-1}, respectively, are similar to those observed by other methods (38).

Plasmid construction and vectors. Subcloning procedures, including restriction digestion, gel electrophoresis, isolation and purification of DNA fragments, ligation, and transformation, were performed by standard methods (24). Plasmid DNA was prepared by the method of Humphreys et al. (17). pHM418 (32) was the source of *hip* fragments for construction of plasmids for analysis of transcription and promoter activity.

pDD3, a gift of D. Daniels, was the vector for pKD144 and pKD1877 (Fig. 1). pDD3 has a fragment of *tetA* inserted into a *Bam*HI site flanked on either side by the strong transcription terminator, *rrnB* t1, which releases transcripts 150 nucleotides from either end of the cloning site. In the construction of derivatives of pDD3 the *tetA* fragment is replaced by other fragments to be tested for initiation of transcription (8).

pKO11 (36), the vector for the pGK series of plasmids (Fig. 1), contains the ribosome-binding site and structural sequences of *galK* but lacks a promoter. Expression of *galK* by these plasmids is therefore dependent on promoter activity of the fragments inserted into the cloning region. Insertions can yield transcriptional but not translational fusions with *galK* owing to the presence of stop codons in all three reading frames between the cloning region and *galK*. The host for the pGK series of plasmids was C600K, a *galK* mutant strain. The specific activity of galactokinase (36) and the plasmid copy number (1) were determined by published methods. Promoter activity is expressed as nanomoles of galactose-1-PO_4 per minute per fentomole of plasmid DNA. Plasmids used as controls were pKO11; pRW37, which contains a fusion of a weak promoter and *galK* (42); and pCAP$_1$P$_2$, which contains a fusion of a strong promoter and *galK* (1).

pNO1575 (20), a gift of M. Nomura, was an expression vector for the pDB series of plasmids (Fig. 1). It contains the *lac* promoter and polylinker of pUC9 inserted into the *tet* gene of pBR322.

The construction of the pHM series of plasmids has been described previously (32).

Determination of nucleotide sequence. The dideoxy-chain termination (37) and limited chemical cleavage (25) methods were used for determination of the nucleotide sequence. M13 (27) and pBS (40), a phagemid (Stratagene, La Jolla, Calif.), were the vectors used in directed or shotgun subcloning (28) or exonuclease III deletion subcloning (13) for production of single-stranded sequencing templates.

Analysis of in vivo transcription. For S1 nuclease protection studies, RNA was prepared by repeated hot-phenol extraction of cells from 500 ml of culture in the early phase of logarithmic growth (43). Fragments of *hip* DNA were labeled at the 5′ ends with ^{32}P by a standard method (25), except that a 90-min incubation period was used for the kinase reaction. After a secondary restriction the fragments were separated by gel electrophoresis and purified. Then 0.05 to 0.1 μCi of these DNA fragments and 100 μg of either RNA prepared as described above or yeast tRNA were coprecipitated with ethanol, dried, and resuspended in 20 μl of hybridization buffer [80% deionized formamide, 0.4 M NaCl, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4) 1 mM EDTA]. The mixtures were heated at 80°C for 5 min, slowly cooled to hybridization temperatures that varied between 42 and 50°C, and held at these temperatures for 3 h. S1 nuclease (100 U in 180 μl of 60 mM sodium acetate [pH 4.6], 100 mM NaCl, and 2mM ZnCl) was added.

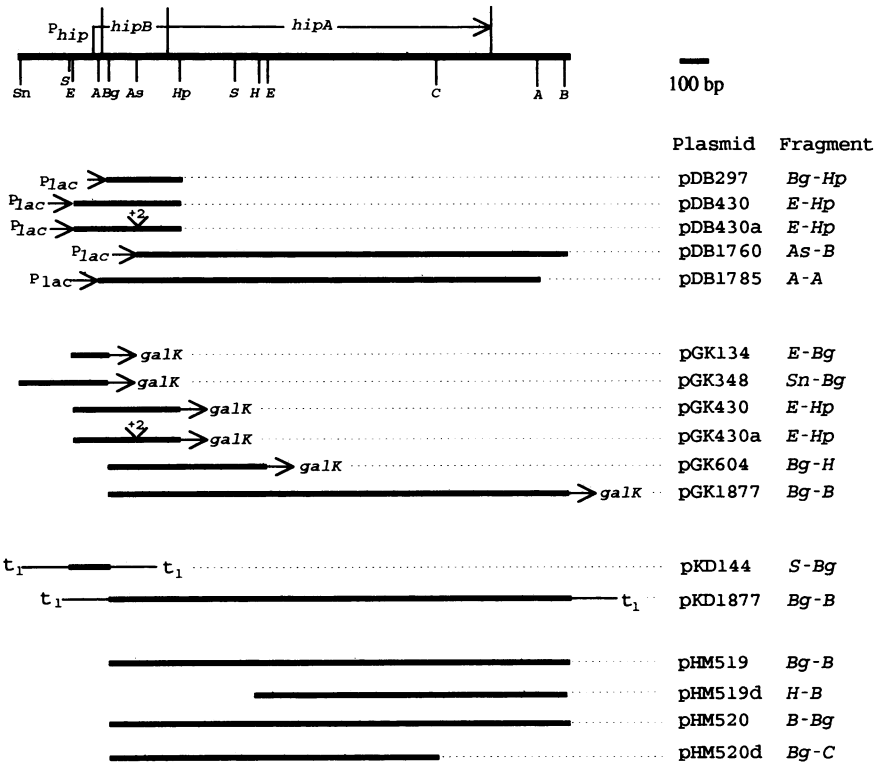

FIG. 1. Plasmids used in this study. The vector for the pDB series of plasmids was pNO1575; that for the pGK series was pKO11; that for the pKD series was pDD3; and that for the pHM series was pACYC177. See Materials and Methods for further details. Abbreviations: *A*, *Afl*II; *As*, *Asu*II; *B*, *Bam*HI; *Bg*, *Bgl*II; *C*, *Cla*I; *E*, *Eco*RV; *H*, *Hin*dIII; *Hp*, *Hpa*I; *S*, *Sau3*AI; *Sn*, *Sna*BI; t_1, *rrnB* t_1 terminator; P_{hip}, *hip* operon promoter; P_{lac}, *lac* operon promoter; *galK*, galactokinase coding region.

After 1 h at 15°C, 10 μg of yeast tRNA was added as a carrier and the nucleic acids were precipitated with ethanol. The precipitates were washed with 75% ethanol, dried, and resuspended in loading buffer (25). Samples containing approximately 0.01 μCi of ^{32}P were analyzed by gel electrophoresis in 8% acrylamide-bisacrylamide (29:1 ratio) containing 7.5 M urea in 1× TBE buffer (24) or were compared with a nucleotide ladder generated from chemical sequencing. Transcripts were visualized on Kodak XAR-5 X-ray film.

In vitro start of transcription. Approximately 1 μg of supercoiled plasmid DNA was preincubated for 5 min at 37°C in a volume of 9 μl containing 170 mM KCl, 22 mM Tris-acetate buffer (pH 7.9), 0.1 mM EDTA, 200 μCi of either [γ-^{32}P]ATP, [γ-^{32}P]CTP, [γ-^{32}P]GTP, or [γ-^{32}P]UTP and each of the other (unlabeled) nucleotides at 0.5 mM, and 1 U of RNasin (Promega Biotec, Madison, Wis.). The γ-labeled nucleotides were made by using the Gamma Prep Synthesis System (Promega Biotec), which yielded specific activities of 3,000 to 6,000 Ci/mmol. A parallel determination was performed with 10 μCi of [α-^{32}P]GTP of at least 400 Ci per mmol (Amersham Corp., Arlington Heights, Ill.). Reactions were initiated by the addition of 0.1 to 1 U of *E. coli* RNA polymerase (Pharmacia, Inc., Piscataway, N.J.) in a volume of 1 μl. The reactions were stopped after 10 min at 37°C by mixing with 75 μl of buffer-saturated phenol and 100 μl of yeast tRNA (0.5 mg/ml). Nucleic acids in the aqueous phase were precipitated with ethanol, dried under vacuum, and resuspended in loading buffer (25). A 10-μl sample of each reaction was analyzed by gel electrophoresis as described in the preceding section.

DNA-directed translation. In vitro transcription-translation assays were performed with cell extracts (Amersham) under conditions described by the manufacturer. The resultant radiolabeled products were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (22).

Nucleotide sequence accession number. The *hip* sequence has been assigned GenBank accession number M61242.

RESULTS

Coding regions of *hip*. The *hip* locus at 33.8 min of the *E. coli* chromosome (32) contains two genes, *hipA* and *hipB*, as determined by DNA sequencing and genetic complementation. *hipB* extends from nucleotides 337 to 600, and *hipA* extends from nucleotides 603 to 1922 (Fig. 2). The stop codon of *hipB* overlaps the first possible start codon of *hipA* by 1 bp. There is a third open reading frame in the opposing direction, designated ORF 3. It starts at nucleotide 2010 and ends at nucleotide 1672 of the strand shown. Review of an earlier analysis of *hip*-containing plasmids (32) revealed that ORF 3 does not complement Hip phenotypes.

In vitro expression of Hip proteins. The protein inferred from the sequence of *hipA* would have a molecular mass of 49,489 Da, which is in close agreement with the approximate size of the protein, 50,000 Da, produced in vitro by plasmids containing this region of *hip* (32). Translation of a protein of this size would have to start at either nucleotide 603 or 624. However, no Shine-Dalgarno sequence (39) is present prior to the start codon at the latter position, but there is a moderately conserved sequence, GGAG, 9 bp prior to the first possible start codon at nucleotide 603.

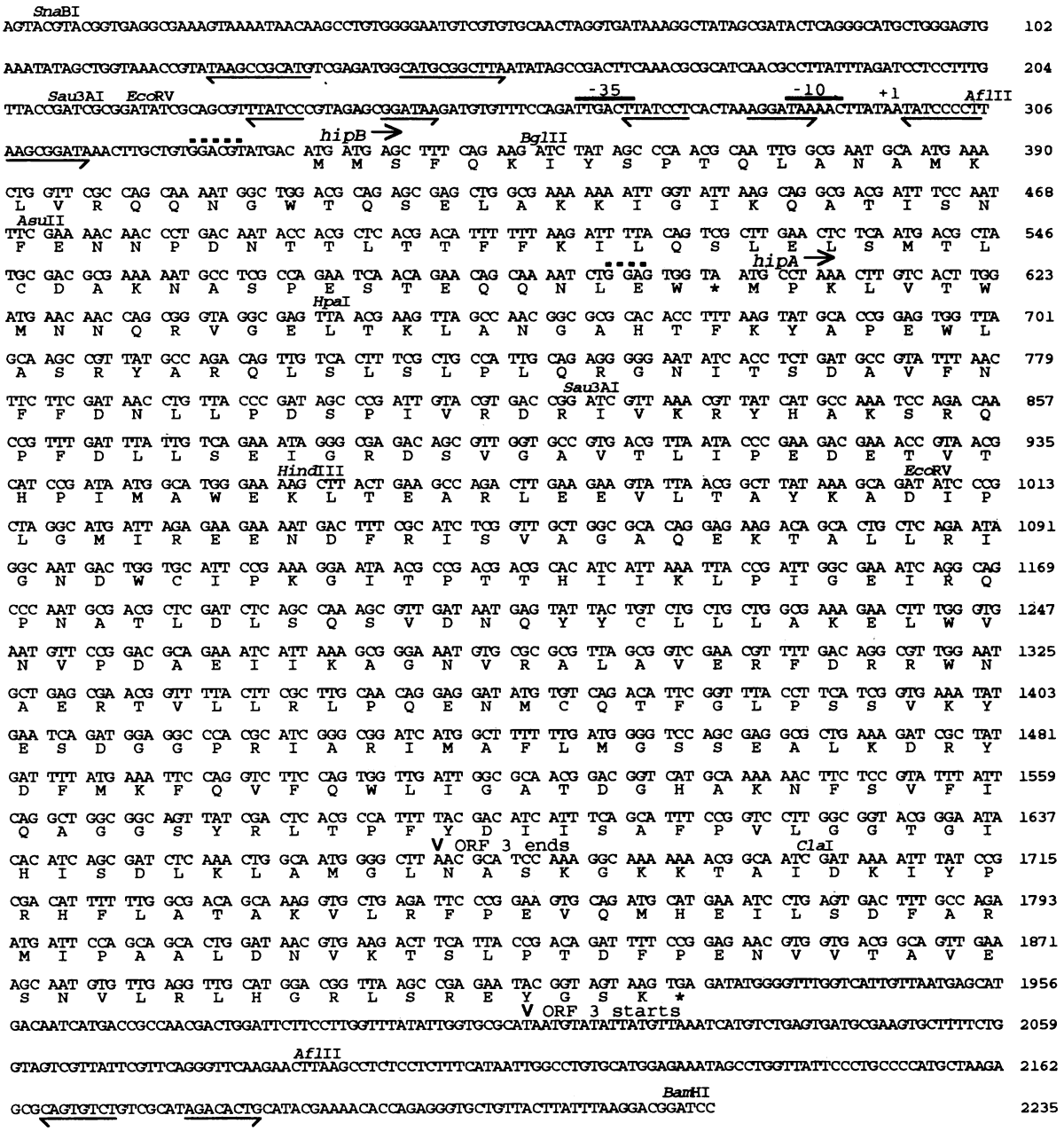

FIG. 2. Nucleotide sequence of the *hip* locus of *E. coli*. The apparent −10 and −35 regions are overlined. Regions of dyad symmetry are underlined. Suspected ribosome-binding sites are overlined with dashes. Asterisks mark the ends of the open reading frames of the strand shown. Carets mark the start and stop of ORF 3 of the opposite strand (not shown). The restriction sites are those used in the construction of plasmids or preparation of probes.

hipB would encode a protein of 10,005 Da. A protein in this size range (Fig. 3) is indeed expressed in an in vitro transcription-translation system by using pDB430, which contains a transcriptional fusion of the *lac* promoter to the *Eco*RV-*Hpa*I fragment spanning *hipB*. pDB430a is identical to pDB430 except that a 2-bp insertion was made at the *Asu*II site of the *Eco*RV-*Hpa*I fragment. This should create a stop codon 48 bp downstream of the insertion, resulting in a protein correspondingly reduced in size (Fig. 3). Estimates of the sizes of proteins in this range from such data are not sufficiently reliable to locate the probable start codon. However, complementation studies (see below) placed the start of translation of *hipB* at either nucleotide 337 or 340.

Complementation of mutant alleles of *hipA* and *hipB*. Plasmids pHM519 and pHM520, in which the only intact open reading frame is *hipA*, complement the Hip phenotypes of *hipA7 recA56* and *hipA9 recA56* strains (32). More recent constructions of this type, pDB1760 and pGK1877 (Fig. 1), also complement *hipA* mutant strains. Loss of complementation accompanies removal of several hundred base pairs from either the 5′ (pHM519d) or 3′ (pHM520d) end of *hipA* (32). Neither pHM519d nor pHM520d produces a Hip phe-

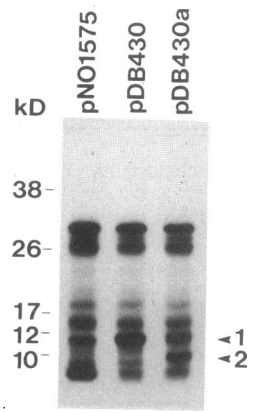

FIG. 3. In vitro synthesis of [^{35}S]methionine-labeled proteins directed by plasmids containing *hip* fragments. Molecular mass markers, a gift of B. Semler, were ^{35}S-labeled poliovirus proteins from a HeLa cell monolayer infected by wild-type poliovirus. Arrowhead 1 indicates a protein encoded by the *Eco*RV-*Hpa*I fragment of pDB430 which overlaps a vector protein. Arrowhead 2 indicates the truncated form of this protein encoded by pDB430a in which the *Eco*RV-*Hpa*I fragment contains a 2-bp insert at the *Asu*II site and, consequently, a frameshift.

notype in a parental background; therefore, the failure of these plasmids to complement is not attributable to an anticomplementary effect. Plasmids containing noncomplementing fragments of the 5′ but not the 3′ region of *hipA* increase the rate of reversion of *hipA* strains; this fragment-specific enhancement of reversion, marker rescue, is *recA* dependent (32).

The Hip phenotype due to *hipB2* in a *recA56* strain, HM1026, is complemented by pGK430, which contains the *Eco*RV-*Hpa*I fragment of the *hip* region. pDB430 also complements. It contains a transcriptional fusion of the *lac* promoter to the *Eco*RV-*Hpa*I fragment. This fragment spans the putative *hip* promoter, *hipB*, and the first 47 bp of *hipA*. A 2-bp insertion at the *Asu*II site of the fragment, as in pGK430a and pDB430a, eliminates complementation. As noted above, the truncated protein predicted to result from such an insertion can be demonstrated for pDB430a (Fig. 3).

A protein of the size encoded by the complementing plasmid, pDB430 (Fig. 3), would require that translation start at nucleotide 337, 340, or 385 (Fig. 2). A start at 385 is unlikely because there is no appropriately placed Shine-Dalgarno sequence (39) preceding it. Furthermore, plasmid pDB297 fails to complement the Hip phenotype of *hipB2* strains. pDB297 contains a transcriptional fusion of the *lac* promoter to the *Bgl*II-*Hpa*I fragment, which lacks the first two possible start codons. Therefore, translation of *hipB* starts at either nucleotide 337 or 340.

Regulatory regions of the *hip* sequence. The sequence was examined for common motifs of bacterial consensus promoters (33). The best match is the only one upstream of the open reading frames on the strand shown (Fig. 2).

There are five regions of dyad symmetry (Fig. 2). The first region has partial homology to repetitive, extragenic sequences of the *E. coli* chromosome (41). The fifth region is found where a transcription terminator might be expected, but it is not followed by the thymidine nucleotides characteristic of *rho*-independent terminators. There are three regions of dyad symmetry in the vicinity of the suspected promoter.

Codon utilization. The utilization of rare (19), infrequent (19), and nonoptimal codons (18) in *hip* is much higher than in the typical genes of *E. coli*. For example, the typical frequency of rare codon utilization is 4%, whereas the frequency of rare codons in the coding regions of *hip* is 11%. The small protein inferred from *hipB* contains 88 amino acids. It lacks histidine and has an equal number of acidic and basic amino acids. The protein inferred from *hipA* contains 440 amino acids. It contains all 20 of the common amino acids. There is an excess of 12 basic over acidic amino acids. Hydropathy profiles (21) reveal no extensive hydrophobic region in either protein, nor does either contain a region suggestive of the signal sequences of membrane or exported proteins (29). A FASTA search (35) of GenBank, version 66, revealed that the nucleotide sequences of *hipA*, *hipB*, and the open reading frame on the opposite strand (ORF 3) have no obvious similarity to any of the nucleotide sequences, nor do the inferred proteins bear extensive resemblance to any other protein.

***hipA* is toxic in *hipB2* strains.** Plasmids capable of expressing *hipB* alone fail to complement *hipA* mutant strains. The converse relationship has not been addressed because plasmids which express *hipA* but not *hipB* fail to transform *hipB2* strains at detectable frequencies. Stable transformants cannot be recovered even if expression of *hipA* is minimized by the lack of a specific promoter, as occurs when the *Bgl*II-*Bam*HI fragment is inserted at vector sites remote from recognized promoters. Plasmids of this type include pGK1877, pKD1877, pHM519, and pHM520. *hipB2* strains can support the replication of plasmids, such as pDB1785, capable of expressing both *hipA* and *hipB*. Plasmids in which *hipA* has been inactivated, pHM519d and pHM520d, are also replicated.

Transcription of *hip*. The transcription start of *hip* was studied by S1 nuclease mapping (Fig. 4a and b) with RNA from strains bearing plasmids pGK134 or pGK430. RNA from both strains protected the *Eco*RV-*Bgl*II fragment of *hip*; however, RNA encoded by the plasmid with the smaller insert (pGK134) provided much more protection than did RNA encoded by pGK430. The products protected from S1 nuclease by RNA from pGK134-bearing cells were run on an 8% denaturing acrylamide gel with a sequence ladder to identify the start of transcription. Two points of possible transcription initiation, at adenosine and cytidine nucleotides separated by 4 or 5 nucleotides, were observed with some heterogeneity at both sites (Fig. 4b). In an attempt to clarify the point of origin, in vitro transcription was analyzed by using γ-labeled ribonucleoside triphosphates. The results of this experiment (Fig. 4c) revealed a primary start at an A residue and a minor start at a T residue. These probably represent the A start and the heterogeneity around that start noted above (Fig. 4b). The in vitro measurement (Fig. 4c) did not detect the C start observed in vivo (Fig. 4b).

The pGK series of plasmids contain transcriptional fusions of various portions of the 5′ region of *hip* with *galK*. Expression of galactokinase by a *galK* mutant strain bearing these plasmids was used to assess promoter activity (Table 2). The *Bgl*II-*Hin*dIII fragment of pGK604, which starts 16 bp downstream of the translation start of *hipB* and extends well into the coding region of *hipA* (Fig. 2), does not have detectable promoter activity. The *Eco*RV-*Bgl*II fragment of pGK134 has moderate promoter activity compared with the activities of well-characterized weak (42) and strong (1) promoters. This fragment spans the suspected promoter region, the start of transcription, and the first 17 bp of the coding region of *hipB*. It also spans the three regions of dyad symmetry which share the core sequence, $TATCC(N)_8$

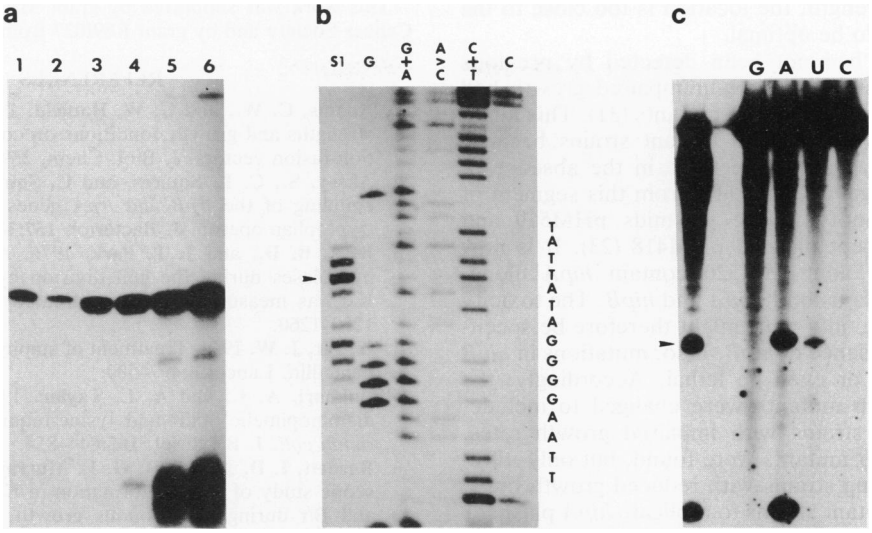

FIG. 4. Initiation of transcription of *hip*. (a) Protection of the *Eco*RV-*Bgl*II fragment of *hip* from digestion by S1 nuclease. Lanes: 1, no RNA; 2, tRNA; 3, RNA from C600K(pGK430) hybridized at 46°C; 4, RNA from C600K(pGK430) hybridized at 50°C; lane 5, RNA from C600K(pGK134) hybridized at 46°C; lane 6, RNA from C600K(pGK134) hybridized at 50°C. (b) S1 nuclease mapping of transcription initiation. The protected portion of the *hip* fragment shown in panel a, lane 6, was run adjacent to a sequence ladder of the fragment. The major start of transcription is indicated by the arrowhead. (c) Identification of the nucleotide at the start of in vitro transcription of *hip* with pKD144 as the template: unmarked lane, [α-^{32}P]GTP; lane G, [γ-^{32}P]GTP; lane A, [γ-^{32}P]ATP; lane U, [γ-^{32}P]UTP; lane C, [γ-^{32}P]CTP. The arrow indicates the 230-nucleotide transcript initiating in *hip* and terminating at *rrnB* t_1 of the vector.

GGATA (Fig. 2). The promoter activity of the *Sna*BI-*Bgl*II fragment of pGK348 is similar to that of the *Eco*RV-*Bgl*II fragment of pGK134. Therefore, the sequence upstream of *Eco*RV does not appear to have a major effect on the promoter. On the other hand, promoter activity is affected by inclusion of sequence downstream of *Bgl*II. The *Eco*RV-*Hpa*I fragment of pGK430 has fourfold-lower activity than does the *Eco*RV-*Bgl*II fragment, from which it differs by including the entire coding region of *hipB* and the first 47 bp of *hipA*. This reduction is partially reversed by a 2-bp insertion at the *Asu*II site (pGK430a), which produces a frameshift and premature translation-terminating codons, the first of which occurs at nucleotide 539. As noted above, this frameshift also eliminates complementation of *hipB* mutant strains.

DISCUSSION

The genetic locus *hipA* (31) had been shown to lie within an 1,877-bp *Bgl*II-*Bam*HI fragment from 33.8 min on the *E. coli* chromosome. Plasmids containing this fragment complement *hipA* mutants and express just-detectable levels of a 50,000-Da protein in an in vitro transcription-translation system (32). Both complementation and expression were independent of the orientation of the fragment even when it had been inserted into vector sites remote from recognized promoters of significant strength. Thus, it was anticipated that both a promoter and a coding region of the appropriate length would be found in the fragment. However, the *Bgl*II-*Bam*HI fragment contains a coding region of the expected length but does not contain the expected promoter. There is no credible homology to known classes of *E. coli* promoters (Fig. 2), and the 5′ region of the fragment has no activity in a sensitive system for the detection of promoters (Table 2). Expression of *hipA* by the *Bgl*II-*Bam*HI fragment must therefore depend on transcripts originating in flanking sequences of the plasmids.

TABLE 2. Promoter activity of fragments from the 5′ region of *hip*

Plasmid[a]	*hip* fragment	Promoter activity[b]
pKO11 (vector)	None	0.002
pGK604	*Bgl*II-*Hin*dIII	0.002
pGK134	*Eco*RV-*Bgl*II	0.213
pGK348	*Sna*BI-*Bgl*II	0.195
pGK430	*Eco*RV-*Hpa*I	0.049
pGK430a	*Eco*RV-*Hpa*I	0.117

[a] See Fig. 1 for a description of plasmids.
[b] Promoter activity is expressed as nanomoles of galactose-1-PO_4 per minute per fentomole of plasmid. See Materials and Methods for further details.

The sequence of an additional 353 bp of DNA upstream of the *Bgl*II site revealed the start of a short open reading frame, *hipB*. This region also includes a sequence with modest homology to the consensus *E. coli* promoter, as well as several other significant features (Fig. 2). First, a portion of the first inverted repeat is similar in sequence to the repetitive elements found between genes at numerous places in the *E. coli* chromosome (41). Comparison of the promoter activities of the *Sna*BI-*Bgl*II fragment of pGK348 and the *Eco*RV-*Bgl*II fragment of pGK134 (Table 2) suggests that the first inverted repeat is not essential. Second, the minimal-length fragment with promoter activity, *Eco*RV-*Bgl*II, contains three inverted repeats; one is just upstream of the promoter, another is in the midst of the promoter, and a third is close to the start of transcription. These could have important roles in the regulation of *hip* expression not only because of their locations, but also because all three have a common core, TATCC$(N)_8$GGATA. This arrangement suggests that the regulation of *hip* expression will prove to have interesting complexities. Although the −35 and −10 regions and the length of the intervening sequence predict a pro-

moter of moderate strength, the location is too close to the start of transcription to be optimal.

Mutations in *hipB* had not been detected by previous genetic analysis, possibly because unimpaired growth had been a criterion in searches for Hip mutants (31). This might have precluded recovery of *hipB* mutant strains because expression of *hipA* appears to be toxic in the absence of *hipB*. Strains with large deletions (11) from this segment of the chromosome cannot tolerate plasmids pHM519 and pHM520, but will accept plasmid pHM418 (23). It is now known that pHM519 and pHM520 contain *hipA* alone, whereas pHM418 contains both *hipA* and *hipB*. The toxicity of plasmids containing only *hipA* might therefore be specifically related to the absence of *hipB*. If so, mutations in *hipB* would impair growth or even be lethal. Accordingly, the search criteria for Hip mutants were changed to include, rather than exclude, strains with impaired growth rates. With this change, *hipB* mutants were found, but only infrequently and only among strains with reduced growth rates. The failure of *hipB* mutant strains to replicate *hipA* plasmids confirms the suspicion that the toxicity of *hipA* depends on a defect in or complete absence of *hipB*.

Biochemical analysis of the toxicity of *hipA* plasmids for mutant *hipB* or Δ*hipB* strains could be valuable in elucidating the physiological role of *hip*. Unfortunately, it has not yet been possible to reduce the expression of *hipA* sufficiently for recovery of the necessary, stable transformants. This toxicity suggests that uncoordinated expression of the *hip* operon leading to a relative excess of *hipA* might be deleterious. If so, *hipA* expression would have to be linked to that of *hipB*. How linkage might be accomplished is suggested by the sequence of *hip* (Fig. 2). The translation stop codon of *hipB* and the probable start codon of *hipA* overlap by 1 bp. Such intercistronic spacing is characteristic of operons which use translational coupling to encode functionally related proteins occurring in fixed ratios (2, 34).

Other features of the sequence and the properties of the promoter are characteristic of a weakly expressed operon. Both *hipA* and *hipB* have increased utilization of nonoptimal (18), rare (19), and infrequently utilized (19) codons, patterns often associated with weakly expressed operons. The sequence of the putative promoter region, particularly its position with respect to the translation start, predicts low activity. This is borne out in the assessment of promoter strength (Table 2). Inclusion of the coding region of *hipB* in the fragment tested for promoter activity causes a substantial reduction of the already low activity. A frameshift in the *hipB* sequence causes partial reversal of the effect. Inclusion of *hipB* has a similar effect on *hip* transcription. A plasmid containing the *hip* promoter plus *hipB*, pGK430, as compared with a plasmid lacking most of *hipB*, pGK134, produces substantially reduced amounts of RNA capable of protecting the promoter region from S1 nuclease (Fig. 4a). The reduction of *hip* promoter function by the *hipB* sequence could be due to repressor activity on the part of the HipB protein. These and other aspects of the regulation of *hip* are being addressed by using single-copy transcriptional fusions in a background from which both *hipB* and *hipA* have been specifically deleted.

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