Cloning, Genetic Characterization, and Nucleotide Sequence of the hemA-prfA Operon of Salmonella typhimurium

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The first step in heme biosynthesis is the formation of 5-aminolevulinic acid (ALA). Mutations in two genes, hemA and hemL, result in auxotrophy for ALA in Salmonella typhimurium, but the roles played by these genes and the mechanism of ALA synthesis are not understood. I have cloned and sequenced the S. typhimurium hemA gene. The predicted polypeptide sequence for the HemA protein shows no similarity to known ALA synthases, and no ALA synthase activity was detected in extracts prepared from strains carrying the cloned hemA gene. Genetic analysis, DNA sequencing of amber mutations, and maxicell studies proved that the open reading frame identified in the DNA sequence encodes HemA. Another surprising finding of this study is that hemA lies directly upstream of prfA, which encodes peptide chain release factor 1 (RF-1). A hemA::Kan insertion mutation, constructed in vitro, was transferred to the chromosome and used to show that these two genes form an operon. The hemA gene ends with an amber codon, recognized by RF-1. I suggest a model for autogenous control of prfA expression by translation reinitiation.

Heme serves two major functions in Salmonella typhimurium and Escherichia coli: respiration and defense against oxygen radicals. It is the prosthetic group of the cytochromes and thus is required for the function of electron transport chains (49). As the cofactor for catalase, heme plays an important role in protecting the cell from toxic oxygen metabolites (29, 30). In enteric bacteria, the heme biosynthetic pathway produces two other tetrapyrroles, siroheme and vitamin B_{12}. Sulfite reductase and nitrite reductase utilize siroheme for similar reactions (58), and sulfite reductase is required for the synthesis of cysteine from inorganic sulfate (for a review, see reference 34). Vitamin B_{12} is known to serve as the cofactor for at least four enzymes in S. typhimurium, including a homocysteine methyltransferase (encoded by metH) that can function in methionine synthesis as an alternative to a B_{12}-independent enzyme (encoded by metE) (12) and ethanolamine ammonia lyase (encoded by the eut locus) (13, 52).

Despite the central role of heme in respiratory energy metabolism, very little is known about most of the genes and enzymes required for heme synthesis or the control of this pathway in genetically accessible bacteria such as E. coli and S. typhimurium. Synthesis of heme is regulated in E. coli and S. typhimurium. For example, synthesis is reduced 10-fold under fermentative anaerobic conditions compared with aerobic respiration (25; T. Elliott and J. R. Roth, Mol. Gen. Genet., in press). Furthermore, mutants with blocks in the heme pathway show substantial overproduction of tetrapyrrole intermediates when grown on limiting amounts of heme (15, 55; unpublished results). It is possible that mechanisms used to regulate heme synthesis are connected to general oxygen control of gene expression, which is poorly understood.

Synthesis of ALA occurs in different organisms by either a C4 or a C5 route. The C4 route employs the enzyme ALA synthase (EC 2.3.1.37) to condense succinyl coenzyme A plus glycine (11, 64), whereas the C5 route proceeds in three steps, starting from glutamate, and requires $tRNA^{Glu}$ and a glutamyl tRNA synthetase (32). Some reports have suggested that ALA synthase activity can be found in extracts of E. coli (31, 63). However, other investigators have been unable to find this activity in extracts of wild-type cells (11, 64; unpublished results). No purification of the enzyme(s) responsible for ALA synthesis in E. coli or S. typhimurium has been reported.

We recently reported a genetic analysis of mutants defective in ALA synthesis (Elliott and Roth, in press), which confirms and extends earlier observations (56, 57). Two genes, hemA and hemL, are required for ALA synthesis in S. typhimurium. Mutants defective in hemA have a more severe auxotrophic phenotype than those lacking hemL. In the context of a C4 route, I imagine that hemA might encode ALA synthase, whose action or synthesis is facilitated by hemL. Here I report the cloning and sequencing of the S. typhimurium hemA gene. The sequence showed that the HemA protein is not similar to known ALA synthases. Although E. coli and S. typhimurium strains carrying hemA on a plasmid overproduce ALA and tetrapyrroles, I could not find ALA synthase activity in crude extracts. At present, the mechanism of ALA synthesis in enteric bacteria remains unclear.

Previously, we had been unable to isolate transposon insertions in the hemA gene by in vivo techniques, and several observations led to the idea that insertions in hemA might not have been recovered because of their polarity on an essential gene downstream. The DNA sequence reported here shows that the prfA gene encoding polypeptide chain release factor 1 (RF-1), which is essential for life, lies downstream of hemA. Genetic studies on the polarity of a hemA::Kan insertion mutation show that the two genes are cotranscribed. This operon organization and features of the DNA sequence between hemA and prfA are incorporated into a model for autogenous regulation of RF-1 expression.

MATERIALS AND METHODS

Bacterial strains. E. coli and S. typhimurium strains used in this study are listed in Table 1. E. coli MH-1 (21) was used as the host for transformation when plasmids were constructed. Plasmids were tested for their Hem phenotype by investigating whether they complemented the hemA mutation present in SASX41B. E. coli TE1335 carries a derivative of the plasmid F'128 (pro⁺ lac⁺); this plasmid has an

TABLE 1. Bacterial strains

Strain	Genotype	Source
E. coli		
HB101	F⁻ λ⁻ hsdS20 (r_B⁻ m_B⁻) recA13 leu-6 thi-1 supE44 lacYl galK2 ara-14 xyl-5 mtl-1 proA2 rpsL20	9
MH-1	araD139 Δ(lac)X74 galU galK hsdR (Str^r)	MC1061 Leu⁺ (21)
CLT43	F⁻ Δ (argF-lac)U169 rpsL150 thiA1 relA1 deoC1 ptsF25 flbB5301 rbsR car-94 srl-300::Tn10 recA56	51
SASX41B	HrfPO2A relA1 spoT1 metB1 hemA41	B. Bachmann (CGSC 4806)
TE1335	trp Δ(lac)X74 Str^r [F'128 (P22 HT105/1 int-201 sieA44)]	This study
S. typhimurium		
TR1810	LT-7 proAB47 (F'128 pro⁺ lac⁺)	J. Roth
TR5877	(SL4213 gal⁺) hsdL6 hsdSA29 (r_{L-T}⁻ m_{L-T}⁺ r_S⁻ m_S⁺) metA22 metE551 ilv-452 trpB2 xyl-404 rpsL120 (Str^r) H1-b H2-e.n,x (Fels2⁻) nml	B. A. D. Stocker
TT7333	hisG9424::Tn10 rho-111(Ts)	J. Roth (27)
TT7334	hisG9424::Tn10 rho⁺	J. Roth (27)
TE768	araC1 DUP[(cob-4)*Tn10*(zdd-1852)]^a	
TE1141	zde-1858::Tn10d-Tet hemA423(Am)	Elliott and Roth, in press
TE1142	zde-1858::Tn10d-Tet hemA424(Am)	Elliott and Roth, in press
TE1145	zde-1858::Tn10d-Tet hemA427(Am)	Elliott and Roth, in press
TE1468	leuA414 (Am) hsdL (r_L⁻ m_L⁺) (Fels2⁻) zde-3634::MudF (Lac⁺)	Elliott and Roth, in press
TE1478	leuA414 (Am) hsdL (r_L⁻ m_L⁺) (Fels2⁻) zde-3634::Mud-P	This study
TE1479	leuA414 (Am) hsdL (r_L⁻ m_L⁺) (Fels2⁻) zde-3634::Mud-Q	This study
TE1480	leuA414 (Am) hsdL (r_L⁻ m_L⁺) (Fels2⁻) zdf-3635::Mud-P	This study
TE1481	leuA414 (Am) hsdL (r_L⁻ m_L⁺) (Fels2⁻) zdf-3635::Mud-Q	This study
TE1749	leuA414 (Am) hsdL (r_L⁻ m_L⁺) (Fels2⁻) recA1	This study
TE2084	araC4 hsdL (r_L⁻ m_L⁺) (Fels2⁻)	This study
TE2470	araC1 DUP[(hemA702::Kan cob-4)*Tn10*(zdd-1852)]	This study
TE2498	LT-2(pBR322)	This study
TE2499	LT-2(pRF1)	70

^a This strain carries Tn10 at the join point of a tandem duplication.

integrated P22 prophage with both an HT (high frequency of transduction) and an int⁻ (integration-defective) mutation. The lysogen was constructed by first forming a P22 prophage in S. typhimurium TR1810. This strain carries a deletion of the chromosomal P22 attachment site, and the prophage has integrated at the P22 attachment site carried on F'128 (26). Lysogenization was accomplished by complementation with an int⁺ helper phage (MS544 is P22 c₂⁻ sieA44 from M. Susskind). Subsequently, the plasmid and integrated prophage were moved to E. coli by conjugation, selecting for transfer of Lac⁺ (26). Both spontaneously and upon induction, strain TE1335 releases phage P22. These phage are all int⁻; thus, TE1335 is likely to be a double lysogen (60). P22 phage was grown in E. coli MH-1 or HB101 carrying recombinant plasmids by zygotic induction as described below. The recA mutant E. coli HB101 and CLT43 strains were transformed with various plasmids and used for analyzing plasmid-encoded proteins by the maxicell method as described below.

Media and growth conditions. E medium (68) supplemented with 0.2% glucose was used as standard minimal medium. NB medium (8 g of Difco nutrient broth with 5 g of NaCl per liter) and LB medium (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) were used as rich media. Difco Bacto-Agar was added at a final concentration of 1.5% for solid media.

Auxotrophic requirements were satisfied by inclusion of the appropriate supplement in minimal medium at final concentrations as specified previously (17). Supplementation with 5-aminolevulinic acid hydrochloride (ALA; Sigma Chemical Co.) was at 200 ng/ml (1.2 μM) in minimal medium and 20 μg/ml (120 μM) in rich medium. Antibiotics were added to final concentrations in rich medium as follows: sodium ampicillin, 30 μg/ml; chloramphenicol, 20 μg/ml;

kanamycin sulfate, 50 μg/ml; and tetracycline hydrochloride, 20 μg/ml. Defined amino acid mix lacking methionine was made as a 200-fold-concentrated stock and added to minimal medium as described previously (48).

The Hem⁻ phenotype was tested by streaking for single colonies on NB agar or NB agar plus 150 μM ALA. Hem⁻ strains form only tiny colonies on NB agar after 3 to 4 days under these conditions (56, 57; Elliott and Roth, in press), whereas they grow normally on NB agar plus ALA.

Transductional methods. Transductional crosses were carried out in S. typhimurium as previously described (18, 28). Phage P22 was grown in E. coli by mixing 0.1 ml each of an overnight culture of strain TE1335 and of a strain carrying a recombinant plasmid, diluting the mixture into 2 ml of LB broth, and shaking it overnight at 30°C. Phage titers were usually 1 × 10⁸ to 5 × 10⁸ PFU/ml. To transduce plasmids into S. typhimurium, 0.05 ml of a lysate was mixed with 0.2 ml of an overnight culture of the restriction-defective strain TR5877 or strain TE1749, incubated 20 min at 37°C, and centrifuged for 1 min in a Microfuge to separate transductants from β-lactamase in the supernatant. Transductants were suspended in 50 mM sodium phosphate, pH 7.0–0.85% NaCl (61) and plated on selective medium. In some experiments, P22 stocks were purified by centrifugation before use.

Manipulation of recombinant plasmids. Plasmids were isolated by alkaline lysis (4). Restriction enzyme digests were carried out in the buffers described by Davis et al. (17). Gel electrophoresis and DNA ligation were performed by using standard techniques (42). E. coli cells competent for transformation were prepared as described previously (41, 45) and stored at −70°C in 15% glycerol (47). Transformation was performed with some of the modifications described by Hanahan (22). After heat shock, cells were grown for 45 min

at 37°C in 10 volumes of SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 1 mM MgSO₄, 1 mM MgCl₂, 20 mM glucose) before being plated on LB–ampicillin (30 μg/ml)–1 mM MgSO₄. This procedure routinely gave 5×10^4 transformants with 10 ng of pBR322 DNA and *E. coli* MH-1 as the host.

Isolation of the *hemA* gene. Attempts to clone the *hemA* gene by selecting for complementation of an *S. typhimurium* *hemA* mutant, using available plasmid libraries carrying *S. typhimurium* genomic DNA, were not successful. I attribute this to instability of the *hemA* gene on high-copy-number plasmids in *S. typhimurium*. To overcome this problem, I used Mud-P22 phages (72) to construct lysogens of P22, in which a locked-in P22 prophage is integrated near the *hemA* gene.

Mud-P22 hybrid phage contain nearly all the P22 genes, but they lack the *immI*-gene 9-*att-int* region. These phage are inserted in the bacterial chromosome via Mu *att* ends, which allow the phage to be substituted at the site of existing Mu insertions or to be introduced at new sites by Mu-mediated transposition. When a Mud-P22 prophage is induced by inactivation of the P22 c_2 repressor, the phage cannot excise because the P22 excision machinery is missing; however, it does replicate in situ. In the ensuing developmental cycle, a lysate is produced which consists mainly of P22 phage particles that carry DNA packaged in several sequential headfuls, starting at the phage *pac* site and extending to the right relative to the phage genetic map. The first headful of DNA includes about one-third of the phage genome (16 kilobases [kb]) and 28 kb of adjacent *S. typhimurium* chromosomal DNA. Subsequent headfuls contain only host chromosomal DNA. Thus, Mud-P22 phage are a type of defective, specialized transducing phage.

I isolated four *S. typhimurium* strains lysogenic for Mud-P22 hybrid phage (TE1479 to TE1482). At each of two sites on either side of *hemA*, I isolated prophages that package in either direction. Two of these four strains produced *hemA* specialized transducing phage after mitomycin C treatment. A lysate of each Mud-P22 strain was tested for the ability to transduce *hemA*⁺ compared with an unlinked marker, *leuA*⁺ (after the particles were treated with purified P22 tail protein, a gift of M. Susskind). The Mud-Q prophage at each site packaged *hemA*⁺ at high frequency. Gel electrophoresis of DNA purified from these particles and digested with *EcoRI* showed several common fragments packaged only by the Mud-Q prophages (data not shown).

Plasmid constructions. DNA purified from particles produced after mitomycin C induction of the *zde-3634*::Mud-Q insertion strain (TE1479; Table 1) was digested with *XbaI* and *BamHI* and ligated to pBR322 plasmid DNA that had been digested with *NheI* and *BamHI*. One resulting plasmid, pTE203, was identified that carries an insert of approximately 10 kb. DNA sequence analysis showed that the insert starts at an *XbaI* site in the Mud-P22 prophage and extends to a *BamHI* site 9.6 kb from the site of the *zde-3634*::Mud-Q insertion in the *S. typhimurium* chromosome. A second plasmid, pTE201, carries a 7.3-kb *HindIII* fragment from the same phage preparation; the insert in pTE201 starts from the single *HindIII* site found in the pTE203 insert and extends to the left (relative to the map in Fig. 1). DNA sequence analysis showed that the left end of the 7.3-kb *HindIII* fragment in pTE201 lies at codon 6 of the *prsA* gene in the sequence reported by Bower et al. (8). Plasmids pTE201 and pTE203 both complement the *hemA41* mutation present in *E. coli* SASX41B.

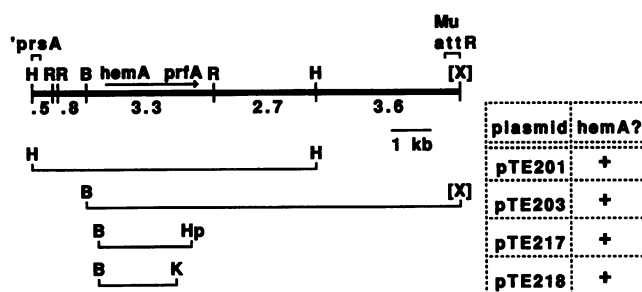Deletion mutants of plasmid pTE203 were constructed by

FIG. 1. Physical map of the *hemA* region. A segment of 10.5 kb of *S. typhimurium* DNA is indicated by the bold line, including a portion of the *prsA* gene and extending to the site of the *zde-3634*::Mud-Q insertion. This map is oriented opposite to the standard genetic map. The *hemA* and *prfA* genes map within the 3.3-kb *Bam*HI-*Eco*RI fragment and are transcribed from left to right. Also shown are the DNA segments present in several plasmids which carry the *hemA* gene. Additional deletion mutants are described in Table 2 and Fig. 4. Restriction sites: R, *Eco*RI; H, *Hind*III; B, *Bam*HI; X, *Xba*I; Hp, *Hpa*I; K, *Kpn*I. Numbers indicate kilobases.

unidirectional exonuclease III digestion (24) followed by linker tailing (36). *Bam*HI and *Sph*I each cleave pTE203 once, resulting in a large DNA fragment with a *Bam*HI end adjacent to the *hemA* gene and an *Sph*I end (resistant to exonuclease III) in the middle of the pBR322 tet region. After digestion for various distances into the *hemA* region, the deletion endpoints were joined to the pBR322 *Sph*I site by using a *Bam*HI linker.

Derivatives of pTE203 were constructed in which all the DNA downstream of the *Kpn*I or *Hpa*I sites in the *prfA* gene was deleted (pTE217 and pTE218). These plasmids were constructed by digesting pTE203Δ43 with *Hind*III plus *Kpn*I or *Hpa*I, followed by polishing with Klenow fragment and ligation. The DNA sequence of the new joints matched that predicted from the specificities of these enzymes.

Plasmids carrying mutant *hemA* amber alleles were constructed as follows. A strain bearing both the *zdf-3635*::Mud-Q insertion and a *hemA* amber mutation was constructed by transduction using transducing phage P22 grown on the *hemA* mutant as the donor and selecting inheritance of the linked Tn*10d*-Tet element. The resulting lysogen was induced with mitocmycin C, and the 6.0-kb *Bam*HI-*Hind*III fragment (Fig. 1) was cloned into pBR322. Subsequently, the 2.4-kb *Bam*HI-*Kpn*I fragment was subcloned to generate the plasmids pTE248 and pTE249. DNA sequence analysis used primers designed for sequencing the wild-type *hemA* gene.

Other plasmids were constructed as described in Table 2. The general method (69) was to isolate appropriate restriction fragments on standard agarose gels and then to dissolve slices of gel containing the desired fragments with sodium iodide. A commercial silica gel preparation (GeneClean; Bio 101) was used to purify the DNA by the recommended procedure of the manufacturer.

DNA sequencing. Double-stranded plasmid DNA (CsCl purified; 2.5 μg) was denatured in 80 μl of 0.2 M NaOH–1 mM EDTA (14) for 5 min at room temperature, neutralized with 24 μl of 7.5 M ammonium acetate, and ethanol precipitated. The DNA pellet was rinsed with 70% ethanol and dried and was then suspended in 7 μl of water. The primer extension method employed Sequenase (U.S. Biochemical Corp.) used according to the instructions of the manufacturer (62) with [α-³²P]dATP (Amersham Corp.; 1,000 to 3,000 Ci/mmol) as the label. Sequencing reactions were analyzed on field gradient (wedge) gels (0.2 to 1 mm;

TABLE 2. Plasmids constructed in this study

Plasmid	Description
pTE201	7.5-kb *Hin*dIII fragment carrying *hemA* in *Hin*dIII site of pBR322 (Fig. 1) *prsA* sequences on *bla* side
pTE203	9.9-kb *Bam*HI-*Xba*I fragment with *hemA* in pBR322 (*Bam*HI and *Nhe*I sites)
pTE203(Δ43)	*Exo*III-generated deletion of pTE203, joins bp −453 of sequence in Fig. 2 through *Bam*HI linker to the *Sph*I site of pBR322 (*Sph*I site not regenerated)
pTE203(Δ40)	Similar deletion to Δ43, ending at bp −38
pTE203(Δ33)	Similar deletion to Δ43, ending at bp 70
pTE203(Δ27)	Similar deletion to Δ43, ending at bp 458
pTE203(Δ42)	Similar deletion to Δ43, ending at bp 982
pTE212	Deletion of plasmid pTE203, joins *Eco*RI site downstream of *prfA* to *Eco*RI site of pBR322
pTE217	Deletion of plasmid pTE203(Δ43), joins *Hpa*I site in *prfA* to *Hin*dIII site of pBR322
pTE218	Deletion of plasmid pTE203(Δ43), joins *Kpn*I site in *prfA* to *Hin*dIII site of pBR322
pTE219	3.3-kb *Eco*RI-*Bam*HI fragment with *hemA* and *prfA* in pHSG415; also has *E. coli araC*
pTE232	Deletion of plasmid pTE203(Δ27), joins *Kpn*I site in *prfA* to *Hin*dIII site of pBR322 (same as pTE218 but carries Δ27)
pTE233	*Exo*III-generated deletion of pTE203 from the 3' side of *hemA*, joins bp 923 through a *Bgl*II linker to the *Eco*RI site of pBR322 (*Eco*RI site not regenerated); this deletion is Δ77
pTE238	Substitute *Bam*HI-*Kpn*I fragment of pTE203(Δ42) into pTE217 (Fig. 3)
pTE245	Substitute *Bam*HI-*Kpn*I fragment of pTE203 into pTE217
pTE246	Substitute *Bam*HI-*Kpn*I fragment of pTE203(Δ40) into pTE217
pTE247	Substitute *Bam*HI-*Kpn*I fragment of pTE203(Δ33) into pTE217
pTE248	Same as pTE245 except *hemA423* (Qam369)
pTE249	Same as pTE245 except *hemA427* (Qam241)
pTE275	pTE219 carrying *hemA702*::Kan (see Materials and Methods for details)

International Biotechnologies) under standard conditions. Gels were fixed in 10% acetic acid, dried, and exposed to X-ray film without a screen.

The deletion mutant derivatives of pTE203 were sequenced by using the oligonucleotide (5'-CAGTAGTAGGT TGAGG-3'), which matches DNA about 50 base pairs (bp) clockwise of the *Sph*I site in pBR322. After most of one strand of the *hemA* sequence had been derived in this way, primers were constructed to close several gaps and to determine the sequence of the second strand. (The sequences of these primers are available on request.) The sequence shown in Fig. 2 was determined on both strands. The junction sequences introduced in constructing pTE217 and pTE218 were determined with the pBR322 *Eco*RI clockwise primer (New England BioLabs, Inc.).

Recombination analysis of deletion plasmids. Deletion mutant plasmids were constructed in *E. coli* and transduced into *S. typhimurium* as described above and then were transduced into a strain carrying the *hemA* mutation to be tested (Table 1), selecting for Amp^r on NB plates containing ALA. Single colonies were purified twice on NB-ampicillin plates containing ALA and grown in duplicate cultures in NB-ALA, and samples were washed with 50 mM sodium phosphate, pH 7.0–0.85% NaCl and plated on NB agar either with or without ALA. The number of Hem$^+$ recombinants ob-

served depended on the time after plating (recombinants continue to arise on the selective plates). Plates were scored after 24 h at 37°C. When Hem$^+$ recombinants were observed, they arose at a frequency of about 10^{-3} compared with the number of colonies on NB agar with ALA, and this value was remarkably consistent for different mutants. In experiments not reported here I detected recombinants at a frequency of 3×10^{-5} for an amber mutation which lies 25 bp from the tester plasmid deletion endpoint.

Maxicell analysis of plasmid-encoded proteins. Plasmids to be analyzed (see Results) were introduced into *E. coli* HB101 or CLT43 by transformation. Maxicells were prepared as described by Silhavy et al. (59) with some modifications (20). Plasmid-bearing strains were grown overnight at 37°C in minimal E medium–0.2% glucose–50 μg of ampicillin per ml–19 amino acids (no methionine)–thiamine, diluted 1:20 in the same medium, and grown at 37°C in a shaking water bath to an A_{650} of 0.2 to 0.4. Cell suspension (10 ml) was pelleted, suspended in 20 ml of 10 mM MgSO$_4$, transferred to sterile plastic petri dishes, and exposed to UV light (200 μW/cm^2) for 15 to 30 s. The optimum time was determined empirically. Under dim light, irradiated cells were pelleted, suspended in 10 ml of medium as described above (minus ampicillin), and then transferred to foil-wrapped tubes to prevent photoreactivation. The tubes were incubated at 37°C for 1 h to allow recovery of viable cells, and then 100 μl of D-cycloserine (50 mg/ml, freshly prepared) was added to the tubes and the incubation was continued overnight at 37°C. Cells were washed twice in 10 mM MgSO$_4$ and suspended in 250 μl of medium (minus ampicillin) and incubated at 37°C for 30 min. A 200-μl sample of cells was then added to 2 μl (20 μCi) of [^{35}S]L-methionine (Amersham; 1,000 Ci/mmol) and labeled for 30 min at 37°C. Following this, the cells were washed with phosphate-buffered saline and suspended in protein gel sample buffer (1% sodium dodecyl sulfate, 10 mM sodium phosphate, pH 6.8, 0.1 M dithiothreitol, 5% β-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue). Samples were electrophoresed through 12% sodium dodecyl sulfate-polyacrylamide gels essentially as described previously (1, 35). After being fixed in 25% isopropanol–10% acetic acid, gels were dehydrated in dimethyl sulfoxide, impregnated with 20% diphenylphenoxazole in dimethyl sulfoxide (5), rinsed with water, dried, and autoradiographed.

Construction of a chromosomal *hemA*::Kan insertion mutation. The *hemA*::Kan insertion mutation (see Results) was first constructed on a plasmid and then was transferred into the *S. typhimurium* chromosome. I used a method described by Matsuyama and Mizushima (43) to construct this mutant. The method uses a plasmid which is temperature sensitive for replication and a color screen to facilitate selection for transfer of an insertion mutation by recombination between a cloned copy of a gene and its chromosomal homolog. In practice, the recombination frequency at low temperature was large enough that temperature shifts were unnecessary. The original method was modified by use of the *araC* gene as the marker for screening.

To use this method I constructed the plasmid pTE219, which consists of three DNA fragments: (i) a 4.9-kb *Eco*RI-*Bam*HI fragment of pHSG415 (23) carrying *bla* and a pSC101 *ori* region whose replication protein functions at 30 but not at 42°C; (ii) a 3.4-kb *Eco*RI-*Bam*HI fragment of pTE201 (Fig. 1) carrying *hemA* and *prfA* of *S. typhimurium*; and (iii) a 1.9-kb *Bam*HI fragment carrying the *E. coli araC* gene (originally derived from pBM1 [39]). The *hemA* gene of pTE219 was then disrupted by insertion of a 1.3-kb *Bam*HI fragment

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-731                                                                                    G GATCCACTGC

-720  CGCAGGCTGT TTAACGGAAT CGGCATCCCG GTGAGTTTGC CGATCATCTC TTCGGCGTCA TCGGCGGTAT AACGCTGGCC TTTGTTATCC

-630  ACCAACTGAA CGTTGCCCGG CTGGGCGTTA AGCTCCAGCT CGGTGCTGCC CAGCGGATTG GTGAGCAGCA GGCGATAGCG ATCCTGTCCG

-540  GTCTGTTGCC AGAAGAAACG CGCATAGACT TTCTGATCAT CTGAGATGTA AGCAAAGGCG CCGCGCGTCT GGTATTGATT CAGATGACGC

-450  ACCTCTTGCT GATGCTGGCG CCACTGAGGG GAATCCGGGC TCTTGCCCGG GCCTTTATGC CCAGGAAGCG TACAGGCGGT GAGAACCAGG

-360  CTTGCTAACG GCAGTAGACG AATCAGGCGA AAATCGGGCA GGGTCATAGT GATGACGAAT CCTTGTGATA CGGTGCAATA ATTGCCTGAT

-270  GGCGTTACGC TTATCAGGCC TGAAAACTGA ACCTGCAGGC TGTAAAAACG TTCGCGCCGC GATTCGGCAA ATTTTTGCTA TCGGTTACAG

-180  TTATAGCCTT TAATGCTAGC GCCGCCTGGT GACATCGTCT ACTTTCAAGT TGTCTTAAAT CATCAAATTA GCGAGCGCTG CCAATTACTC

-90   CAAAAGGGGG CTCTCTCTTT TATTGACCAC GCGCATCCTG TATGATGCAA GCAGACTAAC CATATCAACG CTGGTACTAC TCCCGCAGAC

  1   ATG ACC CTT TTA GCG CTC GGT ATT AAC CAT AAA ACG GCA CCT GTA TCG CTG CGA GAA CGC GTA ACG TTT TCG CCG
      Met Thr Leu Leu Ala Leu Gly Ile Asn His Lys Thr Ala Pro Val Ser Leu Arg Glu Arg Val Thr Phe Ser Pro

 76   GAC ACG CTT GAT CAG GCG CTG GAC AGC CTG CTT GCG CAG CCA ATG GTG CAG GGC GGG GTC GTG CTG TCA ACC TGT
      Asp Thr Leu Asp Gln Ala Leu Asp Ser Leu Leu Ala Gln Pro Met Val Gln Gly Gly Val Val Leu Ser Thr Cys

151   AAC CGT ACA GAG CTG TAT CTG AGC GTG GAA GAG CAG GAT AAC CTG CAA GAA GCG CTG ATC CGC TGG TTA TGC GAT
      Asn Arg Thr Glu Leu Tyr Leu Ser Val Glu Glu Gln Asp Asn Leu Gln Glu Ala Leu Ile Arg Trp Leu Cys Asp

226   TAC CAT AAC CTG AAC GAG GAC GAT CTG CGC AAC AGT CTG TAC TGG CAT CAG GAC AAT GAC GCC GTC AGC CAC CTG
      Tyr His Asn Leu Asn Glu Asp Asp Leu Arg Asn Ser Leu Tyr Trp His Gln Asp Asn Asp Ala Val Ser His Leu

301   ATG CGC GTC GCC AGC GGT CTG GAT TCA CTG GTG CTG GGC GAA CCG CAA ATC CTC GGT CAG GTG AAA AAA GCG TTT
      Met Arg Val Ala Ser Gly Leu Asp Ser Leu Val Leu Gly Glu Pro Gln Ile Leu Gly Gln Val Lys Lys Ala Phe

376   GCG GAT TCG CAA AAA GGC CAC CTT AAC GCC AGC GCG CTG GAG CGA ATG TTT CAG AAG TCT TTT TCC GTC GCT AAG
      Ala Asp Ser Gln Lys Gly His Leu Asn Ala Ser Ala Leu Glu Arg Met Phe Gln Lys Ser Phe Ser Val Ala Lys

451   CGA GTG CGG ACT GAA ACC GAT ATC GGC GCT AGC GCC GTC TCC GTC GCG TTT GCC GCC TGT ACG CTC GCC CGC CAA
      Arg Val Arg Thr Glu Thr Asp Ile Gly Ala Ser Ala Val Ser Val Ala Phe Ala Ala Cys Thr Leu Ala Arg Gln

526   ATC TTT GAA TCG CTC TCG ACG GTC ACC GTA CTG TTA GTT GGC GCG GGC GAA ACC ATT GAA CTG GTG GCG CGT CAC
      Ile Phe Glu Ser Leu Ser Thr Val Thr Val Leu Leu Val Gly Ala Gly Glu Thr Ile Glu Leu Val Ala Arg His

601   CTG CGC GAG CAT AAA GTA CAA AAG ATG ATT ATC GCC AAC CGA ACC CGC GAG CGC GCG CAA GCC CTG GCG GAT GAG
      Leu Arg Glu His Lys Val Gln Lys Met Ile Ile Ala Asn Arg Thr Arg Glu Arg Ala Gln Ala Leu Ala Asp Glu

676   GTA GGC GCT GAG GTT ATC TCG CTC AGC GAT ATC GAC GCC CGT TTG CAG GAT GCC GAT ATT ATT ATC AGT TCG ACC
      Val Gly Ala Glu Val Ile Ser Leu Ser Asp Ile Asp Ala Arg Leu Gln Asp Ala Asp Ile Ile Ile Ser Ser Thr

751   GCC AGC CCG CTG CCG ATT ATC GGT AAA GGC ATG GTG GAG CGC GCA TTA AAA AGC CGT CGC AAC CAG CCG ATG CTG
      Ala Ser Pro Leu Pro Ile Ile Gly Lys Gly Met Val Glu Arg Ala Leu Lys Ser Arg Arg Asn Gln Pro Met Leu

826   CTG GTG GAT ATT GCC GTA CCG CGC GAC GTT GAA CCG GAA GTC GGC AAA CTG GCG AAC GCT TAT CTT TAT AGC GTC
      Leu Val Asp Ile Ala Val Pro Arg Asp Val Glu Pro Glu Val Gly Lys Leu Ala Asn Ala Tyr Leu Tyr Ser Val

901   GAT GAT TTA CAG AGC ATC ATT TCG CAT AAT CTG GCG CAG CGT CAG GCT GCG GCA GTA GAA GCG GAA ACG ATT GTT
      Asp Asp Leu Gln Ser Ile Ile Ser His Asn Leu Ala Gln Arg Gln Ala Ala Ala Val Glu Ala Glu Thr Ile Val

976   GAG CAG GAA GCC AGC GAG TTT ATG GCC TGG CTA CGC GCC CAG GGG GCC AGC GAG ACC ATT CGG GAA TAC CGT AGT
      Glu Gln Glu Ala Ser Glu Phe Met Ala Trp Leu Arg Ala Gln Gly Ala Ser Glu Thr Ile Arg Glu Tyr Arg Ser

1051  CAG TCG GAG CAG ATT CGT GAC GAA CTG ACT ACC AAA GCG CTG TCG GCC CTT CAA CAG GGC GGT GAT GCG CAA GCC
      Gln Ser Glu Gln Ile Arg Asp Glu Leu Thr Thr Lys Ala Leu Ser Ala Leu Gln Gln Gly Gly Asp Ala Gln Ala

1126  ATC TTG CAG GAT CTG GCA TGG AAA CTG ACC AAC CGC CTG ATT CAT GCG CCA ACG AAA TCA CTT CAA CAG GCT GCC
      Ile Leu Gln Asp Leu Ala Trp Lys Leu Thr Asn Arg Leu Ile His Ala Pro Thr Lys Ser Leu Gln Gln Ala Ala

1201  CGT GAC GGG GAT GAC GAA CGC CTG AAT ATT CTG CGC GAC AGC CTC GGG CTG GAG TAG
      Arg Asp Gly Asp Asp Glu Arg Leu Asn Ile Leu Arg Asp Ser Leu Gly Leu Glu STOP

1258  CAGCACACAC CACACTTTTT TACAGGGTGA ATTTACGCCT

1298  ATG AAG CCT TCT ATC GTT GCC AAA CTG GAA GCC CTG CAC GAA CGC CAT GAG GAA GTT CAG GCG TTG CTG GGC GAT
      Met Lys Pro Ser Ile Val Ala Lys Leu Glu Ala Leu His Glu Arg His Glu Glu Val Gln Ala Leu Leu Gly Asp

1373  GCG GGA ATT ATC GCC GAC CAG GAC CGC TTT CGC GCA TTG TCG CGC GAA TAT GCG CAA TTA AGC GAC GTT TCT CGC
      Ala Gly Ile Ile Ala Asp Gln Asp Arg Phe Arg Ala Leu Ser Arg Glu Tyr Ala Gln Leu Ser Asp Val Ser Arg
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1448  TGT TTT ACG GAC TGG CAA CAG GTT CAG GAC GAT ATC GAG ACG GCT CAG ATG ATG CTC GAC GAT CCT GAA ATG CGA
      Cys Phe Thr Asp Trp Gln Gln Val Gln Asp Asp Ile Glu Thr Ala Gln Met Met Leu Asp Asp Pro Glu Met Arg

1523  GAA ATG GCG CAG GAA GAA CTG CGC GAA GCG AAA GAA AAA AGC GAA CAA CTG GAG CAA CAG TTA CAG GTA CTG CTG
      Glu Met Ala Gln Glu Glu Leu Arg Glu Ala Lys Glu Lys Ser Glu Gln Leu Glu Gln Gln Leu Gln Val Leu Leu

1598  CTG CCG AAA GAT CCG GAC GAT GAA CGA AAC GCG TTC CTT GAG GTT CGC GCC GGT ACC GGC GGC GAC GAA GCC GCG
      Leu Pro Lys Asp Pro Asp Asp Glu Arg Asn Ala Phe Leu Glu Val Arg Ala Gly Thr Gly Gly Asp Glu Ala Ala

1673  CTG TTT GCC GGC GAT CTG TTC CGC ATG TAC AGT CGT TAT GCC GAA GCG CGC CGC TGG CGC GTG GAG ATC ATG AGC
      Leu Phe Ala Gly Asp Leu Phe Arg Met Tyr Ser Arg Tyr Ala Glu Ala Arg Arg Trp Arg Val Glu Ile Met Ser

1748  ATG AGC GAA GGC GAG CAT GGC GGT TAT AAA GAG ATC ATC GCC AAA ATC AGC GGC GAC GGC GTG TAT GGC CGA CTG
      Met Ser Glu Gly Glu His Gly Gly Tyr Lys Glu Ile Ile Ala Lys Ile Ser Gly Asp Gly Val Tyr Gly Arg Leu

1823  AAA TTT GAG TCC GGC GGA CAC CGC GTA CAG CGT GTT CCG GCG ACC GAG TCG CAG GGG CGT ATC CAT ACC TCC GCC
      Lys Phe Glu Ser Gly Gly His Arg Val Gln Arg Val Pro Ala Thr Glu Ser Gln Gly Arg Ile His Thr Ser Ala

1898  TGT ACC GTC GCC GTG ATG CCG GAG CTG CCG GAA GCC GAG CTG CCG GAT ATT AAC CCG GCG GAT CTG CGC ATT GAT
      Cys Thr Val Ala Val Met Pro Glu Leu Pro Glu Ala Glu Leu Pro Asp Ile Asn Pro Ala Asp Leu Arg Ile Asp

1973  ACG TTT CGT TCT TCC GGC GCG GGC GGT CAG CAC GTT AAC ACC ACC GAC TCC GCT ATC CGT ATT ACC CAC TTG CCG
      Thr Phe Arg Ser Ser Gly Ala Gly Gly Gln His Val Asn Thr Thr Asp Ser Ala Ile Arg Ile Thr His Leu Pro

2048  ACC GGC ATC GTG GTG GAA TGC CAG GAC GAG CGT TCG CAG CAT AAA AAC AAA GCG AAA GCG CTC TCG GTG CTC GGG
      Thr Gly Ile Val Val Glu Cys Gln Asp Glu Arg Ser Gln His Lys Asn Lys Ala Lys Ala Val Ser Val Leu Gly

2123  GCG CGC ATT CAC GCC GCC GAA ACG GCA AAA CGC CAG CAG GCC GAG GCG TCA ACG CGA CGC AAT CTG CTT GGC AGC
      Ala Arg Ile His Ala Ala Glu Thr Ala Lys Arg Gln Gln Ala Glu Ala Ser Thr Arg Arg Asn Leu Leu Gly Ser

2198  GGC GAT CGC AGC GAT CGT AAC CGG ACC TAT AAT TTC CCG CAG GGG CGC GTG ACC GAT CAT CGT ATT AAT CTG ACG
      Gly Asp Arg Ser Asp Arg Asn Arg Thr Tyr Asn Phe Pro Gln Gly Arg Val Thr Asp His Arg Ile Asn Leu Thr

2273  TTA TAT CGC CTT GAT GAA ACG ATG GAA GGT AAG CTG GAT ATG CTG ATT GAG CCG ATT GTT CAG GAA CAC CAG GCT
      Leu Tyr Arg Leu Asp Glu Thr Met Glu Gly Lys Leu Asp Met Leu Ile Glu Pro Ile Val Gln Glu His Gln Ala

2348  GAC CTG TTA GCC GCC TTA TCC GAG CAG GAA TAA  TG GAT TTT CAG CAC TGG CTG CAT GAG GCG GTA AAC CAG CTC
      Asp Leu Leu Ala Ala Leu Ser Glu Gln Glu     Met Asp Phe Gln His Trp Leu His Glu Ala Val Asn Gln Leu

2422  CGG GAC AGC GAC AGC CCC CGG CGC GAC GCC GAG ATC CTG CTA GAG TAC GTT ACG GGC AAG GGG CGG ACG TAT ATC
      Arg Asp Ser Asp Ser Pro Arg Arg Asp Ala Glu Ile Leu Leu Glu Tyr Val Thr Gly Lys Gly Arg Thr Tyr Ile

2497  ATG GCC TTT GGC GAA ACG CCG CTT ACC GAC GTC CAG CAA CAA CAG CTC GCG GAC CTG CTG CAG CGG CGT AAA CAG
      Met Ala Phe Gly Glu Thr Pro Leu Thr Asp Val Gln Gln Gln Gln Leu Ala Asp Leu Leu Gln Arg Arg Lys Gln

2572  GGC GAA CCT ATT GCG TAC CTG ACG GGC TTA CGC GAA TTC
      Gly Glu Pro Ile Ala Tyr Leu Thr Gly Leu Arg Glu Phe
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FIG. 2. DNA sequence of the 3.3-kb BamHI-EcoRI fragment. The sequence is oriented in the direction of transcription of the hemA gene. The hemA gene has coordinates bp 1 to 1257; the prfA gene extends from bp 1298 to 2380. Underlined sequences are discussed in the text.

carrying the neo gene obtained from pUC4K (67) by using an NheI site at bp 478 of the sequence shown in Fig. 2. The insertion disrupts the hemA gene at codon 160. This neo fragment encodes Kan^r, and the resulting mutation (hemA702::Kan) will be referred to here as hemA::Kan. The resulting plasmid, carrying hemA::Kan, is pTE275. The neo gene is inserted so that transcription originating at the neo promoter reads in the same direction as transcription of the hemA and prfA genes.

The plasmid, pTE275, was first transduced by phage P22 into a restriction-deficient S. typhimurium strain, TE2084, and then introduced into strain TE768. Strain TE768 carries a tandem chromosomal duplication which includes the hemA gene, extending from cysB (33.6 min on the genetic map) to cob (41 min) (Elliott and Roth, in press). It also carries a mutation in the S. typhimurium araC gene (38). A phage P22 lysate grown on TE2084 carrying the plasmid pTE275 was used as the donor in a transductional cross into strain TE768, selecting Kan^r at 30°C on plates which contained 1% arabinose and triphenyltetrazolium chloride (46). Rare Kan^r Ara$^-$ colonies were visualized as red colonies among a large number of white and light pink Ara$^+$ transductants, present at a frequency of about 0.2%. Red colonies were purified and found to be Kan^r Ara$^-$ Amp^s, indicating that the hemA::Kan insertion had been inherited but that the rest of the plasmid had been lost. Putative chromosomal hemA::Kan insertions were then transduced into strain TE768 and characterized as described in Results. The structure of the hemA::Kan insertion was verified by Southern blot analysis of chromosomal DNA purified from the insertion mutants (data not shown).

RESULTS

Isolation of the hemA gene. I used Mud-P22 phages (72) to construct P22 lysogens in which a locked-in P22 prophage is integrated near the hemA gene (strains TE1478-1481) (Table 1) (see Materials and Methods for details). A P22 preparation highly enriched for the hemA gene was used as the source of DNA fragments which were cloned in the plasmid pBR322. Individual plasmid clones carrying different DNA fragments were tested for the ability to complement the Hem$^-$ defect of an E. coli hemA mutant (SASX41B; Table 1). Two plasmids

carrying the *S. typhimurium hemA* gene were identified (pTE201 and pTE203) (Fig. 1; plasmids are also listed in Table 2).

Since the DNA inserted in these plasmids was derived from purified DNA from the *S. typhimurium hemA* region, it is likely that they carry the authentic *hemA* gene rather than a second gene which complements only at high copy number. Additional evidence was provided by the observation that pTE201 and pTE203 and derivatives can recombine with *S. typhimurium hemA* mutants to form Hem$^+$ recombinants that do not carry a plasmid (see below).

A restriction map of the *hemA* region was generated by analysis of pTE201 and pTE203 (Fig. 1). This restriction map is presented with an orientation opposite to that of published genetic maps (*trp* to the right, *his* to the left) (54; Elliott and Roth, in press). Together, these plasmids contain about 10.5 kb of *S. typhimurium* DNA. The plasmid pTE203 also carries 0.5 kb of DNA derived from the Mud-P22 phage including Mu *attR*; the presence of Mu DNA was confirmed by DNA sequencing with a Mu *attR*-specific primer. The left end of the map shows that pTE201 carries DNA including the upstream region and five codons of the *S. typhimurium prsA* gene encoding PRPP synthetase (8). The presence of *prsA* sequences also was confirmed by DNA sequencing (data not shown).

Characterization of deletion mutants. Deletion mutants of pTE203 localized the *hemA* gene (the data are summarized in Fig. 1). Exonuclease III-generated deletion mutants of pTE203 were constructed, starting from the *Bam*HI site near the left end in Fig. 1 and proceeding to the right. Natural restriction sites were also used to localize the gene starting from the right side of the map. The deletion plasmids were tested for the ability to complement *E. coli* SASX41B. Whereas 278 bp could be deleted extending to the right of the *Bam*HI site (Fig. 1) without loss of ability to complement for *hemA* function (plasmid pTE203Δ43) (Table 2), a plasmid with a deletion of 643 bp only complemented weakly (pTE203Δ40). Larger deletions abolished complementation. The smallest DNA fragment that retained *hemA* complementing activity was a 2.1-kb *Bam*HI-*Kpn*I fragment carried by plasmid pTE218 (Fig. 1).

DNA sequence of the *hemA* gene. The DNA sequence of *hemA* was determined by using the deletion plasmids as templates for primer extension. Given the sequence of one strand obtained by this method, oligonucleotide primers were constructed to sequence the complementary strand (Fig. 2). The *hemA* gene is transcribed from left to right relative to the map in Fig. 1. The region sequenced extends from the *Bam*HI site 3.3 kb past the 3' end of *hemA* to an *Eco*RI site. It is numbered so that the *hemA* gene begins with bp 1. The sequence also includes the *prfA* gene (see below).

The sequence contains three long open reading frames. The *hemA* gene is the open reading frame which starts with an AUG at bp 1 and ends with a UAG at bp 1255. (Codons are numbered according to the position of the 5' nucleotide in the sequence). Translation of this open reading frame would result in synthesis of a polypeptide of 418 amino acids and a molecular weight of 46,080. Codon preference analysis showed that the *hemA* gene has a pattern of codon usage similar to that of weakly expressed *E. coli* genes (although the asparagine codon AAC is strongly preferred to AAT—an apparent exception to the general pattern).

Downstream of the *hemA* gene is a second coding sequence which starts with an AUG at bp 1298 and extends to a UAA codon at bp 2378. The polypeptide sequence predicted for this open reading frame differs at only 11 codons
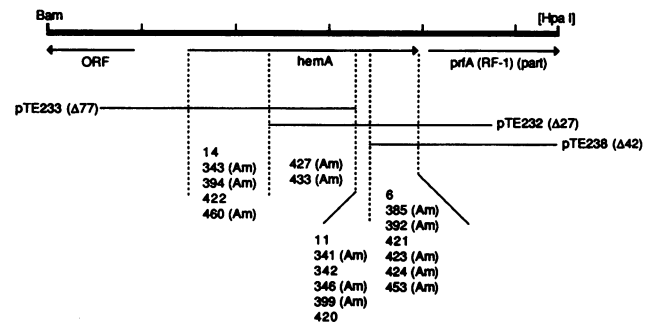

FIG. 3. Recombination mapping of *hemA* mutants. The bold line shows a 2.65-kb region extending from the *Bam*HI to the *Hpa*I sites, and the extent of *hemA* and *prfA* material included in several plasmids is indicated. Twenty *S. typhimurium* mutations were mapped to four intervals in the *hemA* gene, as described in the text and as indicated below the sequence. ORF, open reading frame.

from that deduced for the *E. coli* protein release factor 1 (RF-1) from the sequence of the *E. coli prfA* gene (16, 37). I suggest that this open reading frame is the gene for *S. typhimurium* RF-1 (*prfA*). Translation of this open reading frame would result in synthesis of a polypeptide of 360 amino acids and a molecular weight of 40,416. There is also a short open reading frame downstream of *prfA* which extends to the *Eco*RI site. Finally, there is a third long open reading frame upstream of *hemA* which has the opposite polarity. It starts with an AUG at bp −314 and extends to the *Bam*HI site.

The 418-codon open reading frame is the *hemA* gene. Three observations prove that the open reading frame (bp 1 to 1257) corresponds to the *hemA* gene. First, the plasmid pTE203Δ40, which lost upstream sequences to bp −39, had reduced *hemA* complementation. The deletion in plasmid pTE203Δ33, which extends to bp +69, eliminated complementation. At the 3' end of *hemA*, the plasmid pTE218 lacks DNA downstream of the *Kpn*I site at bp 1649 but retained complementation ability.

Second, plasmids carrying various deletions of the *hemA* region were transferred to *S. typhimurium* and tested for recombination with *hemA* mutations present on the bacterial chromosome (see Materials and Methods for details) (Fig. 3). Twenty chromosomal *hemA* mutants were mapped against three plasmids. All the *hemA* mutants gave Hem$^+$ recombinants with at least one plasmid. Only a background level of Hem$^+$ revertants was seen in control experiments with pBR322. These experiments show that the plasmids carry the *S. typhimurium hemA* gene, and they also indicate that the *hemA* region probably does not hold a second gene required for a Hem$^+$ phenotype, since all 20 mutants of the *hemA* locus tested mapped to this gene. This preliminary deletion map of the *hemA* gene defined four intervals. Strictly interpreted, the data do not assign mutations in the N- and C-terminal intervals to *hemA* but only confine them to a region between the deletion joint within the *hemA* gene and a site within the upstream open reading frame (for the N-terminal group) or within *prfA* (for the C-terminal group). The mutants selected for mapping are not representative; instead, the sample was biased to include amber mutants.

Third, three amber mutations illustrated in Fig. 3 (originally isolated in *S. typhimurium*) were subcloned onto plasmids in *E. coli* and sequenced. Each mutant contains an amber mutation in the open reading frame assigned to *hemA*. The amber mutations (and corresponding amino acid
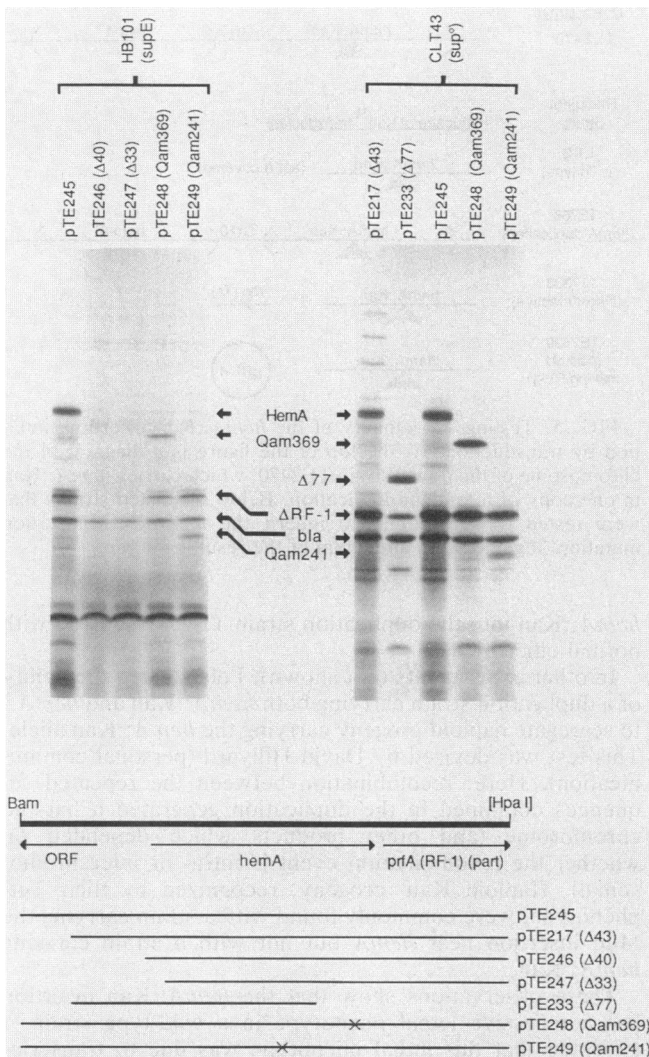
FIG. 4. Maxicell analysis. Polypeptides programmed by various plasmids were labeled in maxicells and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Plasmid-host combinations are indicated above the individual lanes, and maps of the plasmids are presented below the autoradiograms. Sites of amber mutations are indicated (×).

changes) were as follows. In *hemA423* a glutamine codon at amino acid 369 was changed to amber (abbreviated Qam369); the other mutations sequenced and the corresponding amino acid changes were *hemA424* (Wam382) and *hemA427* (Qam241). For the *hemA423* mutant, the entire *hemA* open reading frame was sequenced, and no other changes were found.

Analysis of polypeptides produced in maxicells. Analysis of the proteins produced from plasmids in maxicells showed that two polypeptides of 44 and 45 kilodaltons (kDa) were produced only by strains carrying plasmids with the *hemA* gene. The HemA protein is predicted to have a molecular weight of 46,080. I analyzed the proteins produced in maxicells by several plasmids carrying different DNA fragments from the *hemA* region (Fig. 4). The plasmid, pTE245, carries a DNA fragment extending from the *Bam*HI site (bp −731) to the *Hpa*I site (bp 2007) and directs the synthesis of two polypeptides with apparent molecular masses of 44 and 45 kDa. (These two polypeptides are better resolved in the

original autoradiogram reproduced in Fig. 4.) These polypeptides were assigned to the HemA protein on the bases of their size and the following additional data. Small deletions in this region either 5' [pTE217(Δ43)] or 3' to the *hemA* gene (pTE218; data not shown) do not alter this doublet. However, deletions which remove the predicted promoter region [pTE246(Δ40)] or extend into the N terminus of *hemA* [pTE247(Δ33)] eliminated both bands. A deletion which extends into *hemA* coding sequence from the 3' side [pTE233(Δ77)] and two different amber mutations (pTE248 and pTE249) also eliminated the doublet, and in cells carrying these plasmids, new bands appeared with sizes predicted for truncated versions of the HemA protein.

The origin of two HemA proteins is not clear. The larger protein, which might result from readthrough of the UAG codon at the end of the *hemA* gene, was also seen in a host lacking an amber suppressor mutation (CLT43) as well as in HB101, which is reported to carry *supE*. I have not confirmed directly the presence of the nonsense suppressor in HB101, but only a faint band was observed at the position of full-length HemA in HB101 with the amber mutant plasmids, pTE248 and pTE249. Readthrough of amber mutations might be affected by the presence on these plasmids of a truncated *prfA* gene, but this has not been tested. (See Discussion for further details on this question.)

A cluster of bands (31, 32, and 33 kDa) was labeled in strains with pTE245, which carries 237 codons of *prfA* fused to nine additional codons from pBR322. On the basis of other gels, as well as those shown in Fig. 4, the 32- and 33-kDa bands were assigned to a truncated RF-1 protein on the basis of the following criteria. The polypeptides migrated with an apparent molecular mass only slightly larger than the 27.5 kDa predicted from the DNA sequence. They were eliminated in a plasmid with a deletion that removes a larger part of the RF-1 coding sequence and were replaced by a smaller band of approximately the size predicted from the DNA sequence (pTE218 in Fig. 1 [protein data not shown]). They were also eliminated by a deletion which extends into *hemA* from the 3' side [pTE233(Δ77)]. The relative intensities of the bands varied depending on the host cell background. In the CLT43 host, a 31-kDa band which was not eliminated by the Δ77 deletion was evident, whereas the same deletion plasmid in HB101 gave a pattern similar to the promoter deletion plasmids Δ40 and Δ33, in which all three bands were missing (data not shown). These two hosts also showed variation in the relative intensity of pBR322-encoded proteins. Significantly, deletions which remove the region upstream of *hemA* or extend into the *hemA* coding region (pTE246, pTE247) eliminated the RF-1 cluster.

The *hemA* control region. The AUG codon at bp 1 was assigned as the N terminus of *hemA* because it is the first AUG codon in the long open reading frame encoding *hemA*. This size (418 amino acids, 46.1 kDa) is also consistent with the size of proteins seen in maxicells. However, given the lack of a Shine-Dalgarno homology preceding bp 1, it is possible that some other codon might be used to initiate translation. In considering possible alternative starts, there are few AUG or GUG codons in the region directly upstream or downstream of bp 1 in any frame. None of these has a ribosome-binding site, as judged from the DNA sequence. Potential translational coupling between a peptide in the +1 frame (ending at a UGA at bp 2) is also unlikely, since this reading frame includes multiple termination codons at bp −125, −164, and −170 upstream of *hemA*. This uncertainty will be resolved when the protein sequence is determined for HemA or a fusion protein.

The region upstream of bp 1 (Fig. 2) holds the *hemA-prfA* promoter, as determined by complementation and maxicell expression studies. This region of the sequence showed several hexanucleotides with similar sequence and spacing to *E. coli* and *S. typhimurium* promoter elements. Preliminary experiments indicate that 5' ends of RNA made in vivo map to this region (data not shown).

Region between hemA and prfA. The AUG codon at bp 1298 was assigned to be the first codon of the *prfA* (RF-1) gene by homology of the predicted polypeptide sequence with the *E. coli prfA* gene (16). As with HemA, this sequence is not supported by protein data. The AUG codon at bp 1298 does not have a good match to the Shine-Dalgarno homology in the upstream region, although the sequence GGUG is found 15 nucleotides upstream of the RF-1 AUG. In *S. typhimurium*, the reading frame which ends in an overlapping UGA codon (bp 1299) includes a termination codon upstream at bp 1212 (see Discussion). The predicted amino acid sequence for the *S. typhimurium* RF-1 protein is very similar to that predicted for the *E. coli* RF-1 protein. A correction to the sequence reported recently (37), which removed a frameshift error, is consistent with my results for *S. typhimurium*.

Comparison of S. typhimurium hemA with known ALA synthase genes. Several genes encoding bona fide ALA synthase enzymes have been cloned, and their DNA sequences have been determined (6, 40, 44, 66). As first noted by McClung et al. (44), the predicted amino acid sequence for bacterial enzymes from *B. japonicum* and *R. meliloti* (partial sequence) show striking homology to chicken embryonic liver ALA synthase. This homology extends to the *Saccharomyces cerevisiae* enzyme (66; unpublished results). The predicted amino acid sequence of the *S. typhimurium* HemA protein does not show any of these conserved regions. This suggests that if the *hemA* gene encodes an ALA synthase, it is a different type of enzyme than the ones previously characterized.

A hemA::Kan insertion mutation has a lethal phenotype. As described above, the expression of a truncated RF-1 protein in maxicells required the presence of the *hemA* promoter region, which suggests that *hemA* and *prfA* are cotranscribed. Thus, *hemA* insertion mutations might not have been recovered previously if they have a lethal phenotype due to a polar effect on transcription of the *prfA* gene. To test this model, I constructed a *hemA*::Kan insertion mutation on a plasmid and then transferred the insertion into the chromosome of a strain carrying a duplication of the *hemA* region (see Materials and Methods for details). A diagram of the relevant portion of the chromosome of this strain (TE2470) is shown in Fig. 5.

The *hemA*::Kan insertion was shown to be lethal in single copy by two different tests. First, I tested the ability of the wild-type strain, LT-2, to inherit *hemA*::Kan in a transductional cross compared with a control recipient duplicated for *hemA*, TE768 (Table 3). A phage P22 lysate grown on strain TE2470 was used to transduce LT-2 or TE768, selecting for Kan^r on medium containing ALA. (If LT-2 could inherit *hemA*::Kan, the resulting strain should be an ALA auxotroph.) Although LT-2 was able to inherit a Mud insertion tightly linked to *hemA* from the control donor strain TE1468, Kan^r transductants of LT-2 were observed with P22 phage grown on strain TE2470 only at a frequency of 10^{-3} compared with the TE1468 donor. Such rare Kan^r transductants all were found to have inherited both the *hemA*::Kan insertion and the duplication present in the donor strain (data not shown), and they had a Hem$^+$ phenotype. Transduction of
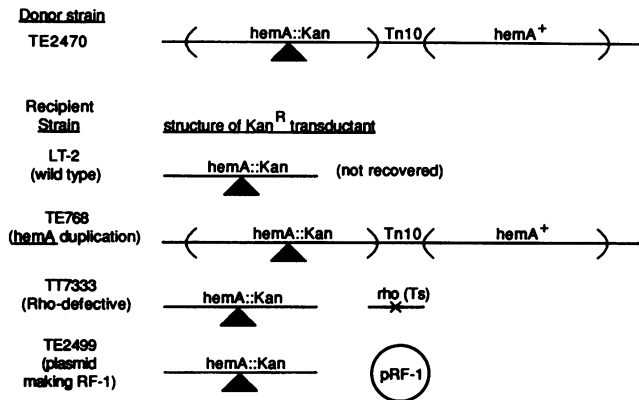

FIG. 5. Testing the lethality of the *hemA*::Kan insertion mutation by transduction. At the top of the figure is a diagram of the chromosome of the donor strain TE2470, which carries *hemA*::Kan in one copy of a tandem duplication. Below are listed strains that were tested for the ability to inherit the *hemA*::Kan insertion mutation, together with a diagram of the resulting strain.

hemA::Kan into the duplication strain TE768 occurred with normal efficiency.

In other experiments (not shown), I also tested the ability of a duplication strain carrying both *hemA*::Kan and *hemA*$^+$ to segregate haploid progeny carrying the *hemA*::Kan allele. This test was devised by David Hillyard (personal communication). Here, recombination between the repeated sequences contained in the duplication generated a haploid chromosome (and other products which depended on whether the recombination event is intra- or interchromosomal). Haploid Kan^r progeny, recognized by their Tet^s phenotype, were commonly found with a strain carrying the Mud insertion near *hemA* but not with a strain carrying *hemA*::Kan.

These observations show that the *hemA*::Kan insertion has a recessive lethal phenotype in a wild-type strain. I expected that this lethal phenotype was due to transcriptional polarity on *prfA* and possibly on other genes downstream. Therefore, I tested whether the *hemA*::Kan insertion could be inherited by LT-2 carrying the plasmid, pRF1, which expresses the *E. coli prfA* gene but does not carry

TABLE 3. Transductional inheritance of *hemA*::Kan^a

hemA::Kan recipient	Description	No. of Kan^r transductants obtained with donor phage P22 grown on:	
		Control strain TE1468 *zde*::Mud-F	TE2470 *hemA*::Kan
LT-2	Wild type	>2,000	3^b
TE768	Duplication	1,284	>2,000
TE2498	LT-2(pBR322)	1,372	3^b
TE2499	LT-2(pRF-1)	1,392	>2,000
TT7334	*rho*$^+$	480	2^b
TT7333	*rho-115*	69	>2,000

^{a} Transductions were performed with 2×10^8 recipient cells and donor phage at a multiplicity of infection of 2.

^{b} Rare Kan^r transductants of LT-2, TE2498, and TT7333 are due to inheritance of two separate fragments: both the *hemA*::Kan insertion mutation and the duplication join point of the donor strain. These transductants were Hem$^+$. When tested further by transduction to Cam^r with donor phage grown on a *trp*::Tn*10d*-Cam insertion which lies within the duplication they remained Trp$^+$.

hemA (70) (Table 3). (This plasmid also includes the open reading frame downstream of prfA.) Strains carrying hemA:: Kan and the plasmid pRF1 were constructed, and these strains grew normally, although as predicted they were auxotrophic for ALA.

If expression of the prfA gene is limited in a hemA::Kan insertion strain by transcriptional polarity, then a rho mutation might also allow a hemA::Kan insertion to be viable in haploid cells, particularly since the Kan insertion is oriented so that the neo promoter might transcribe prfA. This was tested by comparing the ability of isogenic strains carrying either the rho-111 or rho⁺ alleles (strains TT7333 and TT7334) to inherit hemA::Kan in a transductional cross. The crosses were carried out at 30°C, a temperature at which the rho-111 strain grows, although it is defective for rho function (27). The rho mutation specifically allowed inheritance of hemA::Kan, and as expected, the resulting strain was auxotrophic for ALA. This confirms that the lethal character of the hemA::Kan insertion mutation requires wild-type rho function.

In summary, these results show that an insertion mutation in hemA exhibits transcriptional polarity for expression of prfA. I conclude that transcription of prfA, at a level sufficient for growth, requires RNA polymerase to traverse the hemA::Kan insertion mutation. By inference, transcription through the hemA gene is also required for prfA expression in the wild type after transcription initiation at the hemA-prfA promoter.

DISCUSSION

In this report, I describe experiments to characterize the hemA-prfA operon of S. typhimurium. I isolated a P22 specialized transducing phage carrying the S. typhimurium hemA gene, from which about 10.5 kb of DNA was subcloned into pBR322. Complementation analysis (in E. coli) and recombination (in S. typhimurium) used deletion mutants of these plasmids to localize the hemA gene within a 3.3-kb BamHI-EcoRI fragment. The DNA sequence of this fragment was determined. The hemA gene is an open reading frame of 418 amino acids which could encode a polypeptide predicted to have a molecular mass of 46 kDa. It is transcribed counterclockwise with respect to the standard genetic map. DNA sequence analysis of hemA amber mutants confirmed that this open reading frame encodes hemA. Strains carrying a plasmid-borne hemA gene produced two polypeptides with very similar molecular masses in maxicells (44 and 45 kDa). These sizes are consistent with that predicted for the HemA protein. I discuss the possible origin of the two polypeptides below.

In spite of the dramatic overproduction of ALA and tetrapyrroles seen both in cultures and single colonies of E. coli and S. typhimurium strains carrying the cloned hemA gene, I have not been able to find ALA synthase activity in vitro in extracts of these cells. There are many possible explanations for this. However, the HemA protein lacks highly conserved amino acid sequences present in known ALA synthases (in organisms ranging from chickens to bacteria). Thus, if the hemA gene encodes an ALA synthase, that enzyme is not evolutionarily related to the known enzymes. Further work is necessary to understand the mechanism of this key reaction as well as the role played by the HemA protein.

The sequence of an open reading frame directly downstream of hemA is nearly identical to that of an E. coli gene, prfA, known to map near hemA (16). The sequence strongly

suggests that this open reading frame is the S. typhimurium prfA gene. The E. coli prfA gene encodes peptide chain termination factor 1 (release factor 1 or RF-1), which recognizes UAG and UAA chain termination codons and catalyzes release of the finished chain. Another factor, RF-2, recognizes UGA as well as UAA codons. RF-1 is essential for life, probably because amber termination codons cannot be recognized in its absence. Such amber codons are rarely used to terminate translation, but several essential genes do contain them (2). Truncated polypeptides assigned to the amino terminus of the prfA gene were seen in maxicells. Significantly, their expression required the region 5' to the hemA gene, which includes the hemA promoter.

The suggestion that hemA and prfA form an operon was confirmed by characterization of a hemA::Kan insertion mutation constructed in vitro. When transferred to the bacterial chromosome, this mutation conferred a recessive lethal phenotype, but not if E. coli RF-1 was expressed from a plasmid. The viable hemA::Kan mutant strain, with its lethal phenotype corrected by E. coli RF-1, still required ALA, as expected. Thus, expression of hemA itself is not essential for life. The lethal phenotype of hemA::Kan, caused by a failure to express prfA, was also not observed in a strain carrying a rho mutation. This indicated that the rho defect allows expression of the prfA gene from the neo promoter and (perhaps) the hemA promoter. In a hemA:: Kan rho⁺ strain, transcription initiated at these promoters does not reach prfA because of rho-mediated polarity.

The hemA promoter is apparently in the 300 bp immediately 5' to the gene, since an open reading frame extending in the opposite direction starts at bp −312 of the map in Fig. 2. This 311-bp intergenic region presumably contains control signals for the expression of the upstream gene as well as the hemA-prfA operon. Sequences with similarity to E. coli promoters are found in this region, as are several RNA 5' ends seen in primer extension assays, which indicate potential locations for the hemA promoter(s) (unpublished data). Studies are underway to identify sequence elements in this region required for hemA and prfA expression.

Since the hemA and prfA genes are organized in an operon, one might speculate that this could be used to regulate prfA expression. The hemA gene terminates in UAG, which requires the prfA gene product, RF-1, to function as a termination codon. In cells containing RF-1 at a normal concentration, readthrough of amber mutations by the suppression activity of wild-type tRNA occurs at a frequency as high as 2%, depending on the sequence nearby (7). When the effective concentration of RF-1 in vivo is increased (70) or decreased (in uar mutants [53]), readthrough is affected, consistent with in vitro results which show that RF-1 competes with suppressor tRNA species for binding to an amber codon (3, 19). Thus, readthrough of the hemA amber codon ought to occur at a rate inversely proportional to the concentration of RF-1 in the cell. Of course, special sequences or other proteins might stimulate this readthrough.

Regulated readthrough of the hemA amber codon might be used to control prfA expression through translational reinitiation. The sequence of the S. typhimurium and E. coli hemA-prfA intergenic regions is shown in Fig. 6 (the E. coli sequence is from E. Verkamp [personal communication]). The S. typhimurium sequence lacks one nucleotide present in the E. coli sequence. Nevertheless, in both species, ribosomes that read through the hemA amber codon will terminate translation downstream at a UGA codon (recognized by RF-2). In E. coli, this UGA codon overlaps the

probable RF-1 AUG initiation codon. For *S. typhimurium* the UGA codon is 10 nucleotides upstream of the RF-1 AUG codon. For both *E. coli* and *S. typhimurium*, the *prfA* gene has a short (but nevertheless recognizable) sequence complementary to the 3' end of 16S ribosomal RNA (Shine-Dalgarno homology). I suggest that the ribosome-binding site of *prfA* is a poor one and that translation termination in this region facilitates subsequent initiation. Since the rates of transcription and translation of *hemA* are unknown, I am unable to specify the efficiency of coupling that is required. The intracellular concentration of RF-1 in *E. coli* has been estimated to be 500 molecules per cell (33), and in the model this imposes a lower limit on the translation rate allowed for *hemA*. Furthermore, a strict translation reinitiation mechanism, in which RF-1 translation directly utilizes ribosomes that read through the *hemA* UAG codon, only makes sense if both long and short HemA proteins are functional. A requirement for readthrough might also bear on the scale of regulatory control allowed for *hemA* expression. At present I have no data regarding possible regulation at the *hemA-prfA* promoter.

Two proteins encoded by the *hemA* gene were observed in maxicells. The size difference between these two polypeptides (about 1 kDa) is consistent with an extension of 10 amino acids by readthrough of the *hemA* UAG codon. If the heterogeneity seen on gels reflected a difference at the C terminus of the protein, then shortened polypeptides due to amber mutation or 3'-end deletion should be homogeneous in size. In fact, the Qam369 mutant does produce a single protein band of the predicted size. However, the Δ77 deletion, which makes an even smaller polypeptide than Qam369, shows two protein bands. I sequenced the novel joint created in this mutant, and in it the *hemA* gene is fused to a reading frame ending in a UGA codon encoded by pBR322. Thus, the two HemA proteins seen in maxicells may not originate by readthrough. When the N-terminal sequence of the HemA protein is available, that will indicate whether two different protein starts are used, but from the DNA sequence the use of two different translational starts seems unlikely.

The *hemA-prfA* intergenic region also contains sequences similar to factor-independent transcriptional terminators. GC-rich split dyad symmetry or stem-loop elements (71) are indicated by arrows in Fig. 6. Two of these, labeled 1 and 2 in Fig. 6, are followed by runs of uridine residues in the RNA transcript and conform to rules that describe known factor-independent terminators (10). Another stem-loop (labeled 3 in Fig. 6) is similar but not identical to the very stable hairpins described by Tuerk et al. (65). I also noted a region of CA-rich sequence (bp 1258 to 1272) found in *S. typhimurium* but not in *E. coli*. A CA-rich sequence is also found near some sites of *rho*-factor-induced termination.

The geometry of the ribosome-mRNA-RNA polymerase interaction seems to preclude a role for regulated transcription termination in *prfA* expression. Any differential effect of RF-1 activity should depend on the ribosome reaching the *hemA* UAG codon. A ribosome with UAG in its A site should contact nucleotides in the RNA extending about 15 nucleotides downstream of the UAG (50). Since the ribosome and RNA polymerase probably do not bind simultaneously to the same nucleotides, if RNA polymerase is still bound to the transcript it must have proceeded downstream past the run of uridine residues. Transcription termination may decrease the number of transcripts extending into *prfA*, but the fraction of transcripts extended into *prfA* should not be regulated by RF-1 concentration.
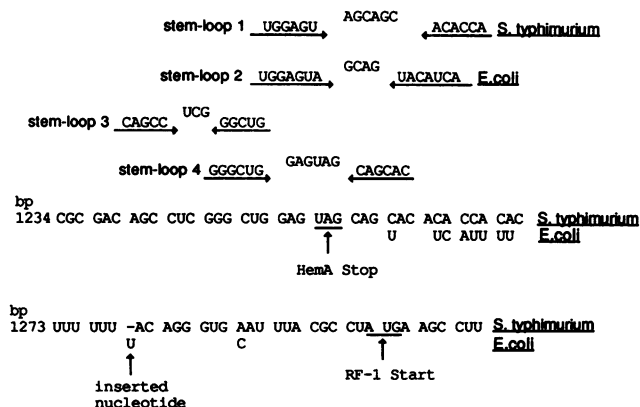

FIG. 6. Structure of the *hemA-prfA* intergenic region. The bottom half of the figure shows the sequence of the mRNA in the region between *hemA* and *prfA*. The *E. coli* sequence (E. Verkamp, personal communication) is indicated below the *S. typhimurium* sequence at the positions where it differs. Note the additional nucleotide present in the *E. coli* sequence. The top half of the figure shows four potential stem-loop structures discussed in the text. Stem-loop 1 is found only in the *S. typhimurium* sequence, while stem-loop 2 is found only in the *E. coli* sequence.

Several *hemA* amber mutations map in the N-terminal part of the gene (Fig. 3). Why don't such mutants exhibit a lethal phenotype due to polarity, as shown by the *hemA*::Kan insertion? Perhaps a similar mechanism to that proposed for the natural *hemA* amber codon also operates in such mutants; a lower concentration of RF-1 protein would allow sufficient translational readthrough by normal tRNAs to alleviate polarity. If RF-1 also negatively controlled transcription initiation upstream of *hemA*, this would contribute to increased RF-1 expression in *hemA* amber mutants. In the *hemA*::Kan insertion mutant, translation of the *neo* gene terminates with multiple UAA codons; thus, RF-2 will terminate *neo* translation leading to polarity on *prfA* expression.

Does the sequence or operon structure of *hemA* give any clue to its function in ALA and heme synthesis? As mentioned above, the HemA protein is clearly not a typical ALA synthase, and at present there is no strong biochemical evidence to favor either a C4 or a C5 mechanism. Furthermore, we need to explain the role of the *hemL* gene, which is also required for ALA synthesis. No persuasive arguments are apparent. However, it may be relevant that the C5 pathway utilizes two elements of the protein synthetic machinery: tRNA^{Glu} and glutamyl tRNA synthetase (32). This might provide (at least) an evolutionary rationale for cotranscription of *hemA* and the gene encoding a peptide chain release factor.

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ADDENDUM IN PROOF

The DNA sequence reported here has been submitted to GenBank (accession no. J04243).

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