Mol Gen Genet (1988) 214:503–508

MGG

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Nucleotide sequence of the *hemB* gene of *Escherichia coli* K12

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Summary. The *hemB* gene of *Escherichia coli* K12, coding for porphobilinogen synthase (PBG-S; syn., 5-aminolevulinic acid dehydratase, ALA-D), was cloned following insertion of an *Eco*RI fragment of plasmid F'13 into the mobilizable vector pCR1. The hybrid plasmid carrying the *hemB* gene was able to complement a *hemB* mutant of *E. coli* K12: not only was the PBG-S activity of the mutant restored after the acquisition of the *hemB* gene, but it was about ten times higher than that of the wild type. Subcloning of the original *Eco*RI fragment (14.6 kb) enabled us to locate the *hemB* gene on an *Nru*I-*Hpa*I fragment of about 1.1 kb. The *hemB* promoter was located toward the *Nru*I end of the fragment, as shown by the use of the pKO promoter-probe series of vectors. Sequencing of the *hemB* gene indicated the presence of an open reading frame (ORF) of 1051 nucleotides, which should correspond to the HemB protein. Primer extension experiments enabled us to identify the 5' end of the *hemB* mRNA, and to deduce the −10 and −35 regions of the *hemB* promoter. Protein synthesis performed by an in vitro coupled transcription-translation system, showed the presence of a protein of about 35 kDa. This is in agreement with the molecular weight of the HemB protein (35.6 kDa), as deduced from the nucleotide sequence of the gene. Comparison of the amino acid sequences of *E. coli* and human PBG-S allowed the detection of several regions of strong homology between the two proteins. Two of these regions correspond, as expected, to the putative zinc-binding and catalytic sites of the human PBG-S.

Key words: *hemB* gene of *Escherichia coli* K12 – Porphobilinogen synthase – Nucleotide sequence of *hemB* gene – Porphyrin synthesis – Highly-conserved region in PBG synthases

Introduction

The *hemB* gene of *Escherichia coli* K12 codes for porphobilinogen synthase (PBG-S; syn., 5-aminolevulinic acid deshydratase, ALA-D; EC 4.2.1.24), the second enzyme in the heme biosynthesis pathway (Granick and Beale 1978). The enzyme has been studied in a variety of organisms from bacteria (Nandi and Shemin 1968, 1973) to plant (Liedgens et al. 1983; Maralihalli et al. 1985; Shibata and Ochiai 1976) and animal cells (Anderson and Desnick 1979; Coleman 1966; Wu et al. 1984), and in all cases it seems to have an octameric structure, composed of identical subunits (M_r 31 – 35 × 10^3). The elegant work of Gibbs and Jordan (1986) identified the lysine-containing sequence, M-V-K-P-G-M, at the active site of human PBG-S, and the binding of zinc to essential SH groups of the enzyme was shown by Barnard et al. (1977); Tsukamoto et al. (1979); Gibbs and Jordan (1981). Recently, rat and human PBG-S cDNA have been isolated (Bishop et al. 1986; Wetmur et al. 1986a) and the human PBG-S gene has been sequenced (Wetmur et al. 1986b). After the work reported here was completed, a note was published on the cloning of the *hemB* gene of *E. coli* K12 by complementation of a *hemB* mutant obtained from a hemin-permeable derivative of *E. coli* K12 (Li et al. 1988).

Previous mapping of the *hemB* locus of *Escherichia coli* K12 (McConville and Charles 1979; Sasarman and Horodniceanu 1967; Sasarman et al. 1968) showed that it was co-transducible with the *lac* locus, and was located between the *lac* and the *proC* genes. In order to clone the *hemB* gene, we used plasmid F'13 (Low 1972; Hu et al. 1975), which is known to carry the corresponding chromosomal region of *E. coli* K12. Since heme-deficient mutants of *E. coli* K12 transform very poorly (Sasarman et al. 1987), the cloning of the *hemB* gene was performed in two steps, as in the case of the *hemB* gene (Sasarman et al. 1987). Here, we report the results of the molecular cloning and sequencing of the *hemB* gene of *E. coli* K12.

Materials and methods

Strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Media. Strains, except the *hemB* mutant, were routinely maintained on BHI agar (Difco) supplemented, when required, with the appropriate antibiotics (10 μg/ml tetracycline, 50 μg/ml ampicillin, 40 μg/ml kanamycin or 150 μg/ml streptomycin). The *hemB* mutant was grown on BHI broth (Difco) without aeration and anaerobic conditions were not required for growth. Davis minimal agar (Difco) or Simmons Citrate agar (Difco) with appropriate supplements (40 μg/ml amino acids, 20 μg/ml purines and pyrimidines, 4 μg/ml vitamins) were used as synthetic media. McConkey agar base (Difco) supplemented with 1% galactose and 50 μg/ml ampicillin was employed for experiments with the pKO promoter-probe series of vectors. YT medium (Miller 1972) supplemented with 0.1 mM IPTG (isopropyl-

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Table 1. List of bacterial strains and plasmids

	Relevant characteristics	Derivation	Source or reference
Derivatives of *Escherichia coli* K12			
SAS1844LP	*recA56 srlC300*::TN*10* derivative of Hfr Cavalli		This laboratory (Sasarman et al. 1987)
SAS1548	*his*$^+$, *recA* derivative of CGSC4539	Details of derivation will be provided on request	This study
CGSC4539	*pyrC ilv met his purE proC pdx cyc xyl lacY str tsx* $(\lambda)^-$ F^-		K.B. Low
SASX411JD	*hemB* mutant of SAS1548	Spontaneous mutant (neomycin selection)	This study
SAS1553JD	SASX411JD (pSAS1346JD)		This study
SAS1941JD	SAS1844LP (pSAS1313JD)		This study
SAS1942JD	SASX411JD (pSAS1313JD)		This study
SAS2017YE	SASX411JD (pSAS1343YE)		This study
SAS2018JD	SASX411JD (pSAS1344JD)		This study
SAS2020YE	SASX411JD (pSAS1341YE)		This study
Plasmids			
pSAS1346JD	F′*hemB*$^+$; (*lac-proC*) derivative of F′13	Spontaneous deletion of F′13	This study
pSAS1313JD	pCR1::*hemB*$^+$Km^r	Insertion of a 14.6 kb *hemB*$^+$ fragment in the *Eco*RI site of pCR1	This study
pSAS1323	pUC12::*hemC*$^+$Amp^r	Insertion of a *Bam*HI-*Pst*I *hemC*$^+$ fragment in pUC12	This laboratory
pSAS1341YE	pUC18::*hemB*$^+$Amp^r	Insertion of a 1.1 kb *Nru*I-*Hpa*I *hemB*$^+$ fragment in pUC18	This study
pSAS1343YE	pRK290: *hemB*$^+$Tc^r	Insertion of a 1.1 kb *Nru*I-*Hpa*I *hemB*$^+$ fragment in the low copy number vector pRK290	This study
pSAS1344JD	pBR325: *hemB*$^+$Amp^{r}Cm^r	Insertion of a 6.0 kb *Bgl*II *hemB*$^+$ fragment in pBR325	This study

β-D-thiogalactophyranoside) and 40 μg/ml X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) was used for cloning with M13mp18 and M13mp19 vectors.

Genetic techniques. Conjugation and mobilization experiments were carried out by a simplified technique, consisting in the inoculation of logarithmic phase cultures of both donor and recipient strains in 2 ml of fresh BHI broth. After 2 h of incubation at 37° C, the cells were plated on appropriate selective media. Bacterial transformation was performed essentially as described by Mandel and Higa (1970).

Enzymes and chemicals. Restriction endonucleases and DNA modification enzymes were purchased from Bethesda Research Laboratory (BRL), Pharmacia-PL Biochemicals and New England Biolabs. Various chemicals used in cloning, DNA sequencing and other molecular biology experiments were from Sigma, BRL, Pharmacia-PL Biochemicals and Boehringer-Mannheim. L-[^{35}S]-methionine was purchased from Amersham and [α-^{32}]dNTPs were from ICN.

Isolation of plasmid DNA. Large plasmid DNA was obtained according to the procedure of Hansen and Olsen (1978). For large scale isolation of small plasmid DNA, the procedure of Birnboim and Doly (1979) was used. Purification of plasmid DNA was performed by buoyant density centrifugation in CsCl gradients containing ethidium bromide (Maniatis et al. 1982). The boiling procedure of Holmes and Quigley (1981) was used for rapid analysis of recombinant plasmids.

Agarose gel electrophoresis of DNA. Plasmid and restriction fragments were analyzed by electrophoresis in 0.5% to 1.5% agarose gels as described by Maniatis et al. (1982). Restriction fragments were isolated from agarose gels after freezing in the presence of phenol, as recommended by Silhavy et al. (1984).

Enzyme assay. Bacteria were grown aerobically in BHI broth to an OD of 0.5 at 600 nm. PBG-S activity in sonic extracts of bacteria was assayed by the procedure of Sassa (1982), confirmed by Jordan (see Sassa 1982), which has already been used for *E. coli* by Li et al. (1988). Protein content was determined by the biuret method (Gornall et al. 1949) with Human Serum Albumin standard (Human Protein standards, Date Diagnostics).

DNA sequencing and computer analysis. Restriction fragments were cloned into M13mp18 or M13mp19 vectors (Norrander et al. 1983) and sequenced by the chain termination procedure of Sanger et al. (1977). The nucleotide and amino acid sequences were analyzed using the data base programs of Staden (1982) and software tools from the Institute de Recherches Cliniques de Montréal (IRCM).

Preparation of RNA. RNA was extracted and purified from bacterial cells as described by Aiba et al. (1981).

Primer extension experiments. Mapping of the 5′ end of the *hemB* mRNA obtained in vivo, was performed by primer extension experiments, as described by Vögtli and Hütter (1987), with the following modification suggested

by one of us (A. Sasarman, unpublished). We used the same unlabeled oligonucleotide for primer extension and for the sequencing of the corresponding DNA region by the dideoxy chain termination method of Sanger et al. (1977); labeling of the synthesized fragment was realized by including an [α-^{32}P]-labeled deoxynucleotide in the mixture of the four dNTPs. A similar modification of the primer extension method was published recently by Aldrich and Chakrabarty (1988).

Analysis of plasmid-encoded proteins. Proteins encoded by the different recombinant plasmids were detected by using an in vitro coupled transcription-translation system (Amersham). The proteins were separated on 15% polyacrylamide gels according to Laemmli (1970).

Results and discussion

Cloning of the hemB *gene*

Cloning of the *hemB* gene was performed in two steps, since heme-deficient mutants of *E. coli* K12 are difficult to transform (Sasarman et al. 1987). *Sal*I and *Eco*RI restriction fragments from F'*hemB*, a derivative of F'13 (Dymetryszyn 1985) were isolated from agarose gels and ligated to bacterial alkaline phosphatase-treated pCR1 vector. The ligation mixtures were used to transform SAS1844LP, a *recA* derivative of Hfr Cavalli. Transformants obtained from the *Sal*I or *Eco*RI digests were crossed to the *hemB* mutant SASX411JD, in order to detect the presence of the *hemB* gene by complementation. The use of an Hfr recipient in the first step greatly facilitated the subsequent mobilization of the vector toward the *hemB* mutant. Positive complementation was indicated by the formation of large colonies on rich media, in contrast to the dwarf colonies formed by the heme-deficient mutants (Sasarman et al. 1968). This result was subsequently confirmed by determination of the PBG-S activity in cell extracts of the HemB$^+$ transconjugants. Only one HemB$^+$ transconjugant was obtained (SAS1941JD) carrying a 14.6 kb *Eco*RI fragment (Fig. 1) inserted in the pCR1 vector (plasmid pSAS1313JD). Direct transformation of the *hemB* mutant by plasmid pSAS1313JD (pCR1: *hemB*$^+$) confirmed the presence of the *hemB* gene, though the rate of transformation was very low.

Subcloning of the initial *Eco*RI fragment (Fig. 1) by using pBR325 and the pUC series of vectors enabled us to localize the *hemB* gene to a 1.1 kb *Nru*I-*Hpa*I fragment.

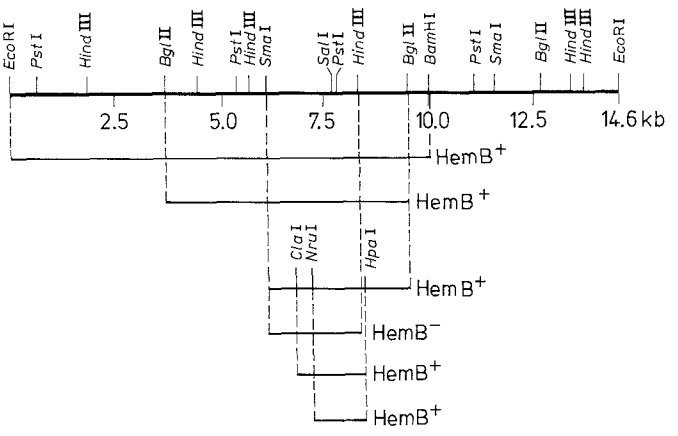

Fig. 1. Complementation of the *hemB* mutant SASX411JD. HemB$^+$, complementation by the corresponding fragment; HemB$^-$, absence of complementation

This same fragment was still able to complement the *hemB* mutant SASX411JD, when carried by the low copy number vector pRK290 (Fig. 1).

Results similar to ours were obtained recently by Li et al. (1988), who cloned the *hemB* gene of *E. coli* K12 by complementation of a *hemB* mutant obtained from a hemin-permeable derivative of *E. coli* K12. Such mutants have already been obtained in the past (McConville and Charles 1979) and have the advantage of being easier to manipulate due to their improved growth in the presence of hemin.

Use of the promoter-probe pKO series of vectors

In order to detect promoter activity of various segments of the *Nru*I-*Hpa*I insert, subfragments of the latter were obtained by digestion with various restriction endonucleases and cloned into the pKO promoter-probe series of vectors. Only one of the subfragments, namely *Nru*I-*Sau*3AI, of about 180 bp, gave a positive result when inserted in the orientation *Nru*I → *Sau*3AI. This indicates that the transcription of the *hemB* gene is oriented from the *Nru*I site toward the *Hpa*I end of the fragment, which was confirmed by subsequent sequencing of the gene.

PBG-S activity of the hemB$^+$ *clones*

The presence of the *hemB* gene in transconjugants and transformants was confirmed by the determination of the PBG-S activity in cell extracts, by the method recommended

Table 2. Porphobilinogen (PBG) synthesis as a function of incubation time of cell homogenates in the presence of Δ-aminolevulinic acid

Bacterial strain	Relevant genotype	Time of incubation at 37° C (min)			
		30	60	90	120
SASX411JD	*Escherichia coli* K12 *hemB411*	0.000	0.000	0.000	0.000
SAS1548	*Escherichia coli* K12 *hemB*$^+$ (wild type)	0.013	0.025	0.045	0.055
SAS1553JD	*hemB411*/F'*hemB*$^+$	0.020	0.038	0.050	0.066
SAS2017YE	*hemB411*/pRK290:*hemB*$^+$	0.033	0.053	0.063	0.085
SAS1942JD	*hemB411*/pCR1:*hemB*$^+$	0.143	0.255	0.358	0.473
SAS2018JD	*hemB411*/pBR325:*hemB*$^+$	0.305	0.545	0.748	0.980
SAS2020YE	*hemB411*/pUC18:*hemB*$^+$	0.751	1.133	–	–

For details see Materials and methods; results are expressed as micromoles PBG synthesized per milligram protein

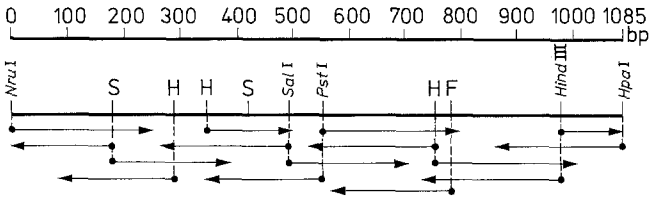

Fig. 2. Sequencing strategy and restriction map of the *hemB* gene. F, *Hin*fI; H, *Hpa*II; S, *Sau*3A. There are more *Sau*3A and *Hin*fI restriction sites than represented

by Sassa (1982), which was used recently for *E. coli* by Li et al. (1988). PBG-S activity, which was absent in the *hemB* mutant, was not only restored after the acquisition of the *hemB*$^+$ gene by the transconjugants and transformants, but was much higher than in the wild type (Table 2). The only exception is represented by strain SAS2017YE (*hemB411*/pRK290: *hemB*$^+$), where the *hemB* gene is carried by the low copy number vector pRK290 and PBG-S activity is comparable to that of the wild type. This difference is therefore probably due to a gene dosage effect since PBG-S activity was much higher in strains where the *hemB* gene was carried by a high copy number vector. The observed differences in PBG-S activities of strains SAS1942JD, SAS2018JD and SAS2020YE, where the *hemB* gene is carried by the high copy number vectors pCR1, pBR325 and pUC18 respectively, are probably again due to a gene dosage effect, since activities are roughly proportional to the known copy number of the respective plasmids.

Nucleotide sequence of the hemB *gene and mapping of the 5′ end of the mRNA*

The sequencing of the *hemB* gene was performed by the dideoxy chain termination method of Sanger et al. (1977). Both strands were sequenced starting from the *Nru*I site (position 1) to the *Hpa*I site (position 1085). The sequencing strategy is presented in Fig. 2, and the sequence itself in Fig. 3. Analysis of *Nru*I-*Hpa*I fragment in both directions, revealed the presence of a major open reading frame (ORF) of 1051 bp oriented in the direction *Nru*I → *Hpa*I. The orientation of the ORF corresponds to the orientation of the transcription of the *hemB* gene as deduced by the use of the pKO promoter-probe vectors, and, the size of the ORF is compatible with a protein of about 35 kDa as found for PBG-S from other organisms (see Introduction). Since the second largest ORF in the same orientation has only 279 bp, the only possible ORF for the *hemB* gene is the major ORF of 1051 bp, oriented from the *Nru*I to the *Hpa*I site.

At the 5′ end of the major ORF there are two potential initiation codons (positions 47 and 80 respectively) preceded by Shine-Dalgarno sequences. In order to be able to determine the ATG codon at which protein synthesis was initiated we decided to map the 5′ end of the *hemB* mRNA. Mapping of the 5′ end of the *hemB* mRNA by primer extension showed that the transcription initiates at position 53 (Fig. 4), immediately downstream from the first ATG codon (Fig. 3). Hence only the second ATG codon is located inside the *hemB* transcript and can represent the start codon for the PBG synthase. As the stop codon is located at position 1052, we can deduce a molecular weight of about 35.6 kDa for the PBG synthase. This is in agree-

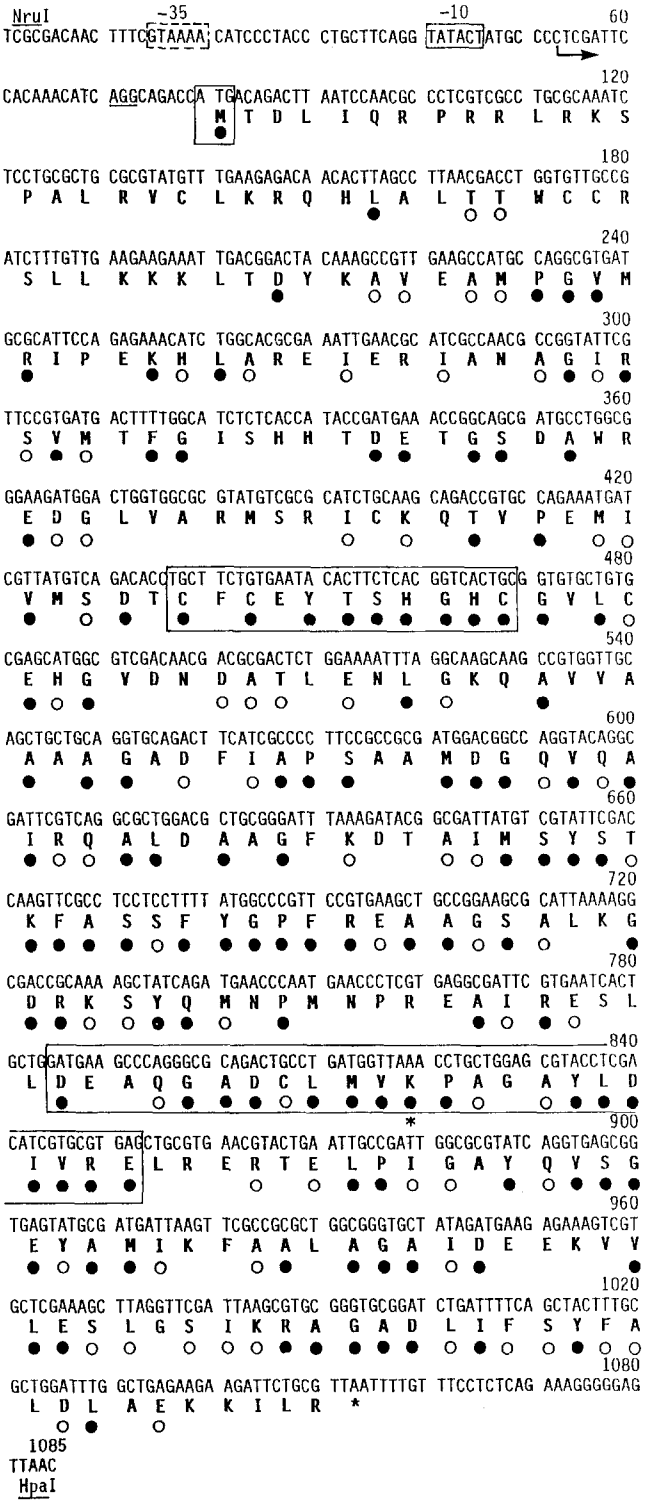

Fig. 3. Nucleotide sequence of the *hemB* gene and the decuded amino acid sequence of the HemB protein. Residues identical to the human ALAD (Wetmur et al. 1986b) and highly conservative substitutions are indicated by *closed* or *open circles*, respectively. Highly conservative substitutions were determined according to Dayhoff et al. (1972) using a relative rate of acceptance of point mutations greater than 40. The putative zinc-binding site and the region containing the active lysine (Gibbs and Jordan 1986) are *boxed;* the active lysine is indicated by an *asterisk*

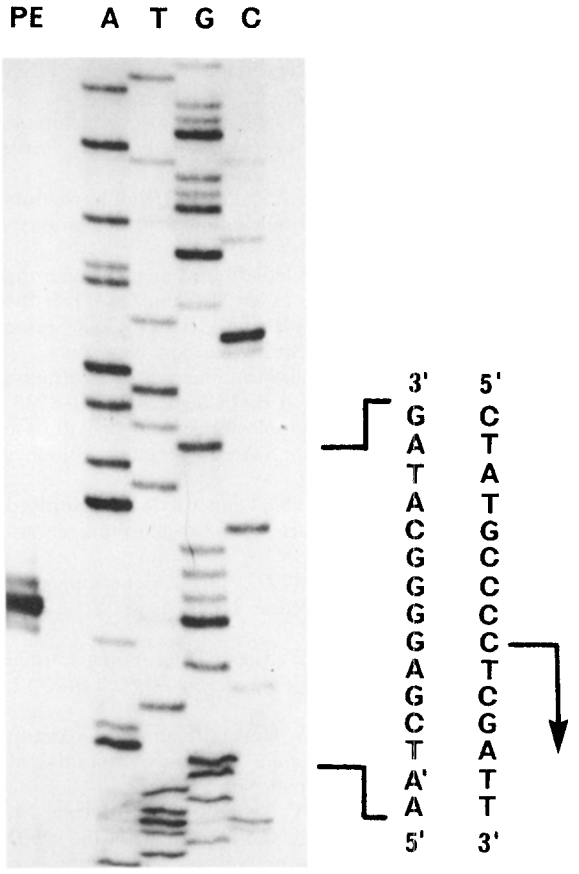

Fig. 4. Mapping of the 5′ end of the *hemB* mRNA by primer extension (left lane). Right lanes represent the sequencing of the corresponding region of DNA, by using the same oligonucleotide as for primer extension. The *arrow* at the right indicates the major start point and the direction of transcription

ment with the results obtained by the in vitro DNA-directed translation system, as shown below.

The results of the primer extension experiments also allowed us to determine the approximate location of the −10 and −35 sequences of the *hemB* promoter. The Pribnow box (−10) is very typical (TATACT), differing only by one nucleotide from the consensus sequence (TATAAT). The −35 sequence GTAAAA is rather atypical, compared with the consensus sequence TTGACA, and only the study of point mutations and deletions could allow a better definition of its position.

In vitro protein synthesis directed by the hemB *gene*

Plasmid DNA from various subclones was used in an in vitro prokaryotic DNA-directed translation system (Amersham), followed by electrophoresis in 15% SDS-polyacrylamide gels. Several molecular weight markers, including the HemC protein, M_r 33857 (Thomas and Jordan 1986) were used in order to evaluate the size of the HemB protein. The experiments showed that all $HemB^+$ subclones encoded the same protein which was slightly heavier than the *hemC* gene product (Fig. 5). Hence the molecular weight of the PBG-S subunit should be of approximately 35 kDa, in agreement with the values deduced from the sequence of the *hemB* gene (35.6 kDa). Our results confirm the data obtained recently by Li et al. (1988) for the PBG-S of *E.*

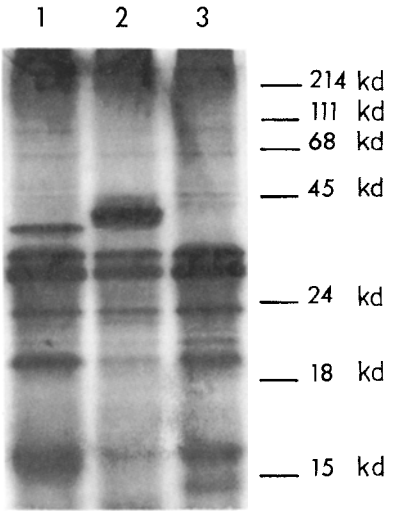

Fig. 5. Identification of the *hemB* gene product by in vitro DNA-dependent translation. The proteins were labeled and electrophoresed as described in Materials and methods. Lane 1, pSAS1323 ($hemC^+$); lane 2, pSAS1341YE ($hemB^+$); lane 3, pUC18. HemC protein was used as molecular weight standard (33857 kDa)

coli K12, and are similar to the values reported earlier for PBG-S subunits from other sources (see Introduction).

The appearance of a second lighter band, almost fused to the band corresponding to the HemB protein (Fig. 5), is not due to the use of an in vitro technique, since a similar pattern was obtained with the Maxicell method (results not shown). We cannot find an explanation for the appearance of this second band; however an early termination of the transcription cannot be excluded.

Homology between the E. coli *and the human PBG-S*

The amino acid sequence of the *hemB* gene product (324 amino acids) was compared with the human PBG-S sequence (330 amino acids) published by Wetmur et al. (1986b) (see Fig. 3). The two aligned protein sequences have 37% identical residues (closed circles); by adding the highly conserved amino acid substitutions (open circles), as described by Dayhoff et al. (1972), the percentage of homology is brought to 58%. Sequence homology is not uniformly distributed along the two molecules but is concentrated in a few regions of strong homology. Two of these regions, with known functional significance, are boxed. The first region, starting at nucleotide 437, corresponds to the putative zinc-binding site (Barnard et al. 1977; Tsukamoto et al. 1979; Gibbs and Jordan 1981) and contains the two active cysteine residues and also histidine (amino acids 120 to 130). The second region, starting at nucleotide 794, contains the lysine residue (amino acid 247) identified by Gibbs and Jordan (1986) at the active site of the human PBG-S. As expected, the sequence containing the active lysine in the human PBG-S is very similar to that found in *E. coli* K12. Of course, these structural homologies should be confirmed by appropriate biochemical determinations of the active site of bacterial PBG-S.

The carboxy-terminal regions of the two proteins are well conserved (except for the 5 last residues), and this seems to have some functional implication, since a truncated PBG-S, lacking the last 27 amino acids (obtained by using the internal *Hin*dIII restriction site), does not comple-

ment the *hemB* mutant SASX411JD, even in a high copy number vector (Fig. 1).

Acknowledgements. We gratefully acknowledge the valuable help of Denis Tardif in maintaining our collection of strains. We thank S. Kaplan for providing plasmids pRK290 and pRK2013. This study was supported by grant MT4997 from the Medical Research Council of Canada to A.S. and by grant CAFIR-132 from the Université de Montréal. Y.E. and M.D. were the beneficiaries of studentships from the Medical Research Council of Canada.

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Communicated by H. Hennecke

Received January 4, 1988 / June 6, 1988