JOURNAL OF BACTERIOLOGY, Sept. 1987, p. 4257–4262
0021-9193/87/094257-06$02.00/0

Vol. 169, No. 9

Molecular Cloning and Sequencing of the *hemD* Gene of *Escherichia coli* K-12 and Preliminary Data on the Uro Operon

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DNA of plasmid pSAS1002TH (F′ *ilv*$^+$ *hemD*$^+$ *hemC*$^+$ *cya*$^+$) was used to clone the *hemD* gene of *Escherichia coli* K-12. Due to poor transformability of the heme-deficient mutants, the restriction fragments of the F′ plasmid were first cloned into a mobilizable derivative of pBR322, pSAS1211LP, which was then mobilized into a *hemD recA* mutant (*E. coli* SASX419AN). One recombinant plasmid, carrying a *Hin*dIII fragment of about 5 kilobases (kb), was shown to complement the *hemD* mutant and also a *cya* mutant of *E. coli* K-12, as well as a *hemC* mutant of *Salmonella typhimurium* LT2. Further subcloning of the insert enabled us to locate the *hemD* gene to a *Bam*HI-*Pst*I fragment (~2.3 kb) which also carried the *hemC* gene. The *hemD* gene occupies a region close to the *Pst*I end, since the deletion of a 0.6-kb fragment from this end resulted in loss of the ability to complement the *hemD* mutation. The use of the promoter-probe vector pK01 and the results of complementation showed that the *hemD* gene was transcribed under physiological conditions from the same promoter as the *hemC* gene, the direction of transcription being *hemC-hemD*. This allows us to define a new polycistronic operon of *E. coli* K-12, for which we propose the designation Uro operon. Sequencing of the *hemD* gene showed the presence of an open reading frame (ORF) of 738 nucleotides which could code for a protein with a molecular weight of 27,766, which should correspond to the *hemD* protein; the ORF starts with the last nucleotide of the *hemC* gene, the two genes having different reading frames. An ORF of at least 480 base pairs follows the *hemD* gene after a few nucleotides. The corresponding gene X, the function of which is unknown, might represent a third member of the Uro operon.

The *hem* genes of *Escherichia coli* K-12 are responsible for the synthesis of heme, the prosthetic group of cytochromes, catalase, hemoglobin, and myoglobin (9). Due to its participation in the formation of cytochromes, heme represents an essential element of cellular respiration (14).

The role of porphyrins is not limited to respiration, since the chlorophyll and corin pathways, found in photosynthetic and vitamin B_{12}-producing organisms, respectively, also derive from porphyrins (9). Hence, porphyrins play a fundamental biological role in all living organisms from bacteria to animals, with the exception of a few anaerobic organisms (9).

The biosynthetic steps leading to the formation of heme are outlined in Fig. 1. The *hemD* gene encodes for the uroporphyrinogen-III cosynthase (E.C. 4.2.1.75), the enzyme responsible for the conversion of hydroxymethylbilane to uroporphyrinogen III (3, 12). The latter represents the last common intermediate of the three pathways, i.e., the heme, chlorophyll, and corin pathways.

Cloning of the *hemD* gene of *E. coli* K-12 was complicated by the fact that heme-deficient mutants of this organism are poorly transformable (unpublished data). Consequently, the cloning of the *hemD* gene was carried out in two steps, using a mobilizable vector. The results of the cloning and sequencing of the *hemD* gene and preliminary study of its expression allowed us to identify a new polycistronic operon of *E. coli* K-12, the Uro operon.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in the present study are listed in Tables 1 and 2.

Media. Strains were routinely maintained on brain heart infusion agar (Difco Laboratories, Detroit, Mich.), supplemented, when required, with the appropriate antibiotics (10 μg of tetracycline or 50 μg of ampicillin per ml). MacConkey agar base (Difco), supplemented with 1% galactose and 50 μg of ampicillin per ml, was used for experiments with the promoter-probe vector pK01. Phenol red agar base (Difco), supplemented with 1% maltose, was used for the study of the Cya character. YT medium (22), supplemented with 0.1 mM isopropyl-β-D-thiogalactopyranoside and 0.004% X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside), was used for cloning with M13mp18 and M13mp19 vectors.

Enzymes and chemicals. Restriction endonucleases and DNA modification enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.), Pharmacia-P.L. Biochemicals, and New England BioLabs, Inc. (Beverly, Mass.). Various chemicals used in cloning, DNA sequencing, and other molecular biology experiments were from Sigma Chemical Co. (St. Louis, Mo.), Bethesda Research Laboratory, and Boehringer Mannheim Biochemicals (Indianapolis, Ind.). L-[^{35}S]methionine was purchased from Amersham Corp. (Arlington Heights, Ill.), and α-^{32}P-nucleotide triphosphates were from ICN Pharmaceuticals, Inc. (Irvine, Calif.).

Plasmid DNA isolation. Large-scale isolation of small plasmid DNA was performed by the procedure of Birnboim and Doly (4), after amplification of plasmid DNA by chloramphenicol (170 μg/ml). Extraction of large plasmid DNA was performed by the method of Hansen and Olsen (10).

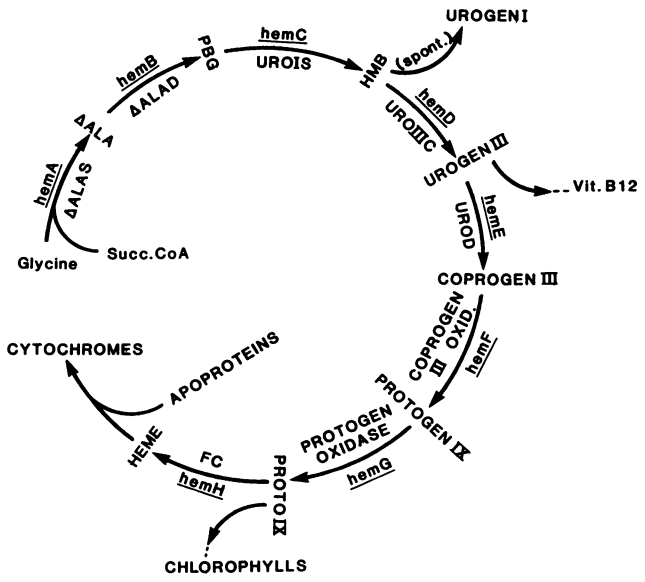

FIG. 1. Simplified scheme of the biosynthesis of heme (3, 9). Succ. CoA, Succinyl coenzyme A; Δ-ALA, δ-aminolevulinic acid; PBG, porphobilinogen; HMB, hydroxymethylbilane; UROGEN I and III, uroporphyrinogen I and III; Vit. B12, vitamin B_{12}; COPROGEN III, coproporphyrinogen III; PROTOGEN IX, protoporphyrinogen IX; PROTO IX, protoporphyrin IX; Δ-ALAS, δ-aminolevulinic acid synthase; Δ-ALAD, δ-aminolevulinic acid dehydratase; UROIS, uroporphyrinogen-I synthase (porphobilinogen deaminase); UROIIIC, uroporphyrinogen-III cosynthase; UROD, uroporphyrinogen-III decarboxylase; COPROGEN III OXID, coproporphyrinogen-III oxidase; FC, ferrochelatase.

Purification of plasmid DNA was achieved by buoyant density centrifugation in a CsCl gradient containing 0.05% ethidium bromide. For rapid analysis of recombinant plasmids, the method of Holmes and Quigley (13) was used.

Agarose gel electrophoresis of plasmid DNA. Plasmids and restriction fragments were analyzed by electrophoresis in 0.7 or 1.5% agarose gels as described by Maniatis et al. (18). Restriction fragments were isolated from agarose gels after freezing in the presence of phenol, as recommended by Silhavy et al. (34).

Restriction endonuclease digestions and ligation. Restriction endonuclease digestions of plasmid DNA were performed at 37°C (except *Sma*I at 30°C) in 10 mM Tris hydrochloride (pH 7.4)–10 mM $MgCl_2$–1 mM dithiothreitol, supplemented when required with either 50, 100, or 150 mM NaCl or 10 mM KCl, as recommended by the supplier for each enzyme. Ligation was carried out as described by Silhavy et al. (34).

Southern blot hybridization. Southern blot hybridization was performed as described by Maniatis et al. (18).

Cloning experiments. The cloning of the *hemD* gene was performed as recommended by Maniatis et al. (18) except that a mobilizable derivative of pBR322 (5), designated pSAS1211LP, was used, due to poor transformability of the heme-deficient mutants. The recombinant plasmids were first transformed into an Hfr strain (Hfr Cavalli or its *recA* derivative SAS1844) and were then mobilized by conjugation into the *E. coli hemD* mutant strain SASX419AN. Further subcloning experiments were undertaken to locate the *hemD* gene better. Appropriate DNA fragments, obtained by restriction endonuclease digestion, were inserted into the pUC series of vectors (24, 38), which were transformed directly into the *hemD* mutant. To detect false-positive complementation results, due to the high copy number of the vector, positive fragments were also tested after ligation in the low-copy-number vector pRK290 (8).

Determination of the direction of transcription. The direction of transcription was determined by the use of the promoter-probe vector pK01 (20). The DNA fragments were inserted in the pK01 vector in both orientations, and the clones were analyzed on galactose-MacConkey agar for the formation of Gal^+ colonies.

DNA sequencing. Nucleotide sequencing by the dideoxy chain termination method of Sanger et al. (30) was carried out using phages M13mp18 and M13mp19 (38) and the synthetic 17-mer primer, purchased from Boehringer Mannheim Biochemials or Pharmacia-P.L. Biochemicals.

In vitro plasmid DNA-directed translation. Proteins encoded by the different recombinant plasmids were detected by using an in vitro coupled transcription-translation system purchased from Amersham Co. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins was carried out as described by Laemmli (16). For all experiments, supercoiled plasmid molecules containing the appropriate insert were used.

RESULTS

Cloning of the *hemD* gene. DNA of plasmid pSAS1002TH (F′ *ilv*$^+$ *hemD*$^+$ *hemC*$^+$ *cya*$^+$), digested with either *Hin*dIII or *Bam*HI restriction endonuclease, was used as the starting

TABLE 1. List of bacterial strains: *E. coli* K-12 derivatives

Strain	Genotype or phenotype	Derivation	Source
SAS1844	*recA56 srlC300*::Tn*10* derivative of Hfr Cavalli	Transduction: P1(JC10240) × Hfr Cavalli	This laboratory
Hfr Cavalli	*metB1 rel-1* Hfr PO2A		W. Hayes
JC10240	*thr-300 ilv-318 rpsE300 recA56 srlC300*::Tn*10* Hfr PO45		G. Drapeau (original A. J. Clark strain)
SAS1845	SAS1702 (pSAS1002TH)		This laboratory
SAS1702	*his*$^+$ *recA1* derivative of CSH57B	Derived in several steps from CSH57B[a]	This laboratory
CSH57B	*leu purE trp his met* (*metA* or *metB*) *ile argG thi ara lacY xyl mtl gal tsx rpsL*		CSHL
SASX419AN	*hemD419 pro-36 metE lacY1 gal-6 mtl-2 recA1* λ^r *rpsL9 recA1 rel-1*(?) (λ)$^-$ F$^-$	Derived in several steps from PA6021	This laboratory
PA6021	*thr-1 leu-6 thi-1 argH1 purE43 pro-36 trp-42 lacY1 xyl-7 mtl-2 ara-13*(?) *malA1 gal-6 tonA2*(?) λ^r *rpsL9 rel-1*(?) (λ)$^-$ F$^-$		F. Jacob

[a] Details of the derivation will be provided on request.

TABLE 2. List of bacterial plasmids

Designation	Genotype or phenotype	Derivation	Source
pSAS1002TH	F′ *ilvA*$^+$ *hemD*$^+$ *hemC*$^+$ *cya*$^+$	HfrAB313 × F$^-$ (*ilv*)[a] (interrupted mating)	This laboratory
pSAS1211LP	Amp^r Tc^r ColE1^{imm} Mob$^+$	Insertion of ColE1 in the *Eco*RI site of pBR322	This laboratory
pSAS1302AN	*hemD*$^+$ *hemC*$^+$ *cya*$^+$ Amp^r ColE1^{imm} Mob$^+$	—[b]	This study
pSAS1338CG	*hemD*$^+$ *hemC*$^+$ *cya*$^+$ Amp^r ColE1^{imm} Mob$^+$	—[b]	This study

[a] This strain was derived in several steps from *E. coli* K-12 PA6021 and is not conserved in our collection.
[b] Inserts in pSAS1302AN and pSAS1338CG are identical, but oriented in the opposite direction (see Results).

material for the cloning of the *hemD* gene. The fragments were inserted into the appropriate restriction site of the mobilizable vector pSAS1211LP, and after ligation, the DNA was used to transform competent cells of Hfr Cavalli or of its *recA* derivative SAS1844. Approximately 1,200 ampicillin-resistant transformants were obtained, 562 of them being tetracycline sensitive; among the latter, 333 originated from *Hin*dIII restriction fragments and 228 originated from *Bam*HI fragments.

The tetracycline-sensitive recombinant plasmids were transferred by mobilization in the mutant strain SASX 419AN to detect complementation of the *hemD* mutation. Only three transformants, all derived from the *Hin*dIII fragments, showed positive complementation (formation of large colonies and catalase production, as compared to the Cat$^-$ dwarf colonies of the mutant). Preliminary restriction mapping of the *hemD*$^+$ recombinant plasmids revealed that two of them had undergone rearrangements; these were not studied further. The third *hemD*$^+$ recombinant plasmid, designated pSAS1302AN, did not contain rearrangements, at least as far as the vector was concerned, and was used for further characterization of the *hemD* locus.

The cloned *Hin*dIII fragment carrying the *hemD* gene was about 5 kilobases in length, as determined by agarose gel electrophoresis, and comigrated with *Hin*dIII fragment F (sixth fragment in length) of plasmid pSAS1002TH. To confirm the identity of the two fragments, we compared their restriction maps and the carried markers. In addition, Southern blot analysis was performed by using the cloned fragment as a probe. A positive signal was only obtained with the *Hin*dIII F fragment of plasmid pSAS1002TH, thus confirming their homology. For further confirmation, the *Hin*dIII F fragment of the F′ plasmid pSAS1002TH was cloned after isolation from the agarose gel. As expected, the recombinant plasmids obtained by insertion of the *Hin*dIII F fragment in the mobilizable vector complemented the *hemD* mutant, and one of these, designated pSAS1338CG, was used for further studies. Comparison of the restriction maps of plasmids pSAS1302AN and pSAS1338CG showed that their inserts were identical, but oriented in opposite directions. The two plasmids also complemented a *cya* mutant of *E. coli* K-12 and a *hemC* mutant of *Salmonella typhimurium* (data not shown), and hence the cloned *Hin*dIII fragment carried the *hemD*, *cya* (27), and *hemC* (35) genes. This is in agreement with the close location of the three genes in *S. typhimurium* LT2 (31) and allows for the precise location of the corresponding genes on the chromosomal map of *E. coli* K-12 (2, 7, 19).

To further define the location of the *hemD* gene on the *Hin*dIII fragment, the latter was digested with various restriction endonucleases and the subfragments were recloned into the high-copy-number pUC series of vectors. The presence of a functional *hemD* gene on the subfragments was detected by direct transformation of the *hemD* mutant as described in Materials and Methods. When a positive complementation was obtained, the corresponding insert was recloned into the low-copy-number vector pRK290 and the complementation test was repeated.

The smallest fragment still able to complement the *hemD* mutation was a *Pst*I-*Eco*RI fragment of 1.9 kilobases (Fig. 2). However, when the same fragment was introduced into the pRK290 vector, no complementation was observed. This shows that the *Pst*I-*Eco*RI fragment does not carry all the necessary information for full expression of the *hemD* gene under physiological conditions, i.e., when only a few copies are available in the cell. For full expression of the *hemD* gene under physiological conditions, the *Eco*RI-*Bam*HI fragment (~0.4 kilobase), adjacent to the *Eco*RI end was also required (Fig. 2).

The recent sequencing of the *hemC* gene (35) showed that this fragment contains, among others, the *hemC* promoter, which initiates the transcription of the gene from the *Bam*HI toward the *Eco*RI site. Hence we concluded that the expression of the *hemD* gene is controlled by the *hemC* promoter and, consequently, that the *hemC* and *hemD* genes form a polycistronic operon (23). In this operon the *hemC* gene occupies the proximal end and the *hemD* gene occupies the distal one, the orientation of the operon being from the *Bam*HI toward the *Pst*I site. The distal position of the *hemD* gene was confirmed by the loss of the *hemD* complementing ability of the insert after the deletion of a 0.6-kilobase *Pst*I-*Sal*I fragment from the *Pst*I end (Fig. 2).

The presence of a unique major promoter, controlling the transcription of the *hemC* and *hemD* genes in the direction indicated above, i.e., from the *Bam*HI to the *Pst*I site, was confirmed by the use of the pK01 vector system (data not shown). The promoter was located in the *Eco*RI-*Bam*HI fragment and, as expected, directed the transcription from the *Bam*HI toward the *Eco*RI site. A very weak promoter, the expression of which was barely visible on MacConkey-galactose agar plates after several days of incubation, was detected in the *hemC* structural gene. This promoter was probably responsible for the complementation of the *hemD*

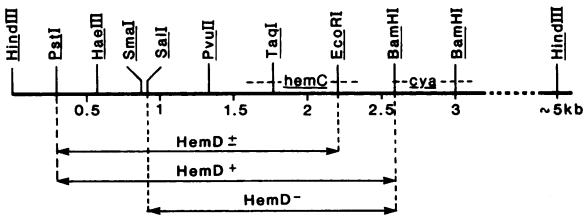

FIG. 2. Results of the complementation studies. HemD$^+$, Complementation of the *hemD* mutation by the corresponding fragment, regardless of the copy number of the vector; HemD$^\pm$, complementation of the *hemD* mutation, only when the fragment is carried by a high-copy-number vector; HemD$^-$, absence of complementation.

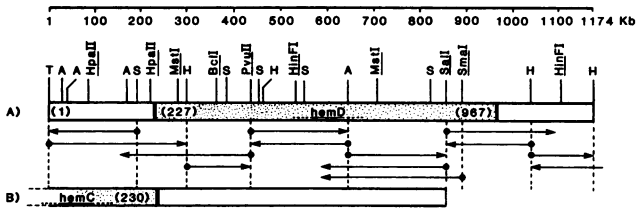

FIG. 3. Sequencing strategy and restriction map of the *hemD* gene. (A) Region sequenced by us; (B) region sequenced by Thomas et al. (35). A, *Alu*I; H, *Hae*III; S, *Sau*3A; T, *Taq*I.

mutant by the *Pst*I-*Eco*RI fragment when it was carried by a high-copy-number vector. Experiments are under way to locate this promoter and to understand its function.

Sequencing of the *hemD* gene. The sequencing of the *hemD* gene and of the flanking DNA regions was performed by the dideoxy chain termination method of Sanger et al. (30). Both strands were sequenced from the *Taq*I site, internal to the *hemC* structural gene, to the *Hae*III site, close to the *Pst*I site (1,174 nucleotides). The strategy of the sequencing is presented in Fig. 3, where the region of the *hemD* gene sequenced by Thomas et al. (35) is also indicated. One major open reading frame (ORF) starting from nucleotide 227 and ending 738 nucleotides further was identified (Fig. 4). This ORF should represent the *hemD* gene, since it extends beyond the *Sal*I site, which is located in the *hemD* structural gene as mentioned above (Fig. 2).

The initiator codon for this ORF is ATG (Fig. 4), as already mentioned for an unknown ORF starting at this point (35). The ATG codon is formed by the last nucleotide of the *hemC* gene and the two first nucleotides of the *hemC* stop codon. This situation is analogous to that found in the Trp operon, where stop codons and initiator codons overlap in the case of the *trpE trpD* and *trpB trpA* pairs, whose products are associated in multisubunit enzyme complexes (39). Though porphobilinogen deaminase, the product of the *hemC* gene, is considered an independent enzyme, the pattern of the *hemC-hemD* intergenic region may well reflect the close cooperation of the two enzymes (12), analogous to a multisubunit enzyme complex.

The ATG codon is preceded by a Shine-Dalgarno sequence (33) located about 10 nucleotides upstream. The stop codon at the end of the *hemD* gene is TAA (nucleotide 965), followed after a short interval by a second TAA codon. At some distance after the two stop codons, a new ORF of at least 480 base pairs is found, preceded by a good Shine-Dalgarno sequence (Fig. 4). This may represent a third member of the Uro operon, since no functional promoter could be found preceding the putative gene X, and its expression, as suggested by preliminary results, was dependent upon the Uro promoter (data not shown).

The amino acid sequence of the *hemD* gene, as estimated on the basis of the identified ORF, allowed us to determine the molecular weight of the corresponding protein to be 27,766. This value was compared with the results obtained in a preliminary study of the expression of the *hemD* gene in an in vitro DNA-directed translation system. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the *hemD* protein obtained by in vitro translation showed the presence of a protein having a molecular weight of about 27,000 (results not shown). Under the same experimental conditions, the truncated protein X, including 17 amino acids from its fusion with the vector, had a molecular weight of about 19,000.

DISCUSSION

Several *hem* genes of various organisms have already been cloned, and a few were even sequenced (6, 15, 17, 26, 35, 37), but this report is the first describing the cloning and sequencing of a *hemD* gene. The cloning of the *hemD* gene allowed us to identify precisely the location of the gene on the chromosome of *E. coli* K-12; in our initial genetic mapping of the *hemD* gene, we simply indicated that it was located between the *ilv* and *cya* genes (7).

The idea of a Uro operon formed by the closely located *hemC* and *hemD* genes of *S. typhimurium* LT2 (29, 31, 32), and possibly of *E. coli* K-12 (2, 7, 19), was first hypothesized in 1979 by A. Sasarman (cited in A. Nepveu, Mémoire M.Sc., Université de Montréal, Montreal, Quebec, Canada, 1981) to explain the absence of accumulation of uroporphyrin I in cultures of actively growing bacteria. This idea was given weight by the results of the cloning of the *hemD* gene of *E. coli* K-12, showing that the *hemC* and *hemD* genes are carried by the same *Hin*dIII fragment.

More recently, Thomas et al. (35), in sequencing the *hemC*

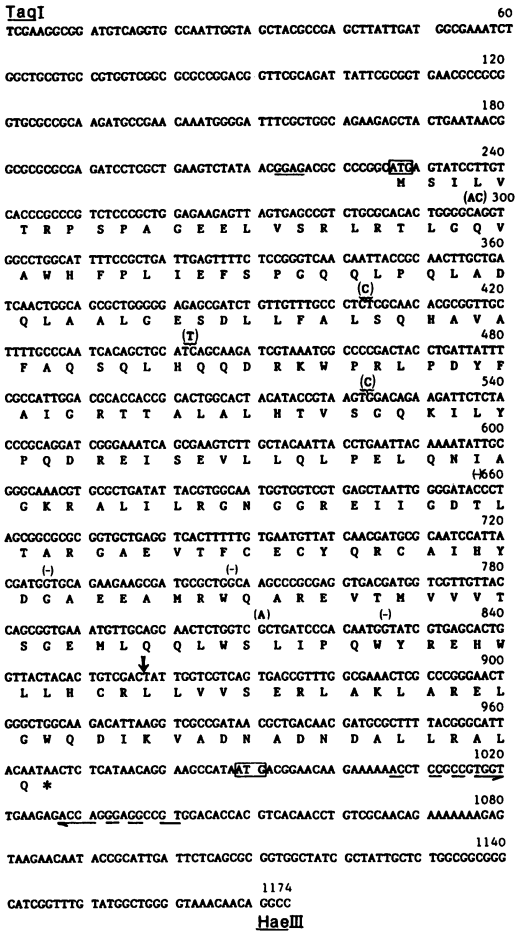

FIG. 4. Nucleotide sequence of the *hemD* gene and the deduced amino acid sequence of the *hemD* protein. Nucleotide sequencing was performed by the dideoxy chain termination method of Sanger et al. (30). Discrepancies between our sequencing results and those obtained by Thomas et al. (35) are indicated in parentheses above our sequence. A vertical arrow points to the end of the region sequenced by Thomas et al. (35). Only the proximal region of the sequence of the putative protein X is shown.

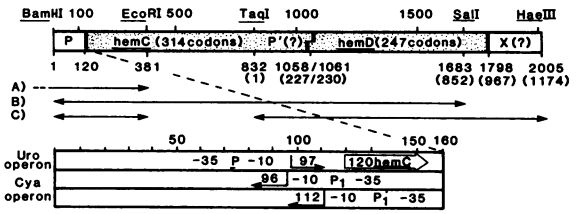

FIG. 5. A tentative scheme of the Uro operon (1, 27, 28, 35; this study). P, Uro promoter; P'(?), a very weak promoter of the *hemD* gene, located in the *hemC* structural gene; X(?), a putative third member of the Uro operon (only the proximal end of the gene X is represented). Nucleotides are numbered at the beginning according to Thomas et al. (35), but starting from nucleotide 1235 and running to the end (nucleotide 2005), the nucleotides are numbered according to our results, due to the observed discrepancies between the two sequences (see Discussion). (A) Region sequenced by Roy et al. (28) and Aiba (1); (B) fragment sequenced by Thomas et al. (35); (C) fragments sequenced by us (only the sequence of the second fragment, carrying the *hemD* structural gene, is shown in our study). The enlargment shows the overlapping of the transcription initiation regions of the Uro and Cya operons. The thin arrows indicate the actual starting points for the corresponding mRNAs.

gene, noted the presence of an ORF of 297 nucleotides starting from the last nucleotide of the *hemC* gene and speculated that an additional unknown protein could be produced under the control of the *hemC* promoter. As the authors had sequenced 631 of the 741 nucleotides that constitute the *hemD* gene, we compared the two sequences to elucidate the discrepancy between the sizes of the two ORFs. Several differences were observed (see Fig. 4), which could account for the diminution of the corresponding ORF from 738, as reported by us (Fig. 4), to 297 nucleotides, as reported by Thomas et al. (35).

The *Bam*HI-*Eco*RI fragment containing the *hemC* regulatory region (Fig. 5) has already been sequenced four times (references 1, 28, and 35 and the present work [data not shown]; see also Fig. 5). In addition to the work of Roy and Danchin (27) and Roy et al. (28), Aiba (1) analyzed by S1 mapping the transcription start of what is now being considered as the *hemC* and consequently the Uro promoter. Aiba also indicated (1) the −10 and −35 regions of the promoter (11), which are identical with those reported later by Thomas et al. (35). An interesting feature, observed by the last authors, is the presence of three repeats of a pentanucleotide in a short region of the promoter that might have a role in regulation (35).

The presence of a Uro operon in *E. coli* K-12 consisting of the *hemC* and *hemD* genes is in contrast with the situation of the other *hem* genes, which are scattered along the chromosome (2). The scattering of the *hem* genes is not limited to *E. coli* K-12 but is also found in other gram-negative bacteria (29, 31). In contrast, the situation is different in gram-positive organisms, where the *hem* genes are grouped in one or a few clusters (21, 25, 36). The existence of a Uro operon as opposed to the scattering of the other *hem* genes in *E. coli* may be the result of a need for the coordinate expression of the *hemC* and *hemD* genes to avoid porphyrin accumulation.

A tentative scheme of the Uro operon, on the basis of previous contributions (1, 27, 28, 35) and of our results, is presented in Fig. 5. The nucleotides are numbered at the beginning according to the system of Thomas et al. (35), but from nucleotide 1235 to the end (nucleotide 2005) the nucleotides are numbered according to our results, due to the observed discrepancies between the two sequences. The size of the structural genes of the Uro operon is 1,679 nucleotides, consisting of 942 nucleotides for the *hemC* gene and 741 nucleotides for the *hemD* gene. There is an overlap of four nucleotides of the two genes, including the stop codon. The *hemD* gene is followed by an intergenic region of 21 base pairs and by an ORF of at least 480 base pairs, the function of which is unknown; this might represent a third member of the Uro operon, as suggested by preliminary data.

An enlargement of the transcription initiation region of the Uro operon in Fig. 5 shows that it overlaps with the corresponding region of the Cya operon (1, 27, 28), which is transcribed in the opposite direction. This may result in a lower expression from the P1 Cya promoter as mentioned by Aiba (1), but it is premature to speculate on the implications of this overlap for the expression of the Uro operon.

ACKNOWLEDGMENTS

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, and G. Drapeau for the gift of strain JC10240.

This study was supported by grant MT 4997 from the Medical Research Council of Canada to A.S. The other authors were the beneficiaries of studentships from the Medical Research Council of Canada (A.N., Y.E., and M.D.) or from the Ministry of Education (FCAR) of Quebec (C.G.).

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