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Spermidine Biosynthesis in *Escherichia coli*: Promoter and Termination Regions of the *speED* Operon

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Two enzymes, *S*-adenosylmethionine decarboxylase and spermidine synthase, are essential for the biosynthesis of spermidine in *Escherichia coli*. We have previously shown that the genes encoding these enzymes (*speD* and *speE*) form an operon and that the area immediately upstream from the *speE* gene is necessary for the expression of both the *speE* and *speD* genes. We have now studied the upstream promoter and the downstream terminator regions of this operon more completely. We have shown that the major mRNA initiation site (I_a) of the operon is located 475 base pairs (bp) upstream from the *speE* gene and that there is an open reading frame that encodes for a polypeptide of 115 amino acids between the I_a site and the ATG start codon for the *speE* gene. Downstream from the stop codon for the *speD* gene is a potential hairpin structure immediately followed by an mRNA termination site, t. An additional mRNA termination site, t', is present about 110 bp downstream from t and is stronger than t. By comparing our DNA fragments with those prepared from this region of the *E. coli* chromosome by Kohara et al., we have located the *speED* operon on the physical map of the *E. coli* chromosome. We have shown that the orientation of the *speED* operon is counterclockwise and that the operon is located 137.5 to 140 kbp (2.9 minutes) clockwise from the zero position of the *E. coli* chromosomal map.

The biosynthesis of spermidine in *Escherichia coli* is carried out by two enzymes, *S*-adenosylmethionine decarboxylase (13, 24, 32) and spermidine synthase (2), which catalyze the following two reactions: (i) *S*-adenosylmethionine → decarboxylated *S*-adenosylmethionine + CO_2 and (ii) decarboxylated *S*-adenosylmethionine + putrescine → spermidine + methylthioadenosine.

We have previously cloned (13, 27, 28) and sequenced (25) the DNA fragment containing two adjacent open reading frames containing *speE* (864 base pairs [bp]), which encodes spermidine synthase, and *speD* (792 bp), which encodes a proenzyme form of *S*-adenosylmethionine decarboxylase. The *speE* gene lies upstream from the *speD* gene. Deletion experiments (28) indicated that the 124-bp region immediately upstream from the *speE* gene contains the area necessary for the expression of both the *speE* and *speD* genes.

In our current work we have studied the promoter and terminator regions of the *speED* operon more completely. We have determined the major initiation site and two termination sites of the mRNA transcribed from the operon and have shown that the operon is considerably larger than we had expected on the basis of our previous sequence and deletion experiments (25, 28).

We have previously shown by genetic mapping studies that *speD* is located at ca. 2.7 min on the genetic map of *E. coli* (26). Recently, Kohara et al. (12) have published the physical map of the entire *E. coli* chromosome, permitting a more definitive localization of specific genes. A comparison of their restriction maps with the data obtained in our current work on the *speED* operon established the specific location of the *speED* operon on the physical map of the *E. coli* chromosome as 137.5 to 140 kbp from the zero position of the map (i.e., 2.9 min on the genetic map).

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MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are shown in Table 1. Strains 71-18 (15) and HB101 (3) are wild-type strains (with respect to the *speE* and *speD* genes). EWH319 contains deletions in *speA*, *speB*, *speC*, *speD*, and *speE* (11, 26, 28); hence, this strain contains no putrescine or spermidine when grown in purified media. Strain HT551 contains deletions in *speE* and *speD* but not in *speA*, *speB*, or *speC* (28). Thus, this strain contains putrescine but no spermidine.

Cultures were grown at 37°C with shaking in either LB broth or a minimal medium (30) with the supplements needed. Ampicillin was added at a concentration of 50 to 100 μg/ml for the growth of strains containing plasmids with the Amp^r marker. For experiments measuring the effect of exogenous polyamine, spermidine was present in the culture medium for at least three generations. All cultures were harvested within the period of logarithmic growth.

The original source of all of the *speED*-containing DNA preparations used in our studies was clone pLC37-29 of the Clarke-Carbon collection (6, 7, 19, 27). Many of the plasmids used in this paper were derived from the plasmids described in our previous publications (13, 27, 28). For some of the current studies, an additional plasmid which contained a 5-kbp *E. coli* fragment, pSPD41, was constructed; this fragment consisted of the *speE* and *speD* genes and ca. 0.8 kbp upstream from the *speE* gene and 2.4 kbp downstream from the *speD* gene. This 5-kbp fragment was obtained from pLC37-29 by digestion with *Sal*I. After gel electrophoresis, the fragment containing the *speED* operon was identified by hybridization with a radioactive RNA probe synthesized by in vitro transcription (8) from the *E. coli* fragment present in pSPD22, i.e., the fragment containing most of the *speED* operon (Table 1). Ligation of this fragment into the *Sal*I site of pUC8 resulted in the new plasmid pSPD41.

For studies on the upstream promoter areas, plasmids

TABLE 1. Strains and plasmids used

E. coli strain or plasmid	Description	Source or reference(s)
Strain		
71-18	Δ(*lac-pro*) (F′ *lacI*^{q}ZΔM15 *pro*$^+$)	15
HT551	Δ(*speE-speD*) *zad-220*::Tn*10 panB6* Δ(*lac-pro*) (F′ *lacI*^{q}ZΔM15 *pro*$^+$)	28
HB101	F′ *hsdf20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	3
EWH319	F′ Δ(*speA-speB*) Δ*speC* Δ(*speE-speD*) *hr-1 proA2 galK2 mtl-1 xyl-5 ara-14 rpsL25 supE44* λ$^-$ *thi-1 lacY1*	11, 26, 28
Plasmid		
pLC37-29	Clarke-Carbon plasmid; contains *speED* operon	6, 19, 27
pSPD22	Contains 124-bp fragment upstream from the *speE* gene, the *speE* gene, and part of the *speD* gene in pUC8 vector	28
pSPD33	Contains 2.7-kbp *Sal*I-*Pst*I DNA fragment (Fig. 8, line B) in pUC8 vector; this insert contains 0.8 kbp upstream from the ATG start site for *speE*, both *speE* and *speD* genes, and 80 bp downstream from the TAA stop codon for *speD* (Fig. 1 and 2 and nucleotide sequence reported in reference 25)	This paper
pSPD41	Contains 5-kbp *Sal*I fragment (Fig. 8, line B) in pUC8 vector	This paper
pKK232-8	Contains promoterless CAT gene and the Amp^r gene (from Pharmacia)	5
pSPD37	Contains the promoter P_1 (*Ssp*I-*Hpa*I fragment from −81 to −21 in Fig. 1) upstream from the CAT gene in pKK232-8	This paper
pSPD38-2	Contains the promoter P_2 (*Afl*II-*Ssp*I fragment from −132 to −82 in Fig. 1) upstream from the CAT gene in pKK232-8	This paper
pSPD38-3	Contains the *Bst*EII-*Afl*II fragment (−317 to −133 bp in Fig. 1) placed upstream from the CAT gene in pKK232-8	This paper
pSPD39	Contains the promoter area of P_a and P_b (*Ssp*I-*Bst*EII fragment from −643 to −318 in Fig. 1) upstream from the CAT gene in pKK232-8	This paper
pT7-5	Modification of pT7-1 (22) in which the orientation of the β-lactamase gene is reversed with respect to the T7 promoter	S. Tabor (unpublished plasmid)
pSPD45	Contains the *Ssp*I fragment (from −643 to −82 in Fig. 1 containing the upstream open reading frame) inserted immediately downstream from the T7 promoter in pT7-5	This paper

pSPD37, pSPD38-2, pSPD38-3, and pSPD39 were constructed as described in Table 1.

For expression of the upstream open reading frame, plasmid pSPD45 was constructed by inserting the *Ssp*I fragment (−643 to −82 in Fig. 1) into the plasmid pT7-5 immediately downstream from the T7 promoter (Table 1).

Enzyme assays. The putative promoter sequences P_1, P_2, and P_a with P_b (Fig. 1) were tested for promoter activity by measuring the activity of chloramphenicol acetyltransferase (CAT) (5, 9, 18) in cells carrying plasmids with these sequences upstream from the promoterless CAT gene (Tables 1 and 2).

The cell pellet harvested from 25 ml of the culture was washed with Tris chloride buffer (0.1 M, pH 7.8) and suspended in 0.5 ml of the buffer. After sonication and centrifugation, the supernatant fluid was collected; the CAT assay was then carried out as described by Neumann et al. (18). The cell extract was diluted in 0.1 M Tris chloride buffer (pH 7.8), and a 50-μl sample was placed in a 20-ml scintillation vial containing 200 μl of 1.25 mM chloramphenicol in the same Tris chloride buffer. The reaction was initiated by the addition of 10 μl of [^{14}C]acetyl coenzyme A (4 mCi/mmol; final concentration, 0.1 mM). This reaction mixture was gently overlaid with 5 ml of Econofluor (Dupont, NEN Research Products). The vial was incubated at room temperature and counted at 10-min intervals in a scintillation counter. With this technique only the radioactivity in the acetylated derivatives of chloramphenicol was detected, since the labeled substrate, acetyl coenzyme A, is not soluble in the water-immiscible Econofluor phase. One unit of enzymatic activity is defined as the formation of 1 nmol of acetylchloramphenicol per min.

Spermidine synthase and *S*-adenosylmethionine decarboxylase were assayed as previously described (13, 28, 32). Protein was determined by the method of Bradford (4).

Nucleotide sequencing and DNA manipulation. Nucleotide sequencing was carried out by the dideoxynucleotide method of Sanger et al. (21) as modified by S. Tabor and C. C. Richardson (23), i.e., with the use of [α-^{35}S]dATP and modified T7 DNA polymerase (Sequenase; U.S. Biochemical Co.). Preparation, isolation, ligation, and transformation of various nucleotide fragments were carried out by standard procedures.

Determination of mRNA start sites by S1 nuclease mapping and primer extension. On the basis of our previous data (28), the 124-bp fragment upstream from the ATG codon of the *speE* gene appeared to contain the regulatory region for both the *speE* and the *speD* genes. By computer search (17), two putative promoter sequences were determined to be present in this region, and thus we assumed that mRNA synthesis probably starts within this region. For S1 mapping to determine the mRNA start sites in this region, a DNA probe (consisting of the nucleotides between the single asterisk and double asterisks in Fig. 1 [bp −124 to +52]) was prepared in which the 5′ end at position +52 (*Rsa*I site) was labeled with ^{32}P as described by Maxam and Gilbert (14). The DNA probe (20 to 30 ng, 10^5 cpm) was hybridized with the total RNA (ca. 140 μg) isolated from various strains of *E. coli* in early logarithmic phase. S1 nuclease digestion as well as gel electrophoresis were then carried out as described by Wek and Hatfield (31). For the wild-type strain, which only has the chromosomal *speE* and *speD* genes, about 1/12 of the final reaction product was loaded on the gel; however, for the plasmid-containing strains which have a high copy number of the *speE* and *speD* genes, only 1/60 or 1/110 of the final reaction product was loaded on the gel (see Fig. 4).

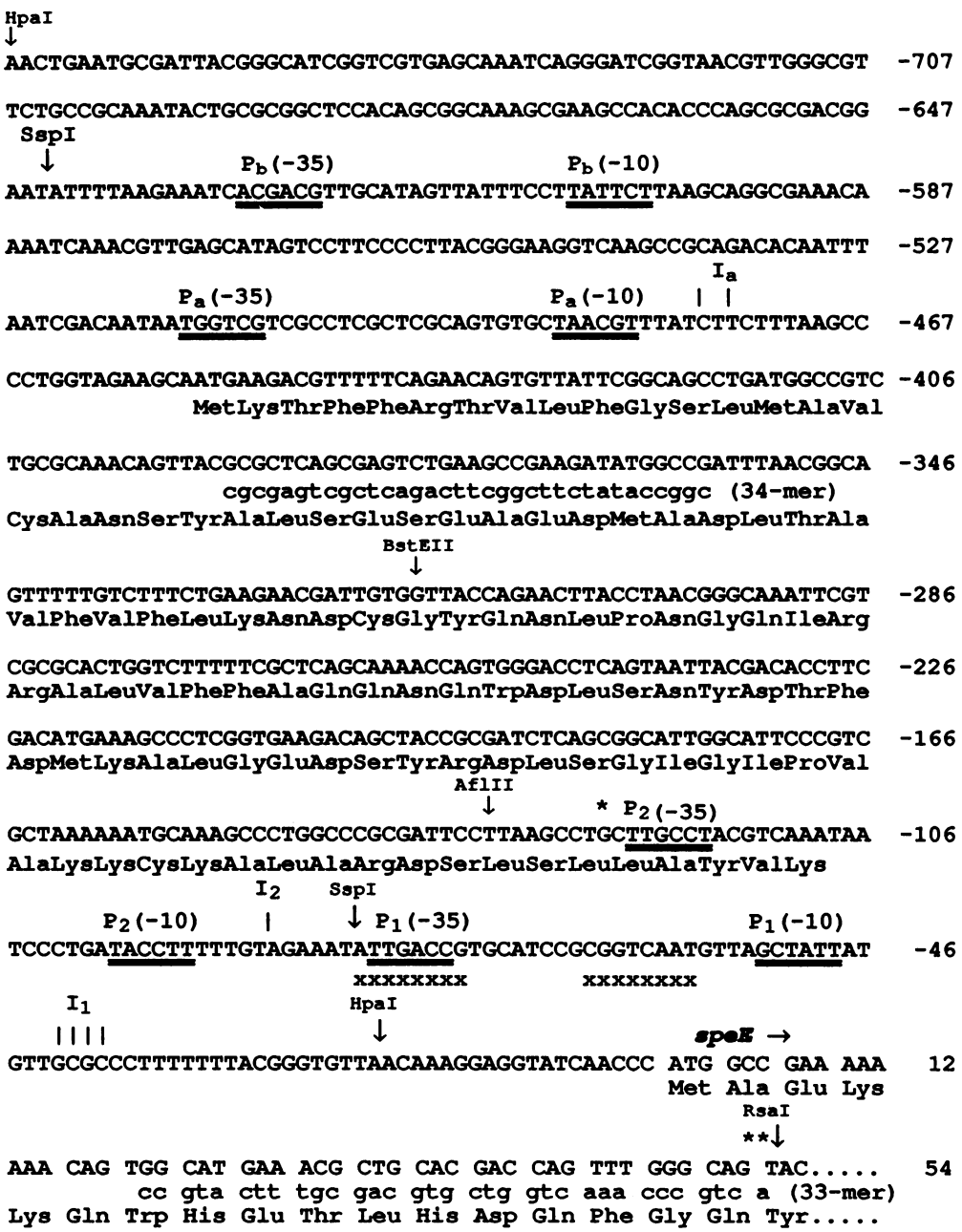

FIG. 1. Nucleotide sequence of the DNA upstream from the gene for *speE*. The numbering of the nucleotide bases is expressed relative to the ATG start site of the *speE* structural gene, defined as position 1. The −10 and −35 regions of four putative promoters (P_1, P_2, P_a, and P_b) analyzed by the computer search program are indicated. *Afl*II, *Bst*EII, *Hpa*I, *Rsa*I, and *Ssp*I restriction sites are also indicated. The fragment between the single asterisk and double asterisks was used to make a DNA probe in the S1 mapping experiment to determine the mRNA start sites I_1 and I_2 in the region of P_1 and P_2. The 33-mer (+52 to +20) and 34-mer (−357 to −390) (indicated in lowercase letters) were used in the primer extension experiments to detect the mRNA initiation site I_a (see Fig. 5 and 6).

We also determined the initiation site of mRNA by primer extension mapping (16). The 33-mer and the 34-mer (indicated by lowercase letters from +52 to +20 and from −357 to −390 in Fig. 1) were synthesized with a 381A DNA synthesizer (Applied Biosystems) and labeled with ^{32}P at the 5′ end (14). In each experiment, the labeled primer was hybridized with the total RNA (50 μg) from *E. coli* strains in 10 mM Tris chloride buffer (pH 8.5) containing 10 mM $MgCl_2$ at 55°C for 30 min and then extended by avian myeloblastosis virus reverse transcriptase in the presence of four unlabeled deoxynucleoside triphosphates at 42°C for 90 min. The size of the extended products was determined by electrophoresis on a sequencing gel parallel to sequencing ladders of the DNA from plasmid pSPD41 starting with the same primer (see Fig. 5 and 6).

Determination of mRNA termination sites by S1 mapping. The S1 mapping procedure was the same as described above, except that a different probe was used. The DNA probe was prepared from the 238-bp fragment between the *Hae*II site (73 bp upstream from the TAA stop codon for

TABLE 2. Promoter activity of P_1, P_2, and P_a with P_b determined by the expression of the CAT gene[a]

Medium and strain[b]	Promoter	CAT activity[c]
LB		
HB101(pKK232-8)	None[d]	0
HB101(pSPD37)	P_1	14.6
HB101(pSPD38-2)	P_2	4.5
HB101(pSPD39)	P_a + P_b	14.6
HB101(pSPD38-3)	None[e]	0.2
Purified medium		
EWH319(pKK232-8)	None[d]	0
EWH319(pSPD37)	P_1	32.7
EWH319(pSPD38-2)	P_2	29.3
EWH319(pSPD39)	P_a + P_b	45.0

[a] Cultures were grown either in LB medium or in a purified medium to a cell density of ca. 2×10^8 to 3×10^8 cells per ml. The medium was supplemented with 100 μg of ampicillin per ml.

[b] Plasmids are described in Table 1.

[c] Specific activity for CAT is expressed as nanomoles of acetylated chloramphenicol formed per milligram of protein per minute under the conditions described by Neumann et al. (18).

[d] No insert.

[e] See Table 1. The insert does not contain P_1 or P_2 but includes the 185 bp of nucleotides immediately upstream from the P_2 area.

speD, not shown in Fig. 2) (25) and the *Eco*RI site (162 bp downstream from the TAA codon [Fig. 2]), which was labeled with [α-^{32}P]cordycepin-5′-triphosphate at the 3′ end of the transcribed strand (at the *Hae*II site) (29).

Translation of the upstream open reading frame in the T7 promoter-expression system. This experiment was carried out by the unpublished method of S. Tabor and C. C. Richardson as used previously (25). Plasmids pSPD45 and pT7-5 (Table 1) were cloned into strain HT551. The cells were grown at 37°C in the minimal medium with 50 μg of ampicillin per ml. Three milliliters of each cell culture (optical density at 550 nm, 0.5) was infected with M13mGP1-2 and treated with isopropylthiogalactoside (1.5 mM) for 1 h and then with rifampin (200 μg/ml) for 1 h. [^{35}S]methionine (10 μCi/ml) was added to each culture, and 0.85-ml samples were added to 0.15 ml of 50% trichloroacetic acid after 2 and 15 min. After centrifugation, each pellet was washed twice with 100% ethanol, dried in a Speed-Vac, and dissolved in 30 μl of sample loading buffer. After being boiled for 5 min, 1 μl of each sample was analyzed by electrophoresis on a 10 to 15% gradient PhastGel (Pharmacia, Inc.).

RESULTS

Nucleotide sequence of the DNA upstream from *speE* and downstream from *speD*. The nucleotide sequence of the coding sequences of *speE* and *speD* and the immediate upstream (124 bp) and downstream (80 bp) areas have been reported in our previous paper (25). A longer region upstream from *speE* and a longer region downstream from *speD* were sequenced in our current studies and are presented in Fig. 1 and 2. The nucleotide sequences have been deposited in GenBank (accession number J04247).

An open reading frame upstream from the *speE* gene. As shown in Fig. 1, in the region upstream from the *speE* gene (−453 to −109 bp) there is an open reading frame coding for a 115-amino-acid protein. This coding region is in frame with the *speE* and *speD* open reading frames. Upstream from the ATG start codon of this region there are two putative promoter sequences (P_a and P_b in Fig. 1) and a putative ribosome-binding site. An area of dyad symmetry lies downstream from the TAA stop codon of this open reading region (xxxxx in Fig. 1).

Plasmid pSPD45, containing this open reading frame

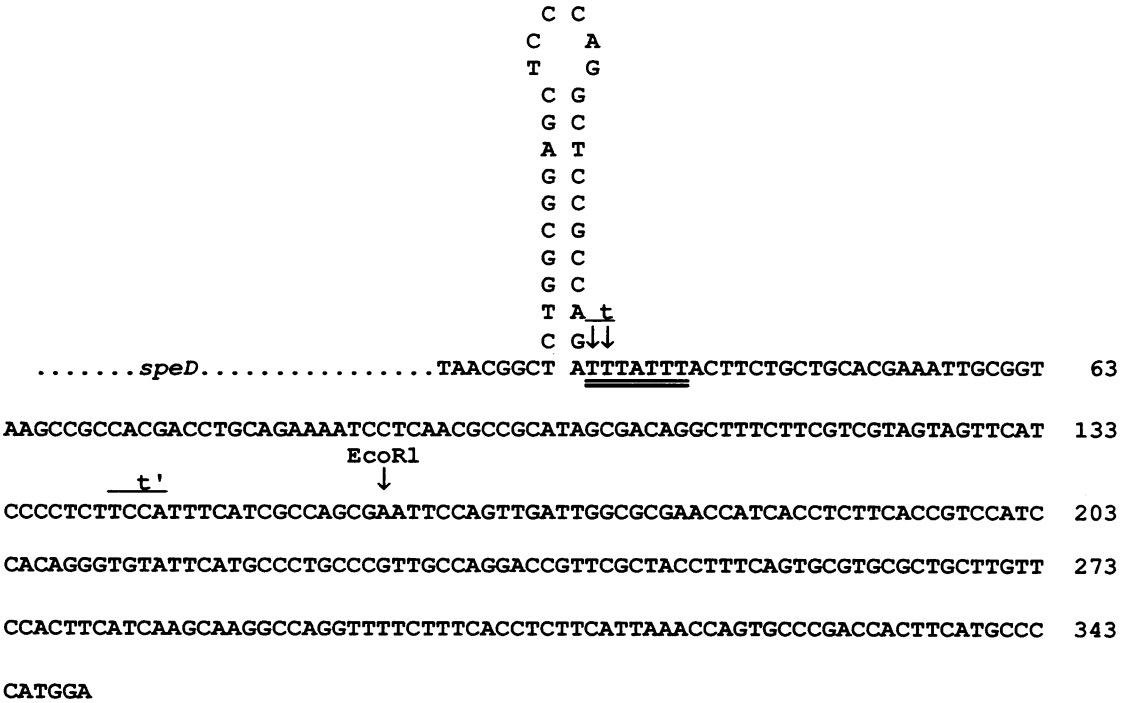

FIG. 2. Nucleotide sequence downstream from the TAA stop codon of the *speD* gene. The nucleotides are numbered with the first one downstream from the stop codon (TAA) as +1. t and t′ indicate the mRNA stop sites determined by S1 nuclease mapping (see Fig. 7). t, positions 33 and 34; t′, region around position 142.

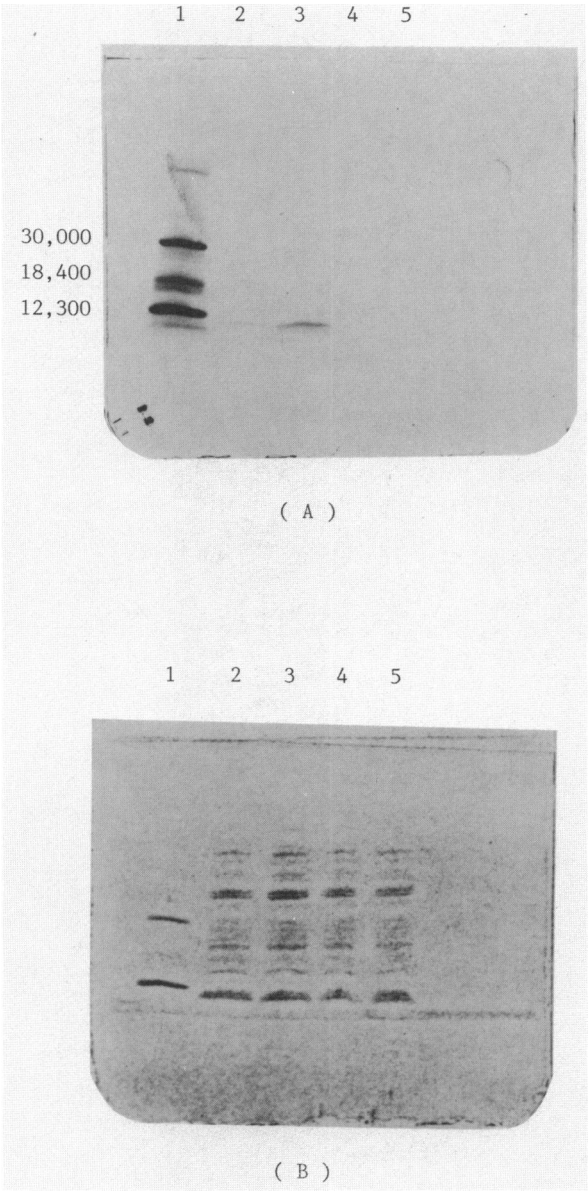

FIG. 3. Translation of the upstream open reading frame in the T7 promoter-expression system. Autoradiograph (A) is from the Coomassie brilliant blue-stained gel (B). The experiment was performed as described in Materials and Methods. Lanes: 1, ^{14}C-labeled molecular weight standards (12,300 [cytochrome *c*], 18,400 [lactoglobulin A], and 30,000 [carbonic anhydrase]); 2, HT551 (pSPD45) labeled for 2 min; 3, HT551(pSPD45) labeled for 15 min; 4, HT551(pT7-5) labeled for 2 min; 5, HT551(pT7-5) labeled for 15 min.

placed immediately downstream from a T7 promoter, was cloned in strain HT551, and labeling experiments with [^{35}S]methionine showed that a protein with a molecular weight of about 12,000 was synthesized in this T7 promoter-expression system (Fig. 3).

No open reading frame is present on the other strand in this region.

Putative promoter regions for the *speED* operon. Examination of the upstream nucleotide sequence by a computer search (17) shows that there are four putative promoter sequences, designated P_1, P_2, P_a, and P_b. Each promoter has a −10 and a −35 region indicated by a double underline in Fig. 1. To determine whether these putative promoters did indeed have promoter activity, the DNA fragments containing P_1, P_2, or P_a plus P_b were inserted upstream of the promoterless CAT gene in plasmid pKK232-8 (Table 1). The promoter activity was determined by measuring the CAT activity in the strains containing these plasmids (Table 2). CAT activity was not found in the absence of these promoter inserts, but good activity was found when P_1, P_2, or P_a with P_b was present.

No CAT activity was observed when another DNA fragment, such as the *Bst*EII-*Afl*II fragment (−317 to −132 in Fig. 1), was placed upstream from the CAT gene (plasmid pSPD38-3 in Table 2).

Transcriptional initiation of the operon in vivo. From deletion experiments in our previous work (28), the 124-bp region immediately upstream from the *speE* gene contained sequences that are necessary for the expression of both *speE* and *speD* genes. Two putative promoter sequences (P_1 and P_2) were found when the sequence was studied with the computer search (17). To locate the mRNA start site in this region, S1 nuclease mapping was carried out (Fig. 4). For these experiments, the labeled DNA probe, prepared as described in Materials and Methods, was hybridized with the same amount of total RNA (140 μg) prepared from HT551(pSPD22) (lane 1), from HT551(pSPD33) (lane 2), and from wild-type strain 71-18 (lane 3). For the wild-type strain, which contains only the chromosomal *speE* and *speD* genes, more reaction product (1/12 of the total) was loaded on the gel; for strains with plasmids, less reaction product (1/60 for lane 1 and 1/110 for lane 2) was loaded on the gel. Under these conditions, the mRNA started at position I_1 in all three strains (i.e., 41 and 42 bp upstream from the ATG start codon for the *speE* gene). A weak mRNA start site at position I_2, which is 45 bp upstream from I_1, was seen in the plasmid-containing strains but not in the wild-type strain.

There was also a strong band at the tops of lanes 2 and 3 in Fig. 4; indeed, this band was much stronger than the band at I_1 in lane 3 (wild-type strain without the plasmid). Primer extension experiments were also carried out, since it was possible that the top bands represented the mRNA readthrough from a start site further upstream (i.e., beyond the limit of the probe used in S1 nuclease mapping); the results showed that this is indeed the case.

The first primer extension experiment (Fig. 5) showed that the mRNA in all three strains does start from the further-upstream region, at position I_a. For this experiment, the amount of the reaction product applied on the gel from the wild-type strain (lane 1) was twice as much as that from the strains containing the plasmids (lanes 2 and 3). The I_1 start site, consistent with that found in S1 mapping, was also seen in lanes 2 and 3 but not in lane 1. To localize more precisely the mRNA start site I_a, another primer extension experiment (Fig. 6) was performed with a primer further upstream than that used in Fig. 5. Figure 6 shows that the mRNA start site I_a is located downstream from the −10 region of the promoter P_a (also indicated in Fig. 1). These primer extension experiments thus showed that most (if not all) of the in vivo transcription from the chromosome (i.e., in the wild-type strain) is initiated at the I_a site.

mRNA termination sites of the *speED* operon. Immediately downstream from the stop codon TAA of the *speD* gene, there is a region of dyad symmetry (hairpin structure) with a high GC content; this is followed by a TTTATTT sequence (Fig. 2). These characteristics are consistent with a rho-independent termination site (20). Using S1 nuclease mapping techniques (Fig. 7), we have been able to show that a

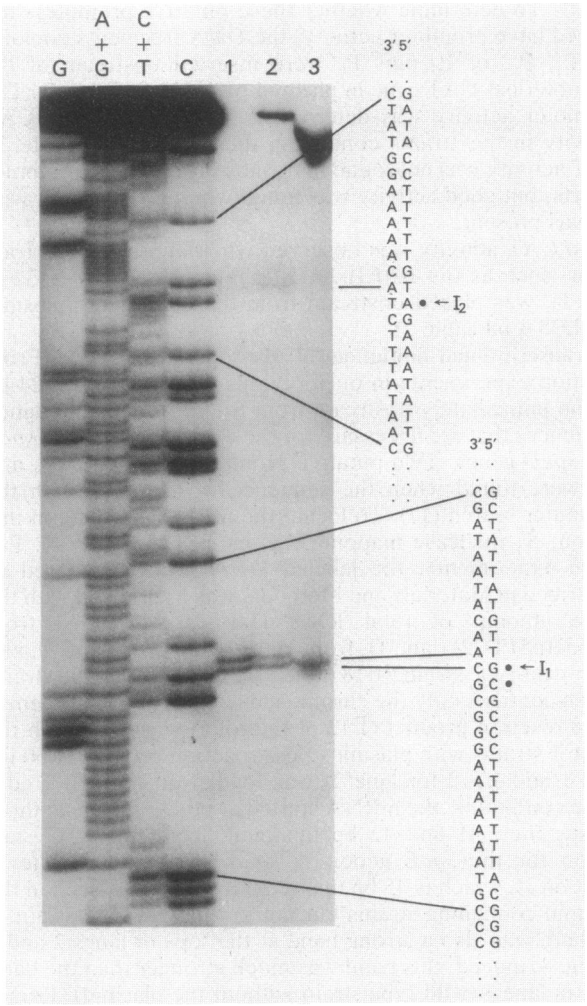

FIG. 4. S1 nuclease mapping of the mRNA start sites in the 124-bp region immediately upstream from the *speE* gene. A 178-bp fragment (between * and ** in Fig. 1) was used as a DNA probe with the 5′ end labeled at the *Rsa*I site (**). This probe was hybridized to 140 μg of the total RNA from the following strains: lane 1, HT551(pSPD22) (this plasmid contains the *speE* gene and the 124-bp adjacent upstream region); lane 2, HT551(pSPD33) (this plasmid contains both the *speE* and *speD* genes plus an additional 0.8 kbp upstream from the ATG start codon for *speE*); and lane 3, 71-18 (wild-type strain; no plasmid). After S1 nuclease treatment, the protected DNA probe was analyzed by electrophoresis on an 8% polyacrylamide–urea gel in parallel with the products of Maxam-Gilbert sequencing reactions of the same DNA probe. The two mRNA start sites, I_1 and I_2, are indicated. (It should be noted that one base was destroyed in producing the Maxam-Gilbert fragments.) A strong band is shown at the tops of lanes 2 and 3.

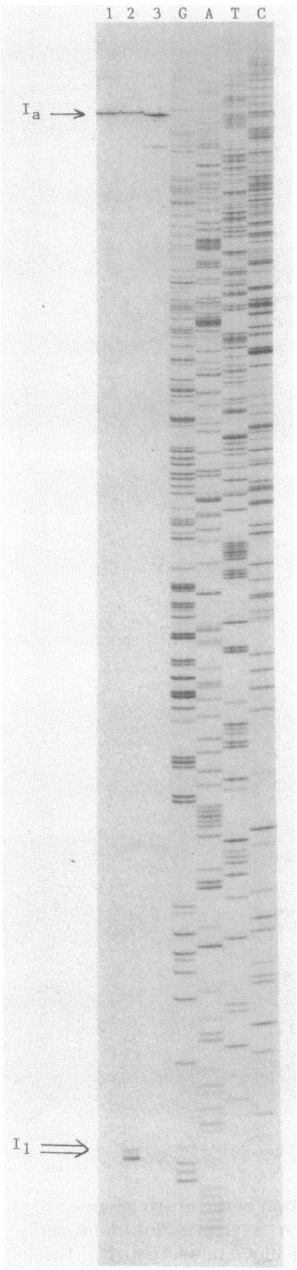

FIG. 5. First primer extension experiment to map the mRNA start site. A 5′-end-labeled 33-mer (+52 to +20 in Fig. 1) which is complementary to the mRNA in the *speE* gene was hybridized to 50 μg of the total RNA from wild-type strain 71-18 (lane 1), HT551 (pSPD22) (lane 2), or HT551(pSPD33) (lane 3) (see Table 1 for plasmid descriptions). The primer was extended by avian myeloblastosis virus reverse transcriptase in the presence of four unlabeled deoxynucleoside triphosphates. The products were analyzed on a 5% sequencing gel with the sequencing ladders of the DNA from plasmid pSPD41 starting with the same 33-mer. The mRNA start site I_a was present in all three lanes. Another mRNA start site, I_1, was detected only in lanes 2 and 3 in this experiment; the position of I_1 is consistent with that of I_1 determined by S1 mapping (Fig. 4).

termination site, t, is present at this hairpin region. We also observed another termination site, t′, located about 110 bp downstream from t and stronger than t.

Localization and orientation of the *speED* operon on the chromosome. The publication by Kohara et al. (12) of the physical map of the entire *E. coli* chromosome permitted us to localize the *speED* operon more definitively than in our earlier studies. For this purpose, we determined the restriction map of the 5-kbp fragment that contains the *speED* operon (Fig. 8, line B) with the same restriction enzymes used by Kohara et al. We then compared this restriction map with the restriction map reported by Kohara et al. for this region of the *E. coli* chromosome (Fig. 8, line A). The results showed that the *speED* operon is located at 137.5 to 140 kbp clockwise from position zero of the chromosomal map (ca. 15 kbp clockwise from the *ace* gene). Since the total chro-

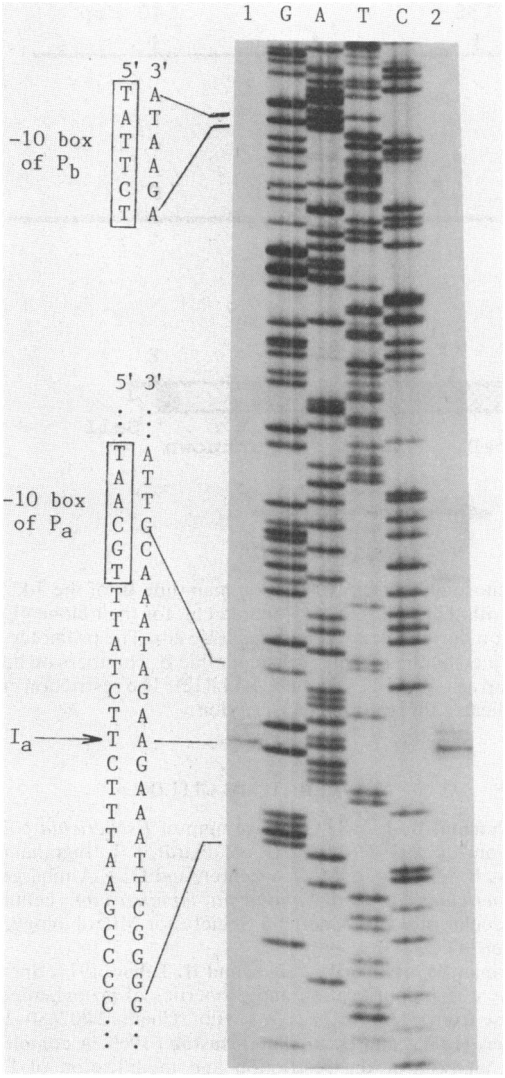

FIG. 6. Second primer extension experiment to map the mRNA initiation site. A 5'-end-labeled 34-mer (−357 to −390 in Fig. 1) which was complementary to the mRNA in the beginning of the upstream open reading frame was hybridized to 50 μg of the total RNA from the wild-type strain 71-18 (lanes 1 and 2). The primer was extended by avian myeloblastosis virus reverse transcriptase in the presence of four unlabeled deoxynucleoside triphosphates. The product was analyzed by electrophoresis on a 5% sequencing gel with the sequencing ladders of the DNA from plasmid pSPD41 starting with the same 34-mer. The positions of the −10 boxes of the putative promoters P_a and P_b are indicated. The precise position of the mRNA initiation site I_a is indicated (also shown in Fig. 1) downstream from the −10 region of the promoter P_a. However, no mRNA start site was detected immediately downstream from the −10 region of P_b.

mosomal map has ca. 4,720 kbp (12), position 137.5 to 140 is equivalent to 2.9 min on the usual chromosomal map (1); thus, *speED* is ca. 0.3 min from the *ace*EF operon (which is located at 2.6 min) (10). The data also show that the orientation and transcription of the *speED* operon are counterclockwise.

Effect of added spermidine on the levels of *S*-adenosylmethionine decarboxylase and spermidine synthase. We studied the effect of added spermidine on the culture of a wild-type strain (71-18) that did not contain any plasmid. The addition

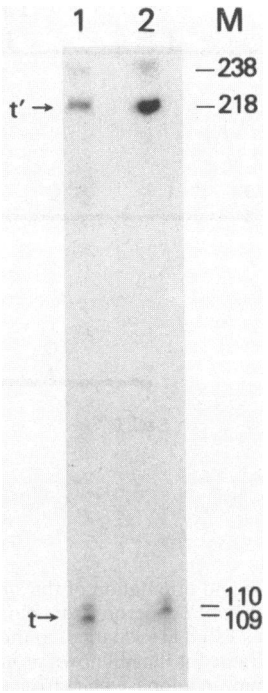

FIG. 7. Determination of the mRNA termination sites of the *speED* operon by S1 nuclease mapping. A 238-bp fragment, extending from the *Eco*RI site in Fig. 2 to a *Hae*II site located 76 bp upstream from the TAA stop codon (not shown in Fig. 2), was 3' end labeled at the *Hae*II site and used as a DNA probe. This probe was hybridized to the total RNA from HT551(pSPD41) (lane 1) and from wild-type 71-18 (lane 2). After S1 nuclease digestion, the protected DNA probes were analyzed on a 5% polyacrylamide–urea gel. mRNA stop sites t and t' are indicated. Size markers on the right (M; prepared by determining the sequence of a defined but unrelated DNA) indicate the number of nucleotides in the protected bands.

of spermidine resulted in a 40 to 60% reduction in enzyme activities (Table 3).

DISCUSSION

In previous studies we found that the two genes required for spermidine synthesis in *E. coli*, *speD* (*S*-adenosylmethionine decarboxylase) and *speE* (spermidine synthase), form an operon. We also showed that the immediate upstream region was required for expression of these genes.

We have now extended this work to a more complete study of the upstream region. We have found that upstream from the *speE* gene there is a 345-bp open reading frame following two putative promoter regions (P_b and P_a). Immediately downstream of the P_a promoter there is a strong initiation site (I_a). Between this open reading frame and the *speE* gene, there are two additional promoter regions (P_2 and P_1), and a weak initiation site (I_1) is located immediately downstream from P_1. No mRNA transcripts corresponding to the other two putative promoter sites are present. We conclude that the major promoter of the operon is P_a and that the major mRNA for the entire operon starts at the initiation site I_a.

We do not know the function of the upstream open reading frame, although we have been able to show that the open reading frame encodes a protein of 12,000 M_r in a T7 promoter-expression system.

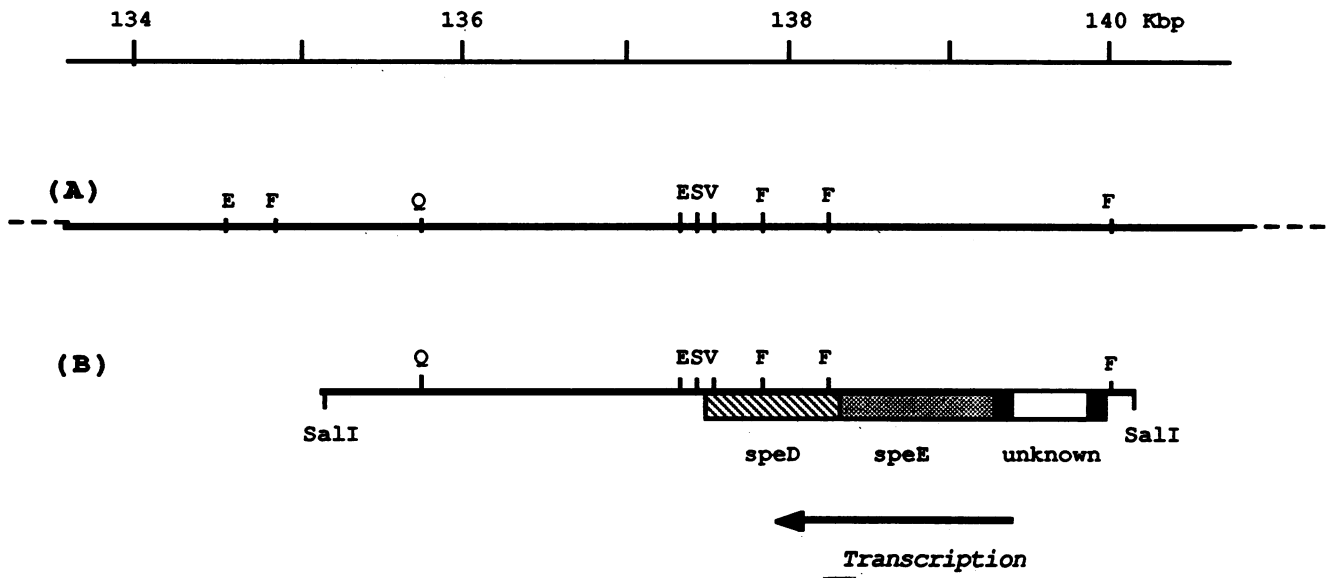

FIG. 8. Localization and orientation of the *speED* operon on the *E. coli* chromosomal map. A restriction map (line B) of the 5-kbp DNA fragment containing the *speED* operon (from our plasmid pSPD41) is compared with that reported by Kohara et al. for their clone 4E11 (line A). The restriction sites listed are identical to those obtained for the *speED* operon during our earlier studies (25, 28). The promoter regions (■), the open reading frame for the unknown protein, and *speE* and *speD* are indicated by the boxed areas under line B. Numbers on the upper line represent the distance in kilobase pairs from the origin of the chromosomal map as reported by Kohara et al. (12). The restriction enzymes used were *Eco*RI (E), *Eco*RV (F), *Pst*I (S), *Pvu*II (V), and *Kpn*I (Q). Arrow indicates direction of transcription.

Two transcriptional termination sites were observed in the region downstream from the *speD* gene. One of them, t, has the characteristics of a rho-independent termination site, but the other, t′, does not. The termination at the t′ site seems to occur much more strongly than that at the rho-independent terminator; however, we do not know how t′ is functioning.

Spermidine does not exert a strong regulatory effect on this operon since growth of a wild-type strain in medium containing spermidine resulted in only a 40 to 60% decrease in the activities of both enzymes.

We have determined that the position of this operon in the physical map of *E. coli* is 137.5 to 140 kbp; the orientation is counterclockwise.

TABLE 3. Effect of exogenous spermidine on the levels of *S*-adenosylmethionine decarboxylase and of spermidine synthase in strain 71.18 (wild type)[a]

Enzyme	Activity (nmol/mg per min)		% Inhibition
	Without spermidine	With spermidine (5 mM)	
S-Adenosylmethionine decarboxylase	0.48	0.20	58
Spermidine synthase	0.36	0.22	39

[a] Culture was grown in a purified medium.

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We thank Yuji Kohara and his colleagues for sending us clone 4E11 from the *E. coli* bank described by Kohara et al. (12) as well as for providing us with a more detailed list of the restriction sites used in Fig. 8. We also thank S. Tabor and C. Richardson for providing the M13 bacteriophage and plasmids containing the T7 promoter.

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