Expression of the *Proteus mirabilis* Lipoprotein Gene in *Escherichia coli*

EXISTENCE OF TANDEM PROMOTERS*

Gee Ching and Masayori Inouye‡

From the Department of Biochemistry, State University of New York at Stony Brook, Stony Brook, New York 11794

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The lpp gene from *Proteus mirabilis* was cloned onto pBR322 and expressed in *Escherichia coli*. The *P. mirabilis* lpp gene is unique in that it has two tandem promoters transcribing two mRNAs that differ in length by approximately 70 nucleotides at their 5′-ends. The two mRNAs thus encode the identical lipoprotein. The *P. mirabilis* prolipoprotein has a 19-amino acid signal peptide and a 59-amino acid lipoprotein sequence. In spite of the substantial differences in the amino acid sequence from the *E. coli* prolipoprotein, the *P. mirabilis* prolipoprotein is normally modified and processed in *E. coli*, and the resultant lipoprotein is assembled in the *E. coli* outer membrane as is the *E. coli* lipoprotein.

The lipoprotein is a major outer membrane protein in various enteric bacteria (1; see also review Ref. 2). The lpp genes of *Escherichia coli* (3), *Serratia marcescens* (4), *Erwinia amylovora* (5), and *Morganella morganii* (6) have been cloned, and their nucleotide sequences have been determined. In all these bacteria, the lipoprotein is synthesized as a precursor prolipoprotein containing a 20-amino acid-long signal peptide and a 58-amino acid-long mature sequence. The cleavage of the signal peptide requires a glyceride modification of the cysteine residue at the cleavage site (7, 8). This cleavage can be specifically inhibited by an antibiotic, globomycin (9, 10).

One of the lipoprotein molecules is known to be covalently linked to the peptidoglycan layer (2).

*Proteus mirabilis* is distantly related to *E. coli*. Its DNA has a low G + C content (39%) and shares little homology (6%) with *E. coli* DNA (11, 12). We have previously shown that the lipoprotein from *P. mirabilis* was very weakly cross-reactive with anti-*E. coli* lipoprotein serum (1) and that the level of lipoprotein in *P. mirabilis* was considerably less than that of *E. coli* (13). Recently, we cloned the *P. mirabilis* lpp gene into pBR322 and determined the nucleotide sequence of its coding region (14). The coding sequence shows that the *P. mirabilis* prolipoprotein consists of a 19-amino acid signal peptide and a 58-amino acid lipoprotein. Its amino acid sequence is substantially different from those of the other enterobacterial lipoproteins. Therefore, it is of great interest to study the expression of the *P. mirabilis* lpp gene in *E. coli*.

In this paper, we attempted to express the *P. mirabilis* lpp gene in *E. coli* and to identify the *P. mirabilis* lipoprotein promoters. We found that in spite of the substantial structural differences from the *E. coli* prolipoprotein, the *P. mirabilis* lpp gene was expressed in *E. coli* and the *P. mirabilis* lipoprotein was normally assembled in the *E. coli* outer membrane. We also found that the *P. mirabilis* lpp gene had two tandem promoters, both of which were functional in *E. coli*.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes were purchased from Bethesda Research Laboratories and New England Biolabs. S1 nuclease, nuclelease-free bovine serum albumin, and Klenow enzyme were obtained from Bethesda Research Laboratories and T4 polynucleotide kinase and *E. coli* RNA polymerase from Boehringer Mannheim. Rifampicin was purchased from Sigma. Globomycin was obtained from Dr. M. Arai (Sankyo Pharmaceutical Co., Japan).

**Bacterial Strains, Phage, and Plasmids**—*P. mirabilis* (15), *E. coli* SB221 (F′ lacI′ recA leuB6 lacY trpE5 lpp+ lac3 lacI′ pro+), *E. coli* JA221 (F′ lacI′ recA leuB6 lacY trpE5 lpp+/F′ lacI′ lac3 lacI′ pro+), and *M. morganii* (6) were used. The following plasmids were used: pGC-01 carrying the *M. morganii* lpp gene (6) and pYM140 carrying the *E. coli* lpp gene (17). The construction of the *P. mirabilis* lpp-carrying plasmid, pGC9-25, and the transformation of pGC9-25 into *E. coli* SB221(F′ lacI′) were described previously (16).

**Protein Labeling Experiments**—*E. coli* SB221(F′ lacI′) carrying pGC9-25 was grown at 37 °C in M9 medium supplemented with 0.4% glucose, 2 µg of thiamine/ml, 200 µg of MgSO₄·7H₂O/ml, and 50 µg each of tryptophan, leucine, and ampicillin/ml. At a cell density of approximately 2.5 × 10⁶ cells/ml, globomycin was added to a final concentration of 100 µg/ml. After 20 min incubation, 200 µCi of [2,3,4,5-3H]arginine (43.5 Ci/mmol; Amersham Corp.) was added to 10 ml of the culture, and the mixture was incubated for 30 s. The labeling was terminated by addition of 10 ml of ice-cold stop solution (40 mM sodium phosphate (pH 7.1), 0.8% formaldehyde, and 2 µg of arginine/ml). The procedures for the membrane preparation, immunoprecipitation with anti-*E. coli* lipoprotein serum, and SDS-polyacrylamide gel electrophoresis were described previously (6).

**Sodium laureyl sarcosinate solubilization** (18) was used to separate the outer and cytoplasmic membrane protein fractions. Total membrane was prepared from a 10-ml culture of *E. coli* SB221(F′ lacI′) carrying pGC9-25 that had been labeled with [3H]arginine for 15 min in the absence of globomycin; this fraction was resuspended in 100 µl of 10 mM sodium phosphate buffer (pH 7.0). Fifty µl of the membrane suspension was mixed with 150 µl of water and 200 µl 1% sodium sarcosinate. The mixture was kept at room temperature for 30 min and centrifuged at 105,000 × g for 30 min at 4 °C. The pellet outer membranes were washed with 10 mM sodium phosphate buffer (pH 7.0), and the supernatant fluid was precipitated with 400 µl of cold 10% trichloroacetic acid. After 60 min incubation in an ice bath, the trichloroacetic acid precipitate (cytoplasmic membrane protein) was collected by centrifugation and washed twice with methanol/ether (1:1). To isolate the peptidoglycan-lipoprotein complex, purified peptidoglycan-lipoprotein complex, purified peptide...
doglycan was obtained by the method of Braun and Sieglin (19). The purified pepdoglycan was treated with 20 μg of lysosome in 20 μl of 10 mM sodium phosphate buffer (pH 7.0) at 37 °C for 3 h.

**S1 Mapping and Northern Blot Hybridization**—To prepare total cellular RNA, a 40-ml cell culture was grown in LB broth (ampicillin was added to a concentration of 50 μg/ml for E. coli strain harboring pGC9-25) at 37 °C to an OD600 of 0.4. The cells were collected by centrifugation and lysed with 3 ml of hot lysis solution (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 350 mM NaCl, 2% SDS, 7 μl 7.0) (17). The lystate was extracted three times with 3 ml of chloroform/phenol (1:1) and three times with 3 ml of chloroform. Three volumes of ethanol and one-tenth volume of 3 M sodium acetate (pH 5.2) were then added to the lystate to precipitate RNA. The RNA precipitate was dissolved in 200 μl of TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA). The mixture was incubated at 37 °C for 25 min. The S1 terminal digestion reaction was carried out at 75 °C for 15 min and then rapidly transferred to a 40 °C water bath and incubated for 3 h (in some experiments, a hybridization temperature of 35 or 45 °C was used instead). After incubation, 300 μl of ice-cold S1 buffer (30 mM sodium acetate (pH 4.6), 50 mM NaCl, 1 mM ZnSO4, 5% glycerol) and 150 units of S1 nuclease were added. The digestion was carried out at 37 °C for 10 min. The S1 digestion reaction was stopped by adding 5 μl of 0.25 M EDTA and 10 μg of yeast tRNA, and the mixture was precipitated with 1 ml of 100% ethanol. The resultant precipitate was washed twice with 70% ethanol, and the supernatant was dissolved in 20 μl of hybridization buffer (80% formamide, 0.4 M NaCl, 0.4 M NaHPO4, 1 mM EDTA). The solution was heated at 75 °C for 15 min and then rapidly transferred to a 40 °C water bath and incubated for 3 h (in some experiments, a hybridization temperature of 35 or 45 °C was used instead). After incubation, 300 μl of ice-cold S1 buffer (30 mM sodium acetate (pH 4.6), 50 mM NaCl, 1 mM ZnSO4, 5% glycerol) and 150 units of S1 nuclease were added. The digestion was carried out at 37 °C for 10 min. The S1 terminal digestion reaction was stopped by adding 5 μl of 0.25 M EDTA and 10 μg of yeast tRNA, and the mixture was precipitated with 1 ml of 100% ethanol. The resultant precipitate was washed twice with 70% ethanol, and the supernatant was dissolved in 20 μl of hybridization buffer (80% formamide, 0.4 M NaCl, 0.4 M NaHPO4, 1 mM EDTA). The solution was heated at 75 °C for 15 min and then rapidly transferred to a 40 °C water bath and incubated for 3 h (in some experiments, a hybridization temperature of 35 or 45 °C was used instead). After incubation, 300 μl of ice-cold S1 buffer (30 mM sodium acetate (pH 4.6), 50 mM NaCl, 1 mM ZnSO4, 5% glycerol) and 150 units of S1 nuclease were added. The digestion was carried out at 37 °C for 10 min. The S1 terminal digestion reaction was stopped by adding 5 μl of 0.25 M EDTA and 10 μg of yeast tRNA, and the mixture was precipitated with 1 ml of 100% ethanol. The resultant precipitate was washed twice with 70% ethanol, and the supernatant was dissolved in 20 μl of hybridization buffer (80% formamide, 0.4 M NaCl, 0.4 M NaHPO4, 1 mM EDTA). The solution was heated at 75 °C for 15 min and then rapidly transferred to a 40 °C water bath and incubated for 3 h (in some experiments, a hybridization temperature of 35 or 45 °C was used instead). After incubation, 300 μl of ice-cold S1 buffer (30 mM sodium acetate (pH 4.6), 50 mM NaCl, 1 mM ZnSO4, 5% glycerol) and 150 units of S1 nuclease were added. The digestion was carried out at 37 °C for 10 min. The S1 terminal digestion reaction was stopped by adding 5 μl of 0.25 M EDTA and 10 μg of yeast tRNA, and the mixture was precipitated with 1 ml of 100% ethanol. The resultant precipitate was washed twice with 70% ethanol, and the supernatant was dissolved in 20 μl of hybridization buffer (80% formamide, 0.4 M NaCl, 0.4 M NaHPO4, 1 mM EDTA). The solution was heated at 75 °C for 15 min and then rapidly transferred to a 40 °C water bath and incubated for 3 h (in some experiments, a hybridization temperature of 35 or 45 °C was used instead). After incubation, 300 μl of ice-cold S1 buffer (30 mM sodium acetate (pH 4.6), 50 mM NaCl, 1 mM ZnSO4, 5% glycerol) and 150 units of S1 nuclease were added. The digestion was carried out at 37 °C for 10 min. To reduce the turbidity, the sample was cooled to room temperature, 4 μl of the loading buffer (50% glycerol, 1 mM EDTA, 0.05% xylene cyanol, 0.05% bromphenol blue) was added, and the sample was subjected to electrophoresis to separate the DNA fragments. A 300-bp XbaI-MspI fragment (+24 to +323 of the E. coli lpp gene (6)) from pGC-01 and a 423-bp XbaI-MspI fragment (+24 to +446 of the E. coli lpp gene (3)) from pYM140 were used as the hybridization probes for the M. morganii lpp mRNA and E. coli lpp mRNA, respectively.

**In Vitro Transcription**—The following fragments were prepared from pGC9-25 and used as the truncated templates in the transcription reaction: a 572-bp EcoRI-RalI fragment (−295 to +77 of P. mirabilis lpp gene), a 245-bp EcoRI-AluI fragment (−295 to −50), and a 127-bp AluI-RalI fragment (−50 to +77) (see Figs. 1 and 2). The single-round transcription reaction was carried out according to Kajitani and Ishihama (24) with the following modifications. The reaction mixture contained 0.6 pmol of DNA, 0.9 units of E. coli RNA polymerase, 0.15 mM each unlabeled ATP, GTP, and CTP, 0.016 mM unlabeled UTP, 5 μCi of [α-32P]UTP (3000 Ci/mmol; Amersham Corp.), and 1 μg of rifampicin/ml. The same buffer system was used except that it contained 1 mM KCl instead of NaCl. The DNA template and RNA polymerase were preincubated together at 37 °C for 15 min prior to the addition of ribonucleotides and rifampicin. The transcription reaction was carried out at 37 °C for 10 min. The samples were subjected to electrophoresis on an 8% polyacrylamide, 8 μm urea gel. The in vitro transcription products of the λDNA (77-base and 193-base (25)) and the MulI-HindIII fragment (−96 to +142) of the crp gene (26) were used as size markers.

**RESULTS**

**Cloning and DNA Sequence of the P. mirabilis lpp Gene**—We have previously cloned a 1.1-kb fragment carrying the entire P. mirabilis lpp gene into the EcoRI site of pBR322 (14). The resultant plasmid, designated pGC9-25, is shown in Fig. 1. The nucleotide sequence encompassing the entire P. mirabilis lpp gene, together with the deduced amino acid sequence, is shown in Fig. 2.

We have previously shown that the cloning of the E. coli lpp gene into pBR322 was unsuccessful due to the lethal overproduction of the lipoprotein (27). However, the promoters of the P. mirabilis lpp gene appeared to be much weaker in E. coli than that of the E. coli lpp gene. Therefore, we were able to clone the P. mirabilis lpp gene into pBR322.

Expression of the P. mirabilis lpp in E. coli—It has previously been demonstrated that E. coli SB221/F' lacI* totally lacks the E. coli lipoprotein (6, 16). In order to examine whether the P. mirabilis lipoprotein was expressed in E. coli, E. coli SB221/F' lacI* cells harboring pGC9-25 were labeled with [3H]arginine for 30 s in the presence and the absence of globomycin (100 μg/ml). In the absence of globomycin, a protein species (lane 1 in Fig. 3A) could be immunoprecipitated with anti-E. coli lipoprotein serum (lane 2 in Fig. 3A) was observed at position α. When globomycin was added, this species disappeared and a new species appeared at posi-
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**FIG. 2. Nucleotide sequence encompassing the P. mirabilis lpp gene.** The translation initiation and termination codons, and the Shine-Dalgarno sequence are boxed. The -35 and -10 regions of the P2 and P1 promoters are underlined and indicated by the numbers -35 and -15, respectively. The putative transcription initiation site is indicated by a large arrow and is deduced from the E. coli lpp mRNA (36). The first nucleotide of transcript I determined by S1 mapping (Fig. 4A) is numbered as +1. The transcription initiation sites of transcript II described in the text are indicated by small arrows. The transcription direction of the P3 promoter is indicated by a broken arrow; the exact location of the transcription initiation site is not known. The amino acid sequence deduced from the nucleotide sequence is also shown.

**Fig. 3. Characterization of the P. mirabilis lipoprotein produced in E. coli carrying pGC9-25.** A, SDS-polyacrylamide gel electrophoresis of the total membrane fraction prepared from cells labeled with [3H]arginine for 30 min. A portion of the solubilized membrane fraction was also treated with anti-E. coli lipoprotein serum. Lane 1, the membrane fraction from cells labeled in the absence of globomycin; lane 2, the immunoprecipitate from the membrane used in lane 1; lane 3, the membrane fraction from cells labeled in the presence of globomycin. B, SDS-polyacrylamide gel electrophoresis of the sarcosinate-treated total membrane fraction from cells labeled with [3H]arginine for 15 min in the absence of globomycin. Lane 1, the cytoplasmic membrane fraction; lane 2, the outer membrane fraction. C, SDS-polyacrylamide gel electrophoresis of the lipoprotein-peptidoglycan complex fraction from cells labeled with [3H]arginine for 15 min in the absence of globomycin. Lane 1, the lysozyme-treated peptidoglycan; lane 2, the untreated peptidoglycan; lane 3, the control membrane fraction labeled with [3H]arginine in the absence of globomycin. Arrows with letters a and b indicate the positions of the P. mirabilis lipoprotein and prolipoprotein, respectively.
The presence of two *P. mirabilis* lpp mRNAs of different sizes was further confirmed by Northern blot hybridization. Two RNA species were clearly detected by using the *P. mirabilis* lpp gene as the probe for both RNA preparations from *E. coli* harboring pGC9-25 and *P. mirabilis* (lanes 1 and 2, respectively, in Fig. 5). These two RNA species were not observed in total cellular RNA from *E. coli* JA221 lpp/′lac′ containing pGC9-25 and yeast tRNA when hybridized to the same probe (data not shown). Based on their sizes, we concluded that the larger species was transcript II and the smaller species was transcript I. In *P. mirabilis*, transcript II was the major species of lpp mRNA as it was in the S1 mapping assay (Fig. 4). In *E. coli* carrying pGC9-25, transcript I was present in slightly larger amounts than transcript II, which contradicts the result from the S1 mapping assay (Fig. 4B). The reason for the discrepancy in the ratio of transcripts II to I in *E. coli* between the S1 mapping assay and Northern blot hybridization is not known. In addition to the two lpp mRNAs, there were two bands of larger size seen in the Northern blot of *E. coli* harboring pGC9-25. These bands were not observed in *P. mirabilis* and were thought to be the plasmid pGC9-25 DNA that was present in the total cellular RNA preparation, since they disappeared after deoxyribonuclease treatment (data not shown).

**FIG. 4. Analysis of the 5′-ends of the *P. mirabilis* lpp RNA by S1 mapping.** Total cellular RNAs from *E. coli* SB221/F′lac′ containing pGC9-25 and from *P. mirabilis* were prepared and used for S1 mapping as described under “Experimental Procedures.” A, the determination of transcription initiation site. Lane 1, the A + G sequencing reaction of the 5′-32P-labeled DdeI-HindIII fragment used as the DNA probe (see Fig. 1); lane 2, 95 μg of RNA from *E. coli* SB221/F′lac′ containing pGC9-25 was hybridized to the 32P-labeled DNA probe at 40 °C for 3 h and treated with 150 units of S1 nuclease. The C residue corresponding to the position of the first nucleotide of transcript I as numbered as +1 (see Fig. 2). B, the effect of hybridization temperatures on the ratio of the two transcripts. The following RNAs were hybridized to the same DNA probe and treated with 150 units of S1 nuclease: lane 1, 95 μg of RNA from *P. mirabilis*, lane 2, 95 μg of RNA from *E. coli* SB221/F′lac′ containing pGC9-25 hybridized at 35 °C for 3 h; lane 3, 95 μg of RNA from *P. mirabilis*; lane 4, 95 μg of RNA from *E. coli* SB221/F′lac′ containing pGC9-25 hybridized at 45 °C for 3 h; lane 5, 150 μg of RNA from *E. coli* JA221 lpp ′/F′lac′ (no plasmid) hybridized at 35 °C for 3 h; lane 6, the DNA probe, alone. I and II refer to the two transcripts produced from the *P. mirabilis* lpp gene (see Fig. 2).

**FIG. 5. Analysis of the *P. mirabilis* lpp RNA by Northern blot hybridization.** Total cellular RNAs from *E. coli* SB221/F′lac′ harboring pGC9-25 and from *P. mirabilis* were prepared, subjected to gel electrophoresis under denaturing conditions, and analyzed by Northern blot hybridization as described under “Experimental Procedures.” Lane 1, RNA from *E. coli* SB221/F′lac′ harboring pGC9-25; lane 2, RNA from *P. mirabilis*. I and II refer to the transcripts shown in Fig. 4A. Letters a, b, and c indicate the positions of the 187-bp RsaI-XbaI DNA fragment, 520-bp HindIII-DdeI DNA fragment, and 581-bp HindIII-XbaI DNA fragment, respectively, that were used as size markers.

In Vitro Transcription—To determine if transcripts I and II arise from two tandem *P. mirabilis* lpp promoters that are active in *E. coli*, a single-round transcription in vitro was carried out as described under “Experimental Procedures.” As shown in Fig. 6, a 372-bp EcoRI-RsaI fragment (−295 to +77; Figs. 1 and 2) from pGC9-25 carrying both putative lpp promoters gave rise to two transcripts with lengths of 77 and 150 bases (lane 2) in good agreement with the initiation sites determined in Fig. 4A; transcript I is initiated at position +1 and transcript II at position −69 (or −71; see Fig. 2).

In addition to the 77-base and 150-base transcripts, transcripts with length of approximately 60 bases were also observed (lane 2, Fig. 6). The same transcripts were also synthesized from a 245-bp EcoRI-AluI fragment (−295 to −50; Figs. 1 and 2) which lost the 127-bp 3′-fragment carrying the promoter for transcript I (designated P1) (lane 1, Fig. 6). Change in the length of the 5′ sequence (upstream of the lpp promoters) of the DNA template caused change in the length of these transcripts (data not shown). These results indicate that there is another promoter (designated P3) upstream of the *P. mirabilis* lpp promoters which directs transcription in the direction opposite to that of the *P. mirabilis* lpp gene (see Fig. 2). When a 127-bp AluI-RsaI fragment (−50 to +77; Figs. 1 and 2) carrying only the P1 promoter was used as the
that these promoters are active in the tandem promoters present in the P. mirabilis lpp gene, only the 77-base transcript was synthesized (see lane 1). The samples were run on an 8% polyacrylamide, 8 M urea gel. The following templates were used: lane 1, the 245-bp EcoRI-AluI fragment (−295 to −50) of the P. mirabilis lpp gene, see Figs. 1 and 2); lane 2, the 372-bp EcoRI-RsaI fragment (−295 to +77); lane 3, the 127-bp AluI-RsaI fragment (−50 to +77). Numbers 90, 77, and 150 indicate the length of the transcripts. I and II refer to the initiation sites of transcripts I and II, respectively (see Fig. 4).

**Fig. 8. Alignment of P. mirabilis lpp P1 promoter with other lpp promoters.** The DNA sequence of P. mirabilis lpp P1 promoter is compared to the lpp promoter sequences of M. organii (6), Erwinia amylovora (5), S. marcescens (4) and E. coli (3). The translation initiation codon, ATG, and Shine-Dalgarno sequence, GAGG, are underlined. The transcription initiation site is indicated by an arrow. The −10 and −35 regions of the P1 promoter are indicated by open circles, and the same regions of other enterobacterial lpp genes are indicated by closed circles. The sequences that do not match each other are illustrated outside the aligned sequences by open triangles. Nucleotides that are different from those of E. coli and M. organii are indicated by * and +, respectively. The upper P. mirabilis lpp sequence is compared to M. organii lpp sequence and the lower P. mirabilis lpp sequence is compared to the other three lpp sequences. Recent evidence suggests that the −10 region of the E. coli lpp gene is AATACT instead of the previously reported TGTAAAT (37). This prompts us to think that the −10 region of the M. organii lpp gene could be AATATC instead of TGTAAAT, but the exact −10 sequence is unknown at present.

**DISCUSSION**

In contrast to all the enterobacterial prolipoproteins studied so far, the M. mirabilis prolipoprotein consists of a 19-amino acid signal peptide and a 59-amino acid mature lipoprotein sequence (3–6, 14). In addition to 26-amino acid substitutions, the P. mirabilis prolipoprotein has a deletion of 1 amino acid at position −17 of the signal peptide, an insertion of 2-amino acid residues, serine and asparagine, immediately downstream of the signal peptide cleavage site, and a deletion of a lysine residue between positions 40 and 41 of the lipoprotein sequence as compared to the E. coli prolipoprotein (14). In spite of these differences in the amino acid sequence, the P. mirabilis prolipoprotein expressed in E. coli is normally modified and processed, and the resulting mature lipoprotein is assembled in the E. coli outer membrane as is the E. coli lipoprotein. The highly conserved regions within the P. mirabilis are likely to play important roles in the assembly of the lipoprotein in the E. coli outer membrane. These highly conserved regions include the signal peptide, the sequence around the signal peptide cleavage site, the sequence between positions 43 and 52, the tyrosine residue at position 57, and the C-terminal lysine residue (14). Furthermore, the entire lipoprotein structure is highly conserved because nearly half of the amino acid substitutions are replaced by functionally similar amino acid residues.

The existence of two tandem promoters in the P. mirabilis lpp gene, designated P1 and P2, which give rise to the two RNA transcripts, I and II, respectively, was clearly demonstrated. Both promoters are active in E. coli. From the S1 template, only the 77-base transcript was synthesized (lane 3, Fig. 6). These results clearly demonstrate that there are tandem promoters present in the P. mirabilis lpp gene and that these promoters are active in the E. coli transcription system as well.

**Presence of Tandem Promoters in Other lpp Genes—**To determine if tandem promoters also exist in other enterobacterial lpp genes, total cellular RNA preparations from M. organii and E. coli JA221 lpp+/F' lacIq were analyzed by Northern blot hybridization as described under "Experimental Procedures." As shown in Fig. 7, M. organii contains two lpp sequence-specific transcripts differing by approximately 100 nucleotides in length, whereas E. coli JA221 lpp+/F' lacIq has only one. Since the M. organii lpp gene seems to have only one transcription termination site (6), it is likely that the M. organii lpp gene also contains two tandem promoters, which give rise to the two transcripts.
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Fig. 9. Possible secondary structures of the P. mirabilis lpp mRNAs. Bases are numbered as shown in Fig. 2. The first nucleotide of transcript I is numbered as +1 and that of transcript II as -71. The termination codon, UAA, is boxed. Transcript I shares stem-loop Structures I-V with transcript II. The \( \Delta G \) values for the six stem-loop structures shown (I-VI) are calculated according to Tinoco et al. (38) to be -19.4, -9.5, -14.0, -8.4, -14.2, and -19.6 kcal, respectively.

mRNA mapping experiments (Fig. 4A), the -35 regions of the P1 and P2 promoters are identified as -30CTGTAT-25 and -10TTGCTT-59, respectively (see Fig. 2), which show some homology to the consensus sequence for the E. coli -35 region TTGACA (31, 32). The -10 regions are -11CATACT-6 for the P1 promoter and -10CATATT-7-7 or -10TATTGT-75 for the P2 promoter, which also show homology to the E. coli -10 region consensus sequence, TATAAT (31, 32). It appears that the P. mirabilis lpp P1 promoter and its flanking regions show some degree of sequence homology to the promoters of the other four lpp genes (Fig. 8), while the P. mirabilis lpp P2 promoter does not appear to share any significant homology to the P. mirabilis P1 promoter or to any sequence upstream of the P. mirabilis P1 promoter regions of the other four lpp genes.

The expression of the P. mirabilis lpp gene is thus controlled by two tandem promoters. The prominent production of transcript II over transcript I seen in P. mirabilis grown in a rich medium (Figs. 4B and 5) suggests that the P2 promoter is more active than the P1 promoter under these growth conditions. However, the P1 promoter appears to be more active in E. coli carrying pG9-25 under the same growth conditions (Fig. 5) as well as in an E. coli cell-free system (Fig. 6). The expression of the lpp gene by tandem promoters was also found in M. morganii but not in E. coli (Fig. 7). Existence of tandem promoters has been shown for genes influenced by catabolite repression such as the gal operon (33), genes that are nitrogen-regulated such as the glnA gene (34), and genes required to produce a large amount of the product such as the genes for ribosomal RNA (35). The tandem lpp promoters may have similar roles or another as yet undetermined role.

Possible stem-loop structures of transcripts I and II are shown in Fig. 9. It is noteworthy that the first 70 nucleotides of transcript II can form a stem-loop structure with a \( \Delta G \) of approximately -20 kcal (Structure VI, Fig. 9). This Structure VI may have an important role in the stability of transcript II, or have a regulating role for the lpp gene expression. The two lpp mRNAs share the other five secondary structures. Structures I and II are common to all the other enterobacterial lpp mRNAs. Structure II has the termination codon UAA (boxed in Fig. 9) at or near the loop (\( \Delta G = -9.8 \) kcal for P. mirabilis, -10.8 kcal for M. morganii, -8.8 kcal for E. amylovora, -15.5 kcal for S. marcescens, and -11.3 kcal for E. coli). Structure I probably acts as a rho-independent transcription termination signal (\( \Delta G = -19.4 \) kcal for P. mirabilis, -21 kcal for E. amylovora, S. marcescens, and E. coli, and -9.4 kcal for M. morganii). The total \( \Delta G \) is approximately -85 and -65 kcal for transcripts II and I, respectively.

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