Identification of Prolipoprotein Signal Peptidase and Genomic Organization of the \textit{isp} Gene in \textit{Escherichia coli}\textsuperscript{*}

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The product of the \textit{isp} gene of \textit{Escherichia coli}, i.e., the prolipoprotein signal peptidase, was identified by both \textit{in vivo} pulse labeling experiments using a high expression \textit{\lambda}I\textsubscript{P} promoter vector and by an \textit{in vitro} transcription/translation coupled system. The molecular weight of prolipoprotein signal peptidase was estimated to be approximately 18,000 by its mobility on polyacrylamide gel electrophoresis and was found to be the same as that of SPase II purified from the wild-type cells. Analysis of SPase II activities in strains containing various subclones, deletion derivatives generated from plasmid pMT521, and analysis of protein products in a strain harboring an \textit{ileS}-\textit{isp}-fused gene generated from plasmid pMT521, and analysis of protein expression XPL promoter vector and by an \textit{in vitro} transcription/translation coupled system. We also present evidence that the \textit{ileS} and \textit{isp} genes are indeed transcribed on the same mRNA, and we report the existence of an internal promoter for the \textit{isp} gene which resides in the \textit{StuI}-HincII region of the \textit{ileS} coding sequence.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Media—\textit{E. coli} K12 strains E699 (Hfr C, pps), J5506 (F \textsuperscript{*} \textit{kip}/254, pps his proA argB thi gal lac xyl mtl tac), CS412 (C600, proA\textsuperscript{R}, hsdR\textsuperscript{M23} /endA hsdR\textsuperscript{M23}), and N4830 (P-L Biochemicals, Cleve) were used. Deletion derivatives of the plasmid pMT521 (4) were constructed and described in Fig. 5. L broth was employed throughout this study except for labeling experiments. M9 minimal medium supplemented with 0.2% glucose and 5% proteose peptone beef extract broth was used for labeling experiments of strain N4830. Cells were shifted to 42 \textdegree C 1 h prior to labeling with [\textsuperscript{35}S]methionine for 15 min, harvested, washed once, and resuspended in M9 minimal medium containing 0.2% glucose.

Subcloning of the \textit{isp} Gene under the \textit{\lambda}I\textsubscript{P} Promoter—The 0.8-kb \textit{BglII} fragment of pMT521, containing the entire \textit{isp} gene and 170 bp of its 5\textsuperscript{'}, and 140 bp of its 3\textsuperscript{'}, flanking regions, respectively, was isolated and inserted into the \textit{HpaI} site of the plasmid pPLA (P-L Biochemicals), which contains the \textit{\lambda}I\textsubscript{P} promoter upstream of the \textit{HpaI} site, to obtain plasmid pMT401. Strain N4830 was transformed with pMT401 and was induced for the \textit{isp} gene expression by shifting the growth temperature from 30 to 42 \textdegree C.

\textit{In Vitro} Transcription/Translation Coupled System—An \textit{in vitro} transcription/translation coupled system using the S-30 fraction of \textit{E coli} (Coden, Houston, TX) was carried out according to the procedure described by Zubay (13). The supercoiled form of plasmid DNA was prepared by CsCl density gradient centrifugation and used as the template (2 \mu g of DNA/25 \mu l of the reaction mixture). After 40 min of incubation at 37 \textdegree C, \textit{in vitro} protein synthesis was terminated by the addition of 10% trichloroacetic acid. [\textsuperscript{35}S]Methionine-labeled gene products were analyzed by SDS-polyacrylamide gel electrophoresis.

Construction of an \textit{ileS}-\textit{isp} Hybrid Gene—The EcoRV-XbaI fragment from pMT521 (Fig. 1) containing the \textit{ileS}-\textit{isp} genes was cloned into the M13 mp10 phage vector at the SmaI-XbaI site. An oligodeoxynucleotide partially complementary to the junction region was synthesized using a model Sam I One Automated Synthesizer from Biosearch Inc. (San Rafael, CA). This oligomer has the sequence 5\textsuperscript{'},-GATTGA CATCCGGCGA AACTTAC-3\textsuperscript{'}; it was used to create a sequence alteration at the \textit{ileS}-\textit{isp} junction by the method of Zoller and Smith (14). The phage carrying the desired mutation was identified by hybridization, and the nucleotide sequence of the mutant was confirmed by direct sequence analysis using the dyeodeoxyribonucleotide chain-termination method of Sanger et al. (15). This site-directed deletion mutagenesis removed the termination codon of \textit{ileS} and the putative initiation codon of \textit{isp}. The resulting clone has the following sequence at the junction: 5\textsuperscript{'},-CCCTAATTTGTCGCGGAGT-3\textsuperscript{'}; encoding Arg-Lys-Phe-Ala-Pro-Ser, which would direct the synthesis of an \textit{IleS-Lsp} hybrid protein by replacing the fMet of the \textit{Lsp} protein with the functions of these genes are related to warrant a coordinate regulation.

In this paper, we describe the identification of SPase II molecules both by pulse labeling experiments \textit{in vivo} and by employing an \textit{in vitro} transcription/translation coupled system. We also present evidence that the \textit{ileS} and \textit{isp} genes are indeed transcribed on the same mRNA, and we report the existence of an internal promoter for the \textit{isp} gene which resides in the \textit{StuI}-HincII region of the \textit{ileS} coding sequence.

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\textsuperscript{1} The abbreviations used are: SPase II, prolipoprotein signal peptidase; P\textsubscript{\textit{\lambda}I\textsubscript{P}}, promoter for tetracycline resistance gene in pBR322; SDS, sodium dodecyl sulfate; bp, base pair(s); kb, kilobase pairs.
Pro and linking the COOH-terminal Ala of the IleS protein to the second residue Ser of the Lsp protein. The 20-kb mutation-containing fragment (\textit{BamHI}-XbaI) from the RF phage DNA was excised and cloned back into pMT521 in place of the corresponding wild-type sequence to generate the clone containing the fused gene.

**Assay of Prolipoprotein Signal Peptidase and Globomycin Sensitivity of \textit{E. coli} Cells—**Prolipoprotein signal peptidase activity was assayed as described previously, using total cell homogenate prepared by lysozyme-EDTA-Nikkol treatment (4). The globomycin sensitivity of \textit{E. coli} cells harboring various plasmids was determined in microtiter plates as described previously (16).

**Protein Analyses of Total Cell Extracts—**Overnight cultures in L broth (1 ml) were harvested in microfuge tubes. Cell pellets were suspended in Laemmli’s solubilizing buffer (17) and boiled for 5 min to solubilize total cell proteins. After centrifugation to remove peptido-oglycan sacculus, aliquots of samples were applied to a SDS-polyacrylamide gel (17) for analyses of protein patterns.

**RESULTS**

*Identification of the ileS and lsp Gene Products Both In Vivo and In Vitro—*We have previously isolated plasmid pLC3-13 carrying the SPase II gene (\textit{lap}, see Ref. 4) from the Clarke-Carbon collection (18). Plasmid pMT521 was obtained by subcloning a 4.5-kb DNA fragment of pLC3-13 into pBR322, and its physical map is shown in Fig. 1. Genetic mapping (6, 10) has indicated that the \textit{isp} gene is located near \textit{dapB} at 0.5–0.6 min of the \textit{E. coli} map. A transducing phage \textit{\lambda}dapB2 containing \textit{E. coli} \textit{dapB} region has been isolated (19), and several subclones of \textit{\lambda}dapB2 into pBR322 have been previously constructed (Fig. 1, Refs. 20, 21). A comparison of the physical maps of \textit{\lambda}dapB2, pGM21, and pMT521 suggests that pMT521 contains the \textit{ileS} (the structural gene for isoleucyl-tRNA synthetase) as well as the \textit{isp} gene. Our DNA sequence data of pMT521 have confirmed that the \textit{ileS} gene is located immediately upstream of the \textit{isp} gene (7).

The gene products encoded by several plasmids were examined by the \textit{in vitro} transcription/translation coupled system described by Zubay (13). Plasmid pMT522 is a deletion derivative of plasmid pMT521 (see the following section). As shown in lane 3 of Fig. 2, two \textsuperscript{35}S-labeled polypeptides (indicated by arrows) were detected in the \textit{in vitro} products programmed by pMT521 DNA. These two \textsuperscript{35}S-labeled bands were also detected in the \textit{in vitro} translation products encoded by pLC3-13 plasmid DNA (lane 6), but not in that encoded by pMT522 DNA (lane 4) or by pBR322 DNA (lane 5). The molecular weight of isoleucyl-tRNA synthetase has been reported to be 114,000 (22), which is similar to that of the larger polypeptide detected in lanes 3 and 6. The molecular weight of the smaller band was estimated to be 18,000, which is similar to the molecular weight of the \textit{isp} gene product deduced from DNA sequencing data (7). Several bands appeared in the high molecular weight range in lane 3 of Fig. 2 most likely correspond to truncated IleS polypeptides; similar bands appeared in lane 4 of Fig. 2 might be fused gene products between \textit{ileS} and vector.

For the identification of the amplified \textit{ileS} and \textit{isp} gene products in \textit{vivo}, the crude extracts of \textit{E. coli} cells harboring plasmid pMT521 were analyzed by SDS-polyacrylamide gel electrophoresis. As shown in lanes 2 and 4 of Fig. 3, a polypeptide with \textit{Mr} of 110,000 was amplified in \textit{E. coli} cells harboring plasmid pMT521. The molecular weight of this polypeptide is in good agreement with that reported for isoleucyl-tRNA synthetase. An elevated level of isoleucyl-tRNA synthetase activity was also detected in cells containing pMT521 (Table 1). At the present time, the reason why a greater extent of accumulation of the IleS protein band in strain J5505 than that in strain E609 is not clear. On the other hand, overproduction of the \textit{isp} gene product was not detected by Coomassie Brilliant Blue staining of the gel in either J5505 or E609 cells carrying plasmid pMT521. In order to obtain higher amplification of the \textit{isp} gene product, we subcloned the \textit{Real-Real} fragment (0.8 kb) containing the \textit{isp} gene (see Fig. 6) into the \textit{HpaI} site of plasmid pPLA (P-L Biochemicals) which contains the \textit{Ap} promoter upstream of the \textit{HpaI} site. Plasmid pMT491 was obtained and was used to transform strain N4830 which contains the temperature-sensitive allele of the \textit{\lambda} repressor gene (C187). After a 4-h
induction of the cloned \( \text{isp} \) gene by shifting the culture temperature from 30 to 42 °C, SPase II activity was amplified by more than 100-fold over the control culture of N4830(pPLA) which does not contain the insert (data not shown). Even under this highly induced condition, the SPase II polypeptide could not be detected in the crude extract by Coomassie Brilliant Blue staining of SDS-polyarylamide gel. To identify the \( \text{isp} \) gene product in vivo, we constructed several deletion derivatives from pMT521 and Fig. 5, deletion of DNA flanking the single XbaI site (pMT522 and pSYC681) completely abolished the amplification of SPase II activity. This observation suggests that the \( \text{isp} \) gene is located around the XbaI site and is in agreement with the genetic mapping data (6) and the DNA sequence data (7). Plasmids with deletions within the ileS gene (pMT101 and pMT102) still conferred on the host cells about plasmid pPLA (lane 2). The mobility of this band is exactly the same as that of a partially purified SPase II preparation from the wild-type cells (lane 6, shown by arrow) provided by Dr. Paul Ray (Wellcome Research Laboratories, Research Triangle Park, NC). When the crude membrane was extracted with Tris-HCl buffer (pH 8.0), Triton X-100, no obvious difference in band pattern was observed between the membrane prepared from the \( \text{kp} \)-containing clone (lane 3) and that of the control (lane 4). This is likely due to the masking of the SPase II by the high background of other proteins extracted under this condition which co-migrated to the \( M_r = 18,000 \) region of the gel.

Effects of Various Deletions in pMT521 on the Expression of the \( \text{isp} \) Gene—In order to study the genomic organization of the \( \text{isp} \) gene and its flanking sequence cloned on pMT521, we constructed several deletion derivatives from pMT521 (Fig. 5). Both the SPase II activities and the maximum globomycin concentrations which allowed cells harboring these plasmids to grow were determined. As shown in Table II and Fig. 5, deletion of DNA flanking the single XbaI site (pMT522 and pSYC681) completely abolished the amplification of SPase II activity. This observation suggests that the \( \text{isp} \) gene is located around the XbaI site and is in agreement with the genetic mapping data (6) and the DNA sequence data (7). Plasmids with deletions within the ileS gene (pMT101 and pMT102) still conferred on the host cells about...
on a weak kp promoter which is located within the ileS gene on pMT521 functions as a major promoter for the cloned insertions are shown with the respective strain numbers in Table III. Tn5 insertion of Tn5 between the EcoRI and EcoRV sites (the P\textsubscript{1sp} region) contained about 60% Isp expression as in pMT521, which is about the same level of Isp expression exhibited in pMT101 and pMT102. As documented in the accompanying paper (24), there is an open reading frame of 936 bp upstream of the ileS gene (designated gene x); the EcoRV site in pMT521 was located at about 400 bp upstream from the 3′-end of this gene. It should be noted that the HpaI-Stul region appears to have a weak stimulation effect on the expression of the Isp gene. The assay of globomycin resistance of E. coli cells provides a very sensitive measurement of a small difference in Isp expression, because this assay is based on cumulative cell growth. With increasing length in deletion between HpaI and Stul (compare SPase II activities between pMT103 and pMT104 or globomycin sensitivities among pSYC887, pSYC888, pSYC889, and pSYC890), cells harboring these smaller plasmids had become increasingly more sensitive towards globomycin.

**mRNA for ileS and Isp Genes Are on the Same Transcript**—We constructed the plasmid pMT521-7 which contains a deletion between the EcoRI and EcoRV sites (the P\textsubscript{1ile} region) of pMT521 (Fig. 6). Cells harboring the plasmid contained only 15% of SPase II activity as compared to that in pMT521, indicating that P\textsubscript{ile} was functioning as a major promoter for the cloned Isp gene in pMT521. This result is consistent with the DNA sequencing data (7) which demonstrated that the stop codon of the ileS gene overlaps with the initiation codon of the Isp gene, and there is no obvious transcription stop signal between ileS and Isp. This result suggests that the Isp gene on the chromosome is probably transcribed by a promoter located upstream from the ileS gene.

The possibility that mRNAs for ileS and Isp are on the same transcript is further supported by the Tn5 insertion experiment shown in Fig. 5. Tn5 provides a transcription termination signal and inactivates the expression of downstream genes. As shown in Table III, insertion of Tn5 between

### Table II

| Plasmid       | SPase II activity | Globomycin concentration |
|---------------|-------------------|--------------------------|
| pMT521       | 100               | >300                     |
| pMT522       | <5                | 30                       |
| pSYC881      | <5                | 30                       |
| pMT101       | 55                | >300                     |
| pMT102       | 50                | >300                     |
| pMT103       | 64                | >300                     |
| pMT104       | 34                | >300                     |
| pSYC887      | 17                | 120                      |
| pSYC888      | 13                | 100                      |
| pSYC889      | 15                | 80                       |
| pBR322       | <5                | 30                       |

Fig. 6. Localization of the promoter(s) for the Isp gene. The structures of pMT521-7, -16, -18, and -20 are shown. Plasmid pMT521-7 was constructed from pMT521 by deleting the EcoRI-EcoRV fragment. Open bars show pBR322-derived sequence, and filled bars represent E. coli chromosomal DNA; P\textsubscript{ile}, the promoter for the tetracycline resistance gene. The positions of the ileS and Isp genes are also shown.

The deletion of the EcoRV-HpaI region (pMT103) still contained about 60% Isp expression as in pMT521, which is about the same level of Isp expression exhibited in pMT521 and pMT102. As documented in the accompanying paper (24), there is an open reading frame of 936 bp upstream of the ileS gene (designated gene x); the EcoRV site in pMT521 was located at about 400 bp upstream from the 3′-end of this gene. It should be noted that the HpaI-Stul region appears to have a weak stimulation effect on the expression of the Isp gene. The assay of globomycin resistance of E. coli cells provides a very sensitive measurement of a small difference in Isp expression, because this assay is based on cumulative cell growth. With increasing length in deletion between HpaI and Stul (compare SPase II activities between pMT103 and pMT104 or globomycin sensitivities among pSYC887, pSYC888, pSYC889, and pSYC890), cells harboring these smaller plasmids had become increasingly more sensitive towards globomycin.

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pBR322 and various pMT521::Tn5 plasmids are shown. The sites of Tn5 insertions allow cell growth in seven strains of E. coli harboring these plasmids and used for the SPase II assay. Lane 1, CS412(pMT521-15); lane 2, CS412(pMT521-18); lane 3, CS412(pMT521-20); lane 4, CS412(pMT521); lane 5, CS412; lane 6, no enzyme control. PLP, modified prolipoprotein; LP, lipoprotein. SDS-polyacrylamide gel electrophoresis (12.5% phosphate buffer system, Ref. 26) was used.

**TABLE III**

| Plasmid | SPase II activity | Globomycin concentration |
|---------|------------------|--------------------------|
| pMT521  | 100.0            | >300                     |
| Tn5-18  | 10.1             | 200                      |
| Tn5-14  | 10.9             | 200                      |
| Tn5-17  | 6.9              | 30                       |
| Tn5-5   | 5.5              | 30                       |
| Tn5-2   | 5.0              | 30                       |
| pBR322  | 5.0              | 30                       |

P<sub>im</sub> and the *lsr* gene in pMT521 significantly reduced *lsr* expression.

Detection of an Internal Promoter for the *lsr* Gene within the ileS Gene—The plasmid pSYC890 is deleted for *P<sub>im</sub>* together with about ¾ of the ileS gene (Fig. 5). The SPase II activity in *E. coli* cells harboring this plasmid was still about three times higher than that found in the control cells (Table II). In addition, we have reported previously that *E. coli* cells harboring plasmid pSYC901 which contains the StuI-AccI fragment of pMT521 at the Smal site of plasmid pUC8 overproduce SPase II activity (11,687 units/mg protein) in the absence of isopropyl-β-D-thiogalactopyranoside as compared to 25,000 units/mg protein in its presence (7). The latter observation could be explained either by the existence of an internal promoter in the StuI-AccI fragment, or by an incomplete repression of the lac-operator. On the other hand, when the same fragment (StuI-AccI) was inserted into the same site of plasmid pUC9 so that the direction of transcription is opposite to that of the lac promoter in the vector, the resulting plasmid overproduced SPase II activity to similar extents, 10,681 or 10,352 units/mg protein with or without isopropyl-β-D-thiogalactopyranoside induction, respectively. These results strongly suggest the existence of an internal promoter within the COOH-terminal ¾ of the ileS gene.

In order to ascertain this promoter activity for the *lsr* gene expression, we subcloned various lengths of DNA fragments flanking the *lsr* gene into the pBR322 vector devoid of the upstream promoter of the tetracycline resistance gene (Fig. 6). Initially, the EcoRI-StuI region was removed from pMT521 (see Fig. 6) which generated pMT521-20. At the junction of EcoRI-StuI site of pMT521-20, a new EcoRI site was generated. We replaced the EcoRI-XbaI fragment with the HincII-XbaI and ScaI-XbaI fragments containing the *lsr* gene, and obtained pMT521-18 and -16, respectively. In these constructions, the *P<sub>im</sub>* in pBR322 had been removed, and the expression of downstream gene should depend entirely on the activity of the promoter located within the inserted fragment. Plasmid pMT521-20 confers about two times higher SPase II activity on the host cells than the control experiment (Fig. 7). On the other hand, cells harboring pMT521-18 and pMT521-16 did not exhibit enhanced SPase II activities, indicating that there exists an internal promoter for the *lsr* gene and that it resides between the StuI and HincII region located about 400 to 200 bp upstream from the ileS stop codon.

Construction and Expression of an ileS-lsp Fusion Gene—The DNA sequencing data have demonstrated that the stop codon of the ileS gene overlaps with the initiation codon of the *lsr* gene (4) into a high expression vector such as the XPL promoter vector, as reported in this paper. By both *in vivo* and *in vitro* studies using pMT401 and pMT521, respectively, we have identified a 18,000-Da polypeptide as the subunit for SPase II. The mobility of overproduced *in vivo* product in cells harboring pMT401 is the same as that of partially purified SPase II from the wild-type cells and also coincides with the predicted molecular weight of SPase II based on DNA sequence data.

SPase II is an essential gene, and the regulation of its expression poses an interesting problem. The first unexpected finding comes from the DNA sequencing data which indicate...
distal portion of the ileS gene, a Shine/Dalgarano sequence would be required for translation initiation because the lps coding sequence is preceded by a nontranslated sequence. At -13 to -6 bp from the initiation codon of lps is found the GTAAGTTT sequence which is complementary to the 3'-end of the 16S RNA of E. coli (25); this may serve as the Shine/ Dalgarano sequence for the lps gene. The DNA region within the ileS structure, i.e., the HpaI-StuI region, appears to be required for the maximal expression of the lps gene (Table II), suggesting the possible existence of other weak promoter(s) within the ileS gene. The physiological significance of coordinated expression of ileS and lps genes by forming a single transcript and the presence of internal promoter(s) of lps in the ileS gene remain obscure.

The synthesis of a hybrid protein of the expected molecular weight encoded by the ileS-lsp fused gene provides further evidence for the cotranscriptional expression of the ileS and lps genes. In addition, it supports the conclusion based on the nucleotide sequence of the lps gene that SPase II polypeptide does not contain a functional signal peptide at its NH₂ terminus. Otherwise, one might have expected the removal of the ileS portion of the hybrid protein along with the putative signal peptide during insertion of the hybrid protein into the inner membrane. The hybrid protein is found in the membrane fraction of the cell extract as the native SPase II. Apparently, the localization of the hybrid protein, which contains an extra 111,000-Da polypeptide amino-terminal to SPase II, is determined entirely by the sequence in the SPase II portion at the COOH terminus of the hybrid protein. Furthermore, this result indicates that the abilities of the SPase II domains in the hybrid protein to assemble into the membrane and to function enzymatically as SPase II have not been severely affected by the sequence alteration at its NH₂ terminus.

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