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Cotranscription of Two Genes Necessary for Ribosomal Protein L11 Methylation (*prmA*) and Pantothenate Transport (*panF*) in *Escherichia coli* K-12

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Genetic complementation and enzyme assays have shown that the DNA region between *panF*, which encodes pantothenate permease, and *orf1*, the first gene of the *fis* operon, encodes *prmA*, the genetic determinant for the ribosomal protein L11 methyltransferase. Sequencing of this region identified one long open reading frame that encodes a protein of 31,830 Da and corresponds to the *prmA* gene. We found, both in vivo and in vitro, that *prmA* is expressed from promoters located upstream of *panF* and thus that the *panF* and *prmA* genes constitute a bifunctional operon. We located the major 3' end of *prmA* transcripts 90 nucleotides downstream of the stop codon of *prmA* in the DNA region upstream of the *fis* operon, a region implicated in the control of the expression of the *fis* operon. Although no promoter activity was detected immediately upstream of *prmA*, S1 mapping detected 5' ends of mRNA in this region, implying that some mRNA processing occurs within the bicistronic *panF-prmA* mRNA.

Posttranslational modification of several ribosomal proteins is a general phenomenon observed in both prokaryotes and eukaryotes (2). Although much information on the expression of the genes for bacterial rRNAs and ribosomal proteins is available (29), little attention has been given to their posttranslational modifications, which are actually quite numerous. In *Escherichia coli*, proteins such as S5, S18, L12, and EF-Tu are acetylated at their N-terminal residues (L7 is the acetylated form of L12). Other modifications include addition of amino acids to the polypeptide chain, e.g., addition of one to four glutamic acid residues to the C terminal of ribosomal protein S6 (25, 30). However, the most frequent modification is methylation. Several ribosomal proteins, e.g., S11, L3, L7/L12, L11, L16, and L33, as well as EF-Tu and IF3, have N-methylated amino acids at specific positions (reviewed in reference 2). L11 is the most heavily methylated ribosomal protein, with three trimethylated amino acid residues: two N ϵ -trimethyllysines at positions 3 and 39 (19) and an amino-terminal N α -trimethylalanine (18, 35). Methylation of ribosomal protein L11 requires S-adenosyl-L-methionine as a methyl group donor (4) in a co- or posttranslational event.

Mutations called *prmA*, which result in an unmethylated form of L11, have been isolated in different laboratories and by independent procedures (15–17, 27). The *prmA1* locus was mapped by Colson et al. (14) to 71 min on the *E. coli* chromosome. This methylation might be expected to affect the function of L11. However, the *prmA* mutant strains were all phenotypically indiscernible from their wild-type parents, and therefore the function, if any, of this energetically costly methylation of protein L11 is unknown. L11 has been implicated in several aspects of ribosome function and assembly, namely, the stringent response in vivo (6, 43–45) and in vitro, ribosomal subunit association (21, 31, 40), the binding domain of the antibiotic thiostrepton (60), ribosomal protein L16 assembly during 50S subunit reconstitution (9, 23), protein

synthesis termination (7, 58, 59), and the ribosomal GTPase domain (20, 48). Despite all of these suggestive results, the precise role for L11 in the ribosome is obscure. Moreover, mutants apparently lacking L11 are viable (56, 57).

The L11 molecules isolated from *prmA* strains are severely undermethylated. In vitro methylation experiments with ribosomes from the *prmA* strains and with extracts from *prmA*⁺ bacteria and radioactively labeled S-adenosyl-L-methionine show that the *prmA* mutations result in loss of methyl groups from both the N-terminal alanine and the internal lysine residues (5, 14, 15). However, some residual L11 methyltransferase activity can be detected in extracts from strains carrying *prmA1* or *prmA3* mutations (2, 15). This residual activity could account for the lack of a phenotype associated with the *prmA* mutations. Since single mutations apparently result in the absence of both internal and N-terminal methyl groups from L11, it seems possible that the same enzyme is responsible for the methylation of all three amino acids. If this is the case, then the distinctive trimethylation of two different amino acids at three different loci when all of the other lysine residues of the protein are unmodified poses an interesting problem of enzyme specificity. We cannot, however, rule out the possibility that two or more enzymes are necessary and that *prmA* is the genetic determinant of the limiting step which prevents subsequent methylations.

To study the role of methylation on L11 function, we have undertaken a systematic study of the *prmA* gene. Chromosomal DNA fragments carrying the *prmA* gene were isolated in a series of λ transducing phages by complementation of a thermosensitive mutation in the nearby gene *fabE* (now renamed *accB*; encodes the biotin carboxyl carrier protein, a subunit of acetyl coenzyme A carboxylase) (3). The *panF* gene (encodes pantothenate permease) has been mapped to the same chromosome region (62). All of the *accB*⁺ λ transducing phages which carried *panF* were also found to be *prmA*⁺, showing that genes *prmA* and *panF* are closely linked (3). The nucleotide sequence of *panF* has been determined (28). We present here the DNA sequence of *prmA* and an analysis of its transcription.

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TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Genotype ^a	Reference or origin
<i>E. coli</i> strains		
IBPC5321	<i>thi-1 argG6 argE3 his-4 xyl-5 mtl-1 tsx-29 rpsL ΔlacX74</i>	46
MB984	<i>srl::Tn10 recA prmA3</i>	IBPC101 (47) × MB1984 (3) by P1 transduction
JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 λ⁻ Δ(lacproAB) F' (traD36 proAB lacI^q lacZΔM15)</i>	65
MB1541	<i>thr-1 leu-6 trp his-4 argH1 thyA1 thi-1 xyl-7 tonA2 supE44 rpsL9 fic Ts[±] prmA3</i>	3
Plasmids		
pBR322	<i>amp tet</i>	10
pFA	<i>accBC panF amp</i>	3
pAF2	<i>panF prmA orf1 amp</i>	This work
pAF3	<i>panF prmA amp</i>	This work
pRS415	<i>amp 'lacZ</i>	54
pAO1	<i>panF prmA-lacZ amp</i>	This work
pAO2	<i>panF-lacZ amp</i>	This work
pRO1	<i>'panF prmA-lacZ amp</i>	This work
pRO2	<i>'panF prmA-lacZ amp</i>	This work
pUC(PrmA)	<i>plac prmA</i>	This work
Phages		
λ6G3	<i>accBC panF prmA orf1 fis</i>	33
λFA6	<i>accBC panF prmA</i>	3
λRS45	<i>'lacZYA</i>	54
λRS/AO1	<i>panF prmA-lacZ</i>	This work
λRS/AO2	<i>panF-lacZ</i>	This work
λRS/RO1	<i>'panF prmA-lacZ</i>	This work
λRS/RO2	<i>'panF prmA-lacZ</i>	This work

^a A prime denotes that the gene is truncated on that side so that the promoter region is absent.

MATERIALS AND METHODS

Strains and media. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were routinely grown in Luria-Bertani (LB) medium. Synthetic MOPS medium (41) containing 0.2% glucose and 50 μg each of arginine and histidine per ml at 30°C was used in bacterial cultures for measurements of β-galactosidase activities as previously described (39). Plasmids were propagated in *E. coli* JM109 or IBPC5321. λ6G3 is from the *E. coli* library of Kohara et al. (33). Plasmid pUC(PrmA) carries the *prmA* structural gene, between oligonucleotides CHIEN and IFRO (see Fig. 2), cloned downstream of the *lac* promoter. Its construction will be detailed elsewhere (64).

DNA manipulations and sequencing. Restriction endonucleases and other enzymes were obtained commercially. Radiochemicals were purchased from Amersham. Large-scale preparations of plasmid DNA, digestions, and cloning were performed by standard procedures (50). DNA restriction fragments and polymerase chain reaction (PCR)-generated fragments were purified from agarose gels by the freeze phenol method (53) or with Gene Clean (Bio 101). The PCR (50 μl) contained 20 pmol of each oligonucleotide, 10 ng of a plasmid [pAO2, pUC(PrmA), or pAF3] template, 2 U of *Taq* polymerase, 0.2 mM each deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, and 0.01% gelatin. PCRs were generally carried out by the following three steps repeated 20 times: denaturation at 92°C for 1.5 min, annealing at 55°C for 1.5 min, and extension at 72°C for 2 min. Double-stranded DNA of recombinant plasmid pAF3 was directly sequenced on both strands as previously described (51). For the oligonucleotides used as sequencing primers (three for each strand), see Fig. 2. Dideoxy-sequencing reactions using T7 DNA polymerase and α-³⁵S-dATP were ana-

lyzed on 0.4-mm-thick 8% acrylamide gels containing 7 M urea.

mRNA preparation. Total RNA was prepared from strain JM109 or IBPC5321 grown in LB medium. Strains carrying plasmid pAF3 or pFA were grown in LB containing 500 μg of ampicillin per ml. Cells were harvested at an A₆₅₀ of 0.7, washed with 10 mM MgCl₂, and resuspended in 20 mM sodium acetate (pH 4.7)–1% sodium dodecyl sulfate, and RNA was extracted by the hot phenol method (49).

S1 nuclease mapping. To map the 5' ends of *panF* and *prmA* transcripts, probes uniquely labeled at one 5' end were synthesized by PCR. A 20-pmol sample of one oligonucleotide (NAPCO for *panF* and ART for *prmA*) was 5' end labeled with [γ-³²P]ATP (specific activity, 3,000 Ci/mmol) and polynucleotide kinase. The second oligonucleotide was complementary to plasmid vector sequences upstream of the *panF* *prmA* region inserted in the plasmid used as the template for the PCR. The reverse sequencing primer (REV17) was used with plasmid pUC(PrmA) for the *prmA* probe (see Fig. 6) and an oligonucleotide homologous to the *amp* gene of pRS415 (RBP22) with pAO2 for *panF* (see Fig. 4). The S1 probes thus synthesized carried a nonhybridizing DNA extension at the unlabeled oligonucleotide end which permits differentiation between readthrough transcription and reannealing of the probe. The labeled fragments were separated from unpolymerized oligonucleotides on a 1% agarose gel and purified by the freeze phenol method (53).

To map the 3' ends of *prmA* transcripts, a DNA fragment, RUE-IFRO (see Fig. 2), was synthesized by PCR. This fragment was digested by *Hind*III. The *Hind*III-IFRO fragment was purified from a 1% agarose gel with Gene Clean (Bio 101), and the *Hind*III end was labeled with [α-³²P]dATP with the Klenow fragment of DNA polymerase in the presence of 0.2

mM each dCTP, dGTP, and dTTP. This 3' end-labeled DNA fragment was purified by elution from a 5% polyacrylamide gel.

The end-labeled DNA probes, approximately 50,000 cpm per hybridization experiment, were incubated with 10 to 30 μ g of *E. coli* total RNA in 80% formamide–40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)–1 mM EDTA–400 mM NaCl (final volume, 50 μ l). After denaturation at 85°C for 10 min, hybridization was carried out overnight at 56°C (5'-end mRNA mapping) or 52°C (3'-end mRNA mapping) and stopped by transferring the hybridized mixture to 0.4 ml of cold S1 nuclease digestion buffer (30 mM sodium acetate [pH 4.6], 250 mM NaCl, 1 mM ZnSO₄, 5% glycerol) containing 100 U of S1 nuclease (Boehringer GmbH, Mannheim, Germany). The samples were incubated at 37°C for 45 min (5'-end mapping) or at 25°C for 1.5 h (3'-end mapping). S1 nuclease-resistant DNA fragments were analyzed on polyacrylamide gels 1 mm thick containing 7 M urea. The gels were fixed, dried, and analyzed with a PhosphorImager (Molecular Dynamics), which permitted quantitation of S1-protected bands, or autoradiographed with Cronex Hi-Plus amplifying screens.

Reverse transcription. Synthetic oligonucleotides were used for primer extension experiments with avian myeloblastosis virus reverse transcriptase (Boehringer). The oligonucleotides were 5' end labeled with [γ -³²P]ATP and polynucleotide kinase and separated from the labeled nucleotide by electrophoresis on denaturing 20% polyacrylamide gels. Typically, 15 or 30 μ g of *E. coli* total RNA and 0.5 pmol of the labeled oligonucleotide were annealed in a volume of 5 μ l. The reverse transcription reaction was performed as previously described (61). The primer extension products were analyzed on denaturing polyacrylamide gels as described above.

In vitro transcription. Templates were made by PCR with suitable oligonucleotides. The reaction mixture (20 μ l) contained 40 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 100 mM KCl, 1 mM EDTA, 100 μ g of bovine serum albumin per ml, 50 ng of template DNA (e.g., the AGFIN-NAPCO PCR-generated fragment for mapping of the *panF* promoter), and 0.3 to 1 U of the *E. coli* RNA polymerase holoenzyme (Boehringer). After 15 min of preincubation at 37°C, a transcription was initiated by addition of 400 μ M each ATP, CTP, and GTP and 100 μ M [α -³²P]UTP. After 15 min, the samples were precipitated with ethanol and analyzed on denaturing polyacrylamide gels as described above.

Construction of *panF-lacZ*, *prmA-lacZ*, and *panF prmA-lacZ* transcriptional fusions. All fusions were constructed on plasmids in vitro and transferred to λ by in vivo recombination. Plasmid pAO1 (*panF prmA-lacZ*) was constructed by inserting the 2.4-kb *Bam*HI fragment from pAF3, carrying a small fragment of pBR322, the end of *accC* (*fabG*), the entire *panF* gene, and the first 10 nucleotides of *prmA*, into the *Bam*HI site of pRS415 (54). Plasmid pAO2 (*panF-lacZ*) was constructed similarly, by inserting the 1.95-kb *Bam*HI-*Bgl*II fragment from pAF3 (the *Bgl*II site is located at the end of *panF* [see Fig. 1]) into the *Bam*HI site of pRS415. Plasmid pRO1 was derived from pAO1 by deletion of the DNA between the *Eco*RI and *Bgl*II (at the end of *panF*) sites (see Fig. 1). Plasmid pRO2 was made by inserting the 1,530-bp *Eco*RV-to-*Bam*HI fragment from pAF3 into pRS415 digested by *Sma*I and *Bam*HI. The fusions carried by pAO1, pAO2, pRO1, and pRO2 were transferred to λ in vivo by using λ RS45 as previously described (54) to give λ RS/AO1, λ RS/AO2, λ RS/RO1, and λ RS/RO2. Two independent plasmid constructs for each fusion were transferred to λ RS45, and two recombinants from each plasmid were picked, purified, and used to make lysates to

lysogenize strain IBPC5321. Monolysogens were determined and β -galactosidase activities were measured as previously described (39).

L11 methyltransferase activity measurements. Ribosomal protein L11 methyltransferase activity was measured basically as previously described (15). To measure the specific L11 methyltransferase activity of a particular crude extract, bacteria from an overnight culture of *E. coli* cells in LB medium with 500 μ g of ampicillin per ml (for plasmid-carrying strains) were pelleted, resuspended in an appropriate volume of a buffer containing 10 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 60 mM NH₄Cl, and 6 mM β -mercaptoethanol, and broken by sonication. The 25- μ l methylation assay contained 1 *A*₂₆₀ unit of 50S ribosomal subunits of *E. coli* MB1541 (*prmA3*) as the substrate (methyl group acceptor), 25 μ M *S*-adenosyl-L-[methyl-³H]-methionine (3 Ci/mmol; Amersham) as the methyl group donor (a concentration eightfold over the reported *K_m* [3.2 μ M] of the enzyme for *S*-adenosyl-L-methionine) (13), and various amounts of the bacterial extracts as a source of L11 methyltransferase. Reaction mixtures were usually incubated at 30°C for 1 to 30 min, and the hot 5% trichloroacetic acid-insoluble radioactivity was collected on Millipore filters and counted. Protein concentrations in the crude extract were estimated (11), and the final result was expressed in picomoles of methyl groups incorporated per minute of incubation at 30°C per milligram of protein in the crude extract.

RESULTS

Subcloning and identification of *prmA* as the genetic determinant for L 11 methyltransferase. The *prmA* gene was found to be closely linked to the *accB* (formerly *fabE*; encodes the biotin carboxyl carrier protein subunit of acetyl-coenzyme A carboxylase) and *panF* (encodes pantothenate permease) genes at 71 min on the *E. coli* chromosome (3). Of the 75 λ bacteriophages isolated as complementing a thermosensitive mutation in *accB*, the 58 that were *panF*⁺ were also *prmA*⁺, showing that *panF* and *prmA* are likely to be adjacent. The gene *fis*, which encodes the factor for inversion stimulation (Fis), has been mapped to the same region (32). Comparison of the restriction maps of the *accB*, *panF*, *prmA*, and *fis* regions showed that these four genes are carried on Kohara bacteriophage λ 6G3 (33) (Fig. 1). The *accB*, *accC* (formerly *fabG*; encodes the biotin carboxylase subunit of acetyl coenzyme A carboxylase), and *panF* genes have been sequenced (3, 28, 34, 38), as have *fis* (32) and a gene (*orf1*) with an unknown function positioned immediately upstream of *fis* and transcribed from the same promoter (8, 42). Located between *panF* and *orf1* is a region of about 1.0 kb which was not sequenced (Fig. 1). This region is also present on the *accB*⁺ *panF*⁺ *prmA*⁺ transducing phages of Alix (3), while it is absent from plasmid pFA, which complements the *accB* and *panF* mutations but not a *prmA* mutation (3). Thus, the *prmA* gene should lie within the unsequenced DNA region.

We therefore cloned a 4.2-kb *Pvu*II fragment, encompassing this region from λ 6G3 into the *Eco*RV site of pBR322 to give plasmid pAF2. This fragment carries the end (last 136 codons) of the *accC* gene, *panF*, and the *orf1* gene, as well as the unsequenced DNA. A slightly smaller plasmid, pAF3, was made by deleting 1,060 bp which included *orf1* (Fig. 1). This plasmid was introduced into the *prmA3* mutant strain (MB984), and L11 methyltransferase activity was measured on crude extracts. MB984 (*prmA3*) showed about 2.5% of the activity of wild-type *E. coli* IBPC5321 (Table 2). Introduction of plasmid pAF3 led to a considerable increase of L11 methyltransferase activity, showing that plasmid pAF3 comple-

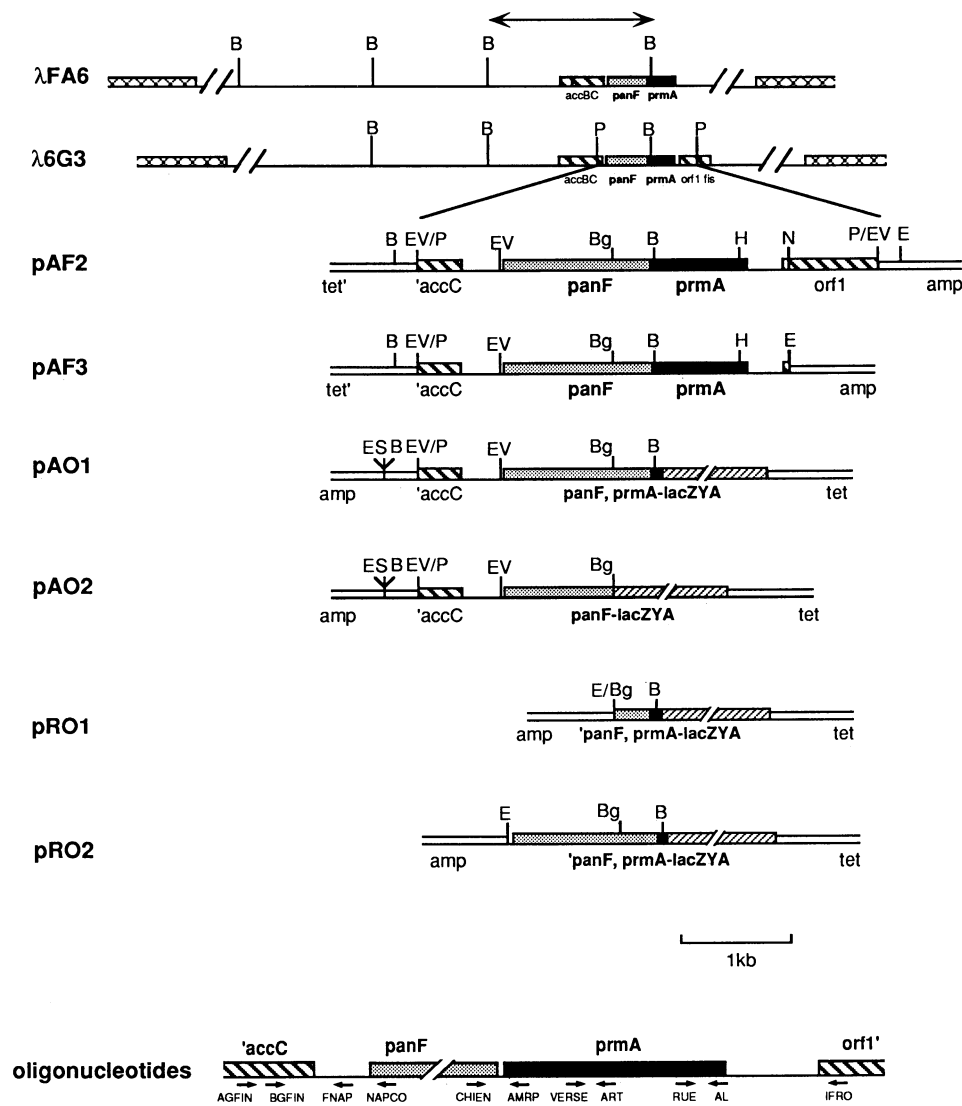


FIG. 1. Subcloning of the *panF* *prmA* region of the *E. coli* chromosome. Restriction maps of λ FA6 and λ 6G3 are shown with the locations of the *accBC*, *panF*, and *prmA* genes. The 4.2-kb *PvuII* fragment of λ 6G3 was cloned into the *EcoRV* site of pBR322 to give pAF2. Deletion of the *NcoI-EcoRI* fragment (by filling in of the sites with the Klenow enzyme and ligation, which recreates the *EcoRI* site) gave pAF3. The different DNA fragments inserted into pRS415 to produce operon fusions with *lacZ*, pAO1, pAO2, pRO1, and pRO2 are shown. ■, *prmA*; ▨, *panF*; ▩, other genes; ▪, *lacZ*; ☒, phage λ ; □, plasmid vector. Abbreviations: B, *Bam*HI; P, *Pvu*II; EV, *EcoRV*; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; N, *Nco*I; S, *Sma*I; E/Bg, hybrid site from *Eco*RI and *Bgl*II; *amp*, ampicillin resistance gene; *tet*, tetracycline resistance gene. \leftrightarrow , 6-kb *Bam*HI fragment of λ FA6 inserted into the *Bam*HI site of pBR322 to give plasmid pFA (3).

TABLE 2. Specific activities of L11 methyltransferase in plasmid-carrying strains

Strain and plasmid	<i>prmA</i> allele on chromosome	Enzyme activity ^a	
		Specific	Relative
MB984(pBR322)	<i>prmA3</i>	0.01	0.02
MB984(pAF3)	<i>prmA3</i>	6.04	13.72
IBPC5321(pBR322)	<i>prmA</i> ⁺	0.44	1.00
IBPC5321(pAF3)	<i>prmA</i> ⁺	7.34	16.68

^a Specific activities are expressed in picomoles of methyl groups incorporated per milligram of protein in the bacterial extract per minute at 30°C.

mented the *prmA3* mutation (Table 2, line 2). Plasmid pAF3 gave a similar level of L11 methyltransferase activity in wild-type strain IBPC5321, resulting in about 15-fold overproduction compared with the same strain carrying pBR322 (Table 2, lines 3 and 4). This level of overproduction, comparable to the pBR322 copy number, is consistent with the idea that the *prmA* locus encodes the structural gene for L11 methyltransferase.

Sequence of the *prmA* gene. The DNA sequence of pAF3 between *panF* and *orf1* was determined (Fig. 2). One long open reading frame (ORF) was found in the region corresponding to the *prmA* gene. The first ATG codon of this ORF is located 11 nucleotides downstream of the termination codon of *panF*. The ORF terminates at a TAA codon 328 nucleotides upstream of the *orf1* gene. The *prmA* gene codes for a polypeptide of 293 amino acids, giving a protein with a

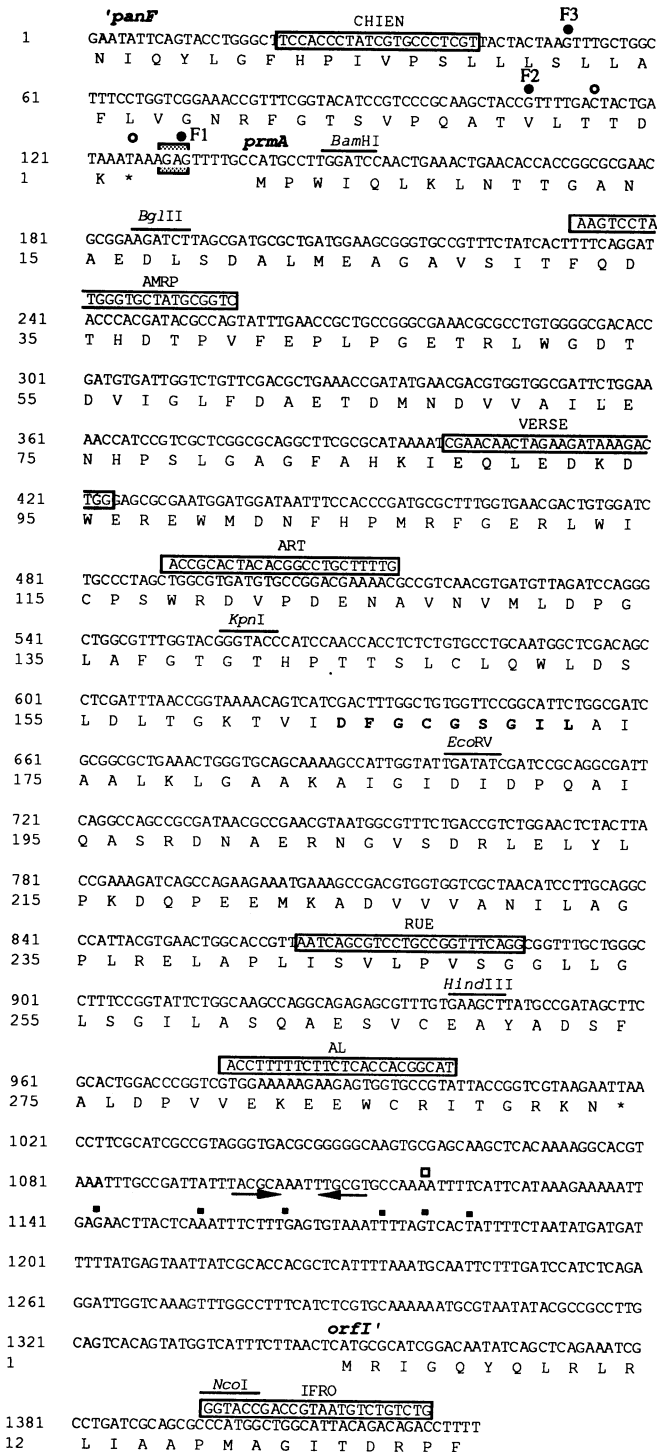


FIG. 2. Nucleotide and deduced amino acid sequences of the *prmA* gene. The boxed sequences show the oligonucleotides used as primers for determination of the nucleotide sequence: CHIEN, VERSE, and RUE on one strand and AMRP, ART, and AL on the other. The sequence presented includes the 3' end of *panF* (28), *prmA*, and the intergenic *prmA-orf1* region (8, 42). Relevant restriction endonuclease sites are indicated. The putative ribosome-binding site of *prmA* is shaded. Stars indicate the termination codons of *panF* and *prmA*. The black circles indicate the 5' mRNA ends mapped immediately upstream of *prmA* by S1 nuclease and reverse transcriptase mapping, and the open circles indicate the 5' ends detected by reverse transcriptase.

molecular mass of 31,830 Da. This is in agreement with the previous biochemical data for the molecular mass of the methyltransferase of 30,000 to 40,000 Da, obtained by sucrose gradient centrifugation (4) and Sephadex gel chromatography (13). Analysis of the *prmA* coding sequence shows no significant homology with other proteins in the DNA-protein data bases. However, a nonapeptide motif characteristic of methyltransferases, DXGXGXGL (where X is any amino acid), as described by Ingrosso et al. (26), is found in *prmA* between amino acids 164 and 172. This pattern of amino acids has been observed in a large number of methyltransferases specific for a wide range of substrates—proteins, nucleic acids, and small molecules. The nonapeptide motif was found associated with two other weakly conserved motifs, primarily in the methyltransferases specific for simple biochemical substrates, e.g., amino acids (26). The other two motifs are not present in the PrmA protein sequence. The presence of the nonapeptide motif is consistent with the idea that *prmA* is the structural gene for the methyltransferase rather than a regulatory protein necessary for its activity.

The *prmA* gene does not show the codon usage characteristic of a strongly expressed protein (22). Upstream of the ATG initiation codon, there is no complementarity, except for GAG, with the 3' end of 16S RNA (52). However, some homology with the "downstream box" (55) associated with high-level expression of certain genes is apparent, 9 of 14 nucleotides at positions 143 to 159 within the *prmA* gene (Fig. 2).

The *panF-prmA* intergenic distance, 11 nucleotides, is not sufficient for a conventional RNA polymerase binding site, and inspection of this region showed no obvious factor-independent transcriptional terminator or homology with the consensus -35 and -10 promoter sequences, suggesting that these two genes are cotranscribed (see below). The short intergenic distance between *panF* and *prmA* might indicate that the two genes are translationally coupled.

Expression of the *prmA* and *panF* genes in vivo. The lack of obvious transcriptional signals on the DNA sequence between *panF* and *prmA* led us to try to localize the *prmA* promoter by using gene fusions: four fragments were cloned into operon fusion vector pRS415 to give plasmids pAO1, pAO2, pRO1, and pRO2 (Fig. 1). pAO2 carries DNA from the end of *accC* to the middle of *panF* (*Bgl*II site; Fig. 1) to give a *panF-lacZ* fusion; pAO1 starts at the same point but extends into the beginning of *prmA* (*Bam*HI site; Fig. 1) to produce a *prmA-lacZ* fusion, while pRO1 and pRO2 carry the same *prmA-lacZ* fusion and all (pRO2) or part (pRO1) of *panF* but lack the sequence upstream of the *panF* structural gene. The operon fusions carried by these plasmids were transferred to λ RS45, giving rise to λ RS/AO1, λ RS/AO2, λ RS/RO1, and λ RS/RO2, which were used to lysogenize IBPC5321. Lysogens of λ RS/AO1 and λ RS/AO2 give moderate β -galactosidase activities, while those of λ RS/RO1 and λ RS/RO2 are near the background value (Table 3). This shows that most *prmA* expression is dependent upon a promoter upstream of *panF*, which could

The squares show the 3' mRNA ends mapped downstream of *prmA* by S1 nuclease, and the major 3' end is indicated by the larger, open square. The nonapeptide motif characteristic of methyltransferases is in boldface. The sequence determined in this work was from nucleotides 60 to 1080. The remaining sequence was taken from references 8, 28, and 42 and is included for clarity. The 3' end of the sequence determined in this work overlaps that previously published (8, 42). Our sequence is in agreement with that of Ball et al. (8), except for a G-C-G change to G-G-C at nucleotide positions 1049 to 1051.

TABLE 3. β -Galactosidase activities in strain IBPC5321 carrying different *panF-lacZ* or *prmA-lacZ* fusions on λ lysogens

Lysogen	Insert ^a	Activity ^b \pm SD
λ RS/AO1	<i>panF prmA-lacZ</i>	40 \pm 2
λ RS/AO2	<i>panF-lacZ</i>	156 \pm 10
λ RS/RO1	' <i>panF prmA-lacZ</i>	1.2 \pm 0.2
λ RS/RO2	' <i>panF prmA-lacZ</i>	3.5 \pm 0.3
λ RS45	' <i>lacZ</i>	1.0 \pm 0.2

^a A prime indicates that the gene is truncated so that the promoter is absent.
^b Activities are expressed in the units defined by Miller (39). Means of four independent cultures are shown.

be the *panF* promoter itself. The low level of λ RS/RO2 activity could indicate that there is a weak promoter activity in the 5' region of the *panF* structural gene. However, it can account for only a minor amount (less than 10%) of *prmA* expression compared with that from the *panF* promoter region.

Localization of the *panF* promoter. The intergenic region between *accC* and *panF* is shown in Fig. 3. We used a combination of techniques, S1 nuclease protection, primer extension, and in vitro transcription, to localize the *panF* promoter within this sequence.

The probe for S1 nuclease protection experiments as the probe, PCR-generated fragment RBP22-NAPCO, which was synthesized with plasmid pAO2 DNA as the template (Fig. 4C). The NAPCO oligonucleotide was 5' end labeled with [γ -³²P]ATP and polynucleotide kinase before polymerization. With this probe and total *E. coli* RNA extracted from a wild-type strain carrying or not carrying plasmid pFA, we detected a major S1-resistant DNA fragment about 340 nucleotides long (Fig. 4A). This located the 5' end of the *panF* mRNA to the series of T residues around nucleotide 263 (B1 of the sequence in Fig. 3). A weaker band of about 395 nucleotides which corresponds to a 5' end around nucleotide 213 (B2 in Fig. 3) was also detected. Primer extension using

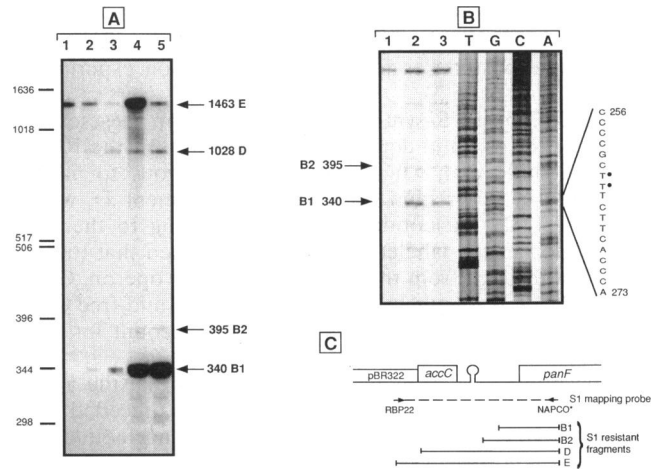


FIG. 4. Mapping of the 5' RNA ends upstream of *panF*. (A) S1 analysis. The probe was the 1,463-bp RBP22-NAPCO PCR-generated fragment ³²P labeled at NAPCO. It was hybridized with RNA made from JM109 grown in LB (lanes 2, 15 μ g; lane 3, 30 μ g) or JM109(pFA) grown in LB with 500 μ g of ampicillin per ml (lane 4, 15 μ g; lane 5, 30 μ g) or with 30 μ g of tRNA (lane 1). The S1-resistant products were electrophoresed on a 5% acrylamide-7 M urea gel. The positions of labeled DNA molecular size markers (in base pairs) are shown on the left, and the locations of the major protected bands B1, B2, D, and E are noted. (B) Primer extension. Total RNA isolated from either IBPC5321 (lane 1, 30 μ g of RNA) or IBPC5321(pAF3) (lane 2, 30 μ g of RNA; lane 3, 15 μ g of RNA) was used for primer extension with the 5'-end ³²P-labeled NAPCO oligonucleotide. The two major extension products, 340 and 395 nucleotides, B1 and B2, are indicated. Lanes T, G, C, and A are sequencing reactions with pAF3 DNA and the NAPCO oligonucleotide as the primer and represent the sequence of the noncoding strand. The sequence around the major 5' mRNA end, shown on the right, corresponds to the sequence of the coding strand. The 5' ends of the transcripts within this sequence are shown by dots. The numbers refer to the nucleotide positions of Fig. 3. (C) Diagrammatic interpretation of the combined mapping results.

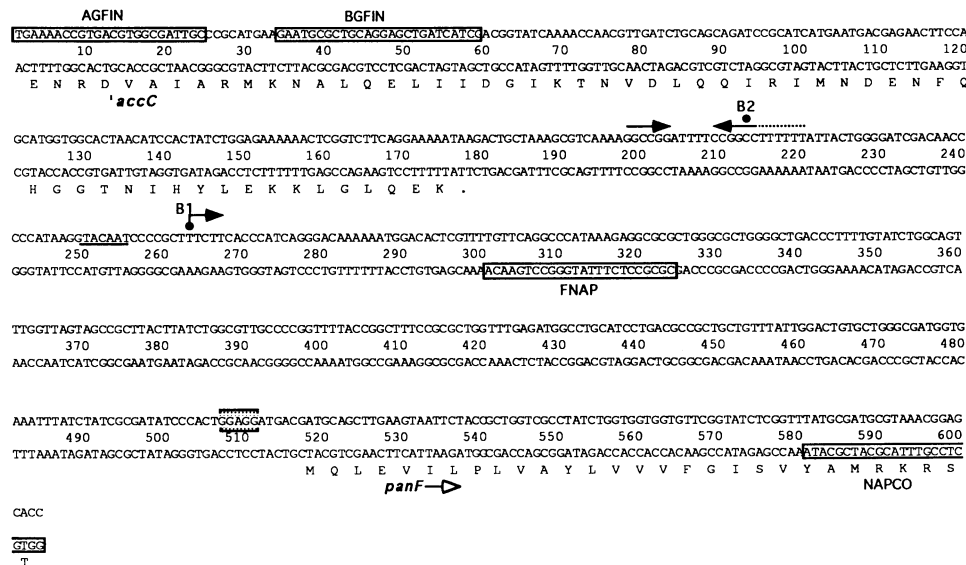


FIG. 3. The promoter region of *panF*. The sequence shows the 3' end of *accC* and the beginning of *panF* taken from references 28 and 34. The sequences of the oligonucleotides used in the promoter-mapping experiments are boxed. The locations of the mRNA 5' ends (B1 and B2) upstream of *panF* are shown by the black dots. The possible -10 box of promoter B1 is underlined. A stem-loop structure after *accC* is indicated by convergent arrows. A potential ribosome-binding site for *panF* is shaded.

reverse transcriptase and the 5'-end-labeled NAPCO oligonucleotide on total *E. coli* RNA extracts detected 5' mRNA ends at the same position as found by S1 protection experiments (Fig. 4B, lanes 1 to 3).

The 1,463-bp PCR-synthesized probe used in these experiments carried plasmid-derived DNA sequences at the RBP22 oligonucleotide end, which are not homologous to mRNA transcripts in this region. Protection of fragment D, with a length of 1,028 nucleotides and corresponding to the *accC* *panF* region of the labeled probe, demonstrated that there is some readthrough from the upstream (*accBC*) operon. Quantitation of these bands in mRNA from a plasmid-free strain suggests that readthrough from *accBC* can account for 10 to 20% of *panF* transcription.

Inspection of the sequence of Fig. 3 shows that the 5' end localized to nucleotide 263 (position B1) is preceded by a possible -10 consensus sequence, TACAAT (nucleotides 250 to 255), but no sequence homologous to the -35 consensus sequence is located at a reasonable distance (16 to 19 bp) upstream. The second 5' mRNA end corresponding to the longer transcript was located at nucleotide 213 (position B2). It falls near a series of T's at the base of a stem-loop structure which could be a transcription terminator for the upstream *accC* gene. Reverse transcriptase, notoriously sensitive to secondary structures within an mRNA, might stop at this site and produce an artifactual 5' end. The fact that the same 5' end was detected by the S1 protection technique, which is less sensitive to secondary-structure artifacts, suggests that the 395-nucleotide band detected does, indeed, correspond to a discrete mRNA species present *in vivo*. Since we found readthrough transcripts from *accBC* into *panF*, it is possible that the B2 RNA is derived from these longer transcripts rather than due to a *de novo* transcription start.

To resolve this problem, we performed *in vitro* transcription with [α - 32 P]UTP on the unlabeled AGFIN-NAPCO fragment (Fig. 3). The transcription products observed are shown in Fig. 5A, lane 1: the strongest transcript (band A) is about 220 nucleotides long, the one near the top of the gel (band C) is a copy of the full-length template, and two or three RNA transcripts of 350 to 420 nucleotides (position B) are also evident.

The 220-nucleotide RNA (A) is a transcript made in the opposite direction (compared with the direction of transcription of *accC*, *panF*, and *prmA*). This was demonstrated by using another PCR-generated fragment between oligonucleotides BGFIN and NAPCO as the template for *in vitro* transcription (Fig. 3). Band A was replaced by a band 34 nucleotides shorter. The cluster of RNA transcripts of about 350 to 420 nucleotides (B) was unaffected, while the full-length template transcript (C) became slightly shorter (data not shown). These experiments localized the 5' end of the backward transcript to around nucleotide 220 on the sequence of Fig. 3, i.e., within the putative transcription terminator for *accC*. We are currently investigating whether this transcript exists *in vivo*.

Transcripts in the size range of 350 to 420 nucleotides (B) are consistent with the size of the S1-protected fragments and primer extension products (340 and 395 nucleotides) detected with *in vivo* mRNA. To confirm that the *in vitro* transcripts found in position B on Fig. 5A, lane 1, really correspond to the 5' ends mapped in Fig. 4, we performed reverse transcriptase mapping on the unlabeled products of *in vitro* transcription of the AGFIN-NAPCO template. The primer used was the 32 P-labeled FNAP oligonucleotide, which hybridizes nearer to the *panF* promoter region than does NAPCO (Fig. 3). The primer extension products obtained are shown on Fig. 5, lane 2. Two major bands about 64 and 112 nucleotides long, which

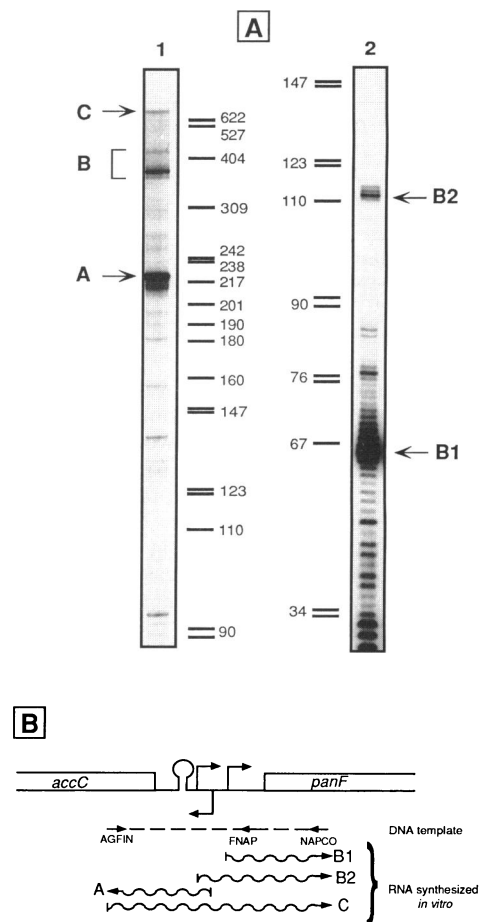


FIG. 5. Determination of the promoter upstream of *panF* by *in vitro* transcription. (A) Lane 1, *in vitro* transcription on the PCR-generated NAPCO-AGFIN fragment with [α - 32 P]UTP. The origin of the three major transcripts (A, B, and C) is discussed in the text. Lane 2, unlabeled *in vitro* transcription products from the NAPCO-AGFIN fragment were subjected to primer extension performed with 5'-end 32 P-labeled oligonucleotide FNAP as the primer. The extension products (B1 and B2) are indicated. The molecular sizes (in base pairs) of labeled DNA markers (pBR322 digested with *MspI*) are indicated. (B) Diagrammatic representation of the transcription results.

correspond to start sites around nucleotides 261 and 212 on Fig. 3, were found. These termini agree extremely well with the location of the 5' ends, B1 and B2, detected previously on *in vivo*-synthesized mRNA. These data are consistent with the existence of two promoter regions for the expression of *panF*. The stronger start site *in vivo* and *in vitro*, B1, coincides with a sequence showing homology to the -10 consensus sequence and seems likely to correspond to a real promoter.

Our data do not allow us to be categorical in our explanation of the B2 transcript. The lack of any promoter consensus sequence and its location within a stem-loop structure makes us suspect that B2 is derived from the *accBC* readthrough transcripts, either as a result of endonucleolytic processing or as an experimental artifact due to the secondary structure in the region. Such a structure could well cause reverse transcriptase to abort prematurely. This is equally possible on the *in vivo*- and the *in vitro*-synthesized transcripts used here. The *in vitro* transcription experiment produced an appreciable amount of a full length, end-to-end transcript (C in Fig. 5A,

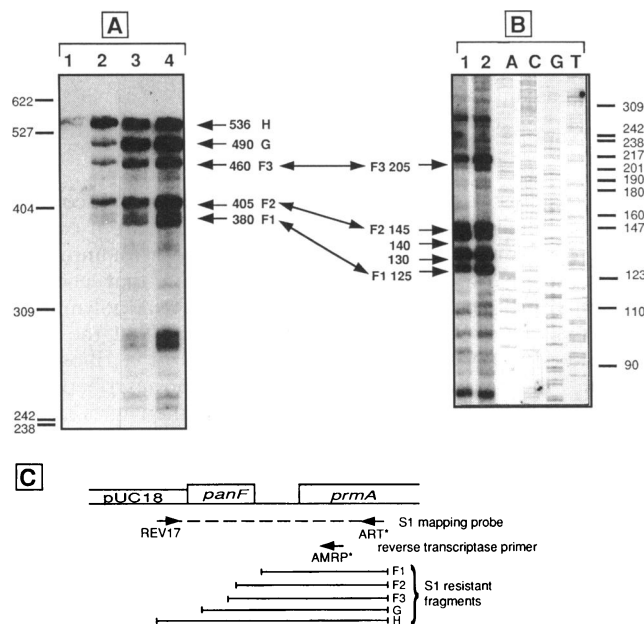


FIG. 6. Mapping of the 5' mRNA ends upstream of *prmA*. (A) S1 analysis. The probe used for S1 mapping was the 536-bp REV17-ART PCR-generated fragment ^{32}P labeled at the ART 5' end. It was hybridized with RNA extracted from strain JM109 (lane 2, 30 μg) or JM109(pAF3) (lane 3, 15 μg ; lane 4, 30 μg) or with tRNA (lane 1, 30 μg). The S1-resistant fragments were electrophoresed on a 6% polyacrylamide-7 M urea gel. The positions of labeled DNA molecular size markers (in base pairs) are shown on the left. The lengths of protected DNA fragments F1, F2, F3, G, and H are indicated on the right. (B) Primer extension. RNA (15 μg in lane 1 and 30 μg in lane 2) isolated from JM109(pAF3) was used for primer extension analysis with 5'-end ^{32}P -labeled oligonucleotide AMRP as the primer. Lanes A, C, G, and T are sequencing reactions with pAF3 DNA and oligonucleotide AMRP as the primer; the sequence corresponds to the noncoding strand. Molecular size markers (pBR322 digested with *Msp*I) are shown on the right in base pairs, and the sizes of the major extension products are indicated on the left. (C) Diagrammatic representation of the combined results.

lane 1) in which the stem-loop structure could also form and block reverse transcriptase. Whatever its origin, B2 is a minor component compared with the B1 transcript.

The *prmA* transcript. The data from the fusions *panF-prmA-lacZ*, *panF-lacZ*, and *prmA-lacZ* suggest that *panF* and *prmA* are cotranscribed. We looked for the polycistronic mRNA by S1 mapping with a long probe covering both genes. We actually detected very little bicistronic *panF-prmA* mRNA but did find evidence for mRNA 5' ends immediately upstream of *prmA*. To confirm the existence of transcripts traversing the *panF-prmA* junction and localize the 5' mRNA ends immediately upstream of *prmA*, an S1 mapping strategy similar to that employed to identify the *panF* mRNAs was used. S1 probe REV17-ART, labeled at the ART oligonucleotide, carries plasmid-derived sequences at the REV17 end which do not hybridize to transcripts from the *panF-prmA* region of the chromosome (Fig. 6C). Use of this probe and mRNA extracted from JM109 carrying or not carrying plasmid pAF3 produced a series of protected fragments with lengths of about 380 (F1), 405 (F2), 460 (F3), and 490 (G) nucleotides, as well as a band corresponding to the reannealing of the probe (H). Some additional, shorter bands were also observed with mRNA from a pAF3-carrying strain (Fig. 6A). The G band corresponds to

protection of the entire *panF-prmA* region of the probe and is therefore equivalent to the bicistronic *panF-prmA* transcript.

Reverse transcriptase mapping with the 5'-end-labeled AMRP oligonucleotide as the primer (Fig. 6C) produced four transcripts in the size range of 125 to 150 nucleotides (approximate sizes: 125, 130, 140, and 145 nucleotides) plus a longer transcript of 205 nucleotides (Fig. 6B, lanes 1 and 2). These 5' ends were precisely located on the sequence of Fig. 2. Comparison with the results of the S1 experiment (Fig. 6A) showed that S1-protected fragment F1 corresponds to the 125-nucleotide reverse transcriptase product, the 145-nucleotide band corresponds to F2, and the 205-nucleotide band corresponds to F3. The 130- and 140-nucleotide transcripts were always detected by primer extension, but their relative intensities varied between experiments, suggesting that they could be artifacts due to secondary structure. To determine whether any of these 5' ends correspond to promoters, we performed in vitro transcription on purified DNA fragment CHIEN-AMRP. We were not able to detect any discrete band, except one corresponding to the full length of the template (data not shown). Since no significant promoter activity was detected in vitro or in vivo in this region, we conclude that the 5' ends of mRNAs F1, F2, and F3 are generated by in vivo processing of the *panF-prmA* transcript.

Comparison of the intensities of the three S1-protected fragments, F1, F2, and F3, with that of the G band, representing the bicistronic *panF-prmA* mRNA, suggests that 75% of the chromosome-derived transcripts are processed (Fig. 6A, lane 2). In pAF3-derived transcripts (Fig. 6, lanes 3 and 4) the processed-to-bicistronic transcript ratio decreased to 50%, possibly because of limiting amounts of the processing enzymes.

3' mRNA extremities downstream of *prmA*. The DNA sequence downstream of *prmA* (Fig. 2) is very AT rich (72% AT if considered from the stop codon of *prmA* to the first codon of *orf1*, of which the central 220 bases are 78% AT) compared with the *prmA* structural gene (54% GC). There is no strong hairpin structure followed by a series of T residues characteristic of prokaryotic terminators in the intergenic *prmA-orf1* region. Two promoter sequences, at least six strong factor for inversion stimulation (Fis)-binding sites, and a cAMP receptor protein (CRP)-binding site have been localized in this AT-rich region, and the DNA exhibits inherent curvature (8, 42).

We used S1 mapping to determine the fate of *prmA* transcripts after the structural gene. The fragment used covered the 3' end of *prmA* and was labeled at the *Hind*III site (Fig. 2). The other, unlabeled end was located within the *orf1* gene (corresponding to oligonucleotide IFRO; Fig. 2). When mRNA from a plasmid-bearing strain [IBPC5321(pAF3)] was used to enhance the amount of *prmA* transcripts, this probe detected a major band of about 185 nucleotides and a series of longer transcripts (Fig. 7A, lanes 4 and 5). The same 3' ends were detectable in RNA extracts prepared from a plasmid-free strain (Fig. 7A, lane 6). The major 3' end (open square in Fig. 2) is located 100 nucleotides downstream of the *prmA* gene and maps to the distal side of a small palindromic sequence which is not, however, followed by a series of T residues (nucleotides 1098 to 1111 of the sequence in Fig. 2). The ladder of bands corresponding to longer transcripts of 200 to 250 nucleotides shows a periodicity of about 10 nucleotides. Thus, these 3' ends on the mRNA correspond to positions on the DNA template separated by about one turn of the B DNA helix (10.5 bp). This spacing suggests that 3' ends are generated by periodic termination of transcription at in-phase positions on the DNA. Since this region is inherently bent because of the presence of

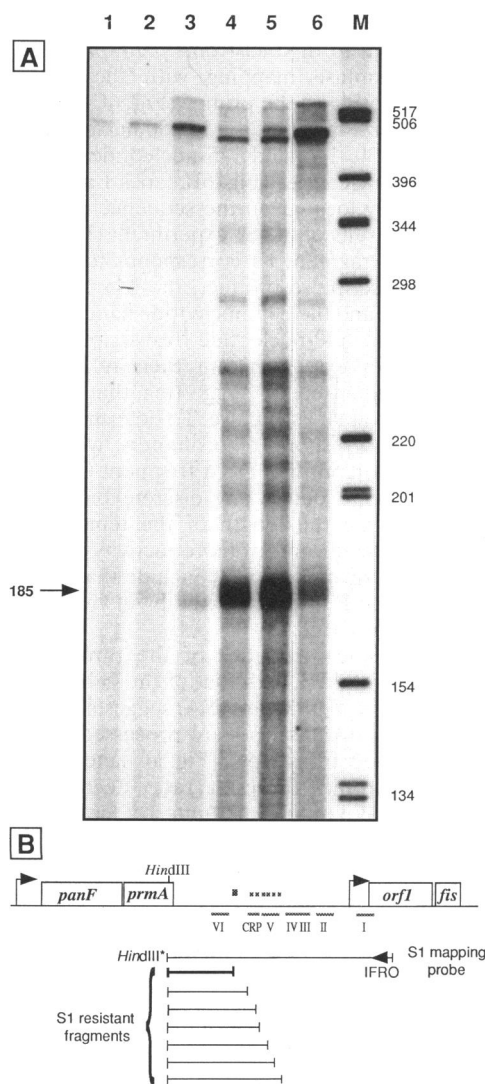


FIG. 7. S1 analysis of the 3' RNA ends of *prmA*. (A) The probe used was the 477-bp *Hind*III-IFRO fragment 3' ³²P end labeled at the *Hind*III end. The probe was hybridized with 15 or 30 μ g of RNA from IBPC5321(pBR322) (lanes 2 and 3), 15 or 30 μ g of RNA from IBPC5321(pAF3) (lanes 4 and 5), 50 μ g of RNA from IBPC5321 (lane 6), or 30 μ g of tRNA (lane 1). The S1-resistant products were analyzed on a 5% polyacrylamide-7 M urea gel. The positions of molecular size markers are indicated on the right in base pairs, and the size of the major S1-resistant fragment is given on the left. (B) Diagrammatic representation of the experiment. CRP, cAMP receptor protein binding site; I to VI, Fis binding sites.

poly(A) tracts and has several Fis-binding sites and a cAMP receptor protein-binding site (Fig. 7B), which are known to affect DNA structure (8), we suspect that the 3' ends observed here are related to the topology of the region rather than due to classical transcription termination. Analysis of the mRNA from the pAF3 plasmid-carrying strain revealed protection of a fragment just shorter than the full-length probe (Fig. 7, lanes 4 and 5). This is because the insert in pAF3, which ends at the *Nco*I site, is slightly shorter than the S1 probe which was synthesized with oligonucleotide IFRO (Fig. 2). The protection of this shorter fragment shows that a certain percentage of RNA polymerase molecules on the plasmid DNA template can

transcribe through the intergenic *prmA-orfI* region into the *orfI-fis* operon. However, the contribution of *panF-prmA* transcription to *orfI-fis* expression in vivo is not likely to be very significant in vivo.

DISCUSSION

Although cotranscription is a convenient strategy for coregulation of genes with a common function, there are a certain number of examples of cotranscription of genes with unrelated functions, e.g., the *rpsU dnaG rpoD* operon that encodes ribosomal protein S21, DNA primase, and RNA polymerase subunit sigma 70 (12). In this work, we show that the gene necessary for posttranslational modification of a ribosomal protein—the methyltransferase of L11—is cotranscribed with the gene for pantothenate permease. Pantothenate is a precursor for coenzyme A. The gene *panF* is necessary only when pantothenate must be taken up from the environment, because wild-type *E. coli* can synthesize pantothenate from β -alanine and pantoate by using the enzyme encoded by *panC* (see Fig. 1 in reference 62). We are currently investigating the implications of coexpression of these two genes, *panF* and *prmA*, which have very different functions.

prmA is the first gene that controls the methylation of a ribosomal protein to be sequenced. A mutation (*prmA*) that affects the methylation of L3 has been isolated and mapped to 50 min on the *E. coli* chromosome (14, 37). We are unaware of information, either genetic or biochemical, concerning the other ribosomal protein methyltransferases. The gene dosage effect on L11 methyltransferase activity observed with the *prmA* gene carried on a multicopy plasmid, the fact that the size of the protein encoded by *prmA* agrees with the molecular weight of the partially purified enzyme (4, 13), and the presence of a short amino acid motif found in many methyltransferases of different specificities (26) argue that *prmA* is the structural gene for L11 methyltransferase. A detailed analysis of mutations within the *prmA* gene and their effect on L11 methylation also support this conclusion (64).

The *prmA* ORF appears to start at the ATG codon 11 nucleotides downstream of the *panF* termination codon. Protein fusions with a truncated *lacZ* gene were constructed at the *Bam*HI site 11 nucleotides downstream of this ATG. The hybrid gene which carries the first four codons of *prmA* in phase with the *lacZ* gene, when present on a plasmid or bacteriophage λ vector, produces blue colonies on 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) plates, showing that the first ATG does function as an initiation codon (63). A second ATG codon, 22 amino acids downstream, shows no better ribosome-binding site. The short distance between *prmA* and *panF*, together with the lack of any recognizable Shine-Dalgarno sequence, except GAG, suggests that the two genes are translationally coupled. Efficient translational coupling has been observed in various bacterial systems in which the termination and initiation codons can overlap or be separated by tens of nucleotides (1, 24, 36).

The data presented here show that, both in vivo and in vitro, genes *panF* and *prmA* are cotranscribed from a promoter located upstream of the *panF* structural gene. The best candidate for the *panF* promoter is that which gives rise to transcript B1 in Fig. 3. Transcripts from B1 carry a 255-nucleotide untranslated leader upstream of the *panF* structural gene. This long mRNA leader might be involved in regulation of expression of the operon, although we have not identified any obvious regulatory elements in this region.

We also detected, by different techniques, a 5' mRNA end located at the base of a stem-loop structure (B2 in Fig. 3). Our

present data cannot unambiguously assign this 5' end to a promoter activity. If it does not correspond to a promoter, then it derives from posttranscriptional processing of the longer transcript which was detected in small amounts. These longer transcripts presumably initiate from the upstream *accBC* operon and must read through the putative terminator located after *accC* (nucleotides 200 to 220 in Fig. 3), which in this case could serve as an mRNA-processing site.

Another potential regulatory device is suggested by the detection of a promoter activity *in vitro* that gives rise to the synthesis of a divergent mRNA in this same region. The length of the transcript detected (A in Fig. 5A, lane 1) locates its start point very near to B2 (Fig. 3) but on the opposite strand. There are no sequences strongly homologous to the consensus -35 and -10 promoter sequences in this region. We are currently investigating whether this promoter is functional *in vivo* and what the length of the putative antisense mRNA is. It is interesting that there is a long ORF of 314 amino acids downstream of this promoter, completely contained within the *accC* structural gene but expressed from the opposite strand.

Despite the lack of any promoter activity, *in vivo* or *in vitro*, expressing a monocistronic *prmA* transcript, we did locate several mRNA 5' ends upstream of the *prmA* structural gene which lie mostly within the 3' end of the *panF* structural gene transcript. The existence of a discrete *prmA* transcript was suggested by Northern (RNA) analysis (63). The use of a probe internal to the *prmA* gene detected basically a smear of hybridizing material with lengths of up to 1,800 nucleotides. There was also a rather diffuse band of about 1,200 nucleotides. This is sufficient to cover the entire *prmA* gene but not both *panF* and *prmA*. The use of a *panF*-specific probe failed to detect this or any other discrete bands. The simplest interpretation of these results is that the bicistronic *panF-prmA* transcript is subject to posttranscriptional processing which generates an mRNA which just covers the *prmA* gene. We are currently investigating this hypothesis. It is interesting that any endonucleolytic processing within the 3' end of the *panF* mRNA will generate a truncated *panF* mRNA incapable of directing the synthesis of a full-size PanF protein. The β -galactosidase activities of the operon fusions (Table 2) provide some indication of the relative expression levels of the two genes. The lower level of β -galactosidase activity of the *prmA-lacZ* operon fusion than of the *panF-lacZ* operon fusion suggests that some down regulation of expression occurs. This effect could be related to the putative processing events that we have detected in the *panF-prmA* mRNA.

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