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Tracking and Elimination of an Interfering Polypeptide Coexpressed with the Vaccinia Virus mRNA Capping Enzyme Overproduced in *Escherichia coli*

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Vaccinia virus (vv) mRNA capping enzyme is composed of a large and a small subunit encoded by genes D1 and D12, respectively. A 38-kDa interfering polypeptide is copurified with the vaccinia virus capping enzyme overproduced in *Escherichia coli*, but the origin of this polypeptide is unknown (P. Guo and B. Moss, 1990, *Proc. Natl. Acad. Sci. USA* 87, 4023–4027). This polypeptide competes with the large subunit in binding to the small subunit during the assembly of the heterodimeric enzyme in the cell, resulting in a reduced yield of the active enzyme. Results from the studies of ribosome-binding site replacement, frame shifting, DNA deletion, and *in vitro* mutagenesis showed that the interfering polypeptide originated from a new translation initiation site within the D1 gene. Transfection of a plasmid containing an internal eukaryotic ribosome binding site into monkey kidney cells infected with vv producing T7 RNA polymerase resulted in the expression of the large subunit up to 30% of total cellular radiolabeled protein; however, the 38-kDa polypeptide was not detected. This finding suggests that the initiation site was recognized only by *E. coli*, not by eukaryotic cells. The Shine–Dalgarno sequence is not found in the corresponding region preceding the putative start codon, indicating that an unusual mechanism for ribosome binding exists. Mutagenesis of the putative initiation codon of the interfering polypeptide from ATG (Met), coding for residue 498 of the large subunit, to ATA (Ile) eliminated the expression of the interfering polypeptide. A stable and active mutant enzyme was expressed in *E. coli* HMS174(DE3) cell without the presence of the interfering polypeptide.

The mRNA capping enzyme of vaccinia virus is a multifunctional heterodimer with the activities of mRNA triphosphatase, mRNA guanylyltransferase, and mRNA (guanine-N^7-)-methyltransferase (1,2). This enzyme catalyzes the generation of eukaryotic mRNA's 5′-terminal cap structure $m^7G(5')pppN$- which is required for translation and stability of mRNA. Recent findings have shown that this enzyme is involved in the initiation of intermediate transcription (3), the termination of early transcription (4), and telomere resolution (5). The large subunit, a 93-kDa polypeptide encoded by the D1 gene (6), has mRNA triphosphatase and mRNA guanylyltransferase activities, and the small subunit, 32-kDa polypeptide encoded by the D12 gene (7,8), may have mRNA (guanine-N^7-)-methyltransferase activity (1,2,8). The C-terminal region of the large subunit may interact with the small subunit. To gain further understanding of the mechanism of mRNA capping, mRNA (guanine-N^7-)-methylation, transcription initiation and termination, and concatemeric DNA resolution, it is essential to overexpress and purify this enzyme for *in vitro* studies. Both genes encoding the vaccinia virus mRNA capping enzyme have been cloned and coexpressed in *Escherichia coli* (8). Both subunits have been assembled into a heterodimer with the enzymatic activities of mRNA triphosphatase, mRNA guanylyltransferase, and mRNA (guanine-N^7-)-methyltransferase (8). The purified enzyme can be used for: (i) 5′-end radioactive labeling of RNA molecules; (ii) capping of mRNA transcribed *in vitro*, thus enabling the mRNA to be translated *in vitro* for protein production; and (iii) biological studies on the mechanism of mRNA capping, methylation, transcription initiation and termination, and resolution of the concatemeric vaccinia virus DNA.

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However, when these two genes were cloned into *E. coli*, a 38-kDa polypeptide was produced and copurified with the functional enzyme (8). Protein sequencing revealed that this polypeptide was encoded by the C-terminal region of the D1 gene and that it contained a methionine on its N-terminus (8). This polypeptide bound to the 32-kDa small subunit competitively with the 93-kDa subunit and was copurified with the small subunit (8). After five steps of purification, levels of the 38-kDa interfering polypeptide were high compared to those of the 93-kDa polypeptide (8). Competition of the 38-kDa polypeptide with the large subunit greatly reduced the capping activity of the enzyme produced in *E. coli*. Assembly of the 32- and 93-kDa subunits into a native enzyme was required for the stability and complete mRNA capping activity (8). A protein fragment with an approximate molecular weight of 55 to 60 kDa was also produced in *E. coli* cells expressing the large subunit. This fragment contained the GMP-binding activity (8). Since the sum of the molecular weight of the 55- and 38-kDa fragments is equal to 93 kDa, which is the size of the large subunit (8), it was originally believed that these fragments were the products of protease cleavage of the large subunits and that the 55-kDa fragment was the N-terminal fragment of the large subunit. The purpose of this study was to investigate the mechanism of the generation of the interfering 38-kDa polypeptide and to eliminate its production in *E. coli* cells that coexpress both subunits, to overproduce the capping enzyme in eukaryotic cells, and to investigate the biological activity of the recombinant and mutant enzymes.

By deletion, site-directed mutagenesis, and the use of a eukaryotic expression plasmid, a new translation initiation codon for the 38-kDa polypeptide within D1 gene was identified. Mutation of Thr–Met at amino acid residues 497 and 498 to Ser–Ile in the D1 gene eliminated the production of the interfering 38-kDa polypeptides. A stable and active mutant enzyme was expressed in *E. coli* HMS174(DE3) cell without the presence of the interfering polypeptide.

MATERIALS AND METHODS

Deletion of the NdeI Fragment from the D1 Gene to Shift the Open Reading Frame (ORF) for the Large Subunit

The 613-bp *Nde*I fragment from the N-terminal part of the D1 gene clone in vector pET3c (9) was deleted. This was accomplished by the religation of the DNA fragment resulting from the digestion of the plasmid pETD1 (8, Fig. 1A) with the restriction enzyme *Nde*I (CATATG), thus generating the plasmid pETD1ND1 (Fig. 1A). This deletion caused a shift in the ORF and resulted in the formation of two stop codons immediately following a new ATG start codon.

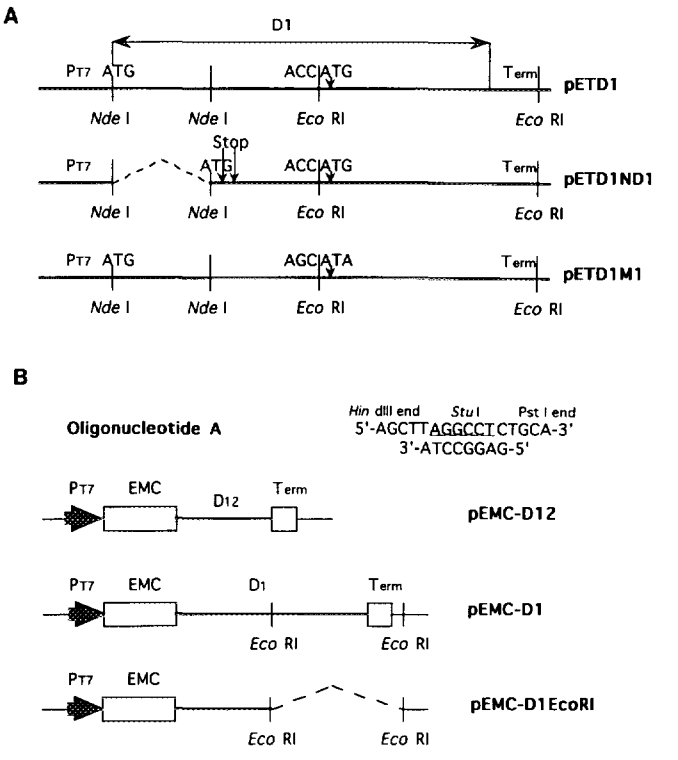

FIG. 1. Structure of plasmids containing *E. coli* (A) or eukaryotic (B) ribosome binding site. Plasmid pETD1ND1 was generated by the deletion of a *Nde*I fragment from plasmid pETD1. Oligonucleotide A is the synthetic double-stranded oligonucleotide used in the construction of plasmid pBlueStu (see text). The dashed lines represent the deleted region. Arrows in (B) show the direction of transcription. P_{T7}, T7 promoter; EMC, Encephalomyocarditis virus 5′ untranslated region; T_{erm}, T7 termination signal.

In Vitro Mutagenesis of D1 Gene

To construct the mutant plasmid pETD1M1, the 1.5-kb *Eco*RI fragment containing the C-terminal part of the D1 gene from the plasmid pETD1 was inserted into the plasmid pBluescript KS(−) (Stratagene). With this plasmid as a template, a PCR fragment was generated with two primers, an M13 forward primer and a synthetic oligonucleotide 664CC (5′-AGAATTCTTAGTTAATGGAGAAATACTTAAACCTAGAATTGATAAAAGCATAAAATATATTAACTC-3′). The synthetic oligonucleotide contained a mutation of ACCATG to AGCATA in codons 497 and 498 of the D1 gene. After digestion with *Eco*RI, the PCR fragment which contained the desired mutation was inserted into the plasmid pETD1EcoRI, which had a deletion of the 1.5-kb *Eco*RI fragment from plasmid pETD1. The resulting plasmid was named pETD1M1 (Fig. 1A). A 1.0-kb *Bgl*II/*Bam*HI fragment from the plasmid PETD12 (8) was isolated and cloned into the *Bgl*II site of plasmid pETD1M1, generating the plasmid pETD12D1M1.

Construction of Plasmids pEMC-D1, pEMC-D12, and pEMC-D1EcoRI

A synthetic double-stranded oligonucleotide A (Fig. 1B) containing the *Stu*I site was inserted into the plasmid pBluescript KS(+) (Stratagene) between the *Hin*dIII and *Pst*I sites, generating a plasmid pBlueStu which preserved the original *Hin*dIII and *Pst*I sites. A *Hin*dIII/*Stu*I fragment which contained the 660-bp 5'-end of the D1 gene was isolated from the *Hin*dIII D fragment of vaccinia virus DNA and ligated into the *Hin*dIII/*Stu*I sites of a plasmid pBlueStu, generating a plasmid pStuD1. An *Nco*I (CCATGG) site around the D1 start codon was created by *in vitro* mutagenesis as previously described (10,11), producing the plasmid pBlueStuNco. The *Hin*dIII/*Stu*I fragment from pBlueStuNco was used to replace the *Hin*dIII/*Stu*I fragment of the plasmid pMG2C which contained a *Hin*dIII/*Nsi*I fragment of the left end of vaccinia virus *Hin*dIII D fragment, producing the plasmid pWD1Nco. A *Nco*I/*Sma*I fragment containing the complete D1 coding sequence was isolated from pWD1Nco and cloned into the *Nco*I/*Sma*I sites of plasmid pTM3 (12), containing an internal eukaryotic ribosome binding site, producing the plasmid pEMC-D1 (Fig. 1B).

A 1.7-kb *Nsi*I fragment containing the complete D12 coding sequence was isolated from the *Hin*dIII D fragment of the vaccinia virus genome and cloned into the plasmid pBluescript KS(+) at the *Pst*I site by ligating the compatible sticky ends to generate the plasmid pBlueNsiD12. This plasmid was utilized to prepare single-stranded DNA for *in vitro* mutagenesis. The orientation of the D12 coding sequence was determined, and the *Bam*HI site of the vector was located at the 3'-end of the D12 coding sequence. An *Nco*I site was created around the start codon by site-directed mutagenesis (10,11). An *Nco*I/*Bam*HI fragment containing the complete D12 coding sequence was isolated and cloned into the *Nco*I/*Bam*HI sites of the plasmid pTM3, producing the plasmid pEMC-D12 (Fig. 1B).

To construct the plasmid pEMC-D1EcoRI, a 1.5-kb *Eco*RI DNA fragment coding for the C-terminal part of the large subunit in plasmid pEMC-D1 was deleted. This was accomplished by religation of the DNA fragment resulting from the digestion of the plasmid pEMC-D1 with the restriction enzyme *Eco*RI (Fig. 1B).

Expression of Capping Enzyme in E. coli

The capping enzyme was expressed by the method of Studier and Moffatt (13), modified by Guo and Moss (8) and Guo *et al.* (14,15). The *E. coli* strains BL21(DE3) and HMS174(DE3) (13) containing an inducible T7 RNA polymerase gene were used as the expression hosts for plasmids pETD1ND1, pETD1M1, and PETD-12D1M1. An overnight culture was diluted 1:100 with Super Broth (for 1 liter: 12 g Trypton, 24 g yeast extract, 12.5 g K_2HPO_4, 3.8 g KH_2PO_4, 5 ml glycerol) and after incubation for 2 h at 37°C, 0.4 mM IPTG was used for induction when samples were prepared for SDS–PAGE. For enzyme activity assays, as described below, no IPTG was used. The bacteria were incubated for an additional 3 h, collected by centrifugation, resuspended in reaction buffer (50 mM Tris–HCl, pH 8.0, 2 mM dithiothreitol, 5 mM $MgCl_2$), and lysed by sonication.

The D1 and D12 genes in the plasmids pEMC-D1 and pEMC-D12 were induced by the following procedure (12). The 30-μl overnight cultures were inoculated into 3 ml Super Broth. After 1 h of incubation at 37°C, glucose was added to a final concentration of 4 mg/ml. The bacteria were grown for an additional 1 h; $MgSO_4$ was then added to a final concentration of 10 mM. The purified phage CE6 was added to a final concentration of 4×10^9 plaque forming units (pfu) per milliliter. After an additional 30 min incubation, cells were precipitated and resuspended in 3 ml M9 media with [^{35}S]methionine (10 mCi/ml). This suspension was incubated for 5 min at 37°C. The labeled proteins were detected by autoradiography after 10% SDS–PAGE.

Overproduction of Capping Enzyme in Vaccinia Virus-Infected Cells

Monkey kidney cell CV-1 grown in Dulbeco's modified Eagle's medium containing 10% fetal bovine serum were infected with vaccinia virus VTF7-3, which expresses the T7 RNA polymerase (13). After 30 min incubation at 37°C with rocking, these infected cells were transfected or cotransfected with the plasmids pEMC-D1, pEMC-D12, or pEMC-D1EcoRI by calcium phosphate precipitation (16). Cells were harvested after 24 h. For detecting the expression of the capping enzyme subunits, 24 h after transfection the cells were deprived of Met for 20 min and then pulse-labeled with [^{35}S]-methionine in Met-deficient modified Eagle medium for 30 min. Cells were lysed with 1% SDS and loaded onto SDS–PAGE. Following electrophoresis and drying, the gel was exposed to X-ray film.

Assay for Enzyme Activity

After sonication in reaction buffer (50 mM Tris–HCl, pH 8, 2 mM dithiothreitol, 5 mM $MgCl_2$), the cell debris, either *E. coli* or CV-1, was precipitated by centrifugation; 0.4 μl of [α-^{32}P]GTP (3000 Ci/mmol, 2 mCi/ml) was added to 10 μl supernatant and incubated for 10 min at 37°C (8). The reaction mixtures were applied to 10% SDS–PAGE and analyzed by autoradiography.

RESULTS AND DISCUSSION

Expression of Mutant Genes in E. coli

In plasmid pETD1, the *Nde*I restriction site (CATATG) at the 5'-end of the *Nde*I fragment contains the

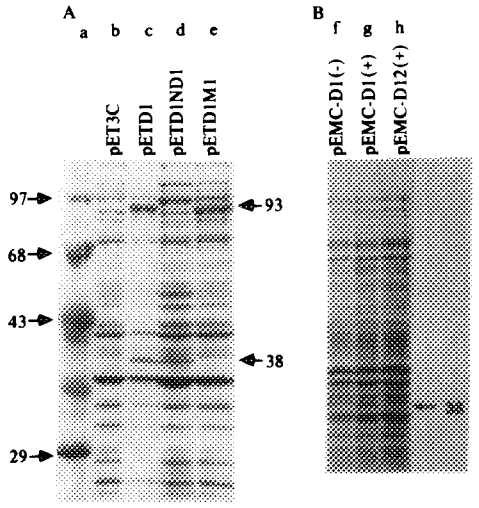

FIG. 2. The vaccinia virus capping enzyme subunits expressed in *E. coli*. (A). *E. coli* transformed with various plasmids was induced by IPTG. Lysates of cells were analyzed by SDS–PAGE and the gels were stained with Coomassie blue. (B). (+) indicates that the *E. coli* cells were induced by phage CE6 (lanes g and h) and (−) indicates that the cells were not induced (lane f). The *E. coli* cells were labeled with [^{35}S]methionine and the gel was dried and exposed to X-ray film. Arrows show the induced 93- and 38-kDa polypeptides. Numbers on the left in (A) indicate the size of molecular weight standards.

ATG start codon of the D1 gene (8). The deletion of the *Nde*I fragment from D1 gene shifted the ORF and the nascent start codon ATG was followed immediately by two stop codons (Fig. 1A). The resulting plasmid pETD1ND1 was transferred to *E. coli* BL21(DE3). The transformed cells were induced with 0.4 mM IPTG and the product was detected by Coomassie blue staining after SDS–PAGE. The 93-kDa products were not expressed, as expected, but the 38-kDa polypeptide was still produced (Fig. 2A, lane d), indicating that the 38-kDa polypeptide was a product expressed from another initiation codon within the coding sequence of 93-kDa polypeptide rather than a cleaved product of the large subunit.

E. coli BL21(DE3) was also transformed with the plasmid pETD1M1, which contained the mutation in codon 498, from ATG(Met) to ATA(Ile), and induced with 0.4 mM IPTG. The 38-kDa polypeptide was not detected in the cells containing this plasmid (Fig. 2A, lane e). The disappearance of the 38-kDa polypeptide after mutation of ATG at codon 498 suggests that this polypeptide was not related to the expression of the 93-kDa ORF and supports the conclusion that the 38-kDa polypeptide was expressed from a different ORF.

Expression of Plasmids pEMC-D1 and pEMC-D12 in E. coli

The D1 and D12 genes were inserted into the plasmid pTM3 so that both genes were preceded by an EMC (12) containing an internal eukaryotic ribosome binding site (Fig. 1B). When the resulting plasmids pEMC-D1 and pEMC-D12 were used to transform the *E. coli* BL21(DE3) or HMS174(DE3) containing an inducible T7 RNA polymerase gene (13), no surviving transformant was recovered. It is possible that the RNA from the EMC region produced by T7 RNA polymerase in leaky expression without IPTG induction (17) was toxic to the host. We attempted to use the *E. coli* hosts of BL21(DE3)plyS and plyE or HMS174(DE3)plyS and plyE (17). These hosts express the gene coding for lysozyme, which could inhibit the T7 RNA polymerase and reduce the leaky expression. However, all attempts at transformation with these strains have failed, suggesting that the RNA transcribed from the EMC region was extremely toxic to *E. coli* cells. To overcome this problem, *E. coli* BL21 (13), which does not contain the T7 RNA polymerase gene, was used as host for the transformation. The transformants were infected with phage CE6, which expresses the gene for T7 RNA polymerase (13).

After infection with phage CE6 in the presence of [^{35}S]methionine, the large and small subunits were not produced in *E. coli* as seen in lanes g and h of Fig. 2B, since *E. coli* did not recognize the EMC eukaryotic ribosome binding sites. However, the 38-kDa polypeptide was detected in cells transformed with pEMC-D1 by autoradiography after SDS–PAGE (Fig. 2B, lane g). No *E. coli* ribosome binding site was present in plasmid pTM3, which was the vector for the construction of plasmid pEMC-D1 and pEMC-D12, indicating that a new *E. coli* ribosome binding site was present within the D1 gene. The ATG codon corresponding to residue 498 of the large subunit was postulated as the start codon of the 38-kDa coding sequence. A ribosome binding site should be present upstream of this ATG. The *E. coli* ribosome binding site has been well characterized. It is a Shine–Dalgarno sequence (AGGAGG) about 7 bases upstream of the ATG. This sequence was not found in the corresponding region which reads ACCTATTAAG TTTATAGCAGAATTCTTAGTTAATGGAGA-AATACTTAAACCTAGAATTGATAAAACCATG. This result indicates that a very unusual mechanism for ribosome binding exists.

Overproduction of the D1 and/or D12 Subunits in Vaccinia Virus-Infected Cells

CV-1 cells, infected with vaccinia virus vTF7-3, were transfected or cotransfected with plasmids pEMC-D1, pEMC-D12, and pEMC-D1EcoRI. Vaccinia virus vTF7-3 expresses the bacteriophage T7 RNA polymerase (12). The plasmids pEMC-D1, pEMC-D12, and pEMC-D1EcoRI all contained a T7 promoter (Fig. 1B). The insertion of the EMC sequence between the T7

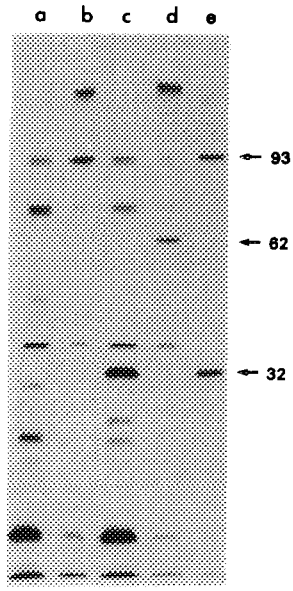

FIG. 3. Expression of vaccinia virus capping enzyme subunits in monkey kidney cells CV-1. Lysates of cells labeled with [^{35}S]-methionine were analyzed by SDS–PAGE and autoradiography. Each lane was transfected with the following plasmids. a, mock; b, pEMC-D1; c, pEMC-D12; d, pEMC-D1EcoRI; e, pEMC-D1 + pEMC-D12. Arrows show the size of proteins expressed by the gene in each plasmid (kDa).

promoter and the gene greatly enhanced the expression of the gene of interest (12). The expression level of the genes was monitored by pulse-labeling the cells with [^{35}S]methionine as detailed in Fig. 3. The genes coding for D1 and D12 as well as the truncated D1 were overproduced up to 30% of the total cellular proteins. No expression of the 38-kDa polypeptide was detected (Fig. 3, lanes b and e), indicating that the initiation codon for the 38-kDa polypeptide was not recognized as a gene in vaccinia virus-infected cells.

GMP Binding Activity of the Gene Products Overexpressed in Vaccinia Virus-Infected Cells

To detect the biological activity of the products, the lysates of vaccinia virus-infected CV-1 cells, which were transfected or cotransfected with plasmids pEMC-D1, pEMC-D12, and pEMC-D1EcoRI, were incubated with [α-^{32}P]GMP and then analyzed by SDS–PAGE and autoradiography (8). Because the vaccinia virus itself can produce the capping enzyme, a lysate of CV-1 cells infected with the vaccinia virus vTF7-3 was used as the background control. Enzymatic activity was assayed by measuring the increased GMP binding activity compared with that of the control. The GMP binding activity in infected CV-1 cells cotransfected with plasmids pEMC-D1 and pEMC-D12 was increased fivefold (data not shown). The increase in GMP binding activity was not detected in infected cells transfected with plasmids pEMC-D1, pEMC-D12, and pEMC-D1EcoRI alone, or cotransfected with plasmids pEMC-D1EcoRI and pEMC-D12. These results are consistent with previous findings that the coexpression of both subunits is required for stability and biological activity of the capping enzyme (8). Moreover, the truncated large subunit expressed from the truncated D1 gene in plasmid pEMC-D1EcoRI, corresponding to the 62-kDa band in lane d of Fig. 3, did not have the GMP binding activity (data not shown). The 363 amino acids at the C-terminus of the large subunit were removed by truncation. The loss of the GMP binding activity of the truncated large subunit indicated that the essential domain was located somewhere beyond residue 481 corresponding to the *Eco*RI deletion site. The results from this study exclude the possibility that both the 55- and the 38-kDa fragments were the products of protease cleavage of the large subunits.

GMP Binding Activity of Mutant Capping Enzyme from E. coli

The plasmid pETD12D1M1 carrying a Thr–Met to Ser–Ile mutation in codons 497 and 498 was constructed by inserting a 1.0-kb *Bgl*II/*Bam*HI fragment from the plasmid PETD12 (8) into the *Bgl*II site of plasmid pETD1M1 (Fig. 1A). When the *E. coli* BL21(DE3) or HMS174(DE3) cells transformed with the plasmid pETD12D1M1 were induced by 0.4 mM IPTG, the overproduced capping enzyme was insoluble due to the aggregation of the protein (8,18). Therefore, IPTG was eliminated and the capping enzyme was expressed by T7 RNA polymerase, which was produced by leaky expression. In this case, the capping enzyme in *E. coli* was soluble.

After breaking the cells with a French Press, cell debris was removed by centrifugation and the supernatant was used in the assay of [α-^{32}P]GMP complex formation. The bacteria HMS174(DE3) containing the plasmid pETD12D1 produced the 55-kDa GMP-binding polypeptide as well as 93-kDa polypeptide (Fig. 4, lanes b and e). The 36-kDa band was detected in *E. coli* BL21(DE3) cells transformed with the plasmid pETD12D1M1, but not in HMS174(DE3) cells containing this mutant plasmid (Fig. 4, lanes c and f). These low molecular weight polypeptides might be derived from the middle region of the large subunit by proteolytic cleavage. The interfering polypeptide was eliminated. Figure 4, lane f, shows that only one polypeptide corresponding to the large subunit was labeled by ^{32}P, indicating that the mutant polypeptide was stable in *E. coli* HMS174(DE3).

In conclusion, the interfering 38-kDa polypeptides

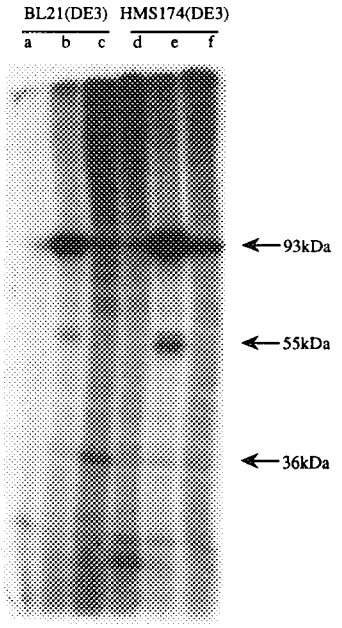

FIG. 4. Protein-GMP complex formation from lysates of *E. coli* haboring plasmids pETD12D1 (lanes b and e), pETD12D1M1 (lanes c and f), and vector pET3c (lanes a and d). Lysates were incubated with [α-^{32}P]GTP, analyzed by SDS-PAGE, and then autoradiographed. Host *E. coli* BL21(DE3) (lanes a, b, and c) was compared with HMS174(DE3) (lanes d, e, and f). Numbers on the right indicate the estimated size of the radioactive bands (kDa). Lane f was the lysate from host HMS174(DE3) harboring plasmid pETD12D1M1. In this lane, only one polypeptide corresponding to the large subunit was labeled by [α-^{32}P]GTP.

were translated from a new initiation site within the *D1* gene and can be eliminated by mutating the new start codon ATG. An active and stable mutant enzyme was expressed in *E. coli* HMS174(DE3).

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