Gene V Protein-Mediated Translational Regulation of the Synthesis of Gene II Protein of the Filamentous Bacteriophage M13: a Dispensable Function of the Filamentous-Phage Genome

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Received 18 June 1991/Accepted 6 November 1991

Introduction of a deletion in the genome of wild-type M13 bacteriophage that eliminates translational repression of M13 gene II by its cognate gene V protein had no effect on phage viability. Furthermore, it was noted that gene V protein of phage IKe, a distant relative of M13, does not function as a translational repressor of its cognate gene II protein. The data strongly indicate that the gene V protein-mediated control of gene II expression in bacteriophage M13 is an evolutionary relic of the ancestral filamentous-phage genome and thus dispensable for proper filamentous-phage replication.

Protein-mediated translational repression is one of the many mechanisms by which *Escherichia coli* and many of its bacteriophages control the expression of their genomes (for a review, see reference 16). Proteins that exert this type of regulation also frequently fulfill a role in gene expression or DNA replication, and translational repression is generally regarded as their secondary function.

In this communication, we report our studies on the function and evolution of translational repression by the single-stranded (ss) DNA-binding protein encoded by gene V of the F-plasmid-specific filamentous bacteriophage Ff, a synonym for the well-known bacteriophages M13, f1, and fd (for a review, see reference 29). Unlike many other bacterial and eukaryotic viruses, filamentous phages do not kill or lyse their host cell. On the contrary, infection essentially can proceed over an infinite number of cell divisions. To establish and maintain such a persistent infection, the phage must be able to adapt its replication cycle to the metabolic growth and division requirements of the host. Gene V protein (gVp)-mediated translational repression of the synthesis of phage-specific DNA replication protein encoded by gene II has been envisioned as one of the mechanisms by which the phage exerts this control (12–14, 25, 26, 28, 46, 48–50).

Multiplication of the Ff genome proceeds according to a rolling-circle type of replication (15). Each replication round is initiated by gene II protein (gIIp), which creates a DNA replication site by introducing a nick in the viral strand of double-stranded replicative-form DNA (RFI) (24). During the first 10 min of infection, the newly synthesized viral strand is converted into duplex RFI. Because RFI is the template not only for phage DNA replication but also for phage gene expression, the concentration of the 10 phage-encoded proteins will increase accordingly. This accumulation continues until the concentration of gVp has reached a critical threshold level. At this concentration, these molecules bind in a cooperative manner to the newly synthesized viral strands, thereby preventing their conversion to RFI and initiating the assembly of ssDNA-containing progeny phage particles at the host cell membrane (1, 30, 36).

Besides its role as a regulator of ssDNA synthesis, genetic and biochemical studies have indicated that gVp is also involved in the translational repression of at least 7 of the 10 phage-encoded proteins (28, 46, 48–50). Whereas regulation of the expression of genes I, II, III, V, and X occurs via specific sequences at the 3' ends of the respective mRNAs (25, 26, 47–50), regulation of genes VII and IX occurs indirectly via the coupling of the translation of these genes to that of gene III (17, 38, 50). The strongest translational repression has been found for gene II mRNA, and the nucleotide sequences involved in this translational regulation have been characterized best (25, 26, 47–50). In vivo gene regulation studies have demonstrated that the efficiency of gVp-mediated repression of the synthesis of gIIp is highly dependent on the sequence of the first 16 nucleotides of gene II mRNA and its relative position with respect to the initiation codon of gene II (25, 47). In vitro studies support this observation. Furthermore, they indicate that these 16 nucleotides encompass the major binding sequence for gVp to gene II mRNA (25, 26). However, note that the nucleotides that are important for gVp-mediated translational regulation are not confined to these 16 nucleotides; nucleotides that are located downstream from position 16 are also involved in translational repression (47).

Since in vitro DNA binding studies also have indicated that gVp has a higher affinity for the DNA analog of the first 16 nucleotides of gene II mRNA than for other deoxyoligonucleotides (26), and since the former DNA sequence is synthesized almost immediately after the onset of rolling-circle-type replication, it has been postulated that this DNA sequence functions as a nucleation site for the cooperative binding of gVp to the nascent viral strands in the DNA-sequestering process (26, 40, 48).

Besides its role in the process of initiation and termination of rolling-circle replication, gIIp has also been shown to stimulate the conversion of the viral strands into RF (12, 13). Repression of the synthesis of gIIp thus presumably contributes to the shift in the life cycle of Ff from the accumulation of RFI DNA and phage-specific proteins to the production of ssDNA for progeny filamentous-phage particles.

To examine whether the viral DNA sequestering model as

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described above is correct and whether translational repression of the synthesis of gVp is an indispensable function of the filamentous-phage genome, we deleted the nucleotide sequence corresponding to the major gVp target site on gene II mRNA both from the Ff genome and from that of a phagemid. Contrary to expectations, we observed that deletion of this sequence has no measurable deleterious effect either on the viability and replication properties of phage Ff or on the production of ampicillin resistance-transducing filamentous-phagemid particles.

To know more about the evolutionary conservation of gVp-regulated translation, we investigated whether the ssDNA-binding protein encoded by phage IKe, a phage that is evolutionarily related to Ff (32), is able to repress the synthesis of its cognate gVp. Evidence is provided that, in contrast to Ff gVp, IKe gVp does not function as a translational repressor.

MATERIALS AND METHODS

Bacterial strains and phages. IKe RF was prepared from phage IKe-infected cells of the E. coli JE2571 (fa gal lac leu mal str thr xyl) (pCU53 tra cam) (41). Gene expression studies were done with E. coli MC1061 araD139 Δ(aral-leu)7697 ΔlacO74 gagU gagK hsr hsm rpsL (2). E. coli BMH71-18mutS Δ(lac-proAB) thi supE mutS215::Tn10 (F' lacZΔ15 lacZAM15 proAB) and MK30-3 Δ(lac-proAB) recA galE strA (F' lacF lacZAM15 proAB) (Boehringer Mannheim) were used for the selection of mutants constructed by the site-directed mutagenesis gapped-duplex approach (21). The ss phage or phagemid DNA for oligonucleotide-directed mutagenesis by the uracil method was prepared in E. coli BW313 (dut ung thi-1 relA spoT1) (F' lysA) (22). After primer extension in vitro, mutant phages or phagemids were selected by transformation of E. coli JM101 supE thi Δ(lac-proAB) (F' traD36 proAB lacZΔ15 lacZAM15) (23) with the mutagenesis mixtures. For the transduction experiments, the helper phages MI3K07 (44), R408 (35), IR1 (10), and M13 wild type were used.

Construction of recombinant plasmids and phagemids. Plasmids and phagemids that were used in this study and whose construction and properties have been described elsewhere are pUC9 (45), pKUN19 (20), pNLG1 (18), and pACYC177, lacZ, pZIIM13, and pGV_M13 (49). Plasmid pGV_IKe contains the region from positions 1275 to 1606 on the IKe genomic sequence (32). It encompasses the Shine-Dalgarno region plus the complete coding sequence of IKe gene V. IKe RF was digested with HaeII, and an 877-bp fragment (positions 729 to 1606) was isolated and inserted into the SmaI site of pUC9. Clones containing gene V in the desired orientation were selected and, after further propagation and isolation, digested with EcoRI and DraI. The fragment that encompasses gene V was inserted between the SmaI and EcoRI sites of pNLG1. To verify the nucleotide sequence of the gene V fragment by the dideoxy method (37), it also was inserted in phagemid pKUN19.

Plasmid pZII_M13 contains a fragment extending from positions 6699 to 45 from the IKe genomic nucleotide sequence (32). This fragment contains the gene II promoter and the first 46 nucleotides, up to codon 12, of gene II mRNA. It was isolated from IKe RF by digestion with AluI and, after being linked to 12-bp BamHI linkers (5'-dCGCGGATCCGG-3'), was inserted into the BamHI site of pACYC177, lacZ. Subsequently, the fragment was cloned into the BamHI site of M13mp9 (23) and its nucleotide sequence was verified.

Site-directed mutagenesis. The sequence coding for nucleotides 2 to 15 of gene II mRNA was deleted by site-directed mutagenesis with the oligonucleotide 5'-GTGGATAATCCAGGGAAGATG-3'. A deletion of this sequence in the nontranslated leader of the fusion gene mRNA (II'/ZM13) was introduced by the gapped duplex method of Kramer et al. (21) in a recombinant M13mp9 phage containing the gene II-derived fragment of plasmid pZII_M13 (49). After verification of the deletion by DNA sequence analysis, the mutated fragment was inserted in pACYC177, lacZ as described previously (48), resulting in pZII_M13Δ2-15. Identical deletions were introduced in phagemid pKUN19, resulting in pKUN19Δ2-15, and phage M13, resulting in M13Δ2-15, by the uracil mutagenesis method of Kunkel (22).

Titrations of transducing particles and phage. The number of ss phagemid DNA molecules that were sequenced during helper phage-induced rolling-circle replication was determined by transduction experiments by making use of the ampicillin marker genes of pKUN19 and pKUN19Δ2-15 (20). Exponentially growing cells of E. coli JM101 harboring either pKUN19 or pKUN19Δ2-15 were superinfected with an Ff helper phage as described previously (20, 44) and incubated at 37°C overnight. To determine the number of infectious phage and phagemid particles by titration, 1 ml of culture was centrifuged for 5 min in an Eppendorf microcentrifuge. The supernatant was transferred to another tube and heated for 10 min at 65°C to kill the remaining bacteria. Appropriate dilutions (10 µl) were then added to 100 µl of a culture of E. coli JM101 cells at an A600 of 1.0 and 500 µl of 2YT medium and incubated at 37°C for 10 min. The mixture was spread on 2YT agar plates containing ampicillin (100 µg/ml) and incubated at 37°C overnight. The number of ampicillin-resistant colonies formed was used to calculate the titer of transducing particles present in the original culture. Similarly, the phage titer was determined by plating serial dilutions of the heated supernatant on E. coli JM101.

Gene expression studies. β-Galactosidase assays and analysis of proteins on sodium dodecyl sulfate (SDS)-polyacrylamide gels were performed as described previously (27, 48). The data of the β-galactosidase assays are given as arithmetic means of at least three independent experiments.

RESULTS

Role of DNA analog of gVp target site on gene II mRNA in rolling-circle replication. In vivo gene regulation studies have demonstrated that the deletion of sequence corresponding to nucleotides 2 to 15 of gene II mRNA completely eliminates the gVp-regulated translation of the fusion gene (II'/ZM13), which is composed of the 5' end of M13 gene II, up to codon 12, and the 5'-truncated lacZ gene of E. coli (Fig. 1), thus providing evidence that this sequence is a prerequisite for the gVp regulated translation of gene II mRNA (25, 47). Furthermore, in vitro binding studies have indicated that this sequence is essential for specific binding of gVp to RNA or to DNA oligonucleotides (25, 26). To examine whether the sequence corresponding to nucleotides 2 to 15 of gene II mRNA is indispensable for the sequestering of ssDNA from rolling-circle replication, we deleted this sequence from the genome of the phagemid vector pKUN19 (20).

To compare the efficiency by which gVp sequesters, during rolling-circle-type replication, the ssDNA of the parental phagemid vector pKUN19 with that of the deletion mutant pKUN19Δ2-15 (see Materials and Methods), we superinfected cells harboring pKUN19 or pKUN19Δ2-15 with an Ff helper phage. Subsequently, the relative number of ss phagemid DNA molecules sequestered was established by titration of the ampicillin resistance-transducing (phage-
mid) particles extruded by the infected cells into the culture medium.

Surprisingly, no differences in phagemid production were observed; i.e., the number of ampicillin resistance-transducing particles per milliliter of culture medium was not significantly different, irrespective of whether the phagemid contained the deletion in the sequence corresponding to the major gVP-binding site on gene II mRNA. Comparison of the relative numbers of biological functional helper phages produced by cells harboring either pKUN19 or pKUN19Δ2-15 also revealed no significant differences. We conclude that in the phagemid DNA the nucleotide sequence that is analogous to the major gVP target site on gene II mRNA is not indispensable for proper sequestering of phagemid ssDNA from rolling-circle-type replication.

**Significance of gVP-mediated translational repression for phage viability.** Zinder and coworkers (8, 25) have elegantly demonstrated that mutations in part of the major gVP target on gene II mRNA are able to suppress the deleterious effect of a particular insertion of four nucleotides in the enhancer domain of the viral strand origin of phage Ff. The primary consequence of these compensatory mutations is that, because of the relief of gVP-mediated translational repression, larger amounts of gIIp are produced, which in turn, by an unknown mechanism, compensate for the deleterious mutations in the origin of viral strand replication.

To investigate the effect of deletion of the major gVP-binding site on gene II mRNA on the viability of M13 wild-type phage, we deleted the sequence corresponding to nucleotides 2 to 15 of M13 gene II mRNA by oligonucleotide-directed mutagenesis using the method of Kunkel (22).

After transfection of the mutagenesis mixture, 12 plaques were picked randomly, and after phage propagation, the nucleotide sequence of the gene II mRNA leader was verified. These analyses revealed that eight plaques contained phage with the intended mutation. This mutagenesis efficiency is similar to that achieved after introduction of the same deletion in phagemid pKUN19, resulting in the generation of pKUN19Δ2-15. Furthermore, comparison of the replication rate and plaque morphology of the mutant phages with wild-type M13 revealed no significant differences, irrespective of whether the host cells were grown on nutrient broth or on minimal medium.

We conclude that translational regulation of gene II mRNA by gVP is a dispensable property of the M13 genome.

**gVP of phage IKe is not a translational repressor.** To answer the question of whether gVP-regulated translation is evolutionarily conserved, we decided to extend our studies to the N-pilus-specific filamentous ssDNA phage IKe. The genomic organizations of M13 and IKe are almost identical, and their nucleotide sequences show an overall homology of 55% (32). The homology of gene V at the DNA sequence level is 58% (31). However, the mechanisms by which these proteins bind to nucleic acids and the three-dimensional structures of at least part of their DNA-binding domains appear to be almost identical (5, 11, 31, 43). Furthermore, the amino acid residues known to be involved in binding to nucleic acids are evolutionarily conserved (5, 11, 43). Although the mRNA leaders of both M13 gene II and IKe gene II are rich in uracil residues, no significant similarities in their primary nucleotide sequences have been observed. Also, the IKe gene II mRNA leader lacks inverted repeats, implying that it does not encompass hairpin structures (Fig. 1) (48, 50).

To examine whether gVP of IKe also regulates at the level of translation the synthesis of its cognate gIIp, we followed a strategy similar to that used in studies on translational regulation of the M13 genes I, II, III, V, and X (48–50). Gene V of IKe was placed under control of the arabinose-inducible promoter of the expression plasmid pING1 (18), resulting in pGVpIKe. A fusion gene, (II'/'Z)IKe, was also constructed by fusion in plasmid pACYC177-lacZ (48) of the 5’ end of IKe gene II, up to codon 12, and the 5’-truncated lacZ reporter gene (Fig. 1). To enable a reliable comparison of the results obtained with the two expression plasmids pGVpIKe and pGVpM13, the production of IKe gVP in cells containing pGVpIKe and grown in the presence of the inducer arabinose was compared with the production of M13 gVP in induced cells containing pGVpM13. As can be deduced from the intensity of the protein bands on the SDS-polyacrylamide gel presented in Fig. 2, the amount of IKe gVP present in induced cells containing pGVpIKe is similar to the amount of M13 gVP present in induced cells containing pGVpM13 (compare lanes 3 and 5).
To investigate whether IKe gVp is able to repress the expression of the (II'/Z)IKe fusion gene, we compared the β-galactosidase activities of cells harboring pGV_{IKe} and pZII_{IKe}, grown in the presence of arabinose. As a control, the expression levels of the (II'/Z)IKe fusion gene were compared in induced and in noninduced cells harboring pZII_{IKe} and pLNG1. The results of the β-galactosidase assays clearly showed that IKe gVp does not significantly repress the expression of its cognate (II'/Z)IKe fusion gene (Table 1).

In a similar way, we examined whether IKe gVp is able to regulate a lacZ fusion whose expression is directed by the transcription and translation initiation signals of M13 gene II, i.e., the gene (II'/Z)M13. and whether M13 gVp is able to regulate the (II'/Z)M13 fusion gene.

The results of the β-galactosidase assays showed that IKe gVp does not repress the expression of the (II'/Z)M13 fusion gene (Table 1). Furthermore, M13 gVp not only efficiently represses the expression of its cognate (II'/Z)M13 gene (48) but also slightly repressed that of the (II'/Z)IKe fusion gene (Table 1).

The SDS-polyacrylamide gel electrophoresis patterns of the proteins present in induced and noninduced cells containing different combinations of recombinant plasmids fully agree with the results obtained from the β-galactosidase assays (Table 1; Fig. 2). From these data, we conclude that gVp-mediated translational regulation of the synthesis of gIIp, as present in Ff, is not conserved in IKe.

**DISCUSSION**

The observation that Ff gVp regulates the synthesis of its cognate gIIp via a specific target site in the gene II mRNA leader has encouraged the idea that the sequestering of viral strands during rolling-circle replication starts at the DNA analog of this site (25, 26, 40, 48). In conflict with this view, we show here that deletion of the sequence corresponding to the major gVp target site on gene II mRNA has no effect on either the efficiency of M13 phage replication or the production of ampicillin resistance-transducing phagemid particles. Thus, we conclude that the DNA analog of the gVp-binding site on gene II mRNA is not essential for the sequestering of progeny viral ssDNA by gVp. Of course, although very unlikely, the possibility cannot be excluded that DNA analogs of other gVp-binding mRNA sequences of M13 (50) serve the role of sequestering of progeny viral ssDNA.

The observation that deletion of the major gVp target site has no influence on the viability or replication properties of the M13 phage demonstrates further that gVp-regulated synthesis of gIIp is a dispensable property. We note that Tuerk and coworkers (42) have made a similar observation with respect to the translational autoregulation of DNA polymerase of bacteriophage T4. These investigators found that mutations that impair translational autoregulation of T4 DNA polymerase have no effect on the phage viability or efficiency of phage replication (42).

The results obtained with the (II'/Z)IKe fusion gene demonstrate that the mechanism of gVp-mediated repression of the synthesis of gIIp, as observed in M13, is not conserved in the distantly related phage IKe. The observation that M13 gVp is also able to weakly repress the synthesis of IKe gIIp suggests that the mechanism of gVp-mediated repression of gIIp already existed in the common ancestor of Ff and IKe, rather than that Ff has acquired this regulation mechanism after divergence of the two phases. Although, under laboratory conditions, gVp-mediated repression of the synthesis of gIIp has no measurable influence on the efficiency of M13 phase DNA replication, it is likely that this regulation mechanism has provided some selective advantage to this common ancestral phage or to Ff in its natural environment.

Although the genomic organization of Ff and IKe is almost identical, a few differences have been noted (32-34). One is the presence of an enhancer of viral strand synthesis in the replication origin of Ff and the absence of this enhancer in IKe (3, 4, 7, 34). It has been shown that mutations in gene II or V of Ff that impair gVp-mediated regulation of gene II are able to relieve the deleterious effect of a particular insertion of four nucleotides in the replication enhancer of Ff (8, 9). It is feasible that, in a similar way, the absence of a replication enhancer in the genome of IKe has favored the loss of gVp-mediated translational repression of the synthesis of gIIp during evolution.

With the aid of nuclear magnetic resonance studies, a number of the amino acid residues of M13 gVp that are involved in binding to ssDNA have been identified (5, 6, 11, 19, 43). It appears that the side chains of residues Tyr-26 and Phe-73 (M13 gVp is 87 amino acids long) stack upon the bases of the nucleotides, while the basic amino acid residues Arg-16, Arg-21, Lys-24, and Lys-46 are involved in electrostatic interactions with the negatively charged phosphate backbone of the DNA. Furthermore, Tyr-41 is involved in dimer-dimer formation. All these residues are conserved in IKe gVp or substituted by a functionally equivalent residue (in IKe gVp, Arg-21 is substituted by Lys, Leu-28 by Ile) (32). The observation that M13 and IKe gVp behave differently in the β-galactosidase assay indicates that M13 gVp-mediated translational repression requires one or more additional amino acid residues elsewhere in the protein that are not conserved in IKe gVp. This conclusion is supported by comparisons of the overall biological activities and the specific translational repressor functions of a series of mutants in M13 gVp (39, 47). These studies have demonstrated that substitution of Glu-5 by Lys, Asp-36 by Gln, Asn-39 by Ile, Ile-47 by Thr, or Pro-58 by Leu strongly impaired translational repressor function, but only weakly affected the sequestering of viral ssDNA from rolling-circle replication. From these data, we suggest that the mechanism by which M13 gVp sequesters viral ssDNA differs from the mechanism by which this protein represses the translation of M13 gene II mRNA. Consistent with the conclusion reached above, three of the five aforementioned residues (i.e., Asn-39, Ile-47, and Pro-58) are not conserved in IKe gVp but are substituted by a Gly, Phe, or Ala residue, respectively (31, 32).
ACKNOWLEDGMENTS

We thank Inge Moelans for the construction of GPGIK, Edwin Janssen and Erik Eckhardt for technical assistance with the other experiments, and Graham Elliott for critical reading of the manuscript.

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