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Synthesis of a bacteriophage MB78 late protein by novel ribosomal frameshifting

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Abstract

MB78 is a virulent phage of Salmonella typhimurium that possesses a number of interesting features, making it a suitable organism to study the regulation of gene expression. A detailed physical map of this phage genome has been constructed and is being extensively studied at the molecular level. Here, we demonstrate the expression of two late proteins of bacteriophage MB78 derived from the same gene as a result of possible ribosomal frameshifting. In vitro transcription-translation yields a major protein that migrates as 28 kDa, whereas in vivo expression using pET expression vectors yields two equally expressed proteins of molecular sizes 28 and 26 kDa. A putative slippery sequence TTTAAAG and a pseudoknot structure, two essential cis elements required for the classical ribosomal frameshifting, are identified in the reading frame. Mutations created at the slippery sequence resulted in a single 28 kDa protein and completely abolished the expression of 26 kDa protein. Thus, we have produced the first evidence that ribosomal frameshifting occurs in bacteriophage MB78 of Salmonella typhimurium. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Bacteriophage MB78; Expression; Frameshift; Minicells; Promoter; Pseudoknot

1. Introduction

Bacteriophage MB78 is a virulent phage of Salmonella typhimurium (Joshi et al., 1982; Srinivasula, 1992). Morphologically, physiologically and serologically, it is different from the well-known temperate phage P22 and related phages as well as a virulent phage 9NA (Murthy, 1987). MB78 cannot multiply in minimal medium containing citrate. The chelating agent EDTA is an effective inhibitor of its DNA synthesis, whereas EGTA and orthophenanthroline have practically no effect on the development of the phage (Verma and Chakravorty, 1987). MB78 is a dominant phage in that it does not allow phages like P22 and 9NA to grow in its presence. MB78 contains a 42 kb linear, double-stranded DNA (molecular weight 28 × 10^6 Da), which replicates through concatemer formation, subsequently converted to full-length phage DNA through 'headful' packaging mechanism. Like P22, MB78 DNA is circularly permuted and terminally redundant (Khan et al., 1991a; Pandey, 1992; Srinivasula, 1992).

It is now known that two proteins can be expressed from a single open reading frame through ‘ribosomal frameshifting’. If the ribosome shifts during translation, one base in either direction, i.e. towards 3' or 5' ends, the reading frame will be changed. During the process of ‘ribosomal frameshifting’, two or more proteins can result, starting from a single initiation codon (Farabaugh and Vimaladithan, 1998). A shift in the 3’ direction (+1 frame shift) has been described in the yeast retrotransposon TY (Belcourt and Farabaugh, 1990), copia-like elements of Drosophila (Saigo et al., 1984) and E. coli release factor 2 (Weiss et al., 1988). Similarly, a shift in the 5’ direction (−1 frame shift) has...
been demonstrated for retroviruses (Jacks et al., 1988; Vickers and Ecker, 1992), luteoviruses (Braunitz and Miller, 1992; Preter et al., 1992), the bacterial transposon IS1 (Sekine et al., 1992) and in potato leafroll virus (Kujawa et al., 1993). A −1 frameshifting event often controls the levels of expression of viral reverse transcriptase relative to viral core proteins in retroviruses. Frameshifting is also known to affect gene expression in coronaviruses and even in a bacterial system (Chamorro et al., 1992). In all these cases, frameshifting occurs as the ribosome passes a seven nucleotide sequence 5′ XXXYYYYZ 3′ (X is A, U or G, Y is A or U, and Z is any nucleotide), known as the ‘slippery site’. Two of the three base pairs between the anticodons of each of the two tRNAs and mRNAs can be maintained after the slip into the −1 reading frame. The slippery sequence is not the only determinent of frameshifting; secondary signals are also required (Larsen et al., 1995; Atkinson et al., 1997). Secondary signals programmed in the mRNA augment shifting at the slippery sequence to give high levels of frameshifting. These signals, called ‘stimulators’, are very diverse. For example, the +1 shift for decoding RF2 (release factor) of E. coli requires two stimulators: one is a UGA terminator at codon 26 flanking the shift site on its 3′ site (Weiss et al., 1988); the other is a Shine-Dalgarno sequence located three nucleotides upstream of the shift site (Weiss et al., 1988). These two stimulators act independently with substantial activity, but their effects are synergistic.

Pseudoknots, a tertiary interaction involving base pairing between two regions of unpaired bases, are also involved in frameshifting. The model for the pseudoknot structure was based on biochemical analysis of the 3′ end of turnip yellow mosaic virus (TYMV) RNA (Pleij et al., 1985; Dumas et al., 1987). In E. coli, ribosomal protein L17 by binding to a pseudoknot structure. The structure resembles a ‘double pseudoknot’ linking a hairpin upstream of ribosome binding site with sequences 2–10 codons downstream of the initiation codon (Tang and Draper, 1989). Pseudoknots also play a role in the structural mimicry of tRNA at the 3′ termini of plant viral RNAs (Pleij et al., 1985). One of the most intriguing functions of the pseudoknot structure in frameshifting occurs during the translation of certain retroviral mRNAs (Jacks et al., 1988; Kujawa et al., 1993; Atkinson et al., 1997). Mutational analyses in mouse mammary tumor virus (MMTV) (Chamorro et al., 1992) and in infectious bronchitis virus (IBV) (Brierley et al., 1992) provide strong evidence for the stimulator structural element being a pseudoknot. The autoregulation of gp32 in phage T4 also involves a pseudoknot (Shamoo et al., 1993). In this investigation, we provide evidence for the first time that one of the two late genes from bacteriophage MB78 is expressed by ribosomal frameshifting.

2. Materials and methods

2.1. Bacterial strains

LT2, a Salmonella strain, was originally obtained from Dr. Myron Levine, Department of Human Genetics, University of Michigan, Ann Arbor, MI. E. coli strain KK2186 was a generous gift from Dr. P. Berget, then at the Department of Biochemistry and Molecular Biology, University of Texas, Houston, TX. All other bacterial strains were purchased commercially from GIBCO-BRL Life Technologies. All the chemicals were obtained from Sigma Chemical Company, St. Louis, USA.

2.2. Purification of bacteriophage MB78 and isolation of its DNA

Phage stocks were prepared as described earlier (Kolla and Chakravorty, 2000). Phage DNA was isolated as per the method described by Maniatis et al. (Sambrook et al., 1989; Kolla and Chakravorty, 2000).

2.3. Isolation of plasmid DNA

Plasmid DNAs were isolated by either alkali lysis method using standard protocols (Sambrook et al., 1989; Kolla and Chakravorty, 2000) or by Qiagen and Promega columns according to the manufacturer’s instructions. DNA from the gels was extracted using Qiagen columns.

2.4. Nested deletions by ExoIII

The deletions were created primarily as described by Henikoff (1987). Briefly, cloned DNA fragment (5–10 µg) was digested with two different restriction enzymes e.g. PstI and BamHI. The enzyme PstI produces a four-base 3′ overhang, resistant to ExoIII activity, while the enzyme BamHI generates 5′ protrusion, which is accessible to ExoIII (Weiss, 1976). After complete digestion with both the enzymes, the DNA sample was deproteinized by extracting with phenol:chloroform and precipitated with ethanol. The DNA pellet was resuspended in 25 µl of 1 × ExoIII buffer and the Exonuclease treatment carried out as per the recommendations of the manufacturer (Promega). Finally, the deleted fragments were ligated and used for completing the nucleotide sequence as well as for in vivo expressions.
2.5. In vitro transcription and translation

The coding region for 26 and 28 kDa proteins was amplified by PCR and cloned in bacterial expression vectors, pET21a and pET28a. Recombinant DNAs were in vitro transcribed-translated in the presence of $^{35}$S-methionine in rabbit reticulocyte lysate with a T7-RNA polymerase-coupled TNT kit (Promega) according to the manufacturer’s recommendations. Briefly, reactions were set up in 50 µl volume in an Eppendorf tube containing 25 µl of rabbit reticulocyte lysate, 2 µl of reaction buffer, 1 µl of amino acid mix minus methionine, 1 µl of RNasin (5 U), 1 µg of template DNA, 4 µl of $^{35}$S-methionine (1,000 Ci/mmol) and 1 µl of T7 RNA polymerase (20 U). The final reaction was made up to 50 µl with sterile distilled water, and the tubes were incubated at 42 °C for 90 min to allow the synthesis of proteins. One to 3 µl samples were applied to SDS-polyacrylamide gels after denaturing in a sample buffer by boiling.

2.6. Sequencing

Nucleotide sequencing of EcoRI ‘F’ fragment was carried out by manual sequencing using Sequenase kit (USB) and also by automated sequencing. Forward and reverse sequencing primers were obtained from Pharmacia (Uppsala, Sweden).

2.7. Preparation of minicells

Minicells were prepared as described by Reeve (1979). E. coli strain DS410, the minicell producing strain, transformed with desired plasmid was grown overnight to stationary phase in 400 ml of terrific broth (Sambrook et al., 1989) in the presence of the relevant antibiotics (ampicillin and tetracycline). The culture was harvested by boiling. Chakravorty, 2000). The frozen minicell suspension (0.1 ml of reaction buffer) was thawed slowly and centrifuged for 3 min in a microfuge. The pellet was suspended in 200 µl of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (Laemmli, 1970), at a constant voltage of 50 V.

2.8. Expression of plasmid encoded proteins

Purified minicells were labeled with $^{35}$S-methionine as described previously (Reeve, 1979; Kolla and Chakravorty, 2000). The frozen minicell suspension (0.1 or 0.2 ml) was thawed slowly and centrifuged for 3 min in a microfuge. The pellet was suspended in 200 µl of M9 minimal medium to which 3 µl of 10.5% (W/V) Difco methionine assay medium were added and incubated at 37 °C for 90 min, to complete the translation of bacterial mRNAs in the minicells, received from the mother cell. Then, 25 µCi of $^{35}$S-methionine were added and incubated for 60 min at 37 °C, followed by incubation of 5 min after the addition of 10 µl of unlabeled methionine (1%). The cells were then centrifuged at 12,000 rpm for 3 min, the cell pellet was washed with 500 µl of 10 mM Tris–HCl, pH 7.6, suspended in 20 µl of the same buffer to which 20 µl of 2 × sample buffer were added. The labeled proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), at a constant voltage of 50 V.

2.9. Fluorography

To detect radio labeled proteins, the gels were fluorographed using water-soluble sodium salicylate (Bonner, 1984). The gel was soaked in methanol, acetic acid, and water (5:1:5) for 60 min, followed by a thorough wash with water (30 volumes of gel), then immersed in 1 M sodium salicylate, pH 7.0 for 1 h with mild shaking. Finally, the gel was transferred to Whatman No. 1 sheet, dried under vacuum and subjected to fluorography.

2.10. Cloning and PCR amplification

The presumed coding regions for 26 and 28 kDa proteins were amplified by PCR using the following forward and reverse primers with overhanging restriction sites:

Forward primer: 5’ CC CGGATCCATGAAATCGTTTTACGTTAC 3’
Reverse primer: $5'$ CCGGAATTCGGCAGGGTT-AGATTT $3'$

The primers were commercially synthesized by GIBCO-BRL Life Technologies (including the primers designed to create mutations at the $3'$ end of the fragment to avoid the frameshift by overlapping PCR). The primers were also used to identify the presence of inserts in the cloned vectors by PCR screening and to determine the nucleotide sequence. The amplified fragments were digested with appropriate restriction enzymes and cloned in-frame into pET21a and pET28a expression vectors at BamHI and XhoI restriction sites. Restriction enzyme analyses and DNA sequencing confirmed the sequence of all the constructs.

2.11. Western blot analyses

Cells were lysed in lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Nonidet P-40) in the presence of protease inhibitors; aprotinin, apopain, and PMSF. Protein estimations were done by Coomassie Plus protein assay reagent kit (PIERCE), a modified Bradford colorimetric method, using bovine serum albumin (BSA) as standard. Total lysates (250-500 μg) were immunoprecipitated by T7 antibody (Novagen), and the proteins were collected using Protein A/G Plus agarose (Santa Cruz Biotechnology) and resolved by SDS-PAGE (10%) and transferred electrophoretically (100 V constant for 1 h) to Hybond ECL nitrocellulose paper (Amersham Life Science). The paper was blocked overnight in 10% non-fat milk, and incubated in 5% non-fat milk with horse-radish peroxidase-conjugated T7-antibody (1:10 000, Novagen) for 1 h at room temperature. After stringent washing, the filter was developed by chemiluminescent ECL, as described (Amersham, Arlington Heights, IL).

3. Results and discussion

3.1. Expression of EcoRI 'F' fragment by the minicell system

In order to understand more about the physiology and genetics of the phage MB78, the EcoRI 'F' fragment (2.3 kb) of the phage was cloned in pUC18 vector (data not shown), and the expression in minicells was examined. The EcoRI 'F' fragment of MB78 codes for four proteins of mass 28, 26, 21 and 11 kDa (Fig. 1, lane 1). The expression of β-lactamase was not strong in cells carrying the EcoRI 'F' vector, indicating that the presence of a strong promoter (Pandey et al., 1997) in the 'F' fragment is interfering with the expression of the β-lactamase gene. To characterize the EcoRI 'F' fragment, the expressed proteins were separated and identified by dot-blot hybridization. The expression of the 28 and 26 kDa proteins was unaffected after the deletion.
of 1518 bp from the original construct (lane 3). The
present study focuses on the characterization of the 28
and 26 kDa proteins. The clone F-1518 expressed 28
and 26 kDa proteins, suggesting that their promoters and
ORFs are present in 793 bp (1518–2311) of the EcoRI
‘F’ fragment. After deletion of 1595 bp, the expression
of both proteins was reduced simultaneously (lane 4),
and no expression was detected after the deletion of
1723 bp (lane 5). These results suggest that the expres-
sion of these two proteins is driven by a common
promoter that resides within 1518–1601 bp and that
both the proteins are possibly expressed from overlap-
ning open reading frames.

3.3. Nucleotide sequence analyses

The nucleotide sequence of the EcoRI ‘F’ fragment
was determined by Sanger’s dideoxy chain termination
method (Sanger et al., 1977) using a set of deletion
mutants produced by EcoIII (Accession No. X87092).
Computer analysis of the nucleotide sequence indicated
the possibility of encoding four proteins that could be
expressed from three ORFs (data not presented). The
ORF for the 28 and 26 kDa proteins starts from 1641
and does not have a stop codon in the ‘F’ fragment; it
appears that the vector stop codon, located adjacent to
EcoRI site, might be used. Analysis of the nucleotide
sequence of EcoRI ‘F’ fragment revealed that expression
of 28 and 26 kDa proteins may have occurred through
ribosomal frameshifting. We analyzed the sequence for
the formation of possible secondary structures, necessary
for the process of classical ribosomal frameshifting
(Fig. 3A). Computation also revealed the presence of a
slippery sequence with a possible downstream pseu-
doknot structure (Fig. 3B). The slippery sequence pre-
sent in bacteriophage MB78 resembles that of the turnip
yellow mosaic virus (Kujawa et al., 1993).

3.4. Effect of 3’ truncation on the expression

We next examined whether the 28 and 26 kDa pro-
teins are expressed from overlapping open reading frames
by ribosomal frameshifting. If they are expressed
from overlapping open reading frames with the same
initiation codon, truncation of the gene from the 3’ end
should yield only a single protein. This part of the gene
has an internal HindIII restriction site, located 138 bp
away from the EcoRI site, at the 3’ end of the fragment.
When this portion is deleted, the coding region will be
reduced to 555 bp, encoding a protein of approximately
22 kDa. To test this, plasmid ▲1518 was truncated
(▲1518/138 H) and used to transform the minicell
producing strain DS410 to observe the expression of
proteins. The expression pattern of the deleted plasmid
is presented in Fig. 4. Deletion of 138 bp from the 3’
end of the EcoRI ‘F’ fragment resulted in complete
abolition of 26 and 28 kDa proteins, but a major protein,
smaller in size (22 kDa, marked with triangle), was
expressed (lane 4). The deletion of 3’ end of the gene,
resulting in the synthesis of a single protein instead of
two proteins, supports the frameshift notion. The pres-
ence of a slippery sequence and a pseudoknot structure
downstream to the putative shift site strengthens the
argument.

Fig. 2. Schematic representation of the different deleted constructs of the EcoRI ‘F’ fragment and the extent of expression, shown arbitrarily with
restriction sites in pUC18 vector. The putative promoter, enhancer sequences and restriction sites are marked arbitrarily. The number of bases
deleted in each construct is also shown on the left-hand side.
Fig. 3. Sequence analysis. (A) Probable secondary structure of mRNA derived from 3' 138 nucleotides. (B) Probable slippery sequence and downstream pseudoknot structure. The slippery sequence essential for the frameshift is marked in a box, and the bold nucleotides represent the stop codon where mutations were created. Nucleotides involved in the pseudoknot structure are connected.

3.5. In vitro transcription and translation

In order to examine the phenomenon of frameshift further, we next performed coupled in vitro transcription and translation using rabbit reticulocyte lysate. The nucleotide sequence starting from ATG to the end of the fragment was amplified by PCR with forward and reverse primers, as described in Section 2.10. The amplified DNAs were cloned in-frame into bacterial expression vectors, pET21a and pET28a, at BamHI and XhoI sites and named KVM21 and KVM28, respectively. These DNAs were used to synthesize proteins by in vitro transcription and translation (Promega). The results are presented in Fig. 5 (lanes 1 and 2). In vitro transcription-translation of the fragment yielded a major protein (90%) of 28 kDa, with the synthesis of a minor protein of apparent molecular mass 26 kDa (arrows). We observed the formation of dimers from 26 and 28 kDa proteins in the absence of reducing agents DTT (data not shown). These results suggest the synthesis of a single major protein by in vitro transcription and translation. However, two proteins at equal levels of expression were observed in E. coli DS410 bacteria (Fig. 1).
Fig. 5. In vitro transcription and translation. The coding region for 26 and 28 kDa proteins was amplified by PCR and cloned in bacterial expression vectors, pET21a and pET28a. Recombinant DNAs were translated in vitro in the presence of [35S]methionine in rabbit reticulocyte lysate, as described in Section 2. One to three microliters of these samples, as indicated, were applied on to SDS-polyacrylamide gels after denaturing in a sample buffer by boiling. A major translated protein product (28 kDa) and a minor protein are marked with arrows. The position of possible dimers is also marked with an arrow. Lanes 3 and 4 represent the translation of empty pET28a and pET21a vectors, respectively.

3.6. Expression of 26 and 28 kDa proteins in BL-21 DE3 bacteria: mutational analysis

To determine whether the in vivo frameshift could occur in the N-terminal or C-terminal region, we purified proteins from KVM21 and KVM28 clones. Vector pET21a carries an N-terminal T7.tag sequence plus an optional C-terminal His.tag sequence, whereas pET28a has an N-terminal His.tag, T7.tag and an optional C-terminal His.tag. These vectors facilitate the purification of expressed proteins in an efficient manner by affinity purification on Ni\(^{2+}\)-affinity resin (Clontech). Recombinant proteins with C-terminal His.tag (pET21a) and C and N-terminal His.tags (pET28a) were expressed in E. coli BL-21 DE3. Purified proteins were separated on a 12% SDS-polyacrylamide gel and transferred on to a nitrocellulose membrane, and Western blotting was performed with T7 specific antibody. A single 28 kDa protein was present in cells expressing C-terminal His.tag in pET21a vector (Fig. 6A), whereas 26 and 28 kDa proteins were present in cells expressing N and C-terminal His.tags in pET28a vector (Fig. 6B). This suggests that frameshift occurs at the C-terminal region, resulting in the appearance of a truncated 26 kDa protein without a C-terminal His.tag in addition to the expression of a full-length 28 kDa protein with a C-terminal His.tag. Mutations were created (deletion of three nucleotides) near the putative
slippery sequence by overlapping PCR and were cloned into pET28a. Recombinant proteins were expressed and separated as described above. We predicted that this mutation should result in the loss of frameshift. As expected the mutated recombinant constructs did not yield the 26 kDa protein, but only the 28 kDa protein (Fig. 6C, lane 1). These results further support ribosomal frameshifting.

4. Conclusions

1. We demonstrated that two late proteins of bacteriophage MB78 could be derived from the same gene as a result of ribosomal frameshifting.

2. Ribosomal frameshifting has been well established in *Escherichia coli* phages T2 (Du et al., 1997), T4 (Grossman and Engelberg-Kulka, 1995) and T7 (Condon et al., 1991; Sipley et al., 1991; Lewis and Matsui, 1996) but not well documented in the case of *Salmonella* bacteriophages except the previously reported observation in the phage P22 (Uomini and Roth, 1974).

3. Two *cis* elements are essential for ribosomal frameshifting (Jacks et al., 1988; Blinkowa and Walker, 1990; Tsuihishi and Kornberg, 1990), but the exact mechanism is not clearly understood. It has been postulated in −1 frameshift that an anti-Shine–Dalgarno-like sequence, present at 5′ to the shift site (Larsen et al., 1994), pairs with the 16S rRNA of the elongating ribosome to make the ribosome pause, resulting in a frameshift. This feature is observed mostly in prokaryotes.

4. An aspect of the current study of bacteriophage MB78 is that more than 35% of the ribosomes appear to be involved in frameshifting. Reports in the literature indicate that the proteins synthesized as a result of frameshifting are much less numerous, to a maximum of 10–20%.

5. Amino acid sequence analysis (e.g. LC/MS, MALDI-TOF) of the 28 and 26 kDa two proteins, encoded by the *Eco* RI 'F' fragment is required to confirm the ribosome frameshift hypothesis from the present study.

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