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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*.

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

Abbreviations: bp, base pairs; IPTG, isopropyl β-thiogalactosidase; kb, kilobase pairs; ORF, open reading frame; PCR, polymerase chain reaction.

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been demonstrated for retroviruses (Jacks et al., 1988; Vickers and Ecker, 1992), luteoviruses (Brault and Miller, 1992; Prufer et al., 1992), the bacterial transposon IS 1 (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 effect 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′ XXXYYYZ 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 determinant 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 S4 represses its own synthesis in addition to the synthesis of other ribosomal proteins viz. S11, S13 and 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. *Pst*I and *Bam*HI. The enzyme *Pst*I produces a four-base 3′ overhang, resistant to *Exo*III activity, while the enzyme *Bam*HI generates 5′ protrusion, which is accessible to *Exo*III (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 × *Exo*III 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 (5U), 1 μg of template DNA, 4 μl of ^{35}S-methionine (1000 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 on to SDS-polyacrylamide gels after denaturing in a sample buffer by boiling.

2.6. Sequencing

Nucleotide sequencing of *Eco*RI '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 examined under a light microscope to observe the formation of long filamentous cells and minicells. When a reasonable number of minicells were visible under the microscope, the culture was chilled on ice for 20 min and then centrifuged at 4°C for 5 min at 2,000 rpm ($675 \times g$) in GS3 rotor of Sorvall RC5C centrifuge. The supernatant was transferred to fresh GS3 cups and centrifuged at 7000 rpm ($5{,}278 \times g$) for 20 min in the same centrifuge. The cell pellet was resuspended in 9 ml of M9 minimal medium, and then 4 ml of the suspension were carefully layered on to 10–30% (w/v) sucrose gradients. The gradients were centrifuged at 4°C for 18 min at 5000 rpm ($4122 \times g$) in the HB4 rotor of the Sorvall RC5C centrifuge.

The top two-thirds of the minicell layer was collected into a 30 ml corex cup using Pasteur pipette. To this, an equal volume of M9 minimal medium was added, and the suspension was centrifuged again at 10 000 rpm ($11\,953 \times g$) for 10 min in the SS34 rotor of the Sorvall RC5C centrifuge. The supernatant was discarded, and the minicell pellet was suspended in 6 ml of M9 medium. The cell suspension was again layered on to sucrose gradient as before. Again, two-thirds of the minicell layer was collected into a 15 ml of corex cup, and an equal volume of M9 was added. The optical density of this minicell suspension was measured at 600 nm in a Hitachi spectrophotometer to determine the volume in which the minicells would be finally suspended to have $A_{600} = 2.0$/ml (2×10^{10} cells/ml). The cells were finally suspended in M9 minimal medium containing 30% (V/V) glycerol, aliquoted into a number of tubes (200 μl in each) and stored at −80°C. The minicells thus stored could be used for at least a year.

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 uCi 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′ CCCGGATCCATGAATCGTT-TTTTACGTTAC 3′

Reverse primer: 5′ CCCGAATTCGGCAGGGTT-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 *Bam*HI and *Xho*I 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% nonfat 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 chemiluminiscent 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 *Eco*RI 'F' fragment (2.3 kb) of the phage was cloned in pUC18 vector (data not shown), and the expression in minicells was examined. The *Eco*RI '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 *Eco*RI 'F' fragment, suggesting 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 *Eco*RI 'F' fragment, sequencing of the fragment was carried out with a nested set of deletions in the target DNA. The clones

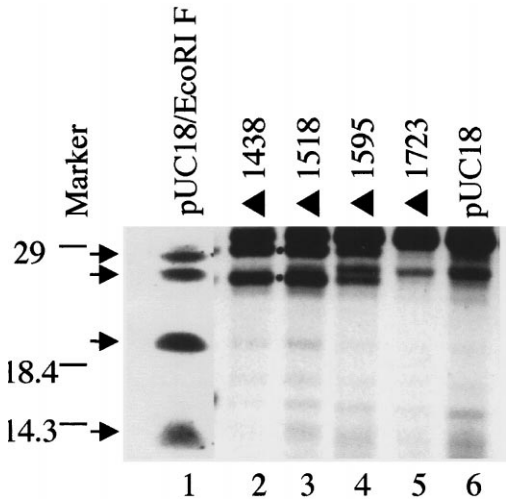

Fig. 1. *In vivo* expression of the minicells. Minicells produced from DS410, transformed with *Eco*RI in pUC18, deletion mutants and pUC18 were labeled with ^{35}S-methionine, as described in Section 2. Samples were denatured and analyzed on 12.5% SDS-polyacrylamide gel followed by fluorography. Lane 1, *Eco*RI F in pUC18; lanes 2–5 represent expression of different plasmid DNAs in minicells; lane 2, F ▲1438; lane 3, F ▲1518; lane 4, F ▲1595; lane 5, F ▲1723; lane 6, pUC18. Molecular weight standards in kDa (BRL: low molecular weight marker) are marked on the left-hand side. Positions of 28 and 26 kDa proteins have been marked with dots (.) between lanes 2 and 3. F ▲ followed by a number indicates the number of bases deleted from the full-length *Eco*RI 'F' fragment.

were named according to the number of bases deleted; for example, ▲1518 means that 1518 bases were deleted, and ▲1601 means that 1601 bases were deleted and so on from the full-length construct. To ascertain the sizes of the inserts, the deleted plasmid DNAs were digested with *Hin*dIII, located 138 bases away from the *Eco*RI site (Fig. 2). The sequentially deleted DNAs were further confirmed by dot-blot hybridization (data not presented). The deleted fragments were cloned into pUC18, and the deletions were confirmed by DNA sequencing.

3.2. In vivo expression of deletion mutants

In order to determine the expression of various deletion mutants, we performed in vivo minicell expression using ^{35}S-methionine [only a few of the sequentially deleted plasmids are presented here (Fig. 1)]. Lane 6 (proteins expressed by vector pUC18) shows two proteins, including 30 kDa β-lactamase protein. In lane 1, four major proteins of molecular weights 28, 26, 21 and 11 kDa expressed from the cloned *Eco*RI 'F' fragment could be seen. Lanes 2–5 show the proteins expressed by different sequentially deleted DNAs. The 21 and 11 kDa proteins could not be seen when 1438 bases were deleted (lane 2), suggesting that their promoters and ORFs (open reading frames) reside within 1438 bp from the original construct. The expression of 28 and 26 kDa proteins was unaffected even after the deletion

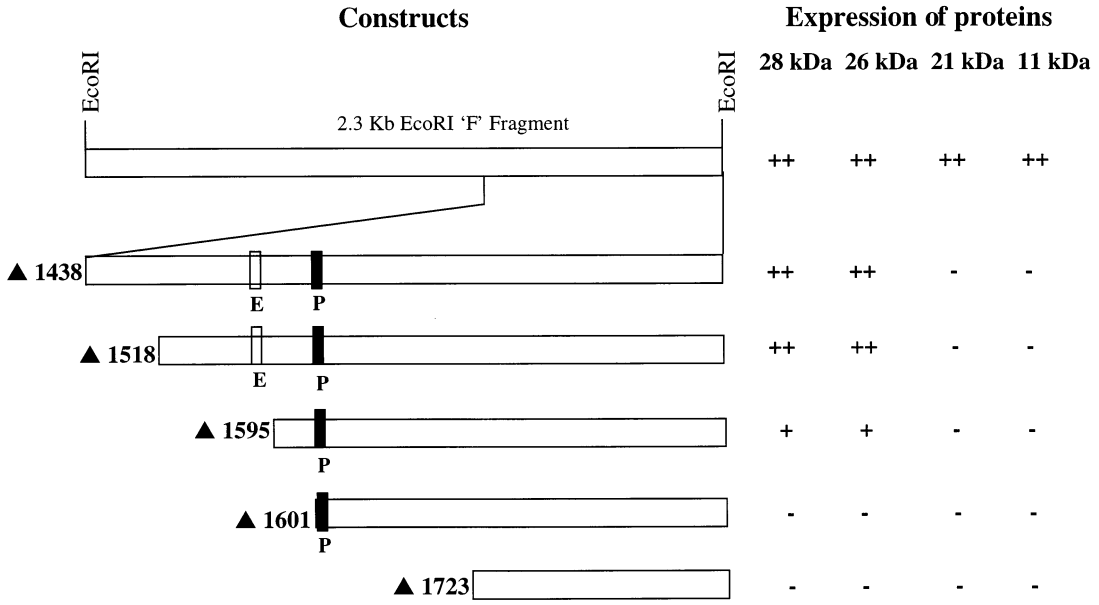

Fig. 2. Schematic representation of the different deleted constructs of the *Eco*RI 'F' fragment and the extent of expression, shown arbitrarily with restriction sites in pUC18 vector. The putative promoter, enhancer sequences and restriction sites are marked arbitrarily. The number of bases deleted in each construct is also shown on the left-hand side.

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 *Eco*RI '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 expression 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 overlapping open reading frames.

3.3. Nucleotide sequence analyses

The nucleotide sequence of the *Eco*RI 'F' fragment was determined by Sanger's dideoxy chain termination method (Sanger et al., 1977) using a set of deletion mutants produced by *Exo*III (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 *Eco*RI site, might be used. Analysis of the nucleotide sequence of *Eco*RI '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 pseudoknot structure (Fig. 3B). The slippery sequence present 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 proteins 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 *Hin*dIII restriction site, located 138 bp away from the *Eco*RI 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 *Eco*RI '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 presence of a slippery sequence and a pseudoknot structure downstream to the putative shift site strengthens the argument.

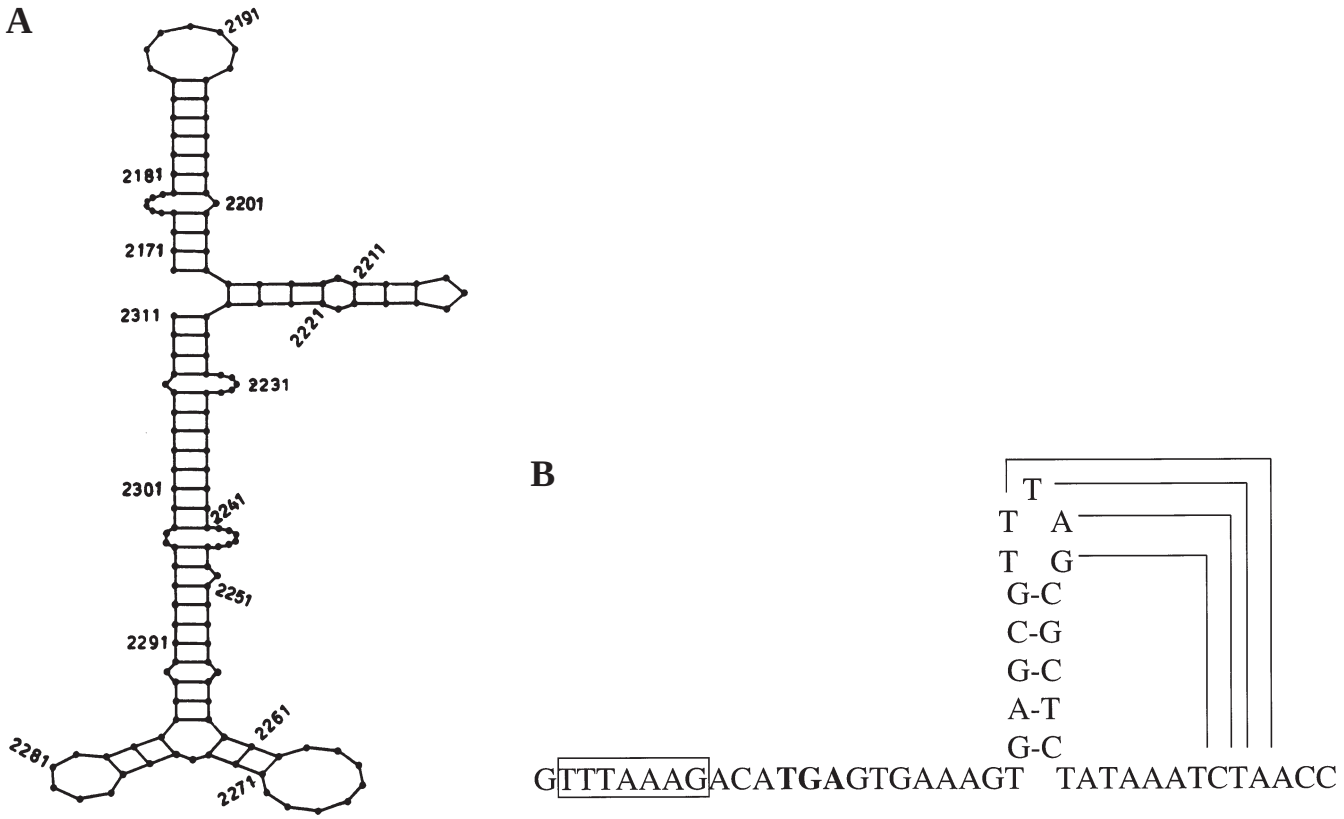

Fig. 3. Sequence analysis. (**A**) Probable secondary structure of mRNA derived from 3′ 138 nucleotides. (**B**) Probable slippery sequence and downstream pseudoknot structure. The slippery sequence essential for the frameshift is marked in a box, and the bold nucleotides represent the stop codon where mutations were created. Nucleotides involved in the pseudoknot structure are connected.

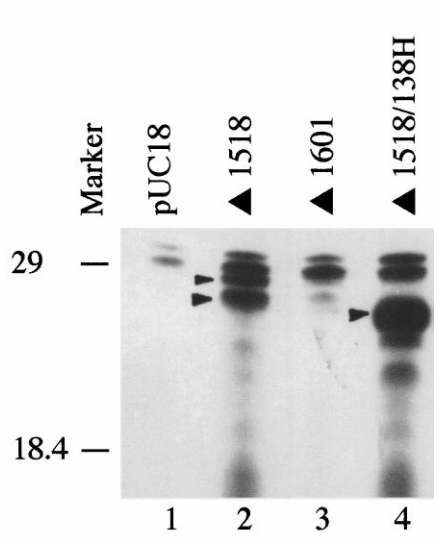

Fig. 4. In vivo minicell expression of truncated 28 and 26 kDa proteins. Minicells were labeled as described in Fig. 1. Lane 1, pUC18 in DS410; lane 2, F ▲1518 in DS410; lane 3, F ▲1601 in DS410; lane 4, F ▲1518/138H in DS410. Molecular weight standards (kDa) are from GIBCO-BRL.

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 *Bam*HI and *Xho*I 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 he 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).

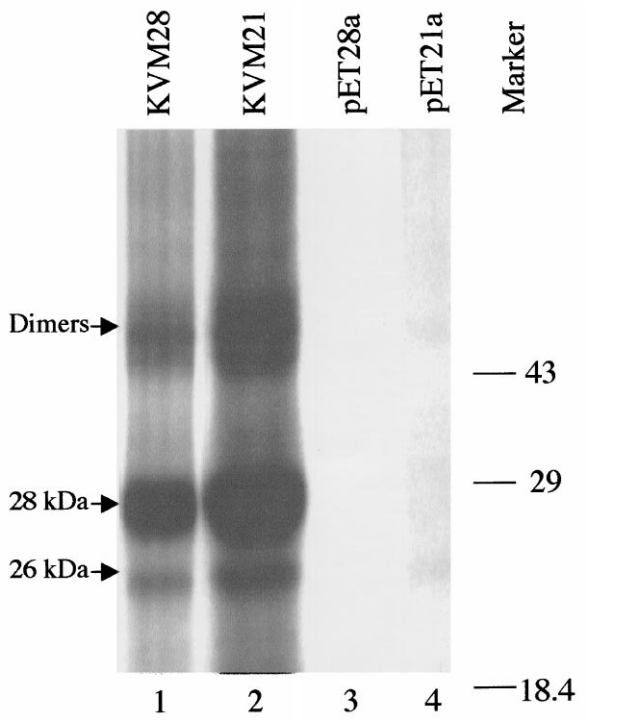

Fig. 5. *In vitro* transcription and translation. The coding region for 26 and 28 kDa proteins was amplified by PCR and cloned in bacterial expression vectors, pET21a and pET28a. Recombinant DNAs were translated *in vitro* in the presence of [^{35}S]-methionine in rabbit reticulocyte lysate, as described 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

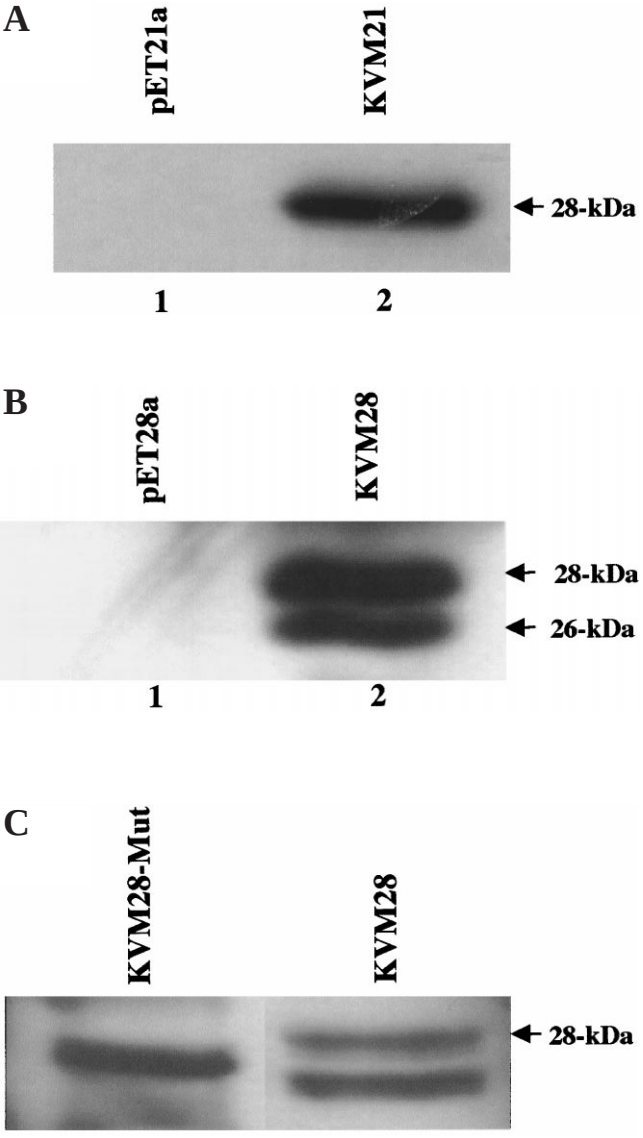

Fig. 6. Expression of 28 and 26 kDa proteins in BL-21 DE3. (**A**) pET21a vector. (**B**) pET28a vector. (**C**) Mutated construct in pET28a. Recombinant constructs and empty vectors were transformed into BL-21 DE3 bacteria, as indicated. Cells were induced in the presence of IPTG, an inducing substance in certain bacteria, at a final concentration of 1 mM and allowed to grow for additional 2–3 h. Subsequently, cells were collected and washed with 1 × PBS and sonicated to lyse in the presence of protease inhibitors: (aprotinin, apopain, and PMSF). The clear supernatant was applied to Ni^{+2}columns and purified as per the manufacturer's instructions (Clontech). Finally, the purified proteins, bound to resin, were denatured by boiling in a suitable volume of 2 × sample buffer and subjected to SDS-PAGE followed by Western blot. The presence of 26 and or 28 kDa proteins was detected using the ECL kit (Amersham). The molecular weight of the proteins is marked with arrows.

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.

3.7. Kinetic expression of 28 and 26 kDa proteins

To examine the functions of the 28 and 26 kDa proteins, kinetic expression of phage MB78 was performed. The LT2 cells (host) were infected with phage MB78 at a multiplicity of infection (m.o.i.) 10 and pulse-labeled with ^{35}S-methionine for 2 min at different times after infection. The labeled proteins were subjected to 12.5% SDS-PAGE followed by fluorography. The kinetic study demonstrated that the two proteins are late proteins of bacteriophage MB78 (Fig. 7). The protein pattern of uninfected cells served as a control. The expression of 28 and 26 kDa proteins was low at 2 and 5 min after infection, but from 10 min onwards, their synthesis increased significantly until cell lysis. It may therefore be assumed that these two proteins are late proteins of phage MB78.

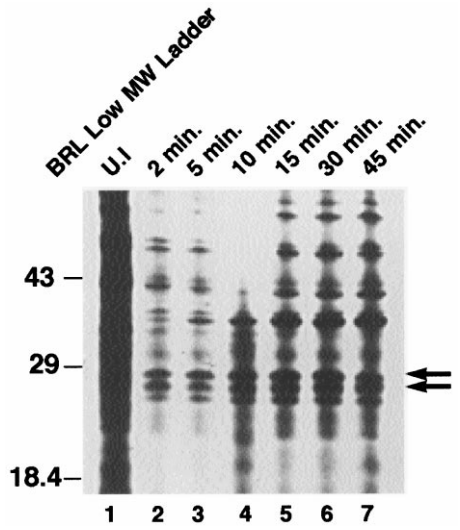

Fig. 7. Kinetic study of 28 and 26 kDa proteins. Exponentially growing LT2 cells in minimal medium (M9) at 37°C were infected with phage MB78 at an m.o.i. 10. The infected cells (400 μl) were pulse-labeled for 3 min at different times after infection. The cells were collected by centrifugation, washed with 500 μl of medium and finally suspended in 25 μl of M9 medium. Samples collected at different times as indicated were lysed in an equal volume of 2× sample buffer and applied on to a 12.5% SDS-polyacrylamide gel. Lane 1, uninfected (UI) LT2 cells; lanes 2–7, cells infected with phage MB78 after 2, 5, 10, 15, 30 and 45 min, respectively. BRL low-molecular-weight markers are represented in the left lane. The 28 and 26 kDa proteins are marked with two arrows.

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 *E. coli* phages T2 (Du et al., 1997), T4 (Groisman and Engelberg-Kulka, 1995) and T7 (Condron 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; Tsuchihashi 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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