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Cloning and Characterization of a Gene Encoding 22 kDa Functional Protein of Bacteriophage MB78

Lalita Gupta* and Maharani Chakravorty
Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, U.P., INDIA

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Functional protein of MB78 bacteriophage having apparent molecular weight of 22 kDa is expressed from 1.7 kb *Hin*dIII G fragment. The nucleotide sequence of this fragment showed two open reading frames of 222 and 196 codons in tail-to-tail orientation separated by a 62-nucleotide intercistronic region. The ORF of 22 kDa protein is present in opposite orientation, i.e. in the complementary strand, preceded by a strong ribosomal binding site and a promoter sequence. Another ORF started from the beginning of the fragment whose promoter region and translational start site lies in the 0.45 kb *Hin*cII U fragment which is located next to the *Hin*dIII G fragment, that has the sequence for DNA bending. 3' end of the fragment has high sequence homology to the EaA and EaI proteins of bacteriophage P22, a close relative of MB78 phage.

Keywords: Bacteriophage MB78 and P22, Minicell expression, Phage gene, *Salmonella*, Tail to tail orientation

Introduction

Bacteriophage MB78, one of the virulent phages of *Salmonella typhimurium* (Joshi *et al*., 1982) is an excellent system to study regulation of gene expression and host-virus interactions (Verma *et al*., 1982). A genomic library of the phage was constructed in M13mp11 vector and different fragments were studied extensively. A detailed physical map of the 42 kb phage genome was constructed (Khan *et al*., 1991; Murty *et al*., 1998). A number of promoter containing fragments have been cloned and a strong promoter has been studied in detail (Zargar *et al*., 1997). An *Eco*RI F fragment of MB78 expressed two late proteins of 26 and 28 kDa derived from the same gene as a result of ribosomal frameshifting (Kolla *et al*., 2000). Interestingly, if the permissive host harbors multicopy plasmid carrying *Eco*RI F fragment, it cannot support development of phage MB78 (Sharma *et al*., 2001). Another 3.9 kb *Sal*I-*Hin*dIII fragment of MB78 expressed four proteins from a polycistronic operon having four overlapping ORF where stop and start codons overlaps and rare initiation codons are also used (Sharma *et al*., 2000) whereas 0.9 kb fragment expressed two proteins and one of them exhibits 57% homology with structural protein of mycobacteriophage (Kolla *et al*., 1999).

The present study is focused on the cloning, sequencing, expression and promoter analysis of a *Hin*dIII G fragment of bacteriophage MB78. This fragment contains two ORF in tail-to-tail orientation and codes for a 22 kDa functional protein.

Materials and Methods

Bacterial strains, plasmids and general manipulations *E. coli* strain KK2186 used for transformation was a gift from Dr. P. Berget then at the Department of Biochemistry and Molecular Biology, University of Texas, Houston, USA. The minicell producing strain of *E. coli* DS410 was provided by Dr. I. B. Holland, University De Paris Sud, Orsay, France. The promoter cloning vector pKK232-8 was provided by Dr. K. Gopinathan, Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India.

Bacteria were routinely grown in LB broth. Antibiotics (ampicillin and chloramphenicol) were added to the media at requisite concentrations and whenever required. The techniques for preparation of DNA and transformation were followed as per standard procedure (Sambrook *et al*., 1989).

Construction of plasmids and subcloning Shotgun cloning and genomic library construction of *Sal*I+*Hin*dIII digested MB78 DNA was carried out between *Sal*I and *Hin*dIII sites of M13mp11 vector.

Abbreviations: bp, base pair; IPTG, isopropyl **b**-thio-galactosidase; kb, kilo base; ORF, open reading frame.

*To whom correspondence should be addressed.
Tel: 301-435-3182; Fax: 301-480-1337
E-mail: lgupta@niaid.nih.gov
EMBL Accession Number: AF349435

From this library, a clone containing 1.7 kb insert was recloned in pUC19, for the purpose of sequencing and expression. For identification of the promoter region, the fragment was further cloned in a promoterless vector (Brosious, 1984) named as pKK232-8. For sequencing and other studies subclones were made using different restriction enzymes.

Southern blotting and hybridization Southern blotting and hybridization of DNA was carried out following standard protocols as described before (Denhardt, 1966; Southern, 1975).

Radiolabeling of DNA *In vitro* labeling of DNA by nick translation was carried out as described before (Rigby *et al*., 1977).

Expression of plasmid coded genes in minicells Minicell producing strain (DS410) of *E. coli* was transformed with the desired plasmid and grown overnight in terrific broth containing relevant antibiotics. Minicells were purified from the culture as described before (Reeve, 1979). and labeled *in vivo* using [^{35}S] methionine. Labeled proteins were analyzed by electrophoresis through 12.5% SDS polyacrylamide gel as described before (Laemmli, 1970). The gel was fluorographed and autoradiographed for detection of labeled proteins. Fluorography of the gel was carried out by using sodium salicylate as scintillator as described before (Bonner, 1984).

Sequencing of DNA DNA sequencing was carried using double stranded templates. The technique was basically based on the dideoxy chain termination method of Sanger (Sanger *et al*., 1977). When sequencing kit was used, the protocols supplied by the manufacture were followed.

Results and Discussion

Cloning and physical mapping of the *Hin*dIII G fragment on the MB78 genome A genomic library of the phage was constructed by shotgun cloning of *Sal*I-*Hin*dIII fragments of phage genome in M13mp11 vector. From this library a clone named as SH12 containing 0.2 kb *Sal*I-*Hin*dIII fragment and 1.7 kb *Hin*dIII fragment was chosen for this study. Preliminary sequencing from *Sal*I site confirmed that 0.2 kb *Sal*I-*Hin*dIII fragment was a part of *Eco*RI C fragment and was already studied in detail (Kolla and Chakravorty, 2000). Therefore, only 1.7 kb *Hin*dIII fragment was recloned in pUC19 vector and referred as pHG12. The position of the fragment in the genomic map was determined from Southern blotting and hybridization experiment. The results presented in Fig.1A show *Hin*dIII digestion pattern of the plasmid DNA (pHG12) containing ~1.7 kb insert (lane 2) and MB78 DNA digested with different restriction enzymes (lane 3~5). The gel (as in Fig. 1A) was Southern blotted and hybridized with ^{32}P-labeled pHG12 DNA. The hybridization pattern (Fig. 1B) suggested that the 1.7 kb fragment has same mobility as *Hin*dIII G fragment (lane 2) and also spans part of *Eco*RI I, D, J and *Cla*I C fragments of MB78. From the physical map of MB78

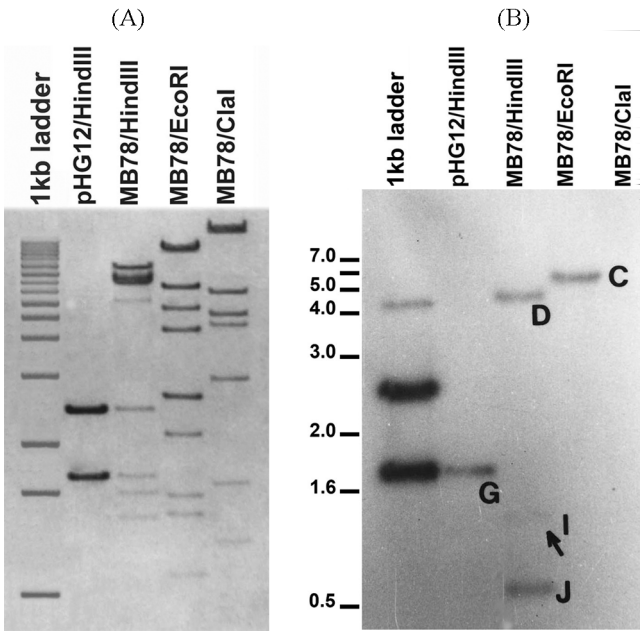

Fig. 1. Gel electrophoresis and hybridization of pHG12 DNA with different restriction digested fragments of MB78 genome. pHG12 DNA digested with *Hin*dIII and MB78 DNA digested with various restriction enzymes (as indicated at the top of the Fig. 1A) was electrophoresed through 0.8% agarose gel. The gel was Southern blotted and hybridized with ^{32}P-labelled pHG12 DNA. MB78 fragments that showed hybridization signal have been marked. (A) Ethidium bromide stained gel and (B) Autoradiogram of the same gel after hybridization.

genome, already constructed in this laboratory (Khan *et al*., 1991), the location of this fragment was determined (Fig. 2). Due to the incomplete digestion of the pHG12 DNA, an undigested top most faint band (4.4 kb) was also visible in Southern blot (Fig. 1B, lane 1).

Identification of gene(s) present in the *Hin*dIII G fragment In order to examine the protein encoded by *Hin*dIII G fragment, the minicells expression system was used as described in Materials and Methods. The rational behind using minicell system was that if the promoter region(s) of the gene(s) is (are) present in the cloned fragment those should be expressed even if they are not in phase with the coding sequence of β-galactosidase of vector pUC19. The minicells were purified and labeled with [^{35}S] methionine. Expressed proteins were analyzed on a 12.5% polyacrylamide gel followed by fluorography and autoradiography. The results presented in Fig. 3 indicate that the *Hin*dIII G fragment expressed only one protein of ~22 kDa (lane 2,). The vector pUC19 expressed only the β-lactamase gene (Fig. 3, lane 1). As pHG12 cloned in pUC19 also expressed β-lactamase gene. The top most band (Fig 3, lane 2) in case of pHG12 is pro β-lactamase, which is often seen in the gel.

Identification of the promoter region in pHG12 From the

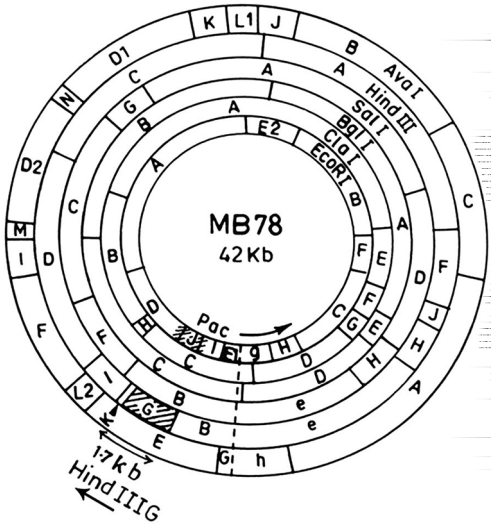

Fig. 2. Physical map of MB78 genome. The physical map of MB78 was constructed to show the location of 1.7 kb *Hin*dIII G fragment. The portion of *Eco*RI D, I and J present in *Hin*dIII G fragment are shown in hatched area.

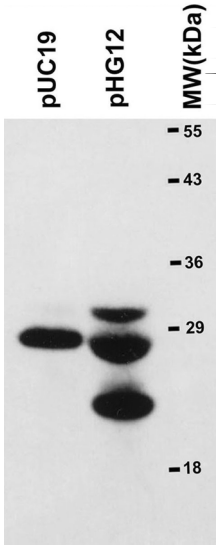

Fig. 3. *In vivo* expression of pHG12 clone in minicells. Minicells producing strain DS410 of *E. coli* was transformed with pUC19 (lane 1) and pHG12 (lane 2). Minicells were purified and labeled with ^{35}S-methionine (as described in Materials and Methods). The labeled proteins were analyzed by electrophoresis through 12.5% SDS-polyacrylamide gel followed by fluorography and autoradiography. The numbers at the right hand side indicate the positions and molecular weight (in kDa) of standard proteins.

in vivo expression of pHG12 (Fig. 3) it was realized that the 1.7 kb *Hin*dIII G fragment has its own promoter that transcribed the gene for the protein of apparent molecular weight 22 kDa. To confirm the direction of transcription and identifying the promoter region within this fragment, the promoter cloning vector pKK232-8 was used which has a

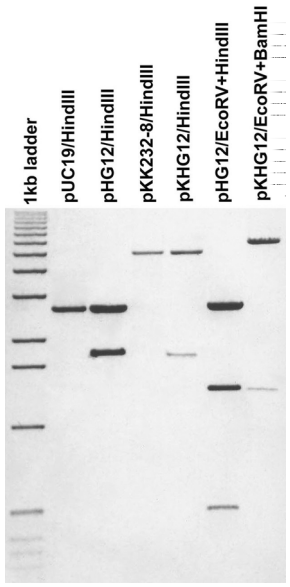

Fig. 4. Confirmation of recloning of *Hin*dIII G fragment from pHG12 and determination of its orientation in pKK232-8. DNA from different plasmids (as mentioned at the top of the figure) was digested with different enzymes, electrophoresed through 0.8% agarose gel and stained with ethidium bromide.

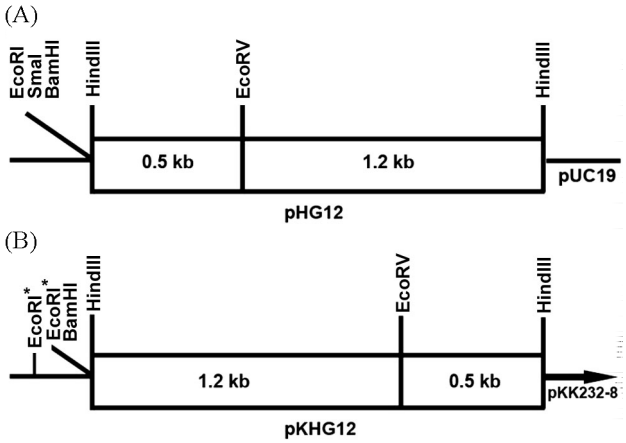

Fig. 5. Schematic representation of the orientation of *Hin*dIII G fragment in pUC19 and pKK232-8. The orientation of *Hin*dIII G fragment cloned in pUC19 (A) and pKK232-8 (B) is shown in the schematic diagram.

promoterless chloramphenicol gene. The pHG12 DNA was digested with *Hin*dIII, cloned at the *Hin*dIII site of pKK232-8 and transforments were selected in 50 µg/ml chloramphenicol and 50 µg/ml ampicilin. The obtained colony was resistant to chloramphenicol which confirmed the presence of promoter region in pHG12 fragment. The resistant clone was selected and referred to as pKHG12. To determine the orientation of *Hin*dIII G fragment in above vector, pKHG12 DNA was digested with different restriction enzymes and digested product was run in 0.8% agarose gel. These results are

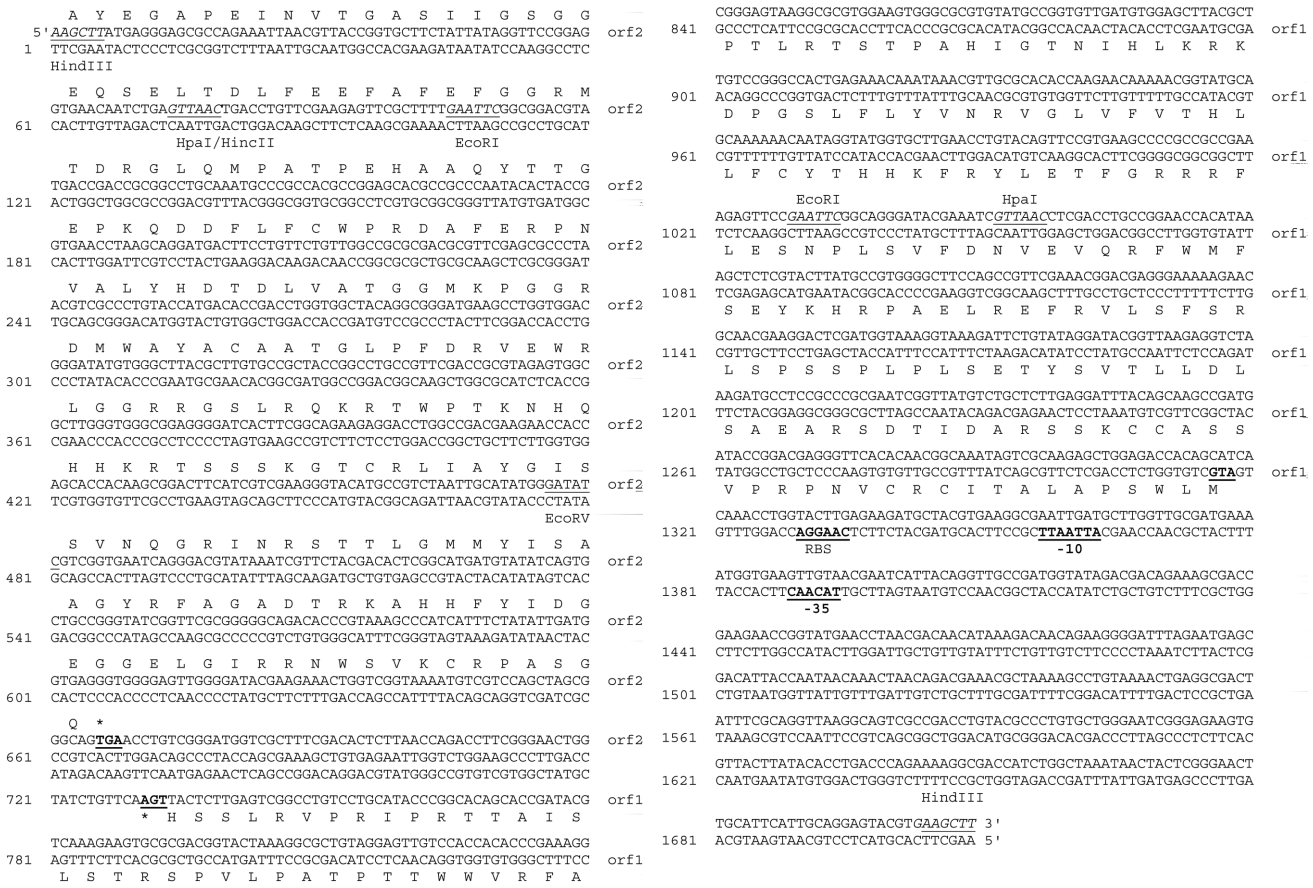

Fig. 6. Nucleotide and deduced amino acid sequence and ORFs present in the *Hin*dIII G fragment. The sequence of the coding strands is presented with nucleotide coordinates on the left and position of the ORFs located on the right. The ribosome binding site (RBS), the putative −10 and −35 regions as well as translation initiation and termination codons have been indicated in bold and underlined. *indicates the termination codons. The important restriction enzyme cleavage sites have been indicated in italics and underlined.

presented in Fig. 4. Digestion of pHG12 DNA with *Hin*dIII and *Eco*RV produced 2.7 kb, 1.2 kb and 0.5 kb fragments (Fig. 4, lane 6) whereas digestion of pKHG12 DNA with *Eco*RV and *Bam*HI (*Bam*HI is a unique site present in multiple cloning site of pKK232-8) produced 5.5 kb and 1.2 kb fragments (Fig. 4, lane 7). This confirmed that the direction of transcription from this promoter is opposite to the orientation of the fragment in pUC19 vector. These results are presented schematically in Fig. 5 which shows the orientation of *Hin*dIII G fragment in pUC19 as well as in pKK232-8. The orientation of *Hin*dIII G frgment is also shown in the physical map of MB78 (Fig. 2). The arrow shows the direction of transcription in MB78 genome.

Nucleotide sequence of the 1.7 kb *Hin*dIII G fragment and its analysis Nucleotide sequence of the *Hin*dIII G fragment of phage MB78 was determined by Sanger's chain termination method as described before (Sanger *et al*., 1977). To complete the sequence of the fragment, different subclones were made by using *Eco*RI, *Eco*RV and *Hpa*I restriction enzymes. After sequencing the total length of the fragment was found to be 1.709 kb. The complete nucleotide sequence along with the predicted amino acid sequence is presented in Fig. 6. The nucleotide sequence (Fig. 6) indicates the presence of two ORFs, the orf1 coding the ~22 kDa protein that appear in the SDS-polyacrylamide gel (Fig. 3), is from the bottom strand. Further analysis suggested that the translational start site of the ~22 kDa protein is ATG, which is preceded by AGGAAC Shine Dalgarno (SD) sequence. Nucleotide sequence analysis of the promoter region revealed the presence of putative −10 and −35 consensus regions (Fig. 6). The termination codon is TGA. The orf1 starts at nucleotide 1318 and terminate at nucleotide 730 from the second reading frame, coding for a protein containing 196 amino acids. The orf2 lies in the complementary upper strand and started from the beginning of the fragment. The promoter region and translational start site for orf2 lies in another 0.45 kb *Hin*cII U (pMC5) fragment that is located next to *Hin*dIII G fragment (Ahmad, 1995; Zargar *et al*., 2001). Earlier experiments in our laboratory confirmed that the direction of transcription of the promoter present in pMC5 is towards the *Hin*dIII G fragment that has a sequence for DNA bending (Ahmad, 1995). These two orfs, one complete and another incomplete, present within *Hin*dIII G fragment are in tail-to-tail orientation (Fig. 6). This type of

orientation is also found in many other cases. For example, the R-M system of *S. infantis* (Karreman and Ward, 1988) and *Eco*RII enzyme system in *E. coli* (Som *et al*., 1987) has tail-to-tail organization. This type of organization definitely has some role in the regulation of gene expression. The 22 kDa protein appears to be a functional protein since it did not produce any positive signal when tested by ELISA by the polyclonal antiserum raised against phage structural proteins (data not shown).

The physical location of *Hin*dIII G was determined by analyzing the nucleotide sequence. The occurrence of a 12 bp (GCTTACGCTTGT) direct repeat at positions 312 and 892 and 7 bp (TGAACCT) sequence 4 times at 182, 666, 985 and 1453 positions (Fig. 6) favors the idea that these sequences are part of regulatory elements. The location of two T stretches, 10 bases apart (947-951 and 963-968 nucleotides) suggest a physiological (or an important) role for this AT-rich region especially because these two stretches are separated by one turn of DNA helix. Such helically phased A tracks are involved in sequence directed DNA bending (Wo and Crothers, 1984). These AT-rich regions may be involved in the opening of DNA strands at promoter and replication origins. All these structural features of this part of the genome (*Hin*dIII G fragment) suggest that this may be an important region of the genome regulating the developmental cycle of the phage MB78. To get an idea about the functional properties of the proteins coded by *Hin*dIII G fragment, the amino acid sequence of the protein was subjected to homology search using Blast search computer-aided program. Nucleotide region from 1543 to 1686 of *Hin*dIII G fragment has high homology with EaA and EaI proteins of bacteriophage P22 (Pedulla *et al*., 2003), a closely related phage to MB78. The *Hin*dIII G nucleotide sequence was submitted to EMBL data base and obtained the accession number AF 349435.

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