

## Association of Mouse Fibrinogen-Like Protein with Murine Hepatitis Virus-Induced Prothrombinase Activity

REBECCA L. PARR,<sup>1</sup> LAISUM FUNG,<sup>2</sup> JEFFREY RENEKER,<sup>1</sup> NANCY MYERS-MASON,<sup>2</sup>  
JULIAN L. LEIBOWITZ,<sup>1,3\*</sup> AND GARY LEVY<sup>2</sup>

*Department of Pathology and Laboratory Medicine<sup>1</sup> and Department of Microbiology and Molecular Genetics,<sup>3</sup>  
University of Texas Medical School at Houston, Houston, Texas, and Department of Medicine,  
The Toronto Hospital, University of Toronto, Toronto, Canada<sup>2</sup>*

Received 9 January 1995/Accepted 2 May 1995

**Previously, we demonstrated induction of a unique macrophage prothrombinase during infection of BALB/cJ mice by mouse hepatitis virus strain 3 (MHV-3). By immunologic screening, a clone representing this prothrombinase was isolated from a cDNA library and sequenced. The sequence identified this clone as representing part of a gene, *musfiblp*, that encodes a fibrinogen-like protein. Six additional clones were isolated, and one clone, p11-3-1, encompassed the entire coding region of *musfiblp*. Murine macrophages did not constitutively express *musfiblp* but, when infected with MHV-3, synthesized *musfiblp*-specific mRNA. *musfiblp* mRNA induction was earlier and significantly greater in BALB/cJ than A/J macrophages. Prothrombinase activity was demonstrated when *musfiblp* was expressed from p11-3-1 in RAW 264.7 cells. These data suggest that *musfiblp* encodes the MHV-induced prothrombinase.**

Mouse hepatitis virus strain 3 (MHV-3), a member of the coronavirus family, causes a strain-dependent spectrum of disease in inbred strains of mice (16, 29). BALB/cJ and C57BL/6J mice develop a fatal hepatitis which is characterized pathologically by sinusoidal thrombosis and associated hepatocellular necrosis (2, 16, 17). C3H mice survive the acute infection and develop a chronic inflammatory disease of the liver; A/J mice are susceptible to infection but do not develop pathological lesions in the liver or biochemical evidence of hepatic necrosis. MHV-3 replicates in all four strains of mice, suggesting that additional factors may be required for hepatic injury. Several lines of evidence suggest that stimulation of the immune coagulation system by MHV-3 participates in the disease process. First, induction of monocyte/macrophage procoagulant activity (PCA) during MHV-3 infection correlates with the severity of the disease (18, 21). Second, there is a genetic linkage between induction of PCA in response to MHV-3 in vitro and in vivo and susceptibility to liver disease (4). Finally, treatment of mice with a monoclonal antibody to the MHV-3-induced PCA, a direct prothrombinase, prevents the lethality associated with MHV-3 infection (8). The MHV-3-induced PCA differs from the recently defined tissue factor, a 47-kDa protein translated from a 2.3-kb RNA, in that the MHV-induced PCA is dependent on prothrombin and fibrinogen for its activity, in contrast to tissue factor, which is dependent only on factor VII for its activity (20, 22, 27). Tissue factor is known to be expressed by monocytes, macrophages, fibroblasts, and endothelial cells but is not induced by MHV-3 infection. To date, only monocytes and macrophages have been shown to express PCA in response to MHV-3 infection (16).

In this study, we report the molecular cloning and sequencing of a cDNA isolated from a library prepared from macrophages which had been infected with MHV-3. The sequence of this cDNA is essentially identical to a previously described sequence corresponding to a gene encoding a mouse fibrino-

gen-like protein (*musfiblp*) (12). *musfiblp* was originally described as a cytotoxic T-cell (CTL)-specific gene which was constitutively expressed. In contrast, in macrophages, *musfiblp* is not normally expressed, but following MHV-3 infection, its expression is upregulated. When the cDNA containing the entire coding region of *musfiblp* was expressed in the RAW 264.7 cell line, a prothrombinase activity was detected by both a one-stage clotting assay and cleavage of <sup>125</sup>I-labeled prothrombin.

### MATERIALS AND METHODS

**Mice.** Female BALB/cJ and A/J mice, 6 to 8 weeks of age, were purchased from Jackson Laboratories (Bar Harbor, Maine) and housed in the animal facilities at the University of Toronto and the University of Texas Health Sciences Center, Houston. They were fed a standard chow diet and water ad libitum prior to and during the studies. Representative animals were tested for prior exposure to MHV by enzyme-linked immunosorbent assay (ELISA) or indirect immunofluorescent assays; anti-MHV antibodies were not detectable by these methods.

**Cells.** Peritoneal macrophages were harvested from A/J and BALB/cJ mice 4 days after intraperitoneal administration of 1.5 ml of 3% thioglycolate (Difco Laboratories, Detroit, Mich.) as previously described (16). Macrophages were greater than 95% pure, as determined by morphology, nonspecific esterase stain, and staining with MAC-1 antibody (8). Viability exceeded 98% by trypan blue dye exclusion. RAW 264.7, a BALB/cJ macrophage-derived continuous cell line, was obtained from the American Type Culture Collection (ATCC) and propagated in Dulbecco's modified Eagle's medium (DME) fortified with 10% fetal bovine serum (24). BSC1 cells, a monkey kidney cell line, and RK13, a rabbit kidney cell line, were obtained from the ATCC and similarly maintained in culture (13, 25).

**Virus.** MHV-3 was grown and titrated as described previously (16). A recombinant vaccinia virus expressing the bacteriophage T3 RNA polymerase (VVT3pol) was generously provided by M. Esteban, Department of Biochemistry, State University of New York Downstate Medical Center, Brooklyn, N.Y., and propagated in RK13 cells and titrated on BSC1 cells (25).

**Production and screening of the cDNA library.** BALB/cJ peritoneal macrophages were infected with MHV-3, and at 6 h postinfection, RNA was extracted with guanidinium hydrochloride and precipitated with ethanol (6). Polyadenylated RNA was selected by chromatography over an oligo(dT)-cellulose column. A random-primed cDNA library was constructed by Stratagene in the vector lambda ZAPII by standard methods (26). The amplified library was screened by plaque immunoscreening with the monoclonal antibody 3D4.3 as the primary immunological reagent and <sup>125</sup>I-labeled staphylococcal protein A as a secondary reagent. Plaques testing positive were diluted, replaqueed, and retested as described above until they were clonally isolated. Bluescript phagemids were rescued by excision from lambda ZAPII by superinfection with the helper phage R408 at a

\* Corresponding author. Mailing address: Dept. of Pathology and Laboratory Medicine, University of Texas Medical School, P.O. Box 20708, Houston, TX 77225. Electronic mail address: leib@casper.med.uth.tmc.edu.

multiplicity of 10. Rescued bacteriophage was then used to infect *Escherichia coli* XL1 cells, and ampicillin-resistant colonies were selected. Plasmid DNA was prepared by using the Wizard miniprep kit (Promega, Madison, Wis.). The presence of inserts was verified by restriction enzyme digestion and agarose gel electrophoresis. The sequence was determined by using a nested deletion (10) and a primer-directed strategy by cycle sequencing on an automated DNA sequencer (model 373A; Applied Biosystems) with dideoxy dye terminator chemistry. A consensus sequence was generated by using the Genetics Computer Group software (2). The GenBank and EMBL databases were searched with the FASTA and BLAST programs. Additional clones corresponding to the *musfiblp* insert within p1360-23 were identified by plaque hybridization. The probe used for screening was synthesized by PCR from a plasmid template, p1360-23, carrying our initial clone, and was then labeled by random priming. The oligonucleotides used for PCR corresponded to positions 860 to 877 and 2084 to 2063 (negative sense) in Fig. 2. Phage was clonally isolated, and plasmids were excised as described above. The sequence was determined and analyzed as described above to produce a full consensus sequence.

**RNA isolation and analysis.** Total RNA was isolated from both MHV-3-infected and uninfected macrophages and cell lines with guanidium hydrochloride (6). RNA (5 to 10 µg) was resolved by electrophoresis through a 0.8% agarose gel in MOPS (morpholinepropanesulfonic acid)-formaldehyde, transferred to a nylon membrane in 20× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, and 1 mM EDTA [pH 7.4]), and UV cross-linked (26). The membrane was prehybridized for 6 h at 42°C with denatured salmon sperm DNA (100 µg/ml in 50% formamide–5× SSPE–5× Denhardt's solution (26)–0.5% sodium dodecyl sulfate [SDS]). A DNA probe identical to that used to rescreen the library was labeled by random priming and added to the hybridization reaction mix, and hybridization was continued for 18 h at 42°C. The membrane was then washed three times at room temperature with 5× SSPE–0.5% SDS for 10 min. Three additional washes were carried out with 1× SSPE–0.5% SDS at 37°C. The membrane was washed three additional times with 0.1× SSPE–1.0% SDS at 65°C and then autoradiographed. Quantitation was performed with a Betagen scanner.

**Transient expression of *musfiblp*.** Replicate cultures of 10<sup>6</sup> RAW 264.7 cells were either infected with MHV-3 at a multiplicity of infection (MOI) of 5, infected with VVT3pol at an MOI of 10, coinfecting with both viruses, or mock infected. Immediately after viral infection, the cells were washed and transfected by the lipofectin procedure (GIBCO BRL) with 10 µg of p11-3-1. The transfection mixture was allowed to incubate for 2.5 h at 37°C. The lipofectin-containing medium was removed and replaced with DME containing 10% fetal bovine serum, and the cells were incubated until 24 h postinfection. The medium was removed, and the monolayers were washed once with DME and frozen in 1 ml of DME at –70°C until assayed for PCA.

**PCA.** The cell lysates were evaluated for PCA expression in a one-stage clotting assay as previously described (14). Following incubation, samples to be assayed for PCA were subjected to three cycles of freeze-thawing to obtain maximal total cellular PCA. Samples were assayed for their ability to shorten the spontaneous clotting time of normal citrated human platelet-poor plasma. Additional studies were performed with human plasmas which were congenitally deficient in coagulation factor VII, X, or II (Helena Laboratories, Beaumont, Tex.) to determine the nature (factor dependence) of the procoagulant. Milliunits of PCA were assigned by reference to a standard curve generated with serial log dilutions of a standard rabbit brain thromboplastin (Dade Division, American Hospital Supply Co., Miami, Fla.). The medium and reagents were without activity.

Factor X and prothrombin were isolated from Cohn fraction III as previously described (23). For assay of cleavage, prothrombin was radioiodinated enzymatically with immobilized lactoperoxidase and glucose oxidase (Enzymobeads; Bio-Rad, Richmond, Calif.) to a specific activity of 6.2 µCi/µg. To 25 µl of cellular homogenates, 10 µl of <sup>125</sup>I-prothrombin and 10 µl of 25 mM CaCl<sub>2</sub> were added. The reaction was allowed to proceed at 37°C for 30 min. Human factor Xa (0.5 µg/ml), in the presence of homogenates of 10<sup>6</sup> unstimulated macrophages, was used as a positive control for <sup>125</sup>I-prothrombin cleavage. Each reaction mixture was electrophoresed on a 10% polyacrylamide gel. Following electrophoresis, the gels were fixed, dried, and subjected to autoradiography.

## RESULTS

**Identification of PCA-specific cDNA clones.** Peritoneal exudate macrophages from BALB/cJ mice were infected with MHV-3, and polyadenylated RNA was purified at 6 h postinfection. Random-primed cDNA was synthesized, and a cDNA library was prepared by using the Stratagene vector lambda ZAPII. The library was initially screened by a plaque immunoassay with the monoclonal antibody 3D4.3, which has been shown previously to be specific for the MHV-3-induced PCA (8). A plaque expressing an immunoreactive protein were selected and retested repeatedly until a clonally isolated phage was obtained. A plasmid carrying the cDNA was propagated after excision and rescue with helper phage R408. The se-

quence of this clone, p1360-23 (1.25 kb), was compared with sequences in the GenBank and EMBL databases and was identical to a portion of exon 2 of *musfiblp*, with the exception of one nucleotide. A series of six additional clones were identified by plaque hybridization with a probe prepared from p1360-23, clonally isolated, and propagated as plasmids (Fig. 1A).

A consensus sequence from portions of these six clones and p1360-23 was assembled by the strategy shown in Fig. 1B. A database search with the sequence of our largest clone, p11-3-1, revealed that the protein encoded by the cDNA sequence of mouse PCA was identical to a gene product from both cDNA and genomic T-cell libraries that encodes a fibrinogen-like protein, *musfiblp* (Fig. 2), which is composed of two exons (12). The clone, p11-3-1, is 2,451 bp in length, encompasses the entire coding region of exon 1 and exon 2 of *musfiblp*, and appears to differ from the published sequence by 1 bp at position 1138 (C→G), which changes an alanine to a glycine in the translated protein. The 5'-most 108 nucleotides of p11-3-1 diverge from the 5' untranslated region of *musfiblp*. A search of the nonredundant sequence database at the National Library of Medicine with the BLASTN program demonstrated that these 108 nucleotides correspond to positions 3658 to 3765 in an uncharacterized 6,379-nucleotide cDNA (GenBank accession number D29640) isolated from a human immature myeloid cell line. For reasons discussed below, we believe that this 108-nucleotide sequence represents a cloning artifact.

**Northern (RNA blot) analysis of PCA mRNA.** To determine if *musfiblp* transcripts were induced by infection of macrophages with MHV-3, macrophages from BALB/cJ and A/J mice were infected with MHV-3, and total RNA was extracted at various times postinfection. RNA was electrophoresed, blotted, and hybridized with a *musfiblp*-specific probe. RNA hybridizing to the *musfiblp*-specific probe was not detected in uninfected macrophages from either A/J or BALB/cJ mice. In macrophages from BALB/cJ mice, *musfiblp*-specific RNA was first detected at 4 h postinfection, peaked at 6 h, decreased thereafter, and returned to baseline by 24 h (Fig. 3). The predominant RNA observed was approximately 4 kb in size, consistent with the previously reported size of *musfiblp* transcripts in CTL. Longer exposures revealed the presence of small amounts of longer transcripts (data not shown). Quantitation by betascanning indicated that an approximately 80-fold induction of the 4-kb transcript was obtained at 6 h postinfection (Fig. 4). In A/J macrophages, a significant but lesser induction of *musfiblp*-specific transcripts was observed. The maximum level of induction, 39-fold, was obtained at a later time, 12 h postinfection (Fig. 3 and 4).

**PCA of transfected cell lysates.** To prove that the gene product of *musfiblp* had prothrombin-cleaving activity, a gene transfer strategy was employed. To express the *musfiblp* protein, we used a macrophage cell line, RAW 264.7, which contained very low levels of *musfiblp* transcripts (data not shown) and expressed no functional PCA, either constitutively, following stimulation with lipopolysaccharide (LPS), or following infection with MHV-3 (Table 1 and Fig. 5). For expression of the *musfiblp* protein, we exploited the presence of an upstream T3 promoter in the p11-3-1 plasmid. RAW 264.7 cells were transfected with plasmid p11-3-1 and infected with a recombinant vaccinia virus expressing the T3 polymerase (VVT3pol). PCA was measured in a one-stage clotting assay and by the ability of the gene product to cleave prothrombin directly to the active moiety, thrombin (14). Uninfected and nontransfected RAW cells expressed low basal PCA (Table 1). No augmentation was observed even following stimulation with MHV-3 or LPS. Infection with VVT3pol by itself or in com-

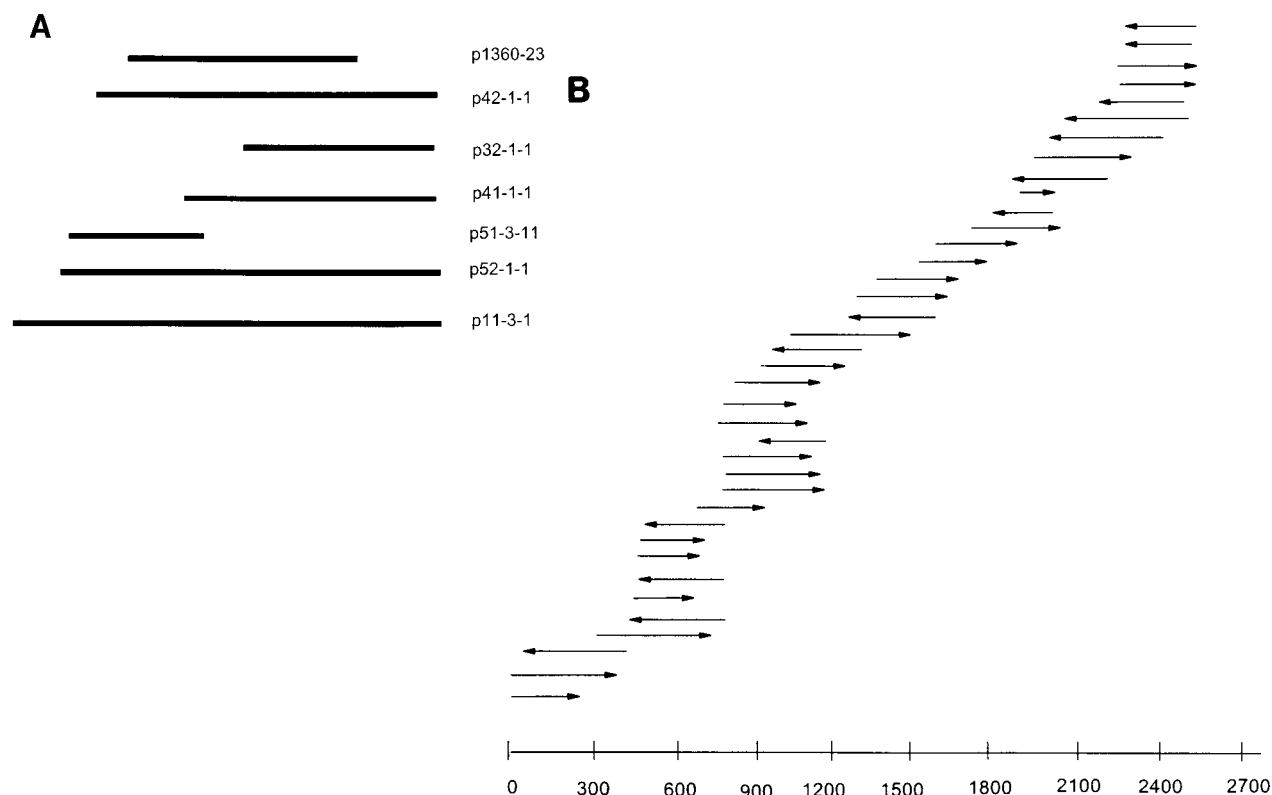


FIG. 1. Schematic map of cDNA clones and the sequencing strategy. (A) Relative size and relationship of each clone. The schematic maps of overlapping cDNA clones representing *musfiblp* were identified from a Stratagene lambda ZAPII library. The library was screened as described in the text, and seven clones were identified. (B) The sequencing was performed with a nested deletion and a primer-directed strategy. Each sequencing reaction is depicted by an arrow showing the direction of sequencing.

ination with MHV-3 resulted in a modest, although statistically significant, augmentation in PCA. Transfection of p11-3-1 in the absence of VVT3pol to drive the T3 promoter resulted in a similar low level of activity. However, the combination of VVT3pol and p11-3-1 induced a marked increase in PCA, suggesting that *musfiblp* expression is necessary for functional PCA. The combination of VVT3pol, p11-3-1, and MHV-3 resulted in a small, reproducible, but statistically not significant further increase in PCA, suggesting that MHV infection may further upregulate PCA, although the mechanism for this is not clear.

To confirm that the PCA was indeed a prothrombinase, the cell lysates were added to  $^{125}\text{I}$ -prothrombin, and cleavage to thrombin was monitored by SDS-PAGE (Fig. 5). The results obtained exactly paralleled those obtained in the one-stage clotting assay. Only RAW 264.7 cells in which the expression of *musfiblp* was driven by VVT3pol (Fig. 5, lanes 5 and 7) demonstrated prothrombin cleavage. Neither infection with VVT3pol (Fig. 5, lane 4) nor transfection with p11-3-1 by itself (lane 6) resulted in prothrombin cleavage. The pattern of cleavage was identical to that observed with the physiologic cleavage observed following addition of factor Xa (Fig. 5, lane 9).

## DISCUSSION

In this study, using a monoclonal antibody to MHV-3-induced PCA (3D4.3), we have isolated a molecular clone corresponding to a mouse fibrinogen-like gene (*musfiblp*) (8, 12).

A series of additional clones was identified, including a clone which contained the entire *musfiblp* coding sequence (p11-3-1). Although the sequence was nearly identical to the published sequence of *musfiblp*, there were two differences. The first was a single base substitution at position 1138, corresponding to an amino acid change of an alanine to a glycine in the protein (Fig. 2). This represents a conservative change which should result in no significant alteration of the protein structure. The second difference was a divergence between the first 108 nucleotides of p11-3-1 from the published *musfiblp* 5' untranslated sequence. *musfiblp* transcripts were shown to be upregulated in MHV-3-infected BALB/cJ and A/J macrophages. However, the kinetics and degree of induction of *musfiblp* RNA were earlier and significantly greater in macrophages from BALB/cJ mice than in macrophages from A/J mice. Furthermore, the kinetics of accumulation of *musfiblp* mRNA in BALB/cJ macrophages paralleled the expression of functional PCA. When the entire coding region of *musfiblp* (p11-3-1) was expressed by a coupled vaccinia virus-bacteriophage system in RAW 264.7 cells, a cell line which did not express functional PCA either constitutively or after infection with MHV, a marked functional PCA response was observed.

The finding of a 108-base divergence from the published sequence was surprising. Two explanations for this divergence are possible. First, these 108 bases could represent a previously undescribed upstream exon within *musfiblp*. A second explanation is that this represents a cloning artifact. Genomic clones encompassing approximately 24 kb of upstream sequence, generously provided by H. Saito (Dana Farber Cancer Institute),

CCCTGCTGACAGGTCAATGATGTGCGAAGGCATCGGGAGCGACGATGGCTGGGTTTCATGTATCGGTAGTAAAGCAGGTTACCGATAATCTTCAGCAGCTCG	100
TCCTCACCGAGCGCCTGGTTCAGGCTGGGCTGCCGCACTGCAAGGATGAGGCTTCTCGTTGGTTGTGGCTGAGTTCTGCCGTCCTCGCTGCCGAGCG	200
M R L P G W L W L S S A V L A A C R A	
GTGGAGGAGCACAACTGACTGAGGGGCTGGAGGATGCCAGCGCCAGGCTGCCCGCGAGGCTGGAGGGCAGCGGGAGGTGCGAGGGGAGCCAGT	300
V E E H N L T E G L E D A S A Q A A C P A R L E G S G R C E G S Q C	
GCCCCTTCAGCTCACCTGCCACGCTGACCATCCAGCTCCCGCGGAGCTGGCAGCATGGAGGAGGTGCTCAAAGAAGTGGGACCCCTCAAGGAAGC	400
P F Q L T L P T L T I Q L P R Q L G S M E E V L K E V R T L K E A	
AGTGGACAGTCTGAAGAAATCCTGCCAGGACTGTAAGTTGCAGGCTGACGACCATCGAGATCCCGCGGGAATGGAGGGAATGGAGCAGAGACAGCCGAG	500
V D S L K K S C Q D C K L Q A D D H R D P G G N G G N G A E T A E	
GACAGTAGAGTCCAGGAACTGGAGAGTCAGGTGAACAAGCTGCCTCAGAGCTGAAGAATGCAAAGGACCAGATCCAGGGGCTGCAGGGGCGCTGGAGA	600
D S R V Q E L E S Q V N K L S S E L K N A K D Q I Q G L Q G R L E T	
CGCTCCATCTGGTAAATATGAACAACATTGAGAACTACGTGGACAACAAAGTGGCAAATCTAACCGTTGTGGTCAACAGTTTGATGGCAAGTGTTCCAA	700
L H L V N M N N I E N Y V D N K V A N L T V V V N S L D G K C S K	
GTGTCCCAGCCAAGAACACATGCAGTCACAGCCGGTTCAACATCTAATATACAAAGATTGTTCCGACCACTACGTGCTAGGAAGGAGAAGCAGTGGGGCC	800
C P S Q E H M Q S Q P V Q H L I Y K D C S D H Y V L G R R S S G A	
TACAGAGTTACCCTGATCACAGAAACAGCAGCTTTGAGGTCTACTGTGACATGGAGACCATGGGTGGAGGCTGGACGGTCTGCAGGCTCGCCTTGATG	900
Y R V T P D H R N S S F E V Y C D M E T M G G G W T V L Q A R L D G	
GCAGACCAACTTCACCAGAGAGTGGAAAGACTACAAAGCCGGCTTTGGAACCTTGAACGAGAATTTGGTTGGGCAACGATAAAAATTCATCTTCTGAC	1000
S T N F T R E W K D Y K A G F G N L E R E F W L G N D K I H L L T	
CAAGAGTAAGGAAATGATTTTGAAGAATAGATCTTGAAGACTTTAATGGTCTCACACTTTATGCTTGTATGATCAGTTTTATGTGGCTAATGAATTTCTC	1100
K S K E M I L R I D L E D F N G L T L Y A L Y D Q F Y V A N E F L	
C	
AAATACCGATTACACATCGGTAACACTACAATGGCACGGGAGGGGATGCCTTTCGCTTTCAGTCGACACTACAACCATGACCTGAGGTTTTTACAAACCCAG	1200
K Y R L H I G N Y N G T G G D A L R F S R H Y N H D L R F F T T P D	
A	
ACAGAGACAACGATCGGTACCCTCTGGGAAGTGTGGGCTCTATTACAGCTCAGGCTGGTGGTTTATTATCTCTCTGCCAACTTAAATGGCAAATA	1300
R D N D R Y P S G N C G L Y Y S S G W W F D S C L S A N L N G K Y	
TTACCACCAGAAATACAAAGGTGTCGTAATGGGATTTTCTGGGGCACCTGGCCTGGTATAAACCAGGCACAGCCAGGTGGCTACAAGTCTCTCTCAA	1400
Y H Q K Y K G V R N G I F W G T W P G I N Q A Q P G G Y K S S F K	
CAGGCCAAGATGATGATTAGGCCAAGAATTTCAAGCCATAAATGTAGTGTTCATCTCTCTGGGCACTACTATCTAAGAGGACGATGAATTCCTTCA	1500
Q A K M M I R P K N F K P	
GCCCTTACCATATGTCTCAGTTTATATTCTTTCTATGGCTAAACATTTCTTTAAAGCTTTACAGCTTTTAGAATAAAGCTGAAAAGATCTAAAAAG	1600
ACTCCTATGTTGCTGTTATATGAGGAATGCTTGAAAGCACTGGAAATATTGACAATTATACATTATAATGCAAAACCTTTCATTTTTATTAGTTGAAAA	1700
GTTTCCTAATATTTTTATTATTTTTATAATAAAAACTAAATATTTCAGCAAGCTAGATTCTATATACGCAAGTTTTATTTTCACTAGGGCTAAATATACA	1800
CATTGAGAATATAACAGTCTTCCAGGTACAACCTGAAAGCCAAGAAGTGTAGTATTATCTTTTCGTCTAAGAAGAAGCTTAAAGCATTTTAGTTCTCAAGA	1900
AGAAGGCAGGGATGGGATGGGGGCCAGGACAATATGTATAGCTAAATGTATTATCTAATGCAAAATATGGCATTAAATACCTAAAAATGTGGTATG	2000
CATAATATATGTCTCTCCCTCTCCAATTGAAAAATAATGTTACCCTGTAGACTTTGGTTTAGTGGTAATTCACTTACTGTTTATAGCCTGTTAGACCGC	2100
GATACAAAAGCTGCTTTATCCTCTCCCTCTGCTCTCTGTGCACAATGGTTTGTGATGTAAGGTGCTAGACTACTGTAAGGTTTCTTGGGGAAAGGCATG	2200
GTAAGGGAAAAACACTGGTTTATATTTTGAAGCCAATCCTAATCCCAAAGCAATACTGTTGTGAGGAGTCAACGTTCTAGGAAGCTGACTTTTCTAG	2300
AACAAATGATTTTATTAGGATGAATTTTGAATTTTAGTTTAAATATTATACATTTTAACTTAAAGCAGTCAATTGCTTATTTCATTCTTAGAAATTCAA	2400
AGAATATATACAATTTTAGCCAAATGTGAGTCCCTGGAATTCCTTACTTGTGGATGTCAAGGGTCACATTAAGGCTCTGGGGCAGTTTCACTCACTT	2500
TATTCTGTGAAAAGGGGTCACCTCAGAGAGAAACAACCAGC	2541

FIG. 2. Consensus nucleotide and predicted amino acid sequences of cDNAs. The sequence shows identity to *musfiblp* except for the boxed first 108 bases and one nucleotide change at position 1138 (from a C to a G), to change the predicted amino acid alanine to a glycine. The codon encoding the glycine in our sequence is indicated by boldface type. The nucleotide at position 1138 is underlined, and the previously reported C at this position is shown above our sequence. The resulting amino acid change is shown below our sequence.

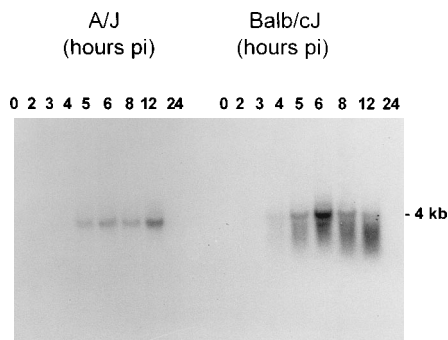


FIG. 3. *musfiblp* RNA expression in macrophages from A/J and BALB/cJ mice following MHV-3 infection. Thioglycolate-elicited peritoneal macrophages (>96% MAC-1 positive) from A/J and BALB/cJ mice were mock infected (time zero) or infected (time 2 to 24 h) with MHV-3 at an MOI of 1. Total RNA was extracted at the various time points postinfection (p.i.) as indicated, and 15  $\mu$ g of total RNA was resolved on a formaldehyde gel, blotted onto Nytran, and hybridized with the *musfiblp* cDNA probe, p1360-23, as described in Materials and Methods. The relative equivalence of the amount and integrity of mRNA in each sample was verified by hybridization with an  $\alpha$ -tubulin cDNA (data not shown).

were examined for the presence of these 108 bases by blot hybridization, and no signal corresponding to the sequence was identified. Additional cDNA clones containing the 108 bases embedded in the center of their sequences have been identified in our cDNA library. These clones contain a single open reading frame, but none of them contain *musfiblp*-related sequences. A search of the GenBank database revealed that the sequences represented by these cDNA clones is almost identical to an otherwise uncharacterized human mRNA isolated from an immature myeloid cell line. Therefore, it is unlikely that these 108 nucleotides are related to *musfiblp*, and they probably represent a cloning artifact.

Examination of the predicted protein structure of the *musfiblp* product showed 36% sequence similarity with the C-terminal portion of the beta and gamma subunits of fibrinogen, similar to other members of the fibrinogen family of proteins (12). In a previous report, it was suggested that the *musfiblp* protein does not contain the amino acid residues corresponding to a thrombin-sensitive site for the release of a fibrinopeptide or a site for cross-linking or interaction with

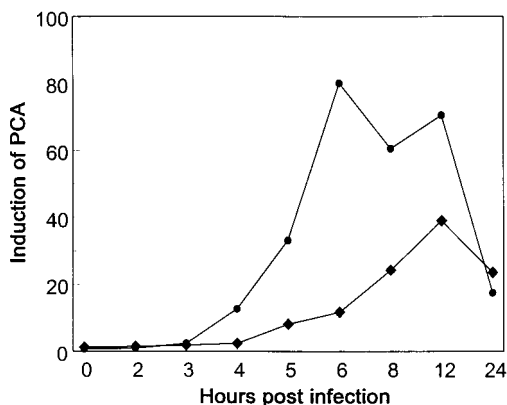


FIG. 4. Induction of *musfiblp* mRNA in MHV-3-infected A/J and BALB/cJ mouse macrophages. Peritoneal macrophages were collected and infected with MHV-3, and total RNA was extracted at various times postinfection. RNA was electrophoresed, transferred to a Nytran membrane as described in the legend to Fig. 3, hybridized with a cDNA probe made from p1360-23, and quantitated with a Betagen scanner.  $\blacklozenge$ , A/J macrophage RNA;  $\bullet$ , BALB/cJ macrophage RNA.

TABLE 1. Induction of PCA by MHV-3<sup>a</sup>

Stimulus	Mean PCA (mU/10 <sup>6</sup> macrophages) $\pm$ SD
Control	16 $\pm$ 2
MHV-3	18 $\pm$ 4
LPS	18 $\pm$ 1
Vaccinia virus vector only	54 $\pm$ 7
Vaccinia virus vector + p11-3-1	795 $\pm$ 143
p11-3-1	83 $\pm$ 40
Vaccinia virus vector + p11-3-1 + MHV-3	1,120 $\pm$ 255
Vaccinia virus vector + MHV-3	85 $\pm$ 35

<sup>a</sup> Data represent the mean  $\pm$  standard deviation of four experiments done in triplicate. Macrophages are the RAW cell line.

platelets (5). It was further suggested that a signal peptide was necessary for transport of the protein into the endoplasmic reticulum, consistent with this being a secreted protein. The authors further suggested that the *musfiblp* product may dimerize or associate with other proteins to form a three-stranded coiled coil. We have previously shown by Western blot that PCA is a disulfide-linked dimer (8). Thus, these observations are compatible. The predicted molecular size, 49 kDa, of the nonglycosylated *musfiblp* protein is in keeping with the approximate 70-kDa size of the prothrombinase expressed in macrophages following MHV stimulation.

Our interest in this molecule is its ability to cleave prothrombin to thrombin independent of coagulation factors XII, VIII, VII, X, and V for its activity, which is characteristic of a direct prothrombinase (27). Previously, we suggested that the MHV-induced prothrombinase was a serine protease, based on inhibitor analysis. Examination of the predicted amino acid sequence of the *musfiblp* product failed to reveal any classical proteinase motifs. However, this is not unprecedented. Recently, the active site of interleukin-1 $\beta$  converting enzyme was determined to be contained within a previously undescribed cysteine proteinase motif (28). The determination of the *musfiblp* proteinase active site awaits site-directed mutagenesis experiments.

Originally, *musfiblp* was isolated from a CTL cell line and was shown to be constitutively expressed in CTL but not in

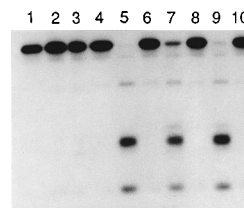


FIG. 5. Expression of *musfiblp* correlates with prothrombinase activity. Homogenates (0.05 ml) of replicate cultures of 10<sup>6</sup> RAW 264.7 cells either infected with MHV-3 alone at an MOI of 5, infected with VVT3pol alone at an MOI of 10, coinfecting with both viruses, or mock infected in normal saline were incubated for 60 min at 37°C with 0.01 ml of <sup>125</sup>I-prothrombin (100 nM) and 0.01 ml of CaCl<sub>2</sub> (25 mM). SDS and EDTA were added, and the samples were electrophoresed on an SDS-10% polyacrylamide slab gel and displayed by autoradiography. Lane 1, <sup>125</sup>I-prothrombin alone; lane 2, <sup>125</sup>I-prothrombin incubated with RAW cells infected with MHV-3; lane 3, <sup>125</sup>I-prothrombin incubated with RAW cells stimulated with LPS for 6 h; lane 4, <sup>125</sup>I-prothrombin incubated with RAW cells infected with VVT3pol; lane 5, <sup>125</sup>I-prothrombin incubated with RAW cells infected with both VVT3pol and p11-3-1; lane 6, <sup>125</sup>I-prothrombin incubated with RAW cells infected with p11-3-1; lane 7, <sup>125</sup>I-prothrombin incubated with RAW cells infected with VVT3pol, MHV-3, and p11-3-1; lane 8, <sup>125</sup>I-prothrombin incubated with VVT3pol and MHV-3; lane 9, <sup>125</sup>I-prothrombin incubated with Russel's viper venom; lane 10, <sup>125</sup>I-prothrombin incubated with mock-infected RAW cells.

B-cell lines, T helper cells, keratinocytes, mouse brain, or fibroblasts. Our data show that *musfiblp* RNA was not constitutively expressed in macrophages but, following MHV infection, was markedly upregulated. Although we did not examine CTL specifically, lymphocytes recovered from popliteal lymph nodes showed only small amounts of constitutively expressed *musfiblp* RNA, and MHV infection did not result in further augmentation (data not shown). Although resistant A/J mice show transcription of *musfiblp*, they express no functional PCA. There are several possibilities that could explain this observation. First, a posttranslational modification may be required for functional activity, and this may not occur in A/J mice. Second, functional activity may require additional factors yet to be identified. Third, it is possible that the sequence of *musfiblp* in A/J mice precludes translation of a functional protein. Previous work has implicated the macrophage as being of central importance in resistance and susceptibility to MHV-3 (1). Furthermore, we have established a correlation between susceptibility to lethal infection and induction of PCA by macrophages (3). The recent production of monoclonal antibodies to PCA in our laboratory and the demonstration that passive transfer of these antibodies to susceptible mice confers resistance to MHV-3 argue strongly for a role for PCA in the pathogenesis of MHV-3 (19). The sequela of prothrombinase production is generation of thrombin. Thrombin is the central bioregulatory enzyme in hemostasis and is responsible for conversion of fibrinogen to fibrin and for activation of coagulation factors V, VII, and XIII (7). In addition, it is a potent activator of platelets, either alone or in synergism with other agents, and may interact directly with endothelial cells, causing vasoconstriction (9) and mediating leukocyte adherence (11), all of which are prominent features of MHV infection (15).

In conclusion, in this study, we have demonstrated that following MHV-3 infection, *musfiblp* RNA is markedly upregulated in macrophages from susceptible BALB/cJ mice. In transfection experiments, we have shown that expression of this gene results in the elaboration of prothrombinase activity. Further experiments on the mechanism of regulation of this gene will lead to further understanding of the role of *musfiblp* in the pathogenesis of MHV-3 infection.

#### ACKNOWLEDGMENTS

This work was supported by a program project grant (PPG11810) from the Medical Research Council of Canada, an NIH grant (AI31069), and a grant from the Council for Tobacco Research.

We thank H. Saito for providing us with three genomic clones of *musfiblp* and M. Estaban for kindly providing VVT3pol. We also acknowledge D. Needleman's helpful advice in designing PCR and sequencing primers.

#### REFERENCES

- Chung, S., S. Sinclair, J. Leibowitz, E. Skamene, L. S. Fung, and G. A. Levy. 1991. Cellular and metabolic requirements for induction of macrophage procoagulant activity by murine hepatitis virus strain 3 in vitro. *J. Immunol.* **145**:271-278.
- Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387-395.
- Dindzans, V. J., P. J. MacPhee, L. S. Fung, J. L. Leibowitz, and G. A. Levy. 1985. The immune response to mouse hepatitis virus: expression of monocyte procoagulant activity and plasminogen activator during infection in vivo. *J. Immunol.* **135**:4189-4197.
- Dindzans, V. J., E. Skamene, and G. A. Levy. 1986. Susceptibility/resistance to mouse hepatitis virus strain 3 and macrophage procoagulant activity are genetically linked and controlled by two non-H-2 linked genes. *J. Immunol.* **137**:2355-2360.
- Doolittle, R. F. 1984. Fibrinogen and fibrin. *Annu. Rev. Biochem.* **53**:195-229.
- Evans, R., and S. J. Kamdar. 1990. Stability of RNA isolated from macrophages depends on the removal of an RNA-degrading activity early in the extraction procedure. *Biotechniques* **8**:357-360.
- Fenton, J. W. 1986. Thrombin. *Ann. N.Y. Acad. Sci.* **485**:5-15.
- Fung, L. S., G. Neil, J. Leibowitz, E. H. Cole, S. Chung, A. Crow, and G. A. Levy. 1991. Monoclonal antibody analysis of a unique macrophage procoagulant activity induced by murine hepatitis virus strain 3 infection. *J. Biol. Chem.* **226**:1789-1795.
- Haver, V. M., and D. H. Namm. 1984. Characterization of the thrombin induced contraction of vascular smooth muscle. *Blood Vessels* **21**:53-63.
- Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**:351-359.
- Jungi, T. W., M. O. Spycher, V. E. Nydegger, and S. Banandum. 1986. Platelet-leukocyte interaction: selective binding of thrombin stimulated platelets to human monocytes, polymorphonuclear leukocytes and related cell lines. *Blood* **67**:629-636.
- Koyama, S., L. R. Hall, H. G. Haser, S. Tonegawa, and H. Saito. 1987. Structure of a cytotoxic T-lymphocyte-specific gene shows a strong homology to fibrinogen  $\beta$  and  $\gamma$  chains. *Proc. Natl. Acad. Sci. USA* **84**:1609-1613.
- Leibowitz, J. L., K. C. Wilhelmsen, and C. W. Bond. 1981. The virus-specific intracellular RNA species of two murine coronaviruses: MHV-A59 and MHV-JHM. *Virology* **114**:39-51.
- Levy, G. A., and T. S. Edgington. 1980. Lymphocyte cooperation is required for amplification of macrophage procoagulant activity. *J. Exp. Med.* **151**:1232-1244.
- Levy, G. A., H. Helin, and T. S. Edgington. 1984. The pathobiology of viral hepatitis and immunologic activation of the coagulation protease network. *Semin. Liver Dis.* **4**:59-64.
- Levy, G. A., J. L. Leibowitz, and T. S. Edgington. 1981. The induction of monocyte procoagulant activity by murine hepatitis virus MHV-3 parallels disease susceptibility in mice. *J. Exp. Med.* **154**:1150-1163.
- Levy, G. A., J. Leibowitz, and T. S. Edgington. 1982. Lymphocyte-instructed monocyte induction of coagulation pathways parallels the induction of hepatitis by the murine hepatitis virus. *Prog. Liver Dis.* **7**:393-409.
- Levy, G. A., P. J. MacPhee, L. S. Fung, M. M. Fisher, and A. M. Rappaport. 1983. The effect of mouse hepatitis virus infection on the microcirculation of the liver. *Hepatology* **3**:964-973.
- Li, C. Y., L. S. Fung, S. Chung, A. Crow, N. Myers-Mason, M. J. Phillips, J. L. Leibowitz, E. Cole, C. A. Ottaway, and G. A. Levy. 1992. Monoclonal antiprothrombinase (3D4.3) prevents mortality from murine hepatitis virus (MHV-3) infection. *J. Exp. Med.* **176**:689-697.
- Mackman, M., J. H. Morrissey, B. Fowler, and T. S. Edgington. 1989. Complete sequence of the human tissue factor gene, a highly regulated cellular receptor that initiates the coagulation protease cascade. *Biochemistry* **28**:1755-1762.
- MacPhee, P. J., V. J. Dindzans, L. S. Fung, and G. A. Levy. 1985. Acute and chronic changes in the microcirculation of the liver in inbred strains of mice following infection with mouse hepatitis virus type 3. *Hepatology* **5**:649-660.
- Nemerson, Y., and R. Bach. 1982. Tissue factor revisited. *Prog. Hemostasis Thromb.* **6**:237-261.
- Ottaway, C. A., R. E. Warren, F. G. Saibil, L. S. Fung, D. S. Fair, and G. A. Levy. 1984. Monocyte procoagulant activity in Whipple's disease. *J. Clin. Immunol.* **4**:348-358.
- Raschke, W., S. Baird, P. R. Nakoinz, and I. Nakoinz. 1978. Functional macrophage cell lines transformed by Abelson leukemia virus. *Cell* **15**:261-267.
- Rodriguez, D., Y. Zhou, J.-R. Rodriguez, R. K. Durbin, V. Jimenez, W. T. McAllister, and M. Esteban. 1990. Regulated expression of nuclear genes by T3 RNA polymerase and *lac* repressor, using recombinant vaccinia virus vectors. *J. Virol.* **64**:4851-4857.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schwartz, B. S., G. A. Levy, D. S. Fair, and T. S. Edgington. 1982. Murine lymphoid procoagulant activity induced by bacterial lipopolysaccharide and immune complexes is a monocyte prothrombinase. *J. Exp. Med.* **155**:1464-1479.
- Thornberry, N. A., H. G. Bull, J. R. Calaycay, K. T. Chapman, A. D. Howard, M. J. Kostura, D. K. Miller, S. M. Molineaux, J. R. Weidner, J. Aunins, K. O. Elliston, J. M. Ayala, J. Casano, J. Chin, J.-F. Ding, L. A. Egger, E. P. Gaffney, G. Limjuco, O. C. Palyha, S. M. Rujau, A. M. Rolando, J. P. Salley, T.-T. Yamin, T. D. Lee, J. E. Shively, M. MacCoss, R. A. Mumford, J. A. Schmidt, and M. J. Tocci. 1992. A novel heterodimeric cysteine protease is required for interleukin-1 $\beta$  processing in monocytes. *Nature (London)* **356**:768-774.
- Virelizier, J.-L., and A. C. Allison. 1976. Correlation of persistent mouse hepatitis virus (MHV-3) infection with its effect on mouse macrophage cultures. *Arch. Virol.* **50**:279-285.