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Cloning, characterization and overexpression of a *Streptococcus pyogenes* gene encoding a new type of mitogenic factor

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A new type of mitogenic factor, termed MF, has been found in the culture supernatant of *Streptococcus pyogenes* and its N-terminal amino acid sequence has been determined. On the basis of this sequence, an *S pyogenes* gene encoding MF was cloned and its nucleotide sequence was determined. The MF gene includes a long, open reading frame with 813 nucleotides capable of encoding the MF precursor protein with 271 amino acids. Removal of the putative 43 residues as a signal peptide results in the mature MF protein with 228 amino acids. The molecular mass of the mature MF is calculated as 25,363 which is consistent with the previously determined value of 25,370 for MF secreted from *S. pyogenes*. Neither nucleotide nor amino acid sequence homology was found between the mature MF and other streptococcal pyrogenic exotoxins, such as SPE A, SPE B and SPE C. The mature MF was recombinantly overexpressed as a fusion protein with glutathione S-transferase in *Escherichia coli*. The recombinant protein showed mitogenic activity in rabbit peripheral blood lymphocytes and immunoreactivity with the rabbit antiserum raised against the secreted MF from *S pyogenes*. These data indicate that a unique gene encoding MF was cloned from *S. pyogenes*.

Streptococcus; Mitogenic factor, Molecular cloning; Nucleotide sequence; Expression; Streptococcal pyrogenic exotoxin

1. INTRODUCTION

Three serologically distinct streptococcal pyrogenic exotoxins (SPEs), SPE A, SPE B and SPE C, have so far been reported. Their genes were cloned and their nucleotide sequences have already been defined [1–4]. These toxins have several biological activities such as pyrogenicity, mitogenicity, enhancement of susceptibility to endotoxic shock, suppression of IgM and enhancement of IgG production [5]. Recent studies have shown that these streptococcal pyrogenic exotoxins and certain enterotoxins from *Staphylococcus aureus* comprise a family of superantigens [6–8], defined as bifunctional molecules binding to MHC class II structure and activating T cells expressing appropriate V_β segments of the T cell receptor [9–12].

During the biochemical studies on SPEs, we found a new type of mitogenic factor, termed MF, as previously reported [13]. In the present paper, we cloned the gene encoding MF and characterized it. It is a novel gene and has no homology to known SPE genes. Using the cloned gene, the overexpression of the recombinant MF was achieved and its mitogenic activity and immunoreactivity with the rabbit antiserum to the secreted MF from *S. pyogenes* were determined.

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The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the following accession number D13428.

2. MATERIALS AND METHODS

2.1. *Bacterial strains*

The *S. pyogenes* strain NY-5 kindly provided by T. Nakahara (Saitama College of Health, Urawa, Japan) was used.

2.2. *Gene detection by PCR*

Three oligonucleotide primers for the polymerase chain reaction (PCR) were designed on the basis of the deduced nucleotide sequence from the determined amino acid sequence of the N-terminal portion of MF [13] (Fig. 1A) and were synthesized as follows: primer 1, 5′-tcgaattcCA(A/G)ACNCA(A/G)GT-3′ (16 mixtures of 19-mer corresponding to amino acids 1–4 of MF); primer 2, 5′-caaagcttA(G/A)NGC(T/C)TC(A/G)TT-3′ (32 mixtures of 19-mer for complementary strand corresponding to amino acids 19–22 of MF); primer 3, 5′-tcgaattcAA(T/C)GA(T/C)GTNGT-3′ (16 mixtures of 19-mer corresponding to amino acids 6–9 of MF). The lower-case letters in these primer sequences indicate the sequence of the *tag* with the restriction enzyme recognition sequences, *Eco*RI for primers 1 and 3 and *Hin*dIII for primer 2, respectively. Genomic DNAs from bacteria were prepared according to the modified SDS-phenol method as already described [14]. PCR was carried out for 50 cycles in a 50 μl reaction mixture containing 200 μM dNTPs, 0.1 ng of genomic DNA and 1.25 units AmpliTaq DNA polymerase (Perkin-Elmer Cetus, CA, USA) using a thermal sequencer (Iwaki Glass Co. Ltd., Japan). The primer concentration was 1 μM and the temperature cycles were as follows: 94°C for 30 s, 37°C for 60 s and 72°C for 10 s. The amplified products were fractionated by agarose gel electrophoresis and determined by staining with ethidium bromide (0.5 μg/ml) The DNA fragment was directly sequenced according to the dideoxy chain termination procedure using a Sequenase Ver 2.0 (United States Biochemical, OH, USA).

2.3. *Restriction enzyme mapping by Southern analysis*

Genomic DNA of the *S pyogenes* strain NY-5 was digested with various restriction enzymes and electrophoresed on a 0.8% agarose gel. The DNA in the gel was denatured and reneutralized followed by transfer to a nylon membrane (Hybond N+, Amersham, Bucking-

hamshire, UK). Hybridization was performed in a hybridization solution (6 × SSC, 5 × Denhardt's, 30% (v/v) formamide, 0.5% (w/v) SDS, 0.5 mg/ml heparin) at 42°C for 16 h. A denatured 5'-^{32}P-end- labeled PCR product from the MF gene was used as a probe. Exposure to X-ray film (Eastman Kodak Co., NY, USA) was done with an intensifying screen.

2 4 *Gene cloning and DNA sequencing*

The genomic DNA of *S. pyogenes* was completely digested with both *Pst*I and *Hin*dIII and electrophoresed on a 0 8% agarose gel. The DNA fragments were extracted from the gel containing the MF gene fragment, which was expected to be about 2.1 kb in size, and ligated into a vector pBluescript SK(+) (Stratagene, CA, USA). *E coli* JM109 cells were transformed by the ligation mixture. The clone, which contained the plasmid capable of encoding MF, was identified by screening with the colony hybridization method using the PCR product DNA amplified from the MF gene as a probe. In order to determine the whole sequence of the MF gene, PCR was performed with either the T7 primer or T3 primer, which is located on the T7 or T3 promoter region in the vector, respectively, and with either the primer 4 or 5 (Fig. 1A). PCR with T3 primer and primer 5 or with primer 4 and T7 primer gave the 1.1 kb or 1 2 kb amplified DNA, respectively. These amplified products were directly sequenced by a dsDNA Cycle Sequencing System (BRL, MD, USA).

2 5. *Overexpression and purification of the recombinant MF protein*

The DNA fragment comprising the coding region for the mature MF protein was amplified by PCR with primers 5'-ctggatccCAAACACAGGTCT-3' (21-mer with a *Bam*HI recognition sequence) and 5'-gcgaattcCTAGCCTTTTGGTAT-3' (23-mer with an *Eco*RI recognition sequence). This fragment was ligated with a pGEX-2T expression vector (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) to obtain the plasmid capable of expressing an MF protein as a fusion protein with glutathione S-transferase (GST-MF). *E coli* cells were transformed by the ligation mixture and several transformants were isolated. The transformant was cultured with Terrific broth [15] at 37°C overnight with shaking. The expression of the GST–MF fusion protein was induced with 5 mM IPTG during the last 3 h of the culture. The cells were harvested by centrifugation and were disrupted by sonication. The bacterial lysates were applied to a prepacked-glutathione Sepharose 4B column (Pharmacia LKB Biotechnology, Uppsala, Sweden). After washing the column with a phosphate-buffered saline (PBS), the bound material was eluted with elution buffer (5 mM glutathione in 50 mM Tris-HCl, pH 8.0). The purity of the fusion protein was analyzed on SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE) as described by Laemmli [16] in a 12% acrylamide gel, followed by staining for protein with a Quick CBB or a Silver stain kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The GST–MF fusion protein was cleaved at the junction region by digesting with human thrombin (Sigma Chemical Co , MO, USA) and separated into two molecules of GST and MF

2.6. *Preparation of anti-MF antiserum*

The MF protein was purified from the culture supernatant of the *S pyogenes* strain NY-5 as previously described [13]. The purified protein solution (10 μg/1 ml) was emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, MI, USA) and injected into multiple sites in the skin of a rabbit. Three weeks later, the emulsion of 10 μg of the same MF protein with an equal volume of a mixture of Freund's complete and incomplete adjuvant was intradermally injected. Two more intradermal injections with the emulsion of MF with Freund's incomplete adjuvant were given at 3 week intervals, and 4 weeks after the final injection, the antiserum was obtained.

2.7. *Double gel immunodiffusion and Western blot analyses*

Micro-double gel immunodiffusion analysis was carried out as described by Ouchterlony [17]. Western blot analysis was performed as follows: the fusion protein was electrophoresed on an SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Trans-Blot Transfer Medium; BioRad Laboratories, CA, USA) The membrane was treated with the rabbit anti-MF antiserum at 1:1,000 dilution in a blocking solution (10% Block-Ace in PBS; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) at room temperature for 30 min. A Vectastain ABC Kit (Vector Laboratories Inc., CA, USA) and a Konica Immunostaining-HRP Kit (IS-50B; Konica Co. Ltd., Tokyo, Japan) were employed for detection of the MF protein.

2.8. *Assay for mitogenic activity*

Mitogenic activity in rabbit peripheral blood lymphocytes was assayed as previously described [13]. [methyl-^{3}H]Thymidine (185 GBq/mmol; Amersham, England) was used. Concanavalin A (Seikagaku Kogyo Co. Ltd., Tokyo, Japan) was used as a positive control for the assay.

3. RESULTS

3.1. *Detection and cloning of the MF gene*

As already reported [13], the sequence of the first 21 N-terminal amino acids of MF secreted from *S. pyogenes* has been determined. In addition to the sequence, as shown in Fig. 1A, two additional residues, Leu-Ala corresponding to the 22nd and 23rd amino acids of the N-terminal of MF, were identified by further amino acid sequencing. To isolate the genomic region encoding this N-terminal of MF by PCR, the three degenerate

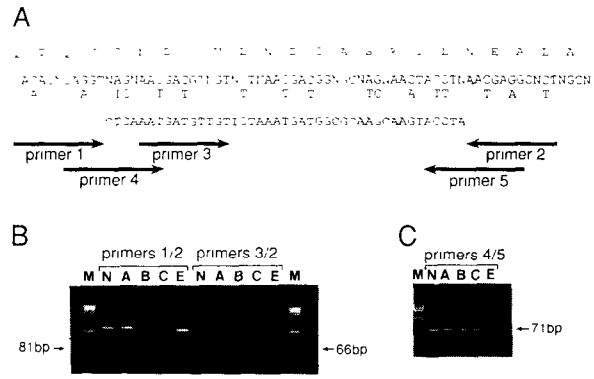

Fig. 1. Determination of the presence of the *S. pyogenes* MF gene by PCR. (A) The N-terminal amino acid sequence of MF, its predicted nucleotide sequence and the location of PCR primers. First row: the defined N-terminal amino acid sequence of the native MF protein secreted from *S pyogenes*. Second row: its predicted nucleotide sequences. Alternative nucleotides are represented by N for the four nucleotides or addition of a nucleotide under the first line. Third row, the determined nucleotide sequence of the DNA fragment amplified from the *S. pyogenes* DNA by PCR with primers 1 and 2. Primers: location of each primer shown by an arrow. Primers 1, 3, and 4 are 5'-primers and primers 2 and 5 are 3'-primers. Fully degenerated primers 1, 2, and 3 contain all sequences enabling one to deduce from each amino acid sequence. The sequence of primers 4 and 5 are described in the text. The sequence of the 3'-terminal 7 or 5 nucleotides of primers 4 or 5, respectively, was designed based on the authentic sequence depicted in the third row. (B, C) Determination of the PCR products by agarose gel electrophoresis. Genomic DNAs from four *S. pyogenes* strains, NY-5 (N) and others (A, B, C), and the *E coli* strain MC1061 (E) were amplified by PCR with primers 1 and 2 (Primers 1/2), 3 and 2 (Primers 3/2) and 4 and 5 (Primers 4/5) and their expected, amplified bands of 81 bp, 66 bp and 71 bp size in length are indicated by arrows, respectively. M: *Hae*III digested ϕX174 DNA size markers.

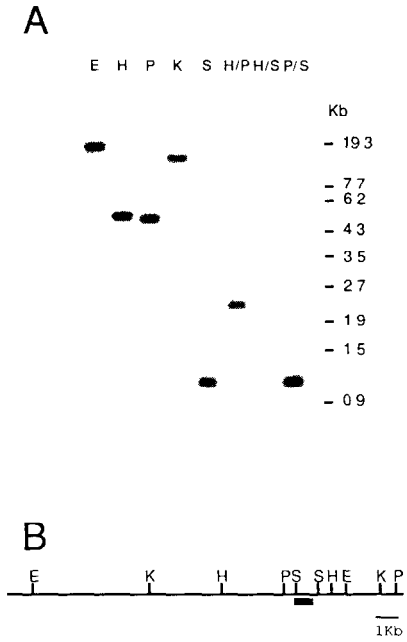

Fig. 2. Southern analysis and the restriction map of the *S pyogenes* DNA fragment bearing the MF gene (A) Southern blot autoradiogram. Genomic DNA extracted from the *S. pyogenes* strain NY-5 was digested singly or doubly with restriction enzymes indicated on the top of each lane. Enzymes *Eco*RI (E), *Kpn*I (K), *Hin*dIII (H), *Pst*I (P), and *Sau*3AI (S) were used. The digested DNAs were fractionated by agarose gel electrophoresis and transferred to a nylon filter, followed by hybridization with the probe of the ^{32}P end-labeled 71 bp MF DNA fragment amplified from the *S. pyogenes* DNA by PCR with primers 4 and 5. The DNA molecular size markers (*Eco*T14I digested lambda phage DNA, Takara Shuzoh Co., Ltd., Kyoto, Japan) are shown at the right. (B) Restriction map of the MF gene. A long horizontal line indicates the genomic DNA involving the region of the MF gene. The location of the MF gene defined by nucleotide sequence analysis is indicated by a thick horizontal bar. Each restriction enzyme site is shown by a vertical bar with an abbreviated symbol for each restriction enzyme as already mentioned. A thin horizontal bar indicates the 1 kb size control.

primers 1, 2, and 3 were designed and synthesized as described in Section 2 and Fig. 1A. PCR with these primers was done with *S. pyogenes* and *E. coli* genomic DNAs as templates (Fig. 1B,C). When used with primers 1 and 2, an expected 81 bp DNA band was amplified from all DNAs of the four *S. pyogenes* strains used but not from the control *E. coli* DNA (Fig. 1B). Additionally, several unexpected DNA bands were also amplified from DNAs of both *S. pyogenes* and *E. coli*, however these bands appeared to be non-specific. When used with primers 3 and 2, an expected 66 bp DNA band was amplified from all DNAs of the four *S. pyogenes* strains but not from the control *E. coli* DNA. Thus, these expected DNA products were directly sequenced. The nucleotide sequence data appeared to show that the sequences of these bands amplified from the four *S. pyogenes* strains were identical and one of three amino acid sequences deduced from its nucleotide sequence was consistent with the defined amino acid sequence of the N-terminal of MF (Fig. 1A). This evidence suggests that these expected PCR products were derived from the authentic MF gene of *S. pyogenes* and supports the fact that *E. coli* does not contain the MF gene. Based on this nucleotide sequence, a new set of primers 4 (5′-tcgaattcCAGGTCTCAAAT-3′, capital letters indicating the nucleotide sequence from the MF gene sequence determined and lower case letters indicating the sequence of the *tag* with the *Eco*RI recognition site) and 5 (5′-ctaagcttCCTCGTTTAGGT-3′, capital letters indicating the nucleotide sequence from the MF gene sequence determined and lower case letters indicating the sequence of the *tag* with the complementary sequence of the *Hin*dIII recognition site) was designed (Fig. 1A) and synthesized. PCR with primers 4 and 5 gave the expected 71 bp DNA band from DNAs of all four *S. pyogenes* strains but not from the control *E. coli* DNA on an agarose gel (Fig. 1C), suggesting that these expected PCR products are derived from the *S. pyogenes* MF gene and again supporting the presence of the MF gene in *S. pyogenes* but not in *E. coli*.

In order to determine the entire MF genomic organization of *S. pyogenes*, Southern analysis with this 71 bp PCR product DNA as a probe was performed. From the results (Fig. 2A), The restriction enzyme map of the genome carrying the MF gene was postulated as shown in Fig. 2B. It shows that the MF gene exists on a 1.1 kb *Sau*3AI–*Sau*3AI DNA fragment and a 2.1 kb *Pst*I–*Hin*dIII fragment includes this *Sau*3AI–*Sau*3AI fragment. Therefore, the 2.1 kb *Pst*I–*Hin*dIII fragment was cloned into the pBluescript vector and its nucleotide sequence was determined by direct-sequencing as described in Section 2. The sequence data (data not shown) indicated that the fragment is 2,099 bp in length and involves one long, open reading frame with 813

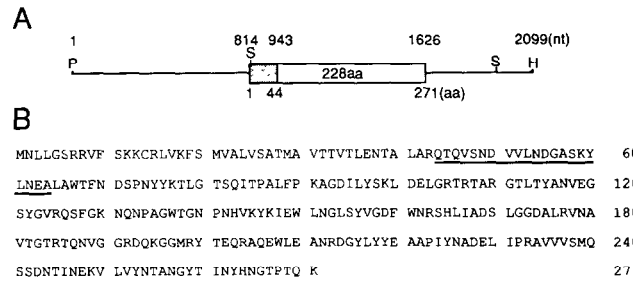

Fig. 3. Genomic structure of the MF gene and the deduced amino acid sequence of MF. (A) Diagram of a *Pst*I–*Hin*dIII fragment containing the open reading frame of MF. The open and shaded boxes represent the regions of the mature MF protein with 228 amino acids and the signal peptide with 43 amino acids, respectively. The numbers of the nucleotide and the amino acid are started from the *pst*I recognition site and the initiation amino acid site, respectively. P, *Pst*I; S, *Sau*3AI; H, *Hin*dIII. (B) Predicted amino acid sequence of the precursor MF protein. The nucleotide sequence data of the MF gene will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases (accession number D13428). The amino acid sequence identical to that of the N-terminal region of the native MF protein secreted from *S. pyogenes* is indicated by underlining.

nucleotides, which has the capacity to encode a polypeptide with 271 amino acids, as shown in Fig. 3A. The identical amino acid sequence to the N-terminal amino acid sequence of the native MF protein secreted from *S. pyogenes* was found in that of this polypeptide from positions 44 to 64 (Fig. 3B), suggesting that the polypeptide is a precursor protein of MF and that removal of the putative 43 residues as a signal peptide results in the mature MF protein with 228 residues. The molecular mass of the mature MF is calculated as 25,363, which is exactly consistent with the previously described value (25,370) for the secreted MF determined by the measurement of ion-spray mass spectra [13]. Its amino acid composition calculated from the deduced amino acid sequence is almost the same as that of MF secreted from *S. pyogenes* (Table I). From these results, we concluded that the clone is from the authentic *S. pyogenes* MF gene.

Comparison of the deduced MF precursor protein sequence with sequences in the GenBank Databases revealed no significant homology with known SPEs, such as SPE A, SPE B, and SPE C, or with other known proteins. This suggests that the MF gene cloned in this study is unique.

Table I

Comparison of amino acid composition between the native and the predicted mature MF amino acid sequences

Amino acid	Predicted value*		Determined value**
	Mol%	Count	Mol%
Gly	9.65	22	10.4
Ala	7.89	18	8.4
Val	6.58	15	6.9
Leu	7.89	18	8.4
Ile	3.51	8	3.7
Ser	5.26	12	4.8
Thr	8.77	20	8.0
Cys	0.00	0	ND
Met	0.88	2	0.0
Asp	5.26	2	
Asn	9.65	22	
Asp/Asn			16.1
Glu	4.39	10	
Gln	4.82	11	
Glu/Gln			9.8
Arg	5.26	12	5.4
Lys	4.39	10	4.8
His	1.32	3	1.6
Phe	1.75	4	2.1
Tyr	7.02	16	5.8
Trp	2.19	5	ND
Pro	3.51	8	3.7

*The values were calculated from the amino acid sequence of the mature formed MF protein deduced from the MF gene cloned.
**The values obtained from the native MF protein secreted from the *S. pyogenes* strain NY-5 as previously described [13].
ND, not determined.

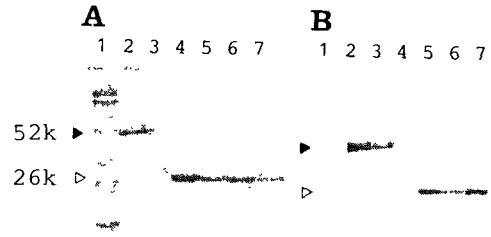

Fig. 4. Characterization of the recombinant MF protein by SDS-PAGE and Western blot analyses. Lane 1, prestained low molecular weight markers (Bio-Rad Laboratories, CA, USA); lane 2, affinity purified GST-MF fusion protein of the clone DB-1 by Glutathione Sepharose 4B column chromatography as described in the text; lane 3, affinity purified GST-MF fusion protein of a clone DJ-6; lane 4, Affinity purified GST protein; lane 5, Thrombin digested GST-MF, which originated from the clone DB-1; lane 6, thrombin-digested GST-MF, which originated from the clone DJ-6; lane 7, the native MF protein purified from the culture supernatant of the *S. pyogenes* strain NY-5 as previously described [13]. Approximately 2 μg protein per lane was applied on lanes 2–7 (A) Coomassie blue staining for proteins, (B) Western blot with the rabbit anti-MF antiserum.

3.2. *Overexpression and characterization of the recombinant MF*

Using the cloned MF gene, the mature MF protein was recombinantly expressed as a fusion protein with GST in *E. coli* as described in Section 2. On SDS-PAGE, the fusion protein and the specimen cleaved by thrombin gave protein bands with molecular masses of 52,000 and 26,000 (Fig. 4A), which correspond to the expected values of the GST-MF fusion protein and the MF or GST protein, respectively. The cleaved GST-MF specimen gave one band, because of the size similarity between the MF and GST proteins. The proteins with the molecular masses of 52,000 and 26,000 reacted with the rabbit anti-MF antiserum on Western blotting, whereas, the GST protein alone did not (Fig. 4B). On the Ouchterlony double gel diffusion test, the cleaved recombinant MF protein corresponding to the mature form and the native MF protein secreted from *S. pyogenes* gave a fused precipitine line against the anti-MF antiserum (Fig. 5). These results indicate that the recombinant MF has the identical antigenicity to the native MF. As shown in Table II, like the native MF, the GST-MF fusion protein specimens showed mitogenic activity in rabbit peripheral blood lymphocytes in a dose-response manner. Moreover, the MF portion cleaved by thrombin but not the GST portion had the mitogenic activity. The recombinant MF protein further purified by ion-exchange chromatography also showed mitogenic activity and the effective dose of the recombinant MF seems to be the same as that of the native MF secreted from *S. pyogenes* (data not shown).

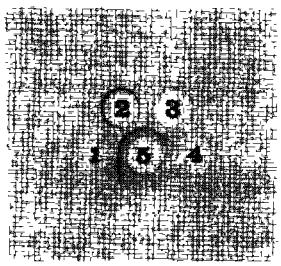

Fig. 5. Comparison of antigenicity of the recombinant MF and the native MF proteins by a micro-double gel immunodiffusion test (Ouchterlony's method). Well 1, thrombin-digested GST–MF fusion protein of the clone DB-1; well 2, the native MF protein purified from the culture supernatant of the *S. pyogenes* strain NY-5; well 3, thrombin digested GST–MF fusion protein of the clone DJ-6; well 4, GST protein alone; well 5, the rabbit anti-MF antiserum.

4. DISCUSSION

We have recently reported the finding of a new type of MF in the culture supernatant of the *S. pyogenes* strain NY-5 and the determination of its N-terminal amino acid sequence [13]. MF shows marked mitogenic activity in rabbit peripheral blood lymphocytes. In this paper, we report the cloning and expression of the *S. pyogenes* MF gene. The nucleotide sequence of the cloned MF gene involves one long, open reading frame. Its deduced amino acid sequence indicates that the MF precursor protein comprises 271 amino acids and removal of the signal sequence with 43 amino acids results in a mature MF protein with 228 amino acids. No cysteine residue is found in the mature MF protein, although one is found in the signal sequence. Neither the nucleotide nor the predicted amino acid sequence of MF shows significant homology with that of SPE A, SPE B or SPE C. No other similar sequence has been reported so far in the GenBank Databases. Using the MF genomic DNA cloned, the mature formed MF protein was recombinantly expressed in *E. coli*. We showed that its recombinant MF has the same mitogenic activity as the native MF secreted from *S. pyogenes* and the identical antigenicity to the native MF. Thus, we concluded that the gene cloned here is from the authentic *S. pyogenes* MF gene and is unique.

It has been reported that the genes for SPE A and SPE C reside on temperate bacteriophages [18,19]. On the other hand, the gene for SPE B is chromosomally encoded [20] and is described as either identical to [21] or related to streptococcal proteinase [3]. The distribution of the genes for SPEs have also been reported by several investigators [22–24]. They described that almost all strains of *S. pyogenes* have the SPE B gene but the genes for SPE A and SPE C were found at a low frequency. Hence, in our investigation, the gene for MF was detected in almost all strains of *S. pyogenes* using our newly developed PCR detection system, as was the SPE B gene (manuscript in preparation). The Southern analysis revealed that the restriction map of the genome bearing the MF gene among the four *S. pyogenes* strains used here was almost the same (data nto shown). These results suggest that the MF gene possibly resides on a chromosome.

McMillian et al. [25] reported the finding of the fourth streptococcal pyrogenic exotoxin, termed SPE D, and described that there is no antigenic cross-reactivity between SPE D and SPE A, SPE B and SPE C. Its molecular mass and its isoelectric point (pI) were reported to be 13,000 and 4.6, respectively. The molecular mass of MF is clearly distinct from that of McMillian's SPE D. The exact pI value of MF has not been examined yet but its amino acid composition postulates that its pI value may be relatively higher than 8.0. These differences suggest that SPE D is not related to MF.

Recently, Itoh et al. [26] also reported the cytoplasmic membrane-associated mitogenic factor produced by *S. pyogenes*. Its molecular mass was reported to be about 10,000–15,000, which is different from that of our MF. As already described, MF is secreted from *S. pyogenes*

Table II

Mitogenic assay of the native and recombinant MF protein specimens

	Final concentration (protein ng/ml)	[^{3}H]Thymidine incorporation (cpm)
Native MF*	2,356	23,367 ± 1,813
	783	23,716 ± 1,979
	236	6,358 ± 1,131
	78 3	2,873 ± 279
PBS		132 ± 0.7
ConA	2,500	10,696 ± 1,765
GST-MF**	13,200	30,853 ± 257
	4,400	9,999 ± 531
	1,470	2,893 ± 345
	489	131 ± 10.7
GST-MF-T***	11,700	42,500 ± 13,602
	3,910	30,157 ± 2,010
	1,300	17,239 ± 1,488
	434	3,509 ± 685
GST-T****	11,800	512 ± 89.1
	3,930	513 ± 110
	1,310	416 ± 73.3
	437	270 ± 31.2
PBS		273 ± 87.8
ConA	2,500	26,278 ± 2,240

*The native MF protein purified from the culture supernatant of the *S pyogenes* strain NY-5.

**The recombinant GST-MF fusion protein purified by Glutathione Sepharose 4B column chromatography as described in the text. GST-MF produced by the *E. coli* clone DB-1 was used. This clone has a single mutation in the coding region resulting in one amino acid change, His^{122} to Arg^{122} (the number being counted from the N-terminal of the mature MF).

***The GST-MF specimens treated with thrombin.

****Thrombin was added in the same concentration (10 units, 3260 NIH units/mg) to the GST protein specimen like the GST-MF specimen was done.

cells but is not membrane-associated. Thus, these data indicate that Itoh's factor is also distinct from MF.

In addition to the mitogenic activity, our preliminary data appeared that both the native and recombinant MF have heat-resistant nuclease activity (unpublished data). Further studies on the biological significance of mitogenic, nuclease, and superantigenic activities in MF remain to be performed.

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