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Immunology

The murine Fc receptor for immunoglobulin: Purification, partial amino acid sequence, and isolation of cDNA clones

(macrophage/cell surface receptor/antigen–antibody receptor)

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ABSTRACT **The murine Fc receptor for IgG (FcγR) was purified to homogeneity by immunoaffinity chromatography from detergent lysates of the macrophage cell line J774. Microsequencing of intact protein yielded a single amino-terminal sequence, which was confirmed and extended to 20 residues by the isolation of an overlapping peptide. The isolation of additional proteolytic fragments obtained by using *Staphylococcus aureus* V8 protease, cyanogen bromide, and lysine C proteinase, facilitated sequence analysis of a total of 119 amino acid residues. Codon usage charts were used to construct oligonucleotide probes based on the amino acid sequences of three nonoverlapping peptides. These probes were used to screen a cDNA library derived from the WEHI-3B myelomonocytic cell line, and a single cDNA clone (pFc24) to which all three probes hybridized was isolated. This clone, containing a 1.02-kilobase cDNA insert, has been characterized by restriction mapping and partial DNA sequencing, and it has been shown to encode the FcγR. The sequence at the 5′ end of the clone contained the coding information for the amino-terminal sequence of the FcγR as well as a putative 13-amino acid signal sequence. The 3′ end of the clone encoded a peptide identified in purified receptor preparations. Thus, the presence of coding information at the 5′ and 3′ ends of this clone suggests that full-length Fc receptor cDNA spans >1 kilobase.**

Receptors for the Fc portion of immunoglobulins (FcR) enable all immunoglobulin classes to bind to various target cells. Unique FcR for different subclasses of immunoglobulins have been found on B lymphocytes and some T cells and on macrophages, neutrophils, mast cells, basophils, and eosinophils (extensively reviewed in ref. 1). In addition, the polymeric immunoglobulin receptor for which cDNA clones have been isolated (2) is detected on hepatocytes (3) and plays a role in the biliary transport of polymeric IgA and IgM. FcR of macrophages and neutrophils take part in phagocytosis and in IgE-dependent cytotoxicity directed against parasites; FcR for IgE (FcεR) on eosinophils also mediate the latter reaction. The FcεR on mast cells and basophils plays a role in the release of chemical mediators. In contrast to FcR of myeloid cells, the function of FcR of lymphocytes has not been fully established. They clearly participate in antibody-dependent cell-mediated cytotoxicity (e.g., on K cells) but also appear to be important in the regulation of the humoral immune response. T lymphocytes (4) and other cells (5, 6) have been shown to release immunoglobulin binding factors (Ig-BF) that are involved in isotype-specific regulation of immunoglobulin synthesis. The Ig-BF bind to the Fc portion of immunoglobulin and show isotype specificity of IgE (7) [cDNA clones encoding rat IgE-BF have been isolated (8–10)], IgA (11), and IgG1 and IgG2 (12). They are thought to be related to FcR, and Daëron and coworkers have recently demonstrated that suppressive IgG binding factors share an epitope with IgG FcR (FcγR) of macrophages, neutrophils, and T and B cells (13).

A rat monoclonal antibody to the murine FcγR has facilitated purification of the macrophage FcγR, which is a heavily glycosylated acidic molecule of 47–70 kDa (14, 15). More recently, it has been shown that the Ly-17 alloantigens are polymorphic forms of the murine FcγR (16, 17) and that the *FcR-1* gene maps to chromosome 1 in the mouse.

Thus, serological and biochemical studies as well as analysis of FcR function indicate that there are many different types of FcR. Since all immunoglobulin classes show structural and sequence homology, it is tempting to postulate that all Fc receptors are also related. To further analyze the relationships between the different FcR, we have obtained partial amino acid and nucleotide sequence of the murine macrophage FcR for IgG.

MATERIALS AND METHODS

Monoclonal Antibodies. The monoclonal anti-Ly-17.2 and Ly-2.1 antibodies have been described (16, 18). $F(ab')_2$ fragments from purified antibody were prepared as described (16).

Purification of FcγR. J774 macrophage cells (2×10^{10}) were harvested from ascites fluid obtained from (CBA × BALB/c)F_1 mice and immediately lysed in cold phosphate-buffered saline (PBS)/0.5% Nonidet P-40 (NP40), pH 7.4, containing 1% aprotinin (Sigma) and 1 mM phenylmethylsulfonyl fluoride (Sigma). An additional 2×10^7 cells were surface-labeled with ^{125}I and pooled with the cold lysate. After lysis for 1 hr at 4°C, the lysates were clarified and incubated for 1 hr at 4°C with anti-Ly-17.2 antibody-conjugated Sepharose 4B (Pharmacia). The immunoabsorbent was washed three times in 0.6 M NaCl/0.0125 M KH_2PO_4, pH 7.4, three times in PBS/0.5% NP40, pH 7.4, and three times in 0.5% deoxycholate/100 mM Tris·HCl, pH 8.0. The immunoabsorbent was packed into a column and the bound material was eluted with 0.5% $NaDodSO_4$/0.1 M triethylamine, pH 11.5, and freeze-dried.

The purity of the FcγR preparations was assessed by $NaDodSO_4$/PAGE (19), and proteins were detected by Coomassie blue staining or by autoradiography.

Protein Sequence Analysis. Protein samples were carboxamidomethylated and ethanol-precipitated prior to sequencing. Briefly, samples were dissolved in 50 mM boric acid/0.1% $NaDodSO_4$/10 mM dithiothreitol, pH 8.0 (NaOH), and heated to 60°C for 1 hr. Iodoacetamide was added to a final concentration of 22 mM and the samples were incubated

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Abbreviations: FcR, receptor(s) for the Fc portion of immunoglobulin; FcγR and FcεR, FcR for γ and ε isotypes; Ig-BF, immunoglobulin binding factors; kb, kilobase(s).

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for 15 min at ambient temperature in the dark. Ice-cold ethanol containing 50 mM HCl was added and the protein was allowed to precipitate at −20°C for 2 hr. The precipitate was collected by centrifugation, dissolved in CF_3COOH, and sequenced by automatic Edman degradation using an Applied Biosystems 470A Sequencer (Foster City, CA) (20, 21). Sequencing and phenylthiohydantoin-derivatized amino acid identification techniques have been described in detail (22).

Preparation of FcγR Peptides. FcγR peptides were obtained by digesting samples of affinity-purified, carboxamidomethylated FcγR with either *Staphylococcus aureus* V8 protease (Miles) (23), lysine C proteinase (according to manufacturer's instructions, Boehringer Mannheim), or cyanogen bromide (CNBr) (24). Peptides were purified by reverse-phase chromatography using a Pharmacia fast protein liquid chromatography (FPLC). The proteinase-digested material was applied to a Pep RPC HR5/5 (C2/C18) reverse-phase column (Pharmacia) while a Pro RPC HR 5/10 (C1/C8) reverse-phase column (Pharmacia) was used to purify CNBr peptides. Peptides were eluted with ascending linear acetonitrile gradients using 20 mM ammonium formate as a buffer and an absorbance trace at 214 nm to detect peptide peaks. Selected peak fractions were then rechromatographed on the same column, again using linear gradients of acetonitrile but in this case containing 0.1% unbuffered CF_3COOH to promote ion suppression.

Preparation of Oligonucleotide Probes. Three oligodeoxynucleotide probes were synthesized by the phosphoramidite method (25) using an Applied Biosystems 380A DNA synthesizer. The oligonucleotide probes were purified from crude mixtures by reverse-phase HPLC and were radiolabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase to 2×10^8 dpm/μg (26).

Isolation and Characterization of cDNA Clones for Macrophage FcγR. A cDNA library (kindly supplied by N. Gough and A. Dunn, Ludwig Institute for Cancer Research, Melbourne, Australia) was constructed in the pJL3 vector (27). Oligo(dT)-primed mRNA derived from the myelomonocytic cell line WEHI-3B, was used to synthesize double-stranded cDNA. The cDNA (>500 base pairs) was tailed with dC and ligated into *Sac* I-cut pJL3, which was tailed with dG. The cDNA inserts cloned in this way are flanked by two *Eco*RI sites allowing excision of the insert from the vector by digestion with *Eco*RI. The library was screened for the presence of FcR sequences using a mixture of the three labeled oligonucleotide probes. Bacterial colonies were plated onto L-ampicillin plates, lifted onto nylon filters (Pall, Glen Cove, NY), and amplified overnight on L-chloramphenicol agar. Filters were treated with 1.5 M NaCl/0.5 M NaOH for 5 min, neutralized for 5 min with 0.5 M Tris/2.0 M NaCl, pH 8.0, then baked at 80°C for 90 min in a vacuum oven. Colony debris was removed by washing filters for 6 hr at 65°C in 3× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/15 mM sodium citrate) containing 0.15% $NaDodSO_4$ and 1 mM EDTA. After 2 hr of prehybridization in 5× NaCl/Cit, 0.1% $NaDodSO_4$, 0.125% nonfat dry milk, 20 mM phosphate buffer (pH 6.8), and 20% formamide at 35°C, filters were hybridized with 50 ng of labeled probe for 15 hr at 35°C. Filters were washed for 1 hr at ambient temperature in 2× NaCl/Cit, 0.1% $NaDodSO_4$, 0.125% nonfat dry milk, followed by 1 hr at 35°C in 1× NaCl/Cit, 0.1% $NaDodSO_4$, dried, then autoradiographed. After three rounds of screening, one positive clone (pFc24) was chosen for characterization. Plasmid DNA from pFc24 was isolated by alkaline lysis (26) and purified on cesium chloride gradients (28).

Southern Hybridization. *Eco*RI-digested plasmid DNA was electrophoresed in 1% agarose and transferred to nitrocellulose membranes (Schleicher & Schüll) as described (26). Hybridization of [γ-^{32}P]oligodeoxynucleotides was performed under the same conditions used for colony hybridization.

DNA Sequencing. cDNA from pFc24 was sequenced by the base-specific cleavage method (29, 30). Sequence obtained by this method was confirmed by the dideoxynucleotide chain-termination method (31) after subcloning cDNA from pFc24 into M13 mp8 and M13 mp9.

RESULTS AND DISCUSSION

Purification and Amino-Terminal Sequencing of Murine FcγR. The FcγR immunoprecipitated from ^{125}I-labeled J774 cells shows a characteristic pattern on $NaDodSO_4$/PAGE—a heterogeneous collection of molecules ranging in size from 47 to 70 kDa (Fig. 1*A*), which has also been identified by others (15, 32). The BALB/c macrophage cell line J774 was chosen as the cellular source from which to purify the FcγR as it has $>5 \times 10^5$ receptor sites per cell (15). An established immunoaffinity chromatography method was used for the FcγR purification (15). However, the anti-Ly-17.2 antibody, which has been shown to define polymorphic forms of the FcγR [based on coprecipitation, two-dimensional gel electrophoresis, and peptide mapping (16)], was used instead of the 2.4G2 antibody, which recognizes an invariant epitope on the FcγR molecule (14). The affinity-purified FcγR eluted from the anti-Ly-17.2 column (Fig. 1*B*) showed an identical pattern on $NaDodSO_4$/PAGE to the FcγR immunoprecipi-

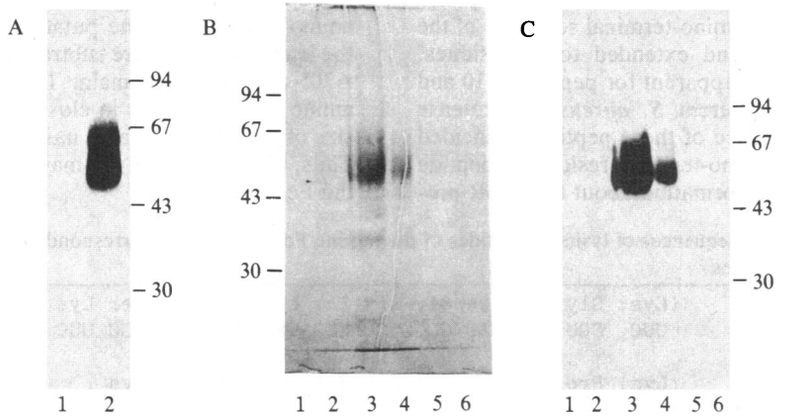

FIG. 1. One-dimensional $NaDodSO_4$/PAGE analysis of the IgG FcR of J774 macrophage cells. Sizes are given in kDa. (*A*) Immunoprecipitation of FcγR from surface-iodinated J774 cells. Immunoprecipitations were performed with anti-Ly-2.1 F(ab')$_2$ (lane 1) or with anti-Ly-17.2 F(ab')$_2$ (lane 2). (*B*) $NaDodSO_4$/PAGE and Coomassie blue-staining of a typical preparation of affinity-purified FcR used for protein sequencing and peptide generation. Lanes 1–6, successive fractions of eluted material from the affinity column. (*C*) Autoradiogram of $NaDodSO_4$/PAGE of affinity-purified ^{125}I-labeled FcR. Lanes 1–6, same as for *B*.

Table 1. The NH_2-terminal sequence of the Fc receptor and amino acid sequences of Fc receptor peptides

Peptide	Sequence
NH_2-terminal	THDLPKAVVKLEPP
L3	KGSLGRTLHQSK
L4	KPVTITVQGPK
L5	KSVRY(G)(G)YSS(S)FCIPK
L9	KAVVKLEPPWIQVLK
V4	ELSTTGGNSG(S)P(V)(K)N
V8	EQTRLSDPVDLGVI
V10	ENTITYSLLKHPE
V11	EAENTITYSLLKHPE
V16	THDLPKAVVKLEP--IQV
V17	THDLPKAVVKLEPPWIQV
CNBr1	MRNKHLNRIVFL(Q/T)N(Y)(K)

Except for the NH_2-terminal, V16, and V17 sequences, the first residue in each peptide sequence is assumed from the known cleavage site of the lysine C (L) and *S. aureus* V8 (E) proteolytic enzymes and CNBr (M). The residues in parenthesis are uncertain assignments and (-) indicates no residue assignment was possible. Amino acids are identified by the single-letter code.

tated from surface-labeled J774 cells (Fig. 1*A*). In addition, trace amounts of material of smaller size were present and probably represent purification of the unglycosylated FcR (37 kDa) and partially glycosylated forms of the FcR (32). An autoradiogram of this gel containing the purified FcγR and a trace of surface-labeled FcγR (Fig. 1*C*) revealed one single-labeled species that completely overlaps the Coomassie blue-stained protein. It is clear, therefore, that the mature form of the FcR was the major species present in the preparation.

Protein Sequencing. The FcγR preparation was subjected to sequence analysis without further purification. A single major amino-terminal sequence of 14 residues was obtained and is shown in Table 1. FcγR peptides were generated by digestion of purified FcγR with lysine C proteinase, *S. aureus* V8 protease, and CNBr. Digests were purified by reverse-phase chromatography using FPLC. Four lysine C peptides, six *S. aureus* V8 peptides, and one CNBr peptide were obtained and sequenced. The amino acid sequences of these peptides is shown in Table 1. Depending on the nature of the digestion, all peptide sequences have an additional residue at position one [either Lys (K), Glu (E), or Met (M), inferred from the specificity of the cleavage reaction], with the exception of V16 and V17, which are the amino-terminal sequences. Several points can be made about the amino acid sequences. First, with the isolation of the overlapping peptides L9, V16, and V17, the amino-terminal sequence of the FcγR has been confirmed and extended to 20 residues. Second, sequence overlap is apparent for peptides V10 and V11, both isolated from different *S. aureus* V8 protease digests of FcγR. The sequence of these peptides coincided exactly except for the two amino-terminal residues of peptide V11. Additional sequence information about the FcγR protein was obtained by cleavage with CNBr. On FPLC purification of the CNBr digest, a major radioactive peak (CNBr1) eluted at 80% CH_3CN. Prior to sequencing, a fraction of this peak was examined by $NaDodSO_4$/PAGE followed by silver staining (33). This peak corresponded to a large glycosylated peptide of 30 kDa (data not shown). However, only enough material was available to sequence 15 residues of the peptide.

Isolation of cDNA Clones. Based on the amino acid sequence of three FcγR peptides (L3, L4, and L5), oligonucleotide probes, which were complementary to the mRNA, were chemically synthesized (Table 2). Rather than constructing a mixture of probes to the amino acid sequence that contains all possible nucleotide sequences, a single probe was constructed for each peptide on the basis of codon usage frequencies. The library was initially screened with a mixture of the three probes and 82 positive clones were selected, plated in triplicate, and probed separately with each of the probes. After a further two screenings, one clone that hybridized strongly to all three probes was chosen for further characterization. Plasmid DNA (pFc24) was isolated from this clone, digested with *Eco*RI to release the cDNA insert, and electrophoresed in a 1% agarose gel. The cDNA insert was estimated to be 1.00 kilobase (kb) (Fig. 2). Southern hybridization experiments of *Eco*RI-digested pFc24 confirmed that all three oligonucleotide probes hybridized to the cDNA insert and not to the vector DNA, pJL3, which is 5.49 kb (Fig. 2). Probe 1 hybridized most strongly to the cDNA insert of pFc24 (Fig. 2, lane B), whereas probe 3 gave the weakest reaction (Fig. 2, lane F).

Nucleotide Sequencing. Nucleotide sequence of the 5′ and 3′ ends of pFc24 are shown in Table 3. The 5′ end of pFc24 contained the coding information for the first 16 amino-terminal residues of the FcγR, identified from amino acid sequencing of the NH_2 terminus and peptides L9, V16, and V17 (Table 1). The NH_2-terminal amino acid sequence was preceded by a stretch of 13 residues beginning with a methionine. The majority of the amino acids in this stretch had hydrophobic side chains characteristic of the signal sequence that precedes the first amino acid residues of membrane proteins (34). It is probable that these 13 amino acids make up the signal peptide of the FcγR. Because of the problems encountered in sequencing over G tails by the dideoxy-sequencing method, the Maxam–Gilbert chemical cleavage method (29, 30) was used to sequence the 3′ end of pFc24 (Table 3). This nucleotide sequence encoded peptides V10 and V11 (Tables 1 and 3), and no poly(A) region was evident in this clone. Since the cDNA insert of pFc24 is ≈1000 base pairs long, its coding capacity should be ≈330 amino acids. When the putative 5′ untranslated region and the leader sequence are subtracted from the coding capacity, ≈305 amino acids remain. The predicted size for this 305 amino acid peptide is in close agreement with the reported size of the unglycosylated nascent FcγR chain (37 kDa) (32). Thus, pFc24 contains the majority of coding information for the FcγR mRNA.

Table 2. Amino acid sequences of lysine C peptides of the murine Fc receptor and corresponding nucleotide sequence of oligonucleotide probes

L3	(Lys)	Gly	Ser	Leu	Gly	Arg	Thr	Leu	His	Gln	Ser	Lys				
Probe 1 3′	UUC	CCU	AGG	GAC	CCU	UCU	UGG	GAC	GUG	GUC	AGG	UUC	5′			
L4	(Lys)	Pro	Val	Thr	Ile	Thr	Val	Gln	Gly	Pro	Lys					
Probe 3 3′	UUC	GGU	CAC	UGG	UAG	UGG	CAC	CUC	CUU	GGU	UUC	5′				
L5	(Lys)	Ser	Val	Arg	Tyr	Gly	Gly	Tyr	Ser	Ser	Ser	Phe	Cys	Ile	Pro	Lys
Probe 2 3′	UUC	AGG	CAC	UCU	AUG	CCU	CCU	AUG	AGG	AGG	AGG	AAG	ACG	UAG	GGU	UUC 5′

Oligonucleotide probes were constructed on the basis of codon usage frequencies and were synthesized to be complementary to mRNA.

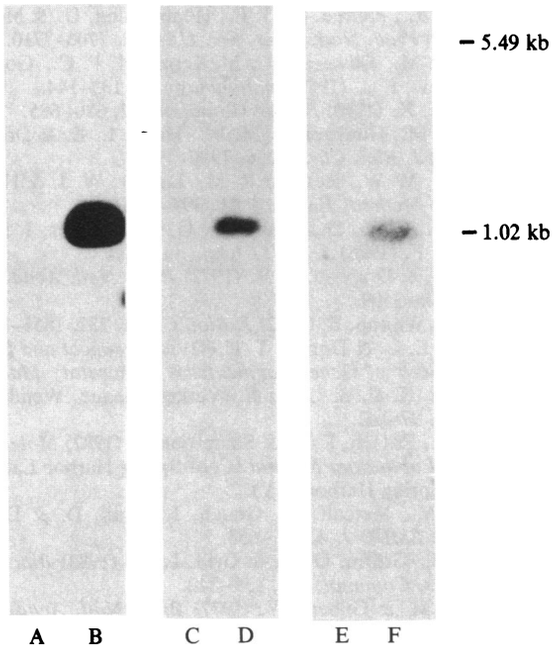

FIG. 2. Southern blot analysis of pFc24 and pJL3. One microgram of pFc24 (lanes B, D, and F) or pJL3 (lanes A, C, and E) was digested with *Eco*RI and electrophoresed in a 1% agarose gel. The DNA was transferred onto nitrocellulose and hybridized with ^{32}P-labeled probe 1 (lanes A and B), ^{32}P-labeled probe 2 (lanes C and D), and ^{32}P-labeled probe 3 (lanes E and F). *Hin*fI fragments of pBR322 DNA were used as markers to estimate the size of the cDNA insert of pFc24 (shown in kb). The relative position of pJL3 is also indicated (5.49 kb).

Homologies with Other Sequences. A computer-assisted search for homologies between FcγR peptides (Table 1) and sequences present in the protein identification resource data base was conducted. These homology searches included examination of a number of immunoglobulins [the concept that receptors for proteins are homologous with their ligands has previously been reported for the polymeric immunoglobulin receptor (2)] and the immunoglobulin binding proteins, the rabbit polymeric immunoglobulin receptor, and rat IgE-BF. Both mutation data matrix and unitary matrix scoring systems were used. Four points were apparent from these comparisons. (*i*) The highest level of sequence homology observed between any FcγR peptide and its closest homologue in the data base was below that required for statistical significance. In part this may reflect the relatively short stretches of peptide sequences available for this comparison. (*ii*) The consistent appearance in different peptide comparisons of sequence segments from the same protein would have been taken as tentative evidence for authentic homology. However, among the sequences listed as FcγR homologues, no one protein sequence appeared more than once. (*iii*) No compelling evidence for homology between an immunoglobulin element and the FcγR was obtained from the computer searches. Several immunoglobulin segments were indeed listed for three of the FcγR peptides (L3, V8, and V10), but the levels of homology observed were not statistically significant. In addition, many totally unrelated protein sequences from a wide variety of species gave the same degree of homology as immunoglobulin segments, which would also argue that these results were not significant. In summary, the FcγR seems unlikely to bear such close homology to immunoglobulin elements as β_2-microglobulin (35), Thy-1 (36), Ly-2 antigens (37), or the rabbit polymeric immunoglobulin receptor (2). Further homology studies with extended cDNA sequences should reveal a relationship between immunoglobulin and the FcγR, if it exists. (*iv*) FcγR is not a fragment or a genetic derivative of the polymeric immunoglobulin receptor or IgE-BF. Thus, the three immunoglobulin binding proteins are structurally quite distinct and may have acquired their respective immunoglobulin binding capacities independently during evolution.

The FcR nucleotide sequences (Table 3) were also compared with the entire data bank. With the limited amount of sequence available, no statistically significant homology was observed.

A significant proportion of the FcR cDNA sequence has still to be determined and six peptide sequences, including those used to make the oligodeoxynucleotide probes, have still to be located. The lack of a poly(A) tail and the presence of a sequence at the very 3′ end of pFc24, which encodes the FcγR peptide V10, indicates that extra coding sequence and the 3′ untranslated region containing the polyadenylylation signal site are missing from pFc24. The availability of cDNA clones encoding the murine FcR will now rapidly lead to its complete primary structure and the appropriate DNA and RNA studies should considerably extend the knowledge of FcR molecules.

Table 3. Nucleotide and predicted amino acid sequence of pFc24

```
A  5′ end                                          −13
                                                   MET leu leu trp thr ala val
 AACTGGACTGTCCATGTGTTCTTCTCACGGACTTTGTGCCAT ATG CTA CTG TGG ACA GCC GTG

                      −1   1                                    10
leu asn leu ala ala gly THR HIS ASP LEU PRO LYS ALA VAL VAL LYS LEU GLU
CTA AAT CTT GCT GCT GGG ACT CAT GAT CTT CCA AAG GCT GTG GTC AAA CTC GAG

PRO PRO TRP ILE
CCC CCG TGG ATC

B  3′ end
        GLU ALA GLU ASN THR ILE THR TYR SER LEU LEU LYS HIS PRO GLU ALA
        GAG GCT GAG AAT ACG ATC ACC TAC TCA CTC CTC AAG CAT CCC GAA GCC

LEU ASP GLU GLU THR GLU HIS
TTG GAT GAA GAA ACA GAG CAC
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Nucleotide sequence of the 5′ and 3′ ends of pFC24 were determined by the Maxam–Gilbert and dideoxy sequencing methods. The putative signal sequence is shown in *A* between amino acid residues −13 and −1 in lowercase letters. The italicized nucleotides in *A* encode the NH_2 amino acids as well as peptides L9, V16, and V17, while those in *B* encode peptides V10 and V11 (see Table 1).

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