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Analysis of Genes Encoding Two Unique Type IIa Immunoglobulin G-Binding Proteins Expressed by a Single Group A Streptococcal Isolate

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An *emm*-like gene (*emmL*) and a *fcrA* gene from group A streptococcal strain 64/14 ($emmL_{64/14}$ and $fcrA_{64/14}$) were amplified by PCR and force cloned into the heat-inducible expression vector pJLA 602. The *emmL* gene encoded a recombinant protein that bound human IgG1, IgG2, and IgG4 in a nonimmune fashion. This is the reactivity profile of a type IIa IgG-binding protein. The $emmL_{64/14}$ gene product was antigenically similar to the previously identified high-molecular-weight type IIa IgG-binding protein of strain 64/14 and had an N-terminal sequence identical to that of the wild-type protein. The *fcrA* gene also encoded a recombinant protein with type IIa functional activity. This protein was similar to the lower-molecular-weight type IIa IgG-binding protein previously isolated from strain 64/14 and was antigenically distinct from the higher-molecular-weight type IIa protein encoded by the $emmL_{64/14}$ gene. The sequences for both genes including the intervening regions are presented. The *emmL* gene demonstrates significant homology to other class I *emm* and *emmL* genes expressed by opacity factor-negative group A streptococcal isolates. The *fcrA* gene was found to be homologous to other *fcrA* genes normally present in opacity factor-positive group A isolates. The sequence upstream of the *fcrA* gene and the intervening sequence between the end of the *fcrA* gene and the start of the *emmL* gene were similar to those reported for other *fcrA* genes.

Analysis of expression of immunoglobulin G (IgG)-binding proteins by group A streptococci has suggested that they may play a critical role in establishing invasive infections in the skin (1, 33). Genetic studies of group A streptococcal IgG-binding protein genes have shown them to display significant homology to other genes associated with the M protein super gene family (14, 18, 19, 45). The genes of both groups are located on adjacent segments of the chromosome and form part of a coordinately regulated *vir* locus (8, 9, 16, 28, 29, 39, 43, 44). A detailed understanding of the role of IgG-binding proteins in streptococcal pathogenesis has been complicated by the heterogeneity of these molecules and the potential for change in expression during laboratory subculture (7, 35). The type II IgG-binding proteins associated with group A streptococci represent the most diverse group of IgG-binding molecules within a single family of bacteria. These molecules have been classified according to their nonimmune functional reactivities with human IgG subclasses and other species of IgG (Table 1). In order to determine the biological implications of expression of distinct forms of these molecules on group A streptococcal pathogenesis, a more detailed characterization of these molecules at the gene level is required.

Antigenic and functional analyses of IgG-binding proteins expressed by a number of fresh clinical group A isolates demonstrate the existence of two major antigenic classes of IgG-binding proteins based on their reactivity with the selected antibody probes anti-IIo and anti-pLOH (36). Each antigenic class contained IgG-binding molecules that differed in their functional IgG-binding profiles (6, 36), and thus a simple relationship between antigenic structure and functional activity has not been established. In general, IgG-binding proteins encoded by *fcrA* genes have been recognized by the anti-pLOH antibody probe and are usually associated with opacity factor-positive (OF^+) group A isolates (6). The anti-IIo antibody probe does not demonstrate significant reactivity with these proteins but recognizes a distinct family of functionally heterogeneous IgG-binding molecules that are usually expressed by OF^- group A isolates. A small number of clinical and laboratory isolates of group A streptococci express two or more antigenically distinct IgG-binding molecules (36).

Recent studies in our laboratory have demonstrated that a group A isolate, strain 64/14, passaged in mice was unusual in that it expressed three distinct IgG-binding proteins (27). Two of these proteins displayed identical profiles of reactivity with human IgG1, IgG2, and IgG4 as well as reactivity with horse, pig, and rabbit immunoglobulins; however, these activities were mediated by antigenically distinct proteins. One of these proteins reacted with an anti-IIo antibody probe, while the other reacted with the anti-pLOH antibody probe (6, 27). The third IgG-binding protein reacted exclusively with human IgG3 and failed to react with either of the antibody probes (36).

In order to explore the structure-function relationship of the two antigenically distinct IgG1-, IgG2-, and IgG4-reactive or type IIa IgG-binding proteins expressed by strain 64/14, an attempt was made to clone the genes encoding each protein from a λgt11 chromosomal library of this strain. The gene encoding the lower-molecular-weight type IIa-binding protein was identified and successfully cloned into pUC18 and expressed in *Escherichia coli* (27). This gene, encoded within plasmid pLOH, was found to be similar to another IgG-binding protein gene, the *fcrA* gene of streptococcal group A isolate CS110, previously cloned and sequenced by Heath and Cleary (18, 19). The recombinant protein expressed by pLOH was functionally and antigenically indistinguishable from the lower-molecular-weight ~32,000- to 35,000-M_r wild-type type IIa protein doublet present in CNBr extracts of strain 64/14 (27).

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TABLE 1. IgG-binding profile of different type II Ig-binding proteins[a]

Functional designation	Human IgG1	Human IgG2	Human IgG3	Human IgG4	Rabbit IgG	Pig IgG	Horse IgG
IIo	✓	✓	✓	✓	✓	✓	✓
II'o	✓	✓	✓	✓	✓		
IIa	✓	✓		✓	✓	✓	✓
IIb			✓				
IIc	✓			✓	✓	✓	✓

[a] Adapted from previously presented data based on the reactivity of heat extracts (35) and CNBr extracts (36) of group A streptococci.

Extensive screening of the λgt11 library of strain 64/14, however, failed to identify a phage expressing the higher-molecular-weight type IIa protein (27).

Other studies carried out in our laboratory have documented an increase in expression of the high-molecular-weight type IIa IgG-binding protein following passage in mice (33, 37) or rotation in human blood (34). This pattern of enhanced expression of a group A streptococcal surface protein following biological pressures is characteristic of that reported for group A streptococcal M proteins (12). Evidence that certain class I M proteins reactive with the anti-IIo antibody probe, e.g., M1 or M12, can also be IgG-binding molecules has also been reported (6, 39, 43). Strain 64/14 has proved to be M protein untypeable by using conventional serological assays. Furthermore, a number of previous studies have indicated that M protein-like sequences can be toxic in *E. coli* (24) and could potentially explain our earlier failure to identify a clone encoding an IgG-binding M-like protein in chromosomal libraries of strain 64/14. Taking all of this circumstantial evidence together, we speculated that the *emmL* gene of strain 64/14 ($emmL_{64/14}$) might encode the high-molecular-weight type IIa IgG-binding protein. To test this possibility, a PCR-based strategy developed by Podbielski and colleagues (29–31) to amplify and force clone M-like protein genes and *fcrA* genes from chromosomal DNA of group A streptococci has been used with strain 64/14. The results of these studies, presented herein, indicate that the M-like protein gene $emmL_{64/14}$ encodes the high-molecular-weight type IIa IgG-binding protein recognized by the anti-IIo antibody probe. As predicted, the *fcrA* gene product is indistinguishable from the pLOH gene product previously cloned from a chromosomal library of strain 64/14 and encodes the lower-molecular-weight type IIa IgG-binding protein recognized by the anti-pLOH antibody probe. These studies demonstrate that PCR amplification of M protein-related genes of group A streptococci is an efficient method to analyze these proteins for immunoglobulin-binding potential.

MATERIALS AND METHODS

Bacteria. Group A streptococcal strain 64/14 is an M protein-untypeable OF^- isolate that was passaged in mice 14 times, as described previously (37). This strain was grown overnight at 37°C as stationary cultures in Todd-Hewitt broth. Approximately 2 g (wet weight) of bacteria per liter was recovered.

Immunoglobulin. Human IgG was a gift from the Sandoz Corporation (East Hanover, New Jersey). Human IgG myeloma proteins of each subclass were obtained from the immunoglobulin committee of the World Health Organization-International Union of Immunologic Societies.

Labeling of proteins. Proteins were radiolabeled with ^{125}I (Amersham Corp., Chicago, Ill.) by the lactoperoxidase method with enzymobeads (Bio-Rad, Richmond, Calif.). A PD-10 desalting column (Pharmacia Fine Chemicals, Piscataway, N.J.) was used to separate labeled proteins from free iodine. The specific activity of all radiolabeled proteins was approximately 0.3 mCi/mg.

CNBr extraction of type II IgG-binding proteins. Group A streptococcal strain 64/14 was grown overnight at 37°C in Todd-Hewitt broth and treated with CNBr under conditions described previously (27). Briefly, approximately 2 g (wet weight) of washed bacteria was resuspended to a final volume of 5 ml in phosphate-buffered saline (PBS). An equal volume of a CNBr solution (30 mg/ml in 0.2 M HCl) was then added to the bacterial suspension, and the mixture was rotated for 8 to 15 h at ambient temperature. The reaction mixture was then centrifuged at 10,000 × *g* for 15 min, and the resulting supernatant was filtered through a 0.2-μm-pore-size filter to remove any remaining bacteria. The filtrate was dialyzed against four or five changes of 0.1 M HCl, and the CNBr-free extracts were then neutralized by the addition of 1.5 M Tris-HCl (pH 8.8).

Generation of anti-type II antibodies in chickens. Monospecific antibodies were prepared in chickens by using affinity-purified type II IgG-binding proteins as immunogens, following the protocol described previously (36). Essentially, each chicken was injected intramuscularly or subcutaneously with an immunogen containing approximately 50 μg of affinity-purified type II protein emulsified in complete Freund's adjuvant. Three weeks later, the chickens were injected with approximately 50 μg of the same immunogen emulsified in incomplete Freund's adjuvant. Eggs were collected from the chickens, and immunoglobulins were extracted from egg yolks as described previously (38). The immunogen used to prepare the anti-type IIo antibody was an ~47,000-M_r protein band cut from a sodium dodecyl sulfate (SDS)-polyacrylamide gel of a heat extract of isolate A928 (34). The immunogen used to prepare the anti-pLOH antibody was an ~32,000-M_r protein band cut from an SDS-polyacrylamide gel of a sonicate of *E. coli* containing pLOH (27). This band contained the recombinant 64/14 FcrA protein (27).

The production of antibody was monitored by the ability of yolk extracts to inhibit binding of ^{125}I-labeled affinity-purified type II protein to immobilized human IgG following the procedure described previously (38). For preparation of ^{125}I-labeled specific antibody probe, the anti-type II antibody preparations were first affinity purified by binding to and selective elution from a column of the corresponding immobilized type II IgG-binding protein immunogen.

Polyacrylamide gel electrophoresis and Western blotting (immunoblotting) techniques. Protein samples were denatured by being boiled for 5 min in 0.5 M Tris-HCl (pH 6.8) containing 2% SDS (wt/vol), 5% β-mercaptoethanol (vol/vol), 10% (vol/vol) glycerol, 0.01% (wt/vol) bromphenol blue. Denatured proteins were electrophoresed on 12% polyacrylamide slab gels at 50 V for 16 h according to the method of Laemmli (23). Prestained molecular weight (indicated in parentheses) standards (Bio-Rad) containing phosphorylase *b* (106,000), bovine serum albumin (80,000), ovalbumin (49,500), carbonic anhydrase (32,500), soybean trypsin inhibitor (27,500), and lysozyme (18,500) were included in each SDS-polyacrylamide gel assay.

The proteins were electrophoretically transferred to nitrocellulose (Bio-Rad) by a modification of the method described by Towbin et al. (46). Briefly, SDS slab gels were presoaked for 30 min in 25 mM Tris–192 mM glycine–20% methanol (pH 8.3), assembled into a high-intensity-field transblot system

TABLE 2. List of oligonucleotides for amplification and sequencing of $fcrA_{64/14}$ and $emm_{64/14}$

Oligonucleotide function and designation[a]	Sequence (5′ to 3′)	Nucleotide position[b]	Description of target site	Mean temp (°C)[c]
Cloning				
F4	AAA TAA GGA GTA AAC AAT GTC	171–191	5′ end of *fcrA*	40.1
F10	TTT TTG CTG TTT AAC CGT TC	1540–1521	Downstream of *fcrA* transcription termination site	47.6
B13	AAA AAT AAG GAG CAA ATA ATG G	1558–1579	5′ end of *emm*	47.4
B3	AGC TTA GTT TTC TTC TTT GCG	3162–3145	3′ end of *emm*	48.4
Sequencing				
A11	GAT TCC AGA AGC GAT TAT TG	1730–1749[d]	3′ portion of *virR*	46.7
F5	TGG ATT AGC AAA CAC AAC TGA	279–299	3′ portion of *fcrA* signal peptide	48.3
F6	ATT TCT TCA GTC TTA GAG GCA	728–708	Region immediately upstream of *fcrA* repeats	45.4
F8	GGT TTT TGA GCT CCT GAA G	1148–1130	PGTS-rich domain of *fcrA*	46.4
F9	GCT TCA GGA GCT CAA AAA C	1129–1147	PGTS-rich domain of *fcrA*	46.5
B5	CCT GTT TTT AAT TTT CTA AGC	1628–1608	5′ portion of *emm*	43.4
J1	TGC TGA TGA ACT TCG TCG TG	1743–1762	N-terminal half of *emm*	51.9
J3	AGA GAA AAG CAA TTG GTA CA	1935–1954	N-terminal half of *emm*	44.2
J5	GTT GAC TAT CCA GAT TTC TGA	2142–2162	N-terminal half of *emm*	43.2
J7	TAT CTC CAA AAA ATG CTA AAC	2504–2524	Region upstream of *emm* C-repeat section	44.0
J2	CAC GTG ATG CGT CCA AGT C	2593–2575	C-repeat section of *emm*	51.9
B24	AGA AGA AGC AAA CAG AAC AT	2748–2767	Cell wall-associated section of *emm*	45.3
B21	TCC AGG TAA AGG TCA AGC AC	2976–2995	PGTS-rich domain of *emm*	49.2

[a] Oligonucleotides used to generate PCR products for cloning and for sequencing. All oligonucleotides except B21, B24, J1, J2, J3, J5, and J7 have been described previously (29).
[b] The nucleotide position numbers refer to the $frcA_{64/14}$ and $emm_{64/14}$ sequences as presented in this article.
[c] Mean temperatures were calculated by using the OLIGO software.
[d] The position numbers refer to those of the *virR12* sequence (8a).

(Bio-Rad), and electrophoresed in that buffer at 70 V for 3 h. The nitrocellulose blots were washed four times with 250 ml of 50 mM veronal buffer (VBS, pH 7.4) containing 0.15 M NaCl, 0.25% gelatin, and 0.25% Tween 20 (VBS-gel-Tween) to saturate the remaining protein-binding sites on the nitrocellulose. Each wash was carried out for 15 min at ambient temperature with agitation. The nitrocellulose membranes were incubated for 3 h at ambient temperature in 25 ml of VBS-gel-Tween containing 3×10^5 cpm of the appropriate ^{125}I-labeled probe per ml. The nitrocellulose membranes were then washed four times (for 15 min each time) with 250 ml of VBS containing 0.01 M EDTA (pH 7.2), 1 M NaCl, 0.25% gelatin, and 0.25% Tween 20 on a rocking platform at ambient temperature. The membranes were dried and autoradiographed by using Kodak XAR-5 film and Kodak X-Omat intensifying screens at −70°C for 1 to 3 days.

General nucleic acid techniques. For preparation of genomic DNA, strain 64/14 was grown anaerobically at 37°C in Todd-Hewitt broth (Difco) supplemented with yeast extract at 5 g/liter. Genomic DNA was isolated according to the procedure of Martin et al. (25). Preparation of plasmid DNA from *E. coli* was done as described by Zhou et al. (49). DNA ligation, transformation of *E. coli* with recombinant plasmid DNA, restriction enzyme analysis of recombinant plasmid DNA, electrophoresis of DNA fragments in 1% (wt/vol) agarose gels, and Southern blotting of agarose gels were performed by standard techniques (42). Hybridization with digoxigenin-dUTP-labeled (Boehringer, Mannheim, Germany) oligonucleotides and visualization with AMPPD (Boehringer) were done as described previously (22). Synthesis and purification of oligonucleotides and hybridization conditions used were described previously (30, 31). DNA sequencing was performed with purified PCR products as templates. Sequencing was carried out with (α-^{35}S)-dATP (Amersham, Braunschweig, Germany) and the T7 sequencing kit (Pharmacia, Freiburg, Germany) according to the instructions of the manufacturer. Sequences were analyzed with the aid of the PC GENE (IntelliGenetics, Mountain View, Calif.) and OLIGO (National Bioscience, Plymouth, Minn.) programs.

PCR techniques. PCR assays (41) were run in a 50-μl format for product preparation with a Trio-Block thermocycler (Biometra, Göttingen, Germany). Assays for analytical purposes or product cloning contained the following final concentrations for reactivity: 50 mM KCl, 10 mM Tris buffer (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM (each) nucleotide, 500 nM (each) primer, 200 to 1,000 ng of template DNA, and 2.5 U of *Taq*-Polymerase (GIBCO BRL). When PCR products were used as hybridization probes, dig-dUTP was also added to the assay to yield a final concentration of 0.5 to 5 μM. Assays for product sequencing contained each primer at a lower final concentration of 50 to 100 nM. Each assay was overlaid with 50 μl of mineral oil and denatured for 5 min at 94°C. Then the reaction was initiated by using the hot-start protocol of Erlich et al. (11), and the mixture was subsequently subjected to 27 to 35 cycles of 0.8 min at 94°C, 0.8 min at 52°C, and 0.8 to 2.5 min (depending on product length) at 72°C per cycle. PCR products for direct sequencing were generated by using one primer biotinylated at its 5′ end (biotinamidite; Clontech, ITC, Heidelberg, Germany).

Electrophoretic analysis of PCR yield was performed by applying 10 μl of the assay mixture directly onto a 1% agarose gel without further purification. PCR products for cloning purposes were separated from the mineral oil as outlined by Whitehouse and Spears (47), treated with proteinase K (10), digested with *Bam*HI and *Eco*RI, and finally purified by agarose gel electrophoresis and electroelution from the gel prior to the ligation step.

The PCR primers used to amplify the *fcrA* and $emmL_{64/14}$ genes and the oligonucleotides used to sequence the amplified gene products are listed in Table 2.

Expression of recombinant protein. The host strain for recombinant pJLA602 plasmid (Medac, Hamburg, Germany), *E. coli* DH5α (GIBCO BRL, Eggenstein, Germany), was cultured aerobically at 30°C on disk sensitivity testing agar (Unipath, Wesel, Germany) supplemented with 50 mg of ampicillin per liter. For preparation of recombinant proteins, the *E. coli* strain was cultured aerobically at 30°C in Luria-Bertani broth supplemented with 50 mg of ampicillin per liter until the optical density at 550 nm reached 0.7. Then the culture was transferred into a water bath and was kept at 40°C for an additional 2-h incubation period (30). The bacterial pellets were harvested, and the expressed recombinant proteins were recovered in the supernatant fraction after the bacteria were heated in SDS containing sample buffer at 100°C for 10 min and analyzed by Western blotting techniques.

Analysis of opsonophagocytosis in human blood. Bactericidal assays were carried out by measuring the survival of group A streptococci incubated in human blood. Briefly, a stationary culture of strain 64/14, grown in Todd-Hewitt broth, was sedimented by centrifugation and resuspended in sterile PBS. The suspension was diluted to contain 10^2 to 10^3 CFU/ml at A_{550}, and 100 μl was added to a tube containing 100 μl of a dilution of preimmune or immune chicken serum. Freshly drawn, lightly heparinized human blood (400 μl) was added to each tube, and the tubes were rotated end over end at 37°C for 3 h. Molten 0.7% Todd-Hewitt agar (3 ml) was added to each tube, and the contents were poured onto a Todd-Hewitt agar plate. The number of CFU remaining after 3 h of incubation in human blood was determined, following overnight incubation at 37°C.

Nucleotide sequence accession number. The EMBL accession number for the sequences described in this paper is X72932.

RESULTS

PCR amplification and cloning of the *emmL* and *fcrA* genes of strain 64/14. Preliminary analysis of the *vir* locus of strain 64/14, by previously described PCR methods (29), demonstrated that this isolate contained a characteristic large virulence locus, i.e., a *virR* gene followed by an *fcrA* gene, an *emmL* gene, an *enn* gene, and finally a C5a peptidase gene. By using previously developed PCR primers (Table 1), the *fcrA* and *emmL* genes of strain 64/14 were amplified selectively and force cloned into an appropriate expression vector. The overall strategy for amplifying and cloning the *fcrA* and *emmL* genes from strain 64/14 is summarized in Fig. 1.

This approach yielded a 1.2-kb PCR fragment for the *fcrA* gene and a 1.7-kb fragment for the *emmL* gene of strain 64/14. The amplified fragments were force cloned into the multiple cloning site of pJLA602, and potential clones were screened by isolation of plasmid, by digestion with *Eco*RI and *Bam*HI, and by analysis of the insert by Southern blot analysis as described in Materials and Methods. Once a plasmid containing the desired insert had been identified, recombinant proteins were expressed in *E. coli* DH5α as described in Materials and Methods.

The recombinant proteins were then analyzed for functional reactivity by Western immunoblotting. The results presented in Fig. 2 demonstrate that the major recombinant product of the $emmL_{64/14}$ gene was an ~52,000-M_r protein that reacted with human IgG1, IgG2, and IgG4 but not IgG3. The proteins were also found to be reactive with rabbit, horse, and pig IgG (data not shown). This profile of immunoglobulin-binding activity is characteristic of the high-molecular-weight type IIa protein previously identified in heat and CNBr extracts of this strain (27).

The functional reactivity of the *fcrA* gene product with different subclasses of human IgG was also tested (Fig. 2). This recombinant protein demonstrated reactivity with human IgG1, IgG2, and IgG4, mediated by a lower-molecular-weight ~33,000-M_r protein (Fig. 2). A minor reactive band corresponding to an ~28,000-M_r protein with similar reactivity was also identified. The ~33,000-M_r protein was also found to be reactive with rabbit, horse, and pig IgG (data not shown). The results presented in Fig. 2c demonstrate that neither of the recombinant proteins bound human IgG3. The CnBr extract of the wild-type protein contained an ~33,000-M_r doublet protein, which bound only IgG3. This corresponds to the previously identified type IIb IgG-binding protein of strain 64/14 (27, 48). Taken together, the results presented in Fig. 2 suggest that the $emmL_{64/14}$ gene encodes the previously described high-molecular-weight type IIa IgG-binding protein of strain 64/14 and the *fcrA* gene encodes the lower-molecular-weight type IIa protein (27). If this prediction were correct, these two proteins should be antigenically distinct molecules—the $emmL_{64/14}$ gene product should react with the anti-IIo antibody probe but not the anti-pLOH antibody probe while the *fcrA* gene product should demonstrate the opposite profile of antigenic reactivity (27).

In the next series of experiments, the recombinant proteins were analyzed for antigenic reactivity. The results presented in Fig. 2e and f demonstrate that the major ~52,000-M_r recombinant product of the $emmL_{64/14}$ gene, as well as the lower-M_r reactive fragments were recognized by the anti-IIo antibody probe but not by the anti-pLOH antibody probe. The M_r of the recombinant protein was ~3,000 higher than the corresponding wild-type protein (M_r of ~49,000) present in the CNBr extract of strain 64/14. Treatment of the recombinant proteins with CNBr results in their comigration with the high-molecular-weight type IIa protein in CNBr extracts of the wild-type organism (data not shown).

The ~32,000-M_r *fcrA* gene product was recognized by the anti-pLOH antibody probe and demonstrated a low level of reactivity with the anti-IIo antibody probe (Fig. 2e and f). This reactivity with the anti-IIo antibody probe was not observed in this molecular weight range in the CNBr extract of strain 64/14. The ~32,000- to 35,000-M_r doublet in CNBr extracts of strain 64/14 reacted exclusively with the anti-pLOH-antibody probe (Fig. 2e and f, lanes 1). The reactivity of the recombinant FcrA protein with the anti-IIo antibody probe was retained when the recombinant protein was treated with CNBr (Fig. 3c). These observations suggest that there may be a limited number of conserved epitopes that are shared by the two molecular weight forms of type IIa proteins which can be detected under certain experimental conditions. The antibody may be reacting with conserved regions of the membrane anchor region, and the results may be explained by differences in the susceptibility of individual methionine residues to CNBr cleavage in cell-bound wild-type proteins and soluble recombinant forms of the protein.

Neither recombinant protein displayed reactivity with control normal chicken antibody probes nor was any reactivity detected with control *E. coli* preparations carrying a pJLA602 construct containing an irrelevant insert (data not shown). Taken together, the results presented in Fig. 2 demonstrate that $emmL_{64/14}$ and $fcrA_{64/14}$ encode distinct forms of type IIa IgG-binding proteins, although evidence for a limited number of epitopes shared by the two recombinant proteins has emerged.

Functional and antigenic analysis of $emmL_{64/14}$ and $fcrA_{64/14}$

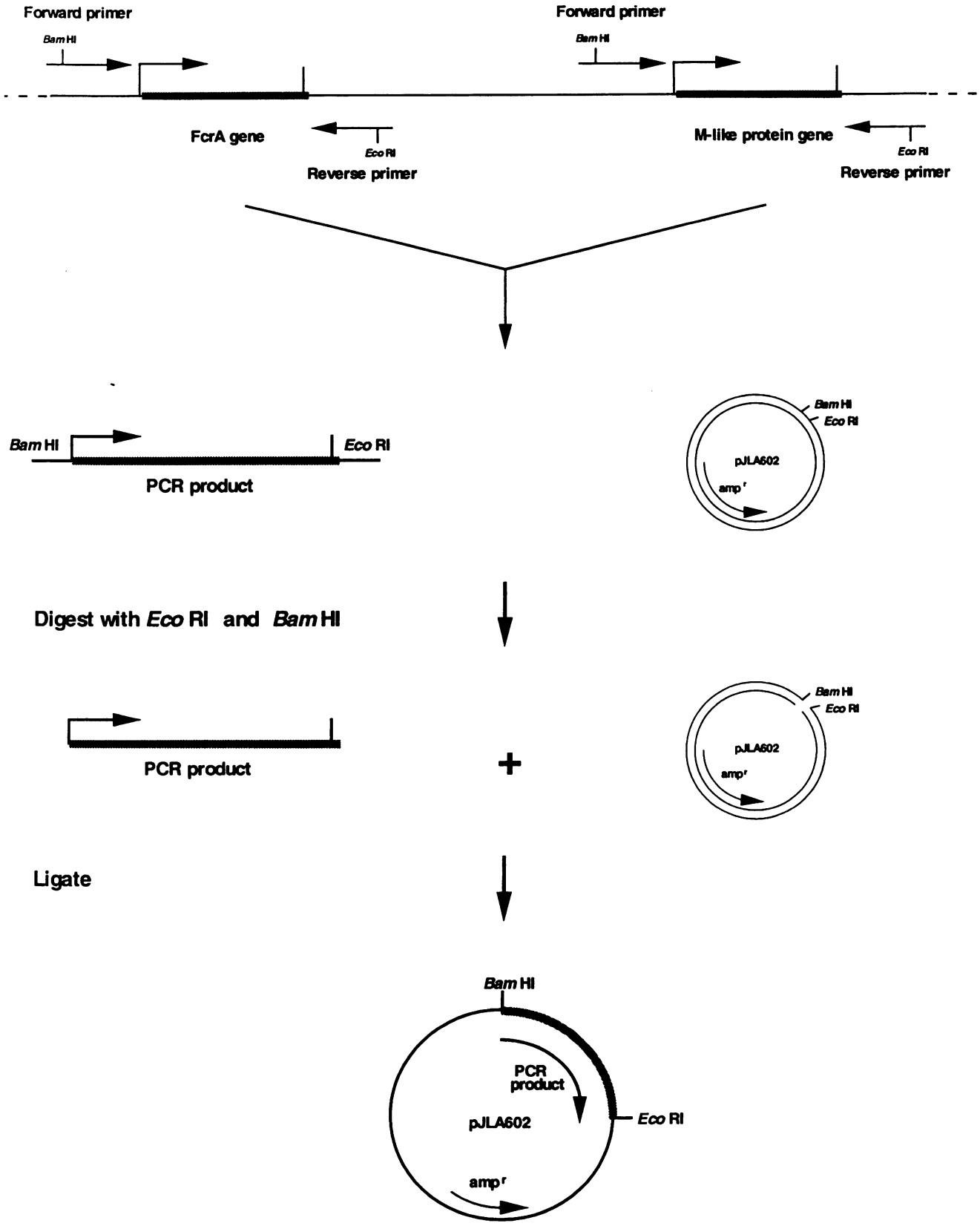

FIG. 1. Schematic representation of strategy to clone the *fcrA* and *emmL* genes of strain 64/14.

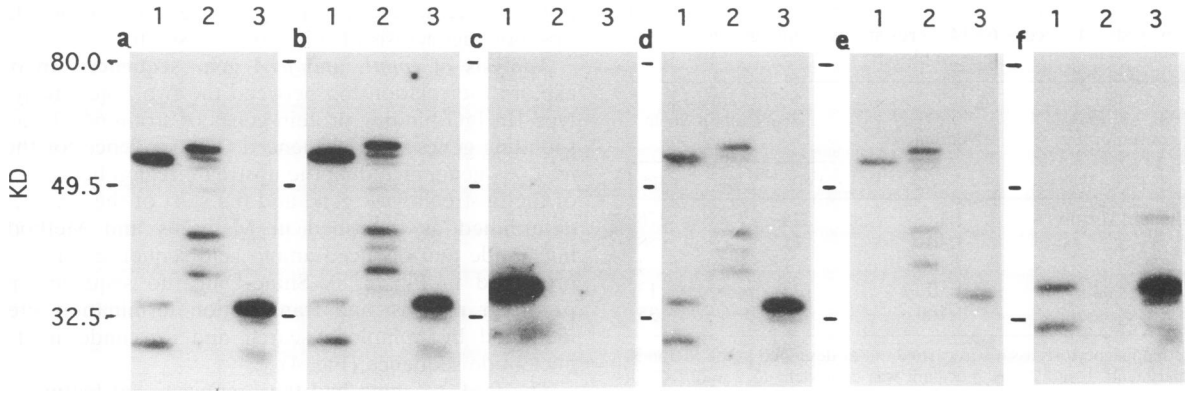

FIG. 2. Western immunoblot analysis of recombinant proteins expressed by the cloned $emmL_{64/14}$ and $fcrA_{64/14}$ genes. Lanes: 1, CNBr extract of group A 64/14 isolate; 2, recombinant proteins expressed by *E. coli* transformed with pJLA602 containing the $emmL_{64/14}$ gene; 3, recombinant proteins expressed by *E. coli* transformed with pJLA602 containing the *fcrA* gene of 64/14. The probe used was either ^{125}I-labeled human IgG1 (a), IgG2 (b), IgG3 (c), or IgG4 (d) or ^{125}I-labeled chicken antibody to a type IIo IgG-binding protein (anti-IIo) (e) or to a recombinant type IIa IgG-binding protein (anti-pLOH) (f). For precise details of probes and experimental conditions, see the text. Molecular size markers (in kilodaltons) are indicated on the left.

gene products. Streptococcal M proteins exist in two major antigenic classes (12). An association between the antigenic class of IgG-binding protein and the antigenic class of M protein expressed by the same isolate has been noted (6). Usually, IgG-binding proteins reactive with the anti-IIo antibody probe are present on strains expressing a class I M protein (6). Bessen and colleagues have reported that the class I M protein contains a conserved epitope that is reactive with a specific monoclonal antibody, 10B6 (2). In the next series of experiments, the M protein nature of the $emmL_{64/14}$ and *fcrA* gene products was studied with the specific class I M protein monoclonal antibody probe. In addition, certain M proteins and M-related proteins have been reported to bind fibrinogen (6, 12, 26, 43). Consequently, the fibrinogen-binding activities of wild-type extracts and recombinant proteins were also analyzed. Since the results presented in Fig. 2 demonstrate that there was a difference in the antigenic reactivity profile of the anti-IIo antibody probe with the recombinant FcrA protein and the corresponding wild-type protein in CNBr extracts of strain 64/14, both recombinant proteins were pretreated with CNBr to ensure that similar fragments were being compared in this experiment.

A CNBr extract of strain 64/14 and the two recombinant proteins, following treatment with CNBr, were separated on SDS–10% polyacrylamide gels under reducing conditions, transferred to nitrocellulose by electroblotting, and probed for reactivity with (i) a radiolabeled form of mouse monoclonal antibody 10B6 (2), (ii) an appropriately subclass-matched mouse monoclonal antibody with an irrelevant specificity, (iii) a radiolabeled anti-IIo antibody probe, and (iv) radiolabeled human fibrinogen. The results of these studies (Fig. 3) demonstrate that the pattern of antigenic reactivity with the class I M protein-specific monoclonal antibody 10B6 was similar to that of the chicken anti-IIo-antibody probe. The 10B6 antibody recognized the ~49,000-M_r high-molecular-weight type IIa-binding protein present in CNBr extracts of strain 64/14 as well as the CNBr-treated $emmL_{64/14}$ gene product. This antibody also displayed significant reactivity with the CNBr-treated, ~32,000-M_r *fcrA* gene product but displayed no significant reactivity with the wild-type extract in the same molecular

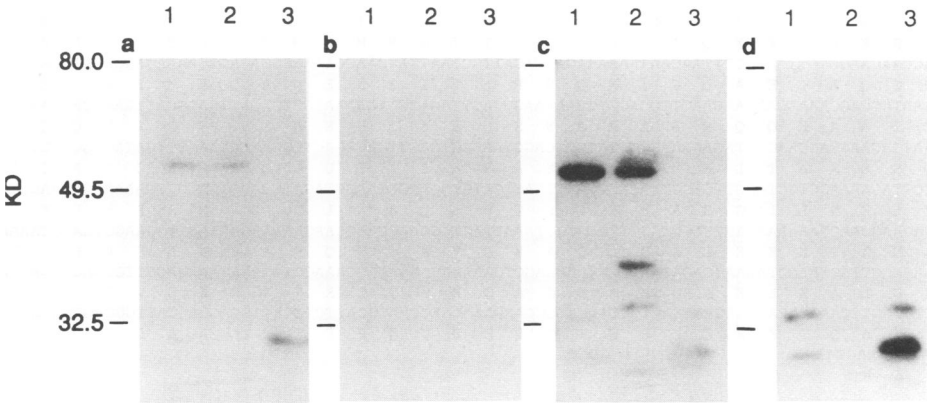

FIG. 3. Analysis of recombinant proteins expressed by the cloned *emmL* and *fcrA* genes of strain 64/14 for M protein-like characteristics. Lanes: 1 to 3, as defined in the legend to Fig. 2, except that the samples were pretreated with CNBr. The probe used was ^{125}I-labeled monoclonal antibody 10B6 (a), ^{125}I-labeled control monoclonal antibody (b), ^{125}I-labeled chicken antibody to type IIo IgG-binding protein (c), or ^{125}I-labeled human fibrinogen (d). Molecular size markers (in kilodaltons) are indicated on the left.

TABLE 3. Effect of addition of chicken antibodies on the ability of group A streptococcus 64/14 to resist opsonophagocytosis in human blood[a]

Source of serum	Dilution	CFU after 3-h incubation in whole blood[b]	
		Expt 1	Expt 2
Normal chicken serum	1:1	224	276
αIIo	1:10	15	8
	1:100	1	4
Anti-pLOH	1:10	123	13
	1:100	25	35

[a] The opsonophagocytosis assay was carried out as described in the Methods.
[b] For experiments 1 and 2, the inoculum was equivalent to 2 and 7 CFU, respectively, at time zero.

weight range. There was a similar pattern of reactivity with the anti-IIo antibody probe before and after treatment of the *emmL* or *fcrA* gene product with CNBr (compare Fig. 2d and 3c), and this pattern was similar to that observed with monoclonal antibody 10B6 (compare Fig. 3a and c).

The results of the studies using human fibrinogen as the probe are presented in Fig. 3d. The high-molecular-weight ~50,000-M_r CNBr-treated product of the $emmL_{64/14}$ gene demonstrated a low level of reactivity with fibrinogen. By contrast, the CNBr-treated recombinant FcrA protein or the corresponding lower-molecular-weight type IIa protein in a CNBr extract of strain 64/14 demonstrated strong reactivity with fibrinogen.

On the basis of the M protein-like properties of the $emmL_{64/14}$ and $fcrA_{64/14}$ gene products, the ability of group A isolate 64/14 to be phagocytosed by human blood in the presence of either normal chicken serum or a polyclonal chicken antibody to either of these proteins was tested. The results of these studies, presented in Table 3, suggest that polyclonal antibodies to either of these proteins could facilitate opsonophagocytosis of this group A isolate.

Analysis of *emmL* and *fcrA* gene sequences. In order to explore the relationship between the two functionally similar type IIa IgG-binding protein genes of strain 64/14, the corresponding genes were sequenced. The sequence for the entire $fcrA_{64/14}$ gene, including the upstream region between the end of the upstream *virR* gene and the start of the *fcrA* gene, was determined as described in Materials and Methods. The nucleotide and deduced amino acid sequence for $fcrA_{64/14}$ is presented in Fig. 4. A Shine-Dalgarno sequence, putative promoter regions, and transcription termination sites were identified by homology search and are underlined in the nucleotide sequence (Fig. 4).

The $fcrA_{64/14}$ gene had the organizational features characteristic of other *fcrA* genes that have been cloned and sequenced (18, 19, 26, 30, 32). There was a typical signal sequence present at the amino terminus followed by three repeat segments encoding 35 amino acids found in the central part of the gene sequence. These repeat regions showed ~50 to 75% amino acid sequence homology to each other and were followed by the conserved portions of the cell wall-spanning and membrane anchor regions which are characteristic for cell surface-associated receptors of gram-positive bacteria. The putative amino acid sequence of the $fcrA_{64/14}$ gene, as expected, was homologous to those of other *fcrA* genes (Fig. 5). The homology to the *mrp-4* (*fcrA 4*) gene sequence was the greatest (>93% homology), while the *fcrA49* and *fcrA76* sequences displayed approximately 75 and 70% homology, respectively (13, 15, 19). The upstream noncoding sequences of all four *fcrA* genes were found to be highly conserved (Fig. 6a), as were the noncoding sequences downstream of the *mrp* (*fcrA 4*) and *fcrA49* genes (Fig. 6b). The corresponding sequence for *fcrA76* is not available.

The $emmL_{64/14}$ gene was also sequenced, and the resulting nucleotide and deduced amino acid sequence is presented in Fig. 7 and includes the upstream sequence between the end of

```
      10        20        30        40        50        60        70        80        90       100       110
TAGGATTTCAGACGTCATGGTAAGAAAAAAGAATCATTTACAGCCCATAGAGGTAAGGTCAAAAGCTGAAAACAGCTCAAAAAAACTGACCTTTACCTTTTGGCTTTTTTTATTTAGAAT  120
                                                                                          -35                 -10
AATTTTATTGGAGAGATGCTTAATTTTAAGCACAATTCTTAGAAATTGAGAAATAAGGAGTAAACAATGTCTAAAAGAAATCCAAACAAACACTACTCACTGAGAAAGTTAAAAACAGGT  240
                                                          SD        M  S  K  R  N  P  N  K  H  Y  S  L  R  K  L  K  T  G
ACTGCATCAGTAGCAGTAGCTTTGACAGTTTTGGGGACTGGCTTAGCAAACACAACTGATGTAAAGGCTGAGAGTCGTGGTTACCAGGTTCCTCCTCGTGTGTTACTGCCAGGTAAAGAA  360
 T  A  S  V  A  V  A  L  T  V  L  G  T  G  L  A  N  T  T  D  V  K  A  >E  S  R  G  Y  Q  V  P  P  R  V  L  L  P  G  K  E
GCTAACAAAGTATTCGAAGAGCGCAAAGCCTTGGAAAAACAAGCACGTGAACTGGGTGACACTATTAACCACATGTCACAAACCATTAGCGAGCAAAGCCGCAAGATTGCAGCATTAAAG  480
 A  N  K  V  F  E  E  R  K  A  L  E  K  Q  A  R  E  L  G  D  T  I  N  H  M  S  Q  T  I  S  E  Q  S  R  K  I  A  A  L  K
TCTGAAGCAGAACTTAAAAACCAACAAGCCCTTGAAGCTTTAACAATAAAACAGAGCAGATTGCTAATTACCAACGAAAACGCACAGTTAAAAGAAGCCATTGAAGGTTATGTGCAAACT  600
 S  E  A  E  L  K  N  Q  Q  A  L  E  A  L  T  I  K  Q  S  R  L  L  I  T  N  E  N  A  Q  L  K  E  A  I  E  G  Y  V  Q  T
ATCCAAAACGCTAGTCGTGAAATCGCAGCAAAACAACAAGAACTTGCAGCTGCAAAAAGCCAGTTAGAGGCAAAAAATGCTGAGATTGAGGCATTGAAACAACAAGATGCCTCTAAGACT  720
 I  Q  N  A  S  R  E  I  A  A  K  Q  Q  E  L  A  A  A  K  S  Q  L  E  A  K  N  A  E  I  E  A  L  K  Q  Q  D  A  S  K  T
GAAGAAATTGCTAAATTGCAATCAGAAGCAGCAACTTTAGAAAACCTCCTAGGTTCAGCTAAGCGTGAGTTGACTGACTTGCAAGCTAAGCTTGATACAGCAACTGCTGAAAAAGCAAAA  840
 E  E  I  A  K  L  Q  S  E  A  A  T  L  E  N  L  L  G  S  A  K  R  E  L  T  D  L  Q  A  K  L  D  T  A  T  A  E  K  A  K
CTAGAATCACAAGTAACAACCTTAGAAAACCTCCTAGGTTCAGCTAAGCGTGAATTGACTGATCTGCAAGCTAAGCTAGATCGAGCTAACGCTGAAAAAGAAAAGCTCCAATCACAAGCA  96
 L  E  S  Q  V  T  T  L  E  N  L  L  G  S  A  K  R  E  L  T  D  L  Q  A  K  L  D  R  A  N  A  E  K  E  K  L  Q  S  Q  A
GCAGCCCTAGAAAAACAACTAGAAGCAACTAAAAAAGAGTTAGCTGATTTACAGGCTAAATTAGTCGCAACCAACCAAGAAAAAGAAAAGTTAGAAGCTGAAGCAAAAGCTCTTAAAGAG 1080
 A  A  L  E  K  Q  L  E  A  T  K  K  E  L  A  D  L  Q  A  K  L  V  A  T  N  Q  E  K  E  K  L  E  A  E  A  K  A  L  K  E
CAATTAGCAAAACAAGCTGAAGAGCTTGCTAAGCTAAAAGCAGATAAAGCTTCAGGAGCTCAAAAACCAGATACCAAACCTGGCAATAAAGAGGTTCCAACAAGACCATCACAAACAAGA 1200
 Q  L  A  K  Q  A  E  E  L  A  K  L  K  A  D  K  A  S  G  A  Q  K  P  D  T  K  P  G  N  K  E  V  P  T  R  P  S  Q  T  R
ACAAACACTAATAAAGCTTCTATGGCTCAAACAAAGAGAGAATTACCGTCAACAGGCGAAGAAACAACCAACCCATTCTTCACTGCAGCAGCATTGGCAGTGATCGCATCTGCAGGCGTA 1320
 T  N  T  N  K  A  S  M  A  Q  T  K  R  E  L  P  S  T  G  E  E  T  T  N  P  F  F  T  A  A  A  L  A  V  I  A  S  A  G  V
TTTGCCCTAAAACGCAAAGAAGAAAACTAA
 F  A  L  K  R  K  E  E  N
```

FIG. 4. Nucleotide sequence of the *fcrA* gene of the group A streptococcal strain 64/14. The sequence is shown starting from the stop codon of *virR* upstream of *fcrA* and ending with a stop codon of *fcrA*. All start and stop codons are printed in bold letters. The −35 and −10 boxes and the Shine-Dalgarno sequence (SD) of the *fcrA* promoter were located by a homology search and are underlined in the nucleotide sequence. The deduced amino acid sequence is shown under the corresponding codons of the nucleotide sequence. The first residues of the mature protein is marked by >.

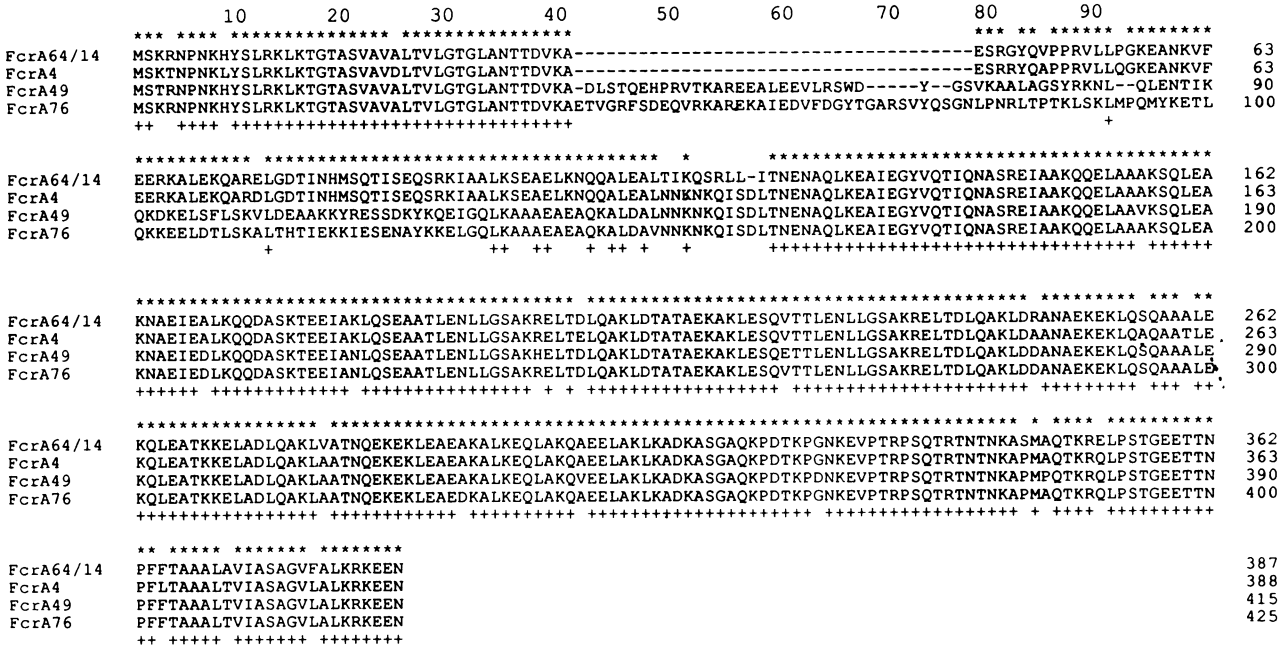

FIG. 5. Amino acid sequence alignment of FcrA proteins. The sequences were taken from the studies by O'Toole et al. (26) for Mrp4 (FcrA4), Podbielski et al. (30) for FcrA49, and Heath and Cleary (19) for FcrA76. Dashes were introduced for maximum alignment. Homology of identical positions between FcrA 64/14 and Mrp4 (FcrA4) are marked by an asterisk (*) above the sequences and for all sequences by a plus (+) beneath the sequences.

the *fcrA* gene and the start of the *emmL* gene. An unequivocal N-terminal sequence for the first 33 amino acids had previously been determined for the high-molecular-weight type IIa protein isolated from strain 64/14 by heat extraction (5). This sequence corresponded exactly to the sequence predicted from the nucleotide sequence (Fig. 7). The $emmL_{64/14}$ sequence shows a typical signal sequence as well as putative promoter regions and transcription termination sites, which were iden-

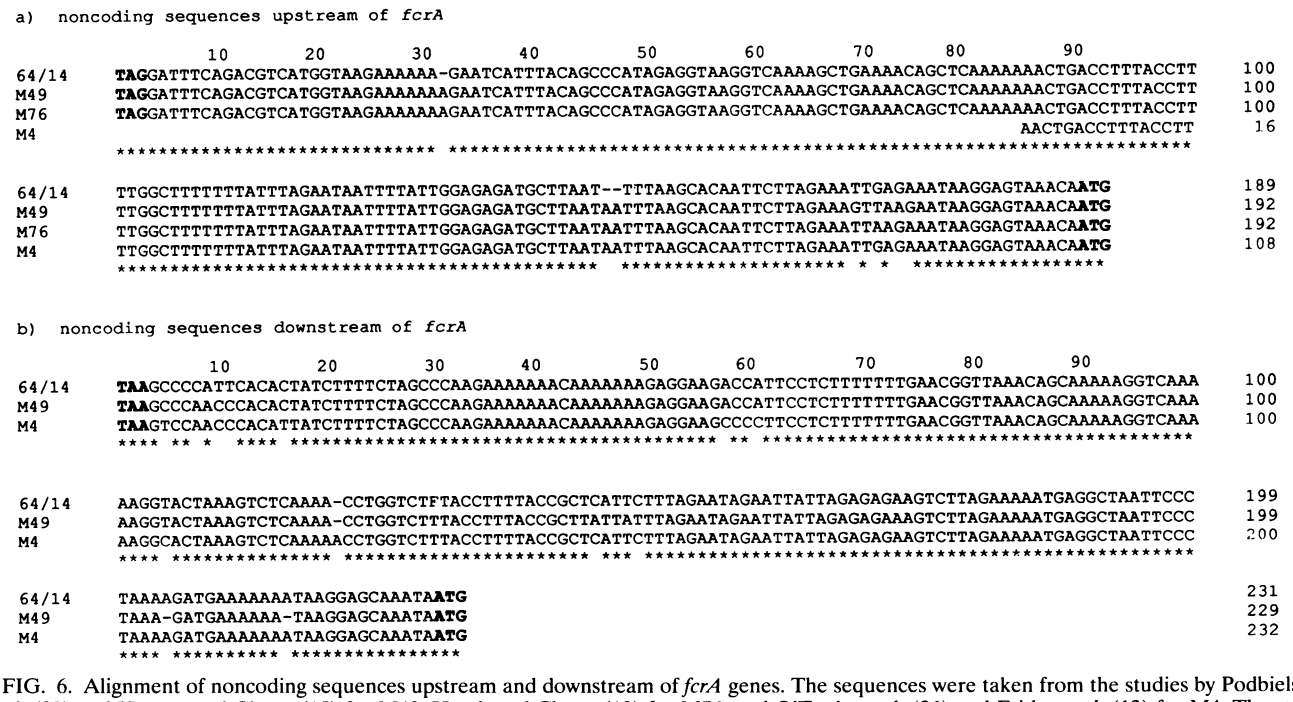

FIG. 6. Alignment of noncoding sequences upstream and downstream of *fcrA* genes. The sequences were taken from the studies by Podbielski et al. (30) and Haanes and Cleary (15) for M49, Heath and Cleary (19) for M76, and O'Toole et al. (26) and Frithz et al. (13) for M4. The start and stop codons of *fcrA* genes and adjacent genes are printed in bold types. Dashes were introduced for maximum alignment. Homology at identical positions is marked by asterisks.

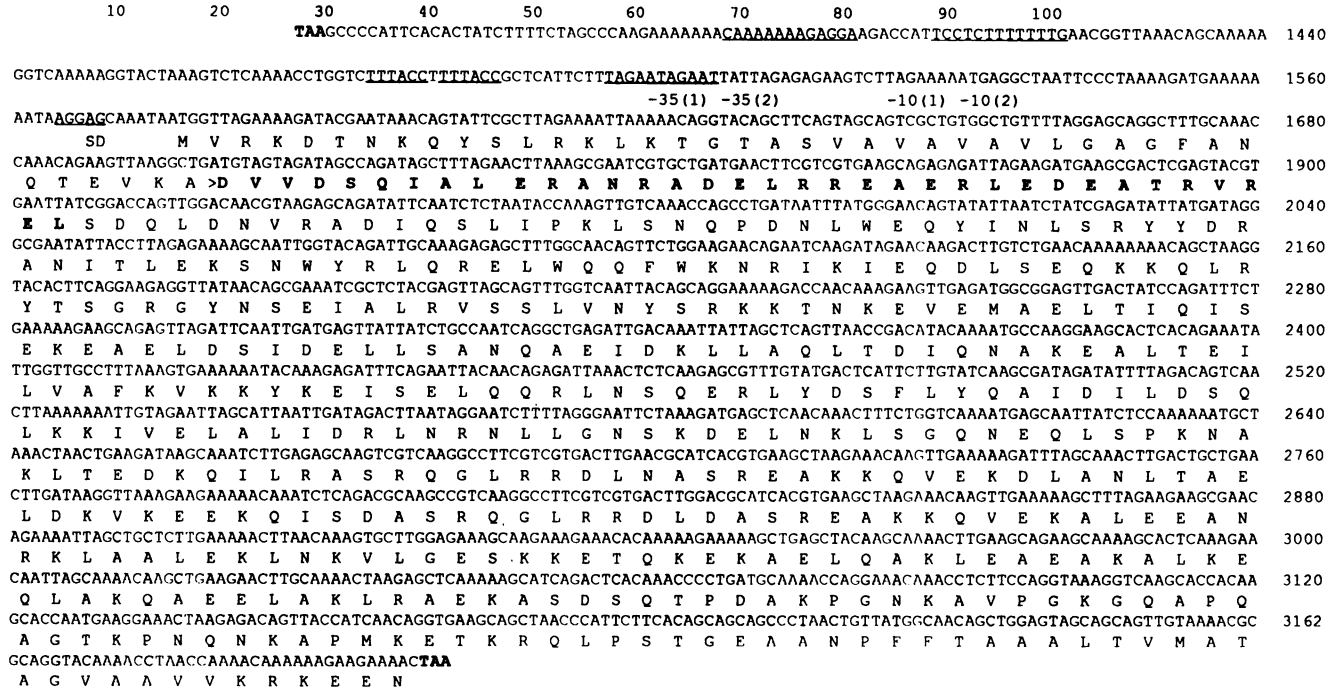

FIG. 7. Nucleotide sequence of the *emmL* gene of group A streptococcal strain 64/14. The sequence is shown from the stop codon of *fcrA* upstream of $emmL_{64/14}$ and ending with the stop codon of $emmL_{64/14}$. Since the *fcrA* and $emmL_{64/14}$ genes are contiguous, the numbering of nucleotides from this figure continues directly from those of the sequence shown in Fig. 4. All start and stop codons are printed in bold letters, and the numbering of nucleotides continues from the *fcrA* sequence shown in Fig. 2. The −35 and −10 boxes and the Shine-Dalgarno sequence (SD) for the two putative *emmL* promoters were located by a homology search and are underlined in the nucleotide sequence. The deduced amino acid sequence is shown under the corresponding codons of the nucleotide sequence. The first residues of the mature protein is marked by >. The N-terminal sequence of the high-molecular-weight type IIa IgG-binding protein has been sequenced previously (5), and the residues corresponding to the deduced amino acid sequence are shown in bold type.

tified by homology search and are underlined in the nucleotide sequence. $emmL_{64/14}$ also contained the typical C repeat regions characteristic of class I M proteins (4, 15, 20, 30).

The putative amino acid sequence for $emmL_{64/14}$ was compared with the available sequences for two other class I M proteins, M6 and M12 (Fig. 8). These sequences were highly homologous in the C repeat regions (Fig. 8a), the cell wall-spanning region (Fig. 8b), the PGTS-rich domains (Fig. 8c), and the membrane anchor and polar tail region (Fig. 8d). The $emmL_{64/14}$ gene, like the emm_6 gene, encoded two highly conserved repeat regions, while the M12 sequence contained four such regions. In the cell wall-spanning region, in the PGTS-rich region, and in the membrane anchor regions, the $emmL_{64/14}$ gene was highly homologous to the other class I M protein genes. Taken together, the sequence data presented in Fig. 6 through 8 provide evidence that $emmL_{64/14}$ has characteristics of a typical class I M protein gene and follows a typical *fcrA* gene flanked by a conserved intervening sequence.

A comparison of the sequences of $emmL_{64/14}$ and $fcrA_{64/14}$ revealed 70.7% similarity in the sequence encoding the signal peptide, 19.1% similarity in the N-terminal section of the gene, and 25.6% similarity for the C-terminal section. The PGTS-rich domain and cell wall-associated portion of both genes demonstrated 37.7 and 82.1% similarity, respectively. These similarities were identified with the PALIGN program of PC GENE. With the Garnier program for secondary structure analysis of the deduced $emmL_{64/14}$ sequence, 78 of the first 140 N-terminal residues of the gene encoding the mature protein adopt an extended coil or turn conformation. This is in marked contrast to other *emm* and *emmL* genes that are largely alpha helical in this region (12).

DISCUSSION

Analysis of IgG-binding proteins expressed by group A streptococci has proved difficult because of the tendency of organisms to lose expression of these proteins during laboratory subculture (7, 35). In an attempt to select a group A isolate expressing stable high levels of IgG-binding proteins, group A isolate 64 was passaged sequentially 14 times in mice, and a variant, 64/14, that expressed high levels of IgG-binding proteins was recovered (37). Two IgG-binding proteins could be solubilized from this strain by heat treatment at neutral pH (48). One protein, an ~50,000-M_r molecule present in heat extracts reacted with human IgG1, IgG2, and IgG4, as well as with horse, rabbit, and pig IgG. This functional binding profile was designated type IIa (48). The second protein (M_r, ~35,000) reacted efficiently with only human IgG3 and was designated type IIb.

Subsequent studies demonstrated that strain 64/14, when treated with CNBr, resulted in solubilization of three unique IgG-binding proteins (27). Two of these proteins had identical type IIa functional reactivity (27) but were antigenically distinct and differed in molecular weight. The higher-molecular-weight type IIa protein corresponded in size and antigenic properties to the type IIa protein originally isolated from heat extracts (5). The gene encoding one of these proteins was successfully identified within a λgt11 chromosomal library of

FIG. 8. Amino acid sequence alignment of the conserved C-terminal portions of selected class I M proteins. The sequence of Emm12 containing four C-repeat regions was taken from the study by Robbins et al. (40); the sequence of Emm6 was taken from the study by Hollingshead et al. (20). The designations of sections and domains within the C-terminal of the M protein follow the nomenclature proposed by Bessen and Fischetti (4). Homology at identical positions is marked by asterisks.

strain 64/14 and cloned into pUC18 (27). The resulting pLOH plasmid expressed a type IIa IgG-binding protein corresponding in size and antigenic properties to the lower-molecular-weight type IIa protein recovered in CNBr extracts of strain 64/14 (27). The gene within the pLOH plasmid was closely related to the *fcrA* gene (pDH56) isolated from another group A streptococcus, CS110 (17–19), and the expressed proteins from pLOH and pDH56 were antigenically and functionally indistinguishable (27). Attempts to identify the gene encoding the second, higher-molecular-weight, type IIa-binding protein from within the λgt11 chromosomal library of strain 64/14 were unsuccessful (17). The third IgG-binding protein present in CNBr extracts of strain 64/14 corresponded in functional and antigenic properties to the previously characterized type IIb IgG3-binding protein from strain 64/14 (5, 27, 48).

Identification of the high-molecular-weight type IIa IgG-binding protein in this study as the product of the *emmL* gene of 64/14 is consistent with the reported changes in expression of this molecule when this isolate was subjected to passage in human blood or mice (33, 34). Previous studies have indicated that group A streptococci exposed to similar biological pressure increase expression of their surface M protein (12). In addition, recent studies by Schmidt and Waldström (43), Retnoningrum et al. (39), and Boyle and Raeder (6) provided evidence that certain class I M proteins also displayed IgG-binding potential. These proteins have also been shown to be antigenically related on the basis of their reactivity with a monospecific chicken antibody to a type IIo IgG-binding protein (6). Taken together, these studies are consistent with the high-molecular-weight type IIa protein of strain 64 being the product of the *emmL* gene of this serologically M protein-untypeable strain.

Recent PCR-based strategies for amplification and forced cloning of group A streptococcal *emmL* and IgG-binding protein (*fcrA*) genes have been developed by Podbielski and colleagues (29–31). This approach was applied to amplification and cloning of *emmL* and *fcrA* genes of strain 64/14, and the resulting recombinant proteins, expressed by using the heat-inducible vector pJLA602, were characterized. The expressed recombinant proteins from each gene displayed the functional binding profiles of type IIa IgG-binding proteins. The $emmL_{64/14}$ gene product displayed all of the functional and antigenic properties of the previously described high-molecular-weight type IIa protein (27). Furthermore, the predicted N-terminal amino acid sequence was identical to the previously reported N-terminal sequence of the isolated wild-type protein (Fig. 7). The predicted C-terminal sequence for $emmL_{64/14}$ demonstrated sequence homology to corresponding regions of other *emm* or *emmL* class I genes previously sequenced. The *emm* or *emmL* genes from group A isolates expressing class I M proteins in studies by Hollingshead and colleagues (20), Bessen and Fischetti (4), Haanes and Cleary (15), Podbielski et al. (30), and O'Toole et al. (26) had defined characteristic C repeat regions and PGTS-rich regions, in addition to cell wall and membrane anchor regions. All of the characteristic class I M structural features were present in EmmL 64/14 (Fig. 8).

The recombinant Emm 64/14 protein was also found to react with a monoclonal antibody that Bessen et al. had previously demonstrated identified a conserved epitope in class I M proteins (2, 3). The product of the *emmL* 64/14 gene demonstrated only weak reactivity with fibrinogen (Fig. 3d). These findings further underscore the range of functional heterogeneity displayed by closely related protein members of the group A streptococcal M protein supergene family (6, 12, 26, 43). Chicken antibody to the high-molecular-weight type IIa-binding protein but not normal chicken serum was found to facilitate opsonophagocytosis of strain 64/14 in human blood (Table 2), further confirming the M protein nature of this molecule.

The functional, antigenic, and genetic analysis of IgG-binding proteins expressed by group A isolate 64/14 has revealed a number of unusual features. First, 64/14 is one of a very limited number of strains of group A streptococci that express multiple functional forms of IgG-binding proteins belonging to each antigenic family of IgG-binding molecules (6). In general, OF^- isolates expressing a class I M protein also express an IgG-binding protein reactive with the anti-IIo antibody probe, while OF^+ isolates, expressing a class II M protein, express an IgG-binding protein reactive with the anti-pLOH antibody probe (*fcrA* gene products) (6). The *fcrA* gene of strain 64/14 was found, as predicted, to encode the lower-molecular-weight type IIa IgG-binding protein. The nucleotide sequence of the gene encoding this protein was similar to that of *fcrA* genes cloned and sequenced from other group A isolates (Fig. 5) but displayed limited homology to the $emmL_{64/14}$ gene sequence, except in the cell wall-associated and membrane anchor regions. All FcrA proteins cloned and sequenced to date have similar functional immunoglobulin-binding profiles. The $fcrA_{64/14}$ gene displayed >93% homology with the *mrp4* (*fcrA4*) gene from strain AP4 (26). In general, *fcrA* genes are associated with OF^+ isolates that express class II M proteins. The finding that $fcrA_{64/14}$ is adjacent to a typical class I M protein gene represents an additional example of a *vir* locus that has characteristics of both OF^+ and OF^- isolates (21, 29, 30).

The identification of the high-molecular-weight type IIa IgG-binding protein of strain 64/14 as also being the *emmL* gene product provides an additional example of an M-like protein capable of binding IgG. This molecule was also antigenically related to other class I M proteins that display immunoglobulin-binding activities (6). The IgG-binding properties of this hybrid molecule (which is reactive with the anti-IIo antibody probe) were unusual in that it is the only M or M-like protein that has failed to react with any IgG3 myeloma tested (data not shown). These functional variations among antigenically related members of the M protein supergene family provide further evidence for the heterogeneity of this family of molecules. The development of rapid PCR-based methods to clone and express *emmL* and *fcrA* genes and the ability, by using CNBr extraction procedures and western blotting techniques, to monitor the IgG-binding expression of two functionally related genes within the virulence locus now provide the opportunity to study regulation of these genes under a variety of different biological pressures. This, in turn, should facilitate an understanding of the structure-function relationship of these proteins and provide a basis for understanding their potential role in streptococcal infection.

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