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DNA Sequence of the Serum Opacity Factor of Group A Streptococci: Identification of a Fibronectin-Binding Repeat Domain

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The serum opacity factor (SOF) is a group A streptococcal protein that induces opacity of mammalian serum. The serum opacity factor 22 gene (*sof22*) from an M type 22 strain was cloned from an EMBL4 library by screening for plaques exhibiting serum opacity activity. DNA sequencing yielded an open reading frame of 3,075 bp. Its deduced amino acid sequence predicts a protein of 1,025 residues with a molecular weight of 112,735, a size that approximates that of the SOF22 protein isolated from both the original streptococcal strain and *Escherichia coli* harboring the cloned *sof22* gene. The molecule is composed of three domains: an N-terminal domain responsible for the opacity reaction (opacity domain), a repeat domain with fibronectin-binding (Fn-binding) activity, and a C-terminal cell attachment domain. The C-terminal end of SOF22 is characterized by a hexameric LPXTGX motif, an adjacent hydrophobic region, and a charged C terminus, which are the hallmarks of cell-bound surface proteins found on nearly all gram-positive bacteria. Immediately upstream of this cell anchor region, SOF22 contains four tandem repeat sequence blocks, flanked by proline-rich segments. The repeats share up to 50% identity with a repeated motif found in other group A streptococcal Fn-binding proteins and exhibit Fn-binding activity, as shown by subcloning experiments. According to deletion analysis, the opacity domain is confined to the region N terminal to the repeat segment. Thus, SOF22 is unique among the known Fn-binding proteins from gram-positive bacteria in containing an independent module with a defined function in its N-terminal portion. Southern blot analysis with a probe from this N-terminal region indicates that the opacity domain of SOF varies extensively among different SOF-producing M types.

Serum opacity factor (SOF) is a product of *Streptococcus pyogenes* named for its ability to generate opalescence of sera from several mammalian species (59). Several studies have suggested that SOF and mammalian sera interact via the high-density lipoprotein fraction of serum (35, 48, 51, 52). In essence, the reaction process involves cleavage of the apolipoprotein AI (Apo AI) component, with the subsequent aggregation of the high-density lipoprotein (51), which, in turn, yields serum opacity.

SOF was first described as an extracellular product of group A streptococci; however, subsequent studies showed that it is actually more abundant in the membrane fraction of the streptococcal cell (24, 60), providing evidence that SOF exists as both a cell-associated and a cell-free species. In several aspects, SOF was found to be related to another cell surface molecule, the antiphagocytic M protein. First, SOF and M protein phenotypes were found to cofractionate in biochemical experiments (25) or cosegregate in genetic analyses (11, 25). Second, it was found that SOF and M protein covary (60). This is illustrated by the finding that antiserum to bacteria of a particular M serotype has the capacity to neutralize the opacity reaction induced by strains of only the homologous serotype (60). The specificity of this reaction also provides the basis of an alternative typing scheme for SOF-producing group A streptococci (30, 40). Finally, the presence or absence of SOF correlates with the two classes of M protein, which may be distinguished by variance of a short epitope in the otherwise conserved portion of the molecule located close to the cell wall surface (5, 6). In an analysis that included about 60 M types, it was found that nearly all class II M types produce SOF, while class I M types rarely exhibit this activity (6). Although the correlations of SOF and M protein were described in detail phenotypically and used in classification of group A streptococci, their molecular basis was never examined. Hence, one of the questions left unanswered was whether the serum opacity reaction is associated with M protein or represents the activity of a separate molecule.

Among surface proteins of gram-positive bacteria, the fibronectin-binding (Fn-binding) proteins are responsible for adhesion to host epithelial cells (17, 26). Accordingly, Fn-binding proteins may provide the bacterial cell with the means to initiate the infection process (17, 26, 27, 39). Fn-binding proteins have been identified in *Staphylococcus aureus* (17, 32, 56), class I (SOF^-) *S. pyogenes* (26, 55, 57), and *Streptococcus dysgalactiae* (38, 39). Sequence analysis of these proteins revealed that they are large cell surface proteins, with a predicted size range of 73 to 122 kDa. The domain architecture of these molecules is similar: a divergent N-terminal portion which constitutes up to 80% of their sequence, followed by three to five homologous tandem Fn-binding repeats of from 32 to 43 residues each (26, 32, 38, 55, 56). In at least two cases, protein F from *S. pyogenes* class I and FnBPB from *S. aureus*, a region of approximately 50 residues N terminal to the tandem repeats has also been implicated as essential for maximal Fn-binding activity (32, 55). A putative cell wall-spanning segment is located C terminally to the repeats, followed by a typical gram-positive cell attachment motif (43).

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TABLE 1. Summary of group A streptococcal strains and corresponding hybridization data

Class	Strain	M type	Signal[a]	Band(s) (kb)[b]	SOF[c]
II	D734	22	+	1.55	+
	B234	22	+	3.20, 0.55	+
	B401	22	+	3.20, 0.55	+
	F312	22	+	3.20, 0.55	+
	B344	2	+	1.60	+
	B512	4	+	1.55	+
	D691	11	+	1.20, 0.21, 0.16	+
	D474	13	+	1.60	+
	D742	13	+	2.50, 0.90	+
	B737	49	+	0.40	+
	D976	51	+	0.80, 0.60, 0.23	+
	D398	60	+	0.16	+
	A956	62	+	0.20	+
	D459	63	+	0.20	+
	D794	66	+	0.22	+
I	D710	1	−		−
	B788	5	−		−
	D471	6	−		−
	S43	6	+	0.80, 0.60, 0.22	−
	A374	12	+	1.90	−
	D469	14	−		−
	1RP284	24	−		−
	D617	30	−		−
	D466	37	−		−
	D421	41	+	0.20	+
	D463	41	−		−
	D432	54	−		−
	D442	55	−		−
	D735	NT[d]	−		−
	D739	NT[d]	−		−

[a] Presence of the hybridizing restriction fragments in Southern blot analysis using the probe mpSOF22.2d976.
[b] Sizes of the *Sau*3AI restriction fragment(s) that hybridized with the probe.
[c] Ability of analyzed strains to produce SOF activity.
[d] NT, nontypeable.

Our cloning of the *sof* gene from type M22 streptococci has afforded us the opportunity for the first time to characterize an SOF protein at the primary structure level. We find that SOF22 consists of an N-terminal opacity domain, which is adjacent to a domain of sequence repeats that bind fibronectin. These Fn-binding repeats are homologous to those of other Fn-binding proteins of streptococci and staphylococci. In the carboxy-terminal region, SOF contains the signature characteristics of cell surface molecules from gram-positive bacteria. Primary and secondary structure analyses show that SOF is not an M protein or M protein-like molecule. However, the analysis of the *sof22* promoter sequence suggests that *sof* transcription may be regulated by the same *trans* activator as the *emm* gene coding for the M protein. Finally, a survey of SOF^+ (class II) and SOF^- (class I) bacteria for *sof*-homologous sequences demonstrates that this genetic locus is highly polymorphic among different streptococcal M serotypes.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. Group A streptococcal strain D734 (M type 22) was the parental strain for the *sof22* gene. This and all other group A streptococcal strains used in this study were from the Rockefeller University collection and are described in Table 1. *Escherichia coli* LE392 (8) served as the host for lambda phage EMBL4 (18). XL1-BLUE (9) (Stratagene Cloning Systems, La Jolla, Calif.) was the host strain for M13 mp18/19 (61) and pUC9.2 (61).

Chemicals and enzymes. Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs, Inc. (Beverly, Mass.). Alkaline phosphatase (from calf intestine), human fibronectin, and a random-primed DNA labeling kit were obtained from Boehringer Mannheim Corp. (Indianapolis, Ind.). DNA sequences were determined using the Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, Ohio). Unidirectional deletions of M13mp18/19 clones were generated with the Cyclone 1 Biosystem (International Biotechnologies, Inc., New Haven, Conn.). $Na^{125}I$ and radionucleotides [α-^{32}P]dATP and α-^{35}S-dATP were obtained from New England Nuclear (Boston, Mass.). DNA oligomers were purchased from Operon Technologies (Alameda, Calif.) or U.S. Biochemical Corp. PCRs were achieved using the GeneAmp PCR reagent kit (The Perkin-Elmer Corp., Norwalk, Conn.). Heat-inactivated horse serum was purchased from Life Technologies, Inc. (Gaithersburg, Md.). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.), unless otherwise indicated.

SOF assays. SOF expressed by colonies of strain D734 and other streptococcal strains was detected by growing bacteria on SOF assay agar, composed of Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.), 50% heat-inactivated horse serum, and 0.9% Oxoid ion agar no. 2 (Colab Laboratories, Chicago Heights, Ill.). Sterile horse serum was added 1:1 to 2× Todd-Hewitt broth in 1.8% ion agar, melted and prewarmed to 56°C. Recombinant phage plaques expressing SOF activity were screened by the filter overlay method, using the SOF assay agar in which Luria broth replaced Todd-Hewitt broth. Briefly, phage lawn replicas were made on sterile nitrocellulose filters, after which they were placed on the surface of SOF assay agar for 10 to 12 h at 37°C. After the filters were removed, the positions of SOF-positive plaques were determined under indirect lighting. Serum opacity activity in solutions, including supernatants of phage or bacterial cultures, was measured by the tube method as previously described (24).

To visualize SOF protein bands after sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), the gel (10%) was placed on SOF assay agar containing 0.02% merthiolate and incubated for 12 h at 37°C. SOF protein was detected as opaque bands in the SOF assay agar and photographed under indirect lighting.

Extraction of SOF from streptococci. Late-log-phase cultures of strain D734 were washed once with 200 mM sodium phosphate buffer, pH 7.5, at 4°C and resuspended in 1/100 of the original culture volume in the same buffer. SOF was then extracted by incubating the bacterial suspension for 2 h at 37°C, followed by centrifugation and filtration of the resulting supernatant through a 0.45-μm-pore-size nitrocellulose filter (Schleicher & Schuell, Inc., Keene, N.H.). SOF was precipitated from the supernatant at 60% saturated ammonium sulfate and collected by centrifugation (31,500 × *g*, 10 min). The precipitate was resuspended in 100 mM Tris-HCl, pH 7.5, and dialyzed in 4 liters of the same buffer for 48 h at 4°C.

Cloning procedures. Streptococcal chromosomal DNA was released from the bacterium and prepared by the phage lysin extraction method as previously described (53). Strain D734 chromosomal DNA, used in creating recombinant libraries, was either partially digested with *Sau*3AI or completely digested with *Eco*RI. The *Sau*3AI digest was dephosphorylated with calf intestinal phosphatase and ligated to lambda phage EMBL4 arms digested with *Bam*HI and *Sal*I. The *Eco*RI digest was ligated to dephosphorylated EMBL4 arms prepared by *Bam*HI and *Eco*RI cleavage. Ligation mixtures were packaged in vitro using Gigapack II gold packaging extracts (Stratagene Cloning Systems). Unamplified libraries were plated on strain LE392 and screened for recombinant phage plaques expressing an SOF phenotype. Four positive phage clones (LSOF22.1 to LSOF22.3 and LSOF22.4 from the *Sau*3AI and *Eco*RI libraries, respectively) were isolated and plaque purified.

Subcloning procedures for SOF expression studies and DNA sequencing of *sof22*. A 2,560-bp *Eco*RI-*Sal*I fragment, containing the entire 2,546-bp insert of phage LSOF22.1, was electrophoretically purified and ligated to both M13 mp19 and mp18 digested with both *Eco*RI and *Sal*I, creating mpSOF22.1 and mpSOF22.2, respectively. Ligation mixtures used to transform XL1-Blue yielded numerous recombinant plaques, as detected by plating on SOR assay agar. Sets of nested deletions from both M13 clones for DNA sequencing were then prepared by T4 polymerase reaction using the Cyclone I kit. The orientation of *sof22* in mpSOF22.1 and mpSOF22.2 was such that the gene was shortened from its 3′ and 5′ sides, respectively. The nested deletion clones of mpSOF22.1 were named according to the deletion size in base pairs. For example, mpSOF22.1d180 is 180 bp shorter than parental mpSOF22.1. To sequence the 3′ portion of *sof22*, an ~3,100-bp *Sac*I-*Eco*RI fragment (see Fig. 2a) was subcloned from LSOF22.4 into both M13 mp19 and mp18, creating mpSOF22.3 and mpSOF22.4, respectively.

DNA sequence and sequence analysis. Single-stranded templates of M13 mp18/19 clones were prepared for chain termination sequencing as previously described (50). DNA sequences of mpSOF22.1- and mpSOF22.2-derived deletion clones were determined by using the M13 −20 forward universal primer, Sequenase 2.0, and α-^{35}S-dATP. The 3′ portion of *sof22*, cloned in mpSOF22.3 and mpSOF22.4, was sequenced by primer walking.

DNA sequence data were aligned by using the STADEN program package (Roger Staden, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom). This package and Eugene software packages (Baylor College of Medicine) were used to predict structural features of the deduced SOF22 protein. The GenBank database was accessed to establish regions of homology between SOF22 and other known proteins. The hydropathy and secondary structure plots were obtained by using algorithms by Kyte and Doolittle (36) and Garnier et al. (19), respectively.

Subcloning of the *sof22* repeat region. The replicative form of mpSOF22.3 served as a template for amplification of the sequence coding for repeats R2, R3, and R4 of *sof22*. The 5′ primer, CCCAAGCTTCAGGAAAATAAAGAT, designed to place the coding sequence in frame with the upstream *lacZ* gene fragment, corresponded to bases 2641 to 2658 (see Fig. 3) and a *Hin*dIII site (underlined), while the 3′ primer, CGGGATCCGCTCGTTATCAAAGTGG, consisted of nucleotides 3002 to 2986 (see Fig. 3) and a *Bam*HI site (underlined). The PCR consisted of 20 cycles of a three-step reaction (1 min at 94°C, 3 min at 55°C, and 3 min at 72°C), employing the GeneAmp PCR reagent kit, native *Taq* DNA polymerase, and a DNA Thermal Cycler (The Perkin-Elmer Corp.). The products of expected size from three independent PCRs were pooled, purified by phenol-chloroform extraction, digested with *Hin*dIII and *Bam*HI, and isolated from a low-melting-temperature agarose gel. Purified DNA was then ligated to the *Hin*dIII and *Bam*HI sites of pUC9.2, creating the clone pFNBR22.1.

Fn-binding studies. The recombinant SOF22 repeat region was prepared from the whole-cell lysates of clone pFNBR22.1, separated by SDS-PAGE, and electroblotted to nitrocellulose. The blots were then blocked by incubation in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, containing 150 mM NaCl, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 60 mM KCl, 0.5% Tween 20, 0.04% NaN_3, and 0.5% bovine serum albumin, pH 7.4, for 2 to 3 h at room temperature. Subsequently, blots were then probed for 3 to 4 h at room temperature in the same buffer containing ^{125}I-fibronectin adjusted to 3×10^5 cpm/ml. Blots were then washed three times with blocking buffer, dried, and exposed to Kodak Blue Brand film in the presence of an intensifying screen for 24 to 36 h at −70°C.

Radioiodination of fibronectin was achieved by using Iodobeads (Pierce Chemical Co., Rockford, Ill.). The labeled protein was separated from free iodine by filtration through Sephadex G-25 (PD-10; Pharmacia LKB Biotechnology Inc.) and equilibrated in 100 mM phosphate-buffered saline, pH 6.5. The specific activity of the iodinated fibronectin was 5×10^5 cpm/μg.

DNA hybridization. Streptococcal chromosomal DNA was digested to completion with restriction enzymes and electrophoresed in 0.6 or 1.0% agarose gels prior to transfer to Hybond membranes (Amersham Corp., Arlington Heights, Ill.). DNA probes, consisting of either restriction fragments or PCR products, were isolated from preparative low-melting-temperature agarose gels and then radiolabeled with [α-^{32}P]dATP, using randomly primed DNA labeling kits (Boehringer Mannheim Corp.).

Blots were incubated in a prehybridization solution (6× SSC [1× SSC is 150 mM NaCl plus 15 mM sodium citrate], 0.5% SDS, 100 μg of denatured salmon sperm DNA per ml, 5× Denhardt's solution, 50% formamide) at 37°C for 2 h and then hybridized with radiolabeled probes in a hybridization solution (6× SSC, 0.5% SDS, 100 μg of denatured salmon sperm DNA per ml, 50% formamide) at 42°C overnight, as described previously (49). Blots that physically mapped the *sof22* and *emm22* loci were washed under high-stringency conditions (twice in 0.1% SSC–0.1% SDS for 30 min at 65°C) that allowed for less than 5% base-pair mismatch. Probes used for restriction mapping *sof22* were inserts from the M13 deletion clones mpSOF22.1d1762 and mpSOF22.2d1160, corresponding to nucleotides 1 to 781 and 1160 to 2543, respectively (refer to Fig. 3 for sequence positions). Blots detecting *sof22* homologs in other group A streptococcal strains were sequentially washed under both high- and low-stringency conditions. Whereas high-stringency washes allowed for less than 5% mismatch, relaxed wash conditions (twice in 0.2% SSC–0.5% SDS for 30 min at 37°C) allowed up to 20% mismatch (42). The probe in the latter experiments was the insert from the M13 clone mpSOF22.2d976, corresponding to an internal *Sau*3AI fragment (bp 976 to 2546).

Nucleotide sequence accession number. The *sof22* sequence accession number in GenBank is U02290.

RESULTS

Extraction of serum opacity activity from *S. pyogenes*. To determine if the opacity reaction is attributable to a specific protein and to judge the feasibility of cloning the gene, SOF was extracted with phosphate buffer at 37°C from whole M type 22 streptococci (strain D734). The extract was analyzed by SDS-PAGE under denaturing conditions and detected by an SOF gel overlay assay. As shown in Fig. 1, this method isolated and detected at least two closely migrating species, with molecular masses of ~100 kDa, that are capable of independently producing an SOF even after the denaturing effects of SDS-PAGE.

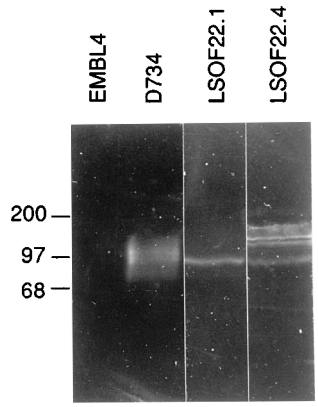

FIG. 1. SOF gel overlay assay of the SOF22 protein from streptococcal strain D734 and recombinant phage lysates. EMBL4, vector lysate; D734, released form of the SOF22 protein extracted from streptococcal strain D734. Recombinant phage lysates: LSOF22.1, *Sau*3AI clone harboring a 2,456-bp insert; LSOF22.4, *Eco*RI clone, containing a 9-kb fragment that holds the whole *sof22* ORF. Recombinant phage crude lysates were loaded onto the gel after treatment with SDS-PAGE sample buffer. Molecular mass standards (in kilodaltons) are indicated.

Cloning of *sof22*. A strategy based on SOF activity was devised to clone the gene responsible for the opacity activity. Accordingly, two streptococcal genomic libraries, containing either *Sau*3AI or *Eco*RI restriction fragments of strain D734 chromosomal DNA, were constructed in the EMBL4 vector and screened for recombinant phage clones exhibiting a serum opacity phenotype. A filter overlay detection method was designed to detect SOF protein expression in phage plaques. A group of four positive clones, LSOF22.1 to LSOF22.3 from the *Sau*3AI library and one (LSOF22.4) from the *Eco*RI library, were plaque purified and confirmed as serum opacity-producing phages. Crude lambda phage lysates of clones LSOF22.1 to LSOF22.3 were all found to contain a recombinant protein (rSOF22) with serum opacity activity that corresponded roughly in size to the SOF extracted from streptococci (100 kDa; Fig. 1). In contrast, phage LSOF22.4 expressed at least four different molecular species, ranging from 100 to ~175 kDa in size, each with serum opacity activity (Fig. 1). An EMBL4 phage lysate did not exhibit any detectable serum opacity activity.

The differences in both the numbers and mobilities of the major reactive species of rSOF22 expressed by recombinant clones LSOF22.1 and LSOF22.4 were found to parallel those in the insert sizes of these clones. The LSOF22.1 clone from the *Sau*3AI library contained a 2.5-kb *Sau*3AI streptococcal genomic fragment flanked by EMBL4 linkers and *Sal*I stuffer fragments. Phage LSOF22.4 harbored an *Eco*RI fragment of ~9.0 kb which contained the *Sau*3AI fragment found in LSOF22.1 (Fig. 2). Thus, with the codon requirements for the expression of a 100-kDa protein, we presumed that phage LSOF22.1 encoded a truncated *sof22* gene and phage LSOF22.4 contained the entire *sof22* locus. Because one species of the SOF protein released from the streptococci comigrated with one of the SOF species expressed by LSOF22.1, we also presumed that at least one form of the SOF molecule released from the streptococci is smaller than the native cell-bound form. For the most part, these presumptions were later verified by sequencing the subclones of these phages.

A physical map of the region around the *sof22* gene (Fig. 2a) was determined from two sets of experiments. One was a restriction analysis of the clones LSOF22.1 and LSOF22.4, and

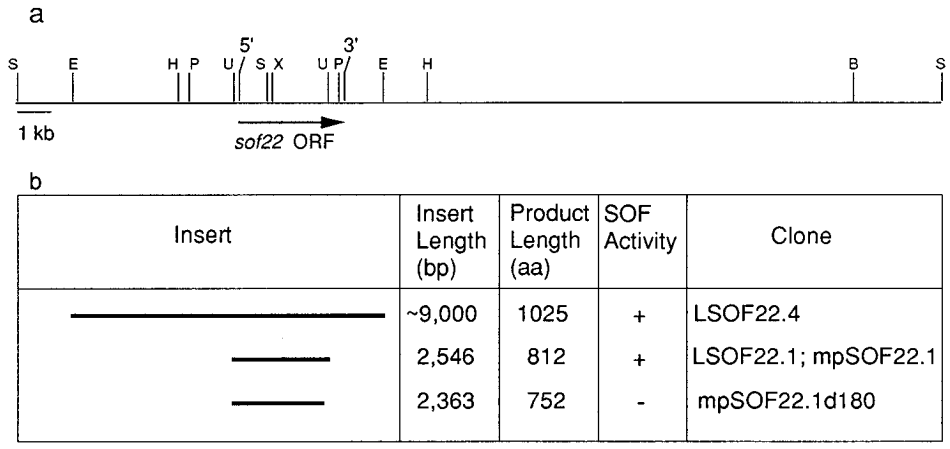

FIG. 2. (a) Restriction map of the *sof22* locus and surrounding regions on the chromosome of D734. The arrow denotes the *sof22* ORF. 5′ and 3′, termini of the *sof22* ORF. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sac*I; X, *Xho*I; U, two *Sau*3AI sites that represent the boundaries of the 2,546-bp fragment cloned in LSOF22.1, mpSOF22.1, and mpSOF22.2. (b) Localization of the region containing the opacity domain of the *sof22* gene. Bars represent the inserts in various clones, aligned with the restriction map in panel a. The numbers next to the bars show the sizes of the inserts in base pairs and the number of residues predicted for the translated SOF22 product contained in the inserts. The SOF activity of the clones is represented by + or −. The clone mpSOF22.1d180 is mpSOF22.1 with a 180-bp deletion.

the other was a Southern blot analysis of D734 genomic DNA digested with various combinations of restriction enzymes, using two probes homologous to the sequences upstream (mpSOF22.2d1160) and downstream (mpSOF22.1d1762) of the *Xho*I and *Sac*I sites (Fig. 3).

Analysis of the *sof22* gene sequence. The strategy used for sequencing *sof22* is described in Materials and Methods. The nucleotide sequence with its deduced amino acid sequence is shown in Fig. 3. The nucleotide sequence begins at the *Sau*3AI site (position 1) and ends at position 3240. Restriction sites *Xho*I, *Sac*I, and *Pst*I and one *Sau*3AI site (the 3′ end of the *Sau*3AI LSOF22.1 insert) are indicated in the figure. The *sof22* open reading frame (ORF) starts at position 113 with the alternate start codon UUG (34) and ends at position 3187. Although there were a few standard start codons (AUG) downstream of the proposed UUG codon, none of the deduced protein sequences beginning at these positions contained a hydrophobic signal sequence that would allow for the translocation of SOF from the cytoplasm (Fig. 4c). The signal sequence cleavage site, predicted by the method of Von Heijne (58), is between A_{34} and S_{35}. On the basis of the proposed signal sequence cleavage site, the mature SOF22 is 991 amino acids in length, with a calculated molecular mass of 109,651 Da. The precursor protein of 1,025 amino acids has a predicted molecular size of 112,735 Da. The calculated size for the mature protein is about 59 kDa smaller than the apparent size of the largest product from the LSOF22.4 clone by SDS-PAGE analysis.

Analysis of the deduced amino acid composition of the mature rSOF22 protein revealed that the most abundant amino acids were lysine (10.5%), serine (9.3%), threonine (8.2%), and glutamine (8.2%), with the majority of threonines and serines clustered in the N-terminal 150 amino acids. Secondary structure analysis by the method of Garnier et al. (19) predicts a protein consisting of 39.3% helix, 28.2% random coils, 18.2% beta sheet, and 14.3% turns (Fig. 4b). Consistent with the low helix potential for the molecule, analysis of the sequence employing the "Matcher" algorithm (14) showed no significant seven-residue periodicity of hydrophobic amino acids (45), excluding the possibility of a fibrillar coiled coil structure.

A block diagram of the SOF22 (Fig. 4a) and conformational analysis (Fig. 4b and c) show some key features of the molecule. These include two strongly hydrophobic regions, one within the N-terminal 30 amino acids of the signal sequence, and one at the C terminus, in the position of the putative membrane-spanning segment (Fig. 4a and c). C terminal to the hydrophobic segment (at the C terminus) are seven charged and three polar residues. Three residues upstream from the C-terminal hydrophobic domain is a hexameric LPXTGX motif (15) with a conservative change of threonine to serine. These C-terminal features are typical for surface proteins found on gram-positive bacteria (Fig. 5) and have been shown to be important for anchoring these molecules within the cell wall (16, 54).

Through the analysis of internal homology, four tandem repeats were detected (residues 820 to 956; Fig. 3 and 6a), which are flanked by two proline-rich stretches (Fig. 4a). The two central repeats of 39 and 40 residues each and the last repeat of 30 residues have the highest identity (59 to 100%), while the first repeat, containing 28 amino acids, has only 36% identity with the consensus central repeats R2 and R3. The N-terminal proline-rich region (residues 780 to 807) is 27 amino acids long and contains 10 prolines. The C-terminal proline region (residues 964 to 982) is 19 amino acids long and contains six prolines. Because of its proximity to the C-terminal anchor motif, the latter region, like other proline-rich regions in surface molecules of gram-positive bacteria, is probably located within the peptidoglycan segment of the cell wall (16, 43).

Localization of the opacity domain of SOF22. The sequence necessary for the SOF activity of SOF22 was defined by deletion analysis. The whole *sof22* gene, coding for a protein of 1,025 amino acids (including the leader sequence), was expressed from clone LSOF22.4 (Fig. 2b and 3). Subclones containing C-terminal deletions were tested for SOF activity. Clones encoding the N-terminal 812 amino acids (LSOF22.1 and mpSOF22.1) were SOF positive. Further 3′-terminal deletions revealed that the next smaller clone (clone mpSOF22.1d180), encoding a 752-residue product, had no SOF activity (Fig. 2b). All smaller clones, each decreasing in size by an average of 150 bp, showed no SOF activity (data not shown). Accordingly, the sequence between amino acids 752 and 812 includes the residues that are necessary (but not sufficient) for the opacity reaction.

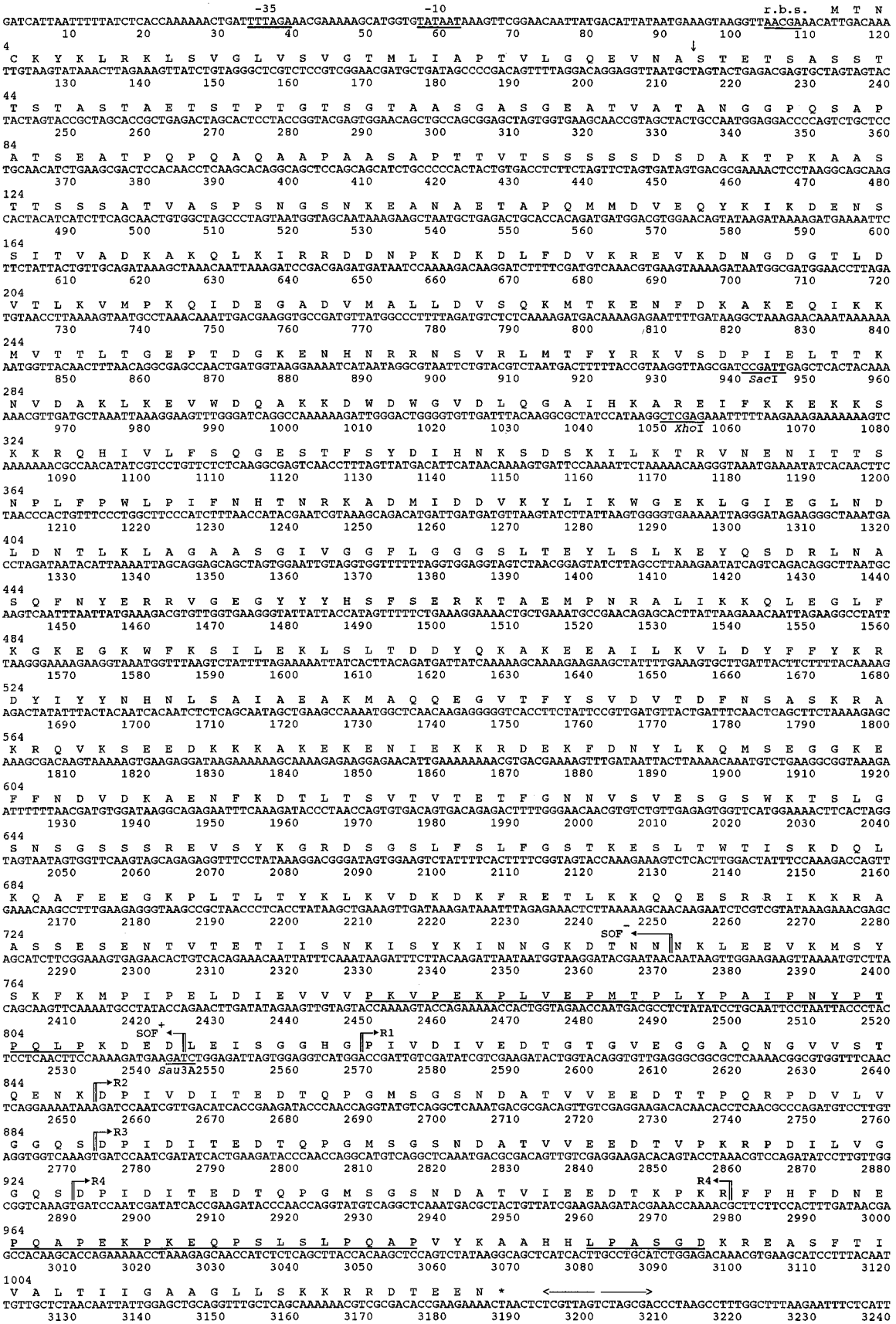
-35
-10
r.b.s.
M T N
SOF
R1
R2
R3
R4
SacI
XhoI
Sau3A

FIG. 3. Nucleotide sequence of *sof22* gene from strain D734 and deduced translation product. The nucleotide sequence begins with the *Sau*3AI site; the 5′ boundary of the 2,546-bp insert in LSOF22.1 clone (Fig. 2b). Underlining indicates the location of the putative promoter boxes (−35 and −10), the ribosome binding site (rbs), and restriction sites *Xho*I, *Sac*I, *Pst*I, and *Sau*3AI (3′ boundary of *Sau*3AI fragment in LSOF22.1). The arrows above the DNA sequence at bp 3193 to 3208 denote the putative transcription termination signal. In the protein sequence underlining identifies the proline-rich regions and the LPASGD motif. The vertical arrow between amino acids A_{34} and S_{35} marks the location of the putative signal sequence cleavage site, and the asterisk identifies the stop codon; R1, R2, R3, and R4 mark the four sequence repeats. Angled arrows labeled SOF^- and SOF^+ identify the C termini of truncated rSOF22 protein produced by clones LSOF22.4/mpSOF22.1 and mpSOF22.1d180, respectively (Fig. 2b).

Fn-binding activity of the SOF22 repeats. Comparison of the mature SOF22 protein with the GenBank translated data base yielded the highest degree of homology with the Fn-binding proteins from staphylococci and streptococci (Fig. 6b). With the exception of FnBA from *S. dysgalactiae*, which is 47% identical to SOF22, the homology within this group of proteins was primarily localized to the Fn-binding repeat regions of the respective molecules. Comparisons of repeats from the different Fn-binding proteins with the repeat R2 of SOF22 show from 32 to 57% identity with sequences in the repeats found in Fn-binding proteins of several gram-positive bacteria (Fig. 6b).

On the basis of the presence of a conserved motif, ED(T/S)($X_{9,10}$)GG($X_{3,4}$)(I/V)D(F/I), found in Fn-binding repeats of gram-positive bacteria (41), we suspected that the SOF22 repeats could potentially bind fibronectin. To address this hypothesis, we subcloned the region of *sof22* encoding repeats R2, R3, and R4 and expressed it in *E. coli*. Blots with ^{125}I-labeled fibronectin showed a positive band in the lysate of the *E. coli* containing the clone, while the control lysate of the strain containing the vector alone gave no Fn-binding signal (Fig. 7).

The Fn-binding repeat fragment migrated on an SDS-polyacrylamide gel as a single band with an apparent relative molecular mass of 30 kDa, a value nearly twice that calculated for the fragment, 16.103 kDa (Fig. 7). A similar discrepancy was reported for a fragment consisting of Fn-binding repeats from protein F (55). In that case, the calculated molecular mass of the product was 23.160 kDa, while the value determined by SDS-PAGE was 41 kDa.

Homology of *sof22* to other streptococcal *sof* genes. Probe mpSOF22.2d976, homologous to a 1,553-bp internal *Sau*3AI fragment, located within the N-terminal region of SOF22 responsible for SOR activity (nucleotides 993 to 2546; Fig. 8b), was used for Southern blot analysis. Chromosomal DNA *Sau*3AI digests of 15 SOF^+ and 15 SOF^- streptococcal strains that belong to different M types were examined in this experiment (Fig. 8; Table 1). This probe detected only the sequence encoding the opacity domain of SOF22 from strain D734, under high-stringency conditions (data not shown). Under reduced stringency (allowing 20% mismatch), hybridization patterns obtained with DNA from non-M22 SOF-producing strains were different from that of D734. Unexpectedly, the signals obtained from strains of the same M type differed among themselves. For instance, both the intensity of the signal and the restriction fragment pattern (length and number) differed for two M type 13 strains. In a second case, two different patterns were obtained within the four M type 22 strains analyzed; B234, B401, and F312 exhibited one pattern, and D734 exhibited another (Fig. 8a; Table 1). Of 15 class I strains analyzed by Southern hybridization, one each of the two M6 and M41 strains and an M12 strain gave positive signals. However, only one of these (M41 strain D421) gave a positive SOF reaction. In summary, despite these few exceptions, the probe comprising the opacity domain of SOF22 distinguishes between SOF^+ and SOF^- organisms.

DISCUSSION

SOF was originally described as an extracellular product of a limited number of strains of group A streptococcal species (59). Subsequent attempts to extract SOF from the streptococcal cell by a variety of means, including use of urea (21, 35, 47, 48), deoxycholate (28, 60), and phage-associated lysin (37),

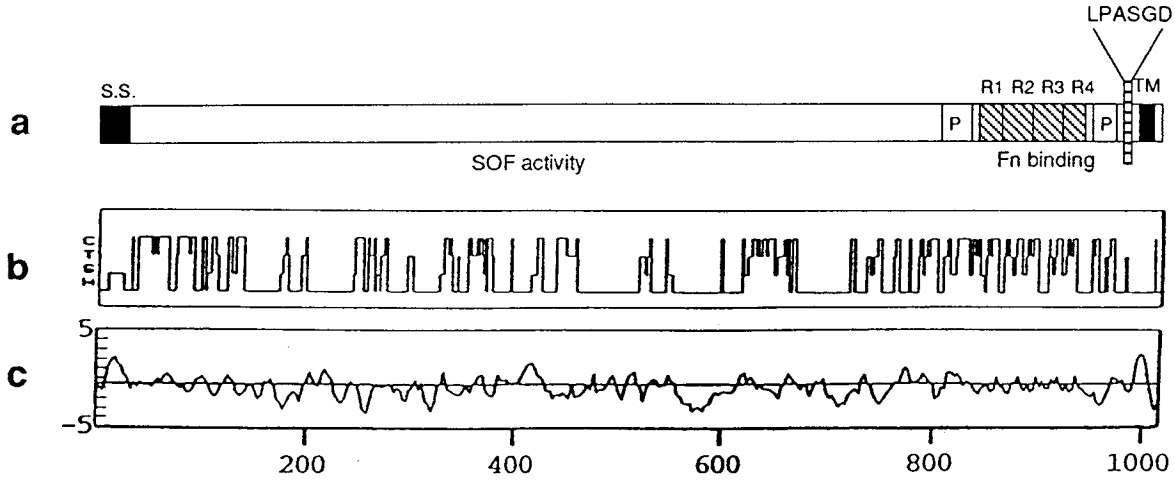

FIG. 4. (a) Block diagram of the SOF22 protein. Symbols: S.S., signal sequence; P, proline-rich region; TM, membrane-spanning segment; R1, R2, R3, and R4, repeats; LPASGD, LPXTGX motif. Boxes: black, hydrophobic sequences; hatched, repeated sequences; white, unique sequences. (b) Secondary-structure prediction. C, random coil; T, β-turn; S, β-sheet; H, α-helix. The four levels of the plot correspond to the likelihood that a region has the indicated secondary structure. (c) Relative hydropathy of SOF22. Hydrophobic domains are located above the central line, and hydrophilic domains are indicated below. The *x* axis for each plot represents the amino acid number of the SOF22 sequence.

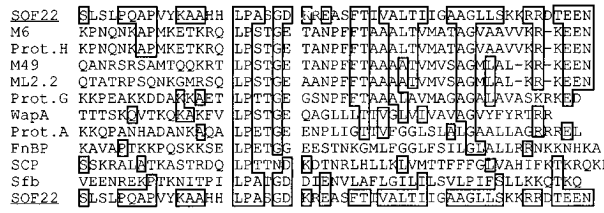

FIG. 5. Comparison of the C-terminal end of the SOF22 protein with the C-terminal anchor region of surface proteins from gram-positive bacteria. M6, M6 protein from *S. pyogenes* (29); Prot. H, protein H from *S. pyogenes* (20); M49, M49 protein from *S. pyogenes* (23); ML2.2, M-like protein 2.2 from M2 *S. pyogenes* (7); Prot. G, protein G from group G streptococci (33); WapA, wall-associated protein A from *Streptococcus mutans* (12); Prot. A, protein A from *S. aureus* (22); FnBP, Fn-binding protein from *S. aureus* (56); SCP, C5a peptidase from *S. pyogenes* (10); and Sfb, Fn-binding protein from *S. pyogenes* (57). Note the conservative replacement of T with S in the LPXTGX motif of SOF22. The LPXTGX motif is separated from the rest of the sequence with a space. Amino acids identical with those in SOF22 are boxed. Dashes represent gaps introduced to maximize identities.

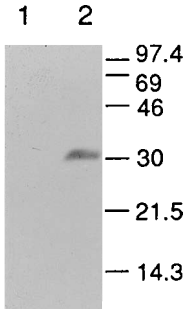

FIG. 7. Fn-binding activity of an *E. coli* clone expressing SOF22 repeats. Whole-cell lysates obtained from *E. coli* transformed with vector pUC9.2 (lane 1) or with clone pFNBR22.1 containing repeats R2 to R4 (lane 2) were subjected to SDS-PAGE, blotted onto nitrocellulose, and treated with ^{125}I-fibronectin as described in Materials and Methods. Molecular mass standards are indicated in kilodaltons.

showed that SOF activity was also predominantly present in the cell wall and membrane fractions. We were able to confirm that opacity factor activity is present in both lysin digest supernatants and the membrane pellet (unpublished data). Furthermore, we show that serum opacity activity is both found in streptococcal culture supernatant and extractable from streptococcal cells with neutral buffer. In this regard, the expression of SOF is in sharp contrast to earlier findings with other gram-positive surface molecules such as M protein from *S. pyogenes* and protein A from *S. aureus*. These proteins are not generally found in the growth media and are not easily removed from the cell with chaotropic agents (13, 54). A notable exception to this rule is the type 1 M protein of strain AP1 (2).

SOF22 exhibits C-terminal features necessary for proper translocation to the cell surface: an LPXTGX motif with a conservative replacement of threonine to serine followed by a hydrophobic region and a short, charged C terminus (54). It has been proposed that this conserved hexameric sequence may represent a site for attachment to and/or cleavage from the peptidoglycan of the gram-positive cell wall (44, 54). In turn, this hypothesis offers an explanation for a released SOF22 molecule with a smaller molecular weight than that deduced from the gene sequence; release of SOF may be the result of a specific proteolytic cleavage within the C-terminal

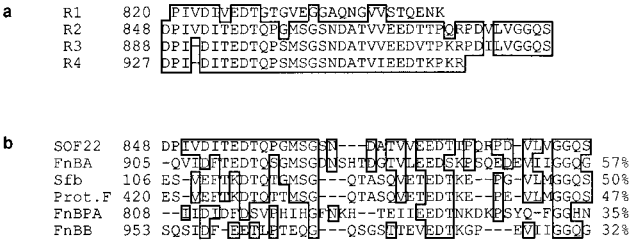

FIG. 6. (a) SOF22 repeats. The four sequence repeats of SOF22 are aligned, and residues that are identical in two or more repeats are boxed. Dashes are inserted to maximize the identities. (b) Alignment of the SOF22 repeat region with repeated motifs from gram-positive Fn-binding proteins. FnBA and FnBB, Fn-binding proteins A and B from *S. dysgalactiae* (38); Sfb, streptococcal Fn-binding protein from an M23 strain of *S. pyogenes* (57); Prot. F, streptococcal Fn-binding protein from an M6 strain of *S. pyogenes*; FnBPA, Fn-binding protein A from *S. aureus* (56). Amino acid identities between the SOF22 repeat and one or more aligned proteins are boxed. The number preceding each line represents the position of the repeat motif in the amino acid sequence; the percent identity is calculated with respect to the SOF22 sequence. Dashes represent gaps introduced to maximize identities.

region of this molecule. Although this is a plausible mechanism for this size discrepancy, clear evidence of sequence and/or enzyme specificity has not yet been established. Alternatively, multiple forms of SOF may be the result of differential expression of SOF from either one genetic locus or two related loci. With regard to the latter possibility, tandem arrays of cell surface binding protein genes have been described, including M and M-like genes of group A streptococci (23) and Fn-binding protein genes in both *S. dysgalactiae* (38) and *S. aureus* (32).

Relative to other cell surface proteins of *S. pyogenes*, which range in size from a reported low of ~42 kDa for PAM, plasminogen-binding protein (4), to a high of ~125 kDa for SCP, C5a peptidase (10), the SOF22 protein, with a deduced molecular mass of ~110 kDa, is among the largest reported. Like other streptococcal cell surface proteins, SOF22 is composed of three functional domains: tandem repeats sandwiched between a variable N terminus and the cell attachment domain. The N-terminal domain, which constitutes about 80% of the molecule, exhibits SOF activity. Our C-terminal nested deletion analysis indicates that the C-terminal boundary of this domain is between residues 752 and 812, a portion which comprises one of the two proline-rich regions. The extent of the N-terminal region necessary for this activity remains to be defined. The tandem repeat region is composed of four repeats, a portion of which (R2, R3, and R4) is sufficient for Fn-binding activity.

The organization of SOF is strikingly similar to that of the Fn-binding proteins from gram-positive bacteria (32, 38, 55–57). In each example, an extensive N-terminal domain lies adjacent to the homologous Fn-binding repeats. In contrast to other Fn-binding proteins, the nonrepetitive N-terminal region of SOF22 constitutes a catalytic domain of a defined character. In this regard, SOF22 is more akin to other streptococcal proteins, C5a peptidase (SCP) from *S. pyogenes* (10) and the glucosyltranferase GTF-I of *S. sobrinus* (1), which exhibit a large N-terminal domain with catalytic activity linked to C-terminal repeats. However, SOF22 is the only Fn-binding protein described to date in which the functions of both the N-terminal and repeat domains have been identified.

In the group of three sequenced Fn-binding proteins from *S. pyogenes*, SOF22 is distinct from the other two, Sfb and protein F. These two proteins, isolated from class I (SOF$^-$) strains, are closely related to each other (55, 57). In contrast, SOF22 is homologous to them only in the repeat region, with an identity of 50% (Fig. 6b). Furthermore, SOF22 differs from protein F by having a significantly larger N-terminal domain that exhibits

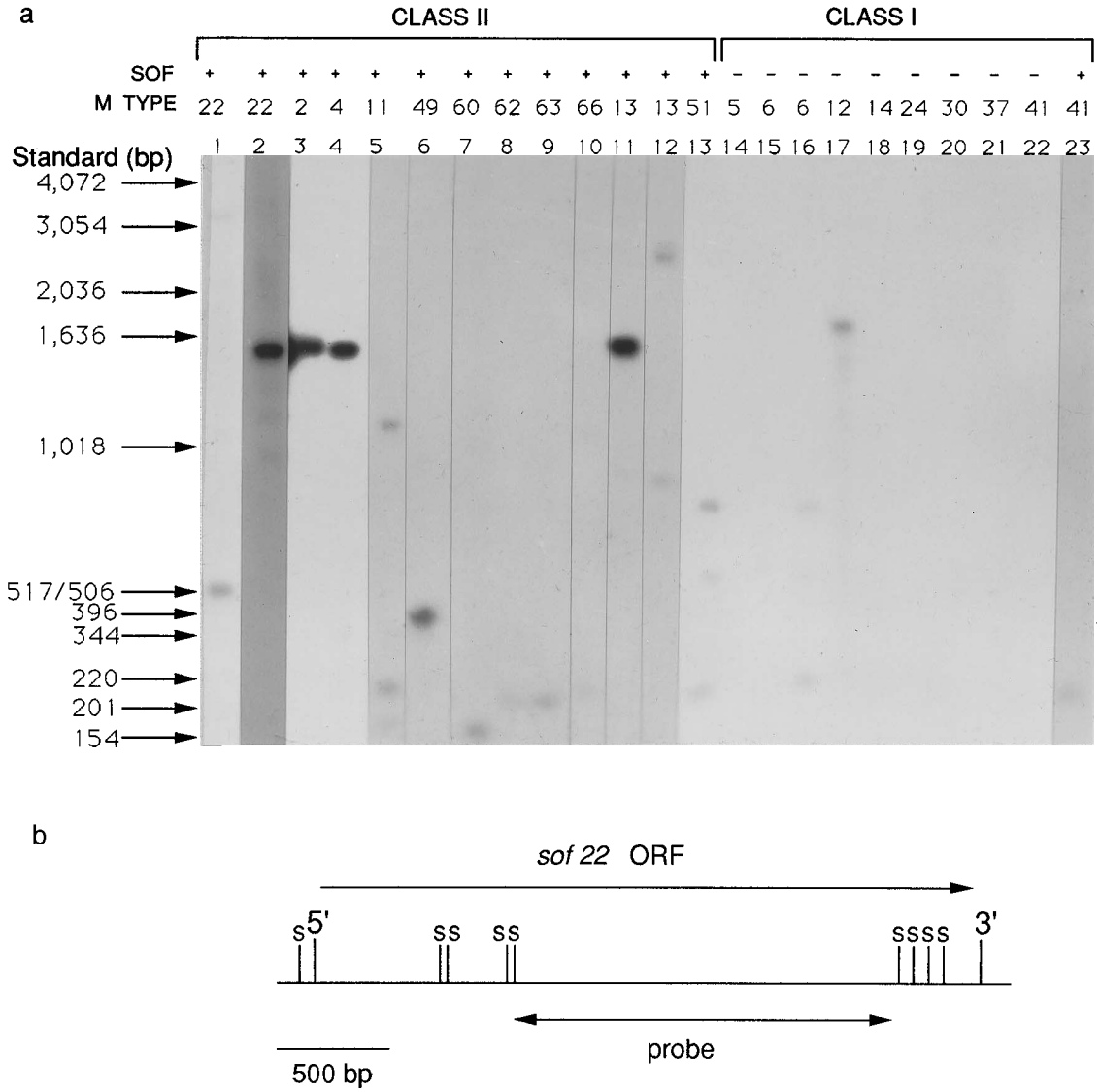

FIG. 8. Relationship of *sof22* to other streptococcal *sof* genes. (a) Autoradiogram of *Sau*3AI hybridization patterns of representative streptococcal strains of several class I and class II serotypes. Washes were done under conditions that allow 20% mismatch. Probe mpSOF22.2d976 was cloned from M22 strain D734. This probe is homologous to the *sof22* intragenic *Sau*3AI fragment spanning nucleotides 993 to 2546 of the sequence shown in Fig. 3. The exposure of the autoradiogram was 10 h, with the exception of the D734 lane, which was exposed for 30 min to decrease the intensity of the signal. Lanes: 1, B234 (M22); 2, D734 (M22); 3, B344 (M2); 4, B512 (M4); 5, D691 (M11); 6, B737 (M49); 7, D398 (M60); 8, A956 (M62); 9, D495 (M63); 10, D794 (M66); 11, D474 (M13); 12, D742 (M13); 13, D976 (M51); 14, B788 (M5); 15, D471 (M6); 16, S43 (M6); 17, A374 (M12); 18, D469 (M14); 19, 1RP284 (M24); 20, D617 (M30); 21, D466 (M37); 22, D463 (M41); 23, D421 (M41). Five class I strains that were negative are not included, and only one of three M22 strains with the same pattern is shown. (b) The *Sau*3AI map of the *sof22* locus from strain D734 showing the location of the probe used in panel a. S, *Sau*3AI restriction site; 5′ and 3′, limits of the *sof22* ORF.

the opacity activity. Thus, SOF22 may be considered a novel type of Fn-binding protein of *S. pyogenes*, found in class II (SOF^+) group A streptococci.

Recent studies on SOF activity suggested that it was a pepsin-like aspartyl protease, specifically cleaving Apo AI of high-density lipoprotein (51, 52). In preliminary studies examining the SOF activity of the opacity-active recombinant fragment of SOF22 (residues 1 to 812), we were able to confirm its cleavage effect on ApoA1 by observing a size shift of Apo AI by SDS-PAGE (unpublished data). However, sequence analysis was unable to verify that SOF is a pepsin-like aspartyl protease since it lacks the two consensus sequences, FDTGS and DTGTS (separated by approximately 200 amino acids), common in this class of enzymes (3).

Reports describing a correlation between M type and the ability to produce SOF (6, 60), as well as the cosegregation of SOF and M protein (11, 25), suggested either a functional association between these two phenotypes or a genetic linkage of their coding sequences. Consistent with both models was the finding that SOF^+ strains were amenable to typing by virtue of SOF specificity that paralleled that of M type (30, 40, 60). Thus, it is noteworthy that sequence analysis of SOF22 suggests little relationship between SOF and all M proteins thus far described. The issues of cosegregation and covariation of M

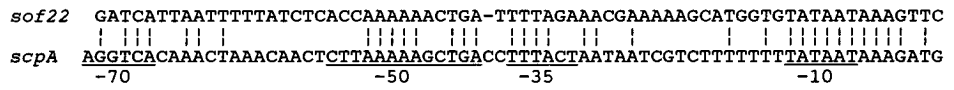

FIG. 9. Alignment of the promoter regions of *scpA* and *sof22*. The 5′ noncoding sequences of *scpA* (10) and *sof22* were compared manually. One gap (dash) was introduced into the *sof22* sequence to increase the identities. The putative −35 and −10 boxes and VirR elements are underlined.

protein and SOF occurring as a function of genetic linkage have been addressed by Southern analysis of the parental M22 strain. Preliminary results obtained with *sof22* and *emm22* probes showed no cross-hybridization of identical restriction fragments (unpublished data). Restriction maps of the *sof22* and *emm22* loci indicate that these two genes are not closely linked and position the *emm22*-like gene at least 15 kb from *sof22* (unpublished data).

Another model that would account for the reported mutual loss of M and SOF activities could be the existence of a common transcriptional regulatory molecule, the mutation of which would lead to the observed SOF^- M $protein^-$ phenotype. One candidate for such a role is VirR/Mry, reported to positively regulate *emm*, *enn*, *fcrA*, and *scpA* in *trans* (46). Support for this hypothesis is seen in the sequence of the *sof22* promoter, which has homology with the upstream regulatory region of *scpA*. A comparison of the *sof22* promoter with that of *scpA* (Fig. 9) shows homology within the −50 and −70 elements of the proposed *vir* regulon consensus sequence as well as within the −35 and −10 boxes. The −35 box of *sof22* differs from the *vir* consensus sequence in the fifth and sixth positions, which are also the least conserved among the *vir*-regulated genes (46). On the basis of these observations, we anticipate that *sof22* gene expression would be altered in an *mry* (*virR*)-deficient genetic background.

Inhibition of SOF activity has been used as a complementary method for typing SOF^+ strains of *S. pyogenes* (40). In a comprehensive serological study (30) it was shown that the inhibition of the opacity reaction parallels the M-type specificity of the typing antisera. This suggests that variability within the opacity activity domain of SOF is responsible for the serotype-specific inhibition of the opacity reaction. This hypothesis was addressed by employing an intragenic probe specific to the opacity domain in Southern analysis of 15 SOF-producing strains belonging to 11 different M serotypes. In support of the notion that the opacity domain is variable, we found that the *Sau*3AI fragment (bp 993 to 2543) failed to show >95% homology with any of the strains tested except the parental M22 strain D734 (Fig. 8a). However, under conditions that allowed up to 20% mismatch of this probe, the restriction patterns and signal intensities varied among the different M types. The only exceptions were strains A956 (M62) and D459 (M63), which both showed the same banding pattern. Surprisingly, restriction polymorphism was also detected among strains of the same M type. Also surprising was the finding of three positive strains among the 15 class I strains tested (Fig. 8a and Table 1). This suggests that *sof* or *sof*-like sequences may exist in some strains that belong to this class of group A streptococci. On the other hand, the fact that the majority of the strains from this class did not show a signal suggests that *sof*-related sequences are either absent or of too low homology to be identified by the method used. The fact that all class II SOF^+ strains tested gave a positive signal with the probe derived from *sof22* suggests that these *sof* genes are closely related and probably originate from one ancestral gene.

Although preliminary, the implications of the Southern analysis are twofold: (i) SOF is highly variable among SOF-producing strains, similar to M protein which varies among more than 80 serotypes, yet maintains a common antiphagocytic activity, and (ii) SOF is able to retain a common enzymatic function despite extensive antigenic variation. Thus, having in mind that M protein is divided into a variable type-specific region and a conserved region (31), it is of interest to determine if the architecture of SOF has evolved similarly. Further clarification of the boundaries of the variable and conserved domains of SOF will require additional analysis of other SOF^+ strains, using specific DNA probes to defined regions of the opacity domain and the Fn-binding repeats. Likewise, opacity neutralization studies with monoclonal antibodies directed against SOF will facilitate definition of the antigenic and functional domains of the SOF protein. Additional sequence data for other SOF molecules will also help to elucidate the mechanism of creating antigenic diversity of SOF.

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