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Heterogeneity of the Streptokinase Gene in Group A Streptococci

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A molecular epidemiological study was conducted to determine the distribution of the streptokinase gene in group A streptococcal strains of different M types and in other streptococcal species. Plasmid pNC1, containing only the internal coding sequence of the streptokinase gene from group C streptococcal strain H46A, was used as a DNA probe in colony and Southern hybridization experiments. Only the pathogenic group A, C, and G streptococci contained a streptokinase gene; 12 other Lancefield group strains did not. A total of 134 group A strains, including 61 M types and 6 T types, were tested. Although only 62% (83 of 134) of the strains tested showed positive streptokinase activity by the casein-plasminogen overlay assay, all strains contained the streptokinase gene as evidenced by strong hybridization with the pNC1 probe. Southern blot DNA hybridizations were carried out with 101 strains of group A streptococci. The restriction enzymes *Hin*dIII and *Hae*III were used to digest the genomic DNA. Six hybridization patterns were observed after *Hin*dIII digestion. Double hybridization bands appeared in all of the patterns, which indicated the existence of a highly conserved *Hin*dIII site. More complex hybridization results were obtained after *Hae*III digestion. Twelve hybridization patterns were observed; three were characterized by a single hybridization band, and nine were characterized by double bands. Variations in hybridization patterns were observed in strains of both the same and different serotypes. The overall results at the gene level indicate that there is considerable heterogeneity among the streptokinases of group A streptococci, consistent with previous findings of immunological and chemical differences among streptokinases of group A streptococci.

The pathogenic streptococci produce a number of extracellular products, one of which is streptokinase. The primary biological activity known to be associated with streptokinase is the specific activation of human plasminogen (17). Streptokinase binds to plasminogen stoichiometrically (1) and triggers a conformational change and self-cleavage that results in the formation of the serine protease plasmin. Once present, plasmin can enzymatically cleave fibrin and fibrinogen. Streptokinase has been referred to as a streptococcal spreading factor (6) because of its ability to effect the lysis of clots and fibrin deposits which limit areas of infection.

Immunological cross-reactivities among streptokinases produced by different streptococcal strains are normally observed; however, not all streptokinases are identical. As early as 1953, Weinstein demonstrated that streptokinases produced by different strains of group A streptococci were antigenically different (16). Two streptokinases, SK-A and SK-B, with different mobilities on gel electrophoresis were isolated from two group A streptococcal strains by Dillon and Wannamaker (2). In a more detailed analysis of the streptokinases produced by six group A strains, Gerlach and Köhler reported on streptokinases with different isoelectric points and different amino acid compositions (3, 4). Isostreptokinases, with similar molecular weights but different isoelectric points, were also identified in a type 1 group A strain (5). Recently, monoclonal antibodies have been used to demonstrate the existence of antigenic variations among streptokinases of different origins (K. H. Johnston, E. S. Kulisek, J. B. Zabriskie, A. M. Bergholm, and S. E. Holm, Abstr. Proc. Lancefield Int. Symp. Streptococci Streptococcal Dis., 1987).

The streptokinase gene (*skc*) from a group C streptococcal strain has been cloned and sequenced (10, 12). In addition, altered forms of *skc* which contain only internal sequences have been constructed (11). In this study, we used a specific internal probe of *skc* to examine the heterogeneity of streptokinases at the molecular level among various streptococci.

MATERIALS AND METHODS

Bacterial strains. A total of 134 group A streptococcal strains were used. Among them, 8 strains were clinical isolates from Canada (M types 1, 2, 4, 9, 12, 22, 27, and PT2841); 8 were clinical isolates from England (M types 6, 12, 28, 41, 73, 76, 78, and 1/80); 1 M type 22 strain was a clinical isolate from the Federal Republic of Germany; 1 M type 23 strain was a clinical isolate from France; 2 strains were clinical isolates from Japan (M types 8 and 63); 14 were clinical isolates from New Zealand (M types 4, 22, 49, 53, 57, 60, and 75); 10 were clinical isolates from Thailand (T types 3/13/B3264, 4/28, 5/12/27, 6, and 8/25/Imp19); 6 were obtained from the Centers for Disease Control, Atlanta, Ga. (M types 3, 12, and 15 and T types 2 and 3/13/B3264); 11 strains of M type 49 were from Minnesota; 73 strains were obtained from the collection from Rockefeller University, generously provided by K. Johnston (M types 3, 4, 5, 6, 8, 9, 11, 12, 13, 14, 15, 17, 18, 22, 23, 24, 25, 28, 29, 30, 31, 32, 33, 34, 36, 37, 38, 39, 40, 41, 42, 43, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 66, and 67 and T type 3). Two nontypable clinical isolates were also used. The sources and numbers of strains from other groups were reported previously (8).

DNA probe. Plasmid pNC1, with an insert of 1,056 base pairs (bp) and containing only the internal coding sequence of the *skc* gene from group C streptococcal strain H46A, was used as a DNA probe in all DNA hybridization experiments. A nick translation kit purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., was used to label plasmid pNC1 with [α-^{32}P]dATP. The labeling procedure

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TABLE 1. DNA hybridization with pNC1 DNA probe

Strains	Hybridization	
	Positive	Negative
Group A streptococci	M1, -2, -3, -4, -5, -6, -8, -9, -11, -12, -13, -14, -15, -17, -18, -22, -23, -24, -25, -27, -28, -29, -30, -31, -32, -33, -34, -36, -37, -38, -39, -40, -41, -42, -43, -46, -47, -48, -49, -50, -51, -52, -53, -54, -55, -56, -57, -58, -59, -60, -61, -62, -63, -66, -67, -73, -75, -76, -78, -1/80, -PT2841; T2, -3, -3/313/B3264, -4/28, -5/12/27, -6, -8/25/Imp19; nontypable strains	None
Other groups of streptococci	C, G	B, D, F, H, K, L, M, N, O, P, R, U, *Streptococcus pneumoniae*
Other bacteria	None	*Peptostreptococcus* spp., *Staphylococcus aureus*

was carried out according to the instructions of the manufacturer.

Genomic DNA isolation. Cultures of group A streptococcal strains were grown overnight in 10 ml of Todd-Hewitt broth at 37°C; 20 ml of prewarmed Todd-Hewitt broth was then added, and the cultures were allowed to grow for an additional hour. Glycine powder was added to the cultures to a final concentration of 0.5%, and the cultures were allowed to grow for another hour at 37°C. After centrifugation, the cells were suspended in 2 ml of lysis solution (mutanolysin [5 U/ml], lysozyme [5 mg/ml], 12.5% sucrose, 10 mM Tris [pH 7.6], 1 mM EDTA) and incubated at 37°C with gentle shaking for 2 h. The cell suspensions were then treated with protease at a concentration of 100 μg/ml and incubated at 37°C with gentle shaking for 30 min. To rupture the cells, 40 μl of 20% *n*-lauroyl sarcosine solution was added to each cell suspension. A clearing appearance indicating cell lysis usually resulted in 15 min at 37°C. The DNA solutions were then extracted with phenol and chloroform and precipitated with ethanol. Each DNA pellet was suspended in 100 μl of TE buffer (13), incubated with RNase A (100 μg/ml) at 65°C for 30 min, and stored at 4°C. A 10-μl amount of the DNA preparation was usually used for Southern blot DNA hybridization.

Colony hybridization. Group A streptococcal strains were inoculated within a 25-mm^2 area on a Todd-Hewitt agar plate supplemented with 1% horse serum and 0.5% glycine. Usually 50 strains, including positive and negative controls, were inoculated on one agar plate. The inoculated plates were incubated at 37°C for 60 min before being overlaid with a piece of sterile nitrocellulose filter. The plates were then inverted and incubated at 37°C overnight. The nitrocellulose filters were carefully removed from the plates and placed on top of 3MM filters (Whatman, Inc., Clifton, N.J.) saturated with mutanolysin (5 U/ml) and lysozyme (5 mg/ml), incubated at 37°C for 2 h, transferred to 3MM filters saturated with protease (5 mg/ml), and incubated at 37°C for 60 min. The nitrocellulose filters were then treated for the indicated times at room temperature with sets of 3MM filters saturated with 10% sodium dodecyl sulfate (5 min), alkaline solution (0.5 N NaOH, 1.5 M NaCl) (10 min), neutralization solution (1.5 M NaCl, 1 M Tris [pH 8.0]) (10 min), and 2× SSPE (0.3 M NaCl, 20 mM NaH_2PO_4 [pH 7.4], 2 mM EDTA) (10 min). The nitrocellulose filters were dried at 80°C for 30 min to fix the DNAs.

Southern blot DNA hybridization. Each genomic DNA preparation from the various strains of group A streptococci was digested separately with *Hae*III and *Hin*dIII. The digested DNAs were separated in a 0.8% agarose gel in 0.5× Tris borate buffer (13) and transferred to nylon membranes by Southern blot (15) or Vacublot (14) as stated in the literature. The membranes were dried at 80°C for 30 min to fix the DNAs. All membranes were hybridized with the DNA probe pNC1 (11), using the procedure described by Maniatis et al. (13). The hybridization and washing temperatures were 65°C.

Streptokinase assay. A 10-ml soft agarose mixture consisting of 0.8% agarose, 1% skim milk, 500 μg of human plasminogen, 150 mM NaCl, and 50 mM Tris (pH 8.1) was poured on top of plate cultures of the tested strains. The plates were incubated at 37°C for 2 to 8 h. Positive streptokinase activity was indicated by a clearing zone around the colonies.

RESULTS

Hybridization of *skc* to streptococcal strains. The DNA probe used in this study was pNC1, a 1,056-bp internal fragment of *skc* (11). All strains were initially tested by the colony hybridization method, and the results were then confirmed by the Southern blot DNA hybridization procedure. Only group A, C, and G strains of 15 Lancefield groups tested gave positive hybridization signals (Table 1). Weak hybridizations were observed with group B and P strains and one group D strain; no hybridization was observed with *Streptococcus pneumoniae*, *Staphylococcus aureus*, or any of the anaerobic streptococci tested. All of these strains were also tested for ability to produce an active extracellular streptokinase; only the group A, C, and G strains showed positive activity in the casein-plasminogen assay.

Hybridization of *skc* to group A strains. All group A strains tested (from 61 M types and 6 T types as well as 2 nontypable strains) gave intense hybridization signals with the *skc* probe. When tested for the ability to produce streptokinase, only 62% (83 of 134) of all group A strains proved positive as detected by the casein-plasminogen assay method.

Digestion of DNA from various strains with *Hin*dIII followed by Southern blot hybridization with the *skc* probe produced six different hybridization patterns (Fig. 1, Table 2). All of the strains exhibited two hybridization bands, which indicated the presence of an internal *Hin*dIII site in all M- and T-type strains tested. The predominant hybridization pattern, consisting of bands at 2.6/2.5 kilobases (kb), was found among 34 M-type and 6 T-type strains. The other five hybridization patterns and their serotype strains were as follows: 4.3/2.6 kb (16 M and 1 T type), 2.6/1.9 kb (7 M

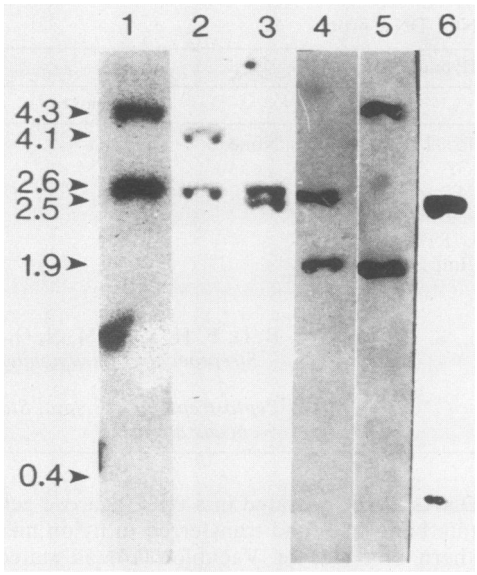

FIG. 1. Autoradiogram of *Hin*dIII hybridization patterns. Lane 1, 4.3/2.6 kb (serotype M3, -4, -9, -11, -12, -14, -15, -18, -32, -39, -40, -47, -50, -60, -62, and -75 and T3/13/B3264 strains); lane 2, 4.1/2.6 kb (serotype M23 strain); lane 3, 2.6/2.5 kb (serotype M4, -6, -8, -12, -13, -14, -22, -27, -28, -29, -33, -36, -37, -38, -41, -42, -43, -46, -49, -51, -52, -53, -56, -58, -59, -61, -63, -66, -67, -73, -76, -78, -1/80, and -PT2841 strains and T2, -3/13/B3264, -4/28, -5/12/27, -6, and -8/25/Imp19 strains); lane 4, 2.6/1.9 kb (serotype M2, -5, -24, -25, -30, -31, -34, and -57 strains); lane 5, 4.3/1.9 kb (serotype M17 strain); lane 6, 2.6/0.4 kb (serotype M1, -49, and -55 strains).

types), 2.6/0.4 kb (3 M types), 4.3/1.9 kb (2 M types), and 4.1/2.6 kb (1 M type).

Digestion of DNA from various strains with *Hae*III followed by Southern hybridization with the *skc* probe revealed

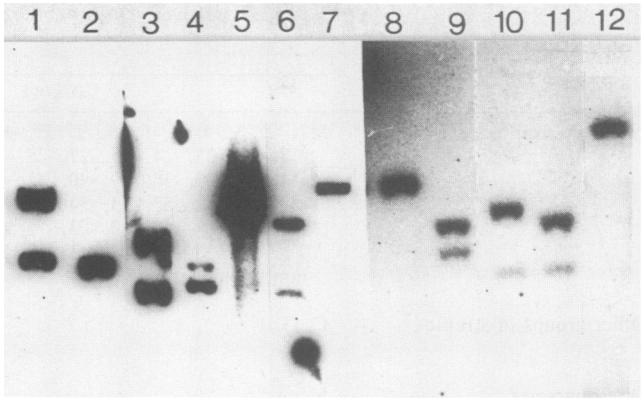

FIG. 2. Autoradiogram of *Hae*III hybridization patterns. Lane 1, 1.3/0.8 kb (serotype M63 strain); lane 2, 0.8 kb (serotype T4/28 strain); lane 3, 1.0/0.7 kb (serotype M43 and -52 strains); lane 4, 0.8/0.7 kb (serotype M51 strain); lane 5, 1.2/0.12 kb (serotype M55 strain); lane 6, 1.2/0.7 kb (serotype MPT2841 strain); lane 7, 1.6/0.4 kb (serotype M53 strain); lane 8, 1.6 kb (serotype M1, -11, -18, -24, -33, -34, -37, -49, -53, -56, -57, and -76 strains); lane 9, 1.2/1.0 kb (serotype M2, -3, -4, -9, -12, -32, -36, -40, -42, -47, -49, and -75 strains); lane 10, 1.4/0.8 kb (serotype M38 and -41 strains and T4/28 and -6 strains); lane 11, 1.2/0.8 kb (serotype M6, -50, -59, -60, -62, and -73 strains); lane 12, 2.1 kb (serotype M3, -5, -4, -8, -13, -14, -15, -17, -22, -23, -25, -27, -28, -29, -30, -31, -39, -46, -58, -61, -66, -67, -78, and -1/80 strains and T2 and -3/13/B3264 strains).

12 hybridization patterns (Fig. 2, Table 2). Three patterns consisted of a single hybridization band: 2.1 kb (23 M and 2 T types), 1.6 kb (12 M types), and 0.8 kb (1 T type). The other nine patterns consisted of two hybridization bands, which indicated the presence of an internal *Hae*III site. The hybridization patterns included 1.2/1.0 kb (12 M and 1 T type), 1.2/0.8 kb (6 M and 2 T types), 1.4/0.8 kb (2 M types), and 1.3/0.8, 1.2/0.7, 0.8/0.7, 1.6/0.4, and 1.2/0.12 kb.

TABLE 2. Hybridization patterns generated from *Hin*dIII and *Hae*III digestion

*Hae*III (kb)	*Hin*dIII (kb)					
	2.6/2.5	4.3/2.6	2.6/1.9	4.3/1.9	2.6/0.4	4.1/2.6
1.2/1.0	M12, -36, -42, -49; T8/25/Imp19	M3, -4, -9, -12, -32, -40, -47, -75	M2			
2.1	M4, -8, -13, -14, -22, -27, -28, -29, -46, -58, -61, -66, -67, -78, -1/80; T2, -3/13/B3264	M3, -14, -15, -39; T3/13/B3264	M5, -25, -30, -31	M17		M23
1.6	M33, -37, -53, -56, -76	M11, -18	M24, -34, -57		M1, -49	
1.2/0.8	M6, -59, -73; T3/13/B3264, -5/12/27	M50, -60, -62				
1.4/0.8	M38, -41; T4/28, -6					
1.3/0.8	M63					
1.2/0.7	MPT2841					
1.0/0.7	M43, -52					
0.8/0.7	M51					
1.6/0.4	M53					
0.8	T4/28					
1.2/0.12					M55	

TABLE 3. Hybridization patterns of strains with the same M or T type

Serotype	Hybridization (kb)		Source (no.) of strain(s)
	*Hae*III	*Hin*dIII	
M3	2.1	4.3/2.6	United States (1)
	1.2/1.0	4.3/2.6	Trinidad (1)
M4	2.1	2.6/2.5	Canada (1)
	1.2/1.0	4.3/2.6	New Zealand (1)
M12	1.2/1.0	4.3/2.6	England (1) Canada (1) United States (2)
	1.2/1.0	2.6/2.5	United States (2)
M14	2.1	4.3/2.6	Guadeloupe (1)
	2.1	2.6/2.5	United States (1)
M49	1.2/1.0	2.6/2.5	APSGN (14)[a]
	1.6	2.6/0.4	Scarlet fever (2)[b]
M53	1.6/0.4	2.6/2.5	New Zealand (1)
	1.6	2.6/2.5	New Zealand (1)
T3/13/B3264	1.2/0.8	2.6/2.5	Thailand (1)
	2.1	2.6/2.5	Thailand (2)
	2.1	4.3/2.6	United States (1)
T4/28	1.4/0.8	2.6/2.5	Thailand (1)
	0.8	2.6/2.5	Thailand (1)

[a] Originated from New Zealand, United States, Chile, Czechoslovakia, Trinidad, England, Egypt, Israel, and Columbia.
[b] Originated from New Zealand.

The hybridization pattern of each M- or T-type strain was not always the same (Table 3). For example, among four M type 12 strains obtained from different sources there were three hybridization patterns, and among 16 M type 49 strains there were two patterns. Interestingly, one of the hybridization patterns was associated with 14 strains (obtained from nine countries) isolated from patients known to have acute poststreptococcal glomerulonephritis (APSGN). The other hybridization pattern was associated with 2 M type 49 strains isolated from patients with scarlet fever. These results indicate that there is heterogeneity of the streptokinase gene even among strains of the same serotype. Whether a particular hybridization pattern among such strains can be reliably associated with a particular disease is yet to be determined.

DISCUSSION

The results from this study indicate that the streptokinase gene and ability to produce extracellular streptokinase are found only in the pathogenic group A, C, and G streptococci. There appears to be considerable heterogeneity of the streptokinase genes among these organisms, an observation that is supported by the finding that there is only 90% identity between the nucleotide sequences from group A and group C streptokinase genes (T. T. Huang, H. Malke, and J. J. Ferretti, Mol. Microbiol., in press). Among group A strains, there is also significant heterogeneity of the streptokinase gene in strains of both the same and different serotypes. This heterogeneity at the gene level provides an explanation for the previous findings of immunological and chemical differences of streptokinases reported by a number of investigators (2–5, 16; Johnston et al., Abstr. Lancefield Int. Symp. Streptococci Streptococcal Dis., 1987). Of the six hybridization patterns generated from *Hin*dIII digestion, each had at least one internal *Hin*dIII site. Whether the internal *Hin*dIII sites of these patterns are located at the same positions relative to the coding sequences cannot be resolved without the actual sequencing data. However, the location of the internal *Hin*dIII sites on both the sequenced *ska* gene (the streptokinase gene from M type 49 group A streptococcal strain NZ131; Huang et al., in press) and the *skc* gene are 826 bp (12) downstream from the translation initiation codon. Moreover, the partial sequencing data of the cloned group A streptokinase gene from an M type 1 strain reveal the presence of an internal *Hin*dIII site at the same position as that of *ska* and *skc*. The perfect alignment of the internal *Hin*dIII sites between *ska* and *skc* indicates the preservation of this *Hin*dIII site between different groups and probably within group A strains as well.

The restriction map of pMF1 (10), which carries *skc*, reveals the *Hin*dIII hybridization pattern for group C streptococcal strain H46A to be 2.6/1.9 kb, with the 1.9-kb fragment preceding the 2.6-kb fragment and the internal *Hin*dIII site lining up with that of *ska*. The seven different M types (M2, -24, -25, -30, -31, -34, and -57) that fall into the pattern of 2.6/1.9 kb may have followed the same genomic arrangement as that found for *skc*.

The 0.4-kb hybridization band that appeared in the hybridization pattern of 2.6/0.4 kb indicates a possible second internal *Hin*dIII site. Four strains tested in this study (one M1, two M49, and one M55) belonged to the 2.6/0.4-kb *Hin*dIII pattern. The available sequencing data for an M type 1 streptokinase gene show the existence of a second *Hin*dIII site 400 bp downstream from the first (F. Walter, personal communication). The sequencing data for *ska* have also shown the presence of a potential *Hin*dIII site with the sequence TAGCTT, 397 bp downstream from the known *Hin*dIII site (AAGCTT). It requires only a transition of T to A to make the potential *Hin*dIII site another internal *Hin*dIII site, which may account for the 2.6/0.4-kb hybridization pattern observed with the one M1, two M49, and one M55 strain.

Twelve different hybridization patterns have been generated from *Hae*III digestion. The complexity of the hybridization results may be due to fewer recognition bases required (4 bp) for the enzyme. Throughout the sequence of *ska* there are numerous potential *Hae*III sites, which would require only one codon change to become the recognition sequence of *Hae*III (GGCC). Therefore, a hypothetical codon change, through either transition or transversion, could account for the occurrence of those hybridization patterns observed with *Hae*III digestion. Nevertheless, the actual location of these *Hae*III sites cannot be determined without further investigation.

Some differences were observed in the hybridization patterns of strains belonging to the same M type (Table 3). In the case of M type 12 and 14 strains, *Hin*dIII digestion generated different hybridization patterns; on the other hand, different *Hae*III hybridization patterns were observed in M type 53 and T type 4/28 strains. There were also hybridization patterns that showed differences in both *Hin*dIII and *Hae*III digestions. Two different sets of hybridization patterns were generated from the two M type 4 strains, three sets of patterns were observed with the four T type 3/13/B3264 strains, and two sets of patterns were observed with M type 49 strains.

The question of why all group A strains do not produce an active extracellular streptokinase has not been resolved.

Although the casein-plasminogen overlay method is a sensitive assay, some strains may simply produce too little streptokinase to be detected. It is also possible that regulatory factors play a role in turning off streptokinase production, although no information is available concerning factors which control the expression of streptokinase or any other streptococcal extracellular product. Given the heterogeneity of *ska*, critical variations may have occurred such that some strains no longer produce an active streptokinase.

Recently, Johnston and Zabriskie (9) reported that the nephritis strain-associated protein is a streptokinase. Of particular interest in this study is the finding that hybridization patterns of 14 M type 49 strains obtained from patients with APSGN in 13 geographic areas of the world were all the same. Two M type 49 strains not associated with APSGN were found to have different hybridization patterns. It would be of interest to determine whether these strains have the potential to induce APSGN in an experimental animal model system such as that described by Holm (7). The possibility exists that hypervariable regions of the streptokinase protein are similar among streptokinases produced by nephritogenic strains of streptococci, a characteristic that can be ascertained once other streptokinase sequences become available.

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