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Novel Genomic Rearrangement That Affects Expression of the *Streptococcus pyogenes* Streptolysin O (*slo*) Gene

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A RecA-independent chromosomal rearrangement in the upstream region of the streptolysin O (*slo*) gene of *Streptococcus pyogenes* which affects *slo* expression was identified. PCR analysis was used to demonstrate that this kind of rearrangement was found in several strains of different lineages. Chromosomal loci involved in the recombination were found to be 746 kb apart on the 1.85-Mb-long chromosome. The primary structure of the splicing region, the reproducibility of the rearrangement, and the fact that reconstructed recombinant molecules fused to *erm* and *lacZ* reporter genes affected their expression indicate that this event is not accidental but may play a role in the expression of the *slo* gene. In addition, the product of the recombining DNAs, including the splicing site, does not follow any example of a known recombination mechanism. The implications of this rearrangement for *slo* expression are discussed.

Gene order is a stable characteristic of all organisms analyzed, and the genetic map, the embodiment of this order, is expected to be shared by all members of a species. However, the discovery of transposable elements showed that the concept of fixed genomes slowly evolving by point mutations is an oversimplification. Indeed, it became clear that gene rearrangements, including not only those related to transpositions, are endemic in bacteria. DNA rearrangements in bacteria are classified into two groups: accidental or unprogrammed DNA rearrangements and programmed DNA rearrangements (3). Accidental rearrangements derive from a plethora of events at the chromosome, such as repair, transpositions, and insertion or excision of plasmids, phages, or other foreign DNA. Most of these rearrangements are detrimental to the cell. However, at the population level, where survival of the few means little to the survival of the whole, the overall picture attains a new quality and biological justification. By permanently generating cells with varied phenotypes, a mixed population of organisms is better equipped to survive drastic environmental changes even though the genetic cripples maybe poorly adapted to the current environment (5). On the other hand, programmed DNA rearrangements are part of a genetic program, and the outcome of such rearrangements is largely predictable; i.e., amplification or deletion of genes, assembly of genes from gene segments, and DNA rearrangements that alter gene expression.

The group A streptococcus (GAS) *Streptococcus pyogenes* is the causative agent of numerous human diseases, including pharyngitis, scarlet fever, erysipelas, impetigo, necrotizing fasciitis, and toxic shock-like syndrome. Additionally, GAS can also provoke the nonsuppurative sequelae of rheumatic fever and acute glomerulonephritis. From a genetic point of view, GAS represent a paradigmatic case that defies the standard definition of the species as it relates to bacteria. More than 100 serological groups based on the antiphagocytic M protein have been identified so far among *S. pyogenes* isolates. This remarkable polymorphism is paralleled at the genetic level; pulsed-field gel electrophoresis analysis of chromosomal DNA demonstrated a variety of restriction fragment patterns not only between different M types but also among strains belonging to the same M group (16, 28, 29). The most recent data obtained from GAS sequencing projects suggest that these differences are prevalently due to nucleotide sequence polymorphism, although certain differences in gene content have also been observed (6, 25; W. M. McShan et al., unpublished data).

The flexibility of genomes and examples of DNA rearrangements and their role in gene regulation have been documented in different prokaryotes (3, 5, 9, 11). In this study, we report on a novel type of rearrangement in the chromosome of *S. pyogenes* that, under laboratory growth conditions, influences the expression of *slo*, the gene responsible for synthesis of the cytolytic exotoxin streptolysin O.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids used in this study are described in Table 1. *S. pyogenes* strains were grown in Todd-Hewitt medium (THY) containing 0.2% yeast extract, and when needed, horse serum was added to a final concentration of 5% (vol/vol). Unless otherwise stated, the following antibiotics were added to THY when needed: erythromycin (0.1 μg/ml), tetracycline (12.5 μg/ml), and kanamycin (40 μg/ml). *Escherichia coli* was grown in Luria-Bertani broth (LB). Both THY and LB were solidified when necessary with 1.5% agar. When required, the following antibiotics were added to LB: ampicillin (100 μg/ml), kanamycin (40 μg/ml), tetracycline (12.5 μg/ml), and erythromycin (500 μg/ml).

Transformation of bacterial strains. The plasmids and linear DNA were introduced into *S. pyogenes* strains by electrotransformation (13, 23). Transformation of *E. coli* was performed by the standard $CaCl_2$ method as described before (15).

PCR analysis. The oligonucleotide primers used are described below. Template DNA was purified by CsCl-ethidium bromide gradient centrifugation. In most cases, 40 cycles of amplification were carried out in a Pelkin-Elmer DNA thermocycler with strand denaturation (1 min at 94°C), annealing (1 min at 59°C), and elongation (1 min at 72°C). The total volume of the PCR mixture was usually 50 μl, and it consisted of *Taq* polymerase (1 U), *Taq* polymerase buffer

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Escherichia coli K-12		
JM109	F′/*traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$B$^+$/el4 (McrA$^-$) Δ(*lac-proAB*) *thi gyrA96* (Nal^r) *endA1 hsdR17*(r$^-$ m$^+$)) *relA1 supE44 recA1*	33
DH5α	F′/*endA1 hsdR17* (r$^-$ m$^+$) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacIZYA-argF*)*U169 deoR* (ϕ*80dlac* Δ[*lacZ*]*M15*)	32
Streptococcus pyogenes		
NZ131 (M49)	Clinical isolate	Laboratory collection
364 (M3)	Clinical isolate	Laboratory collection
455 (M5)	Clinical isolate	Laboratory collection
SF370 (M1)	Clinical isolate	Laboratory collection
NY5 (M10)	Clinical isolate	26
OK50	NZ131 with 102-bp-long deletion in Ex region	This work
OK94	Obtained by homologous recombination between plasmid pOK66 and chromosomal (NZ131) *slo* upstream region	This work
OK95	Obtained by homologous recombination between plasmid pOK67 and chromosomal (NZ131) Ex sequences	This work
OK96	Obtained by homologous recombination between pOK67 and chromosomal (NZ131) *slo* upstream region	
OK86, OK87, OK88, OK89, OK90, OK91, OK103	Strains obtained by integration of pCAMP17-based constructs OK73, pOK74, pOK77, pOK78, pOK83, pOK84, and pOK102, respectively, into chromosome of NZ131	This work
Plasmids		
pUC18 & pUC19	Recombinant *E. coli* vectors	33
pT7Blue	Vector designed for cloning and sequencing of PCR products	Novagen, catalog no. 69820-1
pGEM-T Easy	Vector designed for cloning and sequencing of PCR products	Promega Corp., catalog no. 1360
p7erm	Insertional vector in streptococci; origin of replication from pUC18	14
p7tet	*erm* gene in p7Erm replaced with *tet*	14
pCAMP17	Recombinant plasmid for construction of *lacZ* reporter fusions; it integrates into serine tRNA gene of *S. pyogenes* chromosome	8
pOK18	Recombinant plasmid carrying proximal part of *slo* gene and its upstream region on a *Hin*dIII fragment cloned into the *Hin*dIII site of vector pALTER-1	This work
pOK23	Recombinant plasmid harboring reconstructed fusion of Ex and *slo* DNAs, contains mutated translation initiation codon of *slo* gene	This work
POK23-1	Same as pOK23 with recreated *slo* gene ATG initiation codon	This work
pOK66	Recombinant plasmid carrying approximately 650 bp of the *slo* gene upstream region fused to the *erm* reporter gene	This work
pOK67	Recombinant plasmid carrying 539 bp of Ex DNA region fused to the *erm* reporter gene via 190 bp	This work
pOK73, pOK77, pOK78, pOK83, pOK84, pOK102	Recombinant plasmids carrying *slo* gene upstream region of different lengths fused to *lacZ* reporter gene of vector pCAMP17	This work
pOK74	Product of P98 × P99 PCR on pOK23 as a substrate, cut with *Pst*I and *Bam*HI, and cloned into pCAMP17	This work

(1×), Mg^{2+} (2 mM), deoxynucleoside triphosphates (200 μM each), and primers (1 μM each). Following amplification, a 10-μl aliquot was analyzed by agarose gel electrophoresis. When needed, the PCR products were purified with a PCR purification kit (Qiagen Inc.)

DNA manipulations and sequencing. Plasmid isolation, restriction, and ligation, Southern transfer and hybridization, and other recombinant DNA techniques were performed by standard procedures (20). Plasmids from *E. coli* were also isolated with a plasmid isolation kit (Qiagen Inc.). For cloning PCR fragments, specialized cloning vectors with T-nucleotide overhangs, pT7 Blue (Novagen) and pGEM-T Easy (Promega), were used. All sequencing reactions were performed by the dideoxy termination method (21).

Construction of deletion covering the recombination active site in the Ex region. The deletion, covering 102 bp around the junction point of Ex DNA (temporary designation for "element X," later found to map within Spy1528 [see Fig. 3]), was constructed as illustrated in Fig. 1. Two DNA fragments of 970 bp and 870 bp at distances of 65 bp and 37 bp from the junction point, respectively, were produced by PCR on NZ131 chromosomal DNA as the substrate. The primers used were designed to create suitable restriction sites (*Eco*RI, *Kpn*I, *Xba*I, and *Hin*dIII) at the ends of the amplified fragments (Fig. 1A). After being cut with appropriate restriction enzymes and purified, the fragments were cloned in two consecutive steps into the multiple cloning site of plasmid pOK26, a derivative of pUC18 with an erythromycin resistance gene located within the multiple cloning site (not presented). The construct obtained, pOK28, was treated with *Eco*RI and *Hin*dIII enzymes, and the insert was separated from the vector by gel electrophoresis, extracted from the gel, and introduced by electrotransformation into recipient strain NZ131 (Fig. 1C). Chromosomal DNA from one of the erythromycin-resistant transformants (strain OK50) was isolated, and the presence of the insert containing the 65-bp- and 37-bp-long deletions of Ex DNA encompassing a stretch of DNA involved in recombination with the *slo* DNA was confirmed by sequencing; the chromosomal DNA harboring the insert was amplified with flanking primers P21 and P36, and the fragment obtained was cloned into pT7 and sequenced (Fig. 1D).

Construction of fusions with *erm* as a reporter gene. The *erm* gene was first amplified by PCR from plasmid p7erm and cloned into the *Kpn*I and *Bam*HI sites of the insertion vector p7tet, creating plasmid pOK97 (see Fig. 2A). In the following step, the native upstream region of the *slo* gene and the recombinant Ex/*slo* fragment were amplified by using as a substrate chromosomal DNA from strain NZ131 (Fig. 2B1) and plasmid pOK23 carrying the recombined Ex/*slo* fragment (Fig. 2B2), respectively, and cloned into the *Eco*RI and *Kpn*I sites of the pOK97 vector, resulting in plasmids pOK66 and pOK67, respectively. The recombinant Ex/*slo* fragment in pOK23 was obtained by PCR on chromosomal DNA from strain NZ131 with primers 151 and 152 (Fig. 3). As presented (Fig. 2), *Eco*RI and *Kpn*I restriction sites were introduced into the amplified fragments by mutagenic oligonucleotides containing these restriction sites. The correctness of the nucleotide sequence was confirmed by DNA sequencing (Fig. 2C).

In the final step, plasmids pOK66 and pOK67 were introduced by electro-

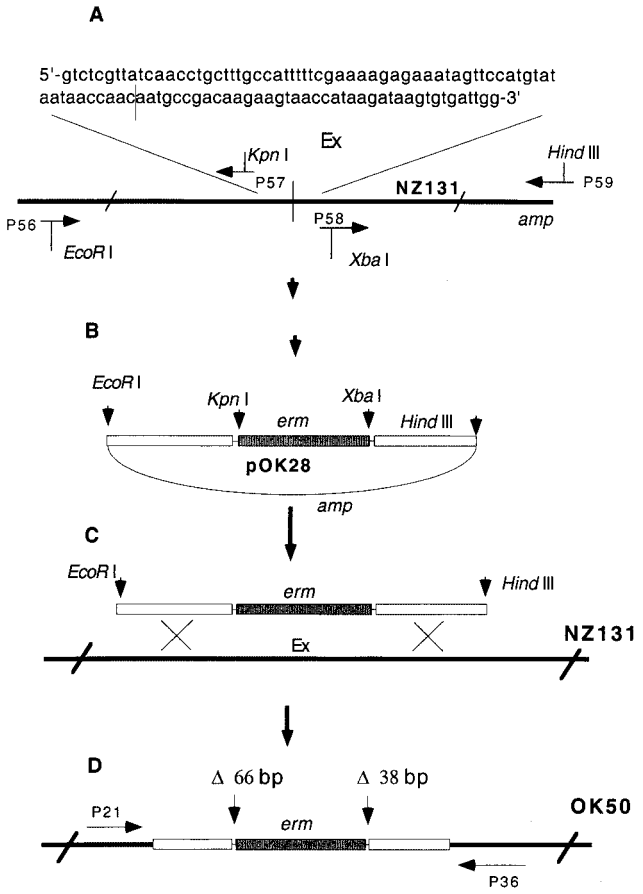

FIG. 1. Construction of 102-bp-long deletion in Ex region of strain NZ131. The deleted stretch of the chromosome (the sequence at the top) is the site in the Ex region that is involved in recombination with the *slo* DNA. The thin vertical line in the chromosome and in the sequence marks the recombination site. Horizontal arrows depict primers used for PCR. The figure is not drawn to scale. For details, see Materials and Methods.

transformation into the competent recipient NZ131. Plasmid pOK66 can integrate into the chromosome in only one way, via the *slo* region (Fig. 2D). On the other hand, plasmid OK67 can produce two genetic constellations, depending on whether it has been integrated into the chromosome via *slo* (Fig. 2E) or via the Ex region (Fig. 2F). The genetic constellation of the final constructs upon insertion of recombinant plasmids into the NZ131 chromosome was confirmed by PCR with the appropriate primers. Finally, growing the constructs under nonselective conditions in THY broth without erythromycin demonstrated that the segregation of the *erm* marker was lower than 0.05%.

β-Galactosidase assay. β-Galactosidase was determined as described before (7) by a modification of the original procedure (15). Overnight cultures of *S. pyogenes* were diluted approximately 20 times in fresh THY medium and then grown to mid-logarithmic phase (approximately 0.2 at an optical density of 600 nm). Cells were harvested by centrifugation, resuspended, and made permeable to *O*-nitrophenyl-β-D-galactopyranoside (ONPG) by adding 20 μl of chloroform per sample and vortexing for 30 s. β-Galactosidase activity was determined at a temperature of 37°C by measuring ΔE at 420 nm to detect *O*-nitrophenyl released from ONPG and was calculated as Miller units, proportional to enzyme activity per cell.

Selected oligonucleotides used in PCRs. The following oligonucleotides (shown in the 5′-3′ direction) were used as PCR primers: P21, CACACGGTA CTGGGGACGCATATTTTCG; P36, TTCCCACAAGTGAGGTATTCAAC TA; P56, GTAAACAACTGGGAATTCCAATTGC; P57, CGCCGGTACCTT TTAAAGACGTGATGCGTC; P58, ATGCTCTAGACATATCCCCC TCCTA TTTTG; P59, CGACAAGCTTCCATCAAACATCGCTTAGAC; P62. CTCTC ACAAATACAGAAAAAAGC; P68, AAGCTTGCCATTTTATTTAAACCG; P69, TTTATATCAGTTAAGCTTGCC; P70, TTGGTTATTATACATGGAAC TATTTC; P71, GTTGGTTATTATACATGGAACTAT; P73, TCAAAGAATT CTCTAAAGCTGTCTTAGCAG; P74′, TTCTTGAATT- CCTTAAGTTAGT CATTTGTTCC; P76, AAAAGGGTACCTTCTAAAATGTTTCTATTG; P82, TTAAGACATAAACGTCTTCAAAGCCAGC; P83, TCTTGATAGTGAGCA AATTCTCCTAATTTG; P90, AGAAGTTATTTTAGAGTATGGGGTTTA; P91, TATTGAAAAATACTTTGTGACCTTCAC; P97, ATGACTCTGCAGT AACTGGTCGCTTCAAAATTAAG; P98, GGACACTGCAGCAAGTCTGG ATACAGCAAAAAAAG; P99, GGCGCCGGATCCTTTTTATTAGACATGT CCTTCATACC; P125, CAAAGAGTGGCGGAGCTTCATTGCTGACAC; P126, CAAATGAGCAACCAAAGCCAGAAAGTAGTGAG; P144, TGTTTT TCCGTGGTCAACGTGG; P145, TTTTCTGCAGATTCTAGTGGC; P151, CAAGGTCATTGGAATCCATGGCACG; and P152, GTCATTGATTTCTTC AGTGTGATCTTCTTCGCTC.

RESULTS

Evidence of chromosomal rearrangement. The first suggestion or indication of a recombination event between two unlinked chromosomal loci was obtained inadvertently during construction of a local restriction map upstream of the *slo* gene of *S. pyogenes* strain NZ131 (M49). A PCR experiment with primers P21 and P22 was designed to amplify and clone a fragment from chromosomal DNA spanning the region immediately upstream of the *slo* gene and the DNA we thought was contiguous to the *slo*-carrying fragment. The reaction produced a fragment of the expected size that contained a *Hin*dIII site found previously at the end of the known sequence of the *slo* gene (Fig. 4A, lane 4) (10). However, Southern blotting and hybridization demonstrated that the DNA beyond the *Hin*dIII site must be at least 35 kb away from the *slo* locus (results not presented) and certainly beyond the range for standard PCR. Furthermore, hybridization of the *slo* DNA and this unknown DNA isolated from strains NZ131 (M49) and SF370 (M1) with pulsed-field gel electrophoresis, chromosomal fragments used in the construction of the *S. pyogenes* genome physical map (29) suggested that the two loci in these strains may be as much as one third of the chromosome away from each other (A. Suvorov, personal communication). This estimate is in accord with the data obtained recently from the genome sequence of stains SF370 (M1), MGAS8232 (M18), and NZ131 (M49) (6, 25; W. M. McShan et al., unpublished data); the distance between the two loci on the chromosome of about 1.852 Mb turned out to be approximately 746 kb. The product of the PCR amplification with primers P21 and P22 was cloned into the pT7Blue vector and sequenced.

The primary structure of the recombinant molecule showed that the chromosomal locus, temporarily named element X (Ex), is spliced to the upstream region of the *slo* gene a few nucleotides upstream of the *slo* native promoter and 189 bp upstream from the beginning of the *slo* gene (Fig. 3) (10, 22). Interestingly, an extra C was found at the recombination site (Fig. 3). Another feature of the recombinant fragment is that eight nucleotides upstream of the junction, the Ex DNA carries a promoter-like structure consisting of an *E. coli* −10 TATAAT consensus sequence and, 17 nucleotides upstream, a TCGAAA sequence identical to one of the −35 sequences found in the *oriC* region of *Bacillus subtilis* (18). It is also interesting that two TTGCCATTTT decamers, found 18 times in the chromosomes of strains SF370 (M1) (6), NZ131 (M49) (W. M. McShan et al., unpublished data), and MGAS8232

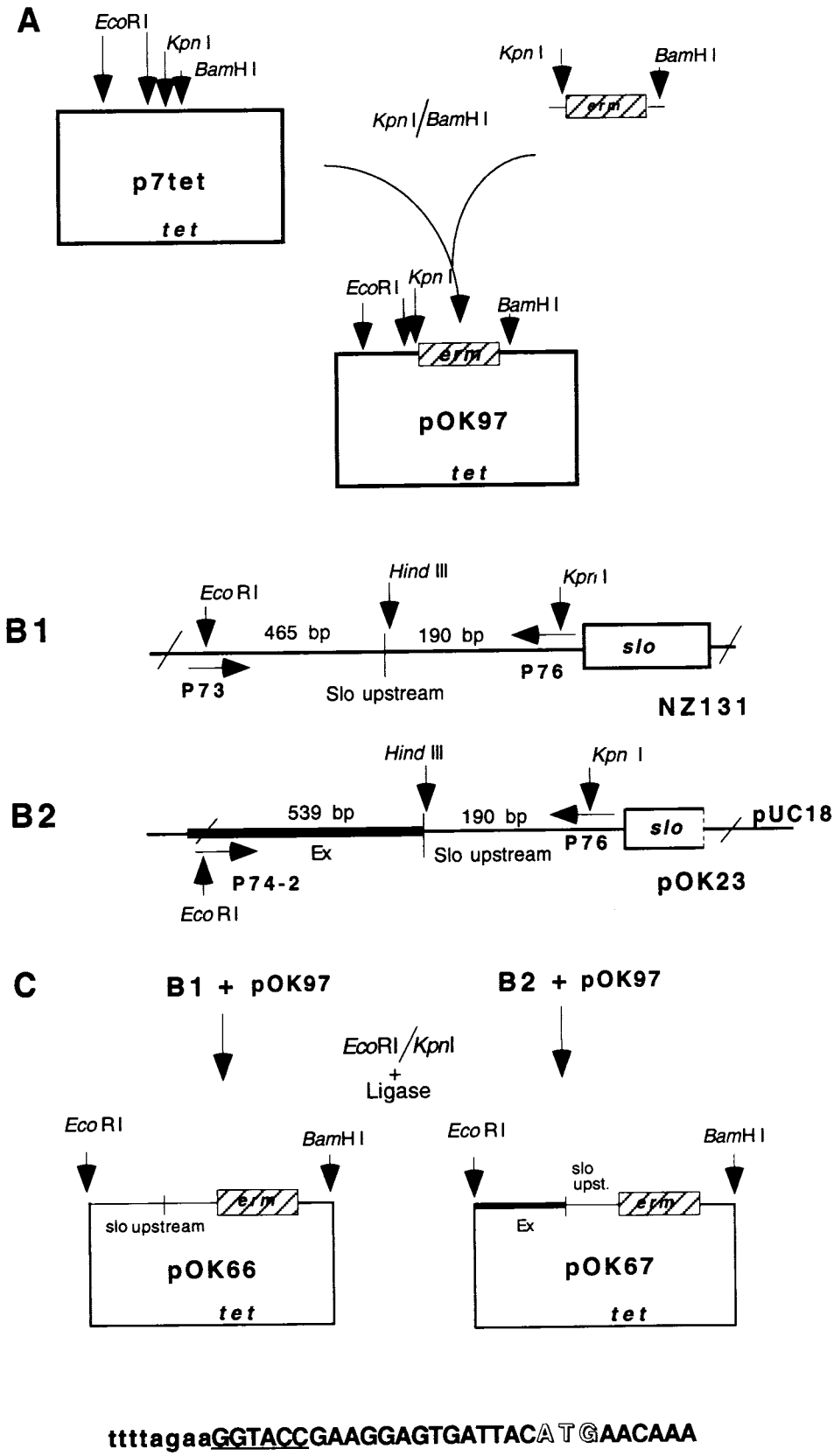

FIG. 2. Construction of transcription fusion strains with *erm* fused to fragments of different composition upstream of the *slo* gene. The sequence at the bottom of section C illustrates the fusion between the upstream region of the *slo* gene and the *erm* gene. The *slo* sequence is presented in lowercase letters, and the *erm* sequence is given in capital letters, the *Kpn*I site is underlined, and the ATG initiation codon of the *erm* gene is presented in open letters. The picture is not drawn to scale. For more details, see Materials and Methods.

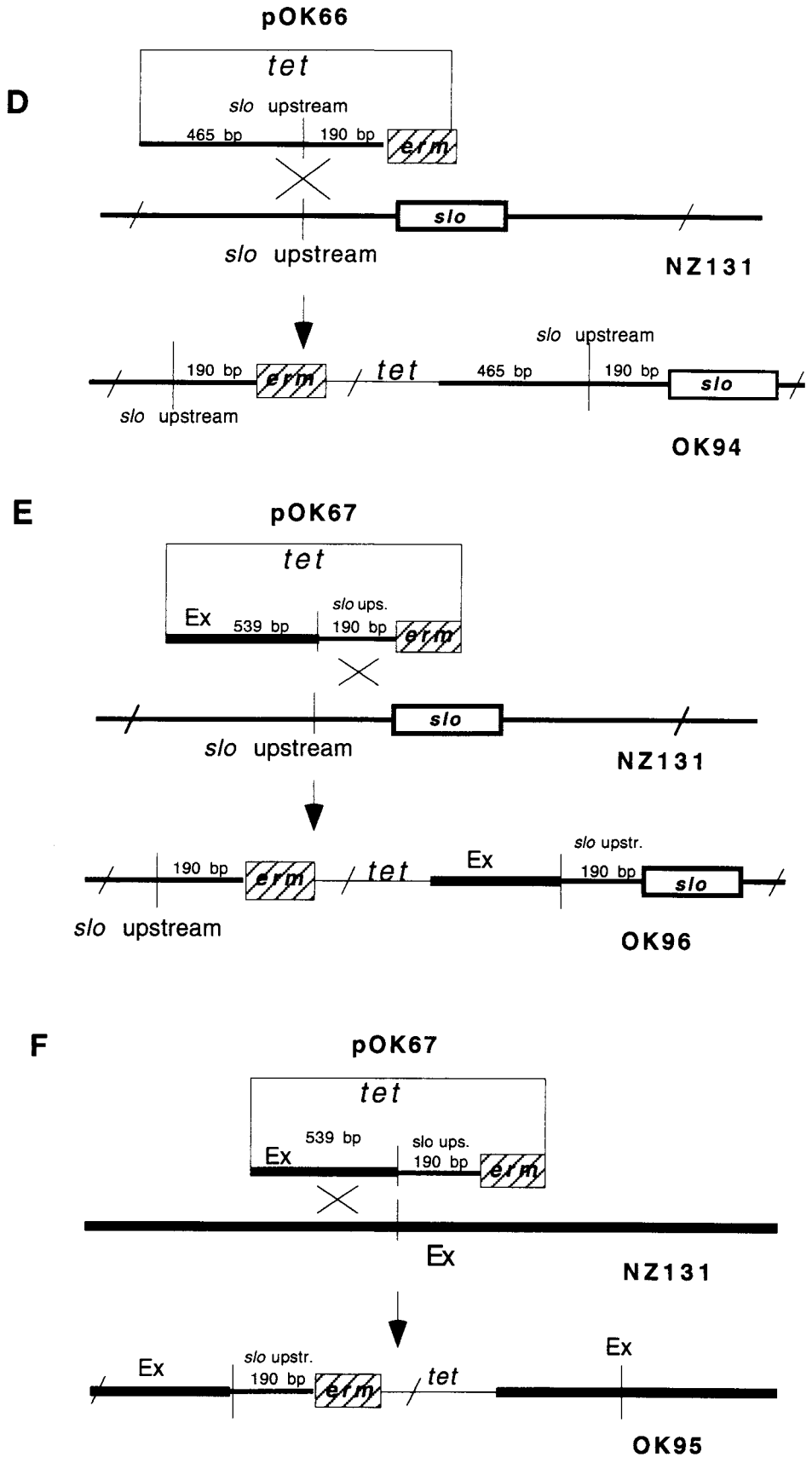

FIG. 2—*Continued*.

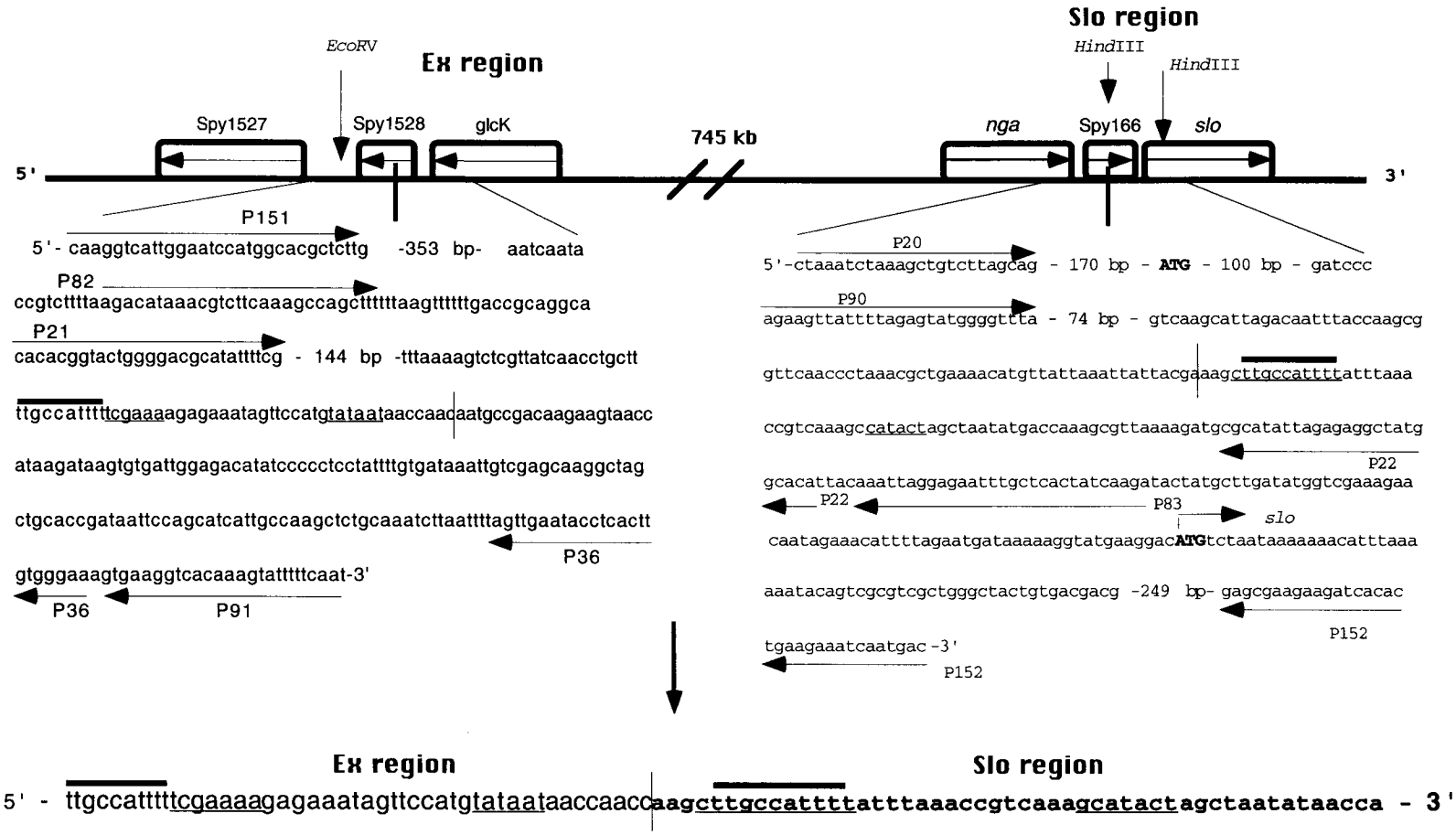

FIG. 3. Primary structure of the Ex and *slo* loci around the sites involved in mutual recombination and the product of their recombination. Note an extra C at the junction of the recombinant molecule. The arrowed lines represent PCR primers, while thin plain lines signify the *slo* promoter and the putative Ex promoter. The arrowed lines inside the boxes mark the orientations of the genes. Vertical lines denote the recombination site between the Ex and *slo* DNAs. The nucleotide decamers are depicted by thick lines above the sequence. *nga* is the genetic symbol for the gene coding for NAD-glycohydrolase, *slo* denotes the gene for streptolysin O, while Spy1527 and *glcK* represent genes highly homologous to *ylaG*, the *Bacillus subtilis* gene coding for elongation factor G and the gene coding for glucose kinase, respectively (6). The open reading frame Spy1528 represents a gene coding for a conserved hypothetical protein showing a low degree of identity to a cell division protein from *B. subtilis;* it could also easily be a stretch of noncoding DNA (6). Similarly, Spy166 is a very short open reading frame with no similarity to any known gene (6, 25; W. M. McShan et al., unpublished data). Initiation codon of the *slo* gene and stop codon of the *nga* gene are given in bold capital letters. The figure is not drawn to scale.

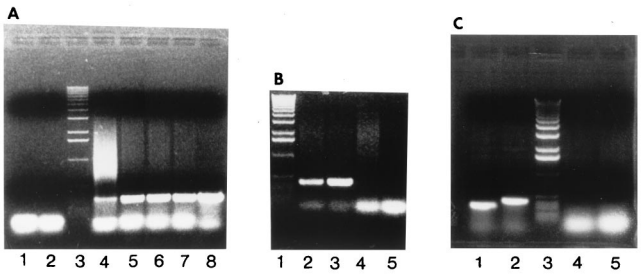

FIG. 4. Electrophoretic profile of PCR products with primers pairs P21 × P22 and P82 × P83 on chromosomal DNA from different strains. (A) Products of PCR with primer pair P21 × P22 on chromosomal DNA from strains NZ131 (lane 4), NZ131 Rec$^-$ (lane 5), M1 (strain 370) (lane 6), M3 (strain 364) (lane 7), and M5 (strain 455) (lane 8). Lanes 1 and 2 are controls without chromosomal DNA, and lane 3 is molecular size standard (1-kb standard; Promega). (B) Lane 1, molecular size standards; lane 2, P82 × P83 on NZ131; lane 3, P82 × P83 on NZ131 Rec$^-$; lane 4, P82 × P83 on OK50; lane 5, control with no DNA. (C) Lane 1, P21 × P22 on NZ131; lane 2, P82 × P83 on NZ131; lane 3, molecular size control; lanes 4 and 5, controls with no DNA.

(M18) (25), are brought together by recombination into a proximity of only 50 nucleotides.

The nucleotide sequence that we determined for both the Ex region and the *slo* regions corresponded well to the sequence of the *slo* region of streptococcal strain Richards (M3) (10, 11) and to the nucleotide sequence of the *slo* and Ex regions from strains SF370 (M1), MGAS8232, and NZ131 (M49) (6, 25; W. M. McShan et al., unpublished data). The results of these analyses positioned the recombination site 189 bp upstream of the *slo* gene and 326 bp downstream from *nga*, the gene encoding NAD glycohydrolase, and between *glcK* and Spy1527, two genes showing similarity to glucose kinase and GTP binding protein, respectively, in the Ex region (Fig. 3) (6).

Figure 3 shows that the recombination site in the Ex region maps within the short open reading frame (Spy1528) which represents a gene encoding a conserved hypothetical protein with a low degree of identity to a cell division protein from *B. subtilis*. Given the size of the gene (387 bp) as well as its low identity value (E value = $8e^{-13}$ [1]), Spy1528 could easily be a stretch of noncoding DNA between Spy1527 and *glcK*. Similarly, Spy166 is a very short open reading frame (354 nucleotides) with no similarity to any known gene (6, 25; W. M. McShan et al., unpublished data). The fact that the *slo* promoter lies within this sequence (22) suggests that this open reading frame may also be a stretch of noncoding DNA.

The requirement for Ex DNA in the recombination event with *slo* DNA was verified in an experiment with an Ex deletion derivative of strain NZ131. The deletion, covering 102 bp around the junction point of Ex DNA, was constructed in strain NZ131 as described in Materials and Methods and illustrated in Fig. 1. Chromosomal DNA from this construct (OK50) was used as a substrate in PCR with a pair of primers, P82 and P83 (see below). No characteristic fragment like the one found with DNA from strain NZ131 could be observed. (Fig. 4B).

Recombination event is RecA independent and seems to be ubiquitous among GAS strains. In order to further characterize the recombination event that takes place in the *slo* region, chromosomal DNA isolated from a *recA* mutant of strain NZ131 (30) was used as a substrate in PCR assays. Chromosomal DNAs isolated from representatives of three streptococcal M types, M1 (strain SF370), M3 (strain 364), and M5 (strain 455), were also included in this analysis to investigate whether the observed phenomenon is specific for M49 or is also present among other M types of GAS. The results of PCR experiments with the P21 and P22 primers (Fig. 4A) show that the recombination event creating the Ex/*slo* hybrid DNA is RecA independent and that it is widely present, possibly omnipresent, among GAS. The nature of the fragments obtained was also verified by sequencing. The nucleotide sequence obtained from the Ex/*slo* splicing region as well as the rest of the amplified fragments was virtually identical to the sequence obtained previously for strain NZ131, including an extra C at the recombination site (results not presented).

Given the extreme sensitivity of PCR, special precautions were taken during isolation of chromosomal DNA from strains other than RecA$^+$ NZ131 for possible contamination with the previously obtained fragment P21 × P22. First, all DNAs, including DNA from strain NZ131, were used as substrates in PCRs in which a new pair of primers, P82 × P83, slightly outflanking the segment of DNA amplified by the pair P21 × P22, was used (Fig. 3). In this way it was ensured that neither possible contaminating P21 × P22 fragment nor the same fragment cloned into vectors pT7Blue or P7erm could be amplified. The PCR products obtained in all reactions, when analyzed by gel electrophoresis, demonstrated the expected increase in molecular size of approximately 80 nucleotides relative to the fragment obtained with primers 21 and 22 (Fig. 4C). An additional PCR (P82 × P152; Fig. 3) was also performed, producing a fragment of the expected size (results not presented).

DNA sequence analyses of the fragments obtained in both PCRs (P82 × P83 and P82 × P152) was the same (again, including the extra C at the Ex/*slo* junction) compared to the sequence obtained previously with DNA fragments from P21 × P22 PCRs. Finally, a test for possible contamination with P21 × P22 fragments cloned into the pT7Blue and p7Erm vectors was tested with primers which flank multiple cloning sites of these vectors (primers T7 and M13 Forward for pT7Blue and T7 and SP6 for p7Erm). None of the chromosomal DNAs produced any detectable fragment in these PCRs (results not presented). We conclude that the amplified P21 × P22 fragments are authentic and that, similar to the hybrid fragment obtained in the original experiment with strain NZ131 (M49), they illustrate an in vivo recombination event in the GAS M types 1, 3, and 5 as well as in the Rec$^-$ derivative of NZ131 (M9).

Detection of recombinant fragments between the *slo* and Ex DNAs is not the result of a coincidental in vitro hybridization of these two chromosomal segments. The possibility was considered that the detected recombined DNA did not reflect an in vivo recombination event, but rather might have resulted from an in vitro hybridization of the *slo* and Ex chromosomal fragments in the course of PCR amplification. Provided that these two fragments harbor shorter or longer homologous DNA stretches, hybrid molecules could have been established either between single-stranded homologous DNA stretches at the recessed ends of the double-stranded fragments or be-

tween partial overlaps between single-stranded molecules. Double-stranded hybrids could readily serve as a substrate for the PCR amplification, while partially overlapping single-stranded molecules would require an earlier conversion into a fully double-stranded form by the extension of the internal 3′OH ends.

Although homologous sequences that would provide substrates for such reactions have not been found in the recombined section of Ex and *slo* DNAs (Fig. 3), control experiments were performed in the following way. Fragments of Ex and *slo* DNA of approximately 1 kb carrying the presumptive point of recombination were obtained from plasmid clones carrying these DNAs. The fragments obtained were separated by gel electrophoresis and purified from the gel as described above. The fragments were mixed at a 1:1 molar ratio and used as substrates for PCR amplification at 1, 10, 50, and 100 ng of DNA per reaction. No product was observed with primer pairs 21 × 22 and 82 × 83 (results not presented). We conclude that the observed recombinant Ex/*slo* fragments are not the result of a coincidental in vitro hybridization of these two chromosomal segments, but are rather the consequence of their prior in vivo recombinational fusion.

Quantification of recombination event. The frequency of the described recombination event was determined by PCR. Original preparations of DNA isolated from strains NZ131 (M49) and SF370 (M1) were adjusted to an approximate concentration of 200 to 300 ng/μl and subsequently diluted up to 10^{10}-fold by serial dilution in TE (Tris-EDTA) buffer. The first PCR, used as a positive control and reference point, was done with a pair of primers (P155 × P125) flanking a stretch of genetically stable DNA of an approximately 0.5-kb-long structural part of the *slo* gene (10). The highest dilution of both DNAs at which fragments were detected on the gel after PCR amplification was 10^8; PCR performed with DNA diluted 5×10^8 times did not produce a visible fragment (results not presented).

As expected, PCR amplification with primers P82 and P83, used in this study to detect the recombination event between Ex and *slo* DNA (Fig. 4), produced visible fragments at lower dilution values. Interestingly, the values were not the same for the two DNAs: the highest dilution of NZ131 DNA at which the amplified fragment was visible was 10^3, while the highest dilution of SF370 DNA that allowed synthesis of a detectable amount of the fragment was 10^4 (results not presented). We conclude that the frequency of rearrangement in strain NZ131 was 10^{-5} in the population, while the frequency of the rearrangement in strain SF370 was higher (10^{-4}). The observed lower frequency values may have resulted from DNA shearing leading to the separation of Ex and *slo* loci in the subpopulation of chromosomal fragments. However, such shearing of 0.5-kb-long standard DNA is not to be expected. The numbers obtained in this type of experiment are always an approximation; it is difficult to expect that two pairs of primers will bind to their respective targets with the same affinity.

Search for the other terminus. To understand the nature of the recombination event, an attempt was made to identify the other end of the detected rearrangement. In this pursuit, it was first hypothesized that the rearrangement might be an intragenomic transposition. Although contraindicated by the generation of an extra C at the splicing region that does not fit any of the mechanical models of transposition as well as by the lack of any transposase genes in the vicinity of the recombining DNAs, this belief was based on the fact that the two recombining loci were found to map far apart on the chromosome.

The first model tested was that the *slo* gene might be part of a transposon that had been inserted into the target sequence within the Ex DNA (Fig. 5A). The alternative hypothesis was that the Ex "transposon" was inserting itself into the target sequence upstream of the *slo* gene (Fig. 5B). To test these hypotheses, primers were designed for PCR based on the assumption that the termini of both "transposons" were composed of either inverted or direct repeats. The known nucleotide sequences at one end of the hypothesized transposons, i.e., DNA stretches immediately flanking the splicing point of the Ex/*slo* recombinant DNA, served as a reference for designing these primers. To minimize difficulties due to the possible incomplete identity of the known ends and the projected ends, a pair of partially overlapping primers differing in their 3′OH ends were designed for each end of the putative Ex and *slo* transposons. The reactions, performed under various experimental conditions, produced either no PCR products or bands which differed vastly from the expected sizes (results not presented). After several additional unsuccessful attempts, it was concluded that the observed rearrangement possibly was not due to a standard bacterial transposition event.

Although most Rec-independent inversion or deletion-generating events are characterized by shorter or longer repeats around recombination sites (28), the possibility that the rearrangement might be a consequence of either inversion or deletion of the chromosomal segment between the Ex and *slo* loci was also considered (Fig. 5C and D). At the time we performed these experiments, the relative orientations of the *slo* and Ex loci on the chromosome were not known and the distance between them was not firmly established, so both possibilities had to be taken into account.

The experimental strategy employed to ascertain this possibility was basically the same as that exercised in a search for transposons. In the case of deletion, the PCR primers were designed to detect an excised, circular chromosomal fragment (Fig. 5D). Again, no PCR fragments were detected to verify the occurrence of inversion or deletion.

Rearrangement represses *slo* gene expression. To investigate the possible effect of recombination on the activity of the nearby *slo* gene, sets of fusion strains with *erm* and *lac*Z reporter genes were constructed. Figure 2 illustrates the construction of the first set of strains with different *slo* upstream regions fused to an *erm* reporter gene. Resistance to erythromycin in these strains was tested on THY plates containing erythromycin by inspecting growth after overnight incubation at 37°C and also by measuring growth in THY broth supplemented with erythromycin. Strains OK94 and OK96, in which the *erm* gene is fused to the natural upstream region of the *slo* gene (Fig. 2D and E), produced individual colonies on plates containing 0.1 μg of erythromycin per ml. Surprisingly, strain OK95 harboring the recombinant Ex/*slo* fragment with the putative Ex promoter upstream of the *erm* gene (Fig. 2F) was sensitive to erythromycin at 0.1 μg/ml (results not presented). The same pattern of strain sensitivity to erythromycin was reproduced in an experiment in which bacterial growth was

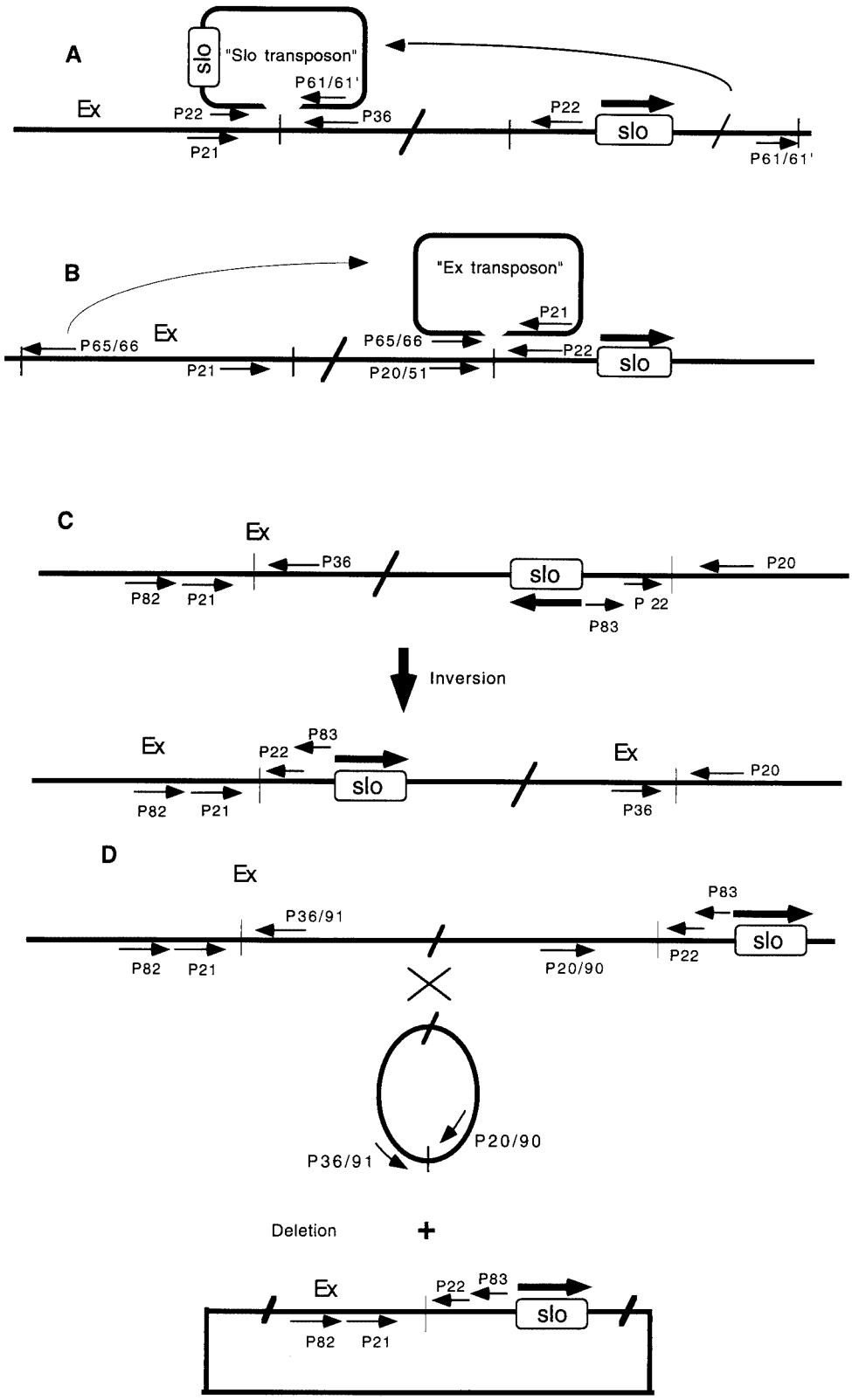

FIG. 5. Hypothetical models of chromosomal rearrangement between the *slo* and Ex chromosomal loci by a transposition of the "*slo* transposon" (A), by a transposition of the "Ex transposon" (B), by an inversion event (C), and by deletion (D). Thin arrows denote oligonucleotide primers used in PCR, while thick arrows indicate the transcriptional polarity of the *slo* gene. The primers 68, 69, 70 and 71 (not presented) are inverse complements of primers P61, P61′, P65, and P66, respectively (see Materials and Methods); they were used to test the possibility that the hypothetical transposons are flanked by direct repeats. The figure is not drawn to scale.

monitored in THY broth supplemented with 0.1 μg of erythromycin per ml (results not presented).

In a parallel experiment, a set of strains containing a protein fusion with *lacZ* as a reporter gene were constructed. Similar to previous constructions, the fragments fused to the *lacZ* gene were obtained by PCR amplification with mutagenic oligonucleotides containing *Pst*I and *Bam*HI restriction sites. The fragments amplified from strain NZ131 chromosomal DNA contained the proximal part of the *slo* gene and different lengths of the *slo* upstream region (Fig. 6). The fragment amplified from pOK23-1 contains the Ex/*slo* recombinant fragment (Fig. 6). pOK23-1 is a derivative of pOK23 (Fig. 2) that carries a mutated form (ACTG) of the *slo* gene initiation codon (not shown). A wild-type initiation codon was recreated by PCR from pOK23 with the mutagenic primer P99. Primer P99 also introduces a flanking *Bam*HI restriction site, while the other primer, P98, creates a *Pst*I restriction site.

Upon treatment with *Pst*I and *Bam*HI enzymes, the fragments were cloned into the *Pst*I- and *Bam*HI-treated vector pCAMP17. As shown (Fig. 6), the *Bam*HI restriction site is located inside both the *slo* and *lacZ* genes, so that the products obtained at the splicing region between the *lacZ* gene and the *slo* gene included the Shine-Dalgarno sequence, ATG initiation codon, and the first four codons of the *slo* gene. A special advantage of the pCAMP17 vector is that besides the *lacZ* reporter gene and the *erm* selective marker, it also harbors the *S. pyogenes* phage T12 genes for integrase and its specific attachment site (8). These characteristics enable pCAMP17 to simulate a temperate pathway of phage T12, i.e., to integrate into a specific attachment site in the *S. pyogenes* chromosome (8, 14). Finally, integration of pCAMP17 into the chromosome is irreversible because excision of phage T12 also requires the enzyme excisionase, which is lacking in this system (14).

Preliminary measurements with strain OK86 showed that *slo*, i.e., the *lacZ* gene, is expressed practically at the same level from the early log to the stationary phase in THY broth, so the samples for measuring β-galactosidase activity were taken at the end of the log phase. The strains carrying increasing sections of the *slo* upstream region demonstrated low but reproducible expression of the reporter gene (Fig. 6). The negative control strain NZ131 with no plasmid (results not presented) as well as strain OK103 with a deletion of the −35 sequence of the *slo* promoter (22) demonstrated no β-galactosidase activity. Similar to strain OK95 with the *erm* reporter gene (Fig. 2F), the strain carrying the recombinant Ex/*slo* upstream region (OK87) showed no β-galactosidase activity. Interestingly, strain OK88, in which the natural *slo* upstream sequence terminates at practically the same nucleotide as in strain OK87 but is spliced at the breakpoint not with Ex DNA but with the vector pCAMP17 DNA, demonstrated a higher level of β-galactosidase activity than strain OK86, harboring the longest segment (573 bp) of the natural *slo* upstream region (Fig. 6).

DISCUSSION

In this study we describe the detection and partial characterization of a rearrangement involving two *S. pyogenes* chromosomal loci separated by 745 kb on the 1.85-Mb-long chromosome. By its nature, the event is RecA independent and seems to be ubiquitous among GAS. The phenomenon was detected by PCR, and its functional consequence on expression of the *slo* gene was monitored by fusing reconstructed recombinant molecule with the *erm* and *lacZ* reporter genes.

The structural aspect of this rearrangement remains obscure for the moment. The experimental strategies applied in the quest for the other terminus, i.e., design of appropriate primers for PCR, were grounded in structural models of genomic rearrangements such as transpositions, inversions, and deletions (2, 4). With this method, we found neither inversion nor deletion as a basis for the rearrangement. The failure to identify an inversion is not surprising because the same genetic polarity of the recombining loci was found postfestum during genome sequencing of strains M370, MGAS8232, and NZ131 (6, 25; W. M. McShan et al., unpublished data).

Constant generation of a G/C pair at the Ex/*slo* junction only seemingly indicates a classical transposition event almost always characterized by duplication of the target sequence. However, this extra G/C does not fit into any known mechanism of target duplication following transposition (2). We also did not succeed in describing a mechanism by which an extra G/C pair would be generated via the conjugative transposon insertion pathway, known to generate not only base substitutions but also frameshift mutations in the course of homogenization of mismatched staggered ends (4). The fact that the chromosomal regions in the neighborhood of both *slo* and the Ex recombination sites do not demonstrate any resemblance to transposons (6, 25; W. M. McShan et al., unpublished data) also speaks against a transposon model. The failure to demonstrate the transposon nature of the rearrangement might have been caused by larger than expected divergence in homology of the postulated transposon termini. Designing more alternative primers for PCR experiments, particularly various nucleotides at the 3′OH end, might help in further testing of this possibility. Finally, there is a possibility that this rearrangement might be related to transposons which diverge widely from the common models. The most extreme example is the reported case of transposition without transposase, in which a block of donor DNA is inserted into the target sequence without the involvement of any known transposase. In addition, the described transposable element lacks typical transposon ends, and insertion of a transposon is not associated with characteristic duplication of the target sequence (19).

Aside from transposition models, the structural requirements in genomic rearrangements such as direct or inverted repeats or monotonous nucleotide stretches seem not to be universally required. The recent report on capsular phase variation in *Streptococcus pneumoniae* clearly shows that the event is due to an apparently random formation of duplications and their subsequent precise excision within a heterogeneous nucleotide sequence of the *cap3A* gene (31).

The reproducibility of the recombination event and the characteristics of the sequences around the junction between the Ex and *slo* DNAs argue that this rearrangement is not a stochastic, pathological event, but a chromosomal rearrangement of some biological significance. Our failure to detect the relevant PCR fragments in the search for the other terminus and the failure to observe amplified fragments in several PCR experiments with primers located in either the *slo* or Ex locus paired with primers around the chromosome (results not presented) indicate that background recombination between dis-

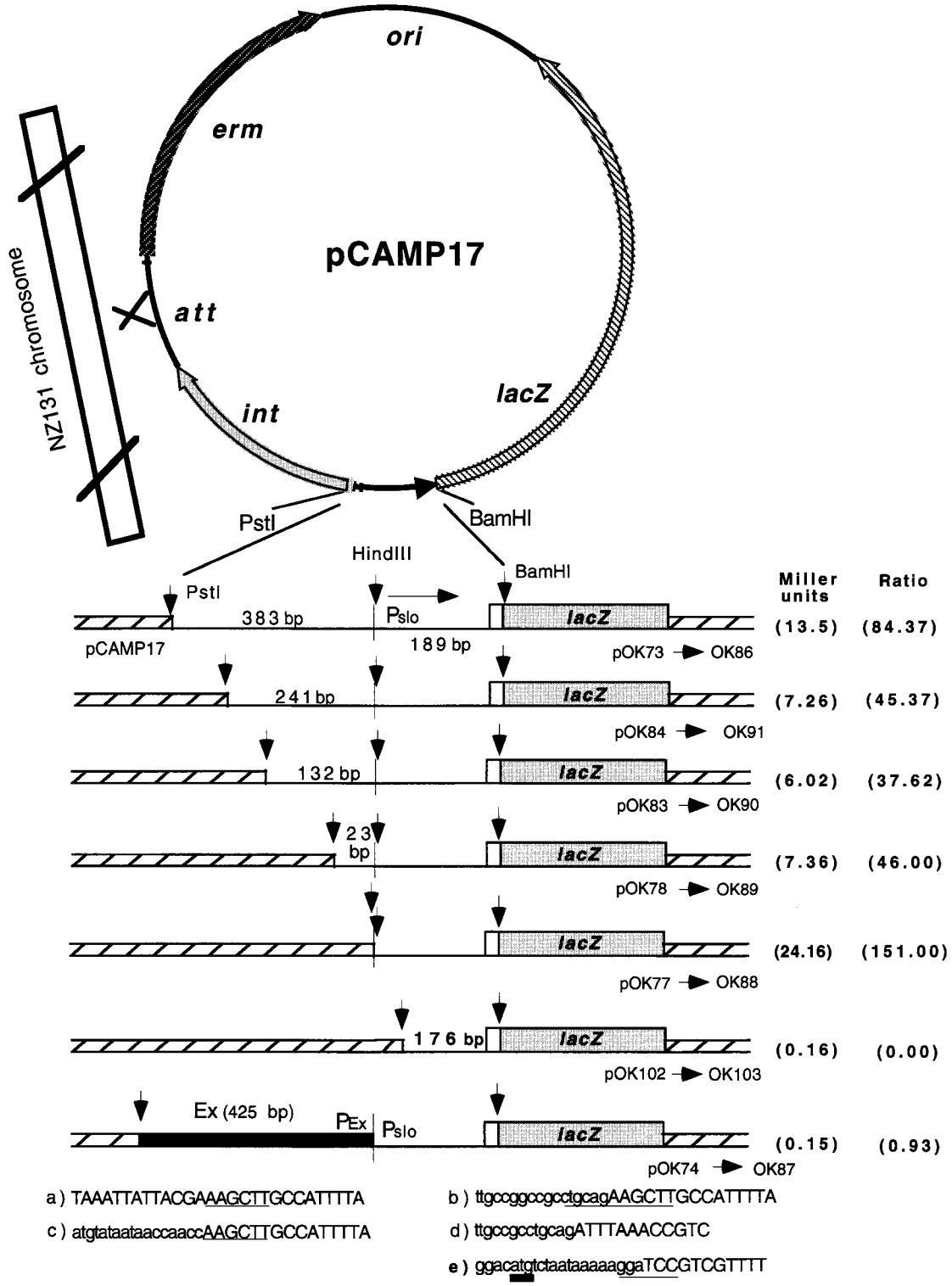

FIG. 6. Plasmid constructs with *lacZ* reporter gene fused to the proximal part of the *slo* gene and fragments harboring different constructs of the *slo* upstream region. The vertical line represents the recombination point between Ex and *slo* DNAs. Pslo and Pex denote the native *slo* promoter (22) and the hypothetical promoter situated on Ex DNA, respectively. The correctness of the nucleotide sequence in all constructs was verified by DNA sequencing. Strains OK86 to OK103 were generated by introducing plasmids pOK73 to pOK102, respectively, into the recipient strain NZ131. The genetic configuration of the plasmids with fusion upon their integration into the chromosome of NZ131 was verified by PCR with appropriate primers. The nucleotide sequences at the bottom denote the fusion point between the *slo* upstream region and pCAM17 DNA or Ex DNA in the presented constructs. (a) Strains OK86, OK91, OK90, and OK89; (b) strain OK88; (c) strain OK87; (d) strain 103; (e) nucleotide sequence at the junction (*Bam*HI site) between the proximal part of the *slo* gene (first 17 bp; open box) and the *lac*Z gene (shaded box) shared by all fusions. *Hin*dIII, *Pst*I, and *Bam*HI restriction sites are underlined. The translation initiation codon of the *slo* gene is underlined with a thick line. The values in the last column demonstrate the ratio between the β-galactosidase activity presented in the first column and the β-galactosidase activity obtained in strain OK103 with a deleted *slo* promoter (22).

tant chromosomal loci, as opposed to the Ex/*slo* recombination event, is a rare event. Finally, positioning two decamers into a proximity of only 50 nucleotides (Fig. 3) argues in favor of the rearrangement as a nonstochastic event. The TTGCCATTTT decamer appears 18 times in all three sequenced strains SF370, MGAS8232, and NZ131 (6, 25; W. M. McShan et al., unpublished data). The average distance between decamers in these strains is about 103 kb, while minimal and maximal distances between two decamers are approximately 4.2 kb and 280 kb, respectively. As computed from the chromosomal sequence of strain SF370, the TTGCCATTTT sequence is statistically expected only 3.4 times (17), which also argues against the random nature of this decamer and its coincidental positioning of two copies in the recombinant.

The results obtained with the *erm* (Fig. 2) and *lacZ* (Fig. 6) reporter genes for monitoring the effect of the rearrangement on the expression of the *slo* gene in a certain way confirmed the assumption about the nonrandom nature of the Ex/*slo* rearrangement. The rearrangement did produce an effect on expression of the *slo* gene, although the effect was quite opposite to what was expected, that positioning of the putative Ex promoter upstream of the *slo* gene would boost the expression of that gene in the recombinants (strain OK95, Fig. 2; strain OK87, Fig. 6). In both reporter systems, joining of the putative Ex DNA promoter to the *slo* gene upstream region resulted in a complete lack of expression of the reporter genes. The fact that the clone containing the identical segment of the *slo* gene upstream region joined not to the Ex DNA but to the pCAMP17 vector DNA (Fig. 6, strain OK88) showed significant expression of the reporter gene argues against the possibility that recombination immediately upstream of the *slo* gene native promoter (22) was detrimental to the function of that promoter. The lack of β-galactosidase activity in the strain with the deleted −35 sequence of the native *slo* promoter (Fig. 6) (22) shows that the activity of the reporter gene in strain OK88 is not a consequence of some spurious transcription coming from the pCAMP17 vector.

One of the possible answers for the difference in expression of the *lacZ* gene in strains OK87 and OK88 is that the native *slo* promoter might be a superhelicity-sensitive promoter, its activity being affected in different ways by the nearness of Ex DNA (OK87) and pCAMP17 DNA (OK88). A similar explanation is that the applied experimental system is unsuitable, i.e., that in strains OK87 and OK95, both native and putative Ex promoters are inhibited by structural constraints induced by inappropriate superhelicity of the chromosomal region in which they integrate. However, plasmid pOK67 generated strain OK95 by integrating itself into the Ex region (Fig. 2F), in contrast to pOK74, which integrated at a completely different site on the chromosome, the gene for serine-tRNA (Fig. 6) (14). It is hard to envision that only in the strains having Ex DNA joined to the *slo* DNA (OK87 and OK95), as opposed to other strains (e.g., OK96 and OK88), the local topology is affected so as to impede functioning of the promoters. In addition, the activity of promoters of the *cfa* gene (8) and the *speA* gene (J. Liu and J. J. Ferretti, unpublished data) monitored by the same system demonstrated normal β-galactosidase activities.

A recent important report on a type III-like secretion system in *S. pyogenes* may offer an alternative explanation for the biological meaning of the Ex/*slo* rearrangement (12). In that work, a model is presented in which the effector NAD-glycohydrolase, coded by *nga* (*spn*), the gene immediately upstream of the *slo* gene, is transported through the Slo pore into the target cell. It also suggests that the *slo* gene is a part of an *nga-slo* operon transcribed from a promoter immediately upstream of the *nga* gene, implying their common regulation of transcription (12). Consequently, the Slo protein is always physiologically connected to NAD-glycohydrolase, providing the secretion function. However, in our independent study (22), we showed that the *slo* gene, although being mostly transcribed from the strong *nga* promoter, also possesses its own promoter. This promoter shows weak activity, at least under in vitro experimental conditions (growth in THY), which explains the low values obtained with the *lacZ* reporter gene clones not containing the upstream *nga* promoter (Fig. 6) (22). We also found that the *slo* promoter is negatively regulated by the 19-kb-distant gene *sloR* (alias Spy0146 [6, 22]). These data suggest that the regulatory network of the *nga-slo* operon is more complex and that the products of the *nga* and the *slo* genes may also have other, independent roles in pathogenesis. The Ex/*slo* recombination that splits the *nga-slo* operon and physiologically separates NAD-glycohydrolase from Slo introduces a new variable into this genetic system.

The frequency of rearrangement, as measured in vitro by PCR, is low (10^{-4} in strain SF370 and 10^{-5} in strain NZ131). The detected frequency of recombination in these experiments may not be too low, since a quantitative study by Simpson and Cleary (24) showed that the gene for M12 protein reversibly switches between on and off states at a "high" frequency, from 10^{-3} to 10^{-4}, which is close to the frequency of Ex/*slo* rearrangement found in strain SF370 (10^{-4}). Thus, the observed rearrangement may belong to a plethora of still undefined genetic switches and rearrangements that cause instability of GAS strains. In addition, the Ex/*slo* rearrangement might be advantageous under certain physiological conditions, and mutants harboring the rearrangement could quickly become a significant or dominant part of the population.

We still do not know the extent of the chromosomal perturbation caused by the rearrangement. More genes may be involved, repression of the *slo* gene being only part of a more complex picture. Further experiments, including immortalization of the described rearrangement as a priority, are necessary for complete understanding of this phenomenon.

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