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Dual Control of Streptokinase and Streptolysin S Production by the *covRS* and *fasCAX* Two-Component Regulators in *Streptococcus dysgalactiae* subsp. *equisimilis*

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Synthesis of the plasminogen activator streptokinase (SK) by group A streptococci (GAS) has recently been shown to be subject to control by two two-component regulators, *covRS* (or *csrRS*) and *fasBCA*. In independent studies, response regulator CovR proved to act as the repressor, whereas FasA was found to act indirectly as the activator by controlling the expression of a stimulatory RNA, *fasX*. In an attempt at understanding the regulation of SK production in the human group C streptococcal (GCS) strain H46A, the strongest SK producer known yet, we provide here physical and functional evidence for the presence of the *cov* and *fas* systems in GCS as well and, using a mutational approach, compare the balance between their opposing actions in H46A and GAS strain NZ131. Sequence analysis combined with Southern hybridization revealed that the *covRS* and *fasCAX* operons are preserved at high levels of primary structure identity between the corresponding GAS and GCS genes, with the exception of *fasB*, encoding a second sensor kinase that is not a member of the GCS *fas* operon. This analysis also showed that wild-type H46A is actually a derepressed mutant for SK and streptolysin S (SLS) synthesis, carrying a K102 amber mutation in *covR*. Using *cov* and *fas* mutations in various combinations together with strain constructs allowing complementation in *trans*, we found that, in H46A, *cov* and *fas* contribute to approximately equal negative and positive extents, respectively, to constitutive SK and SLS activity. The amounts of SK paralleled the level of skc_{H46A} transcription. The most profound difference between H46A and NZ131 regarding the relative activities of the *cov* and *fas* systems consisted in significantly higher activity of a functional CovR repressor in NZ131 than in H46A. In NZ131, CovR decreased SK activity in a Fas^+ background about sevenfold, compared to a 1.9-fold reduction of SK activity in H46A. Combined with the very short-lived nature of *covR* mRNA (decay rate, 1.39/min), such differences may contribute to strain-specific peculiarities of the expression of two prominent streptococcal virulence factors in response to environmental changes.

The presence of the gene for the plasminogen activator streptokinase (SK) is a stable trait in group A streptococci (GAS) and human isolates of group C and G streptococci (GCS and GGS, respectively). In addition to its omnipresence in these organisms (11), the linkage relationships of this gene are preserved in a chromosomal region in which it is interspersed among five unrelated genes transcribed in the opposite direction (6, 7, 19). Its product, translated from a 440-codon monocistronic mRNA (16, 19), has no known homologues outside the genus *Streptococcus.* This protein, originally termed streptococcal fibrinolysin (3, 30), binds plasminogen with high affinity and, after activation of the zymogen (33), causes not only fibrino(geno)lysis but also the hydrolysis of various other substrates of the active enzyme, plasmin. The absence of fibrin in spreading streptococcal lesions has long been associated with the action of streptokinase as a virulence factor that contributes to the invasiveness of the pathogen, a notion supported by the failure of knockout mutants for the streptokinase gene to acquire cell-associated plasmin activity in the presence of plasminogen (15).

SK activities found in cell-free culture fluids can vary considerably among strains, ranging from exceedingly low fibrinolytic activities seen in, e.g., GAS strain NZ131 (≈3 U/ml in stationary-phase cultures) to very high activities (≈80 U/ml) as measured in the culture medium of the GCS strain H46A. In fact, in his early studies of SK-plasminogen interactions, Christensen (3) chose the latter strain as the source of the protein because H46A produced the most active fibrinolytic filtrates among more than 100 strains tested.

Recently, three lines of investigations have shed some light on the regulatory systems involved in the expression control of the SK gene. First, Northern hybridization analysis of the SK gene (*skc*) region of H46A revealed that of the six genes examined, *skc* is transcribed most abundantly (19) from a core promoter region resembling *Escherichia coli* σ^{70} consensus promoter sequences. With a TG motif one base upstream of the −10 region, this promoter qualifies as a promoter with an extended −10 region that directs transcription initiation predominantly at a G located 32 bases upstream of the *skc* translational start site (8). The −35 regions of *skc* and the oppositely oriented *lrp* gene adjacent to *skc* are separated by 202 bp of intergenic sequence that is intrinsically bent (9). Circular permutation analysis combined with computer modeling placed the bending center at position −98 relative to the major transcription initiation site of *skc.* Despite sequence differences in the intergenic regions between GCS and GAS, this bending locus is also present in NZ131, the curvature maps

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revealing a high degree of congruence at homologous positions (9).

The functional significance of the sequence features in the *skc-lrp* intergenic regions was analyzed with nested deletions that were inserted as single-copy promoter-*'lacZ* transcriptional fusions into the chromosome. Reporter gene activities revealed that full *skc* promoter activity depends strongly on the region containing the bending locus. Interestingly, deletion of the bending locus did not alter *skc* promoter strength in the heterologous *E. coli* background, where *skc* is generally highly expressed from its own promoter (8, 17, 21). In addition, reporter gene constructs containing the wild-type promoter upstream regions from H46A and NZ131 were exchanged in allele swap experiments between the GCS and GAS strains, with results showing strikingly that reporter gene activity expressed from the GAS construct is upregulated in the GCS strain background and, vice versa, expression of the GCS construct is downregulated in the GAS background. Taken together, these results show that the host genetic background dictates SK gene expression levels and suggest the existence of a *trans*-acting factor(s) with strain-specific activity that contacts the bending locus, thereby modulating SK gene expression (17).

Evidence for the existence in GAS of regulators that control *ska* expression has recently come from two additional lines of investigation. Several groups of investigators from different laboratories have identified the two-component signal transduction system CovRS, also designated CsrRS, as a pleiotropic repressor that negatively regulates the transcription of at least five virulence genes, among them *ska* (5, 10, 13, 20). Specifically, mutational analysis has also included strain NZ131 among the GAS strains in which *ska* expression is repressed by CovRS (27). Very recently, another group of investigators (12) has discovered, in GAS, the *fasBCA* two-component system that encodes, in addition to the response regulator (FasA), two histidine protein kinases (FasB and FasC). This system, which acts growth phase dependently and requires the RNA product of the *fasX* gene for activity, regulates *ska* expression positively (12). In NZ131, the four genes of the *fas* operon exhibit differential transcription modes, depending on nutritional conditions. Under amino acid starvation conditions, polycistronic *fasBCAX* mRNA accumulates, whereas under normal conditions, *fasX* is preferentially transcribed separately from one of its two operon-internal promoters, P_2 (28).

The *skc* gene found in the GCS strain H46A is the first SK gene that was cloned and sequenced (14, 16). As this strain has also been exploited in industrial SK production for fibrinolytic therapy, it is of obvious interest to understand the mechanism(s) which controls the synthesis of this protein in H46A. Here we show that the *cov* and *fas* systems also function in this strain. Whereas their actions in GAS have been studied independently of one another, we analyzed the balance between their opposing actions and show that wild-type H46A is actually a derepressed mutant for Skc production. As the *cov* and *fas* systems also target the streptolysin S (SLS) operon, *sag* (5, 10, 12, 22), we included the control of SLS production in our analyses.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The GCS strain H46A and the GAS strains NZ131 and SF370 were grown in ambient air at 37°C without agitation in brain heart infusion (BHI) broth (Difco) or chemically defined medium (CDM) buffered with 26 mM morpholinepropanesulfonic acid (18). The complete genomic sequence of SF370 (6) allowed derivation of oligonucleotide primers for the synthesis of specific PCR-generated genes or gene fragments from the streptococcal chromosomal DNAs as templates. *E. coli* JM109 was used as the host for plasmid constructions and was grown at 37°C in rotary flasks in standard Luria broth (LB). Concentrations of antibiotics used were as follows: chloramphenicol, 3 μg/ml for streptococci and 100 μg/ml for *E. coli*; erythromycin, 2.5 μg/ml for streptococci and 200 μg/ml for *E. coli*; kanamycin, 100 μg/ml for streptococci and 50 μg/ml for *E. coli*; and spectinomycin, 100 μg/ml for both streptococci and *E. coli*.

Nucleic acid techniques. Plasmid isolation, DNA fragment isolation and ligation, electrotransformation of *E. coli*, random-primed DNA labeling, Southern hybridization, and DNA sequencing by the dideoxynucleotide chain termination method were performed by standard procedures (25) or according to the manufacturer's recommendations. Chromosomal DNA from streptococci was isolated by the method of Caparon and Scott (2). Electrotransformation of streptococci was carried out as described previously (8). PCR, performed with *Pwo* DNA polymerase (Peqlab Biotechnologie, Erlangen, Germany), was used to amplify DNA fragments for gene isolation, sequence analysis, insertion mutagenesis, DNA probe production, and confirmation of the constructed plasmids and streptococcal insertion mutants. Total RNA isolation from streptococci, Northern hybridization of streptococcal RNA, and determination of mRNA chemical half-lives have been described in detail elsewhere (17). Hybridization reactions were quantified by PhosphorImager (Fujix BAS1000) analysis with software TINA 2.08c from Raytest (Straubenhardt, Germany).

Construction of *cov* plasmids. Plasmid pM$covR_{SF}$, containing the complete SF370-derived *covR* gene, including its promoter, was constructed by generation of an amplicon with primers *covR*F1 (ccggaattcCAAGGGTTGTTTGATGAATA) and *covR*R1 (ccggaattcATGACTTATTTCTCAC) (lowercase letters indicate sequences added to facilitate cloning.) The *Eco*RI cleavage product of this DNA was cloned into pMG36e (31) with JM109 as the host. The resultant plasmid, pM$covR_{SF}$, was isolated from JM109 and introduced into H46A by electrotransformation, and transformants were selected with erythromycin.

To construct pVA$covRS_{NZ}$, containing the complete *covRS* genes from NZ131, including the promoter of the operon, NZ131 template DNA was amplified with primers *covR*F1 (see above) and *covS*R1 (cgcctgcagCTTAAGCTACTCTAACTCTC). The *Eco*RI plus *Pst*I cleavage product of this amplicon was inserted into pVA8912 (26), and plasmid transformants of JM109 were selected with erythromycin. The resultant pVA$covRS_{NZ}$ plasmid was linearized with *Eco*RI and used for ligation with an *Eco*RI fragment containing the 3′ end of the *dexB* gene from H46A to form pVA$covRS_{NZ}$dI and pVA$covRS_{NZ}$dII containing *'dexB* in either orientation. These suicide plasmids, unable to replicate in streptococci, were introduced into H46A, and erythromycin-resistant transformants were confirmed by appropriately primed PCR to result from insertion of the plasmids into the resident *dexB* gene of the H46A chromosome (19).

Construction of plasmid pFC1 containing an internal $covR_{NZ131}$ fragment in the vector pFW5 (selective marker, spectinomycin resistance; GenBank accession number U41082) has already been described (27). Likewise, NZ131 *covR*::pFC1 isolates bearing *covR* truncated at codon 174 were characterized previously (27). For complementation, these strains were transformed with pVA $covRS_{NZ}$, selecting for spectinomycin plus erythromycin resistance.

Construction of *fas* plasmids. For insertional inactivation of $fasA_{H46A}$, two different fragments internal to the $fasA_{H46A}$ coding region were amplified by PCR with the following primers: *fasA*F1B (cgcggatccCCAGCAGACACGCATAGAATC), *fasA*R2H (cgcaagcttCGGTGCTACTGCTTGAATCTCAG), *fasA*F2B (cgcggatccCTTGAATTAGCTGCAGCTATTCG), and *fasA*R1H (cgcaagcttCACAAGGTAGGATCTATGGC). The *Bam*HI plus *Hin*dIII cleavage products of the amplicons were cloned in pFW5 (see above), pFW15 (erythromycin resistance; GenBank accession number U50977), or pFW14 (chloramphenicol resistance; GenBank accession number U50981; identical to pFW8) to form pFW5*fasA1*, pFW15*fasA1*, and pFW14*fasA2*, respectively. Electrotransformation of H46A with the latter two mutagenic plasmids and verification of the transformants resulted in strains H46A*fasA*::pFW15*fasA1* and H46A*fasA*::pFW14 *fasA2*, carrying *fasA* alleles interrupted at codon positions 194 (*fasA1*) and 205 (*fasA2*), respectively. Plasmid pFW15 *fasA1* was also used to construct the corresponding *fas* insertions in NZ131*covR*::pFC1. Plasmid pFW5*fasA1* served to inactivate *fasA* in H46A*dexB*::pVA$covRS_{NZ}$dII.

For complementation of the insertionally inactivated $fasA_{H46A}$ gene, plasmid pFasAX_{SF} was constructed, which contains the complete coding sequences of $fasAX_{SF370}$, the expression of which is driven by promoter P32 of the vector pMG36 (kanamycin resistance) (31, 32). The $fasAX_{SF370}$ genes were amplified by

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Streptococcus dysgalactiae subsp. *equisimilis*	Human serogroup C	
H46A	Cov^- (K102 amber) Fas^+	3; this study
H46A(pM*covR*$_{SF}$)	Cov^+ Fas^+; Em^r	This study
H46A*dexB*::pVA*covRS*$_{NZ}$dI	Cov^+ Fas^+; Em^r	This study
H46A*dexB*::pVA*covRS*$_{NZ}$dII	Cov^+ Fas^+; Em^r	This study
H46A*fasA*::pFW15*fasA1*	Cov^- Fas^-; Em^r	This study
H46A*fasA*::pFW14*fasA2*	Cov^- Fas^-; Cm^r	This study
H46A*fasA*::pFW15*fasA1*(pFasAX$_{SF}$)	Cov^- Fas^+; Em^r Km^r	This study
H46A*dexB*::pVA*covRS*$_{NZ}$dII, *fasA*::pFW5*fasA1*	Cov^+ Fas^-; Em^r Spc^r	This study
Streptococcus pyogenes	Human serogroup A	
NZ131	Cov^+ Fas^+	J. J. Ferretti
NZ131*covR*::pFC1	Cov^- Fas^+; Spc^r	27
NZ131*covR*::pFC1, *covR*::pVA*covRS*$_{NZ}$	Cov^+ Fas^+; Spc^r Em^r	This study
NZ131*covR*::pFC1, *fasA*::pFW15*fasA1*	Cov^- Fas^-; Spc^r Em^r	This study
Plasmids		
pMG36, pMG36e	Vectors; Km^r and Em^r respectively	31
pM*covR*$_{SF}$	pMG36e containing *covR*$_{SF370}$ under its natural promoter; Em^r	This study
pVA8912	Suicide vector; Em^r	26
pVA*covRS*$_{NZ}$	pVA8912 containing *covRS*$_{NZ131}$ under its natural promoter; Em^r	This study
pVA*covRS*$_{NZ}$dI	pVA8912 containing *covRS*$_{NZ131}$ under its natural promoter and the 3′ end of *dexB*$_{H46A}$ (orientation I) for genomic integration into H46A; Em^r	This study
pVA*covRS*$_{NZ}$dII	As pVA *covRS*$_{NZ}$dI but 3′ end of *dexB*$_{H46A}$ in orientation II; Em^r	This study
pFW5, pFW14, pFW15	Vectors; Spc^r, Cm^r, Em^r, respectively	24
pFW5*fasA1*	pFW5 containing an internal fragment of *fasA*$_{H46A}$; Spc^r	This study
pFW15*fasA1*	As pFW5 *fasA1*; Em^r	This study
pFW14*fasA2*	pFW14 containing an internal fragment of *fasA*$_{H46A}$; Cm^r	This study
pFasAX$_{SF}$	pMG36 containing *fasAX*$_{SF370}$ under vector promoter P32; Km^r	This study

PCR with primers *fasA*F3 (tcccccgggGACAATTGTTAGAAAGGAGATAA AG) and *fasX*R3 (cgcaagcttGACGTCAGCTACTTATCCCTG). Amplicon DNA restricted with *Sma*I plus *Hin*dIII was inserted into pMG36, and pFasAX$_{SF}$ was primarily established in JM109. Plasmid pFasAX$_{SF}$ DNA was then used to transform H46A*fasA*::pFW15*fasA1*, with selection for erythromycin plus kanamycin resistance.

To facilitate comprehension, the relevant characteristics of the strains and plasmids used in this study are compiled in Table 1.

Probes used for Southern and Northern hybridizations. Probes were generated with the following primer sets: *covR*F1 and *covR*R1 (see above) for *covR*; CCAGCAGACACGCATAGAATC and CACAAGGTAGGATCTATGGC for *fasA*; CAGTATGGCTTTATTCTATGCTACC and GGAATTCATGATACTGCGAATCAC for *fasC*; GTCGCCATTGCTAATTGATCCTC and GTTGTGCTTCAAGAGCTGCCTC for *fasB*; GAGAGCAATAACATTTTAGGAC and GACGTCAGCTACTTATCCCTG for *fasX*; ggcgccaagcttTTTTAGCTCCATAGCCATTCC and ccgtggTTATTTGTCGTTAGGGTTATCAGG for *ska* and *skc*; GCTACTAGTGTAGCTGAAACAAC and CCTGAGACGTTAGCATCAAGAAG for *sagA*; and CAAGTCAACAGTGGAGAGAAC and CGGTAGTGTTATGAAGGATGAC for *mga*.

Protein activity assays. The plasminogen activation assay on microtiter plates as described by Tewodros et al. (29) was used to measure SK activity in BHI culture supernatant fluids of the various wild-type and mutant strains. The release of *para*-nitroaniline from the chromogenic substrate H-D-valyl-leucyl-lysin *p*-nitroaniline (Sigma) was measured at an optical density at 405 nm (OD_{405}) over time, and activity rates were calculated from the linear parts of absorbance versus time plots. SK activities were also visualized as caseinolytic zones on agarose well plates containing plasminogen and casein as described previously (14). Standard streptokinase came from Sigma and its definition was used to determine specific activities.

SLS activity was measured with cultures grown in BHI containing 10% horse serum. The SLS activity assay of culture supernatant fluids was performed with rabbit erythrocytes according to Heath et al. (10). The release of free hemoglobin was measured at an OD_{540} in the presence of cholesterol (10 μg/ml) to inhibit streptolysin O and in some samples in the presence of trypan blue, an inhibitor of SLS, to control the specificity of the assay.

Nucleotide sequence accession numbers. The nucleotide sequences of the *cov* and *fas* genes from *Streptococcus dysgalactiae* subsp. *equisimilis* strain H46A have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AY075106 and AY075107, respectively.

RESULTS

Identification of the *covRS* locus in *S. dysgalactiae* subsp. *equisimilis*. In search of *Streptococcus pyogenes covRS* homologues in strain H46A, the heterologous primer pair *covR*F1 and *covS*R1, designed on the basis of the genomic sequence of SF370 (6), was used to produce by PCR from the chromosomal DNA of H46A a weak DNA band corresponding in size to the entire *covRS* locus of SF370 (≈2.5 kb). Sequence determination of this DNA with walking primers covered 1,141 nucleotides counting from the *covR*F1 forward primer. This sequence revealed a high degree of homology to the three *S. pyogenes covRS* operons sequenced so far from different strains, SF370 (6), Manfredo (http://www.sanger.ac.uk), and DLS003 (13). Our sequence represented the entire *covR*$_{H46A}$ gene, including its promoter region, the very short intergenic region between *covR*$_{H46A}$ and *covS*$_{H46A}$ (5 nucleotides), and the first 59 codons of *covS*$_{H46A}$.

The coding sequences of *covR*$_{H46A}$ and *covR*$_{SF370}$ and the derived amino acid sequences showed 84 and 98% identity, respectively. Similarly, the partial sequence of *covS*$_{H46A}$ was 88% and the deduced amino acid sequence was 95% identical to the corresponding sequences from SF370. Most importantly, the *covR*$_{H46A}$ sequence contained a translational stop codon at codon position 102, which altered the corresponding AAA triplet present in all known GAS *covR* genes to read TAG in H46A. Sequence analysis of this position done repeatedly with

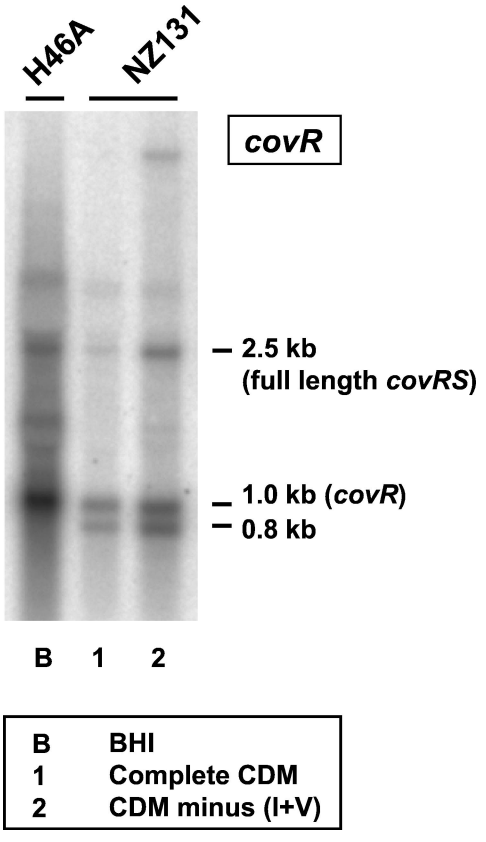

FIG. 1. Detection of *covRS* mRNA in H46A and NZ131 incubated in the indicated media. Shown is a Northern blot of total RNA probed with *covR* (boxed).

independently isolated H46A chromosomal DNAs confirmed that this mutation, which requires 2 base substitution events, is a genuine H46A trait rather than being introduced during DNA manipulations. Given that the full-size CovR protein contains 228 amino acids, its truncated form in H46A, presumably missing the DNA-binding domain, was highly likely to be functionally inactive. In the context of this work, however, we refer to H46A with the identified amber mutation in *covR* as the wild type.

Similar to the high degree of coding sequence conservation, the $covRS_{H46A}$ promoter, resembling *E. coli* σ^{70} promoters, was found to be identical in both the −10 region (TATACT) and the −35 region (TTGAAA) to the corresponding GAS promoters. Likewise, the putative transcriptional start site at a G eight bases downstream of the −10 hexanucleotide was identical to that determined for GAS strain MGAS166 (20). However, the relatively long sequence between the −10 region and the translational start codon, which is almost identical among the GAS strains, differed from them in 79 of 212 base positions.

Properties of *covRS* mRNA. Similar to what has been found for transcription of $covRS_{DLS003}$ (13), Northern hybridization analysis of $covRS_{H46A}$ mRNA probed with *covR* revealed cotranscription of the two genes in the GCS strain as well (Fig. 1). However, in addition to the 2.5-kb dicistronic transcript, a prominent 1.0-kb transcript corresponding in size to monocistronic *covR* mRNA was detected in both H46A and NZ131. Since no terminator sequence was visible downstream of *covR*, this transcript might result from degradation of the dicistronic message, as might a distinct 0.8-kb RNA seen in NZ131 but not in H46A (Fig. 1). Loss of about 200 nucleotides from the 5′ end would still allow functional activity of *covR* mRNA, as judged by the retained ribosome-binding site.

The stability of *covR* mRNA was determined in NZ131 rather than H46A because the nonsense mutation in $covR_{H46A}$ could lead directly to decreased message stability. Decay kinetics were measured in comparison to those of *ska* and *mga* mRNAs during a time period within which new synthesis of mRNA was inhibited by rifampin (Fig. 2). The chemical half-lives of the three messages amounted to 0.5, 2.5, and >10 min, respectively, corresponding to decay rates of 1.39/min, 0.28/min, and <0.07/min, respectively. This indicates the extremely short-lived character of *covR* mRNA in contrast to *mga* mRNA, which proved to be very stable. It is interesting that the mRNA half-lives of two important pleiotropic regulatory genes can differ more than 20-fold.

Identification of *fasCAX* genes in *S. dysgalactiae* subsp. *equisimilis*. Using the SF370-based heterologous primer sets for *fasA*, *fasX*, and *fasAX* (see the Materials and Methods section), we were able to produce from H46A chromosomal DNA three amplicons, the DNA sizes of which agreed with those predicted from the *fasAX* sequence of SF370 (580, 322, and 1,095 bp). Using standard PCR conditions, we failed, however, to produce any amplicons that corresponded to the proximal *fasBC* genes from H46A template DNA when SF370-specific primers for these genes were employed. With temperature gradient PCR and $fasB_{SF370}$-specific primers (see Materials and Methods), a 600- to 700-bp fragment was obtained that deviated from the predicted length of 931 bp. Sequence analyses of this DNA ruled out its relationship to $fasB_{SF370}$ but revealed homology to the Spy1148 gene product of SF370, a predicted ATP-binding protein of an ABC transporter (6).

Evidence for the existence of a $fasC_{SF370}$ homologue and for linkage of *fasCAX* in H46A was obtained by Southern hybridization analysis. The gene-specific probes used included both heterologous and homologous DNAs, namely $fasC_{SF370}$, $fasA_{SF370}$, $fasA_{H46A}$, $fasX_{SF370}$, and $fasX_{H46A}$. All probes detected hybridizing H46A DNA fragments and, as controls, SF370 DNA fragments, with $fasC_{SF370}$ giving the weakest signal with H46A DNA. Hybridizing $fasCAX_{H46A}$ DNA was associated with a ≈3.5-kb *Msp*I fragment, a ≈2.2-kb *Sty*I fragment, and a ≈6.5-kb *Hae*II fragment (Fig. 3). For comparison, $fasCAX_{SF370}$ DNA has a length of 2.3 kb (6).

Determination of the complete coding sequences of the $fasAX_{H46A}$ genes by primer walking revealed a high degree of homology to the corresponding genes from SF370. The $fasA_{H46A}$ gene and deduced protein showed, respectively, 81 and 90% sequence identity to $fasA_{SF370}$, and the sequence of $fasX_{H46A}$ was 95% identical to that of $fasX_{SF370}$. Taken together, these results provided physical evidence for the presence of homologues of the $fasCAX_{SF370}$ genes in H46A but failed to detect a proximal *fasB* gene encoding a second sensor kinase.

Functional analysis of *covRS* in H46A. To answer the question of whether or not heterologous *covR* or *covRS* genes are capable of complementing in *trans* the putatively inactive

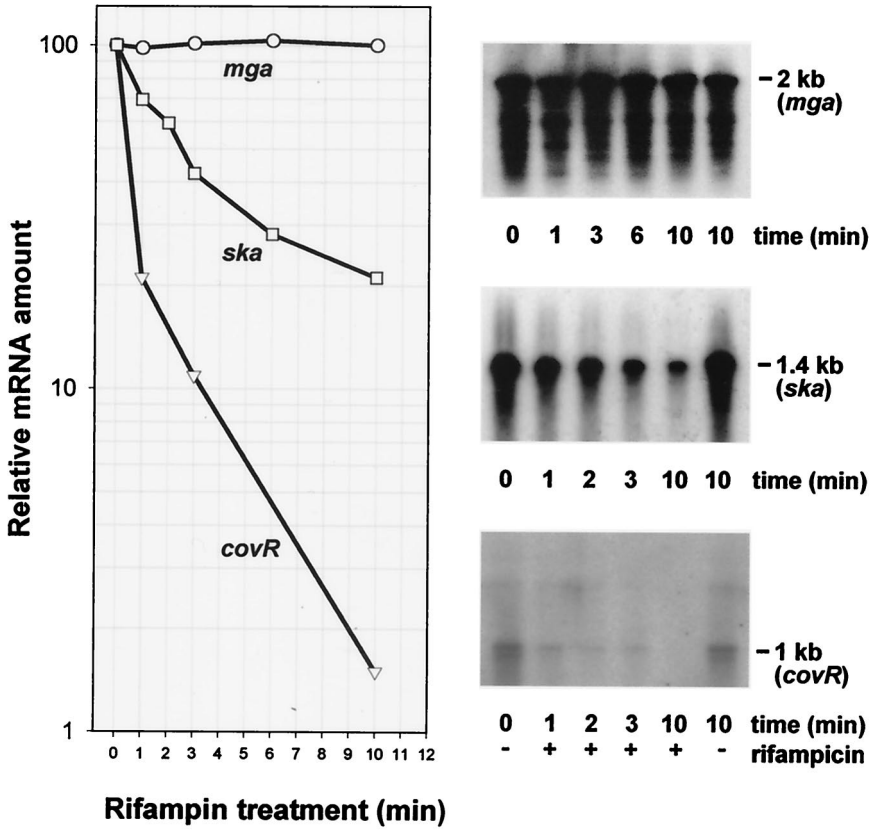

FIG. 2. Determination of mRNA half-lives of the indicated NZ131 genes under conditions in which new mRNA synthesis is inhibited by rifampin. Estimation of decay kinetics of the mRNAs (left) is based on Northern blots, representative results of which are shown on the right.

covR$_{H46A}$ gene, two complementation systems were established by transformation of H46A. In the first system, functional *covR*$_{SF370}$ was delivered in multicopy plasmid form by the construction of H46A(pM*covR*$_{SF}$). In the second system, *covRS*$_{NZ131}$ was introduced into H46A in single-copy form by integration into the resident *dexB* gene in either orientation to form H46A*dexB*::pVA*covRS*$_{NZ}$dI and H46A*dexB*::pVA*covRS*$_{NZ}$dII. The three transformed strains were used to determine the amounts of SK and SLS in culture supernatant fluids relative to the amounts of these proteins in comparable cultures of the original H46A strain.

Depending on the particular transformant strain examined, repression of SK production ranged from 68 to 48%, and that of SLS ranged from 75 to 24% (Fig. 4). In general, *cov* activity delivered in plasmid form tended to have a greater repressive effect than that specified by *cov* integrated into the chromosome. Furthermore, in the integrated form, *covRS* was more active in orientation dII than dI, suggesting some *dexB* promoter contribution to the expression level of *covRS* in the dII orientation. We conclude from these results that, regardless of their copy number, the *covRS* genes from GAS were functionally active as repressors of H46A SK and SLS synthesis. Moreover, the heterologous CovS$_{H46A}$ and CovR$_{SF370}$ protein pair is suggested to interact normally in strain H46A(pM*covR*$_{SF}$), as might be expected from the high degree of sequence identity between CovR$_{H46A}$ and CovR$_{SF370}$.

In NZ131*covR*::pFC1 isolates, inactivation of the resident *covR* gene increases SK production about 10-fold (27). At the level of *ska*$_{NZ131}$ transcription, derepression of *ska* increased the *ska* mRNA level about 100-fold (Fig. 5). Complementation of NZ131*covR*::pFC1 with single-copy pVA *covRS*$_{NZ}$ restored

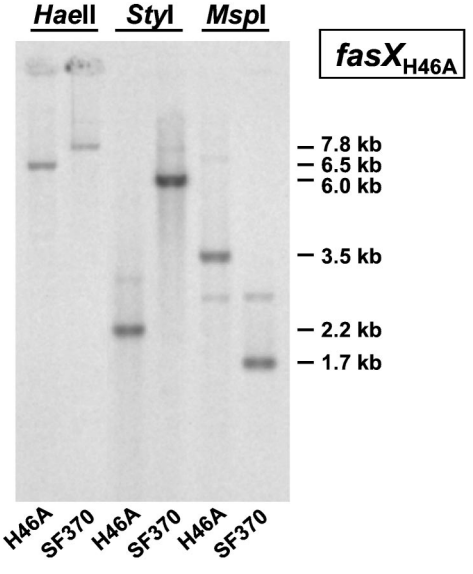

FIG. 3. Detection of the *fasCAX* operon by Southern hybridization of H46A and SF370 chromosomal DNAs restricted with the indicated enzymes and probed with *fasX*$_{H46A}$ (boxed).

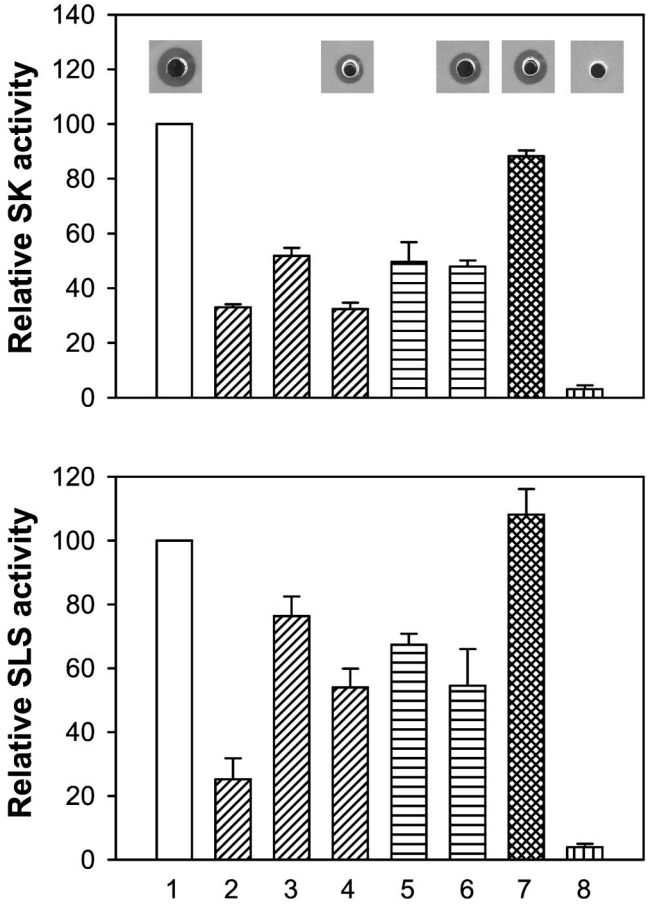

FIG. 4. SK and SLS activities in cell-free supernatant fluids of BHI cultures of the specified H46A wild-type and mutant strains containing comparable numbers of CFU. For SK determination, cultures were sampled after 16 h of growth, whereas log-phase cultures grown to an OD_{600} of 0.30 were used for SLS determination. Relative activities of 100% correspond to 80 U of SK per ml or 0.75 OD_{540} hemolytic units produced by 200 μl of a 1:40 diluted supernatant fluid obtained from an H46A culture. (For comparison, an OD_{540} value of 1.15 corresponds to complete hemolysis produced by the same volume of water.)

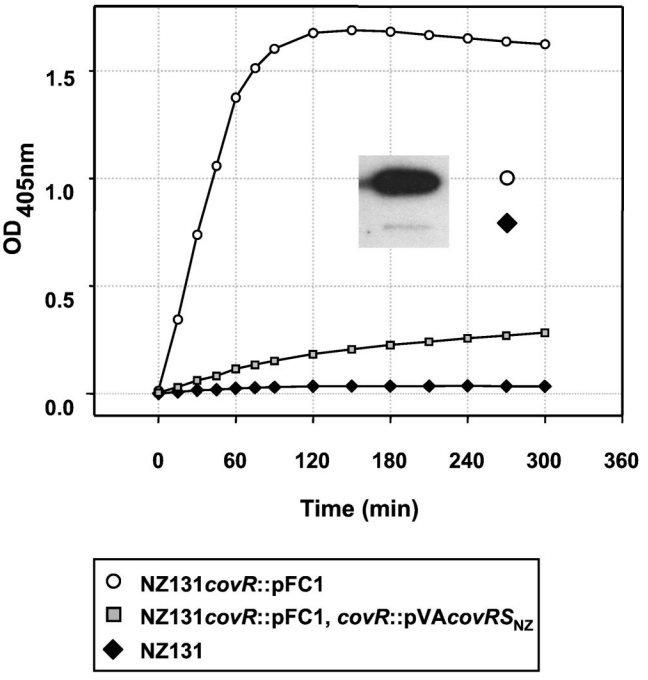

FIG. 5. CovR-regulated SK production by wild-type NZ131, the *covR* mutant, and complemented *covR* mutant strains. SK activities of culture supernatant fluids were compared by measuring plasminogen activation with a plasmin-specific chromogenic substrate as a function of time. The inset shows a slot blot hybridization of total RNA extracted from the indicated strains and probed with *ska*.

the repression of SK production close to the very low NZ131 wild-type level, indicating high CovR repressor activity in this strain (Fig. 5). In comparison, the corresponding complementation system caused strain H46A to retain 40 to 50% of its wild-type SK production level (Fig. 4).

Functional activity of the *fas* system in H46A. We used insertional mutagenesis of $fasA_{H46A}$ to explore whether this response regulator influences the levels of SK and SLS production in H46A. The results showed that, relative to protein activities measured in culture supernatant fluids of H46A, SK activities produced by H46A*fasA*::pFW15*fasA1* and H46A*fasA*::pFW14*fasA2* were reduced by about 50% and SLS activities dropped to an average of about 60% (Fig. 4). Complementation in *trans* of the interrupted *fasA* gene by introduction of plasmid pFasAX$_{SF}$ restored the wild-type levels of both proteins (Fig. 4). We conclude from these data that the *fas* system positively regulates the *skc* and *sag* genes in H46A. Combination of the functional *covR* repressor with an inactivated *fasA* gene in strain H46A(*dexB*::pVA*covRS*$_{NZ}$dII, *fasA*::pFW5*fasA1*) decreased the SK and SLS production capacities of H46A to 3 and 4%, respectively, showing that in the absence of positive regulation, the negative *covR* regulator almost completely abolished the capability of H46A to produce the two proteins (Fig. 4).

The results obtained at the protein level for the combined effects of the *cov* and *fas* regulatory systems correlated with quantitative *skc* transcript analysis. The Northern blot in Fig. 6 shows that inactivation of $fasA_{H46A}$ reduced *skc* mRNA abundance to 79%, and restoration of *covR* activity decreased the amount of *skc* mRNA further to 16% of that seen in the wild type.

Transcriptional analysis of *fas* system in H46A. According to previous results, regulation of *fas* operon transcription in GAS occurs in a complex manner. The transcriptional profile has been found to be growth phase dependent (12), influenced by nutritional conditions (28), and also determined by the presence of at least two operon-internal promoters for *fasX*

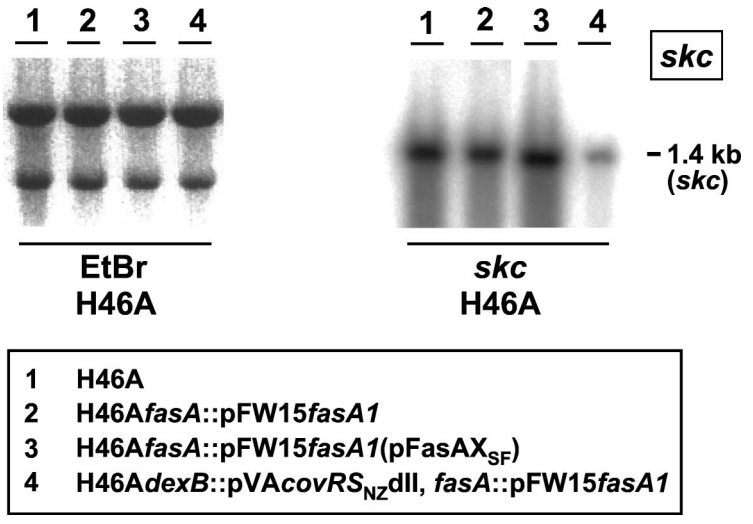

FIG. 6. Transcriptional regulation of *skc* expression by the *cov* and *fas* regulatory systems demonstrated by Northern hybridization of total RNA extracted from the indicated strains and probed with *skc* (boxed). The ethidium bromide (EtBr)-stained gel on the left demonstrates equal loading of the slots and integrity of the 23S and 16S rRNAs.

(12, 28), the relative activities of which are responsive to amino acid availability (28). In agreement with the situation found previously in strain NZ131 (28), H46A RNA isolated from log-phase or stationary-phase cells grown in BHI or CDM and probed with *fasX* yielded only one strongly hybridizing band at 0.2 kb, corresponding to *fasX* RNA produced from the downstream P_2 operon-internal *fasX* promoter (Fig. 7). Probing the same RNAs with a homologous *fasA* or heterologous *fasC*$_{NZ131}$-specific DNA failed to detect any transcripts corresponding to these genes. However, total RNA isolated from strain H46A*fasA*::pFW15*fasA1*(pFasAX$_{SF}$), in which transcription of *fasAX*$_{SF}$ was driven from the strong P32 promoter of the vector pMG36 (32), yielded, in addition to the 0.2-kb monocistronic *fasX* transcript, a prominent 1.1-kb dicistronic transcript corresponding to *fasAX*.

These results corroborate our previous observation that *fasBCAX*$_{NZ131}$ can be cotranscribed but that, in addition to transcriptional linkage, there is strong independent transcription of *fasX* from its P_2 promoter. Expectedly, RNA isolated from H46A*fasA*::pFW15*fasA1* and probed with *fasX* did not show the 1.1-kb *fasAX* transcript. Importantly, this RNA also lacked the 0.2-kb *fasX* transcript (Fig. 7). Its absence cannot be attributed to the polar nature of the *fasA* insertion; rather, it confirms the previous finding of Kreikemeyer et al. (12) showing that *fasX* transcription from its own promoter(s) is dependent on the *fasA* gene product.

SK production levels of H46A and NZ131 strains with comparable genetic backgrounds. The H46A and NZ131 mutant strains constructed for the *cov* and *fas* systems enabled us to compare their relative efficiencies in the two strains and to detect strain-specific differences (Table 2). In a Fas$^+$ background, repression of SK synthesis by CovRS was approximately sevenfold stronger in NZ131 than in H46A where it only halved the SK production. Accordingly, when both systems were active, SK production by H46A exceeded that of NZ131 by a factor of about 13. In contrast, in a Cov$^-$ background, the positive action of the Fas system did not differ substantially between the two strains, promoting SK production by factors of 2 and 1.5, respectively. In the absence of both repression and activation, i.e., in a Cov$^-$ Fas$^-$ background, the remaining SK activity would appear to reflect mainly the strength of *cis*-active elements. This constitutive level of SK expression appeared to be greater by merely a factor of 1.3 in H46A relative to that seen in N131 (Table 2).

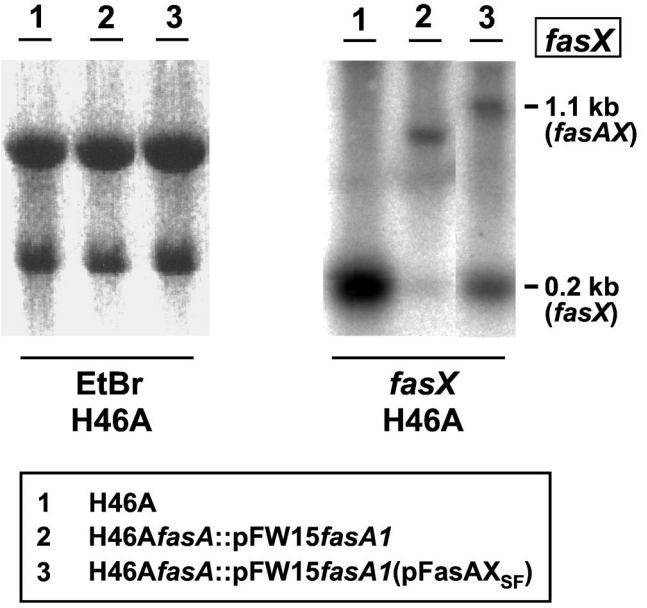

FIG. 7. Cotranscription of *fasAX* and dependence of separate transcription of *fasX* from its own promoter on the presence of FasA, demonstrated by Northern hybridization of total RNA extracted from the indicated strains and probed with *fasX* (boxed). The ethidium bromide (EtBr)-stained gel on the left demonstrates equal loading of the slots and integrity of the 23S and 16S rRNAs.

TABLE 2. Comparison of SK production by strains H46A and NZ131 as a function of the genetic background

Phenotype	SK activity[a] (U/ml)		Activity ratio	
	H46A	NZ131	H46A	NZ131
Cov^+ Fas^+	42	3.2		
Cov^- Fas^+	80	44		
Cov^+ Fas^-	2.4	ND[a]		
Cov^- Fas^-	39	30		
Cov^- Fas^+/Cov^+ Fas^+			1.9	13.8
Cov^- Fas^+/Cov^- Fas^-			2.0	1.5

[a] ND, not determined.

DISCUSSION

In this study, we provided physical and functional evidence for the existence of the *cov* and *fas* two-component regulatory systems in the genome of GCS. In GAS, recent electrophoretic mobility shift and footprinting experiments have revealed the binding sites of CovR in the promoter regions of the regulated genes (1, 20). The footprints in the *ska* promoter region are large, measuring >130 bp and containing AT-rich DNA. The search of common sequence tracts in the footprints of five CovR-regulated genes led to the identification of a conserved 16-bp sequence with runs of A's and T's (20). For the ska_{NZ131} and skc_{H46A} promoter regions, the sequences that matched the 16-bp motif most closely are given in Table 3.

Since *covR* is autoregulated (5), we also inspected the promoter region of $covR_{H46A}$ and found a sequence tract that showed some adherence to the 16-bp motif (Table 3). However, $CovR_{MGAS166}$ was found not to bind to its own promoter region (20), which makes it difficult to understand the mechanism of autoregulation. Moreover, as CovR recognition of its target promoters requires AT-rich DNA segments much longer than 16 bp, direct evidence for the involvement of this motif in CovR binding is missing (20). In a similar vein, comparison of the 16-bp sequence between the promoter regions of skc_{H46A} and ska_{NZ131} does not suggest any explanation of why $CovR_{NZ131}$ represses transcription of ska_{NZ131} more strongly than that of skc_{H46A} (Table 2). The fact that we restored repressor activity in H46A by complementation with the heterologous $covR_{NZ131}$ gene does not seem to explain the strain-specific CovR activities, given the high degree of sequence identity between the CovR proteins of different strains (≈98%). At the target DNA level, the biggest sequence difference between skc_{H46A} and ska_{NZ131} consists in the presence or absence of a 9-bp direct repeat (CATTATCAT) located between the bending center and the −35 region. The homologue of this sequence is present only once in H46A, and this offers a testable hypothesis about its possible involvement in differential CovR binding.

Of considerable interest is our finding that the *covRS* system in H46A is inactive because of a naturally acquired K102 amber mutation in *covR*. A systematic search of spontaneous *covRS* mutations in an in vivo mouse model of GAS infection led Engleberg et al. (4) to the discovery of frameshift, missense, nonsense, and IS*1548* insertion mutations in the gene tandem. These authors also detected mutated *covRS* loci in a high proportion of a panel of clinical GAS isolates (4). Expectedly, spontaneous mutations such as those created by site-directed mutagenesis have proven to enhance the virulence of the mutant strains in animal models of GAS infection (4, 10). The reported hypermutability of the *covRS* system and the idea that the course of a streptococcal infection could be determined by the clonal fluctuation of more virulent subpopulations of the original wild-type strain (4) is worth pursuing and would also need to include human GCS. Given the disparity of the mutations found in the *covRS* locus, the mechanism(s) that renders it hypermutable remains a mystery. An alternative explanation involves random mutation associated with a strong in vivo selection for the loss of *covRS* expression, which could confer a selective advantage on *covRS* mutants.

The number of times that an mRNA is translated, and hence the amount of protein it produces, will be determined by its half-life. This means of gene regulation has not been explored before in the pathogenic streptococci, and our results concerning the stability of *covRS*, *ska*, and *mga* mRNAs bear on this problem. Whereas the half-life of *ska* mRNA falls in the range typical of many bacterial mRNAs, *covRS* mRNA is extremely short-lived, and in contrast, *mga* mRNA is very long-lived. The *mga* and *covRS* pleiotropic regulators have distinct arrays of target genes, *mga* controlling genes related predominantly to the production of cell surface proteins and *covRS*-regulated gene products being released into the extracellular environment. Grossly different stabilities of the messages of these regulators will render the surface proteins more rigidly expressed than the released proteins, the levels of which may quickly respond to changing environmental conditions. As a case in point, the level of *covRS* mRNA but not that of *mga* mRNA increases by an as yet undetermined mechanism during amino acid starvation (27) (see Fig. 1). Thus, unfavorable nutritional conditions will stabilize repression of the *covRS* regulon. As the same conditions will also restrict the expansion of variant subpopulations, potentially short-term environmental changes for the better may induce rapid responses of the CovR regulatory circuit at both the population and gene expression level.

In this study, the *fas* operon of GCS was found to differ from the GAS *fas* system (12) by lacking the proximal *fasB* gene encoding a second histidine protein kinase. Because, in the absence of repression, the $fasCAX_{H46A}$ operon stimulates skc_{H46A} expression to approximately the same extent as the $fasBCAX_{NZ131}$ operon stimulates ska_{NZ131} expression (Table 2), one sensor kinase, FasC, would appear to be sufficient for full activity of the operon. Thus, FasC and FasA appear to be the genuinely mated pair, and FasB would assume an orphan state rather than forming a heterodimer with FasC or sensing

TABLE 3. Similarity of streptococcal promoter-upstream sequences to the 16-bp consensus sequence proposed (20) to be contained in CovR target sequences

Promoter	Sequence	Location	Adherence to consensus (no. of bases/total)
Pska_{NZ131}	TCATTTAAAAACAATT	−64 to −49	10/16
Pskc_{H46A}	TCATTTTAAAAAAATC	−64 to −49	13/16
P$covR_{H46A}$	TTTTATTTGAAAAAAG	−42 to −27	12/16
Consensus	TTATTTTTAAAAAAAC A T A	Variable	

different environmental signals to communicate with FasA, as discussed by Kreikemeyer et al. (12). Thus, since the overwhelming majority of the two-component systems are organized in dicistronic operons, *fasB* accretion may be a secondary feature of the fas_{GAS} system, the possible functional significance of which remains to be explored. Our previous observation that there is some degree of transcriptional independence of $fasB_{NZ131}$ from the downstream genes of the operon (28) might also be taken to lend some support to our notion that the occurrence of *fasB* in the fas_{GAS} operon is a derived feature. In any case, at the sequence level, FasB and FasC are very dissimilar (sequence identity, ≈28%), and it would not be surprising if FasA were able to tell them apart.

The present results confirm for GCS our previous observation with GAS that *fasX* RNA, in addition to its monocistronic form, can occur at lower abundance as part of the *fas* operon transcript. They also extend to GCS the original finding by Kreikemeyer et al. (12) that the high transcription rate of $fasX_{GAS}$ from its own promoter(s) depends on the products of the promoter-proximal *fas* genes, in particular *fasA*. These authors also showed that deletion of $fasX_{GAS}$ alone evokes the same regulatory behavior of the target genes as inactivation of *fasA*, lending strong support to the notion that *fasX* is the effector of the *fas* regulon. As such, $fasX_{H46A}$ RNA appears to act primarily at the transcriptional level, as judged by reduced *skc* mRNA levels in H46A *fas* mutants (Fig. 7). However, a search of complementarity between the 280-bp region upstream of the *skc* start codon and the $fasX_{H46A}$ sequence did not reveal potential target DNA tracts of more than 10 contiguous nucleotides. A similar observation was made as a result of screening the SF370 genome sequence for regions matching the $fasX_{GAS}$ sequence (12). However, long matching regions between the target and the effector might be dispensable, given the possibility that extended secondary-structure formation of the effector RNA (23), target DNA bending (9), or additional protein(s) (23) could assist in binding of *fasX* RNA to its DNA targets.

Seeing that the manner in which the much longer known staphylococcal analogue of *fasX*, RNAIII, regulates target genes still awaits understanding (23), elucidation of the mechanism of *fasX* action may present a formidable problem. For the time being, the present results provide appropriate explanations of differential SK activities in distinct streptococcal isolates and of the previously observed epistatic effects of different host backgrounds in SK allele swapping (17).

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