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The *rec* Locus, a Competence-Induced Operon in *Streptococcus pneumoniae*

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To study competence and the process of transformation (TFN) in pneumococci, we developed a method for isolating TFN$^-$ mutants using insertional inactivation coupled with fusions to the gene for alkaline phosphatase (*phoA*). One TFN$^-$ mutant transformed 2 log units less efficiently than the parent strain. Reconstitution of the mutated region revealed a locus, *rec*, that contains two polycistronic genes, *exp10* and the previously identified *recA* (B. Martin, J. M. Ruellan, J. F. Angulo, R. Devoret, and J. P. Claverys, Nucleic Acids Res. 20:6412, 1992). Exp10 is likely to be a membrane-associated protein, as it has a prokaryotic signal sequence and an Exp10-PhoA fusion localized with cell membranes. On the basis of sequence similarity, pneumococcal RecA is a member of bacterial RecA proteins responsible for homologous recombination of DNA. DNA-RNA hybridization analysis showed that this locus is transcribed as a polycistronic message, with increased transcription occurring during competence. With an Exp10-PhoA chimera used as a reporter, there was a 10-fold increase in the expression of the *rec* locus during competence while there was only minimal expression under growth conditions that repressed competence. The TFN$^-$ mutant containing the *exp10-phoA* fusion produced activator, a small extracellular polypeptide that induces competence, and the expression of *rec* was induced in response to activator. Therefore, the *rec* locus is directly required for genetic transformation and is regulated by the cell signaling mechanism that induces competence.

Streptococcus pneumoniae is genetically transformed by exogenous DNA (2) during a programmed, growth stage-specific event defined as competence (46). Occurring early in logarithmic growth, competence is induced in a cell density-dependent manner by an unknown signaling mechanism that results in the production of an extracellular polypeptide (activator [Act], or competence factor) (45, 48) which in turn induces the production of at least 11 new proteins that are presumably involved in the binding, uptake, and incorporation of exogenous DNA (25). This cascade of events implies a signaling mechanism that triggers the expression of competence-specific genes. With continued bacterial growth the competence state decays, perhaps also triggered by a specific signaling mechanism.

Several classes of mutants with specific defects in the binding, uptake, or incorporation of homologous DNA during the process of transformation have been identified (26). Only a few molecular determinants associated with these types of mutants have been well characterized. The product of the *endA* locus is an exported membrane-bound protein with endonuclease activity (33). It has been proposed that this protein is required for the degradation of one strand of the extracellular DNA in preparation for the translocation of the complementary strand across the bacterial membrane. The *comAB* locus encodes a member of the family of ATP-binding cassette (ABC) proteins that are responsible for the transport of many varied molecules, from large polypeptides to small charged molecules (15). ComA is most like the proteins responsible for the transport of bacterial toxins such as hemolysin from *Escherichia coli* and the adenylate cyclase toxin from *Bordetella pertussis*. On the basis of phenotypic analysis, it has been suggested that ComA is associated with both the production of Act and the response of the bacteria to Act. Another locus, *recP*, encodes a protein with sequence similarity to transketolases (37). Mutations in this determinant prevent homologous recombination of exogenous DNA but do not affect plasmid transfer (35, 38). Recently, a locus, *cia*, which encodes a two-component regulatory system, has been proposed to be involved in the early steps of competence development as well as resistance to penicillin (13). Studies in our laboratory have shown that mutations in two determinants, *plpA* and *ami*, which encode distinct peptide permeases, modulate the process of transformation (30), suggesting that the substrates for these peptide permeases serve as signaling molecules to control this process.

It is clear that factors responsible for the homologous recombination and integration of incoming DNA would be involved in genetic transformation and may be regulated with the induction of competence. In this paper, we report the identification, characterization, and competence-specific regulation of the *rec* locus, which contains two genes. One of these genes, *exp10* (for exported protein [31]), encodes a membrane-associated protein and is located directly upstream of the previously identified *recA* (22).

MATERIALS AND METHODS

Strains and media. The pneumococcal parent strain used in this study was R6x, which is a derivative of the unencapsulated Rockefeller University strain R36A (44). Libraries of pneumococcal mutants containing random insertions of the shuttle vector pHRM100 or pHRM104 were from a previously described work (31). *E. coli* strains used were DH5α, which is F$^-$ ϕ80d*lacZ* Δ(*lacZYA*Δ*M15*) *lacU169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thy-1 gyrA relA1* (Bethesda Research Laboratories), and CC118, which is Δ(*ara leu*)*7697* Δ*lacX74 araD139 phoA20 galE galK thi rpsE rpoB argE recA1* (20). *Streptococcus pneumoniae* and *E. coli* were grown as described elsewhere (31). Pneumococci were grown in liquid media adjusted to pH 8.0 to induce competence or adjusted to pH 6.8 to repress competence.

Mutagenesis and pneumococcal transformation. The pneumococcus-*E. coli* shuttle vectors pJDC9, pHRM100, and pHRM104 were used for gene inactivation, and the last two were also used for the construction of *phoA* gene fusions. The details for using these vectors have been previously described (28, 31).

The initial screening of large numbers of mutants for a decrease in transformation efficiency has been described previously (30). To determine the transformation efficiency of the various mutants (see Fig. 1), strains were grown to an

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optical density at 620 nm (OD_{620}) of 0.1 in liquid medium and frozen at −70°C in 10% glycerol. After thawing on ice, cells were preincubated at 30°C for 20 min. Chromosomal DNA (1 μg/ml, final concentration) from a streptomycin-resistant (Str^r) strain was added to each sample, and the cells were incubated for 30 min at 30°C. After the addition of DNase (10 μg/ml, final concentration) to stop further DNA uptake, the cultures were transferred to 37°C for 90 min to allow the expression of antibiotic resistance. Transformation efficiency was calculated as the number of Str^r transformants divided by the total number of bacteria, expressed as a percentage, and compared with the parent strain, R6x.

To generate a profile of competence (see Fig. 6), samples (1 ml) were taken over time from growing cultures of pneumococci, adjusted to a final concentration of 10% glycerol, and stored at −70°C for subsequent transformation, which was performed as described above.

Recombinant DNA techniques. Techniques for manipulating DNA, including plasmid preparations, restriction endonuclease digests, ligations, transformation into *E. coli*, and gel electrophoresis, were performed according to standard protocols (40). Restriction fragments used in cloning experiments were isolated from agarose gels by using glass beads (Bio 101 or Qiagen). Large-scale plasmid preparations were done with affinity columns (Qiagen) according to the manufacturer's instructions.

Double-stranded DNA sequencing was performed by the method of Sanger et al. (41) by using [α-^{35}S]dATP (New England Nuclear) with Sequenase version 2.0 (United States Biochemical Corp.) according to the manufacturer's instructions. Dimethyl sulfoxide (1%, vol/vol) was added in the annealing and extension steps. The Gene Amp kit (Perkin-Elmer Cetus) was used for the PCR. Oligonucleotides were synthesized at the Protein Sequencing Facility at Rockefeller University. Direct sequencing of PCR products was performed by using the CircumVent Thermal Cycle dideoxy DNA sequencing kit (New England Biolabs) according to the manufacturer's instructions.

Subcellular fractionation. Pneumococci were fractionated into subcellular components by a previously described technique (14, 30). Exp10-PhoA fusion proteins were detected with anti-PhoA antiserum (5′ 3′ Inc.) and visualized indirectly by enhanced chemiluminescence as described previously (30).

Recovery and sequencing of the *rec* locus. The integrated plasmid pBJP1, containing the *Sau*3A fragment from bp 651 to 1192 of *exp10* (see Fig. 1) inserted into the *Bam*HI site of pHRM104, was isolated from SPRU100 by transformation into *E. coli* of the spontaneously excised plasmid, which contaminates chromosomal preparations of DNA. Chromosome walking was used to isolate the downstream region of the *rec* locus. The 541-bp insert was excised from pBJP1 by using the flanking *Kpn*I sites derived from pHRM104 and cloned into pJDC9 (3) to produce pBJP2. This plasmid was shuttled back into pneumococci to produce SPRU108. Chromosomal DNA from SPRU108 was digested with various restriction endonucleases that cut the vector once but not the original fragment. The digested DNA was ligated and transformed into *E. coli*. By this procedure, *Sal*I produced pBJP3, which extended the 3′ region of the original fragment in pBJP2 by 635 bp into the adjacent gene, *recA*, while *Hin*dIII produced pBJP4, which contained an additional 1.7 kb that extended to 330 bp downstream from *recA*.

The region upstream of the original fragment of *exp10* was obtained by inverse PCR of chromosomal DNA. Chromosomal DNA from the parent strain, R6x, was digested with either *Sal*I or *Hin*dIII, ligated, and then amplified by the PCR using a primer to the 5′ end of the original fragment (P6-1: CTA GCC TTT GTT GAC AGA CG) and a primer to *recA* (recA2: GAT TGA CTC AGG TGC AG). Template amplified from the *Sal*I-treated mixture gave a 2.5-kb product, while the *Hin*dIII-treated mixture gave a 2.2-kb product. Conditions for the PCR were 40 cycles of 94°C for 1 min for denaturing, 50°C for 1 min for annealing, and 72°C for 3 min for extension. Primers to the upstream region were made as required for sequencing.

Northern (RNA) analysis. Northern analysis, including RNA preparation and electrophoresis, transfer to nitrocellulose, labeling of DNA probes, and hybridization, was done as described previously (30). The DNA probe used to detect *exp10* transcripts was the 541-bp *Kpn*I fragment from pBJP2. For detection of *recA* transcripts, the DNA probe was a 307-bp *Sac*I-*Sal*I fragment from pBJP3 (see Fig. 1).

Transcription start site of *rec*. RNA was prepared as described previously (34). Primer P6-4 (5′ ATT GTC TCC TAC AGC CG 3′) is 134 bp downstream from the start codon of *exp10*. RNA (15 μg) was precipitated with 2.5 volumes of ethanol together with 10 pmol of primer P6-4 and 20 μg of baker's yeast tRNA. The pellet was dissolved in 10 μl of hybridization buffer consisting of 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.8), 1 mM EDTA, and 80% deionized formamide, heated at 90°C for 2 min, and then incubated at 25°C for 3 h for annealing. After precipitation with ethanol, the pellet was resuspended in 25 μl of reaction mix (50 mM Tris-HCl [pH 8.3]; 50 mM KCl; 10 mM $MgCl_2$; 10 mM dithiothreitol; 0.5 mM spermidine; 0.4 mM [each] dCTP, dGTP, and dTTP; 12.5 μCi [10 nmol] of [α-^{35}S]dATP [New England Nuclear]; 40 U of RNasin [Promega]; and 10 U of reverse transcriptase [Promega]). The reaction proceeded at 42°C for 15 min, and then 0.4 mM dATP was added and incubation continued at 42°C for 1 h. After extraction with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1), the nucleic acids were precipitated with ethanol and resuspended in water. The samples were run on a 6% polyacrylamide next to a sequencing reaction mixture using the same primer, P6-4.

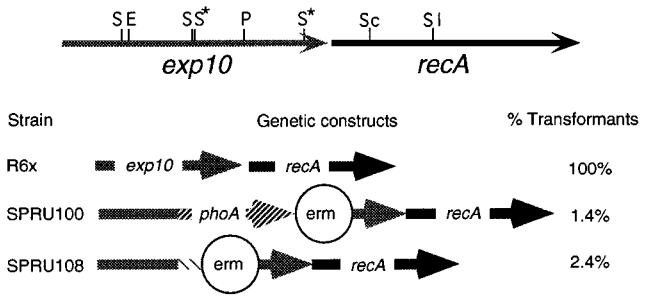

FIG. 1. Restriction map, structural organization, and mutational analysis of the *rec* locus. The original fragment (between asterisks) that was duplicated with the mutagenesis is indicated (*E*, *Eco*RV; P, *Pst*I; S, *Sau*3A; Sc, *Sac*I; Sl, *Sal*I). Insertion duplication mutagenesis was performed as described previously (31). SPRU100 contains pHRM104 integrated into *exp10* at the last *Sau*3A site. The vector-derived truncated gene for *phoA* is in frame with *exp10* at codon 382. SPRU108 contains pJDC9 (3) inserted at the same codon. Transformation efficiency is presented as percent Str^r transformants compared with the parental strain (R6x). Values presented are the average of two datum points, with the standard error of the mean not exceeding 12% of those values. Results are averages of assays performed on two separate occasions. Erythromycin resistance (erm) is encoded by the integrated vector.

Act induction of competence and alkaline phosphatase activity. A crude preparation of Act was prepared as described previously (45). To determine alkaline phosphatase activity, pneumococcus strains were grown to an OD_{620} of 0.2 in liquid medium adjusted to pH 6.8 to repress competence. After centrifugation, cells were resuspended in liquid medium adjusted to pH 8.0 plus 10% glycerol at a concentration of 1.6×10^8 CFU/ml and frozen at −70°C. To 600 μl of thawed cells, 125 μl of crude Act (final concentration, 4 ng/μl) or buffer alone was added and the mixture was incubated at 30°C for 30 min. The cells were immediately centrifuged at 4°C, resuspended in 1 M Tris-HCl (pH 8.0), and assayed for alkaline phosphatase activity as described previously (30). Parallel samples were removed and assayed for transformation to confirm Act induction of competence (data not shown).

Nucleotide sequence accession number. The DNA sequence of *exp10* has been deposited in Genbank under accession no. L36131.

RESULTS

Identification of a transformation-deficient mutant. The use of insertion duplication mutagenesis coupled with translational *phoA* (alkaline phosphatase) fusions to genetically identify and mutate exported proteins in *S. pneumoniae* has recently been reported (31). Furthermore, we have used this technique to successfully characterize the role of two peptide permeases in the process of transformation (30). From a bank of 1,200 pneumococcal mutants that contained random insertions of the *phoA* fusion vectors, we identified a strain, SPRU100, that was transformation deficient in a previously described assay (30). Compared with the parent strain, SPRU100 showed a 99% decrease in transformation efficiency (Fig. 1). A spontaneously excised plasmid, pBJP1, containing the 541-bp duplicated fragment (Fig. 1), was recovered from this strain and transformed into *E. coli* and then back into the parent strain of pneumococcus. This strain was also transformation deficient, showing that the defect in transformation was associated with the disruption of this locus (data not shown). The 541-bp insert of pneumococcal DNA was excised from pBJP1 and placed in a shuttle vector, pJDC9 (3), that does not contain *phoA* to produce pBJP2. This plasmid was transformed into a pneumococcus and the resulting strain (SPRU108) was also transformation deficient (Fig. 1), confirming that a PhoA fusion protein does not cause the decrease in transformation efficiency.

Organization and characterization of the *rec* locus. Recovery and identification of the locus altered in SPRU100 revealed an operon with two open reading frames (ORFs). The first ORF, *exp10*, is 1,256 bp (Fig. 2A) and is immediately followed

A

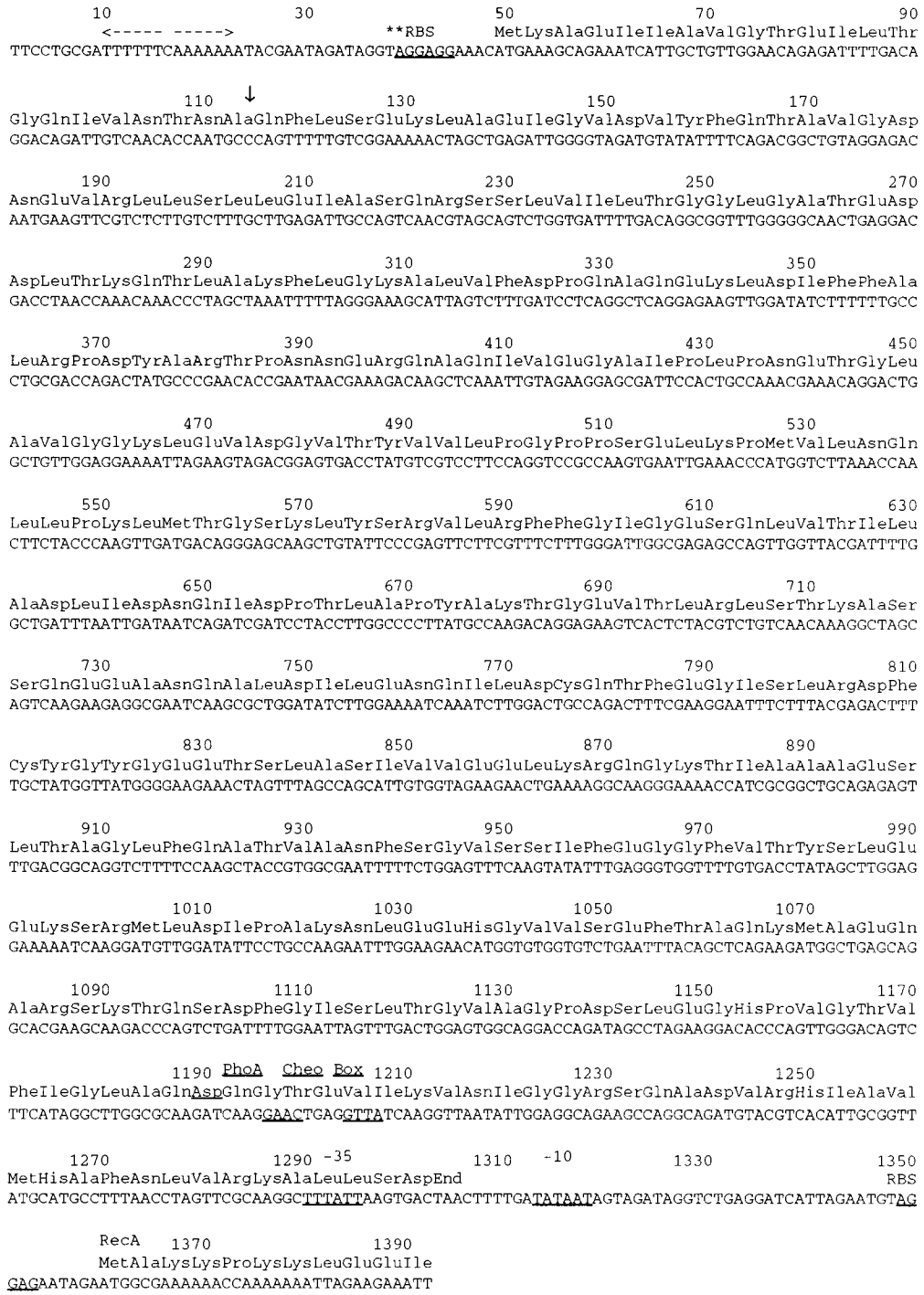

FIG. 2. Sequence analysis of *exp10*. (A) DNA and derived protein sequences of *exp10*. A putative RBS just upstream from the ATG initiation codon for *exp10* is indicated. The transcriptional start sites (asterisks) are shown. A consensus promoter sequence was not detected, but a 13-bp inverted repeat just upstream from the RBS (dashed arrows) is indicated. The 5′ end of the previously identified *recA* is included. A putative RBS, −10/−35 promoter sequence, and a potential *cis*-acting regulatory sequence (6) upstream from the ATG initiation codon for *recA* (underlined), a putative cleavage site (50) at Ala-22 following a signal sequence (↓), and the position of the PhoA fusion at Asp-382 are also depicted. (B) Sequence alignment of Exp10 with gephyrin, MoeA, Orf172 from the MarRAB locus, and an ORF from *E. agglomerans* (E.a.orf). The alignments were performed with the PILEUP program available on version 7 of the Genetics Computer Group (University of Wisconsin) sequence analysis software package (10). Identical or conserved amino acids (uppercase) are indicated. A consensus sequence was derived on the basis of a comparison of each individual sequence. Dots indicate no sequence. Dashes indicate no consensus.

B

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          1                                                   50
    Exp10  mkaEiiavGt EiLtGQIvnT NaqfLsekLa EiGvDVyfqt aVGDNevrLL
 Gephyrin 509.ELlnPed DLLpGkIrDs NRstLlatiq EhGYptINLG IVGDNPddLL
    MoeA 188.ELqlPGq pLgdGQIyDT NRlavhlmLe qlGCEVINLG IIrDdPhaLr
Consensus  ...EL--PG- -LL-GQI-DT NR--L---L- E-G--VINLG IVGDNP--LL

          51                                                  100
    Exp10  slLeiAsqRs slVIltGGlg atEdDlTKQt Lakflgkalv fdpqaqekld
 Gephyrin  nALnEgiSRA DVIItSGGVS mGEkDYlKQV L.586..... ..........
    MoeA  aAFiEAdSqA DVVIsSGGVS vGEaDYTKtI L.265..... ..........
Consensus  -AL-EA-SRA DVVI-SGGVS -GE-DYTKQ- L--------- ----------

          101                                                 150
    Exp10  iffalrpdya rtpnnerqaq ivegaiplpn etglavggkl evdgvtyvvl

          151                                                 200
    Exp10  pgppselkpm vlnqllpklm tgsklysrvl rffgigesql vtiladlidn

          201                                                 250
    Exp10  qidptlapya ktgevtlrls tkassqeean qaldilenqi ldcqtfegis

          251                                                 300
    Exp10  lrdfcygyge etsLaSiVve eLKrqGktIa aAESlTaGLF qatVanfsGv
   Orf172  ......... 19.nLtSalsq rLiAdqlRlT tAESCTGGkL AsAlcaaedt
  E.a.orf  ......... 8.eLsveIga aLKAkGwWVT cAESCTGGLi AkAItdiaGs
Consensus  ---------- ---L-S---- -LKA-G---T -AESCTGGL- A-A-----G-

          301                                                 350
    Exp10  SsiFEgGFVT YSleeKsrML dIpaknLEeh GvVSEftAqk MAeqArsktq
   Orf172  pkFYgaGFVT FtdqAKmkiL sVSqqsLErY sAVSEkVAaE MAtGAierAd
  E.a.orf  SaWFDrGFVT YSnaAKheLL gVSestLEqY GAVSEqVvhE MAlGvlhaAg
Consensus  S--F--GFVT YS--AK---L -VS---LE-Y GAVSE-VA-E MA-GA---A-

          351                                                 400
    Exp10  sDfgISlTGV AGPDslE.Gh PVGTVFiGLA qdqGtevikV niggrsqadV
   Orf172  ADVsIaITGy gGPEGGEdGt PaGTVWFaWh iK.GqnyTAV mhFaGDcEtV
  E.a.orf  ADVaVSVsGI AGPDGGsaek PVGTVWFGFA gKdGrvlTAk qqFsGDrEaV
Consensus  ADV-IS-TG- AGPDGGE-G- PVGTVWFG-A -K-G---TAV --F-GD-E-V

          401             419
    Exp10  RhiAVmhAFn lvrkaLLsd
   Orf172  lalAVrFALa qlLqlLL   172
  E.a.orf  RlqAavFsLq taLreFikn 164
Consensus  R--AV-FAL- --L--LL--
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FIG. 2—*Continued.*

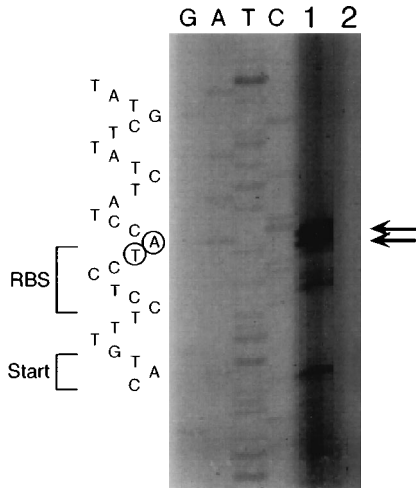

FIG. 3. Primer extension analysis of *rec*. Products of primer extension with RNA prepared from competent R6x cells (OD_{620}, 0.2; pH 8.0) with primer P6-4 (lane 1) or no primer (lane 2) are indicated (arrows). Nucleotide sequence analysis using the same primer is shown (lanes G, A, T, and C). This is the sequence of the complementary strand of *exp10* and is indicated at the left. The two major primer extension products (lane 1) corresponded to the first nucleotide of the consensus RBS sequence and the nucleotide immediately upstream (circles).

by the previously identified *recA* (22). Therefore, this locus is designated *rec*. Analysis of the plasmid pBJP1, recovered from SPRU100, showed that *phoA* from the vector (pHRM104) was fused in frame with *exp10*, leading to the production of a fusion protein (see below). The 5′ end of *exp10* contains a putative ribosome binding site (RBS) 5 bp upstream from the ATG initiation codon. Both a putative promoter and an RBS were found just upstream of *recA*. Sequence analysis up to 330 bp downstream from *recA* did not reveal any significant coding sequences. Inspection of the 3′ end of the published sequence of *recA* (22) suggests a *rho*-independent transcription terminator (data not shown).

Mapping the start site of transcription. The start of transcription of *rec* mapped evenly to two nucleotides located 10 and 11 bp upstream from the ATG start codon (Fig. 3). These are exactly at the RBS and 1 bp upstream (Fig. 2A). No consensus promoter sequence for *rec* was identified at the −10 and −35 regions or further upstream, suggesting an unusual recognition sequence required to initiate transcription of this locus. We did, however, note a perfect 13-bp palindrome located between 17 and 29 bp upstream from the RBS. Further evidence that upstream genes do not form part of the *rec* operon was obtained by sequencing 500 bp upstream from *exp10* (data not shown). A potential transcription terminator is located 34 bp upstream from *exp10* following the 3′ end of an ORF with homology to *lytR* from *Bacillus subtilis* (18).

Analysis of Exp10. Analysis of the derived sequence of *exp10* predicts a 46-kDa polypeptide with a putative 22-amino-acid signal sequence (Fig. 2A). A hydrophobicity plot shows several hydrophobic domains, implying that the translated product associates with cell membranes (Fig. 4). This was confirmed by subcellular localization of the PhoA fusion protein to the membrane fraction (Fig. 4). Comparison of the derived sequence of *exp10* with the most recent version of GenBank (81.0) revealed two regions of sequence similarity to four previously identified proteins (Fig. 2B). The amino terminus of Exp10 showed similarity over a 78-amino-acid stretch to gephyrin and MoeA. The former is a eukaryotic, membrane-associated protein which is responsible for the clustering of glycine receptors in spinal neurons (16, 32), while the latter is a protein of unknown function but by genetic analysis is essential for molybdopterin biosynthesis in *E. coli* (29). The carboxy terminus of Exp10 showed similarity over a 159-amino-acid stretch to a hypothetical 18.3-kDa protein (Orf172) derived from a sequence found in the 3′ region of the *marRAB* operon in *E. coli* and to an ORF from *Enterobacter agglomerans* (35a) (EMBL accession no. S313480). By genetic analysis, the *marRAB* operon has been implicated in both chromosome-mediated multiple-antibiotic resistance and the adaptive response to oxidative stress (1, 7, 8). Like that of *exp10*, the putative reading frame in *E. agglomerans* is also upstream from the *recA* gene.

Competence-dependent regulation of *rec*. Several studies have shown competence-specific regulation of *recA* (formerly called *recE*) in *B. subtilis* (5, 6, 19, 36, 51). To test if this was also the case for the *rec* determinant in pneumococci, we analyzed transcription by Northern analysis and expression by alkaline phosphatase (PhoA) activity in SPRU100, which harbors an *exp10-phoA* fusion.

Competence is a growth stage-specific phenomenon responsive to the hydrogen ion concentration of the growth medium. Bacteria grown in medium at pH 8.0 become maximally competent at a cell density of $\sim 10^7$/ml, while competence is repressed in bacteria grown at pH 6.8 (46). Therefore, we measured the transcription and expression of *rec* in bacteria grown under these two conditions.

A single 2.4-kb transcript corresponding in size to the *rec* locus was detected with DNA probes specific for *recA* or *exp10* in RNA preparations from competent cells (Fig. 5, lanes 1 and 5). In contrast, this transcript was present in low levels in RNA preparations from incompetent cells (Fig. 5, lanes 2 and 6), suggesting low-level constitutive expression and upregulation during competence. A transcription terminator is not apparent

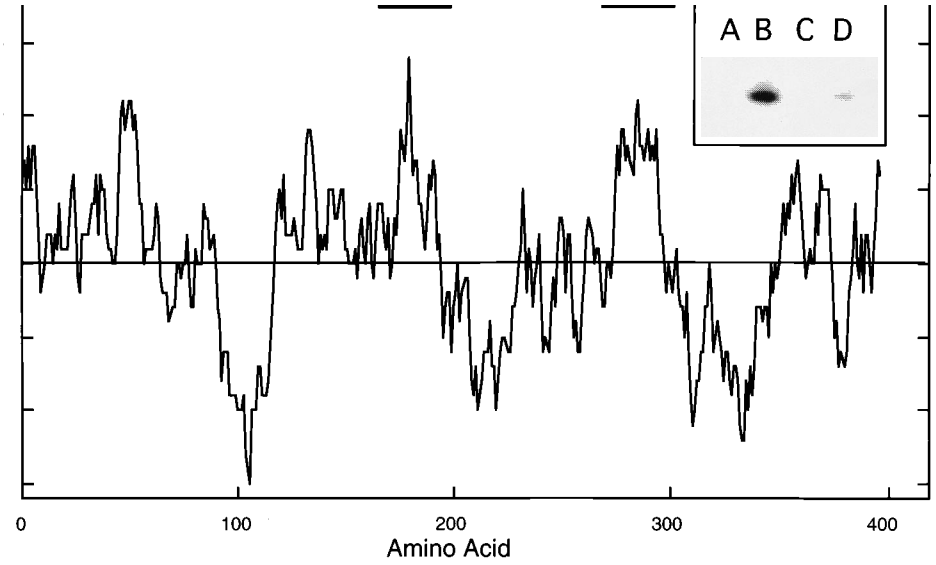

FIG. 4. Hydrophobic profile of Exp10 and subcellular localization of Exp10-PhoA. The Kyte-Doolittle plot (17) is of the derived sequence of *exp10* using a window of 20 residues. Potential membrane-associated regions are indicated at the top of the graph (bars). (Inset) Subcellular localization of Exp10-PhoA detected with anti-PhoA antiserum. Subcellular fractions from SPRU100 (50 μg of total protein) were applied to a sodium dodecyl sulfate (SDS)–8 to 25% polyacrylamide gel and transferred to nitrocellulose for detection with antibody. Lanes: A, culture supernatant; B, membranes; C, cytoplasm; D, cell wall.

after *exp10* (Fig. 2A), supporting readthrough into *recA*. Disruption of *rec* by insertional inactivation of *exp10* in SPRU108 and SPRU100 ablated the competence-induced transcription of *recA* (Fig. 5, lanes 3 and 4) but not the transcription of *exp10* (Fig. 5, lanes 7 and 8). The *exp10*-specific transcripts in lanes 7 and 8 are longer than the inactivated *exp10* gene because of transcription continuing into the integrated vectors. The 2-kb transcript from SPRU108 (Fig. 5, lane 7) is derived from 1.15 kb of *exp10* and 800 bp of pJDC9, while the 2.4-kb transcript from SPRU100 (Fig. 5, lane 8) contains 1.15 kb from *exp10* with the remainder from the truncated *phoA* (1.35 kb). As a control, there was no change in the transcription of *amiA* (a gene encoding a peptide permease) in RNA prepared from bacteria grown under these conditions. Therefore, these results show transcription of the *rec* locus as a polycistronic message and strongly suggest that competence-induced promoter sequences exist upstream of *exp10*, close to the translational start codon, as confirmed by primer extension analysis (Fig. 3).

To generate a profile for *rec* expression during competence, we measured transformation efficiency and PhoA activity in a growing culture of a mutant (SPRU100) harboring an *exp10-phoA* fusion. Figure 6 shows the competence profile of a growing culture of pneumococci with a maximal number of transformants obtained at an OD_{620} of 0.1. In parallel, there was a concomitant 10-fold increase in PhoA activity derived from the *exp10-phoA* fusion that peaked at an OD_{620} of 0.2. Bacteria

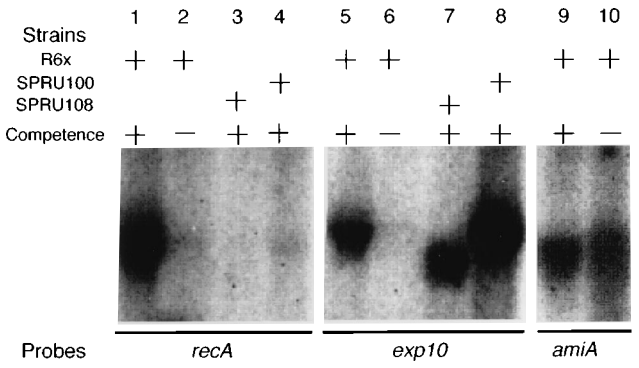

FIG. 5. Transcriptional analysis of *rec* during competence. RNA was prepared from various pneumococcus strains (R6x, SPRU100, and SPRU108; see Fig. 1 for genetic constructs) grown under conditions that induce (lanes 1, 3, 4, 5, 7, 8, and 9) or repress (lanes 2, 6, and 10) competence. These samples were then probed with DNA specific for *recA*, *exp10*, or *amiA* as indicated below the lanes. The transcripts of 2.0 and 2.4 kb derived from mutants SPRU108 and SPRU100 (lanes 7 and 8, respectively) that were identified with an *exp10*-specific probe most likely contain chromosomal and vector-derived sequences.

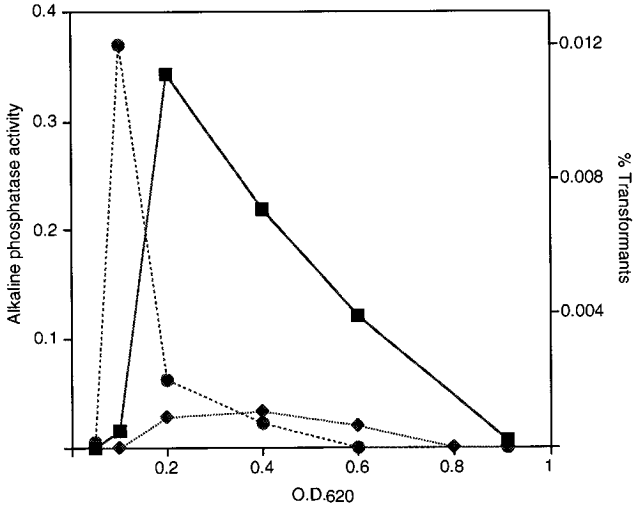

FIG. 6. The expression of *rec* follows the induction of competence. Alkaline phosphatase activity (■ and ◆) and transformation efficiency (●) were determined for growing cultures of pneumococci that harbor an *exp10-phoA* fusion (SPRU100). Parallel cultures were grown under conditions that induce (■ and ●) or repress (◆) competence. Units of enzyme activity have been described previously (30). Transformation efficiency is presented as percentage Str^r transformants per total number of cells. The standard error of the mean did not exceed 10% of any value.

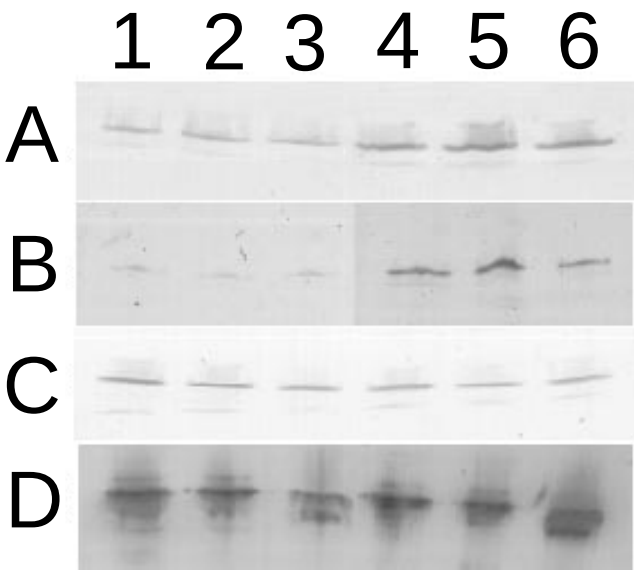

FIG. 7. Competence-induced expression of RecA and Exp10-PhoA. Bacterial cultures were grown under conditions that repress (lanes 1 through 3) or induce (lanes 4 through 6) competence. Samples were taken at various cell densities (lanes 1 and 4, OD_{620} of 0.15; lanes 2 and 5, OD_{620} of 0.20; and lanes 3 and 6, OD_{620} of 0.25), and total cell lysates (50 μg of protein) were applied to SDS–8 to 25% polyacrylamide gels, transferred to nitrocellulose, and probed with specific antibody. (A) R6x probed with anti-RecA antiserum; (B) SPRU100 (*exp10-phoA*) probed with anti-PhoA antiserum; (C) SPRU100 (*exp10-phoA*) probed with anti-RecA antiserum; (D) SPRU121 (*amiA-phoA*) probed with anti-PhoA antiserum. See Fig. 1 for genetic constructs.

grown under conditions that did not engender competence did not produce transformants or induce the expression of *rec*. It is of note that the peak of transformation precedes the maximal expression of *rec*. Since transformation is a multistep process that involves the binding, uptake, and incorporation of exogenous DNA, it is tempting to speculate that activation and/or expression of the elements required for the binding and uptake of DNA precede the increased expression of the *rec* determinant, which is clearly required for the later steps of DNA integration.

To confirm the increased expression of the *rec* locus during competence measured by PhoA activity from the *exp10-phoA* fusion, we measured levels of RecA and Exp10-PhoA from strains grown under incompetent and competent growth conditions by Western (immunoblot) analysis (Fig. 7). Densitometry of the immunoreactive bands from the transblots (analysis not shown) demonstrated a fivefold increase in the amount of RecA (Fig. 7A) and a 10-fold increase in the level of Exp10-PhoA (Fig. 7B) with the induction of competence. In contrast, a competence-induced increase in RecA was not seen in SPRU100 (Fig. 7C), even though the duplication mutagenesis leaves an intact copy of *recA* (Fig. 1). Constitutive expression of *recA* was still observed with this construct. We cannot rule out expression of *recA* derived from a competence-independent promoter just upstream from this gene or due to a polar effect from the inserted vector. Alternatively, there may be a low level of constitutive expression of *rec*. We did not observe an increase in the amount of the AmiA-PhoA chimera grown under these two conditions as a control (Fig. 7D). Therefore, these results also show an increased expression of Exp10 during competence concomitant with an increase in the expression of RecA. We can also conclude that the increased expression of Exp10 during competence is not dependent on a functional Exp10 or an increase in the expression of RecA.

TABLE 1. Act-induced expression of *rec*

Strain	Act[a]	PhoA activity[b]
SPRU100 (*exp10-phoA*)	+	0.44
	−	0.17
R6x (parental control)	+	0.04
	−	0.03

[a] Act was added at a final concentration of 4 ng/μl.
[b] Assays were performed and units of activity are as described previously (30). Each value is the average of duplicate samples, and the standard errors of the means did not exceed 10% of those values. This experiment is representative of two independent experiments.

Act-dependent induction of *rec*. Act is a small extracellular polypeptide that induces genetic transformation when added to incompetent cells (45, 48). To determine if there exists a regulatory circuit between Act and *rec* expression, we added a partially purified preparation of Act to incompetent cells harboring an *exp10-phoA* fusion. Table 1 shows a threefold increase in PhoA activity in response to the addition of Act. To determine if *rec* was required for the production of Act, we purified Act from the mutant strain SPRU100 harboring an insertion in the *rec* locus. This mutant produced Act, which was able to induce transformation of incompetent cells (data not shown). Therefore, a positive-regulatory circuit between Act and the *rec* locus exists, which suggests that *rec* is a late competence-regulated gene induced following the production of Act.

DISCUSSION

Identification of the *rec* locus in *S. pneumoniae* was achieved by assessing a library of mutants with gene fusions to *phoA* for loss of function in an assay for transformation. Mutations in this locus, consisting of two polycistronic genes, *exp10* and the previously identified *recA* (22), produced a transformation-deficient phenotype, presumably by a mechanism that requires Exp10 and RecA for the homologous recombination of exogenous DNA into the chromosome. On the basis of the current work, we can only surmise a role for Exp10 in this process. One possible role for Exp10 could be implied from the homology between Exp10 and gephyrin. Gephyrin is a membrane-associated eukaryotic protein that is thought to link clusters of glycine receptors to microtubules in spinal neurons. By analogy, perhaps Exp10 facilitates the clustering of RecA protein, incoming single-stranded donor DNA, and chromosomal double-stranded DNA at the cell membrane. This model is not without precedence. A complex of proteins that includes RecA has been colocalized with donor DNA and chromosomal DNA to membranes during transformation in *B. subtilis* (42, 43). In pneumococci, a similar protease-sensitive complex that included a protein that apparently protects DNA from endogenous nuclease digestion has been demonstrated with labeled donor DNA (23, 24, 27, 49).

Results presented in this paper clearly show the competence-induced regulation of the *rec* locus. Mapping of the transcription start site and Northern analysis suggest that a competence-regulated promoter exists close to the initiation codon of *exp10*. The lack of any obvious promoter upstream from this site suggests a unique sequence for competence-regulated promoters in pneumococci. A similar transcription start site that mapped to the RBS for the mRNA2 transcript of the *Dpn*II cassette from *S. pneumoniae* has been documented previously (39). The authors of that study suggested that this transcript may have arisen from degradation of mRNA unprotected by

protein synthesis. We do not think that this is possible for *rec*, for the following reasons. Sequencing upstream of *rec* showed a putative transcription terminator located only 34 bp from the start codon of *exp10* following an ORF with homology to *lytR* from *B. subtilis* (18). This suggests that the promoter for *rec* is in this intergenic region, which is supported by the results of both primer extension and Northern analysis.

Repeated sequences have now been found upstream of several genetic elements in pneumococci (21, 34), and it has been suggested that these sequences may participate in the global regulation of elements required for virulence and transformation (21). Sequence analysis up to 500 bp upstream of *exp10* did not reveal any of these repeated sequences (data not shown). Even though the presence of these putative regulatory sequences further upstream cannot be ruled out, it seems likely that competence-specific regulatory elements that control the expression of the *rec* locus do not include these repeated sequences.

The increase in expression of *recA* during competence has also been reported for *B. subtilis*, in which there are sequences upstream of *recA* required for competence-specific expression (11). These sequences are distinct from a *cis*-acting regulatory region that contains an operator binding site (Cheo box) upstream of *recA* and other DNA damage-inducible genes (4–6). Thus, in *B. subtilis*, *recA* expression is induced by both competence development and DNA damage, via two different mechanisms. It is of note that upstream of the pneumococcal *recA* is a consensus sequence for a bacterial sigma-70-dependent promoter as well as a Cheo box (Fig. 2A). This suggests a second level of regulation independent of induction during competence and may parallel those observed in *B. subtilis*. In contrast, *rec-1*$^+$ (*recA* homolog) transcription in the naturally transformable *Haemophilus influenzae* is not increased during the development of competence but only in response to DNA damage (52). It was suggested that during competence, levels of Rec-1 activity may change by a posttranscriptional mechanism. The regulation of *recA* expression in *Neisseria* spp., which are also naturally transformable, has not been studied, although it has been hypothesized that regulation of *recA* expression may occur during competence rather than after DNA damage, as the *Neisseria gonorrhoeae recA* lacks LexA repressor binding sites (12).

The structural organization of the *rec* locus in *S. pneumoniae* is unique, with the location of *exp10* upstream from *recA* and the cotranscription of these genes during competence. This suggests a functional link between transformation and the process of homologous recombination. The region of similarity between the ORF upstream of *recA* in *E. agglomerans*, Orf172 in *E. coli*, and Exp10 suggests a conservation in function between several diverse bacterial species. In *H. influenzae*, there is a gene that is required for transformation, *tfoX*, located upstream from *rec-1*, but this gene is oriented in the opposite direction to *rec-1* (52). Whether *tfoX* affects expression of *recA* has yet to be determined. In several gram-positive and gram-negative bacteria, a conserved gene with a proposed regulatory function exists downstream of the respective *recA* homologs (9). In pneumococci, sequencing up to 330 bp downstream of *recA* did not reveal any coding sequence (our unpublished results).

Previous studies have shown an absolute requirement of protein synthesis for transformation in *S. pneumoniae* (47) that leads to the induction of at least 11 novel proteins during competence (25). Furthermore, this phenotypic change can be induced by the hormonelike substance Act. None of these proteins have been identified or well characterized. Our results from both transcriptional and expression studies of *exp10-phoA* fusions and *recA* clearly demonstrate upregulation of *rec* during the growth stage-specific competence state and show that expression of this locus is induced by Act. Therefore, it seems likely that *rec* is an essential determinant required for transformation and that it is a member of the network of elements globally regulated during competence following stimulation by Act.

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