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# Transient Association of an Alternative Sigma Factor, ComX, with RNA Polymerase during the Period of Competence for Genetic Transformation in *Streptococcus pneumoniae*

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**Natural transformation in *Streptococcus pneumoniae* is regulated by a quorum-sensing system that acts through accumulation and sensing of a peptide pheromone (competence-stimulating peptide [CSP]) to control many competence-specific genes acting in DNA uptake, processing, and integration. The period of competence induced by CSP lasts only 15 min (quarter-height peak width). The recently identified regulator ComX is required for the CSP-dependent expression of many competence-specific genes that share an unusual consensus sequence (TACGAATA) at their promoter regions. To test the hypothesis that this regulator acts as a transient alternative sigma factor, ComX was purified from an *Escherichia coli* overexpression strain and core RNA polymerase was purified from a *comX*-deficient *S. pneumoniae* strain. The reconstituted ComX-polymerase holoenzyme produced transcripts for the competence-specific genes *ssbB*, *cinA*, *cglA*, *celA*, and *dalA* and was inhibited by anti-ComX antibody, but not by anti-$\sigma^{70}$ antibody. Western blotting using antibodies specific for ComX, $\sigma^{70}$, and poly-His revealed a transient presence of ComX for a period of 15 to 20 min after CSP treatment, while RNA polymerase remained at a constant level and $\sigma^{A}$ remained between 60 and 125% of its normal level. ComX reached a molar ratio to RNA polymerase of at least 1.5. We conclude that ComX is unstable and acts as a competence-specific sigma factor.**

Competence for genetic transformation in *Streptococcus pneumoniae* is a transient physiological state that may appear suddenly during the exponential growth phase at a cell density determined, in part, by quorum sensing. Once the process is initiated, competence may reach a maximum in 20 min, and then it is rapidly extinguished (9, 12, 19, 28, 41). This developmental sequence appears to accompany a drastic change in protein synthesis, as pulse-labeling of cellular proteins showed that synthesis of most cellular proteins was switched off, while other proteins appeared specifically at competence (29).

A map of how cells achieve competence is beginning to emerge. It includes two cycles of regulation, the quorum-sensing circuit and the competence-evoking machinery (Fig. 1). The quorum-sensing signal responsible for competence induction is a heptadecapeptide, named CSP (competence-stimulating peptide) (15), which is derived from a ribosomally synthesized precursor (ComC) through cleavage and export by an ATP-binding cassette (ABC) transporter, ComAB (28). CSP is sensed by a putative two-component system comprising the histidine kinase homologue, ComD, and the response regulator, ComE (16, 34). This system activates expression of both *comAB* and *comCDE* operons, forming an autocatalytic circuit (2, 10, 34). It has been suggested that ComE might have dual functions in the regulation of *comAB* and *comCDE*: activation at low doses of the CSP stimulus and repression at high doses (1, 2). ComE binds to sites at the *comAB* and *comCDE* operons, where it is thought to act as a transcriptional activator (44), and it may also recognize a site near another gene, *comX* (23). Unlike *comAB*, *comCDE*, and *comX*, which depend on *comE* for activation but not on *comX*, a large set of competence-specific operons depends on both *comE* and *comX* for expression. Major genes in this set that are involved in transformation and specifically expressed during competence include *ssbB, dalA, ccl, cglABCD, celAB, cflAB, cinA-recA*, and *coi* (7, 11, 22, 26, 35). The genes identified to date in the latter group are associated with a conserved sequence in their putative promoter region, TACGAATA, designated the Cin box or Com box (7, 35).

Sequential expression of some of the competence-specific genes has been observed during the response to CSP (2, 30, 35, 36). Genes of the quorum-sensing circuit, *comAB* and *comCDE*, together with *comX*, exhibit a strong rise-and-fall expression pattern with a maximum approximately 10 min after CSP addition, while the ComX-dependent genes' expression patterns are similar but delayed by approximately 3 min (36). Since ComX has homology to *Bacillus subtilis* $\sigma^{H}$, a member of the $\sigma^{70}$ family of the RNA polymerase sigma subunit, it was hypothesized to be an alternative sigma factor whose brief appearance could account for the transient transcription of other competence-specific genes (23). To test this hypothesis, we purified a ComX derivative expressed in *Escherichia coli*, purified RNA polymerase holo- and core enzymes from *S. pneumoniae*, and examined the specificity of reconstituted polymerase in vitro. Finally, quantitative Western blotting was used to monitor the fate of ComX protein during the response to CSP and to estimate the ratio of ComX to RNA polymerase in vivo.

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## MATERIALS AND METHODS

**Strains, plasmids, culture media, and antibodies.** The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *E. coli* strains (Invitro-

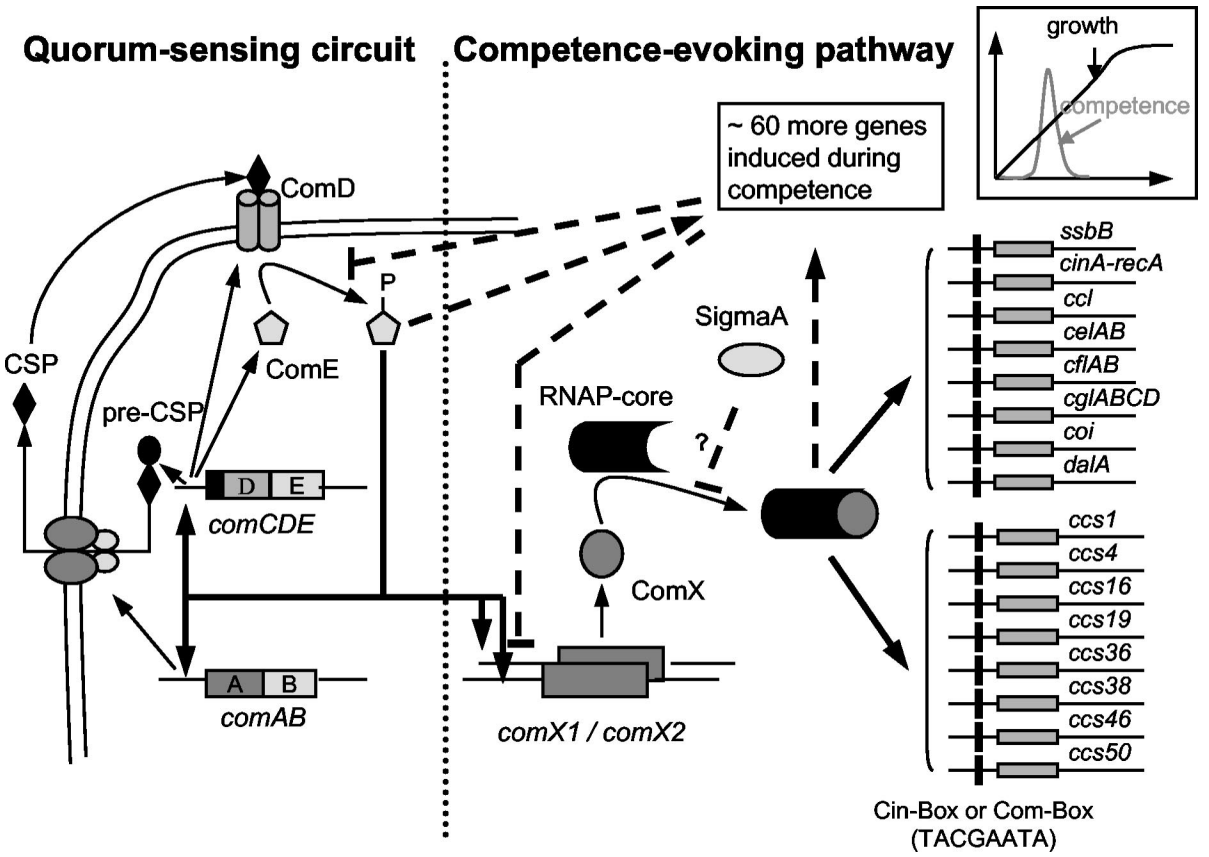


FIG. 1. Model for the regulation of genetic transformation in *S. pneumoniae.* The quorum-sensing system that accumulates CSP and an active form of ComE induces the expression of ComX. ComX is confirmed in this paper to act as an alternative sigma factor and enable core polymerase to transcribe competence-specific genes whose up-regulation is required for competence. The role of $\sigma^A$ remains unclear, but some possibilities are discussed in the text. Solid arrows indicate processing (thin lines) or activation (thick lines) steps that are supported by previous research. Dashed lines, hypothetical links; T bars, negative regulation.

gen) were routinely grown in Luria-Bertani (LB) medium (38). *S. pneumoniae* strains were grown in casein hydrolysate yeast extract medium (CAT) for cell culture and transformation assays, as described previously (23). For selection, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 34 μg/ml for *Escherichia coli* and 2.5 μg/ml for *S. pneumoniae*; novobiocin, 2.5 μg/ml; erythromycin, 0.5 μg/ml; and tetracycline, 0.25 μg/ml. Antibodies used in this study were anti-His (C terminal)-horseradish peroxidase (HRP)-conjugated antibody (1.2 mg/ml; Invitrogen); anti-ComX antibody (0.25 mg/ml; Quality Bioresources); and anti-$\sigma^{70}$ antibody (2G10; Neoclone).

To construct a *comX* expression plasmid, a 477-bp fragment containing the entire ComX coding sequence (bp 477 to 953 in AF161700) was generated by PCR with *Pfu* DNA polymerase (Stratagene) using primers DAM284 and MSL41 and a CP1250 template, purified, and ligated directly to the vector pCRT7/CT-TOPO (Invitrogen) (39). After transforming *E. coli* TOP10F′, clones were selected on LB agar with ampicillin. Plasmid DNA was prepared and analyzed by PCR with primers PL05, PL06, PL08, PL09, and PL12. A plasmid with a correctly oriented insert was designated pXPL01 and was shown to have the predicted insert sequence by sequencing.

To make a gene for a His-tagged ComX, we chose to tag the carboxyl terminus of *comX2* in the *comX1*-deficient strain CPM2 (23). A blunt-ended fragment (339 bp) containing the 3′ end of *comX2* and a six-His extension together with a stop codon was amplified by PCR from CP1250 DNA by using primers MSL27 and MSL43. After insertion of the PCR product into the *Sma*I site of pEVP3 and transformation of *E. coli* DH10B, a clone carrying a chimeric plasmid having the partial *comX*::His6 and *lacZ* on the same strand was identified by digestion with restriction enzymes. The plasmid was integrated into CPM2 by insertion duplication at the *comX2* locus by transformation, resulting in strain CP1288. The integrated structure was confirmed by sequencing both junctions and the whole *comX* fragment containing a six-His tag at its 3′ terminus.

To construct the new strain CP1290, we transformed CPM1 (CP1250 derivative, with 10-His fused to the β′ subunit of RNA polymerase) with chromosomal DNA of the *comX*-deficient strain CPM8. Positive strains were selected on CAT agar containing chloramphenicol, erythromycin, and tetracycline, and mutation structures were verified by PCR.

**Transformation of *S. pneumoniae*.** Cells were cultured in CAT adjusted to 0.2% bovine serum albumin (BSA), 0.5 mM $CaCl_2$, and 10 mM HCl, and were exposed to DNA (0.2 μg/ml) for 55 min after treatment with CSP at an optical density at 550 nm ($OD_{550}$) of 0.05 as described previously (23). Transformants were selected in CAT agar with appropriate drugs. For the competence assay, samples were withdrawn, exposed to 1 μg of CP1500 DNA/ml for 5 min at 37°C (terminated by addition of 20 μg of DNase I [Sigma]/ml), incubated another 55 min at 37°C, and diluted appropriately before plating to determine $Nov^r$ recombinants as described previously (36).

**SDS-PAGE and Western blotting analysis.** *E. coli* cell culture samples (1 ml) were harvested by centrifugation at 14,000 × *g* for 5 min at 4°C, resuspended in gel sample buffer, held in boiling water for 5 min, and analyzed on a discontinuous 15% Tris–glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel with a 4% stacking gel (21, 38). Pneumococcal culture samples (10 ml) were chilled to 0°C, harvested by centrifugation at 14,000 × *g* for 15 min in the cold, washed with 1 ml of cold 1× phosphate-buffered saline (PBS) buffer (10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, 0.2 M NaCl, 3 mM KCl [pH 7.4]), and resuspended in 1 ml of cold TGED buffer (50 mM Tris-HCl [pH 8.0], 5% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol [DTT]) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton X-100, and 5 μg of DNase I/ml. After lysis for 10 min at 37°C and precipitation with 7.2% trichloroacetic acid (3), the precipitate was collected and redissolved in 60 μl of gel sample buffer, adjusted to basic pH with ammonia vapor, boiled for 5 min, and applied to discontinuous 7.5 or 15% Tris–glycine SDS-PAGE gels with 4% stacking gels.

TABLE 1. Bacterial strains, plasmids, and oligonucleotide primers used in this study

| Strain, plasmid, or primer | Relevant characteristics or sequence | Source, reference, or location |
|---|---|---|
| *E. coli* strains | | |
| DH10B | F$^-$ *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara-leu*)*7697 galU galK* λ$^-$ *rpsL* (Str$^r$) *nupG* | Gibco-BRL |
| TOP 10F′ | F′ (*lacI*$^q$) *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str$^r$) *endA1 nupG* | Invitrogen |
| BL21(DE3)pLysS | F$^-$ *ompT hsdS*$_B$ ($r_B^-$ $m_B^-$) *gal dcm* (DE3) pLysS | Invitrogen |
| *S. pneumoniae* strains | | |
| CP1250 | *hex malM511 str-1 bgl-1*; Rx derivative, low β-galactosidase background | 35 |
| CP1500 | *hex nov-r1 bry-r str-1 ery-r1 ery-r2*; donor of point markers | 8 |
| CPM1 | CP1250, but *rpoC*::C-his-10::pEVP3; β′-His$_{10}$ Cm$^r$ | 23 |
| CPM2 | CP1250, but Δ*comX1*::Em; Em$^r$ | 23 |
| CPM8 | CP1250, but Δ*comX1*::Em Δ*comX2*::Tet; *comX1*$^-$ *comX2*$^-$ Em$^r$ Tet$^r$ | 23 |
| CP1288 | CP1250, but Δ*comX1*::Em *comX2*::C-his-6::pEVP3; Cm$^r$ Em$^r$ | This study |
| CP1290 | CPM1, but Δ*comX1*::Em Δ*comX2*::Tet; constructed by transformation of strain CPM1 with CPM8 chromosomal DNA; Cm$^r$ Em$^r$ Tet$^r$ | This study |
| Plasmids | | |
| pCR-T7/CT-TOPO | Linearized vector with 3′-T overhangs and topoisomerase I | Invitrogen |
| pXPL01 | pCR-T7/CT-TOPO derivative, carrying *comX* (477 bp) | This study |
| Primers | | |
| DAM206 | CTGACTTTCTCAAGATAAAAAGCC | In *ssbB* |
| DAM207 | TCAACCTCGTCGTGTTCAGA | Upstream of *ssbB* |
| DAM208 | GACTTCCCAATCCTCTGCAAC | In *amiA* |
| DAM209 | TTTGTGCCAACAAGCCTAAAT | Upstream of *amiA* |
| DAM268 | GAGCACCTGTTGGTGATGAAGAT | Upstream of *cinA* |
| DAM269 | TGTCTTTCGTTATTCGGTGTTCG | In *cinA* |
| DAM284 | ATGATTAAAGAATTGTATGAAGAAG | In *comX* |
| MSL27 | GGACTGGTAGACGATATTCCACG | In *comX* |
| MSL41 | GTGGGTACGGATAGTAAACTCCT | In *comX* |
| MSL43 | CTTAGTGATGGTGATGGTGATGGTGGGTACGGATAGTAAACTCCT | *comX* C- six-His |
| MSL45 | CTTGACCAAGGAAGACTATTTTGC | Upstream of *celA* |
| MSL46 | AGCACCTTTGACATCTACTGTGA | In *celA* |
| MSL47 | CGTTCTTGCCGTATGAAATTAAA | Upstream of *cglA* |
| MSL48 | TGCTTGCCTAATTCTTCAATATC | In *cglA* |
| PL05 | CCGCGAAATTAATACGACTC | In T7 promoter |
| PL06 | AAGCTTCGAATTGCCCTTGT | In V5 |
| PL08 | GACCAAGAAGGCATGCTCTG | In *comX* |
| PL09 | CGCGTTCTAGTTCTTCTTGT | In *comX* |
| PL12 | CCCTCAAGACCCGTTTAGA | In T7 terminator |
| PL26 | GAGATGACTCGAAACAAGGCTCTC | Upstream of *rpoD* |
| PL27 | AACTGCATACCACGACCGACATAG | In *rpoD* |
| PL28 | GTTGAAGAATTAGAAGGCAATAGCA | Upstream of *dalA* |
| PL29 | GGCCAGACCACTGACAATAAC | In *dalA* |

Electrophoresis was carried out at 15 V/cm in Tris-glycine buffer. Gels were then fixed and stained with Coomassie brilliant blue R-250 in methanol-glacial acetic acid, destained in methanol-acetic acid, dried with a vacuum, and scanned by using a Hewlett Packard ScanJet 6200C (38).

Alternatively, the fractionated proteins analyzed on mini-gels were electrotransferred onto a 7- by 8.4-cm polyvinylidene difluoride membrane (Bio-Rad) for 50 to 60 min at 300 mA in transfer buffer (48 mM Tris, 39 mM glycine; pH 9.2). After blocking overnight at 4°C in 20 ml of TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Tween 20) containing 5% powdered nonfat milk (Bio-Rad), the membrane was probed for 2 h at room temperature in TBST with a purified polyclonal antibody specific to ComX (1:1,000 dilution), with a monoclonal HRP-conjugated anti-His tag antibody (1:5,000 dilution) or with a monoclonal anti-$\sigma^{70}$ antibody (1:5,000 dilution). After washing in TBST three times, secondary antibodies in TBST were a peroxidase-labeled anti-rabbit immunoglobulin G antibody (Amersham Pharmacia) for the anti-ComX at a dilution of 1:20,000 or a peroxidase-labeled anti-mouse immunoglobulin G antibody (Amersham Pharmacia) for the anti-$\sigma^{70}$ antibody at a dilution of 1:10,000. No secondary antibody was needed for the anti-His-HRP antibody. After incubation at room temperature for 1 h, membranes were washed with TBST five times before signal detection. The reaction was visualized by using ECL-Plus Western blotting detection substrate reagents (Amersham Pharmacia) and exposure to film (X-Omat FS-1; Kodak). To quantify Western blots, the intensities of bands on the films were scanned and quantified with Alpha-Imager 3.2 (Alpha Innotech) and were adjusted into arbitrary units according to their integrated density value (IDV) for comparison with standards running in the same gel.

**Purification of *S. pneumoniae* RNA polymerase.** RNA polymerase was purified from *S. pneumoniae* strains CPM1 and CP1290. Strains were grown in CAT medium of reduced pH to block endogenous competence induction (9) but allow response to exogenous CSP. To obtain RNA polymerase from noncompetent cultures, cells were collected after growth in 6 liters of CAT with 10 mM HCl to an $OD_{550}$ of 0.05 at 37°C. To obtain polymerase from competent CPM1 cells, 2 liters of culture was rapidly chilled and collected 10 min after addition of CSP to 250 ng/ml at an $OD_{550}$ of 0.05. Cell pellets were washed once with cold 1× PBS and resuspended in 10 ml of cold column buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol) containing 1 mM PMSF, 0.2% Triton X-100, and 5 μg of crystallized DNase I/ml for cell lysis. All further steps were carried out at 0 to 4°C unless otherwise specified. After 5 to 10 min at 37°C, the lysates were clarified by centrifugation (14,000 × *g*; 30 min) and applied to a 2-ml nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) affinity column. Unbound proteins were removed with 20 ml of column buffer containing

20 mM imidazole. Bound RNA polymerase was eluted from the column with 5 ml of column buffer containing 100 mM imidazole and then 5 ml of 250 mM imidazole in column buffer, sequentially. Fractions of 1 ml each were stored at −20°C. Fractions with polymerase detected by SDS-PAGE were pooled (5 ml) and dialyzed twice against storage buffer (50 mM Tris-HCl [pH 8.0], 250 mM NaCl, 50% glycerol, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT) overnight and stored at −20°C. The stock concentration was about 0.5 mg/ml. Enzyme yield in a typical purification was 0.1 to 0.15 mg of RNA polymerase from 1 liter of culture, which amounts to 35 to 40% of total RNA polymerase determined by Western blotting (0.3 to 0.4 mg of cells/liter).

Core RNA polymerase was separated from holoenzyme by phosphocellulose chromatography as described previously (14). A 1.0-mg sample of holoenzyme purified from CP1290 was diluted 10-fold in 6 M urea buffer (50 mM Tris-HCl [pH 8.0], 50 mM KCl, 0.1 mM EDTA, 0.4 mM DTT containing 6 M urea), and applied to a 4-ml phosphocellulose column that had been equilibrated with the same buffer. All further purification steps were carried out at 0 to 4°C unless otherwise specified. After washing the column with the same buffer, proteins were eluted with a 75-ml linear gradient of KCl (50 to 500 mM) in the same buffer containing 6 M urea and stored at 4°C. Fractions containing core polymerase only (identified by SDS-PAGE) were pooled (6 ml) and dialyzed against renaturation buffer (50 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 1 mM DTT, 300 mM KCl, 50% glycerol) and stored at −20 or −80°C. The stock concentration was about 0.3 mg/ml. Core polymerase yield was about 30% of total RNA polymerase applied to the column. Protein concentrations were determined by Bradford assay (Bio-Rad) (4) with BSA as the standard.

**Purification of V5/6H-tagged ComX protein.** For ComX purification, an overnight culture of *E. coli* BL21(DE3)pLysS cells carrying pXPL01 was diluted 1:100 in 1 liter of LB containing ampicillin and chloramphenicol. Growth at 37°C with shaking was monitored until cells reached an $OD_{600}$ of about 0.5 to 0.8. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. All further purification steps were carried out at 0 to 4°C unless otherwise specified. After an additional 4-h incubation at 30°C, the cells were harvested by centrifugation (10,000 × *g*; 30 min), washed with 1× PBS once, and resuspended in 40 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 50 mM NaCl, 5% glycerol, 1 mM DTT, 100 μg of lysozyme/ml, 1 mM PMSF). Cell lysis was achieved by sonication (50% duty; output at 7 for microtip) for 5 to 10 min on ice.

The insoluble fraction containing ComX-V5/6H (a ComX derivative with the V5 epitope and a six-His tag) was collected, washed, and then solubilized with Sarkosyl as described elsewhere (6). Briefly, ComX-V5/6H inclusion bodies were isolated by centrifugation at 10,000 × *g* for 20 min at 4°C, washed twice with 40 ml of TGED buffer containing 2% sodium deoxycholate, and collected by centrifugation. The washed inclusion bodies were then solubilized in 20 ml of TGED buffer with 0.5% (wt/vol) Sarkosyl by incubation for 1 h at 21°C with stirring. After centrifuging to clarify (15,000 × *g* for 30 min), the supernatant was diluted with 30 ml of TGED buffer containing 1 mM PMSF and dialyzed twice against 2 liters of column buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol) containing 0.1 mM PMSF. The centrifuge-clarified dialyzed sample was applied to a 10-ml Ni-NTA agarose column, washed sequentially with 50-ml portions of column buffer containing 20 and 40 mM imidazole, and eluted sequentially with 100 and 250 mM imidazole in 10 ml of buffer. Collected fractions (about 10 ml) were stored at −20°C. Fractions containing ComX-V5/6H (identified by SDS-PAGE) were then combined, dialyzed against storage buffer (50 mM Tris-HCl [pH 8.0], 50% glycerol, 250 mM NaCl, 0.1 mM EDTA, 1 mM DTT), and stored at −20 or −80°C. The stock concentration was about 0.1 mg/ml. The yield of ComX-V5/6H was regrettably lower than expected due to its polymerization during refolding: 5 to 6 mg of purified protein from 1 liter of *E. coli* cells.

**In vitro transcription.** Templates were synthesized by PCR using *Pfu* DNA polymerase and CP1500 template DNA. The primers (Table 1) used and their positions (relative to +1 as the putative mRNA start site suggested by Peterson et al. [36]) were DAM206 and DAM207 (*ssbB*; −145 to +460); DAM208 and DAM209 (*amiA*; −70 to +300); DAM268 and DAM269 (*cinA*; −100 to +365); MSL45 and MSL46 (*celA*; −90 to +265); MSL47 and MSL48 (*cglA*; −85 to +400); PL26 and PL27 (*rpoD*; −146 to +470 relative to +1 as the start of the $\sigma^A$ protein); and PL28 and PL29 (*dalA*; −162 to +452). Sequences of all genes are from the genome sequence of *S. pneumoniae* strain TIGR4 (40).

Holoenzyme or core RNA polymerases (2 μg) with or without purified ComX (0.2 μg) were incubated with templates (0.5 μg) for 15 min at 37°C in a 40-μl volume with a final concentration of 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 20 mM KCl, 0.1 mM DTT, and 0.15 mg of BSA/ml (43). For transcription using monoclonal anti-$\sigma^{70}$ (2G10) antibody or anti-ComX antibody as inhibitors of $\sigma^A$ or ComX activities, 1 μl of each at a dilution of 1:10 in PBS was incubated with polymerase in reaction buffer for 30 min on ice before addition of template DNA (5). Following addition of the ribonucleotides (500 μM final concentration) ATP, CTP, and GTP (Boehringer Mannheim) and 5 μCi of [α-$^{32}$P]UTP (400 Ci/mmol; Amersham) and incubation for 1 min at 37°C, 10 μg of heparin (Sigma) was added to ensure single-cycle transcription. After 5 min more at 37°C, unlabeled UTP (500 μM final concentration) was added. Finally, after 10 additional min at 37°C, reactions were stopped by the addition of 20 μl of Tris-borate-EDTA (TBE)–urea sample buffer (Bio-Rad). The products were heated at 90°C for 5 min and loaded (10 μl) onto a mini-precast 5% polyacrylamide–7 M urea gel (Bio-Rad). Electrophoresis was carried out at 10 V/cm in TBE buffer. Gels were then dried and exposed to film (X-Omat FS-1; Kodak). Specific transcription from the *amiA*, *ssbB, cinA, cglA, celA*, and *dalA* promoters was expected to produce transcripts of 300, 460, 365, 400, 265, and 452 bp, respectively, according to their putative mRNA start sites.

## RESULTS

**ComX protein is regulated and unstable, appearing transiently during induction of competence by CSP.** Previous investigations using DNA microarrays (36) and *lacZ* reporter genes (23) showed that the level of *comX* mRNA increases dramatically within 5 min after exposure of naïve cells to exogenous CSP, reaches a maximum at about 10 min, and decreases rapidly thereafter, while the mRNA for ComX-dependent genes follows a pattern that is similar but delayed by about 3 min. Since ComX is implicated genetically as a key regulator in competence development (23), we hypothesized that synthesis of the ComX protein was regulated and that the protein was unstable. To test this hypothesis, the fate of ComX protein was assayed directly during the CSP response by Western blotting.

A ComX-specific polyclonal antibody was raised by using a conjugated synthetic peptide corresponding to residues 43 to 58 of ComX (REEGLVDDIPRLRKYF, with an additional cysteine at the N terminus for column purification) as an antigen. The anti-ComX antibody was further purified by its affinity to the same peptide. To conveniently detect both ComX protein and the RNA polymerase β′ subunit in the same samples, CPM1, a derivative of wild-type CP1250 with a 10-His-tagged β′ subunit of RNA polymerase (23), was used for Western blotting, while its parent, CP1250, was used in the previous work with mRNA (36). Western blotting using this anti-ComX antibody preparation to estimate ComX levels in crude cell extracts showed that ComX expression patterns in CP1250 and CPM1 were identical, as expected (data not shown).

After initiation of competence induction by a high dose of CSP, samples were chilled and harvested for preparation of cell lysates at 2.5-min intervals. As shown in Fig. 2B, ComX was not detected in noncompetent cells (0 min), showing that the basal level of *comX* gene expression was very low. ComX protein was detected 7.5 min after addition of CSP, preceding the appearance of substantial quantities of mRNA for ComX-dependent genes at 10 min (Fig. 3A), according to the findings of Peterson et al. (36). The intracellular concentration of ComX then increased dramatically, reaching a maximum at 15 min. In the following 7 min, its level dropped rapidly, falling below detection by 25 min.

In separate Western blots, the levels of ComX protein at different times after CSP induction were quantitated by comparing the IDV of experimental samples to a dilution series of a ComX protein standard incorporated in the same gel (Fig.

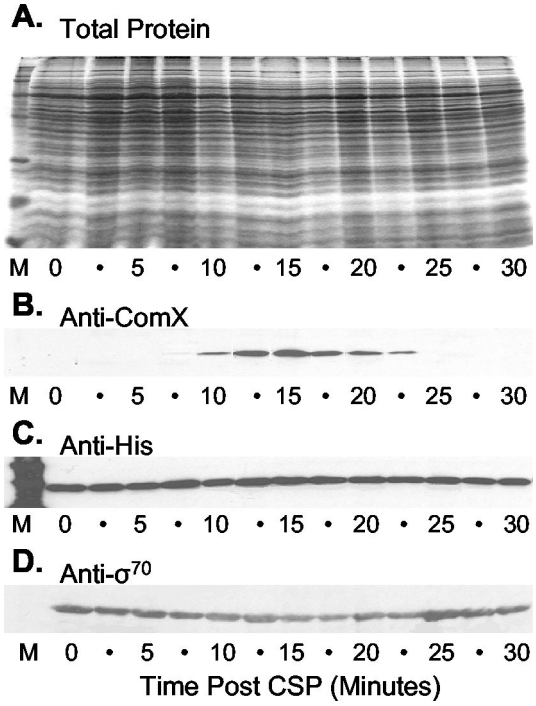


FIG. 2. Evolution of levels of ComX, the β′ subunit of RNA polymerase, and the primary sigma factor ($\sigma^A$) during competence induction. Samples of cell lysates prepared at 2.5-min intervals after CSP treatment were analyzed with SDS–15% PAGE by Coomassie brilliant blue staining (80 μg of total protein) (A) or Western blotting with anti-ComX antibody (20 μg of total protein) (B). Alternatively, samples (20 μg of total protein) were applied to an SDS–7.5% PAGE for Western blotting with anti-His (C terminal)-HRP-conjugated antibody (C) and Western blotting with anti-$\sigma^{70}$ antibody (2G10) (D). M, molecular mass standards (Bio-Rad), as a protein ladder of 200, 116.3, 97.4, 66.2, 45, 31, 21.5, 14.4, and 6.5 kDa.

3B and C). Amounts of ComX ranged from below 0.5 fmol to a maximum of 75 fmol of ComX/μg of total protein. ComX exceeded 25% of its maximal level during 12 min, from 10 to 22 min.

**The levels of $\sigma^A$ and ComX shift during competence induction, but RNA polymerase remains at a constant intracellular concentration.** In a recent analysis of mRNA levels during competence induction, it was observed that the $\sigma^A$ gene, *rpoD*, was induced during competence (Fig. 3A) (36). To describe more directly the events accompanying competence induction, we determined protein levels of a subunit of core RNA polymerase and $\sigma^A$ in parallel with the measurements of ComX protein described above.

The His-tagged β′ subunit of polymerase in strain CPM1 was determined by using an anti-His antibody, while a monoclonal antibody to *E. coli* $\sigma^{70}$ that has been reported to detect many bacterial primary sigma factors (5) was used to monitor the $\sigma^A$ level, as shown in Fig. 2C and D. The level of RNA polymerase β′ subunit remained constant before, during, and after the period of competence, while the level of $\sigma^A$ varied somewhat. Quantitation of $\sigma^A$ obtained by IDV of a Western blot (Fig. 3C) showed that the primary sigma factor decreased about 20 to 40% after addition of CSP, remained at this lower level during competence, and rebounded nearly twofold after 22.5 min to about 125% of its precompetence level. The combined results of these protein determinations and previous mRNA determinations (36) are shown in Fig. 3.

**ComX enables the core RNA polymerase of *S. pneumoniae* to generate transcripts from the competence-specific genes *ssbB*, *cinA*, *cglA*, *celA*, and *dalA*.** ComX was proposed to act as an alternative sigma factor, based on the following characteristics: (i) it can be obtained from RNA polymerase preparations (23); (ii) many genes known to be responsible for competence development depend on ComX for transcription (23, 36); (iii) those genes share a consensus sequence in the promoter region, the Campbell consensus, or the Cin box or Com box, which is quite different from the promoter that is recognized by the primary sigma factor (7, 36), and they lack evident $\sigma^A$ promoter sites; and (iv) ComX shares similarity to *B. subtilis* $\sigma^H$ in the most highly conserved region of the $\sigma^{70}$ family (subregion 2.2), which is known to interact with core RNA polymerase (23). To test this hypothesis more directly by asking whether ComX could replace $\sigma^A$ in a reconstituted holoenzyme, we developed purification procedures for pneumococcal core polymerase and for ComX protein.

Core RNA polymerase was obtained from a CPM1 derivative (CP1290, deficient in both *comX* genes) by using a phosphocellulose column to remove the primary σ factor from a preparation of holoenzyme (Fig. 4A). The six-His-tagged form of ComX in CP1288 supported normal competence levels and induction kinetics (data not shown), suggesting that extension of the C terminus of ComX had no effect on its biological activity. A derivative of ComX with a 30-amino-acid C-terminal extension containing the V5 epitope and a six-His-tag (ComX-V5/6H) was overexpressed and purified using the plasmid pXPL01 in *E. coli* strain BL21(DE3)pLysS. The *comX* sequence corresponds to a molecular mass of 19,874 Da, while the doubly tagged ComX-V5/6H is 3,297 Da larger, allowing their separation as shown in the SDS-PAGE gel in Fig. 3B. Overexpressed ComX-V5/6H was recovered mainly in the insoluble cell fraction (Fig. 4B, lanes 4 and 5) and was not solubilized by the detergents *N*-dodecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate or sodium deoxycholate (data not shown). However, it was successfully solubilized with 0.5% Sarkosyl by following the procedure of Burgess (6). After refolding by dialysis, the protein was recovered from a Ni-NTA affinity column and appeared to be soluble monomer at least 95% pure, based on analysis of the Coomassie brilliant blue R-250-stained gel (Fig. 4B, lane 6).

To study the activity of RNA polymerase associated with either ComX or the primary sigma factor in vitro, single-round runoff transcription assays were used. Polymerase from noncompetent cells ($\sigma^A$ holoenzyme), polymerase from competent cells ([$\sigma^A$ plus ComX]-holoenzyme), or reconstituted holoenzyme (ComX-V5/6H plus core RNA polymerase) were incubated with PCR DNA fragments of the promoter region including the Cin box or Com box of several ComX-dependent genes as templates. After initiation by ribonucleoside triphosphates including [α-$^{32}$P]UTP, reinitiation of transcription was inhibited by a supplement of heparin.

RNA polymerase from noncompetent cells produced transcript from the constitutive promoter of the *amiA* template (Fig. 5A, lane 1) but failed to generate a transcript from the *ssbB* template (Fig. 5A, lane 3). In contrast, polymerase from

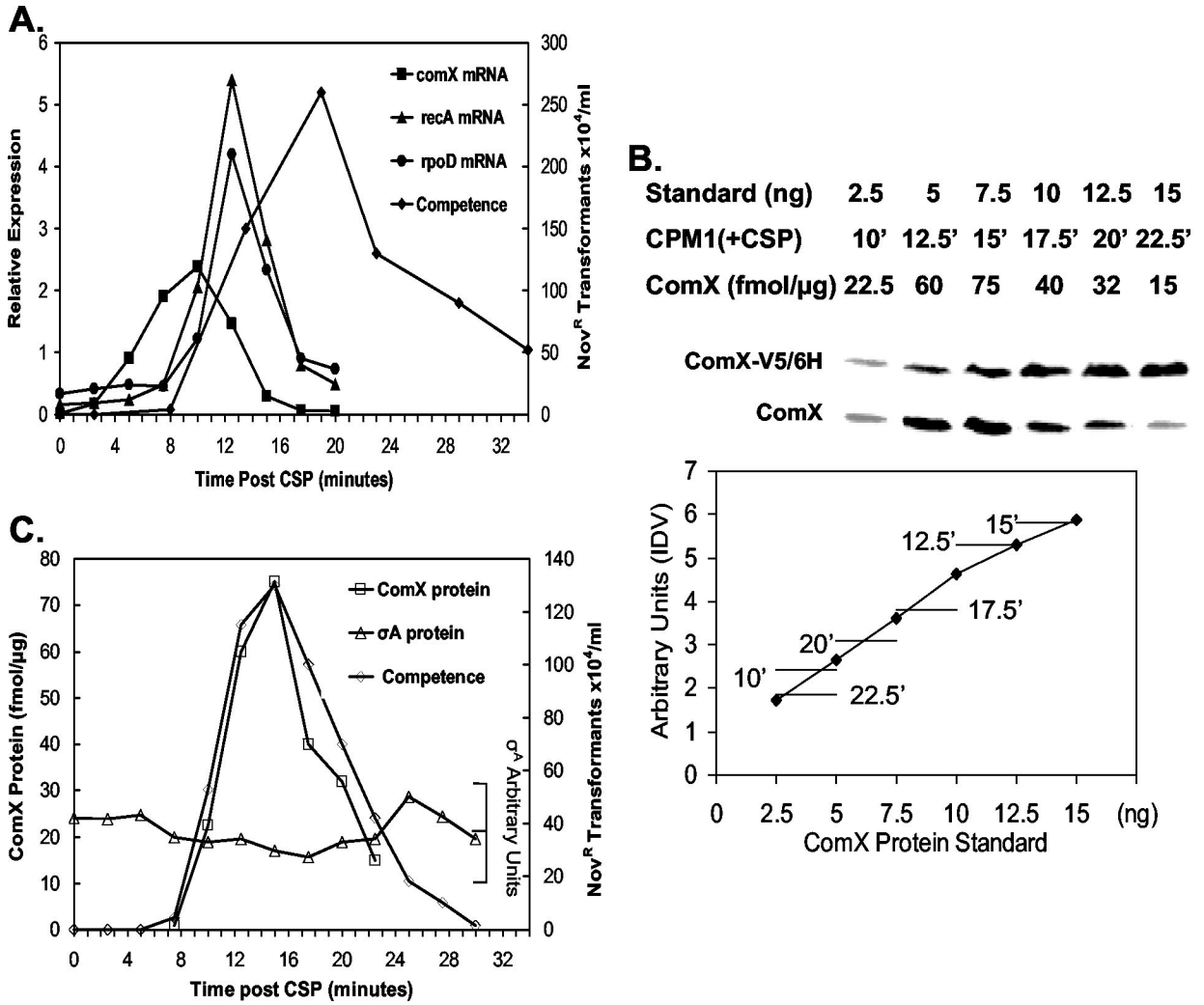


FIG. 3. Comparison of protein and mRNA levels during competence induction. (A) mRNA levels of *comX, recA,* and *rpoD* and competence kinetics from Peterson et al. (36). (B) Quantitation of ComX levels in CSP-induced cells by use of an internal standard of purified ComX-V5/6H. Whole-cell lysates obtained at 10 to 22.5 min post-CSP addition (10 μg of total protein) were analyzed with purified ComX-V5/6H as an internal standard (2.5 to 15 ng) in an SDS–15% PAGE gel. After probing with anti-ComX by Western blotting, the amount of ComX was determined (in femtomoles of ComX per microgram of total protein) by comparison of the IDVs for standards and samples, as shown in panel C. (C) The Western blot shown in Fig. 2D was scanned and adjusted to arbitrary units according to the IDV to quantitate levels of $\sigma^A$. As reference for comparison with the kinetics reported by Peterson et al. (36), competence was determined simultaneously with Western blotting by 5-min exposures to CP1500 DNA, termination with DNase I, and plating for Nov$^r$ transformants.

competent cells did transcribe the *ssbB* template (Fig. 5A, lane 4), confirming the report by Lee and Morrison (23). RNA polymerase reconstituted from core and purified ComX-V5/6H also produced a specific transcript from the *ssbB* template, while neither component produced transcript when used alone (Fig. 5A, lanes 6, 7, and 8).

To determine the specificity of these transcription reactions, two anti-sigma antibodies were employed. Anti-$\sigma^{70}$ monoclonal antibody blocked transcription from the *amiA* promoter by $\sigma^A$-holoenzyme (Fig. 5A, lane 2) but did not block transcription from the *ssbB* promoter by reconstituted ComX-core RNA polymerase (Fig. 5A, lane 9). Conversely, anti-ComX antibody severely inhibited transcription from the *ssbB* template by either reconstituted ComX-core RNA polymerase (Fig. 5A, lane 10) or holoenzyme from competent cells (Fig. 5A, lane 5). These results show that $\sigma^A$ does not contribute to the ComX-dependent transcription.

To show ComX-dependent transcription from other templates containing a Cin-box promoter region, further transcription reactions used *cinA*, *cglA*, *celA*, *dalA,* and *rpoD* promoters and *S. pneumoniae* core polymerase alone, or *S. pneumoniae* core polymerase associated with ComX-V5/6H (Fig. 5B). Anti-$\sigma^{70}$ monoclonal antibody was included in these transcription reactions to preclude activity of any residual $\sigma^A$ in *S. pneumoniae* core polymerase. Transcripts of the predicted length (see Materials and Methods) were obtained for each of those templates except *rpoD*, suggesting that there was no ComX promoter in the region up to 140 bp upstream of the *rpoD* protein start site. We conclude that ComX acts as an alterna-

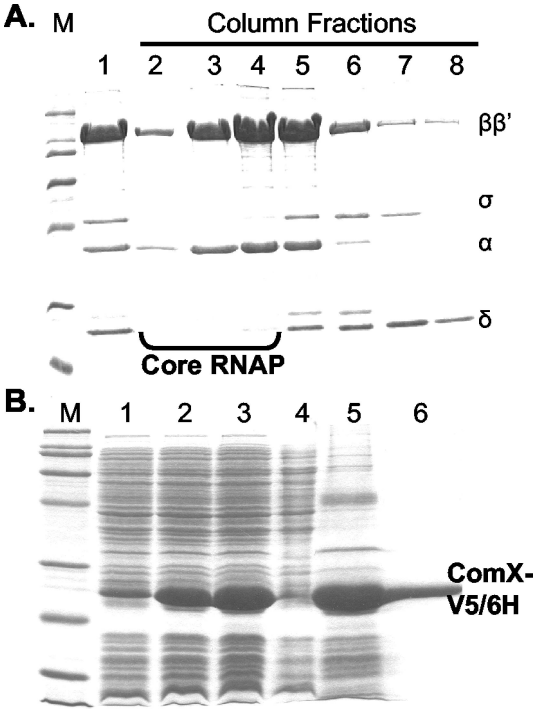


FIG. 4. Purification of core RNA polymerase and ComX protein. (A) RNA polymerase purified from *comX*-deficient strain CP1290 as described in Materials and Methods was fractionated on phosphocellulose to obtain core polymerase. Coomassie blue–SDS-PAGE analysis of the fractionation is shown. Lane M, molecular mass standards, as for Fig. 2; lane1, purified RNA polymerase from CP1290; lanes 2 to 8, KCl gradient fractions eluted from the phosphocellulose column. Fractions of lanes 2, 3, and 4 were pooled, dialyzed, and stored as core RNA polymerase. (B) ComX-V5/6H protein was purified from *E. coli* carrying the expression plasmid pXPL01 by dissolving the insoluble cell fraction with Sarkosyl. SDS-PAGE analysis of protein samples from purification steps is shown as follows: M, molecular mass standards as for Fig. 2; lane 1, uninduced whole-cell extract; lane 2, whole-cell extract after 2-h induction; lane 3, whole-cell extract after 4-h induction; lane 4, soluble fraction of 4-h induced cell extract; lane 5, insoluble fraction of 4-h induced cell extract, dissolved in 0.5% (wt/vol) Sarkosyl; lane 6, refolded protein purified from nickel column. Whole-cell extracts represent the equivalent of 1 ml of cell culture at an $OD_{600}$ of 0.2.

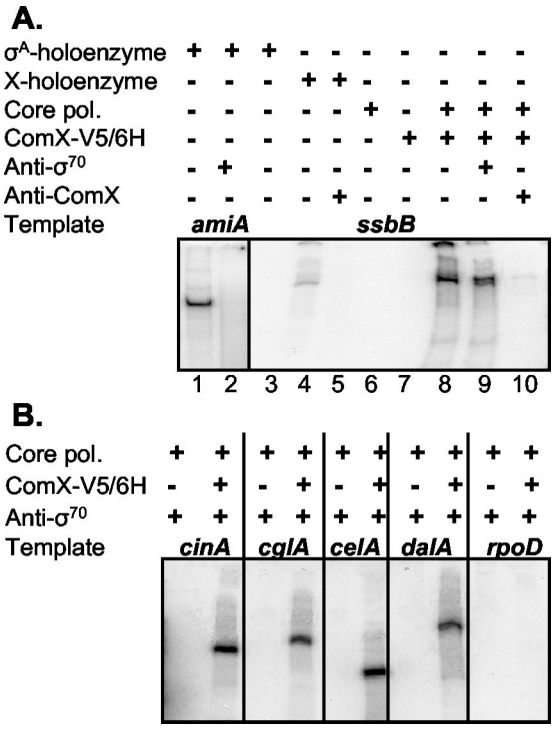


FIG. 5. ComX-dependent in vitro transcription from promoters of competence-specific genes, as shown in autoradiographs of transcripts fractionated on 5% polyacrylamide–7 M urea gels. (A) Templates containing *amiA* promoter (lanes 1 and 2) or *ssbB* promoter (lanes 3 to 10) were incubated in transcription reactions with $\sigma^A$-holoenzyme (lanes 1, 2, and 3), ComX-holoenzyme (lanes 4 and 5), *S. pneumoniae* core polymerase alone (lane 6), ComX-V5/6H alone (lane 7), or *S. pneumoniae* core polymerase plus ComX-V5/6H (lanes 8, 9, and 10). The anti-$\sigma^{70}$ monoclonal antibody (lanes 2 and 9) or anti-ComX antibody (lanes 5 and 10) was preincubated with RNA polymerase before exposure to certain DNA templates, as indicated. (B) Templates containing *cinA, cglA, celA, dalA,* or *rpoD* promoter regions were incubated with *S. pneumoniae* core polymerase alone, or with *S. pneumoniae* core polymerase plus ComX-V5/6H. The anti-$\sigma^{70}$ monoclonal antibody was added to all transcription reactions represented in this panel.

tive sigma factor to allow polymerase to utilize competence-specific promoters.

**At its maximal level, ComX is in excess to RNA polymerase.** The development of competence in *S. pneumoniae* is accompanied by a brief but dramatic change in protein synthesis, as indicated by pulse-labeling patterns (29). As the competence regulator ComX acts as an alternative sigma factor (see above), it is possible that a highly ordered program of temporal gene activation may be governed by the combined activities of the principal holoenzyme, $E\sigma^A$, and a competence-specific holoenzyme containing the alternative sigma factor, ComX. Since it is likely that both sigma factors bind to the same site on core RNA polymerase (24), the shift in protein synthesis suggests that the alternative sigma factor may be present in sufficient quantities to dominate the holoenzyme specificity for a short time. As one step toward analyzing the protein synthesis shift associated with competence in pneumococci, we sought to estimate whether ComX accumulated to a sufficient level to occupy more than a minority of the polymerase holoenzyme complexes.

RNA polymerase purified from *S. pneumoniae* strain CPM1 and ComX-V5/6H purified from *E. coli* were used as standards for comparison of the intracellular levels of RNA polymerase and ComX. A culture was harvested at 15 min after adding CSP, when ComX would reach its maximum, according to Fig. 2B. For quantitative Western blotting of these proteins, both standards and lysate samples were analyzed in the same gel by using anti-ComX antibody for ComX or ComX-V5/6H and anti-His antibody for the β′ subunit of RNA polymerase, as shown in Fig. 6. In five individual experiments, the average molar ratio was 1.72, with a range of 1.58 to 1.85 and a standard deviation of 0.12 (Table 2). Since over 90% of cells are competent at maximal competence following CSP treatment (9, 28, 37), it appears that ComX is at least as abundant as polymerase core. As the total protein of this 15-min sample was measured by the Bradford assay to be 24 μg of total

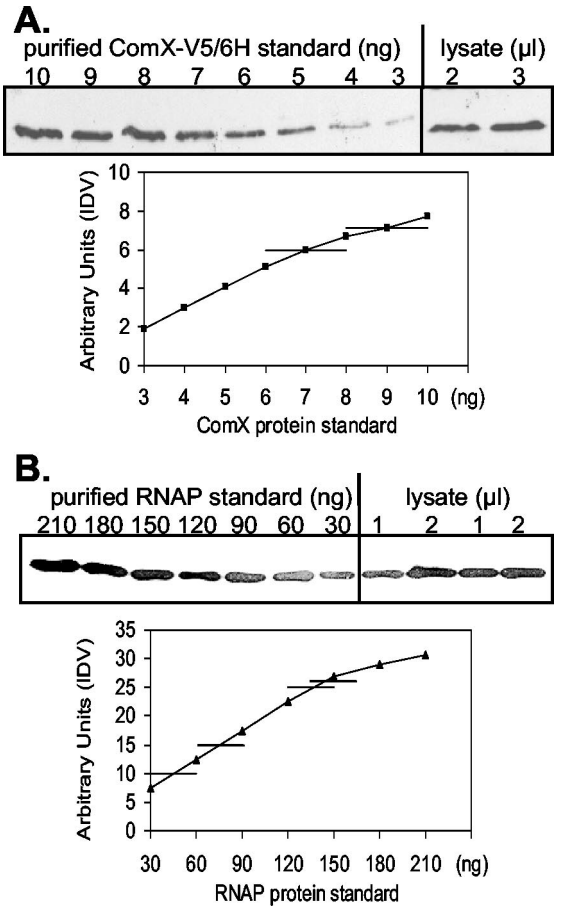


FIG. 6. Abundance of ComX and RNA polymerase. Total proteins extracted from a single competent culture of CPM1 were fractionated in SDS gels containing internal standards of known amounts of purified reference protein ComX-V5/6H (A) or *S. pneumoniae* RNA polymerase (B). After blots were probed with anti-ComX antibody or anti-His-HRP antibody, the intensities of bands on the chemiluminescence films were quantified using Alpha-Imager 3.2 and are shown in arbitrary units proportional to their IDV. Horizontal lines show the relative IDV for cell lysate samples. At the top of each panel is the Western blotting analysis results of standard curves and lysates running simultaneously on the same gel. For detailed calculation of the molar ratio of ComX to RNA polymerase, see Table 2.

protein/ml of culture, and a culture at an $OD_{550}$ of 0.05 contains about $1.5 \times 10^8$ CFU/ml (or $2.7 \times 10^8$ cells [37]), the polymerase estimation corresponds to an RNA polymerase content of about 2,000 molecules per cell or 1.6% of total protein.

## DISCUSSION

The present results establish three new characteristics of the pneumococcal competence regulatory protein, ComX. First, we showed that ComX was unstable and appeared transiently for 15 min preceding the decay of competence. This contrasts with the fate of two other components of the quorum-sensing circuit, the products of *comC* and *comE* (CSP and ComE), which are stable (in contrast to the rapid turnover of their mRNA) and persist at high levels even 30 to 120 min after competence decays to zero (12, 32, 36, 44). Second, we found that the molar concentration of ComX reaches a level in excess of that of RNA polymerase. Third, we demonstrated experimentally that ComX could function in vitro as an alternative sigma factor, interacting with core RNA polymerase to confer new promoter specificity and allow transcription of several ComX-dependent genes. The possibility of contamination of $\sigma^A$ in these preparations of core RNA polymerase was ruled out by showing that a monoclonal antibody against *E. coli* $\sigma^{70}$, which blocked activity of pneumococcal $\sigma^A$-holoenzyme, had no effect on ComX-directed transcription.

While it now appears clear that ComX allows recognition of a new set of promoters in response to CSP, it is less clear what effect the new factor's presence may have on transcription of $\sigma^A$ promoters. We found that the level of RNA polymerase remained constant during competence induction, while the level of $\sigma^A$ appeared to decrease to a minimum of about 60% and to rebound to about 125% after the decay of competence. The period of decreased $\sigma^A$ was coincident with the presence of ComX. In *E. coli*, the level of the primary sigma factor $\sigma^{70}$ ($\sigma^D$) remains nearly constant in both exponential and stationary phases (20). In contrast, the primary sigma factor ($\sigma^A$) of *B. subtilis* decreases slightly when $\sigma^H$ appears during initiation of sporulation (13), similar to the pattern observed here upon induction of ComX. The *rpoD* operon of *B. subtilis* is controlled by at least six promoters, two of which are recognized by E$\sigma^H$-holoenzyme (18). The feedback loop between $\sigma^A$ and

TABLE 2. Relative abundance of RNA polymerase and ComX in competent cells

| Experiment[a] | Total protein (μg/ml) | ComX[b] | | RNAP[b] | | Molar ratio (ComX/RNAP) |
|---|---|---|---|---|---|---|
| | | ng/ml | pmol/ml | ng/ml | pmol/ml[c] | |
| A | 24.4 | 33.3 | 1.67 | 400 | 0.94 | 1.78 |
| B | 28.5 | 31.2 | 1.56 | 370 | 0.87 | 1.79 |
| C | 28.5 | 45.7 | 2.29 | 528 | 1.24 | 1.85 |
| D | 23.9 | 30.0 | 1.5 | 405 | 0.95 | 1.58 |
| E | 16.7 | 21.0 | 1.05 | 277 | 0.65 | 1.62 |
| Avg | | | | | | 1.72 ± 0.12 |

[a] A 10-ml culture of CPM1 was collected 15 min after addition of CSP, centrifuged and washed, lysed in 1 ml of lysis buffer, TCA precipitated, and redissolved in 60 μl of SDS-PAGE sample buffer. Samples were subjected to Western blotting together with standard proteins and probed with anti-ComX antibody and anti-His-HRP antibody, respectively. The results shown here were collected from five such individual assays of four induction experiments.

[b] The content of either RNA polymerase (RNAP) or ComX was determined by comparison to a standard curve, using the IDV of whole-band images from Alpha-Imager 3.2, analyzed as shown in Fig. 6.

[c] RNA polymerase (RNAP) has several subunits: two α, 2 × 34.2 kDa; β, 135.9 kDa; β′, 137.0 kDa; $\sigma^A$, 42.0 kDa; δ, 22.7 kDa; ω1, 11.9 kDa; ω2, 9.0 kDa. We estimate the molecular mass of RNA polymerase from the predicted sequences (40) as 426.9 kDa.

$\sigma^H$ is probably involved in maintaining the proper amount of $\sigma^A$. Although our in vitro transcription experiment showed no ComX-directed promoter near the *rpoD* gene, it remains possible that one is located farther upstream. The small apparent shift in the level of $\sigma^A$ during pneumococcal competence contrasts with the 10-fold increase reported for *rpoD* mRNA (36), suggesting that this message may not be efficiently translated in competent cells.

Lee and Morrison proposed that ComX coordinates both the induction and decay of competence (23). The transient appearance of ComX and its short half-life (~4 min) are consistent with this hypothesis. Interestingly, the autoregulated transcriptional factor ComK in *B. subtilis*, which drives the development of competence, is subjected to regulated proteolysis, accumulating rapidly in cells that become competent and undergoing degradation during the escape from competence (42). The ComX transcriptional and translational expression patterns together suggest that some mechanism stops the transcription of *comX* after 10 min, resulting in the decay of its message and the end of its translation by 15 min. However, as the levels of transcripts of ComX-dependent genes seem to decrease several minutes before significant losses of ComX protein occur, an additional mechanism may also act to stop the transcription of ComX-dependent genes while ComX protein is still present.

We found in this study that there were at least 1.5 to 2 molecules of ComX per molecule of RNA polymerase when ComX was at its maximum. The hypothesis that ComX may replace all $\sigma^A$ during competence induction (23), causing a strong shift in protein synthesis (29), is thus not ruled out by an insufficiency of ComX. In contrast to this model, the RNA polymerase preparations obtained from competent cultures contained both $\sigma^A$ and ComX (23; data not shown). There are two simple explanations for the presence of both $\sigma^A$ and ComX in those preparations. First, the competent cultures might not have been harvested at precisely the time of maximal ComX, or the fraction of competent cells might have been significantly less than 1.0. Second, the affinity of core RNA polymerase for ComX may be so low that there is not enough ComX to completely replace the primary $\sigma^A$. In *B. subtilis*, $\sigma^F$ reaches a peak level about 2-fold higher than that of $\sigma^A$ after the onset of sporulation, but the affinity of core for $\sigma^F$ is found to be about 25-fold lower than that for $\sigma^A$. Thus, the difference in concentration cannot adequately account for the replacement of $\sigma^A$ holoenzyme by $\sigma^F$ holoenzyme, suggesting the existence of an anti-$\sigma^A$ and/or the modification of $\sigma^A$ as possible mechanisms (25). The relative affinities of pneumococcal core polymerase for $\sigma^A$ and for ComX have not yet been determined.

Recently, a ComX homologue in the human pathogen *Streptococcus pyogenes* was shown experimentally to function as an alternative sigma factor ($\sigma^X$) at the promoter sequences TAC GAATA of the genes *cinA* and *femB* in vitro (31). The significance of this observation is unclear, as *S. pyogenes* is not known to be competent for genetic transformation (17) and the role of *cinA* in competence of pneumococci is unknown (26, 33). However, as the *Staphylococcus aureus* homologue to FemB has been identified as a virulence factor (27), it does suggest the possibility that $\sigma^X$-dependent genes may include some required for virulence in *S. pyogenes*.


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