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Identification of a DNA transformation gene required for *com101A*$^+$ expression and supertransformer phenotype in *Haemophilus influenzae*

JAMES J. ZULTY* AND GERARD J. BARCAK†

Department of Biological Chemistry, University of Maryland School of Medicine, 108 North Greene Street, Baltimore, MD 21201

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ABSTRACT **DNA sequencing, RNA mapping, and protein expression experiments revealed the presence of a gene, *tfoX*$^+$, encoding a 24.9-kDa polypeptide, that is transcribed divergently from a common promoter region with the *Haemophilus influenzae rec-1*$^+$ gene. *H. influenzae* strains mutant for *tfoX* failed to bind transforming DNA and were transformation deficient. Primer extension experiments utilizing *in vivo* total RNA from precompetent and competent *H. influenzae* cells demonstrated that transcription of *tfoX*$^+$ increased immediately upon competence induction, suggesting that *tfoX*$^+$ is an early competence gene. Similar experiments showed that the expression of the late competence-specific gene, *com101A*$^+$, was *tfoX*$^+$ dependent. Moreover, expression of plasmid-borne *tfoX*$^+$ in *H. influenzae* resulted in constitutive competence. The addition of cyclic adenosine monophosphate (cAMP) to strains carrying a *tfoX::lacZ* operon fusion resulted in an immediate increase in β-galactosidase activity that correlated with an increase in genetic transformability. Collectively, our results suggest that TfoX may play a key role in the development of genetic competence by regulating the expression of late competence-specific genes.**

Genetic transformation in *Haemophilus influenzae* is a natural process involving regulated gene expression, nucleic acid–protein interactions, macromolecular transport, and genetic recombination. Competence for transformation is induced in growing cultures by physiological change occurring during late-logarithmic-phase growth, by a temporary shift to anaerobic conditions, or by transfer of cells to a chemically defined medium such as MIV (1, 2).

Although the biochemical fate of transforming DNA in *H. influenzae* has been characterized (reviewed in ref. 3), only recently has the molecular cloning of transformation genes allowed the identification of several components of the transformation apparatus. Recently, both the *crp*$^+$ and *cya*$^+$ (adenylate cyclase) genes from *H. influenzae* were cloned and shown to be essential for competence development (4, 5), suggesting that *H. influenzae* cAMP–cAMP receptor protein (CRP) complex may function to regulate the expression of competence-specific genes. Tomb (6) has shown that *por*$^+$, encoding a periplasmic oxidoreductase homologous to DsbA and TcpG, is required for the proper recruitment of polypeptides into the competent cell outer membrane. Components of the translocation machinery, as deduced by the phenotype of mutants, include the products of the recently cloned and sequenced *com101A*$^+$ (7, 8) and *rec-2*$^+$ (9) genes. Rec-2 is homologous with the *Bacillus subtilis comE*-open reading frame (ORF) 3 transformation gene product (9, 10). Both proteins are predicted to have multiple membrane-spanning domains that putatively form the pore through which transforming DNA enters the cell.

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Here we report the identification of an *H. influenzae* transformation gene, *tfoX*$^+$,‡ that when present on a multicopy plasmid confers upon strains constitutive competence. We provide evidence that *tfoX*$^+$ is an early competence gene whose expression is modulated by cAMP–CRP and that TfoX is required for the expression of the late competence gene, *com101A*$^+$ [*comF*$^+$ (11)].

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *H. influenzae* strains KW20 (wild type) and MAP7 [resistant to novobiocin and nalidixic acid (Nov^r, Nal^r)] have been described (12). GBH strains were derived from strain KW20 by transformation. Strain GBH6.6 contains a 2.0-kb *aadA*$^+$ [spectinomycin resistance (Spc^r) (13)] gene insertion at the *Sna*BI site of the *tfoX*$^+$ coding sequence (see Fig. 1). The genetic structure of GBH strains carrying insertion mutations was verified by Southern hybridization.

Plasmids pHKRec (*rec-1*$^+$ *tfoX*$^+$ *kan*$^+$) and pGJB103 (*bla*$^+$ *tet*$^+$) have been described (12). Plasmids pJZMC and pJZMS contain either the promoter region and N terminus of the *tfoX* coding sequence or *tfoX*$^+$, respectively, in pGJB103. Plasmids pJZSSX1.2 and pJZSSX2 contain the 0.7-kb *Sca* I *tfoX*$^+$ DNA fragment in opposite orientations in vectors pTZ19 or pTZ18, respectively. Two pJZSSX1.2 subclones, pX1NE and pX1CE, have undergone C-terminal deletions of *tfoX* initiating at either the unique *Nsi* I site or the unique *Cla* I site, respectively, within the coding sequence (see Fig. 1). Standard recombinant DNA techniques were employed throughout (14).

Culture Conditions. *H. influenzae* strains were grown as described (15). Chromosomal or plasmid DNAs were used to transform MIV-competent *H. influenzae* cells as before (15). For competence-deficient strains, plasmid DNA was introduced by electroporation (16). *H. influenzae* strains containing antibiotic resistance markers were selected at the following final antibiotic (Sigma) concentrations (μg/ml): ampicillin, 10; kanamycin, 10–30; nalidixic acid, 3; novobiocin, 2.5; and spectinomycin, 15.

DNA Sequence Determination. Segments of *tfoX*$^+$ DNA were ligated to M13 bacteriophages (17, 18) and unidirectional deletion derivatives were prepared (19) prior to DNA sequencing (20) both DNA strands (Fig. 1). Computer analysis of DNA and protein sequences was performed with the GENEPRO software package (Riverside Scientific, Bainbridge Island, WA).

Primer Extension and Nuclease S1 Analyses. Total cellular RNA was isolated from 20-ml samples of *H. influenzae* cultures

Abbreviations: CRP, cAMP receptor protein; ORF, open reading frame; IHF, integration host factor; Nal^r, Nov^r, and Spc^r, phenotypic resistance to the antibiotics nalidixic acid, novobiocin, and spectinomycin, respectively; AMVRT, avian myeloblastosis virus reverse transcriptase; IPTG, isopropyl β-D-thiogalactoside.

*Present address: Department of Pharmacy, Union Memorial Hospital, Baltimore, MD 21218.
†To whom reprint requests should be addressed.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. U13205).

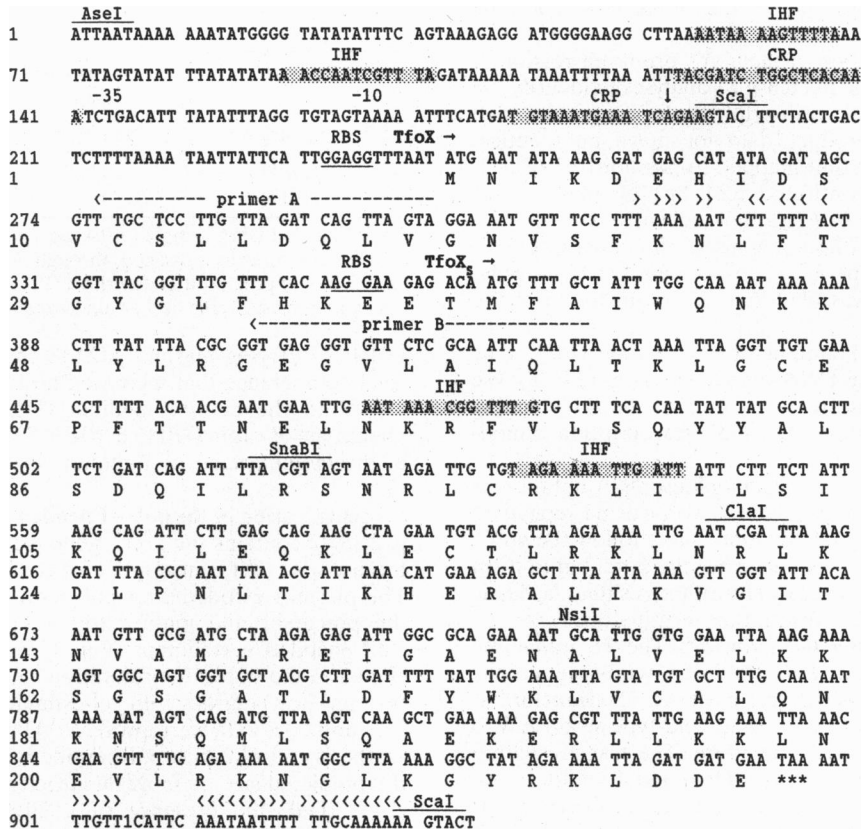

FIG. 1. Nucleotide sequence and deduced amino acid sequence (one-letter symbols below DNA sequence) of the *H. influenzae tfoX*$^+$ gene and protein. The downward-pointing arrow denotes the proposed transcriptional start site for *tfoX*$^+$. Two potential ribosome-binding sites (RBS) are underlined and followed by proposed translational start sites (arrow) for TfoX and TfoX$_S$. Amino acid numbering begins after the first methionine residue of TfoX and ends before the ochre stop codon (***). The dashed line depicts the hybridization sites for primers A and B, which were used to map the 5′ end of *tfoX*$^+$ mRNA. Shaded areas represent stringent matches to the consensus binding domains of the *Escherichia coli* CRP or integration host factor (IHF) proteins. The −35 (CTGACA) and −10 (TAAAAA) hexamers of the proposed *tfoX*$^+$ promoter are indicated. Inverted repeat sequences are indicated by converging chevrons. Landmark restriction endonuclease sites (overlined) are labeled. The numbers at the left indicate either nucleotide or amino acid position.

by a hot phenol method (21). Four oligonucleotide primers, A, B, C, and D, were synthesized (A, B, and C, Macromolecular Resources, Colorado State University, Fort Collins, CO; D, University of Maryland at Baltimore Biopolymers Laboratory). Primers A, B, and D hybridized to the antisense DNA strand of *tfoX*$^+$ at nucleotides 276–300, 400–425, and 170–237, respectively (Fig. 1). Primer D contained an additional 10 nt at its 3′ end nonhomologous to *tfoX*$^+$ sequences for easier determination of nuclease S1 digestion products. Primer C hybridized to the equivalent DNA strand of *com101A*$^+$ at nucleotides 107–131 (figure 3 in ref. 7). All primers were 5′ end-labeled with [γ-^{32}P]ATP to specific activities of at least 5 × 10^6 cpm/μg with T4 polynucleotide kinase (Promega). Primers A, B, and C were used in primer extension reactions as directed by the supplier (Promega kit, technical bulletin no. 113). Primer D was used in high-resolution nuclease S1 mapping experiments (22). RNA concentrations were determined from UV absorbance at 260 nm. Avian myeloblastosis virus reverse transcriptase (AMVRT)-derived primer extension products and nuclease S1 digestion products were resolved on an 8% polyacrylamide denaturing gel alongside a DNA sequencing ladder derived from phage M13. Control experiments, in which levels of input RNA were varied over an 8-fold range, established that primers were always in excess (not shown).

Preparation and Characterization of *tfoX::lacZ* Operon Fusion Strains. Our strategy to prepare operon fusion strains was previously described (12). Briefly, strain KW20 was transformed to kanamycin resistance (Kan^r) with the integrating *tfoX::lacZ* operon fusion plasmid pJZGL (*tfoX::lacZ*, kan$^+$), yielding strain GBH2.1. Strain GBH21.12 is isogenic with strain GBH2.1 except for an *aadA*$^+$ gene insertion within the *crp* gene. The β-galactosidase activity of *lacZ* fusion strains was determined by Miller assay (23) as before (12).

Identification of Plasmid-Encoded Polypeptides. The bacteriophage T7 RNA polymerase-directed expression system of Studier and Moffatt (24) was used with *E. coli* strain BL21DE3*pcnB* and [^{35}S]methionine as the tracer as described (12).

RESULTS

Sequence Analysis of the *H. influenzae tfoX*$^+$ Gene. Previous studies of the *H. influenzae rec-1*$^+$ gene led to the identification of an upstream ORF which we named *tfoX*$^+$ (12). The *tfoX*$^+$ coding sequence extends from nucleotide 244 to nucleotide 897, potentially encoding a 217-amino acid, 24.9-kDa protein with a predicted pI of 9.7 (Fig. 1). Analyses of the *tfoX*$^+$ DNA and deduced amino acid sequences showed no homology (i.e., <30% identity) to sequences found in GenBank, European Molecular Biology Laboratory, SwissProt, and National Biomedical Research Foundation data bases.

Two potential *tfoX*$^+$ ribosome-binding sites (RBS, identified by complementarity to the 3′ end of *E. coli* 16S rRNA because *tfoX*$^+$ is expressed well in *E. coli*), appropriately spaced from two initiation codons in the same reading frame, were identified (nucleotides 233–237 and 350–353; Fig. 1). Experiments

suggested that both RBSs may be functional in *E. coli* (see below).

To determine the location of the *tfoX*$^+$ promoter region we used primer extension and nuclease S1 analyses to identify the 5′ end of the *in vivo tfoX*$^+$ transcript. Both primers A and B gave a single extension product (data not shown, but identical result in Fig. 4*A*, lane 1) that mapped to position 193 (Fig. 1, +1). This result was confirmed (±1 bp) by nuclease S1 mapping using primer D (data not shown). A candidate promoter with hexamers homologous to the conserved −10 and −35 sequences of the *E. coli* σ^{70}-dependent RNA polymerase promoter (25) was identified 22 bp upstream of the transcription start site.

Potential CRP and IHF binding sites (4, 26) were located by using the *E. coli* consensus DNA-binding domains (27, 28). We have no data to demonstrate the activity of the IHF binding domains, but we show below that *tfoX*$^+$ transcription is modulated by cAMP and CRP.

We found no matches to the *Haemophilus* DNA uptake-site sequence (29) in the *tfoX*$^+$ segment. Two inverted repeats at base pairs 901–916 and 917–930 immediately follow the *tfoX*$^+$ coding sequence. The first sequence has the potential to form a stem-loop structure followed by six uridine residues, a classic feature of ρ-independent transcription terminators.

Characterization of the *tfoX* Mutant. The *H. influenzae tfoX*-insertion mutant GBH6.6 exhibited wild-type colony morphology, color, and growth rate patterns. The transformation frequency for strain GBH6.6 and wild-type strain KW20 was determined by the MIV procedure. The transformation frequency was $<10^{-7}$ for strain GBH6.6 and 10^{-2} for strain KW20 (Table 1). The transformation defect of GBH6.6 was further characterized by determining cellular ability to bind and internalize radiolabeled DNA as described (30). Strain KW20 internalized 96% of bound DNA, while GBH6.6 failed to take up more than 0.5% of the DNA (Table 2).

The transformation defect of strain GBH6.6 could be complemented in trans by the cloned *tfoX*$^+$ gene (Table 1). While GBH6.6 carrying either pGJB103 or pJZMC transformed at barely detectable levels with chromosomal DNA, GBH6.6 carrying either of the *tfoX*$^+$ plasmids, pHKRec or pJZMS, transformed at nearly wild-type levels. In addition, the binding and internalization of radiolabeled DNA by strain GBH6.6 carrying pHKRec or pJZMS was comparable to wild-type levels (data not shown). The possibility that, upon establishment of the various plasmids in strain GBH6.6, reciprocal recombination had occurred between plasmid and chromosome was ruled out by a plasmid transformation assay (data not shown).

Constitutive Competence Conferred by Plasmid-Borne *tfoX*$^+$. To demonstrate that the supertransformer phenotype of *Haemophilus* strains reported by Stuy (31) was due to *tfoX*$^+$ and not *rec-1*$^+$ as originally hypothesized, we determined the effect of multiple copies of *tfoX*$^+$ upon transformation. Strain GBH6.6 carrying plasmid pJZMS showed constitutive high-level competence that increased no further upon addition of cAMP to the culture medium (Fig. 2). Interestingly, the phenotype of strain GBH6.6/pJZMS resembles the phenotype of the *H. influenzae sxy-1* mutant strain isolated by Redfield (32).

Table 1. Transformation of *H. influenzae* strains with chromosomal DNA

Strain	Selected antibiotic resistance	Transformation frequency* Nov^r	Nal^r
KW20	Nov^r or Nal^r	2.3×10^{-2}	1.9×10^{-2}
GBH6.6	Nov^r or Nal^r	1.2×10^{-8}	4.3×10^{-8}
GBH6.6/pHKRec	Nov^r	4.0×10^{-3}	
GBH6.6/pJZMS	Nov^r or Nal^r	2.0×10^{-3}	2.0×10^{-3}
GBH6.6/pJZMC	Nov^r	$<10^{-8}$	
GBH6.6/pGJB103	Nov^r	$<10^{-7}$	

*The transformation frequency is defined as the number of antibiotic-resistant colonies divided by the total number of viable cell colonies. The data are the average values from two experiments performed in duplicate.

Table 2. DNA binding and internalization by *H. influenzae* cells after incubation in MIV medium

Strain	Cell-associated DNA,* cpm: No DNase treatment	DNase treatment
KW20	39,985	38,456
GBH6.6	122	40

*A ^{35}S-labeled DNA sample (≈0.4 μg; 7.8×10^5 cpm/μg) was added to 0.5 ml of cells passaged through MIV competence-inducing medium and processed as described (12). The values are the average of two samples; 23 cpm background was subtracted from each sample.

Identification of the *tfoX*$^+$-Encoded Polypeptide(s). Plasmid pJZSSX2 contains the *tfoX*$^+$ gene oriented relative to the T7 promoter so that transcription of the antisense strand occurs. This plasmid encoded three polypeptides that corresponded to the precursor and mature forms of β-lactamase, and the *lacZ*-encoded α fragment (Fig. 3, lane 1). When *tfoX*$^+$ was cloned in the opposite orientation, as in plasmid pJZSSX1.2, two new polypeptides with gel-estimated molecular masses of 24.3 and 20.8 kDa were produced (Fig. 3, lane 3). While the synthesis of a 24.3-kDa polypeptide is in good agreement with the predicted size of TfoX deduced from the DNA sequence (24.9 kDa), the presence of a 20.8-kDa polypeptide was unexpected. While we cannot rule out the possibility that the smaller polypeptide is a product of TfoX proteolysis, closer inspection of the *tfoX*$^+$ DNA sequence revealed the presence of a second potential translation initiation site in the same reading frame as the full-length TfoX polypeptide. This shorter polypeptide, which we named $TfoX_s$, has a predicted size of 20.6 kDa (see Fig. 1). Both of these polypeptides were undetectable when IPTG was omitted from the culture medium (Fig. 3, lane 2). Furthermore, each of the *tfoX* C-terminal deletion plasmids pX1NE and pX1CE also encoded two polypeptides with molecular masses of 23.0 and 19.3 kDa and 19.9 and 15.0 kDa, respectively (Fig. 3, lanes 4 and 5). The sizes of these polypeptides are consistent with versions of TfoX and

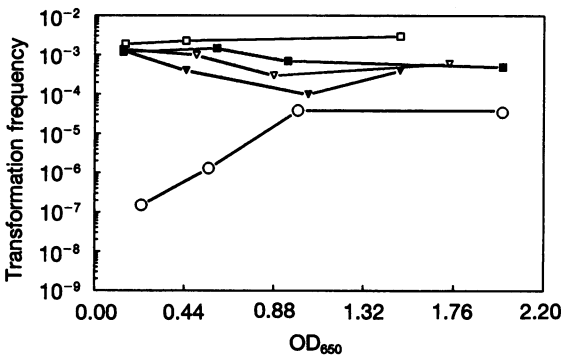

FIG. 2. Transformability of *H. influenzae* strains growing in rich medium. Logarithmic-phase cells were diluted to OD_{650} = 0.05 in sBHI medium (15) supplemented with ampicillin as appropriate. After 40 min MAP7 chromosomal DNA was added to each culture at 5 μg/ml. Simultaneously, cAMP was added to cultures as indicated at a final concentration of 1 mM. At various times cell samples were placed on ice in tubes containing 60,000 units of DNase I for at least 30 min before dilution and plating in duplicate on appropriate agar medium. For plasmid strains, data from two representative experiments are shown. For reference, the response of strain KW20 is also shown. ■ and □, GBH6.6/pJZMS, no cAMP; ▼ and ▽, GBH6.6/pJZMS, cAMP; ○, KW20.

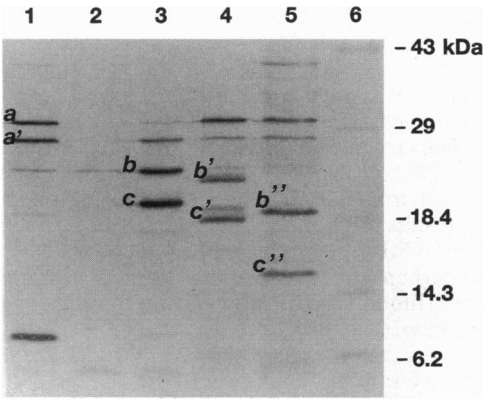

FIG. 3. Isopropyl β-D-thiogalactoside (IPTG)-induced polypeptides encoded by *E. coli* BL21DE3*pcnB* containing various expression plasmids. Proteins were labeled *in vivo* with [^{35}S]methionine. Lanes: 1, pJZSSX2 (*tfoX*$^-$); 2, pJZSSX1.2 (*tfoX*$^+$, no IPTG); 3, pJZSSX1.2 (*tfoX*$^+$); 4, pX1NE; 5, pX1CE; 6, ^{14}C-labeled protein standards. Labeled polypeptides: a, pre-β-lactamase; a', mature β-lactamase; b, TfoX; b' and b'', C-terminal deletion forms of TfoX; c, TfoX$_S$; c' and c'', C-terminal deletion forms of TfoX$_S$. The 9.5-kDa band in lane 1 is likely LacZ α fragment.

TfoX$_s$ that resulted from in-frame fusions between *tfoX* and vector *lacZα* sequences produced during the preparation of the C-terminal deletions.

Transcription of *H. influenzae com101A*$^+$ Is Modulated by TfoX. First we investigated the pattern of *tfoX*$^+$ and *com101A*$^+$ mRNA synthesis in strain KW20 by employing a primer extension assay (Fig. 4*A*). Our results show that upon brief exposure to MIV competence-inducing medium levels of *tfoX*$^+$ mRNA increased about 3- to 4-fold (as determined by densitometry of the autoradiogram). Subsequently, *tfoX*$^+$ mRNA levels steadily declined. As shown previously by Larson and Goodgal (7), *com101A*$^+$ mRNA was temporally regulated, peaking at the 50-min point of competence development.

To determine what effect the loss of TfoX activity might have on *com101A*$^+$ expression, total RNA was prepared from strain GBH6.6. We failed to detect *com101A*$^+$-specific transcripts at any time (Fig. 4*B*, lanes 1–5), even after the exposure time of the autoradiogram was increased 4-fold. To eliminate the possibility of a primer defect, a parallel reaction mixture containing the same labeled *com101A*$^+$ primer was annealed with RNA harvested from strain KW20. The specific *com101A*$^+$ extension products were observed (Fig. 4*B*, lane 6). To eliminate the possibility that the GBH6.6 RNA preparation was defective, reaction mixtures containing the *tfoX*$^+$ primer and RNA isolated from strain GBH6.6 were analyzed. Only the *tfoX* primer extension product was detected when both primers A and C were annealed with RNA prepared from strain GBH6.6 (Fig. 4*B*, lanes 7 and 8). When the same labeled primers were used with strain KW20 RNA (Fig. 4*B*, lane 9) both *tfoX*$^+$ and *com101A*$^+$ extension products were observed. We conclude that TfoX is required for proper expression of *com101A*$^+$ mRNA.

A *tfoX::lacZ* Transcriptional Fusion Is Regulated by cAMP and CRP. We determined the effect of cAMP addition and a *crp*$^+$ gene mutation upon the expression of *tfoX* by using two *tfoX::lacZ* operon fusion strains. As shown in Fig. 5, strain GBH2.1 showed an approximately 3-fold induction in β-galactosidase activity 30 min after the addition of cAMP. At this time, the transformability of strain GBH2.1 was determined to be 100-fold higher than a parallel culture lacking cAMP (data not shown). Interestingly, the same increase in β-galactosidase activity was observed for strain GBH2.1 after 180-min incubation, as the cells entered stationary phase of growth, even in the absence of exogenously added cAMP. cAMP was unable to increase the level of β-galactosidase activity in the *crp* strain, GBH21.12. Furthermore, as this strain reached stationary phase, levels of β-galactosidase failed to rise, supporting the idea that the cAMP effect is CRP mediated.

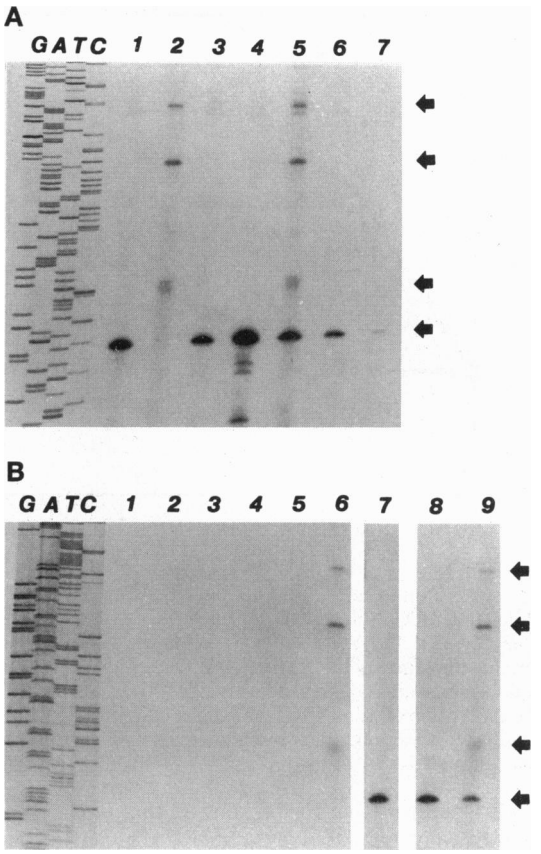

FIG. 4. Primer extension analysis of *in vivo tfoX* and *com101A* transcripts in noncompetent and competent *H. influenzae* strains. DNA sequencing ladders (G, A, T, C; generated with, e.g., M13mp18 and "−40" 17-mer *lac* primer; *A*) were used as a size standards for extension products. (*A*) Primer extension products obtained from noncompetent and competent *H. influenzae* strain KW20. Total RNA was isolated either immediately before cell exposure to MIV competence-inducing medium (noncompetent cell sample; NC) or after cell resuspension (t = 0) and incubation in MIV medium for various times (0–150 min). RNA samples were annealed with primer A, primer C, or a mixture of both primers and elongated with AMVRT. Lanes: 1, *tfoX* primer A, 50-min MIV incubation; 2, *com101A* primer C, 50-min MIV incubation; 3–7, primers A and C, NC, 0-, 50-, 100-, and 150-min MIV incubation. (*B*) Primer extension products obtained from strain GBH6.6 before and during passage in MIV medium. Lanes: 1–5, primer C, NC, 0-, 50-, 100-, and 150-min MIV incubation; 6, primer C used in lanes 1–5 plus RNA prepared from KW20 at 50-min MIV incubation; 7 and 8, primers A and C, 50- and 150-min MIV incubation; 9, primers A and C, KW20 RNA, 50-min MIV incubation. The locations of the 108-nt *tfoX* and 122- to 124-, 162-, and 186-nt *com101A* extension products are marked.

DISCUSSION

We have identified a transformation gene, *tfoX*$^+$, that is essential for competence development and the expression of the late competence-specific gene, *com101A*$^+$, in *H. influenzae*.

Though we were able to identify a candidate σ^{70}-dependent promoter, an atypical feature of this promoter is that transcription initiates 22 bp downstream from the Pribnow box. This suggested to us that *tfoX*$^+$ might require additional factors for optimal expression. Consistent with this idea is our identification of two potential CRP-binding sites in the DNA segment near the *tfoX*$^+$ transcription initiation site (Fig. 1). Furthermore, studies with strains carrying both *tfoX*$^+$ and a

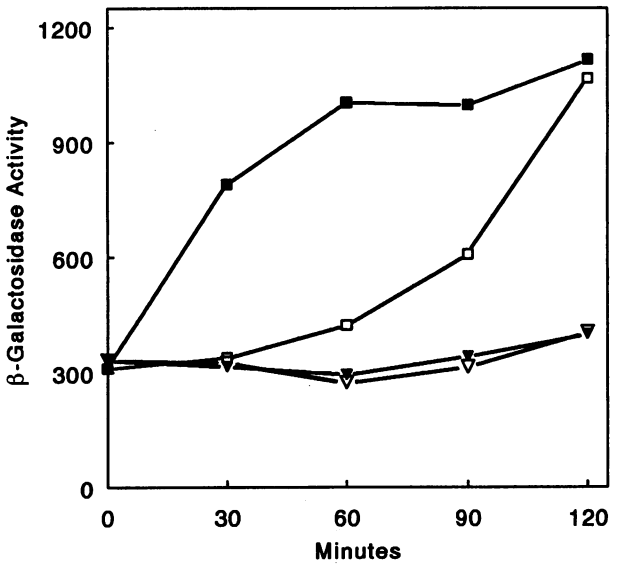

FIG. 5. cAMP-induced transcription of a *tfoX::lacZ* operon fusion is CRP dependent. Cultures of *H. influenzae strains* GBH2.1 (crp$^+$, □ and ■) and GBH21.12 (crp, ▽ and ▼) were grown in sBHI broth with appropriate supplements to OD_{650} = 0.2. The cultures were split in half, and to one sample of each culture cAMP was added (■ and ▼) to 1 mM final concentration. Two samples of each culture were taken at the indicated times and each sample was assayed in duplicate for β-galactosidase activity.

tfoX::lacZ transcriptional fusion showed that *tfoX*$^+$ transcription is positively regulated by cAMP in a CRP-dependent manner (Fig. 5). Although Chandler (4) and Dorocicz *et al.* (5) have demonstrated a requirement for CRP and adenylate cyclase, respectively, for competence development in *H. influenzae*, *tfoX*$^+$ is, to the best of our knowledge, the first transformation gene for which there is direct evidence for transcriptional modulation by cAMP and CRP.

The production of two forms of TfoX in *H. influenzae* could be used by the cell as a mechanism to regulate activity, especially since *tfoX*$^+$ mRNA can be detected in noncompetent cells (Fig. 4*A*). Interestingly, Kofoid and Parkinson (33) have demonstrated that the *E. coli* chemotactic signal transduction gene *cheA*$^+$ encodes two functional polypeptides ($CheA_L$ and $CheA_S$) that differ at their N termini by 97 amino acid residues.

The *tfoX* mutant (GBH6.6) is defective for DNA binding, uptake, and transformation. The strain was also tested for resistance to DNA-damaging agents such as 4-nitroquinoline-1-oxide and UV irradiation and found to be wild type (data not shown). Thus it is likely that general recombination and DNA repair functions are intact and are not responsible for the mutant phenotype.

On the basis of our data (Fig. 4), *tfoX*$^+$ is an early competence gene whose product affects the expression of at least one late competence gene, *com101A*$^+$. We suspect that the expression of other competence genes is also affected, because electron micrographic studies of wild-type and *tfoX* cell lines revealed that *tfoX* cells could not make transformasomes (data not shown). We speculate that the overexpression of *tfoX*$^+$ is responsible for the constitutive competence and supertransformer phenotype we observed in *tfoX*$^+$ plasmid strains. We infer from this that either the amount or the activity of TfoX is limiting for competence development and is tightly regulated by the noncompetent cell. We do not know whether TfoX is acting directly as a positive regulator of gene expression (e.g., as a transcription factor) or indirectly by titrating/inactivating a competence repressor. Alternatively, TfoX might be an RNA-binding protein that is required for the stabilization of competence-specific mRNAs.

Our identification and characterization of *tfoX*$^+$ allows verification of the existence of both early and late competence genes in *H. influenzae*. Similar observations have been made in *Bacillus subtilis* (reviewed in ref. 34). We propose an elementary model for competence development in *H. influenzae*: a response to environmental change (e.g., anaerobiosis, nutrient depletion) results in a transient increase in intracellular cAMP levels. The cAMP–CRP complex stimulates transcription of *tfoX*$^+$ and perhaps other early competence genes. TfoX (and other factors) modulates the expression of the late competence genes (e.g., *com101A*$^+$). In the presence of Por (6) (and other proteins), the transformasome complex is assembled and cells become transformable. Refinements of the model will be possible as we learn more about the biochemistry of TfoX.

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