Chlamydia trachomatis protein GrgA activates transcription by contacting the nonconserved region of σ^{66}

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The bacterial RNA polymerase holoenzyme consists of a catalytic core enzyme in complex with a σ factor that is required for promoter-specific transcription initiation. Primary, or housekeeping, σ factors are responsible for most of the gene expression that occurs during the exponential phase of growth. Primary σ factors share four regions of conserved sequence, regions 1–4, which have been further subdivided. Many primary σ factors also contain a nonconserved region (NCR) located between subregions 1.2 and 2.1, which can vary widely in length. Interactions between the NCR of the primary σ factor of *Escherichia coli*, σ^{70}, and the β' subunit of the *E. coli* core enzyme have been shown to influence gene expression, suggesting that the NCR of primary σ factors represents a potential target for transcription regulation. Here, we report the identification and characterization of a previously undocumented *Chlamydia trachomatis* transcription factor, designated GrgA (general regulator of genes A). We demonstrate in vitro that GrgA is a DNA-binding protein that can stimulate transcription from a range of σ^{66}-dependent promoters. We further show that GrgA activates transcription by contacting the NCR of the primary σ factor of *C. trachomatis*, σ^{66}. Our findings suggest GrgA serves as an important regulator of σ^{66}-dependent transcription in *C. trachomatis*. Furthermore, because GrgA is present only in chlamydiae, our findings highlight how nonconserved regions of the bacterial RNA polymerase can be targets of regulatory factors that are unique to particular organisms.

Chlamydiae are obligate intracellular bacterial parasites, and their hosts range from single cellular eukaryotes to humans (1). In humans, *Chlamydia trachomatis* is the most common sexually transmitted bacterial pathogen (2). *C. trachomatis* is known for its ability to efficiently ascend from the lower genital tract to the upper genital tract, where chlamydial replication can have many devastating consequences, including infertility and pelvic inflammatory disease in women. In addition, some *C. trachomatis* serovars cause eye infection, which is still a major cause of blindness in underdeveloped countries (2).

Chlamydiae have a unique developmental cycle with two alternating cellular forms. The metabolically inactive, infectious elementary body (EB) enters a vacuole through host cell endocytosis (3). Inside the vacuole (termed inclusion), the EB develops into the proliferating but noninfectious reticulate body (RB). As RBs accumulate, they reorganize, in an asynchronous manner, back to EBs, which exit the host cell at the end of the developmental cycle (4). Whereas a typical developmental cycle ranges from 2 to 4 d, the infection may enter a latent state, which is characterized by accumulation of aberrant reticulate bodies and a lack of EB production (5).

The *C. trachomatis* genome is ~1 Mb in size and encodes ~1,000 genes (6, 7). Genome-wide microarray analyses have revealed that ~80% of all genes are expressed a few hours after infection through the remaining developmental cycle. For the remaining genes, some are expressed immediately after chlamydial entry into the cell, and others are not transcribed until a middle or late stage. (8, 9). Furthermore, certain gene transcripts exhibit increases or decreases in abundance as chlamydiae

enter latency (10). These observations suggest that alterations in gene expression manifest at the level of transcription contribute to the development of latency. Nevertheless, due to the limited number of genetic tools available in chlamydiae, only a few transcription regulators have been identified.

Here we describe the identification and characterization of a previously undocumented transcription factor that we call GrgA (general regulator of genes A). GrgA was identified based upon its ability to activate transcription in vitro from the promoter that controls the expression of *def*A, which encodes the peptide deformylase (PDF). We demonstrate that efficient transcription activation of the *def*A promoter by GrgA in vitro requires contact with both DNA and a portion of a nonconserved region (NCR) of the primary σ factor of *C. trachomatis*, σ^{66}. We further show that GrgA can stimulate σ^{66}-dependent transcription in vitro of three other genes that are expressed in vivo during different stages of the chlamydial developmental cycle. Our findings suggest that GrgA is not only a regulator of *def*A expression in vivo, but also a general transcription activator of many σ^{66}-dependent genes. Furthermore, because GrgA is present only in chlamydiae, our findings highlight how regulatory factors that are unique to particular organisms can target nonconserved regions of the bacterial RNA polymerase (RNAP).

Results

Identification of GrgA as a *def*A Promoter-Binding Protein with Transcription Activation Activity. PDF catalyzes the removal of the N-formyl group from the leading methionine of newly synthesized proteins. Most bacterial proteins require the deformylation and subsequent removal of the N-terminal methionine to function properly. Furthermore, even for the small proportion of proteins that can function while carrying the N-formyl methionine, deformylation is necessary for the initiation of regulated degradation (11). Thus, PDF is a potential therapeutic and/or prophylactic target for infectious diseases. Accordingly, small inhibitors of PDF have shown effectiveness against a variety of pathogens, including chlamydiae both in vitro and in vivo (12, 13).

Our goal was to identify additional chlamydial proteins that might serve as potential therapeutic targets. Given that small molecules targeting PDF inhibit chlamydial growth, we sought to identify factors involved in the expression of *def*A, which might themselves serve as potential targets. To accomplish this goal, we used a DNA pull-down assay to identify chlamydial proteins that bound to a DNA fragment containing the *def*A promoter. To do this, we mixed cell extracts isolated from *C. trachomatis*-infected

mouse L cells with streptavidin beads attached to either a biotinylated DNA fragment that carried sequences extending from position −144 to +52 of the *def*A promoter (pdf_long) or a biotinylated DNA fragment that carried sequences from position −54 to +52 (pdf_short). We then analyzed proteins associated with either the beads bound to the pdf_long fragment or the beads bound to the pdf_short fragment by liquid chromatography (LC)–MS/MS. From this analysis we identified *C. trachomatis* proteins with no previously assigned function that were enriched when the pull-down assay was performed with the pdf_long fragment compared with the pdf_short fragment.

We focused our attention on the *Chlamydia*-specific hypothetical protein that showed the highest enrichment in binding the pdf_long fragment compared with the pdf_short. This protein was encoded by an ORF designated CTL0766 in the genome of *C. trachomatis* L2. BLAST analysis did not detect significant homology between the CTL0766 protein and any nonchlamydial proteins, and no bacterial protein motif was identified in a motif search. We expressed and purified a His-tagged derivative of CTL0766 in *Escherichia coli* (Table S1 and Fig. S1 *A and B*) and tested its potential role in the regulation of *def*A gene transcription using an in vitro transcription assay (14) (Fig. 1 and Fig. S2). Addition of CTL0766 stimulated transcription of the *def*A promoter by chlamydial RNA polymerase (cRNAP) in a dose-dependent manner from the wild-type promoter (Fig. 1*A*) as well as two mutant derivatives (Z100 and GR10) (14), which contain single base-pair substitutions that increase basal transcription (Fig. 1 *B* and *C* and Fig. S2). These results demonstrate that the gene product of CTL0766 can activate transcription of the *def*A gene in vitro. As shown below, CTL0766 also acts as an activator of three other chlamydial genes tested. Accordingly, we rename the *C. trachomatis* gene CTL0766 *grg*A (general regulator of genes A).

GrgA Binds to DNA in a Sequence-Nonspecific Manner. To characterize the nature of the interaction between GrgA and the *def*A promoter, we first performed an EMSA using purified GrgA and a ^{32}P-labeled promoter fragment extending from position −144 to +52 of the *def*A promoter. Surprisingly, initial experiments detected no clear shifted band (Fig. S3*A*), even though the same sequence of the *def*A promoter was used for initial pull-down

experiment that led to the identification of GrgA (see above). We reasoned that the inclusion of poly(deoxyinosinic-deoxycytidylic) acid [poly(dI-dC)] in the reaction might have prevented GrgA from binding the *def*A promoter fragment. Consistent with this hypothesis, dose-dependent retardation of the *def*A promoter fragment by GrgA was readily detectable in the absence of poly (dI-dC) (Fig. 2*A*). However, GrgA-bound DNA did not migrate as a clear band in the resolving gel. At lower concentrations (0.2 and 0.3 μM) of GrgA the DNA appeared as smear in the gel, whereas at higher concentrations of GrgA (≥0.6 μM) the protein–DNA complex largely or completely remained in the loading well (Fig. 2*A*). We further performed GrgA pull-down assays to determine what effect shortening the DNA fragment carrying the *def*A promoter had on the binding of GrgA. Accordingly, we generated biotinylated *def*A promoter fragments of different lengths (Fig. 2*B*) that were immobilized to streptavidin-conjugated agarose beads and determined the amount of GrgA that precipitated with equimolar amounts of each DNA fragment. Progressive removal from the 5′-end of the promoter resulted in a steady decrease in the amount of GrgA that was precipitated (Fig. 2 *C* and *D*), revealing a clear correlation between the length of the promoter DNA fragment and the amount of precipitated GrgA. These findings, which are consistent with the enrichment of GrgA association with the pdf_long fragment compared with the pdf_short fragment as detected by MS (see above), suggest that GrgA does not bind preferentially to *def*A promoter sequences, but rather binds DNA in a sequence-nonspecific manner. Consistent with this hypothesis, replacement of the sequences extending from −144 to +5 of the *def*A promoter fragment with unrelated DNA sequence had no effect on the amount of GrgA precipitated (Fig. S3 *B* and *C*). Furthermore, we found that GrgA could bind to DNA located in the 5′-untranslated region of the *def*A gene and to two fragments containing portions of the GrgA ORF (Fig. S3 *D* and *E*). Taken together, data presented in Fig. 2 and Fig. S3 indicate that GrgA binds DNA in a sequence-nonspecific manner.

GrgA-Dependent Transcription Activation Requires DNA Binding. We next pursued the identification of amino acid residues in GrgA that were required for sequence-nonspecific DNA binding. We constructed a series of GrgA mutants with deletions of 50–82 amino acids (Table S1 and Fig. S4 *A and B*) and assessed these mutants' abilities to associate with *def*A promoter DNA using both a pull-down assay (Fig. S4*C*) and an EMSA assay (Fig. 3*A*). GrgA mutants lacking amino acids 1–64 (Δ1–64), 65–113 (Δ65–113), 166–206 (Δ166–206), or 207–288 (Δ207–288) all exhibited DNA binding activities that were identical to that of the wild-type GrgA protein. In contrast, the GrgA mutant lacking amino acid residues 114–165 (Δ114–165) was severely compromised in its ability to bind DNA (Fig. 3*A* and Fig. S4*C*), suggesting that residues 114–165 comprise a portion of GrgA that is essential for sequence-nonspecific DNA binding. Noticeably, this region is rich in positively charged lysine and arginine residues (Fig. S4*D*), which may mediate the binding of GrgA to the negatively charged DNA. Furthermore, the lysine/arginine-rich sequence and a following region appeared to form a helix-turn-helix (Fig. S4*D*), a structural motif characteristic of DNA binding proteins.

We next assessed the effects of the various deletions in GrgA on the ability of GrgA to activate transcription from the *def*A promoter. Transcription activation was essentially that of the wild-type GrgA for the Δ65–113, Δ166–206, and Δ207–288 mutants. In contrast, the Δ114–165 mutant, which could not bind DNA, suffered a significant (~75%) loss of transcription activation activity compared with wild-type GrgA (Fig. 3*B*), suggesting that the ability of GrgA to bind DNA is important for GrgA-dependent transcription activation. In addition, the Δ1–64 mutant, which retained a strong DNA binding activity (Fig. 3*A* and Fig. S4*C*), also displayed a significant (~65%) loss of transcription activation activity (Fig. 3*B*). These results indicate that
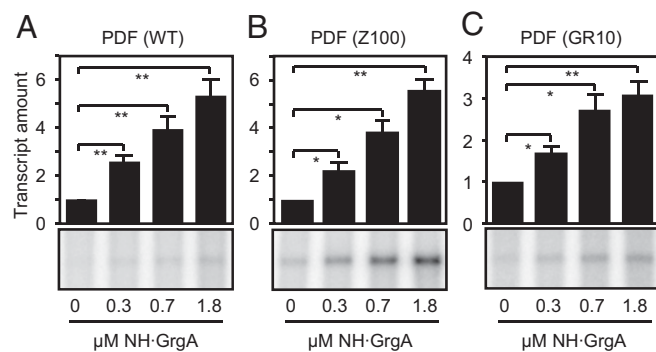

Fig. 1. GrgA stimulates transcription of the *def*A promoter in vitro. Shown are in vitro transcription assays performed using cRNAP, a DNA template carrying the indicated *def*A promoter variant, and the indicated concentration of NH·GrgA. The Z100 promoter derivative (used in *B*) carries a base pair substitution in the promoter −35 element, whereas the GR10 derivative (used in *C*) carries a substitution in DNA upstream of the −35 element (14). Graphs show the averages and SDs for three independent measurements. We note that C-terminally His-tagged GrgA also demonstrated a dose-dependent stimulatory effect on *def*A promoter activity (Fig. S2). Single and double asterisks denote that the difference between control and GrgA-containing reactions were statistically significant ($P < 0.05$ and $P < 0.01$, respectively).
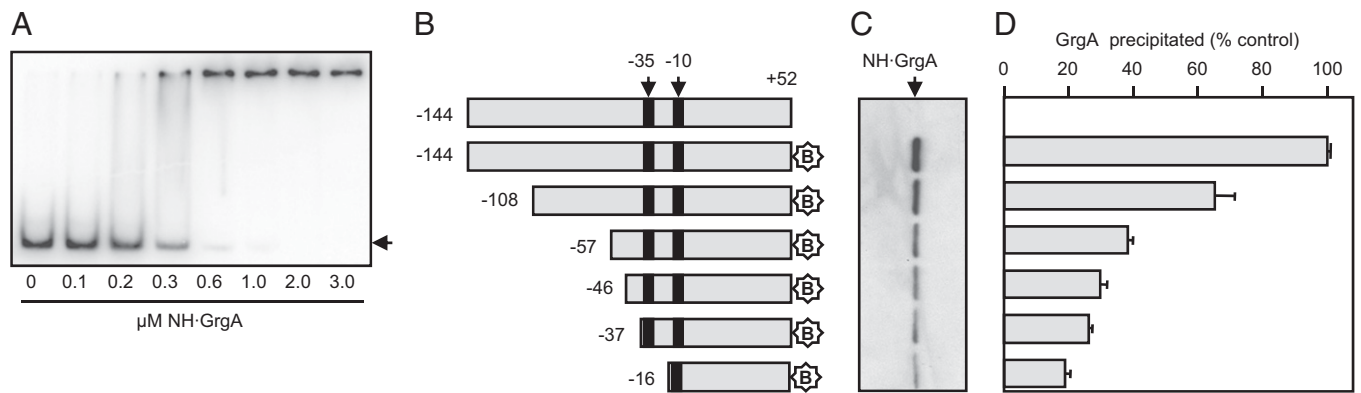
Fig. 2. GrgA binds DNA. (*A*) EMSA assays performed in the absence of poly(dI-dC) using a DNA fragment carrying sequences extending from position −144 to +52 of the *def*A promoter. (*B*) Schematic of the DNA fragments used to precipitate NH·GrgA (star indicates the presence of a 3′ biotin moiety). (*C*) Western blot analysis of the amount of NH·GrgA precipitated by the corresponding DNA fragments in *B*. (*D*) Graph shows the amount of GrgA precipitated as a percentage of that precipitated by the biotinylated −144 to +52 fragment. Plotted are the averages and SDs for three independent measurements.

DNA binding is necessary but not sufficient for GrgA to efficiently activate transcription.

GrgA-Dependent Transcription Activation Requires Interaction with the Nonconserved Region of σ⁶⁶. We have previously demonstrated that a hybrid RNAP holoenzyme consisting of the *E. coli* RNAP core enzyme and chlamydial σ^{66} can transcribe from the *def*A promoter (14). We therefore tested whether or not GrgA could activate transcription of the *def*A promoter in reactions performed using this hybrid holoenzyme. We found that addition of GrgA to transcription reactions performed using the hybrid holoenzyme stimulated transcription (Fig. S5*A*) in a manner similar to the stimulatory effect observed in reactions performed using cRNAP (Fig. 1 and Fig. S2). In contrast, GrgA was unable to activate transcription in reactions performed with *E. coli* RNAP core enzyme reconstituted with *E. coli* σ^{70} (Fig. S5*B*) or *C. trachomatis* σ^{28} (Fig. S5*C*). These findings raised the possibility that GrgA may stimulate transcription through direct interaction with σ^{66}. Consistent with this hypothesis, Strep-Tactin–immobilized, C-terminally Strep-tagged σ^{66} (CS·σ^{66}) was able to precipitate GrgA (Fig. 4*A*); furthermore, N-terminally Strep-tagged GrgA (NS·GrgA) also precipitated C-terminally His-tagged σ^{66} (CH·σ^{66}; Fig. 4*B*).

We next used the GrgA mutants constructed for mapping the DNA binding region (Fig. S4*A*) to determine what residues of GrgA were required for the interaction with σ^{66}. The Δ65–113, Δ114–165, Δ166–206, and Δ207–288 GrgA mutants all retained the ability to interact with σ^{66} (Fig. 4*C*). Importantly, the finding that the Δ114–165 mutant retained the ability to bind σ^{66} indicates that this mutant is stable, providing further support that the reduction in transcription activation observed with this mutant (Fig. 3*B*) is a consequence of its inability to bind DNA. In contrast to the other GrgA mutants, the Δ1–64 mutant, which could efficiently bind DNA (Fig. 3*A* and Fig. S4*C*), did not detectably interact with σ^{66} (Fig. 4*C*). This finding suggests that the inability of the Δ1–64 mutant to efficiently stimulate transcription from the *def*A promoter (Fig. 3*B*) is due to the inability of the mutant to contact σ^{66}.

To further explore the hypothesis that GrgA-dependent transcription activation requires contact with σ^{66}, we sought to identify regions of σ^{66} that were required for the interaction with GrgA. Primary σ factors share four regions of conserved sequence, regions 1–4 (15–18). Furthermore, many primary σ factors carry a NCR between regions 1 and 2 that can widely vary in sequence and length (15–17). We therefore constructed individual *C. trachomatis* σ^{66} fragments (Table S1 and Fig. S6*A*) that comprised σ
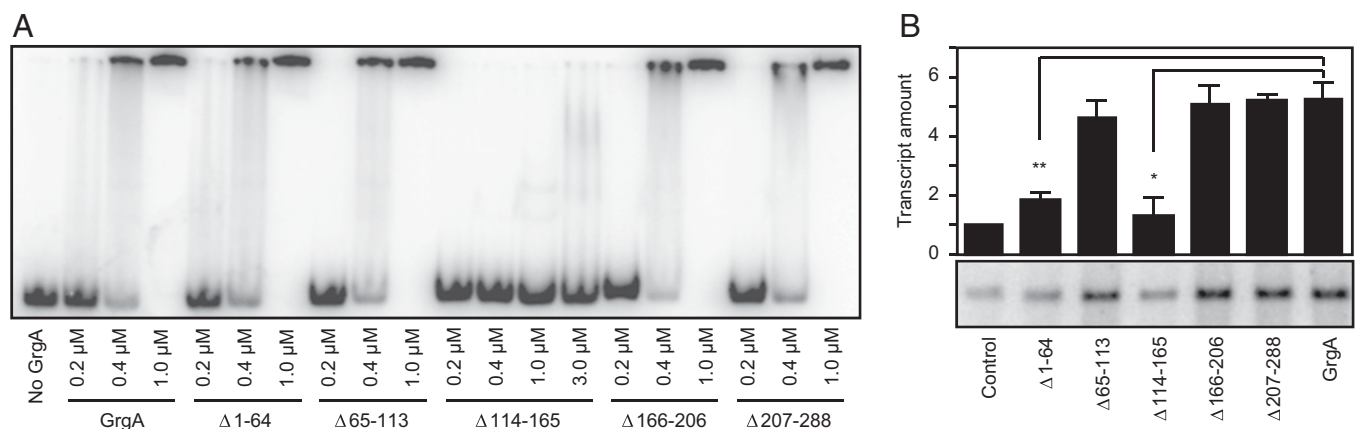

Fig. 3. DNA binding by GrgA is essential but not sufficient for full transcription activation. (*A*) EMSA assays performed using a radiolabeled DNA fragment carrying sequences extending from position −144 to +52 of the *def*A promoter in the presence of the indicated concentrations of wild-type GrgA or the indicated GrgA mutant. (*B*) In vitro transcription assays performed in the presence or absence of 1.8 μM wild-type GrgA or the indicated GrgA mutant. Assays were performed using cRNAP and a DNA template carrying the Z100 *def*A promoter variant. Graph shows the averages and SDs for three independent measurements.
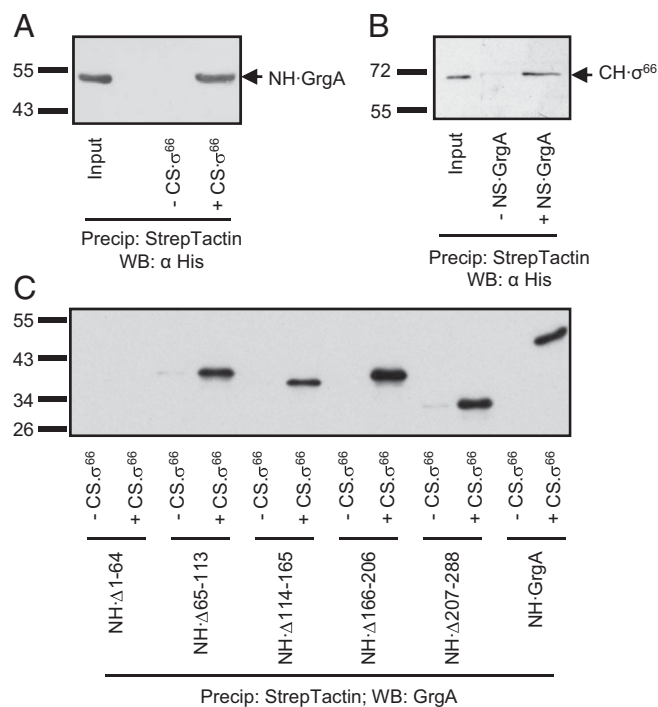
Fig. 4. GrgA binds σ⁶⁶. (A) Precipitation of NH·GrgA by Strep-Tactin–immobilized CS·σ⁶⁶. Shown is a Western blot detecting His-tagged GrgA. (B) Precipitation of CH·σ⁶⁶ by NS·GrgA. Shown is a Western blot detecting σ⁶⁶. (C) Precipitation of wild-type GrgA or the indicated mutant GrgA derivatives by Strep-Tactin–immobilized CS·σ⁶⁶. Shown is a Western blot detecting GrgA variants. Anti-GrgA instead of anti-His was used for detection because the anti-His recognized GrgA variants with greatly varying efficiency, as demonstrated in Fig. S8.

region 1 (residues 1–146), region 2 (residues 303–408), region 3 (residues 386–490), region 4 (residues 473–571), or the NCR (residues 121–322), and attempted to express them in *E. coli*. All of the fragments except the one comprising region 3 were successfully expressed and purified (Fig. S6B). We next determined whether or not we could precipitate these fragments with GrgA, and found that only the fragment encompassing the NCR could precipitate GrgA (Fig. 5A), suggesting that GrgA contacts the σ⁶⁶ NCR.

To further define the residues of the NCR that were important for GrgA binding, we constructed a series of σ⁶⁶ mutants that each contained 40–52 amino acid deletions in the NCR (ΔNCR1, which lacked residues 132–183; ΔNCR2, which lacked residues 184–224; ΔNCR3, which lacked residues 224–268; and ΔNCR4, which lacked residues 269–316; Fig. S6 C and D). Among these mutants, only ΔNCR4 lost the ability to bind to GrgA (Fig. 5B), indicating that residues 269–316 of the NCR of σ⁶⁶ are required for interaction with GrgA.

We next tested the effect of deleting residues 269–316 of σ⁶⁶ on the ability of GrgA to activate transcription of the *def*A promoter. To do this, we performed in vitro transcription assays using a hybrid RNAP holoenzyme consisting of the *E. coli* RNAP core enzyme and either wild-type or mutant σ⁶⁶. Removal of residues 269–316 in σ⁶⁶ had no effect on basal transcription but severely reduced the ability of GrgA to stimulate transcription (Fig. 5C). In contrast, removal of other NCR residues that did not affect the ability of GrgA to bind σ⁶⁶ did not significantly affect GrgA-dependent activation (Fig. 5C). Thus, disrupting the interaction between GrgA and the NCR of σ⁶⁶ by either removing residues 1–64 of GrgA or residues 269–316 of σ⁶⁶ severely impairs GrgA-dependent transcription activation (Figs. 3, 4, and 5C). Taken together, these results establish that the interaction between GrgA

and the NCR of σ⁶⁶ is required for efficient transcription activation by GrgA.

GrgA Is a General Activator of σ⁶⁶-Dependent Genes in Vitro. Given that GrgA bound DNA in a sequence-nonspecific manner and could activate transcription from the *def*A promoter, we next determined if the transcription activation activity of GrgA was limited to the *def*A gene. Accordingly, we assessed the effects of GrgA in vitro on the activities of three additional σ⁶⁶-dependent promoters that are active at different stages of growth: ribosomal RNA promoter P1 (rRNA P1; early), major outer membrane protein A (*ompA*; middle), and histone-like protein A (*hctA*; late) (8, 9, 19, 20). GrgA demonstrated significant stimulatory effects on all of the three promoters (Fig. 6). Furthermore, both the Δ1–64 GrgA mutant (that does not interact with σ⁶⁶) and the Δ114–165 GrgA mutant (that does not bind DNA) exhibited significantly reduced levels of activation at each promoter compared with that observed with wild-type GrgA (Fig. 6). Thus, the
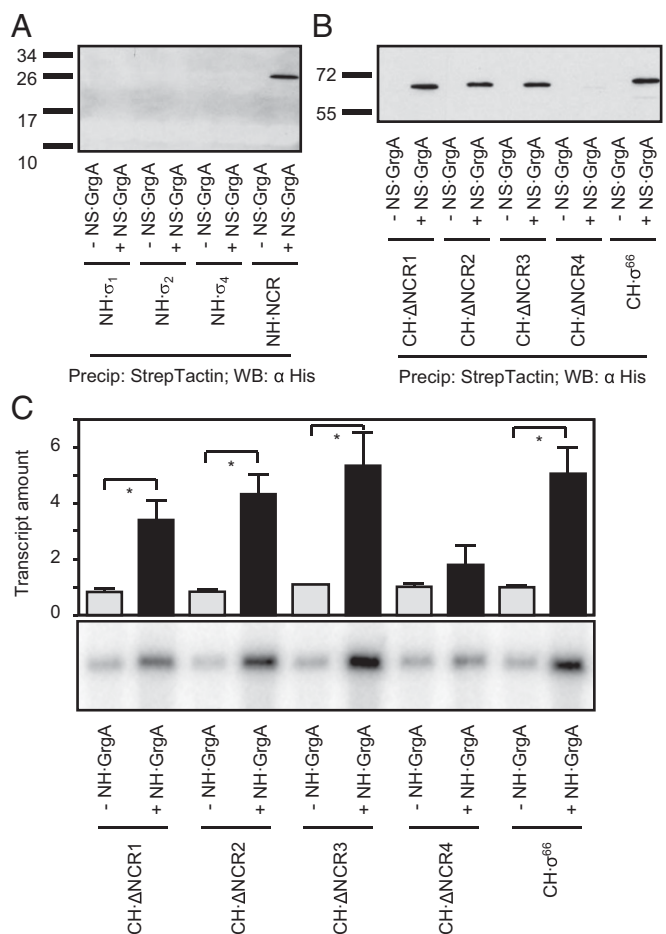

Fig. 5. Interaction between GrgA and the nonconserved region of σ⁶⁶ is required for efficient transcription activation. (A) Precipitation of His-tagged fragments of σ⁶⁶ by Strep-Tactin–immobilized GrgA (NS·GrgA). Shown is a Western blot detecting the His-tagged σ⁶⁶ fragments recovered after precipitation. (B) Precipitation of His-tagged derivatives of σ⁶⁶ by Strep-Tactin–immobilized GrgA (NS·GrgA). Shown is a Western blot detecting the His-tagged σ⁶⁶ derivatives recovered after precipitation. ΔNCR1 lacks residues 132–183; ΔNCR2 lacks residues 184–223; ΔNCR3, lacks residues 224–268; and ΔNCR4 lacks residues 270–316 (Fig. S6). (C) In vitro transcription assays performed in the presence or absence of 1.8 μM wild-type GrgA using a hybrid holoenzyme consisting of *E. coli* core and the indicated σ⁶⁶ derivative. The DNA template used for these assays was the Z100 *def*A promoter variant. Graph shows the averages and SDs for three independent measurements.
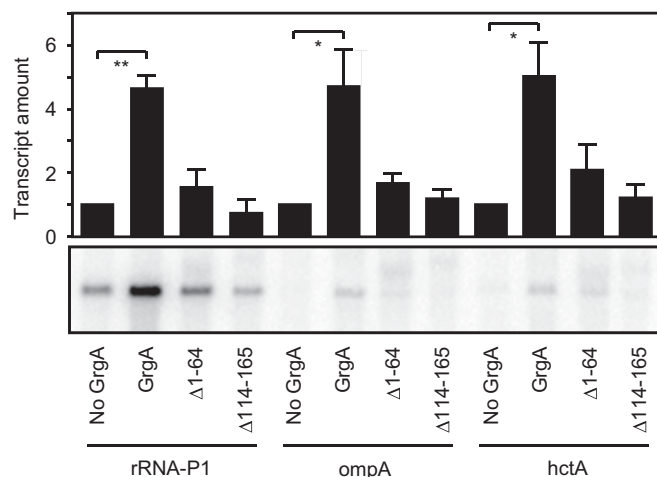
Fig. 6. GrgA is a general activator of σ^{66}-dependent transcription in vitro. In vitro transcription assays performed in the presence or absence of 1.8 μM wild-type GrgA or the indicated GrgA mutant. Assays were done using cRNAP and a DNA template carrying the indicated promoter. Graph shows the averages and SDs for three independent measurements.

GrgA-dependent activation observed in the context of the rRNA P1, *ompA*, and *hctA* promoters, like that observed in the context of the *def*A promoter, requires GrgA to contact the DNA and the NCR of σ^{66}.

Discussion

Here we identify a transcription factor from *C. trachomatis*, GrgA, which activates transcription of several σ^{66}-dependent promoters in vitro by binding DNA and contacting the σ^{66} NCR. These findings strongly suggest GrgA serves as an important regulator of σ^{66}-dependent transcription in *C. trachomatis*.

GrgA Is a General Regulator of σ^{66}-Dependent Transcription. *Chlamydia* is an important pathogen that has a unique developmental cycle with two alternating cellular forms. Knowledge of the extent to which the regulation of transcription contributes to the chlamydial life cycle has been limited, in part, by the lack of suitable experimental tools. In this regard, only a handful of transcription inhibitors (21–25) and three transcription activators (26–28) had been identified in chlamydiae before our study.

Our work identifies GrgA as a previously undocumented transcription factor in *C. trachomatis*. Although we originally identified GrgA on the basis of its ability to stimulate transcription from the σ^{66}-dependent *def*A promoter (Fig. 1), we found that GrgA stimulates transcription from several σ^{66}-dependent promoters that are active at different stages of the chlamydial developmental cycle (Fig. 6). Based upon these in vitro findings, we propose that GrgA functions as a general transcription activator in *C. trachomatis* that up-regulates the expression of a broad spectrum of genes during all developmental phases. Consistent with this proposal, Western blot analysis indicates that GrgA is present in both chlamydial cellular forms, the EB and RB (Fig. S7). Nevertheless, although our in vitro data provide strong evidence that GrgA functions in vivo as an important regulator of σ^{66}-dependent gene expression, a direct test of the functional role that GrgA plays in vivo awaits the development of methods to perform targeted mutagenesis in *Chlamydia*.

GrgA-Dependent Transcription Activation Requires Contact with both DNA and the σ^{66} NCR. We found that efficient GrgA-dependent activation requires GrgA to retain the ability to contact both DNA and the σ^{66} NCR. In particular, we found that a GrgA mutant lacking amino acid residues required for DNA binding (Δ114–165)

and a GrgA mutant lacking amino acid residues required for contact with the σ^{66} NCR (Δ1–64) were significantly impaired for transcription activation (Figs. 3, 4, and 6). The importance of the GrgA–σ^{66} NCR interaction is further indicated by the inability of wild-type GrgA to efficiently activate transcription when reactions were performed with RNAP reconstituted with a σ^{66} mutant lacking amino acids required for the interaction with GrgA (Fig. 5).

Many bacterial proteins have been identified that bind DNA and activate transcription through direct contact with conserved region 4 of the primary σ factor (for review, see ref. 29). To our knowledge, GrgA represents the first DNA binding protein that activates transcription through direct contact with the NCR region of a primary σ factor. Prior studies have identified a role for the NCR of the primary σ factor of *E. coli*, σ^{70}, in modulating both promoter escape and early elongation pausing (30). In particular, interaction between the σ^{70} NCR and the β′ subunit of the *E. coli* core enzyme has been shown to facilitate escape from promoter DNA during initial transcription as well as escape from σ^{70}-dependent pausing during early elongation. We found that GrgA can stimulate transcription with both chlamydial RNAP and with a hybrid RNAP consisting of chlamydial σ^{66} and *E. coli* core (Figs. 1 and 6 and Fig. S5). Furthermore, the NCR of σ^{66} and the NCR of σ^{70} lack significant sequence similarity, suggesting that the NCR of σ^{66} is unlikely to interact with the β′ subunit of the *E. coli* core enzyme. Thus, we consider it unlikely that GrgA activates transcription by influencing interactions between the σ^{66} NCR and the RNAP core enzyme. We propose instead that GrgA activates transcription by stabilizing the binding of RNAP to promoter DNA. Nevertheless, defining the precise mechanism by which GrgA activates transcription awaits further investigation.

In conclusion, we have identified GrgA as a *Chlamydia*-specific transcription activator that exerts its stimulatory effect through interactions with the NCR of σ^{66} and sequence-nonspecific interactions with DNA. Furthermore, because GrgA can stimulate transcription in vitro from several promoters that control the expression of genes that are critical for chlamydial growth, GrgA likely represents a promising antichlamydial target.

Materials and Methods

Purification and Identification of PDF Promoter-Binding Proteins. RBs were partially purified from 12 L of suspension culture and disrupted by sonication. The lysate was clarified by centrifugation. The clarified RB lysate was mixed with biotinylated PDF promoter DNA immobilized to streptavidin-conjugated agarose beads, which were then packed into a column and washed with buffer I (*SI Materials and Methods*) supplemented with 200 mM NaCl. Bound proteins were eluted using buffer I containing 600 mM NaCl. Proteins were mixed with SDS/PAGE sample buffer, resolved by electrophoresis, and digested by trypsin. Tryptic peptides were identified by nanoLC-MS/MS as detailed in *SI Materials and Methods*.

Purification of Recombinant GrgA. For transcription assays and protein–protein interaction assays, N- or C-terminally His-tagged GrgA (Table S1) was purified from *E. coli* extracts prepared in guanidine hydrochloride. Denatured proteins were purified with metal TALON affinity resin and renatured as detailed in *SI Materials and Methods*. Strep-tagged GrgA or σ^{66} were purified using Strep-Tactin beads following manufacturer's instruction.

In Vitro Transcription Assay. The ability of GrgA to regulate transcription from chlamydial promoters was determined using a previously reported in vitro transcription assay (14, 31) with modifications, as detailed in *SI Materials and Methods*.

GrgA–DNA Interaction. EMSA was performed with or without poly(dI-dC). Streptavidin-immobilized biotinylated DNA fragments were used to precipitate GrgA. Alternatively, antibody immobilized GrgA was used to precipitate DNA. Experimental conditions for each of these assays are provided in *SI Materials and Methods*.

Protein–Protein Interaction. A lysate of *E. coli* expressing either NS·GrgA or CS·σ⁶⁶ (Table S1), which contained 50 mM Hepes and 300 mM NaCl, was diluted with equal volume of H$_2$O. A total of 200 μL of the diluted lysate was mixed with 10 μL of Strep-Tactin beads (20 μL suspension) on a nutator for 1 h at 4 °C. The beads were washed 4× with 25 mM Hepes (pH 7.0) containing 150 mM NaCl and 1% Nonidet P-40 (HeNN buffer), and mixed with 5 μg of CH·σ⁶⁶, a CH·σ⁶⁶ mutant, NH·GrgA, or a NH·GrgA mutant for 1 h at 4 °C. After four additional washes with HeNN, His-tagged proteins were resolved by SDS/PAGE and visualized by Western blotting using anti-His or anti-GrgA.

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