Role for GrgA in Regulation of σ^{28} -Dependent Transcription in the Obligate Intracellular Bacterial Pathogen *Chlamydia trachomatis*

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ABSTRACT The obligate intracellular bacterial pathogen *Chlamydia trachomatis* has a unique developmental cycle consisting of two contrasting cellular forms. Whereas the primary *Chlamydia* sigma factor, σ^{66} , is involved in the expression of the majority of chlamydial genes throughout the developmental cycle, expression of several late genes requires the alternative sigma factor, σ^{28} . In prior work, we identified GrgA as a *Chlamydia*-specific transcription factor that activates σ^{66} -dependent transcription by binding DNA and interacting with a nonconserved region (NCR) of σ^{66} . Here, we extend these findings by showing GrgA can also activate σ^{28} -dependent transcription through direct interaction with σ^{28} . We measure the binding affinity of GrgA for both σ^{66} and σ^{28} , and we identify regions of GrgA important for σ^{28} -dependent transcription. Similar to results obtained with σ^{66} , we find that GrgA's interaction with σ^{28} involves an NCR located upstream of conserved region 2 of σ^{28} . Our findings suggest that GrgA is an important regulator of both σ^{66} - and σ^{28} -dependent transcription in *C. trachomatis* and further highlight NCRs of bacterial RNA polymerase as targets for regulatory factors unique to particular organisms.

IMPORTANCE *Chlamydia trachomatis* is the number one sexually transmitted bacterial pathogen worldwide. A substantial proportion of *C. trachomatis*-infected women develop infertility, pelvic inflammatory syndrome, and other serious complications. *C. trachomatis* is also a leading infectious cause of blindness in underdeveloped countries. The pathogen has a unique developmental cycle that is transcriptionally regulated. The discovery of an expanded role for the *Chlamydia*-specific transcription factor GrgA helps us understand the progression of the chlamydial developmental cycle.

KEYWORDS σ^{28} , σ^{66} , CT504, CTL0766, *Chlamydia*, GrgA, transcription, transcription factor

Each year, ~60% of the 2.2 million cases of notifiable infections reported to the Centers for Disease Control and Prevention (CDC) are due to the sexually transmitted pathogen *Chlamydia trachomatis* (1, 2). In addition, because the infection is mostly asymptomatic, the CDC estimates only 1 of 10 people infected with *C. trachomatis* is reported (3). Nonetheless, without antibiotic treatment, the infection often leads to serious complications, such as infertility and pelvic inflammatory syndrome in women. Furthermore, some *C. trachomatis* serotypes cause ocular infection and are still the

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most common infectious microbes associated with blindness in underdeveloped countries (4, 5).

Like other chlamydiae, *C. trachomatis* is an obligate intracellular Gram-negative bacterium that exists in two cellular forms with contrasting properties (6). The small elementary body (EB) is infectious and capable of extracellular survival but is incapable of proliferation. Following binding to a cellular receptor(s), the EB enters a host cell membrane-derived vacuole through endocytosis (7). Within the vacuole, termed inclusion, the EB differentiates into a larger cellular form, termed reticulate body (RB), within several hours. No longer infectious, the RB divides exponentially by binary fission until around 20 h, when a significant portion of RBs redifferentiate back into EBs, while some RBs continue to proliferate (8). Progeny EBs along with residual RBs are released from infected cells following cell lysis. Alternatively, whole inclusions may be released from infected cells (9).

The 1-million-bp *C. trachomatis* genome carries fewer than 1,000 genes (10). Microarray analyses demonstrated that the majority of these genes are transcribed starting a few hours postinoculation throughout the remaining developmental cycle, whereas a small number of genes are transcribed immediately following cell entry and another small set of genes are transcribed only at late stages (11, 12). Transcriptome sequencing (RNA-seq) detected distinct sets of gene transcripts specifically enriched in either EBs or RBs (13), and purified EBs and RBs have been found to transcribe different sets of genes in axenic media (14). These findings suggest that the progression of the chlamydial developmental cycle is transcriptionally regulated.

Transcription is initiated following binding of the RNA polymerase (RNAP) to the gene promoter (15). The bacterial RNAP holoenzyme is comprised of the catalytic core enzyme and a σ factor, which is required for promoter recognition (16). Transcription of the vast majority of *C. trachomatis* genes involves σ^{66} , a homolog of σ^{70} that is often referred to as the housekeeping σ factor in eubacteria (16). Expression of some (but not all) chlamydial late genes depends on σ^{28} . Several genes possess both a σ^{66} promoter and a σ^{28} promoter (17).

GrgA (with gene codes CT_504 and CTL0766 for *C. trachomatis* serovars D and L2, respectively) is a *Chlamydia*-specific transcription activator (18). It was identified as a protein bound to the σ^{66} -dependent promoter of *defA*, which encodes peptide deformylase, an enzyme required for bacterial protein maturation and regulated protein degradation. In addition to *defA*, a midcycle gene, GrgA also stimulates transcription from another midcycle promoter (*ompA*), an early promoter (rRNA P1), and a late promoter (*hctA*), suggesting that GrgA functions as a general activator of σ^{66} -dependent genes (18). In this report, we demonstrate that GrgA also stimulates σ^{28} -dependent gene transcription *in vitro*. Thus, our findings suggest that GrgA plays an expanded role in gene expression during the *C. trachomatis* developmental cycle as a regulator of both σ^{66} - and σ^{28} -dependent transcription.

RESULTS

GrgA physically interacts with σ^{28} . To assess whether GrgA potentially regulates expression of σ^{28} -dependent genes, we determined whether GrgA can interact with σ^{28} . We performed protein pulldown assays using differential epitope tagging. The Strep-Tactin beads, which have affinity for the Strep-tag (19), precipitated NH- σ^{28} (N-terminally poly-His-tagged *C. trachomatis* σ^{28}) in a manner that was dependent on the N-terminally Strep-tagged GrgA (NS-GrgA) (Fig. 1A). Reciprocally, NH-GrgA was pulled down in an NS- σ^{28} -dependent manner (Fig. 1B). The results establish that GrgA can directly interact with σ^{28} .

GrgA has a lower affinity for σ^{28} than for σ^{66} . We next determined the binding affinities of GrgA for both σ^{28} and σ^{66} . We first compared the efficiencies of the σ factors in GrgA binding by performing competitive pulldown assays. As expected, NS-GrgA efficiently pulled down NH- σ^{28} and CH- σ^{66} in separate reactions (see Fig. S1 in the supplemental material). However, in the presence of equal molar concentrations

FIG 1 GrgA physically interacts with σ^{28} . (A) Pulldown of NH- σ^{28} by Strep-Tactin bead-immobilized NS-GrgA. Shown is a Western blot detecting NH- σ^{28} . (B) Pulldown of NH-GrgA by Strep-Tactin bead-immobilized NS- σ^{28} . Shown is a Western blot detecting NH-GrgA.

of NH- σ^{28} and CH- σ^{66} , NS-GrgA pulled down only CH- σ^{66} but not NH- σ^{28} (Fig. S1), indicating that GrgA has a lower affinity for σ^{28} than σ^{66} .

We quantitatively characterized GrgA binding by σ^{28} and σ^{66} with biolayer interferometry (BLI) using the BLItz system, which detects light wavelength shifts at the biosensor tip with an immobilized ligand following binding of an analyte in a real-time manner (20). Whereas representative BLItz recordings using NH-GrgA as a ligand and either CS- σ^{66} or NS- σ^{28} as an analyte are shown in Fig. S2A and B, values of kinetic parameters are provided in Table 1. The CS- σ^{66} analyte yielded a statistically highly significant 25-fold higher association rate constant (k_a) than that of the NS- σ^{28} analyte, suggesting that CS- σ^{66} binds NH-GrgA much faster than NS- σ^{28} . CS- σ^{66} also demonstrated a 3-fold statistically significant increase in disassociation rate constant (k_d), suggestive of moderately higher dissociation from NH-GrgA. Compared to the NH-GrgA-CS- σ^{66} interaction, the NH-GrgA-NS- σ^{28} interaction had a 32-fold higher disassociation equilibrium constant (K_D), indicating that GrgA has a lower overall affinity for σ^{28} than for σ^{66} .

We performed reciprocal BLI using CH- σ^{66} and NH- σ^{28} as ligands and NS-GrgA as the analyte to validate the difference in GrgA binding by the σ factors presented above (Table 1 and Fig. S2C and D). Consistent with the trend in k_a value changes presented above, the NS-GrgA analyte also demonstrated a statistically significantly higher k_a for CH- σ^{66} than for NH- σ^{28} , although the difference is smaller (25-fold versus 3.7-fold). Interestingly, the k_d values reveal that NS-GrgA also dissociates from CH- σ^{66} 6-fold slower than that from NH- σ^{28} . Compared to the CH- σ^{66} -NS-GrgA interaction, the NH- σ^{28} -NS-GrgA interaction had a 28-fold higher K_D , which is nearly identical to the 32-fold higher K_D detected for the NH-GrgA-NS- σ^{28} interaction versus the NH-GrgA-CS- σ^{66} interaction. Thus, competitive pulldown assays and BLI establish that GrgA has a lower affinity for σ^{28} than for σ^{66} .

TABLE 1 GrgA binds σ^{66} with a higher affinity than σ^{28} ^a

Ligand	Analyte	n	k_a		k_d		K_D	
			1/Ms	% control	1/s	% control	M	% control
NH-GrgA	CS- σ^{66}	6	$(1.9 \pm 1.3) \times 10^6$	100	$(9.3 \pm 5.2) \times 10^{-3}$	100	$(6.9 \pm 5.9) \times 10^{-9}$	100
NH-GrgA	NS- σ^{28}	8	$(1.5 \pm 1.7) \times 10^4$; $P = 0.001$	4.0	$(2.8 \pm 0.8) \times 10^{-3}$; $P = 0.001$	30	$(2.2 \pm 1.1) \times 10^{-7}$; $P < 0.001$	3,188
CH- σ^{66}	NS-GrgA	4	$(7.7 \pm 1.3) \times 10^5$	100	$(8.9 \pm 0.6) \times 10^{-3}$	100	$(1.2 \pm 0.1) \times 10^{-8}$	100
NH- σ^{28}	NS-GrgA	6	$(2.1 \pm 0.9) \times 10^5$; $P < 0.001$	27	$(5.6 \pm 1.2) \times 10^{-2}$; $P < 0.001$	629	$(3.4 \pm 2.0) \times 10^{-7}$; $P = 0.013$	2,833

^aBLItz assays were performed with His biosensors using the indicated ligand and analyte pairs. Representative graphs of recordings are shown in Fig. S2. Values of kinetic parameters (averages $\pm$ SDs) were generated by the BLItz Pro software (20). k_a (association rate constant) is defined as the number of complexes formed per second in a 1 M solution of ligand and analyte (1/Ms). k_d (disassociation rate constant) is defined as the number of complexes that decay per second (1/s). K_D (disassociation equilibrium constant), defined as the concentration at which 50% of ligand binding sites are occupied by the analytes, is k_d divided by k_a . n, number of experimental repeats. P values were calculated using 2-tailed Student's t tests.

FIG 2 GrgA stimulates σ^{28} -dependent transcription using *C. trachomatis* RNAP. (A) Transcription from the *C. trachomatis* *hctB* promoter in the pMT1212 report plasmid is dependent on the addition of NH- σ^{28} to the chlamydial RNAP. (B) Gel image showing dose-dependent stimulation of transcription from the *hctB* promoter by GrgA. (C) Averages and SDs for three independent measurements are shown. (B and C) Molar ratios of GrgA to σ^{28} were 0, 10, 20, and 33.

GrgA stimulates σ^{28} -dependent transcription. To determine whether GrgA can stimulate σ^{28} -dependent transcription, we performed *in vitro* transcription assays using pMT1212, a transcription reporter plasmid carrying the promoter of a gene encoding a histone-like protein (*hctB*) in *C. trachomatis* (21). Consistent with previous findings (21), transcription from the *hctB* promoter required the addition of NH- σ^{28} to the *C. trachomatis* RNAP (cRNAP) (Fig. 2A). Interestingly, GrgA demonstrated a dose-dependent stimulatory effect on the transcription from the promoter (Fig. 2B and C). These data suggest that GrgA can increase the expression of genes with a σ^{28} -dependent promoter in addition to genes with a σ^{66} -dependent promoter (18).

Residues 138 to 165 in GrgA are required for binding both σ^{28} and DNA and for activating σ^{28} -dependent transcription. A series of His-tagged GrgA deletion mutants (Fig. S3A and B) were tested for the effects on transcription from the *hctB* promoter (Fig. 3A). Noticeably, GrgA Δ 114-165 was completely defective in activating transcription from the σ^{28} -dependent promoter, whereas GrgA Δ 1-64 also demonstrated a significant 50% loss of transcription activation activity (Fig. 3A). Deletion of other regions (residues 65 to 113, 166 to 266, and 207 to 288) from GrgA had either no or minimal effects on transcription activation (Fig. 3A).

Our previous studies have shown that deletion of residues 114 to 165 disables GrgA's DNA binding, leading to loss of stimulation of transcription from σ^{66} -dependent promoters, whereas removal of residues 1 to 64 disables σ^{66} binding, also causing a defect in activating σ^{66} -dependent transcription (18). Therefore, the results shown in Fig. 3A suggest that (i) DNA binding is also required for σ^{28} -dependent transcription and (ii) the N-terminal σ^{66} -interacting region interacts with σ^{28} as well. Surprisingly, pulldown assays demonstrated that GrgA Δ 114-165 is completely defective in σ^{28} binding, whereas GrgA Δ 1-64 appeared to have only a slightly decreased σ^{28} -binding activity (Fig. 3B).

We performed a series of deletions within the 114-165 region to define the elements required for interacting with either DNA or σ^{28} . Since residues 114 to 138 are predicted to have coiled and stranded structures, whereas residues 139 to 158 are rich in positively charged lysine and aspartate and are predicted to form a helix (Fig. S4), we expected GrgA Δ 114-137 but not GrgA Δ 138-165 to retain DNA-binding activity. Electrophoretic mobility shift assay (EMSA) confirmed this prediction (Fig. 3C). Interestingly, GrgA Δ 114-137 but not GrgA Δ 138-165 also retained σ^{28} -binding activity (Fig. 3D). Not surprisingly, GrgA Δ 114-137 but not GrgA Δ 138-165 retained the capacity to activate σ^{28} -dependent transcription (Fig. 3E). Additional and extensive deletion mutagenesis and functional analyses for the region of residues 138 to 165 failed to (i) separate residues required for σ^{28} binding from residues required for DNA binding (Fig. S5 and S6), and (ii) define a smaller region fully required for binding either σ^{28} or DNA (Fig. S5

FIG 3 Residues 138 to 165 in GrgA are required for binding both σ^{28} and DNA and for activating σ^{28} -dependent transcription. (A) Efficient stimulation of transcription from the *hctB* promoter requires the N-terminal residues 1 to 64 and the middle region (residues 114 to 165). One micromolar full-length GrgA (FL) or the indicated deletion mutant was used in each assay. *p*1 is the *P* value between basal transcription activity (without GrgA) and activity with GrgA (FL or mutant); *p*2 is the *P* value between FL and the deletion mutant (paired *t* tests of three independent experiments); na, not applicable. (B) Pulldown of NH-GrgA full length (FL) and $\Delta 1-64$ but not $\Delta 114-165$ by Strep-Tactin bead-immobilized NS- σ^{28} . GrgA and deletion mutants were detected by Western blotting using a mouse anti-GrgA antibody, because the anti-His used in panels A and B does not recognize $\Delta 1-64$ (18). (C) $\Delta 138-165$ but not $\Delta 114-137$ is defective in DNA binding, like $\Delta 114-165$. Electrophoresis mobility shift assays (EMSA) were performed using a radiolabeled DNA fragment carrying sequences extending from position -144 to +52 of the *defA* gene (18) in the presence of the indicated concentrations of wild-type GrgA or the indicated GrgA mutant. (D) $\Delta 138-165$ but not $\Delta 114-137$ is defective in σ^{28} binding. Protein pulldown and detection were performed as described for panel B, with the exception of detection via anti-His. (E) $\Delta 138-165$ but not $\Delta 114-137$ is fully defective in activating transcription from the *hctB* promoter. See the legend to Fig. 2D for experimental and statistical information.

and S6). These studies suggest that σ^{28} and DNA bind to the same region in GrgA and further confirm that σ^{28} and DNA binding are required for activation of σ^{28} -dependent transcription (Fig. S7).

Residues 1 to 64 in GrgA contribute to σ^{28} binding. Transcription assays showed a 50% loss of activity in activating σ^{28} -dependent transcription in the GrgA $\Delta 1-64$ mutant (Fig. 3A). We used the BLItz system (20) to confirm decreased σ^{28} -binding activity in $\Delta 1-64$. Representative BLItz recordings of binding experiments using full-length NH-GrgA or deletion mutants as ligands and NS- σ^{28} as analyte are shown in Fig. S8A to D, and kinetic parameters are provided in Table 2. Compared to the full-length NH-GrgA, NS- σ^{28} revealed a moderately slowed association with and a moderately accelerated dissociation from NH-GrgA $\Delta 1-64$, as indicated by a nearly 3-fold decrease in the k_a and a 2-fold increase in the k_d (Table 2). These changes resulted in a highly significant 4.8-fold increase in the K_D value. On the other hand, NH-GrgA $\Delta 114-137$, which retains the activity to activate σ^{28} -dependent transcription (Fig. 3E), demonstrated no changes in kinetic parameters for interaction with NS- σ^{28} (Table 2). In contrast to NH-GrgA $\Delta 114-137$, NH-GrgA $\Delta 138-165$ immediately and completely dissociated from NS- σ^{28} upon washing (Fig. S8D), leading to a 1.5×10^6 times higher k_d and a 3.1×10^6 times higher K_D (Table 2), which are fully consistent with pulldown data (Fig. 3D). Furthermore, unlike NS- σ^{28} , which retained a low affinity for NH-GrgA $\Delta 1-64$

TABLE 2 Deletion of amino acids 1 to 64 from GrgA negatively affects σ^{28} binding^a

Ligand	n	k_a		k_d		K_D	
		1/Ms	% control	1/s	% control	M	% control
NH-GrgA	8	$(1.5 \pm 1.7) \times 10^4$	100	$(2.8 \pm 0.8) \times 10^{-3}$	100	$(2.2 \pm 1.1) \times 10^{-7}$	100
NH-GrgAΔ1-64	4	$(5.6 \pm 2.8) \times 10^3$; $P = 0.037$	37	$(5.8 \pm 2.3) \times 10^{-3}$; $P = 0.009$	200	$(1.1 \pm 0.2) \times 10^{-6}$; $P < 0.001$	479
NH-GrgAΔ114-137	4	$(1.5 \pm 0.4) \times 10^4$; $P = 0.947$	98	$(3.5 \pm 0.5) \times 10^{-3}$; $P = 0.128$	124	$(2.5 \pm 0.3) \times 10^{-7}$; $P = 0.658$	112
NH-GrgAΔ138-165	2	$(5.6 \pm 0.1) \times 10^3$; $P = 0.125$	37	$(4.1 \pm 0.3) \times 10^2$; $P = 0.002$	1.5×10^8	$(6.9 \pm 4.5) \times 10^{-2}$; $P < 0.001$	3.1×10^8

^aIndicated His-tagged proteins were immobilized on His biosensors as ligands for the NS- σ^{28} analyte in BLItz assays. Representative graphs of BLItz recordings are shown in Fig. S8. See Table 1 for information regarding kinetic parameters and statistics.

(relative to full-length NH-GrgA), CS- σ^{66} quickly and completely dissociated from NH-GrgAΔ1-64 upon washing (Fig. S8E), which is consistent with pulldown data previously reported (18). Taken together, the BLItz data shown in Fig. S8 and Table 2 indicate that the decreased affinity with NS- σ^{28} in NH-GrgAΔ1-64 is responsible for the partial loss of activity in activating σ^{28} -dependent transcription (Fig. 3A).

The N terminus of σ^{28} is most critical for binding GrgA. We constructed NH- σ^{28} variants with deletion of the N-terminal leader sequence, σ factor region 2, 3, or 4 (unlike the housekeeping σ factor, σ^{28} , does not contain region 1) (Fig. 4A). All deletion mutants (i.e., NH- σ^{28} ΔNL, NH- σ^{28} ΔR2, NH- σ^{28} ΔR3, and NH- σ^{28} ΔR4) were expressed in *Escherichia coli* (Fig. 4B). Noticeably, in pulldown assays, NS-GrgA completely failed to pull down the NH- σ^{28} ΔNL and NH- σ^{28} ΔR2 mutants and pulled down only small amounts of NH- σ^{28} ΔR3 and NH- σ^{28} ΔR4 compared to full-length NH- σ^{28} (Fig. 4C).

In BLItz assays, the rate of association with NH-GrgA varied greatly among the σ^{28} deletion mutants. Whereas NH- σ^{28} ΔNL had essentially the same k_a as full-length NH- σ^{28} , NH- σ^{28} ΔR2 displayed a significant 2-fold reduction in k_a . In contrast, NH- σ^{28} ΔR3 and NH- σ^{28} ΔR4 showed a 3.5-fold increase and a 50% increase in k_a , respectively (Table 3). All mutants demonstrated dramatic increases (10- to 383-fold) in the k_d (Table 3). Consequently, NH- σ^{28} ΔNL and NH- σ^{28} ΔR2 had 345- and 177-fold higher K_D values, respectively, whereas NH- σ^{28} ΔR3 and NH- σ^{28} ΔR4 both demonstrated 7-fold higher K_D values (Table 3). Representative graphs of BLItz recordings are shown in Fig. S9. Taken together, both the pulldown (Fig. 4) and BLI data (Table 3) indicate that the N terminus of σ^{28} (i.e., NL and R2) interacts with GrgA while R3 and R4 stabilize the GrgA- σ^{28} binding. Figure 5 summarizes domain structures of GrgA, σ^{66} and σ^{28} , and modes of interaction between GrgA and the σ factors.

DISCUSSION

As an obligate intracellular bacterium, *C. trachomatis* faces unique challenges at every stage in the developmental cycle (22). Proper expression of certain genes is

FIG 4 N-terminal leader sequence and region 2 of σ^{28} are required for interaction with GrgA. (A) Schematic of σ^{28} and mutants lacking the indicated regions. (B) Western blot showing expression of purified NH- σ^{28} and mutants. (C) Precipitation of NH- σ^{28} and mutants by Strep-Tactin-immobilized NS-GrgA. Shown is a Western blot detecting NH- σ^{28} or mutant. All proteins were resolved via SDS-PAGE and detected using an anti-His antibody.

TABLE 3 The N-terminal leader sequence and region 2 of σ^{28} are required for GrgA binding^a

Analyte	n	k_a		k_d		K_D	
		1/Ms	% control	1/s	% control	M	% control
NH- σ^{28}	3	$(2.2 \pm 0.4) \times 10^4$	100	$(4.7 \pm 0.1) \times 10^{-3}$	100	$(2.2 \pm 0.3) \times 10^{-7}$	100
NH- $\sigma^{28}\Delta 1-13$	3	$(2.4 \pm 0.4) \times 10^4$; $P = 0.649$	109	$(1.8 \pm 0.2) \times 10^0$; $P < 0.001$	38,297	$(7.6 \pm 0.5) \times 10^{-5}$; $P < 0.001$	34,545
NH- $\sigma^{28}\Delta R2$	3	$(1.3 \pm 0.4) \times 10^4$; $P = 0.044$	59	$(5.3 \pm 0.3) \times 10^{-1}$; $P = 0.036$	11,276	$(3.9 \pm 1.1) \times 10^{-5}$; $P = 0.003$	17,727
NH- $\sigma^{28}\Delta R3$	3	$(8.0 \pm 1.6) \times 10^4$; $P = 0.004$	363	$(1.2 \pm 0.2) \times 10^{-1}$; $P < 0.001$	2,553	$(1.5 \pm 0.2) \times 10^{-6}$; $P < 0.001$	714
NH- $\sigma^{28}\Delta R4$	3	$(3.3 \pm 0.5) \times 10^4$; $P = 0.038$	150	$(4.9 \pm 0.3) \times 10^{-2}$; $P = 0.004$	1,043	$(1.5 \pm 0.6) \times 10^{-6}$; $P = 0.018$	714

^aBiotinylated NH-GrgA was immobilized on streptavidin biosensors as the ligand for the indicated analytes in BLItz assays. Representative graphs of recordings are shown in Fig. S9. See Table 1 for information regarding kinetic parameters and statistics.

required for meeting those challenges (11, 12, 23). Surprisingly, only two transcription activators have been identified in *C. trachomatis* (24). GrgA was first identified as a transcription activator for σ^{66} -dependent genes (18). However, the present study has demonstrated that GrgA potentially stimulates expression of σ^{28} -dependent genes as well. Transcription of chlamydial genes is temporally controlled during the developmental cycle (11, 12, 17, 25, 26). Whereas σ^{66} is involved in transcription of most *C. trachomatis* genes, some late promoters are recognized by σ^{28} (17). Microarray studies have shown that synthesis of the σ^{28} mRNA temporally falls behind that of the σ^{66} mRNA (11, 12). Thus, it would be safe to assume that GrgA activates σ^{66} -dependent genes in earlier developmental stages. The high concentration of GrgA in the EB (18, 27, 28) also supports the notion that GrgA plays a critical role in activating transcription of immediate-early and early genes, which are dependent on σ^{66} but not σ^{28} . Protein products of these genes, including (but not limited to) TARP (translocated actin recruiting phosphoprotein), Npt1 ATP/ADP translocase, and Inc proteins (inclusion membrane proteins) are critical for cell entry, acquiring energy from the host cell, and establishing and maintaining a niche for chlamydial growth and development inside the inclusion (11, 29, 30). GrgA may also stimulate expression of genes that are important for EB survival. Although the EB was long regarded as a metabolic inert cellular form, active transcription and translation are readily detectable in isolated EBs (14). The presence of GrgA in RBs suggests that GrgA activates midcycle genes that are required for growth and late genes that are needed for conversion of RBs back to EBs. Significantly, we have shown that GrgA activates transcription from σ^{66} -dependent early, mid-stage, and late gene promoters *in vitro* (18).

Whether or not GrgA also regulates expression of σ^{28} -dependent genes during later developmental stages likely depends on the expression levels of GrgA, σ^{28} , and σ^{66} . If GrgA is limited, σ^{28} would have to be present at significantly higher concentrations than σ^{66} to effectively compete for GrgA. However, in a quantitative whole-proteomic mass spectrometry study by Skipp et al., higher levels of GrgA relative to σ^{66} were

FIG 5 Domain structures of GrgA, σ^{66} and σ^{28} , and modes of interaction between GrgA and the σ factors. R_1 , R_2 , R_3 , and R_4 are regions conserved across bacterial species. In addition to the nonconserved region (NCR) between R_1 and R_2 in σ^{66} , the N-terminal leader sequences, shown in light color, of both σ^{66} and σ^{28} are also nonconserved. Numbers on GrgA indicate positions of amino acid residues.

detected in both EBs and RBs purified from the midcycle, whereas σ^{28} was undetected in either cellular form (28). Thus, GrgA could stimulate transcription from σ^{28} -dependent promoters in addition to σ^{66} -dependent promoters regardless of the molar ratio of the two σ factors. It should be pointed out that another quantitative proteomic study, carried out by Saka et al., failed to detect GrgA in the RBs (27). They also detected a lower concentration of GrgA than σ^{66} (27). Whereas reasons for the discrepancy between the two proteomic studies are unclear, we found that GrgA is readily detectable in RBs (18). Accurate quantification of GrgA and σ factors in different stages of the developmental cycle is needed to elucidate roles of GrgA in the expression of σ^{66} - and/or σ^{28} -dependent genes throughout the developmental cycle.

We used both pulldown assays and BLI to analyze the interaction of GrgA with σ^{28} and σ^{66} . Clearly, owing to its quantitative nature, BLI offers higher sensitivities than protein pulldown assays in studying protein-protein interaction. This led to the conclusion that decreased affinity for σ^{28} in GrgA Δ 1-64, which was ambiguous in pulldown assays, is the most probable cause for a 50% loss of activity in activating σ^{28} -dependent transcription.

We have defined a middle region in GrgA (residues 138 to 165) as a σ^{28} - and DNA-binding domain (Fig. 3 and 5). Extensive deletion mutagenesis in this region failed to divide it into subdomains that bind either σ^{28} or DNA but not both (Fig. S5 and S6). We speculate that multiple positively charged residues (K138, K139, R142, R143, K144, K147, K150, K152, K154-156, R159-161, and/or K164) interact with negatively charged DNA, whereas negatively charged residues (E141, E145, E149, D153, and/or E165) interact with σ^{28} .

GrgA has demonstrated similar but nonidentical properties in activating σ^{66} - and σ^{28} -dependent transcription (Fig. 5). Apparently, sequence-nonspecific DNA binding is required for activating both σ^{66} -dependent transcription (18) and σ^{28} -dependent transcription (Fig. 3A and E and 5 and Fig. S7). However, the N-terminal region (residues 1 to 64) of GrgA has a stronger role in σ^{66} -dependent transcription (18) than in σ^{28} -dependent transcription (Fig. 3A), because this region is absolutely required for GrgA to interact with σ^{66} (18) but plays only a supportive role in binding σ^{28} , which was clearly evident only with BLI (Table 2) but appeared uncertain with pulldown assays (Fig. 3B).

Whereas the major GrgA structural determinants for binding σ^{28} and σ^{66} differ, there is similarity between the GrgA-binding regions in the two σ factors (Fig. 5). The GrgA-binding sequence in σ^{66} is the last portion of the nonconserved region immediately upstream of conserved region 2, whereas the GrgA-binding sequence in σ^{28} also involves the N-terminal nonconserved leader sequence (and the immediately downstream region 2). To the best of our knowledge, GrgA is the only transcription factor that targets nonconserved regions of σ factors (16).

In summary, we have demonstrated that the *Chlamydia*-specific protein GrgA can activate both σ^{66} -dependent transcription and σ^{28} -dependent transcription *in vitro*. Current knowledge suggests that GrgA activates σ^{66} -dependent genes throughout the developmental cycle. Whether or not GrgA also regulates expression of σ^{28} -dependent late genes likely depends on the expression levels of GrgA, σ^{28} , and σ^{66} , because GrgA has a lower affinity for σ^{28} than σ^{66} . To date, GrgA remains the only transcription factor that targets nonconserved regions of σ factors (16).

MATERIALS AND METHODS

Reagents. All DNA primers were custom synthesized at Sigma-Aldrich. The QuikChange site-directed mutagenesis kit and BL21(DE3) ArcticExpress *E. coli* competent cells were purchased from Agilent Technologies. A Q5 site-directed mutagenesis kit and deoxynucleotides were purchased from New England BioLabs. Isopropyl- β -D-thiogalactopyranoside (IPTG) was purchased from Gold Biotechnologies. Talon metal affinity resin was purchased from TaKaRa. The Strep-Tactin Superflow high-capacity resin and D-thiobiotin were purchased from IBA Life Sciences. Coomassie brilliant blue G-250 dye, mouse monoclonal antihistidine antibody (H1029), goat anti-mouse horseradish peroxidase-conjugated antibody (A4416), and EZ-Link sulfo-NHS-LC-biotin were purchased from Sigma-Aldrich. SuperSignal West Pico Plus chemiluminescent substrate was purchased from ThermoFisher Scientific. Dip and Read nickel-nitrilotriacetic acid (NTA) biosensors were purchased from Pall ForteBio. The HNE buffer contained

50 mM HEPES (pH 7.4), 300 mM NaCl, and 1 mM EDTA. The HNEG buffer contained 50 mM HEPES (pH 7.4), 300 mM NaCl, 1 mM EDTA, and 6 M guanidine HCl. The TNE buffer contained 25 mM Tris (pH 8.0), 150 mM NaCl, and 1 mM EDTA. The protein storage (PS) buffer contained 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 mM EDTA, 10 mM MgCl₂, 0.1 mM dithiothreitol (DTT), and 30% (wt/vol) glycerol. The BLITZ buffer contained 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 mM EDTA, 10 mM MgCl₂, and 0.1 mM DTT.

Vectors. Plasmids for expressing His- or Strep-tagged GrgA, σ^{66} , σ^{28} , and their mutants are listed in the Table S1 in the supplemental material. Sequences of primers used for constructing expression plasmids (GrgA deletion mutants, NS- σ^{28}) and a DNA fragment for EMSAs are provided in Table S2. Sequence authenticities of cloned genes and epitope tags in the final vectors were confirmed using Sanger's DNA sequencing service, provided by GenScript Biotech Corporation.

Expression of recombinant proteins and preparation of cell extract for purification. BL21(DE3) ArcticExpress *E. coli* cells transformed with a plasmid for expressing an epitope-tagged chlamydial protein (GrgA, σ^{28} , σ^{66} , or their mutant) (Table S1) were cultured in the presence of 1 mM IPTG overnight at 15°C in a shaker. Cells were collected by centrifugation and resuspended in one of the following buffers: HNE buffer (for purification of native His-tagged proteins), HNEG buffer (for purification of denatured His-tagged proteins), or TNE buffer (for purification of native Strep-tagged proteins). The cells were disrupted using a French press. The cell extract was subjected to high-speed (20,000 $\times g$) centrifugation at 4°C for 30 min. Supernatant was collected and used for protein purification.

Purification of Strep-tagged proteins. Strep-tagged GrgA and σ factors were purified as previously described (19). The supernatant of centrifuged cell lysate was incubated with the Strep-Tactin Superflow high-capacity resin on a Nutator for 1 h at 4°C. The resin was packed onto a column, washed with 30 column volumes of the TNE buffer, and then eluted with the TNE buffer containing 2.5 mM D-desthiobiotin. The elution was collected in 10 fractions. Protein in the fractions was examined following SDS-PAGE and Coomassie blue staining. Fractions with high purity and concentration were pooled and dialyzed overnight against the PS buffer at 4°C and then stored in aliquots at -80°C.

Purification of His-tagged proteins. The supernatant of centrifuged cell lysate was incubated with the Talon metal affinity resin on a Nutator for 1 h at 4°C. The resin incubated with nondenatured cell extract was packed onto a column, washed with 30 column volumes of HNE buffer containing 1% NP-40, and eluted with the HNE buffer containing 250 mM imidazole. The resin incubated with denatured cell extract was packed onto a column, washed with 30 column volumes of HNEG buffer, and eluted with HNEG buffer containing 250 mM imidazole. Examination of protein purity, dialysis, and storage was carried out in the same manner as that for purified Strep-tagged proteins (18).

In vitro transcription assay. In vitro transcription of σ^{28} -dependent promoter was performed as previously described (18). The assay, in a total volume of 30 μ l, contained 200 ng supercoiled plasmid DNA, 50 mM potassium acetate, 8.1 mM magnesium acetate, 50 mM Tris acetate (pH 8.0), 27 mM ammonium acetate, 1 mM DTT, 3.5% (wt/vol) polyethylene glycol (average molecular weight, 8,000), 330 μ M ATP, 330 μ M UTP, 1 μ M CTP, 0.2 μ M [α -³²P]CTP (3,000 Ci/mmol), 100 μ M 3'-O-methyl-GTP, 20 U of RNasin, RNAP, and the indicated amount of GrgA or GrgA mutant. The reactions using cRNAP and σ^{28} contained 1.0 μ l purified cRNAP and 30 nM His-tagged σ^{28} , purified by procedures involving denaturing and refolding as described above. For reactions using eCore and σ^{28} , their concentrations were 5 nM and 30 nM, respectively. The reaction was allowed to continue at 37°C for 40 min and terminated by the addition of 70 μ l of 2.86 M ammonium acetate containing 4 mg of glycogen. After ethanol precipitation, ³²P-labeled RNA was resolved by 8 M urea-6% polyacrylamide gel electrophoresis and quantified with a Storm PhosphorImager and ImageQuant software. Relative amounts of transcripts were presented, with that of the control reaction set as 1 U. Data shown in bar graphs represent averages $\pm$ standard deviations (SDs) from three or more independent experiments. Pairwise, two-tailed Student *t* tests were used to compare data.

EMSA. GrgA-DNA interaction was determined by EMSA as described previously (18). ³²P-labeled DNA fragment containing the *C. trachomatis* *defA* promoter (31) was amplified using a ³²P-labeled 5' primer and an unlabeled 3' primer (Table S2) and purified with a Qiagen column. The GrgA-DNA binding reaction was performed in a total volume of 10 μ l, containing 10 nM promoter fragment, the indicated amount of NH-GrgA, 1 mM potassium acetate, 8.1 mM magnesium acetate, 50 mM Tris acetate (pH 8.0), 27 mM ammonium acetate, 1 mM DTT, and 3.5% (wt/vol) polyethylene glycol (average molecular weight, 8,000). After mixing for 1 h at 4°C, the binding mixture was resolved by 6% nondenaturing polyacrylamide gel. Free and GrgA-bound DNA fragments were visualized on a Storm PhosphorImager (Molecular Dynamics).

Pulldown assays. Twenty microliters of Strep-Tactin Superflow high-capacity resin was washed twice with the HNE buffer and incubated with 50 μ l of Strep-tagged cell extract or purified protein on a Nutator at 4°C for 1 h. The resin was washed three times with HNE buffer containing 1% NP-40 and then incubated with 5 μ g of a purified His-tagged protein (or mutant) on a Nutator at 4°C for 1 h. After 3 washes with the HNE buffer containing 1% NP-40 and a final wash with phosphate-buffered saline (PBS), the resin was eluted using SDS-PAGE sample buffer. All protein was resolved via SDS-PAGE and detected by either Coomassie blue staining or Western blotting using a monoclonal mouse anti-His or a polyclonal mouse anti-GrgA primary antibody and horseradish peroxidase-conjugated goat anti-mouse secondary antibody.

Preparation of biotinylated protein. Purified NH-GrgA was dialyzed against PBS to remove Tris and then incubated with 10 mM EZ-Link sulfo-NHS-LC-biotin for 2 h at 4°C. Excess biotin was removed via two-step dialysis, initially against PBS and subsequently against the PS buffer.

BLI assay. An NTA His or streptavidin biosensor was subjected to initial hydration in BLItz buffer for 10 min before being loaded onto the ForteBio BLItz machine and washed with BLItz buffer for 30 s to obtain a baseline reading. The His biosensor was then incubated with 4 μ l of a His-tagged ligand for 240 s. The concentrations of ligand ranged from 1 to 20 μ M, all of which saturated the His-binding sites on the biosensor. Alternatively, the streptavidin biosensor was incubated with 4 μ l of a biotinylated ligand (10 μ M NH-GrgA, which was sufficient to saturate the binding sites on the biosensor) for 240 s. After a brief wash with BLItz buffer for 30 s to remove excess protein, the biosensor was incubated with 4 μ l of an analyte (purified Strep-tagged protein for the His biosensor or NH- σ^{28} for the streptavidin biosensor) for 120 s to measure association of the ligand-analyte complex. Subsequently, the biosensor was washed with BLItz buffer for 120 s to measure disassociation of the ligand-analyte complex. All BLItz recordings were subsequently fit to a 1:1 binding model using BLItz Pro software (version 1.1.0.31), which generated the association rate constant (k_a), disassociation rate constant (k_d), and disassociation equilibrium constant (K_d) for each interaction.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00298-18>.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

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