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Mutagenesis of Region 4 of Sigma 28 from *Chlamydia trachomatis* Defines Determinants for Protein-Protein and Protein-DNA Interactions▽

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Transcription factor σ^{28} in *Chlamydia trachomatis* (σ^{28}_{Ct}) plays a role in the regulation of genes that are important for late-stage morphological differentiation. In vitro mutational and genetic screening in *Salmonella enterica* serovar Typhimurium was performed in order to identify mutants with mutations in region 4 of σ^{28}_{Ct} that were defective in σ^{28}-specific transcription. Specially, the previously undefined but important interactions between σ^{28}_{Ct} region 4 and the flap domain of the RNA polymerase β subunit (β-flap) or the −35 element of the chlamydial *hctB* promoter were examined. Our results indicate that amino acid residues E206, Y214, and E222 of σ^{28}_{Ct} contribute to an interaction with the β-flap when σ^{28}_{Ct} associates with the core RNA polymerase. These residues function in contacts with the β-flap similarly to their counterpart residues in *Escherichia coli* σ^{70}. Conversely, residue Q236 of σ^{28}_{Ct} directly binds the chlamydial *hctB* −35 element. The conserved counterpart residue in *E. coli* σ^{70} has not been reported to interact with the −35 element of the σ^{70} promoter. Observed functional disparity between σ^{28}_{Ct} and σ^{70} region 4 is consistent with their divergent properties in promoter recognition. This work provides new insight into understanding the molecular basis of gene regulation controlled by σ^{28}_{Ct} in *C. trachomatis*.

Chlamydia trachomatis is a leading causative pathogen of bacterial sexually transmitted diseases and ocular infections (trachoma) in humans (36). The obligate intracellular parasitic feature of *Chlamydia* has hindered genetic and biochemical studies. As a result, little is known about how gene expression is regulated in *Chlamydia*. Recent studies indicate that gene expression takes place coordinately at the level of transcription throughout the developmental cycle of *Chlamydia* (2, 33, 37). Bacterial RNA polymerase (RNAP) is a key enzyme that controls transcription (15). Chlamydial RNAP is identical to the well-studied *Escherichia coli* RNAP in containing subunits $\alpha_2\beta\beta'$ and a σ factor, despite the lack of a recognizable ω subunit (43). The σ factors associate with the RNAP core to form an RNAP holoenzyme that initiates promoter-specific transcription (15, 19, 46). Three known σ factors, σ^{66}, σ^{54}, and σ^{28}, and some transcription regulators have been revealed in the *C. trachomatis* genome (43). Both chlamydial σ^{66} and *C. trachomatis* σ^{28} (σ^{28}_{Ct}), but not σ^{54}, belong to members of the *E. coli* σ^{70} family that share up to four conserved amino acid regions (regions 1 to 4) (26). Chlamydial σ^{66} serves as the primary σ factor that directs transcription of housekeeping genes or most constitutively expressed genes (2, 7, 23, 27, 33), whereas σ^{28}_{Ct} directs transcription of several late-stage genes that are required for differentiation from the noninfectious but metabolically active reticulate bodies to the infectious but metabolically inactive elementary bodies (3, 39, 48). σ^{28}_{Ct} also performs its task of transcription by responding to stressful conditions (39, 50). It is not known how the σ^{28}_{Ct} function is regulated in *Chlamydia*. *C. trachomatis* does not appear to encode an identifiable anti-σ^{28} homologue, which is known as FlgM in *Salmonella* (18, 22). A putative chlamydial RsbW, encoded by the *rsbW* gene, was shown to bind σ^{28}_{Ct} in a glutathione *S*-transferase pull-down assay (A. L. Douglas and T. P. Hatch, personnel communication). However, there is no direct evidence to show an inhibitory effect of RsbW on chlamydial σ^{28}_{Ct}-specific transcription (17, 22). Even less is known about the function of chlamydial σ^{54}, and only two putative σ^{54}-like promoters have been reported (28). Defining the molecular basis of σ factor action is central for understanding the mechanism by which *Chlamydia* completes its unique intracellular developmental cycle (16) and adapts to environmental cues.

Several lines of evidence demonstrate that the DNA-σ and σ-core interactions are essential for the process of transcription initiation and elongation (5, 11, 30, 31, 35). Such molecular interactions are well characterized in *E. coli* σ^{70}-mediated transcription regulation (5, 11, 25). Regions 2 and 4 of *E. coli* σ^{70} recognize the promoter −10 and −35 elements, respectively (4, 11, 34). Region 2 of σ^{70} also interacts with the β′ coiled coil in core RNAP. This interplay is essential for holoenzyme formation (1, 5, 12, 34). Also, the σ^{70} region 4 interacts with the flexible flap structure in the β subunit (β-flap). This interaction is required to properly position σ^{70} regions 2 and 4, allowing for simultaneous contact with the −35/−10 promoter elements. Furthermore, the σ^{70} region 4/β-flap interaction can be a target of transcription factors, such as the anti-σ^{70} factor T4 AsiA (13, 21). By sequence analogy with *E. coli* σ^{70}, conserved region 4 in an alternative σ factor is likely to interact with the β-flap for recognition of the −35/−10 promoters. Indeed, direct interactions of region 4 of *Helicobacter pylori* σ^{28} with the β-flap (6), as well as those of

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region 4 of *E. coli* σ^{38} with the β-flap (27, 36) have been identified. An apparent difference in the strengths of the *E. coli* σ^{38}/β-flap and *E. coli* σ^{70}/β-flap interactions was found, although *E. coli* σ^{38} and σ^{70} recognize similar promoter consensus sequences (19). The question is raised of whether the variability of σ region 4 and the β-flap interaction contributes to promoter recognition in a σ-specific manner.

Previously, we have characterized the range of promoter elements that are recognized by chlamydial σ^{28} (39, 40). This study was performed in order to explore the details of σ^{28}_{Ct} action in the process of transcription. Specifically, we report that certain σ^{28}_{Ct} determinants are required for contact with the chlamydial β-flap of RNAP or the −35 promoter element. Because σ^{28}_{Ct} plays a role in regulation of genes important for late-stage morphological differentiation and the stress response, our data can shed light on molecular mechanisms underlying these processes. Importantly, given the uncertainty about whether a transcription regulator would affect the σ^{28}_{Ct} activity, our ability to define the molecular interactions of the transcription machinery provides us with a useful genetic tool and information to facilitate the discovery and characterization of potential regulatory factors for σ^{28}_{Ct} in *Chlamydia*, a genetically intractable pathogen.

MATERIALS AND METHODS

Bacterial strains and growth. The bacterial strains used are listed in Table 1. *C. trachomatis* was propagated and purified as previously described (38). DH5α or XL1-Blue was used as the host for cloning. *Salmonella enterica* serovar Typhimurium *fliA*, encoding *Salmonella* σ^{28}, mutant strains TH5504 and TH7034 were kindly provided by Kelly T. Hughes (University of Utah). Reporter strains BN317 (34) and KS1 (8) were kindly provided by Ann Hochschild (Harvard University). Reporter strains ZY101 containing the test promoter P*lac_hctB*-35 linked to *lacZ* on an F′ episome were constructed as described previously (45) (see Fig. 6). Briefly, the EcoRI-HindIII-digested DNA fragment carrying the synthetic promoter P*lac_hctB*-35 was cloned into pFW11 via EcoRI and HindIII sites. The resultant plasmid was introduced into strain CSH100, allowing homologous recombination of P*lac_hctB*-35 and *lacZ* onto an F′ episome. Further mating with strain FW102 was performed in order to finalize construction of strain ZY101. In this test promoter, an ectopic chlamydial *hctB* promoter −35 element with the sequence TAAA GTTT was centered at bp −45.5 relative to the transcription initiation site of *lac*. Using a similar strategy, the reporter strain ZY102 was constructed. ZY102 is identical to ZY101, except that it contains a mutated ectopic chlamydial *hctB* promoter −35 element (gAAAGTTT). *E. coli* and *S. enterica* serovar Typhimurium strains were grown in Luria-Bertani (LB) medium at 37°C. MacConkey agar supplemented with 1% lactose (Difco) plus 0.02% arabinose (MacConkey-lactose-arabinose) was used in indicator plates for β-galactosidase activity. When required, medium was supplemented with 25 μg/ml chloramphenicol, 50 μg/ml kanamycin, 10 μg/ml tetracycline, and/or 100 μg/ml carbencillin.

Plasmid construction. The plasmids used in this study are listed in Table 1. The hybrid σ^{28} genes (Fig. 1) were generated by overlapping PCR amplification and recloned into expression vectors pS28H and pES28H (39), creating $pH\sigma^{28}_{Ct\text{-}Ec4}$ and $pH\sigma^{28}_{Ec\text{-}Ct4}$. The hybrid σ^{28} genes are under the control of an arabinose-inducible P_{ara} promoter. Plasmids pACλcI, pBRα, pBRαLN and pACλcI-β-flap (8–10, 25) were kindly provided by Ann Hochschild (Harvard University). pACλcI-β-$flap_{Ct}$ directs synthesis of λcI fused to chlamydial β-flap under the control of a *lacUV5* promoter. pBRα-σ^{28} encodes the *E. coli* N-terminal domain of the α subunit of RNAP (αNTD) fused to the chlamydial σ^{28} region 4 under the control of tandem *lpp* and IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *lacUV5* promoters. pBRα-σ^{66} encodes αNTD fused to region 4 of chlamydial σ^{66}. Plasmid pGHR, carrying promoters of chlamydial *hctB* and the rRNA, was created by inserting the *hctB* promoter region into XbaI-EcoRV-digested pMT504 (44). pGHR together with pGS_9, pGP_{hctB}, pGS_{14}, $pGP_{hctB}\Delta_{up}$, pGP_{tar}, or pGP_{tar+} (40) was used as a supercoiled template in the in vitro transcription assay. Inserts in all constructs were confirmed by restriction mapping and DNA sequencing. Details of primers and procedures for these constructs are available upon request.

Mutant σ^{28}_{Ct} library and genetic screening. For convenience in cloning, a silent mutation (T to A) was introduced at position 723 of the σ^{28}_{Ct} coding region using PCR-based site-directed mutagenesis in order to remove a natural HindIII site in pLF28 (40). The resultant plasmid was named pLF28a. Error-prone PCR was performed with *Taq* DNA polymerase (New England Biolabs) for introducing random mutations of the σ^{28}_{Ct} gene into appropriate fragments of pLF28a. The mutagenized PCR fragments were digested with HindIII-SphI, and a 187-bp DNA fragment containing region 4 of σ^{28}_{Ct} was ligated with the HindIII-SphI-digested fragment of pLF28a. DH5α was transformed with the ligation mixture in order to generate a mutant library of σ^{28}_{Ct} region 4. This mutant library was then subjected to a genetic screen in *S. enterica* serovar Typhimurium *fliA* mutant strain TH7034. The selection strains formed white or pink colonies on MacConkey-lactose-arabinose indicator plates when transformed with mutant σ^{28}_{Ct} (the wild type was red). The plasmids carrying potential mutations were analyzed by restriction enzyme digestion, and the full-length σ^{28}_{Ct} gene was sequenced in order to confirm the presence of mutations.

Expression and purification of wild-type σ^{28}_{Ct} and derivatives. For the production of N-terminally His_6-tagged σ^{28}_{Ct} and derivatives, pS28H and derived plasmids were introduced into *S. enterica* serovar Typhimurium strain TH7034. Cells were grown in LB containing carbenicillin. The fusion proteins were induced by treatment with 0.02% arabinose for 2 h. Proteins were purified under denaturing conditions on an Ni-nitrilotriacetic column (Qiagen), followed by a HiTrap HP column (GE Healthcare), as described previously (42). Purified proteins were renatured by serial dialysis. The final dialysis buffer contained 50% glycerol, 50 mM Tris-HCl (pH 7.9), 0.01% (vol/vol) Triton X-100, 0.1 mM EDTA, 150 mM NaCl, and 0.1 mM dithiothreitol. Protein was stored at −20°C for future use. Protein concentrations were determined using a protein assay kit (USB Corporation) and confirmed by Western blot analysis.

In vivo assay of chlamydial *hctB* promoter activity in *Salmonella*: relative β-galactosidase activity. To examine the effect of mutant σ^{28}_{Ct} proteins on transcription from the chlamydial *hctB* promoter, pS28H or derived plasmids were introduced into *Salmonella fliA* mutant strain TH5504 cells harboring pRV_{hctB}, which carries the reporter *hctB*::*lacZ*. Cells were grown in LB containing carbencillin and chloramphenicol to ensure selection of both plasmids. The protein was induced by the addition of 0.02% (wt/vol) arabinose starting from an optical density at 600 nm of ≈0.3 for 1 hour. Aliquots of cells were collected. Cell lysates in sodium dodecyl sulfate (SDS) loading buffer were electrophoresed on a 10% glycine-SDS-polyacrylamide gel, followed by Western blot analysis. Levels of cellular σ^{28}_{Ct} and RNAP β subunit were measured by Western blot analysis with polyclonal anti-σ^{28}_{Ct} antibody (a gift from Thomas P. Hatch, University of Tennessee) and monoclonal antibody 8RB13 against the β subunit of RNAP (NeoClone) as probes, respectively. The β-subunit band was used as an internal standard for correcting the amount of protein loading. The activity of β-galactosidase was measured as described previously (29) and then normalized to the abundance of cellular σ^{28}_{Ct} protein. The resultant relative β-galactosidase activity was used to evaluate σ^{28}_{Ct} function.

Core-binding assays: coimmunoprecipitation. To test the in vivo core-σ binding, cellular lysates of *Salmonella fliA* mutant TH7034 expressing σ^{28}_{Ct} (from pLF28 or its derivatives) in buffer (10 mM Tris [pH 7.5], 150 mM NaCl) were used for coimmunoprecipitation. To test core-σ binding in vitro, a reaction mixture (15 μl) containing purified σ^{28} proteins (4 pmol) and *E. coli* core RNAP (1 pmol) (Epicenter) in buffer A (10 mM Tris-Cl [pH 7.5], 1 mM β-mercaptoethanol, 0.3 M NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.01% Triton 100, 250 μg/ml bovine serum albumin) was used. Monoclonal antibody B8R13 (1 μl) against the β subunit was incubated with cell lysate or a mixture of σ^{28} and core at 4°C overnight, and then complexes of core and σ were purified using protein A-agarose (Sigma). After three time washes with buffer A to remove unbound σ factor, the bound proteins were separated on a 10% SDS-polyacrylamide gel, followed by Western blot analysis. Bands of the bound σ^{28}_{Ct} and β subunit were probed with polyclonal σ^{28}_{Ct} antibody and β antibody (8RB13), respectively. Protein bands were quantified with Quantity One software (Bio-Rad).

In vitro transcription assays. The σ^{28}RNAP holoenzyme was formed by incubation of a threefold excess of purified His_6-tagged σ^{28} protein or derivatives with *E. coli* core RNAP enzyme (Epicenter) on ice for 15 min. In some cases, the ratio of mutated σ^{28} to core enzyme was increased in order to make σ^{28}-saturated RNAP. σ^{70}RNAP holoenzyme was purchased from Epicenter. The in vitro transcription system contained RNAP holoenzyme, supercoiled plasmids (1 μg), 10 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 5 mM dithiothreitol, and transcription was initiated by adding 400 μM ATP, 400 μM UTP, 1.2 μM CTP, 0.20 μM [α-^{32}P]CTP (3,000 μCi/mmol), and 100 μM 3′-*O*-methylguanosine 5′-triphosphate (GE Healthcare). The reaction was performed as described previously (39). mRNA made by the RNAP holoenzyme was separated and detected on a 6% (wt/vol) polyacrylamide–8 M urea polyacrylamide gel electrophoresis. Signal intensities from autoradiographs were determined with Quantity One software (Bio-Rad).

TABLE 1. Strains and plasmids used in this study

Species and strain or plasmid	Description	Reference or source
C. trachomatis ATCC VR-346	Serovar F/IC-Cal-13	ATCC
E. coli		
KS1	MC1000 F′ *lacI*^{q} integrated with a single copy of promoter p*lac* O_R2-62 controlled by *lacZ* in the chromosome	8
ZY101	MC1000 F′ *lacI*^{q} integrated with a single copy of P*lac_hctB*-35(TAAAGTTT)::*lacZ* on an F′episome	This study
ZY102	MC1000 F′ *lacI*^{q} integrated with a single copy of P*lac_hctB*-35(gAAAGTTT)::*lacZ* on an F′ episome	This study
BN317	MC1000 F′ *lacI*^{q} integrated with a single copy of p*lac*Cons-35C::*lac*Z on an F′ episome	34
S. enterica serovar Typhimurium		
TH7034	Isogenic with wild-type strain LT2; *fliA*5886 [R91C L207P] *fliC*5050::MudJ	K. T. Hughes
TH5504	Isogenic with wild-type strain LT2; *fliA*5647::FRT	K. T. Hughes
Plasmids		
pFW11		45
pS28H	P_{araB}-His_6 chlamydial *fliA*	39
pES28H	P_{araB}-His_6 *E. coli fliA*	39
pH$\sigma^{28}_{Ct\text{-}Ec4}$	P_{araB}-His_6 hybrid *fliA*; encoding σ^{28}_{Ct} residues 1–178 fused to region 4 of σ^{28}_{Ec} residues 166 to 239	This study
pH$\sigma^{28}_{Ec\text{-}Ct4}$	P_{araB}-His_6 hybrid *fliA*; encoding σ^{28}_{Ec} (residues 1–165) fused to region 4 of σ^{28}_{Ct} (residues 179–253)	This study
pRV5	Promoter probe vector containing less *lacZ*; ligation of the XmnI-SphI fragment of pQF50KgroE and the MfeI (blunted)-SphI fragment of pTara; origin of replication of p15A; Cm^r	This study
pRVP$_{hctB}$	pRV5 with promoter of chlamydial *hctB* (−125 to +45)	This study
pQF50KgroE	Promoter reporter vector containing P_{groE}::*lacZ*	24
pTara	Expression vector carrying P_{araB}-T7RNAP	47
pACλcI	P_{lacUV5}-directed synthesis of the λCI protein	8
pACλcI-β-flap	P_{lacUV5}-directed synthesis of the λCI protein fused via three alanines to residues 858–946 of the β subunit of *E. coli* RNAP	25
pACλcI-β-flap$_{Ct}$	P_{lacUV5}-directed synthesis of the λCI protein fused via three alanines to residues 798–887 of the β subunit of *C. trachomatis* RNAP	This study
pBRα	P_{lacUV5}/P_{lpp}-directed synthesis of the α subunit of *E. coli* RNAP	8
pBRαLN	P_{lacUV5}/P_{lpp}-directed synthesis of N terminus (residues 1–248) of the α subunit of *E. coli* RNAP	8, 10
pBRα-σ^{66}	P_{lacUV5}/P_{lpp}-directed synthesis of the αNTD fused to *C. trachomatis* σ^{66} region 4 (residues 468–571 of σ^{66})	This work
pBRα-σ^{70}	P_{lacUV5}/P_{lpp}-directed synthesis of the αNTD fused to *E. coli* σ^{70} region 4 (residues 528–613 of σ^{70})	10
pBRα-σ^{70}D581G	P_{lacUV5}/P_{lpp}-directed synthesis of the αNTD fused to *E. coli* σ^{70} region 4 carrying the D581G substitution.	25
pBRα-σ^{28}	P_{lacUV5}/P_{lpp}-directed synthesis of the αNTD fused to chlamydial σ^{28}_{Ct} region 4 (residues 148–253 of σ^{28})	This study
pBRα-σ^{28}E206G	pBRα-σ^{28} encoding the E206G substitution in the β moiety of the fusion	This study
pBRα-σ^{28}Y214C	pBRα-σ^{28} encoding the Y214C substitution in the β moiety of the fusion	This study
pBRα-σ^{28}E222G	pBRα-σ^{28} encoding the E222G substitution in the β moiety of the fusion	This study
pBRα-σ^{28}Q236L	pBRα-σ^{28} encoding the Q236L substitution in the β moiety of the fusion	This study
pBRα-σ^{28}L243K	pBRα-σ^{28} encoding the L243K substitution in the β moiety of the fusion	This study
pGP$_{hctB}$	Contains a chlamydial-*hctB*-controlled guanine-less cassette and an *E. coli fliC* promoter-controlled guanine-less cassette; Ap^r	40
pGP$_{hctB}\Delta_{up}$	Derivative of pGP$_{hctB}$ with the AT-rich sequence upstream of the promoter −35 element deleted	40
pGS9	Derivative of pGP$_{hctB}$ containing a 9-bp spacer between the −35/−10 elements	40
pGS14	Derivative of pGP$_{hctB}$ containing a 14-bp spacer between the −35/−10 elements	40
pGP$_{tar}$	Contains an *E. coli tar*-controlled guanine-less cassette and an *E. coli fliC* promoter-controlled guanine-less cassette; Ap^r	40
pGP$_{tar+}$	Derivative of pGP$_{tar}$ fusing a DNA sequence upstream of P_{hctB} to the core promoter of P_{tar}	40
pGHR	Contains a P_{hctB}-controlled guanine-less cassette and a chlamydial rRNA P1 promoter-controlled guanine-less cassette	This study
pLF28	Low-copy-number expression vector carrying chlamydial *fliA*; Tc^r	39
pLF28a	Derivative of pLF28 with a silent mutation at nucleotide position 723 to remove the natural HindIII site	This study

RESULTS

Replacement of σ^{28}_{Ct} region 4 with σ^{28}_{Ec} region 4 changes the behavior of RNAP holoenzyme in vitro. Previously, we found that σ^{28}_{Ct}RNAP is permissive in recognizing promoters with altered length between the promoter −35 and −10 elements, and preferentially activates promoters with upstream AT-rich sequences; in contrast the σ^{28}RNAP of *E. coli* (σ^{28}_{Ec}RNAP) does not have such a preference (40). In order to test whether differences in region 4 of σ^{28} were related to the observed disparity, we assessed the complementary functionality of σ^{28}_{Ct} region 4 by

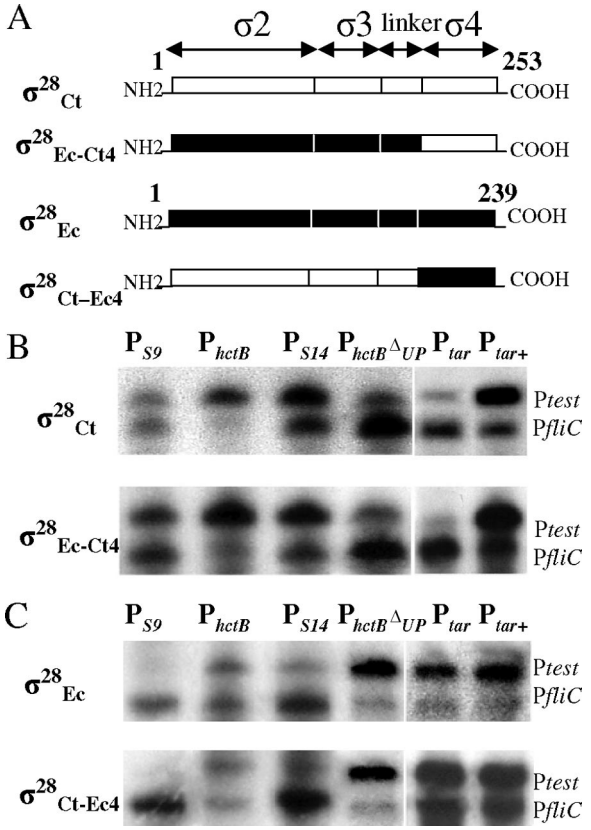


FIG. 1. In vitro promoter selectivity of holoenzymes containing hybrid σ^{28} proteins. (A) Construction of hybrid σ^{28}, showing the organization of σ^{28}_{Ct}, σ^{28}_{Ec}, $\sigma^{28}_{Ec\text{-}Ct4}$ (σ^{28}_{Ec} residues 1 to 165 fused to region 4 of the σ^{28}_{Ct} [residues 179–253]), and $\sigma^{28}_{Ct\text{-}Ec4}$ (σ^{28}_{Ct} residues 1 to 178 fused to region 4 of σ^{28}_{Ec} [residues 166 to 239]). (B) Gel analysis of in vitro transcription products using the RNAP holoenzyme containing σ^{28}_{Ct} or hybrid $\sigma^{28}_{Ec\text{-}Ct4}$ protein. (C) Gel analysis of in vitro transcription products using the RNAP holoenzyme containing σ^{28}_{Ec} or hybrid $\sigma^{28}_{Ct\text{-}Ec4}$ protein. RNAP is designated by the σ^{28} proteins as indicated on the left. Above each lane is shown the test promoter used in the transcription assay. On the right side of each panel are shown transcripts from different test promoters. P_{fliC} serves as an internal control in the same reaction.

making a reciprocal pair of hybrid σ^{28} proteins. Specifically, region 4 of σ^{28}_{Ec} was exchanged with the relevant region 4 of σ^{28}_{Ct} (Fig. 1A). *S. enterica* serovar Typhimurium *fliA* mutant strain TH7034 carrying the constructs directing the synthesis of the hybrid σ^{28} ($\sigma^{28}_{Ec\text{-}Ct4}$ and $\sigma^{28}_{Ct\text{-}Ec4}$) appeared as red colonies on MacConkey-lactose-arabinose indicator plates, suggesting that both $\sigma^{28}_{Ec\text{-}Ct4}$ and $\sigma^{28}_{Ct\text{-}Ec4}$ transcribe from the same *fliC* promoter that was recognized by wild-type σ^{28}_{Ct} (23, 40).

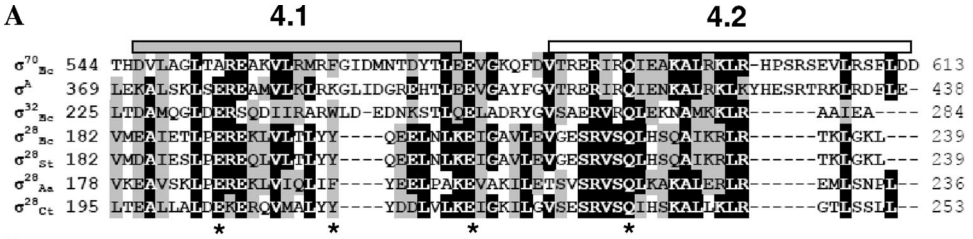
We next tested the in vitro activities of purified $\sigma^{28}_{Ec\text{-}Ct4}$ or $\sigma^{28}_{Ct\text{-}Ec4}$ in the context of a holoenzyme with several defined σ^{28} promoters. We chose the chlamydial *hctB* promoter (P_{hctB}) (native AT-rich upstream sequence plus core promoter of *hctB*), chlamydial *hctB* promoters with spacer lengths of 9 or 14 bp between the −35 and −10 element (P_{S9} and P_{S14}), the core promoter of *hctB* ($P_{hctB}\Delta_{up}$), and the *E. coli tar* promoter (P_{tar}) (native GC-rich upstream sequence plus core promoter of *tar*) and its derivative P_{tar+} (AT-rich upstream sequence of *hctB* plus core promoter of *tar*) (40). In the reaction mixture containing plasmid templates and reconstituted RNAP holoenzymes, the resultant transcripts were dependent on the addition of RNAP holoenzyme. RNAP holoenzyme made from hybrid $\sigma^{28}_{Ec\text{-}Ct4}$, which contains σ^{28}_{Ct} region 4, exhibited higher activities with P_{S9}, P_{hctB}, P_{S14}, and P_{tar+} (P_{test}/P_{fliC} transcript ratio of ≥1) but was weakly active with $P_{hctB}\Delta_{up}$ and P_{tar} (P_{test}/P_{fliC} ration of <1) (Fig. 1B). Such behaviors of $\sigma^{28}_{Ec\text{-}Ct4}$ RNAP are comparable to those of wild-type σ^{28}_{Ct}RNAP. In contrast, reconstituted $\sigma^{28}_{Ct\text{-}Ec4}$RNAP, which contains σ^{28}_{Ec} region 4, conferred strong activities for P_{hctB}, $P_{hctB}\Delta_{up}$, P_{tar}, and P_{tar+}, but it was weakly active with P_{S9} and P_{S14} (Fig. 1C). These actions are in agreement with results from using wild-type σ^{28}_{Ec}RNAP. Taken together, the results obtained with the hybrid σ^{28} proteins indicate that the observed differences in promoter selectivity between σ^{28}_{Ct} and σ^{28}_{Ec} are, at least in part, specified by region 4.

Identification of mutants defective in σ^{28}_{Ct}-dependent transcription in *Salmonella*. We next sought to identify residues in region 4 of σ^{28}_{Ct} that are important for σ^{28}_{Ct}-dependent transcription. A plasmid library carrying mutagenized σ^{28}_{Ct} genes driven from an arabinose-inducible P_{araB} promoter was transformed into *Salmonella fliA* mutant TH7034, which allowed us to screen mutants defective in transcription from σ^{28}-dependent *fliC*::*lacZ*. In this background, only σ^{28}_{Ct}-dependent transcription can be detected, because no functional *Salmonella* σ^{28} exists. Cells were selected for a lower level of σ^{28}-dependent transcription of *lacZ* (white or pink colonies) than for the wild type (red colony) by plating on MacConkey-lactose-arabinose agar. The candidates were further confirmed by measurement of β-galactosidase activity. Approximately 7,000 clones were screened, and four with single-residue substitutions in σ^{28}_{Ct} (E206G, Y214C, E222G, and Q236L) were isolated (Fig. 2). These mutants were further characterized as described below.

Mutations in region 4 of σ^{28}_{Ct} decrease transcription from the chlamydial *hctB* promoter. We compared the effects of σ^{28}_{Ct} mutants on transcription from the σ^{28}_{Ct}-dependent *hctB* promoter in *Salmonella.* Plasmid-encoded wild-type σ^{28}_{Ct} or derivatives were expressed in *Salmonella fliA* mutant strain TH5504 carrying pRV_{hctB}, and σ^{28}_{Ct}-driven transcription from P_{hctB}::*lacZ* was assessed by measurement of β-galactosidase activity as described in Materials and Methods. Relative β-galactosidase activities from strains carrying σ^{28}_{Ct} substitutions Y214C, E222G, and Q236L were significantly lower than that of wild-type σ^{28}_{Ct} (i.e., 7.9%, 25.1%, and 1.0%, respectively) (Fig. 3A). σ^{28}_{Ct} E206G also decreased levels of β-galactosidase activity, to about 41.9% relative to wild-type σ^{28}_{Ct}.

Next, the direct effect of mutant σ^{28}_{Ct}RNAP holoenzyme on transcription from P_{hctB} was examined in vitro. In the presence of both σ^{28}_{Ct}RNAP and σ^{70}_{Ct}RNAP holoenzymes, plasmid template pGHR produced two divergent transcripts: a 152-bp transcript from the σ^{28}-dependent P_{hctB} and a 130-bp transcript from the σ^{66}-dependent rRNA P1 ($P1_{CtrRNA}$) (data not shown). The RNAP containing σ^{28}_{Ct} Y214C or Q236L was severely defective in generating transcripts from *hctB*, showing a yield of less than 10% of the wild-type level (Fig. 3B). The RNAP containing σ^{28}_{Ct} E206G or E222G reduced transcription activity to 79.1% and 49.0% of that of wild-type σ^{28}_{Ct}RANP, respectively (Fig. 3B). None of the mutant σ^{28}_{Ct}RNAPs was able to transcribe from the $P1_{CtrRNA}$. We also

A



B

Regions	Codon substitution	Position in gene of σ^{28}_{Ct}	Substitution in σ^{28}_{Ct}	Relevant position				
				σ^{70}_{Ec}	σ^{A}	σ^{32}_{Ec}	$\sigma^{28}_{Ec}/\sigma^{28}_{St}$	σ^{28}_{Aa}
4.1	GAG→GGG	A617G	E206G	E555	E380	S236	E193	E189
4.1	TAC→TGC	A641G	Y214C	F563	K388	W244	Y201	F197
4.2	GAA→GGA	A665G	E222G	E575	E400	E255	E209	E205
4.2	CAG→CTG	A708T	Q236L	Q589	Q414	Q269	Q223	Q219

FIG. 2. Mutagenesis of σ^{28}_{Ct} region 4. (A) Alignment of the amino acid sequences of region 4 from *E. coli* σ^{70}, *Thermus aquaticus* σ^{A}, *E. coli* σ^{32} (σ^{32}_{Ec}), *Salmonella* σ^{28} (σ^{28}_{St}), σ^{28}_{Ec}, *Aquifex aeolicus* σ^{28} (σ^{28}_{Aa}), and σ^{28}_{Ct} using the CLUSTAL W program. The amino acid sequence of σ^{28}_{Sa} is identical to that of σ^{28}_{Ec}. Regions were defined based on previous studies (26, 41). The number for each amino acid position is relative to that from the start of each protein sequence. Identical or similar residues are shown in black or gray shadow, respectively. Asterisks at the bottom indicate residues undergoing mutation. (B) Isolation of σ^{28}_{Ct} mutants. Mutated nucleotides and the deduced amino acid residues are indicated based on the position of σ^{28}_{Ct} and corresponding residue positions in different σ factors.

noted that the RNAP saturated with mutated σ^{28}_{Ct} did not mount transcription activity in vitro. Moreover, mutated σ^{28}_{Ct}RNAPs did not change their behavior for transcription from P_{S9}, P_{hctB}, P_{S14}, $P_{hctB}\Delta_{UP}$, P_{tar}, and P_{tar+} in vitro (data not shown).

Taken together, these transcription studies indicate that σ^{28}_{Ct} substitutions Y214C and Q236L severely decreased transcription from *hctB*, whereas substitutions E206G and E222G moderately affected *hctB* transcription.

Effect of σ^{28}_{Ct} substitutions on core binding. The abilities of wild-type and mutant σ^{28}_{Ct} to bind core RNAP were compared using coimmunoprecipitations. The levels of mutant proteins that bound to *Salmonella* core RNAP were similar to or slightly higher than the wild-type level. (Fig. 4). As validation, we also examined potential core-binding differences among these proteins using the *E. coli* core and purified recombinant σ^{28}_{Ct} or derivatives in vitro. We found that each of the σ^{28}_{Ct} mutants effectively bound *E. coli* core RNAP by coimmunopreciptation relative to wild-type σ^{28}_{Ct} (data not shown). This confirms that none of the σ^{28}_{Ct} substitutions severely reduced σ affinity for the core RNAP under our test conditions.

Influence of σ^{28}_{Ct} substitutions on the interaction of σ^{28}_{Ct} region 4 with the β-flap. We examined whether substitutions in the σ^{28}_{Ct} region 4 would disrupt protein-protein interaction of σ^{28}_{Ct} region 4 with the β-flap in a bacterial two-hybrid assay (8, 10). This assay involves the use of (i) a reporter strain, KS1, which carries a chromosomal copy of the test promoter p*lac* O_R2-62 linked to *lacZ*; (ii) a plasmid encoding the λcI fused to the β-flap from *Chlamydia* (λcI-β-$flap_{Ct}$) or *E. coli* (λcI-β-flap); and (iii) a second plasmid encoding the N-terminal domain of αNTD fused to σ^{28}_{Ct} region 4 or its derivatives. Interaction of the β-flap with σ^{28}_{Ct} region 4 stabilizes RNAP binding to the test promoter, thus, mediating transcription activation from the test promoter p*lac* O_R2-62::*lacZ*. This can be monitored by measuring β-galactosidase activity (Fig. 5A).

As shown in Fig. 5B, in the presence of the chlamydial β-flap fusion (cI-β-$flap_{Ct}$), the chimera α-σ^{28}_{Ct} activates transcription from p*lac* O_R2-62 up to ~14.0-fold relative to that of the negative control α expressed from pBR-α. Such an increased magnitude compared to no obvious increase in the paired α-σ^{70}/cI-β-flap did not surprise us, because σ^{28}_{Ct} contains a residue at G228 corresponding to σ^{70}D581 (25, 34). Substitution at σ^{70}D581G stabilizes the folded structure of the tethered σ^{70} region 4 moiety and enhances interaction of σ^{70} region 4 with the DNA −35 element (25, 34). In strains coexpressing chimera λcI-β-$flap_{Ct}$ and chimera α-σ^{28}E206G, α-σ^{28}Y214C, or α-σ^{28}E222G, the magnitude of transcription activation from p*lac* O_R2-62 decreased about ~8.3-, ~4.5-, and ~6.9-fold, respectively. The decrease of transcription activation observed is not due to instability of chimera proteins, as the chimeras were able to interact with the *hctB* −35 element in a one-hybrid assay (Fig. 6). In contrast, chimera α-σ^{28}Q236L strengthened the interaction between the β-flap and σ^{28} in the presence of λcI-β-$flap_{Ct}$; transcription from p*lac* O_R2-62 was increased up to ~23.4-fold (Fig. 5B) compared to the wild-type σ^{28}_{Ct} (~14.0-fold increase).

In the presence of an *E. coli* β-flap fusion (cI-β-flap), the α-σ^{28}_{Ct} chimera increased transcription from p*lac* O_R2-62 up to ~8.5-fold, compared to ~14-fold in the presence of the cI-β-$flap_{Ct}$ chimera. Substitutions E206G, Y214C, and E222G in σ^{28}_{Ct} reduced transcription from p*lac* O_R2-62 to ~3.4-, ~3.4-, and ~5.4-fold, respectively. However, the α-σ^{28}_{Ct} Q236L chimera stimulates transcription from p*lac* O_R2-62 ~14.4-fold, compared to a change of ~23.4-fold in the presence of the cI-β-$flap_{Ct}$ chimera (Fig. 5C).

These results indicate that substitution E206G, Y214C, or E222G in σ^{28}_{Ct} weakens interactions between σ^{28}_{Ct} and the β-flap from both *E. coli* and *Chlamydia*. Thus, these residues are involved in interaction of the cI-β-flap with σ^{28} region 4, whereas σ^{28}_{Ct} residue Q236 seems not to be important for β-flap binding. *E. coli* and chlamydial β-flap share 62.2% amino acid identity and 73.8% similarity. The finding that σ^{28}_{Ct}

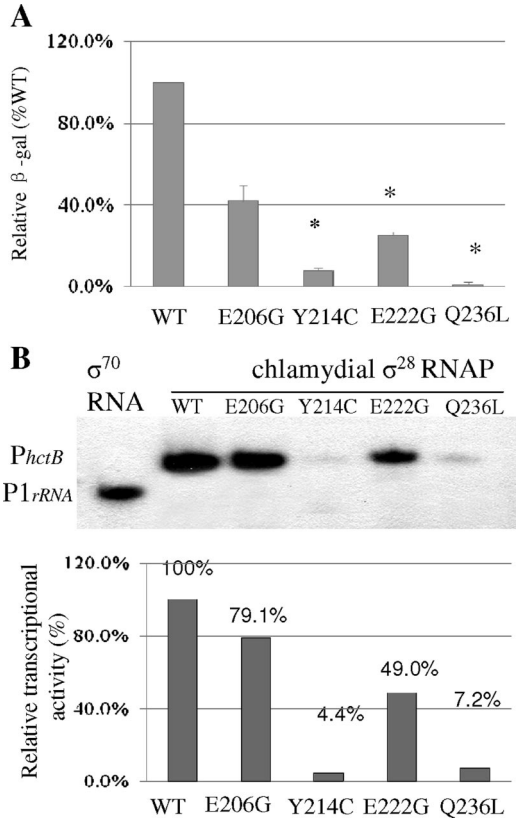


FIG. 3. Effect of single-residue substitution on σ^{28}-dependent transcription from chlamydial *hctB* promoter. (A) Activity of mutated $\sigma^{28}{}_{Ct}$ in vivo using a *lacZ* reporter assay. Cellular β-galactosidase activity was normalized to levels of $\sigma^{28}{}_{Ct}$ determined by Western blot analysis using specific anti-σ^{28} antibody. We previously found that mutation in region 4 of $\sigma^{28}{}_{Ct}$ did not impair its reactivity with the antibody to $\sigma^{28}{}_{Ct}$. The β-galactosidase activity from each strain containing the indicated mutated proteins is represented as a percentage relative to the β-galactosidase activity of the strain with wild-type $\sigma^{28}{}_{Ct}$. Asterisks above the bars indicated significantly reduced activities compared with that of the wild type ($P < 0.05$). (B) Transcripts from single-round transcription assay in vitro. Plasmid pGHR containing two chlamydial promoters (P_{hctB} and $P1_{CtrRNA}$) was used as a template in the presence of σ^{70}RNAP or $\sigma^{28}{}_{Ct}$RNAP as indicated on the top. The transcripts generated from promoters are indicated on the left. The intensity of each transcript band was quantified using Quantity One. The amount of transcript produced by each mutant RNAP is reported as a percentage of the transcription of wild-type $\sigma^{28}{}_{Ct}$RNAP.

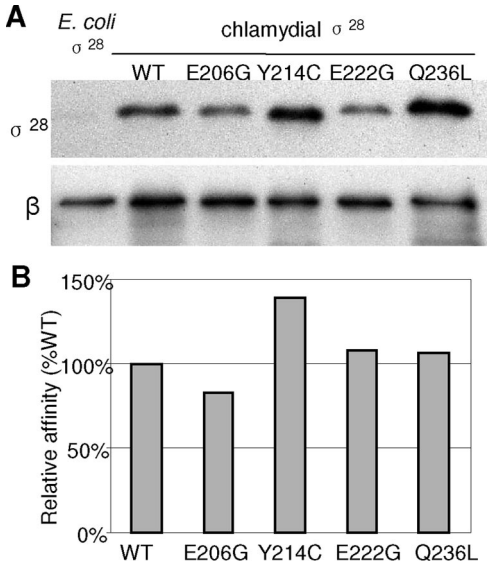


FIG. 4. Binding of σ^{28} and derivatives to core RNAP in *Salmonella*. (A) Immunoblot showing the results of a coimmunoprecipitation assay from *Salmonella fliA* mutant TH7034, which expresses σ^{28} or derivatives as indicated above each lane. A strain expressing *E. coli* σ^{28}, which is identical to *Salmonella* σ^{28}, was the negative control. Cell lysates were subjected to immunoprecipitation using anti-β monoclonal antibody as described in Materials and Methods. Precipitated proteins were separated by PAGE and immunoblotted with anti-σ^{28} antibody or anti-β monoclonal antibody. (B) Relative binding affinity of σ^{28} or derivatives for core. The amount of precipitated σ^{28} protein is reported as a percentage of the level of β in core RNAP.

can bind the *E. coli* β-flap could be relevant to its ability to transcribe from both $\sigma^{28}{}_{Ec}$- and $\sigma^{28}{}_{Ct}$-dependent promoters when it associates with the *E. coli* core enzyme in a heterologous genetic system or in vitro.

Effect of $\sigma^{28}{}_{Ct}$ mutants on binding of the −35 element from the *hctB* promoter. We next examined whether residue substitutions in $\sigma^{28}{}_{Ct}$ region 4 would affect interaction of σ region 4 and the −35 element of the promoter, using a one-hybrid assay (34). This in vivo DNA-binding assay is designed to use a test promoter, which contains an ectopic *hctB* −35 element upstream of the *lac* promoter in strain ZY101, and a plasmid-encoded α-σ^{28} fusion. The direct interaction of σ^{28} region 4 with the ectopic *hctB* −35 element recruits and stabilizes RNAP to the test promoter, causing an increase of reporter gene (*lacZ*) expression. This can be monitored by measuring β-galactosidase activity (Fig. 6A).

We introduced a plasmid encoding chimera α-σ^{28} or its derivatives into the reporter strain ZY101. We chose chimera α-σ^{66} as a negative control, as previous report showed that chlamydial σ^{66} did not recognize the σ^{28}-dependent promoter (48). The σ^{28}L243K mutant bears enhanced transcriptional activity from the *hctB* promoter (Z. Hua et al., unpublished data) and was the positive control. In the presence of α-σ^{28}, transcription activation from the test promoter was observed, and the increase of stimulation occurred in an IPTG dose-dependent fashion (Fig. 6B). Similarly, expression of α-σ^{28}E206G, α-σ^{28}E222G, or α-σ^{28}Y214C was able to activate transcription from the test promoter. Although the stimulations are small (<1.5-fold), these increases are reproducible in all experiments. Chimera α-σ^{28}L243K increased transcription up to 1.8-fold, indicating that this system functioned. In contrast, chimera α-σ^{28}Q236L failed to stimulate transcription from the test promoter (Fig. 6B). Because α-σ^{28}Q236L interacts with chimera λcI-β-flap (Fig. 5), the inability of α-σ^{28}Q236L to interact with the ectopic promoter is unlikely to be related to the poor protein expression. The substitution $\sigma^{28}{}_{Ct}$Q236L disrupted α-σ^{28}/*hctB* −35 interactions, which may be an explanation for our findings. As expected, chimera α-σ^{66} was unable to activate transcription from test promoter in ZY101 (Fig. 6B and C); however, α-σ^{66} stimulated transcription from p*lac*Cons-35C in BN317, which contains a σ^{70}-specific ectopic −35 element (data not shown).

In ZY102, which bears a mutant ectopic *hctB* −35 element

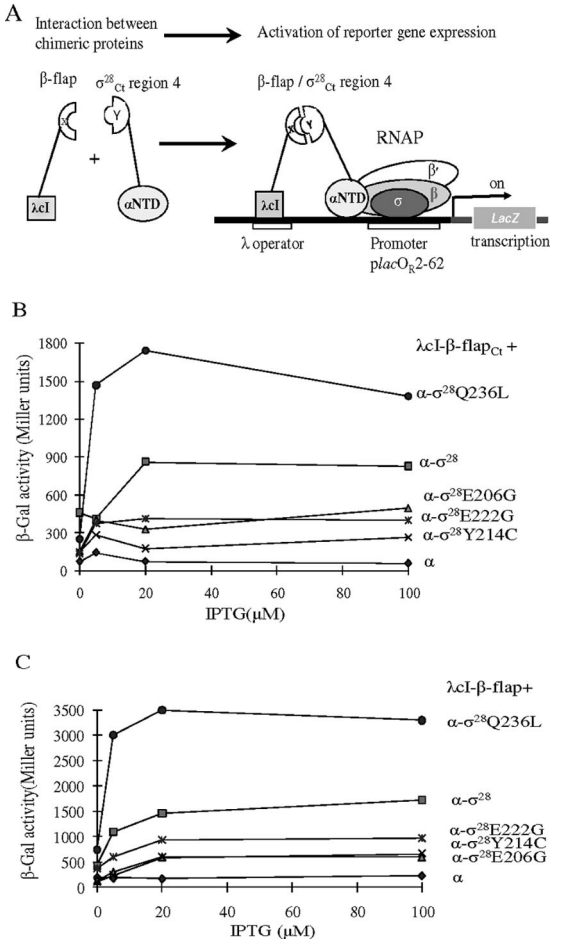


FIG. 5. Bacterial two-hybrid assay: characterization of σ^{28}_{Ct} substitution that affects its interaction with the β-flap. (A) Diagram showing how interaction of the σ^{28}_{Ct} region 4 (in pBRα-σ^{28}_{Ct}) with the β-flap (in pACλcI-β-$flap_{Ct}$ or pACλcI-β-flap) activates transcription from the test promoter p*lac* O_R2–62 located on the chromosome in KS1. (B) Effects of substitution in the σ moiety of the α-σ^{28}_{Ct} chimera on transcription from p*lac* O_R2–62 in the presence of the chimera λcI-β-$flap_{Ct}$. (C) Effects of substitutions in the σ moiety of the α-σ^{28}_{Ct} chimera on transcription from p*lac* O_R2–62 in the presence of the chimera λcI-β-flap. KS1 cells harboring two compatible plasmids, which direct the synthesis of α-σ^{28}_{Ct} or derivatives and λcI-β-$flap_{Ct}$ or pACλcI-β-flap as indicated, were grown in the presence of carbencillin, chloramphenicol, and kanamycin. Protein expression was induced by treatment with different concentrations of IPTG for 1 hour when cultures reached an optical density at 600 nm of 0.3. β-Galactosidase activity was measured in duplicate on at least three independent occasions. Values shown are the averages from one experiment.

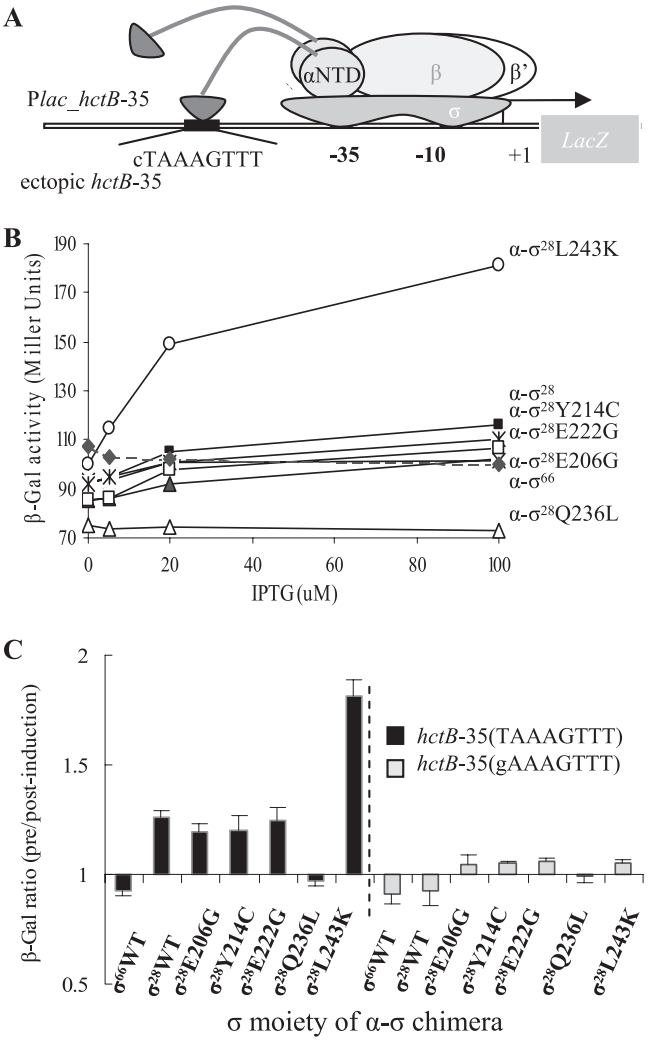


FIG. 6. Bacterial one-hybrid assay: characterization of the σ^{28}_{Ct} substitutions that affect interaction with the *hctB* −35 DNA element. (A) Cartoon illustrating the test promoter with wild-type or mutant ectopic *hctB* −35. The ectopic *hctB* −35 element serves as a binding site for the tethered σ^{28}_{Ct} region 4 moiety. When they interact with each other, transcription from the test promoter is activated, resulting in an increase of reporter *lacZ* (β-galactosidase). (B) Effects of substitution in the σ moiety of the α-σ^{28}_{Ct} chimera on transcription from the test promoter. Strain ZY101 harboring plasmids directing the synthesis of the α-σ^{28}_{Ct} or derivatives was grown in the presence of different concentrations of IPTG as indicated. β-Galactosidase activity was measured in duplicate on at least three independent occasions. Values shown are the averages from one experiment. (C) Change (*n*-fold) in the β-galactosidase activity before and after IPTG (100 μM) induction. Shown are changes of transcription from the test promoter with wild-type *hctB* −35 (TAAAGTTT) (in ZY101, black bars) or mutant *hctB* −35 (gAAAGTTT) (in ZY102, light gray bars). ZY101 and ZY102 harboring plasmids that directed the synthesis of α-σ^{28}_{Ct} or derivatives were grown in the absence or presence of IPTG and assayed for β-galactosidase activity. β-Galactosidase activity was measured in duplicate on at least three independent occasions.

(gAAAGTTT), none of the wild-type and mutant α-σ^{28} chimeras stimulated transcription from the test promoter (Fig. 6C). The first base pair T of the *hctB* −35 element has been shown to be the major determinant for σ^{28}_{Ct} recognition (49). This result confirmed that the observed stimulatory effects of α-σ^{28}_{Ct}, α-σ^{28}E206G, α-σ^{28}Y214C, and α-σ^{28}E222G in ZY101 are a result of the specific interaction of the chimeras with the ectopic *hctB* −35 element. Substitution σ^{28}_{Ct}Q236L interrupts interaction between σ^{28} and the *hctB* −35 element, indicating that σ^{28}_{Ct} Q236 is essential to be in contact with the −35 element.

DISCUSSION

We focused on characterization of several σ^{28}_{Ct} mutants with mutations in region 4 that are defective in σ^{28}-dependent transcription. Since there is no successful genetic system available to manipulate genes in *Chlamydia*, our strategy has been to study σ^{28}-dependent transcription using complementary ap-

proaches. By switching σ^{28}_{Ct} region 4 with σ^{28}_{Ec} region 4, we found that amino acids in σ^{28}_{Ct} region 4 might specify the function of the σ^{28}_{Ct}RNAP holoenzyme for use of an altered spacer length between promoter −35 and −10 elements, as well as preference for the UP-like sequences in vitro (Fig. 1). We also screened for σ^{28}_{Ct} mutants defective in transcription from the σ^{28}-dependent *fliC* promoter in *Salmonella* (Fig. 2). We then examined the effects of four single-residue substitutions in σ^{28}_{Ct} region 4 on transcription from the chlamydial *hctB* promoter both in vitro and in a *fliA* mutant *Salmonella* strain. We found that substitutions σ^{28}_{Ct}, Y214C and Q236L, significantly decreased transcription from the *hctB* promoter, while σ^{28}_{Ct}E206G and -E222G moderately reduced *hctB* transcription (Fig. 3); the promoter specificity was unchanged, and the effect was stronger in vivo than in vitro. A possible explanation for this is that the existence of additional host cell regulatory factors in vivo might lead to a bias for a diminished in vitro effect seen with σ^{28}_{Ct} mutations. It is unlikely that the difference observed is due to the inhibition of FlgM encoded by the surrogate strain of *S. enterica* serovar Typhimurium, because FlgM specifically negatively regulates *Salmonella* σ^{28} but not σ^{28}_{Ct} (22).

The apparent transcriptional deficiency observed might result from σ^{28}_{Ct} mutations that have a reduced affinity of σ with the core RNAP, a disrupted σ/β-flap interaction, and/or an occluded σ/promoter interaction. Our data do not support a direct strong effect of mutant σ^{28}_{Ct} on core affinity, since RNAP saturated by mutant σ^{28} did not increase transcription. Moreover, the mutant σ^{28}_{Ct} effectively bound core RNAP in a coimmunoprecipitation experiment (Fig. 4). However, modest indirect effects cannot be excluded. By taking advantage of the bacterial two-hybrid system, we found that the three substitutions in σ^{28}_{Ct}, E206G, Y214C, and E222G, impaired interaction of σ^{28}_{Ct} with the β-flap from *Chlamydia* or from *E. coli* (Fig. 5). Of these residues, only the corresponding residue σ^{28}_{Ct}Y214 has been mapped and directly contacts the β-flap in the X-ray structure of the *Thermus aquaticus* σ^{A} holoenzyme-DNA complex (34) (Fig. 7A and B). The corresponding residues of σ^{28}_{Ct}E206 and -E222 may indirectly involve such σ/β-flap interactions. The X-ray structure of *Aquifex aeolicus* σ^{28}, free or in complex with FlgM, the anti-σ^{28} factor, does not explain our observations, as both core and DNA-binding domains are buried in the folded compact structure (Fig. 7C) (41, 42). We speculate that σ^{28}_{Ct} is mostly in an active conformation when it associates with the core RNAP in *Salmonella* or in vitro, consistent with their transcription activities. Perhaps substitution E206G, Y214C, or E222G in σ^{28}_{Ct} caused a deficiency of σ^{28}_{Ct} in binding the β-flap, resulting in an unstable or mispositioned region 4 in the σ^{28}_{Ct}RNAP holoenzyme and an inability to simultaneously bind promoter −10 and −35 elements, leading to poor transcription activity. Our results further add support to the idea that RNAP remodeling of σ^{28} region 4 is required to expose the recognition domains of both the −35 binding site and β-flap on σ^{28} (41, 42). Previous studies indicated that the σ^{70} residues E555, F563, and E575 (corresponding to σ^{28}_{Ct} E206, Y214, and E222, respectively) (Fig. 3) have been implicated in interaction with the *E. coli* β-flap (12, 13).

Our data suggest a role of residue Q236 of σ^{28}_{Ct} in binding the −35 element of the *hctB* promoter because (i) substitution

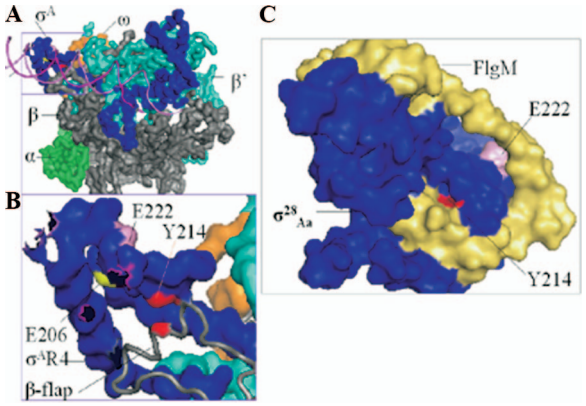


FIG. 7. Positions of residues in substitutions that affect the interaction of σ^{28}_{Ct} region 4 with the β-flap. (A) Locations of residues based on the crystal structures of *Thermus aquaticus* σ^{A} holoenzyme and DNA (PDB ID no. 1I9Z) (32). Shown are the surfaces of α (green), β-flap (gray), β′ (cyan), ω (orange), and σ factor (blue). The DNA promoter (magenta) is shown as a cartoon. (B) Enlarged illustration of region 4 of *T. aquaticus* σ^{A} (labeled with corresponding amino acid numbers in σ^{28}_{Ct}) (see amino acid alignments in Fig. 2) and the β-flap contact. The β-flap (gray) is shown as the cartoon. The DNA promoter is hidden. (C) Locations of residues based on the crystal structures of the σ^{28}_{Aa}/FlgM complex (labeled with corresponding amino acid numbers in chlamydial σ^{28}_{Ct}) (PDB ID no. 1SC5 and 1RP3) (41). Blue, σ^{28}_{Aa}; yellow-brown, FlgM. Residues corresponding to the *T aquaticus* σ^{A} or σ^{28}_{Aa} were identified and mutated to the naturally occurring residues in chlamydial σ^{28}_{Ct} as indicated. This figure was generated using Pymol 0.99rc6 (http://pymol.sourceforge.net).

Q236L in σ^{28}_{Ct} disrupted the interaction of σ^{28}_{Ct} with the *hctB* −35 sequence as determined by the one-hybrid assay (Fig. 6), and the same protein interacted with the β-flap as determined by the two-hybrid assay (Fig. 5); (ii) Q236 is located in the DNA-binding helix-turn-helix motif in of σ^{28}_{Ct}; and (iii) in *T. aquaticus* σ^{A}, the corresponding residue Q414 is structurally positioned on the DNA-binding interface (4). Consistent with our results, Kourennaia et al. (24) reported that the substitution Q269A in *E. coli* σ^{32} (corresponding to σ^{28}_{Ct} Q236) impeded recognition of the −35 element of the *E. coli groE* promoter but did not reduce its ability to bind to the core enzyme. The corresponding residue in σ^{70} (Q589) has not been implicated in contact with a specific DNA base. Instead, the adjacent residue R588 of σ^{70} can help position residue 585 for direct contact with bacteriophage P_{RM} DNA and mediate binding to the DNA activator (20). Presumably, the σ^{28}_{Ct} substitution Q236L, which changes a polar residue to an aliphatic hydrophobic residue, interrupts the side chain contacting the −35 element sequence and/or indirectly affects its ability to bind DNA by distorting or destabilizing the structure of σ^{28}_{Ct} region 4.

Given the favorable effect of the presence of AT-rich sequences upstream of the promoter on σ^{28}_{Ct}-dependent transcription (40), we wondered whether a possible positive influence is induced by the C-terminal domain of the α subunit (αCTD). The interaction of σ region 4 with the αCTD can stimulate transcription from a subset of UP element-dependent promoters by tightening RNAP-promoter associations (14). We failed to detect direct interaction of wild-type or mutant σ^{28}_{Ct} with the αCTD in a two-hybrid assay (Hua et al.,

unpublished data). We still cannot rule out the possibility that this association, if any, might be transient and/or weak, thus making it difficult to detect in vivo.

These studies have allowed us to begin defining the determinants in $\sigma^{28}{}_{Ct}$ that are essential for contact with the β-flap and with the −35 element; both are required for recognition of the −35/−10 promoter. Because there is no overlapping promoter recognition specificity between $\sigma^{28}{}_{Ct}$ and the primary σ^{66} (σ^{70}) (39, 40), it is fair to assume that there might be some differences regarding the σ/core interaction as well as σ/promoter interaction. Supporting this, $\sigma^{28}{}_{Ct}$ Q236 has been shown to be important for the $\sigma^{28}{}_{Ct}$-specific −35 element binding. This function has not been reported for the counterpart residue of *E. coli* σ^{70}. While this study does not directly address whether σ^{28} activity is inhibited by a regulator, observations with several $\sigma^{28}{}_{Ct}$ region 4 mutants defective in contact with the β-flap indicates that inactivation may play a role in chlamydial σ^{28} activity control. In the future, we will use a stabilized or deficient protein-binding mutant of chlamydial σ^{28} in order to facilitate identification and characterization of potential σ^{28} regulators in a two-hybrid assay.

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