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Mutational analysis of *Escherichia coli* σ^{28} and its target promoters reveal recognition of a composite −10 region, comprised of an "extended −10 motif" and a core-10 element

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Summary

σ^{28} controls the expression of flagella related genes and is the most widely distributed alternative σ factor, present in motile gram-positive and gram-negative bacteria. The distinguishing feature of σ^{28} promoters is a long −10 region (GCCGATAA). Despite the fact that the upstream GC is highly conserved, previous studies have not indicated a functional role for this motif. Here we examine the functional relevance of the GCCG motif and determine which residues in σ^{28} participate in its recognition. We find that the GCCG motif is a functionally important composite element. The upstream GC constitutes an extended −10 motif and is recognized by R91, a residue in Domain 3 of σ^{28}. The downstream CG is the upstream edge of −10 region of the promoter; two residues in Region 2.4, D81 and R84, participate in its recognition. Consistent with their role in base-specific recognition of the promoter, R91, D81 and D84 are universally conserved in σ^{28} orthologues. σ^{28} is the second Group 3 σ shown to use an extended −10 region in promoter recognition, raising the possibility that other Group 3 σs will do so as well.

Keywords

Group 3 σ; σ^{28}; extended −10 motif; −10 region; Region 2.4; Region 3.0

Introduction

Bacteria use a family of σ factors to orchestrate transcription. The housekeeping σ, called σ^{70} in *Escherichia coli*, directs core RNA polymerase ($\alpha_2\beta\beta'\omega$) to the vast bulk of promoters active during exponential phase, whereas alternative σ's direct core RNA polymerase to mediate the transcription of regulons required for specific tasks such as response to stress, or mediating growth transitions or development (Gross *et al.*, 1998, Paget & Helmann, 2003). The σ^{70} family of proteins has conserved modular domains with discrete functions. In addition to the N-terminal regulatory domain found only in housekeeping σs, there are three additional domains, each with recognition determinants both for core RNA polymerase and for portions of the promoter (Gruber & Gross, 2003). Thus, Domain 2 recognizes the −10

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region of the promoter; Domain 3 recognizes the "extended −10 motif" immediately upstream of the −10 region; and Domain 4 recognizes the −35 region of the promoter (Campbell *et al.*, 2002, Gross et al., 1998). In addition, Region 1.2 recognizes a motif downstream of the −10 region (Haugen *et al.*, 2006, Feklistov *et al.*, 2006). σs have been divided into four groups based on their phylogenetic relationships and modular structure (Paget & Helmann, 2003, Lonetto *et al.*, 1992, Gruber & Gross, 2003). Group 1 σs (housekeeping σs) are essential and have all domains; Group 2 σs are the most related to Group 1 σs but are not essential. Group 3 σs have Domains 2, 3, and 4. Group 4 σs have only Domains 2 and 4 and comprise the largest group of σs.

Promoter recognition has been extensively studied in σ^{70} and several other Group 1 σs (Siegele *et al.*, 1989, Kenney *et al.*, 1989, Waldburger *et al.*, 1990, Campbell et al., 2002, Sanderson *et al.*, 2003). However, less attention has been devoted 64 to promoter recognition in the alternative σs. σ^{28}, a Group 3 σ, is the most widely distributed alternative σ factor, making it an attractive candidate for study of its promoter recognition. σ^{28} controls expression of flagella-related genes in all motile Gram-negative and Gram-positive bacteria, and plays a role in development in some non-motile bacteria (e.g. Chlamydia) (Yu *et al.*, 2006b, Yu & Tan, 2003, Shen *et al.*, 2006, Chilcott & Hughes, 2000, Serizawa *et al.*, 2004). The conservation of this σ across millions of years of evolution is indicated by the fact that SigD, the σ^{28} orthologue in *B. subtilis*, can substitute for the function of σ^{28} in *E. coli* (Chen & Helmann, 1992). Likewise, the σ^{28} promoter sequence is conserved across organisms (Serizawa et al., 2004, Shen et al., 2006). Bioinformatic analysis of *E. coli* σ^{28} promoters suggests that their consensus sequence is TAAAgttt-N_{11}-GCCGATAA (Zhao et al., 2007). Mutational and biochemical analysis of a strong σ^{28} promoter validated the importance of the TAAA −35 motif and the CGA −10 motif. However, the functional relevance of the highly conserved GC motif of the −10 region remains in question (Yu *et al.*, 2006a, Wozniak & Hughes, 2008).

Additionally, there have been no studies of the amino acid residues in σ^{28} that mediate recognition of the −10 region. The studies in this report clarify both the functionally relevant bases in the −10 region of the promoter and the amino acid residues in σ^{28} partially responsible for recognizing this region. Our studies indicate that both the GC and the CGA motifs are functionally important in the −10 region of σ^{28} promoter, and that this is a composite element. The upstream GC motif is an extended −10 motif recognized by a residue in first helix of Domain 3, whereas the downstream CG is part of the −10 region recognized by residues in Region 2.4 of Domain 2.

Results

Construction of *in vivo* assay system

A robust *in vivo* assay system for identifying the residues in σ that mediate base-specific promoter interactions must be solely dependent on the activity of the particular σ mutant that is being assayed. Such an assay system has two requirements: the cellular regulatory loops that adjust the amount of σ^{28} to compensate for its activity deficit must be removed; and the promoter utilized must be sensitive to the level and activity of σ. We removed the cellular regulatory loops by using a host strain lacking both endogenous σ^{28} and its anti-σ, FlgM (Hughes *et al.*, 1993, Chadsey *et al.*, 1998). We based our promoter library on the core region (−44 to +10) of the *tar* promoter, which has consensus −35 (TAAAGTTT) and −10 (GCCGATAA) sequences (Fig. 1A). Using this promoter ensures that deviations at every position of the promoter can be assayed and that α binding to the UP element will not obscure defects of the mutant σ^{28} in binding to the promoter. Our assay system utilizes two plasmids, one supplying mutant σ^{28} alleles, and the other using variants of the σ^{28} promoter to drive expression of the *lacZ* reporter (Fig. 1B). Expression from the wild-type (wt) *tar*

promoter was <1 Miller unit without induction and ~ 900 Miller units following induction of wt σ^{28} (data not shown). Thus, this assay system has an excellent signal to noise ratio.

Determination of functional −10 promoter sequence of σ^{28}

We examined the effect of base changes at positions −14 to −10 in the *tar* promoter. Every base mutation at the CGA motif resulted in severe defects in transcription, consistent with previous results (Fig. 2A) (Yu et al., 2006a,Wozniak & Hughes, 2008). Additionally, single mutations at the upstream GC motif did not result in a significant decrease in transcription, with exception of −14G to C and −13C to G (Fig. 2B), as has previously been seen by Wozniak and Hughes on the *flgK* promoter (Wozniak & Hughes, 2008). We considered the possibility that only one of the conserved upstream GC bases was required for activity. Indeed, the two doubly mutated promoters that we tested, −14 −13 GC to TT or AA, were both severely defective in expression, exhibiting only 2~5% of the activity of the wt promoter (Fig. 2B). This defect is far greater than that expected from the behavior of the single mutants, each of which exhibited 60–80% of the activity of the wt promoter. The synergistic effect of the double mutants is consistent with the idea that either −14G or −13C but not both is required for high promoter activity.

Identification of residues in σ^{28} potentially important for recognition of the −10 region of the promoter

The studies above demonstrated that both the upstream GC motif and the CGA motif of the −10 region are functionally important. We recently found that the upstream CC motif in the promoters of another Group 3 σ, σ^{32}, constitutes an extended −10 motif (Koo *et al.*, 2009). Moreover, the GC motif in σ^{28} promoters aligns with other extended −10 motifs (Fig. 3A). Together, this led us to direct alanine substitution mutagenesis to σ regions implicated both in extended −10 recognition (Region 3.0 in Domain 3) and in −10 region recognition (Region 2.4 in Domain 2). Selection of amino acid residues for substitution in Region 2.4 was based on sequence conservation among σ^{28} orthologues and a comparison of σ^{70}, σ^{32}, and σ^{28} sequences. For Domain 3, we extensively substituted resides in the first helix, as this region is implicated in extended −10 recognition (Fig. 3B). When compared to wt σ^{28}, six of the twelve substitutions in σ^{28} resulted in a significant decrease in transcriptional activity on wt tar promoter (≤ 40% of wt σ^{28} , Fig. 3C). Three of these were located in Region 3.0 (R91A, R94A and R98A) and three in Region 2.4 (R74A, D81A and R84A). Interestingly, only R74 and D81 corresponded exactly to positions known to be involved in recognition of their cognate promoters of σ^{70}, σ^{32} and *B. subtilis* σ^{H} (Fig. 3B and Daniels et al., 1990). We then tested whether any of the alanine mutants met the genetic criterion for base-specific interaction; namely, suppression of the promoter defects of base changes limited to particular positions in the promoter. This criterion is based on the idea that if the mutant σ no longer recognizes a particular base, then base changes at that position should show a smaller than expected decrease in expression. This criterion has been used successfully both for transcription factors and for other σs (Koo et al., 2009, Hochschild *et al.*, 1986, Hochschild & Ptashne, 1986, Ebright, 1986, Ebright *et al.*, 1984, Siegele et al., 1989).

Identification of base-specific interactions between Region 2.4 of σ^{28} and the −10 promoter region

Two of the three Region 2.4 candidates, D81 and R84, met the criteria for mediating a base specific interaction. Both D81A and R84A had significant defects in transcription from wt *tar* promoter, exhibiting <2% of the transcription mediated by wt σ^{28} (Fig. 3C) and each suppressed all single base changes at a unique position in the promoter. D81A suppressed all single base changes at −11G at least 10-fold and R84A suppressed all single base changes at −12C at least 20-fold (Fig. 4A). The absolute values of β-galactosidase are very low, because both the mutant promoter and the mutant σ^{28} independently give severe

transcription defects. However, the results are very reproducible and significantly above background. In stark contrast, when D81A is assayed at positions other than −11, β-galactosidase activity is at the background level (compare activity at −14,13 or −12 with that at −11; Fig. 4B). Likewise, when R84A is assayed at positions other than −12, β-galactosidase activity is at the background level (compare activity at −14,13 or −11 with that at −12; Fig. 4B). Taken together, these results support the idea that D81 and R84 participate in base specific recognition of the promoter.

R74 is at the boundary between Regions 2.3 and 2.4 and was tested because the analogous position in σ^{70} (W434) and in the Group 3 σ, σ^{32} (W108) were important in −10 recognition and/or melting (Kourennaia *et al.*, 2005, Fenton *et al.*, 2000, Koo et al., 2009, Tomsic *et al.*, 2001). However, R74A did not suppress any mutations in the −10 promoter region. Instead, this 'loss of function' mutant had even less relative activity than wt σ^{28} on promoters that were mutant at the −14G and −13C positions (compare Fig. 4C (R74A) and Fig. 2 (wt)).

Identification of a base-specific interaction between Domain 3 of σ^{28} and the −10 promoter region

Of the three Domain 3 alanine substitution mutants that reduced expression from the wt promoter, only R91 meets the genetic criteria for mediating a base specific interaction. R91A reduces activity to 5% of wt σ^{28} (Fig. 3C) and suppressed almost all changes at −13C and −14G to a variable extent (Fig. 5A). Mutations to A or T at both −13 and −14 and to C at −14 were completely or almost completely suppressed. Only the C−13G change was not suppressed. These data suggest that R91 makes base-specific contacts with both −14G and −13C. In support of this idea, we find that R91A suppressed AA and TT changes at −14,−13 completely (Fig. 5A; about 200% for AA and 100% for TT). Interestingly R91 had previously been implicated as important for the transcriptional function of σ^{28} as it was part of a double mutant (R91C,L207P) isolated to be completely defective in expression from a σ^{28} promoter (Aldridge et al., 2006). In contrast, R91A did not suppress changes at −11 or −12, exhibiting β-galactosidase activity at the background level of the assay (data not shown).

The two other mutants that reduced activity from the wt promoter, R94A and R98A, showed about 40% and 15% of wt σ^{28} activity on wt *tar* promoter respectively. Intriguingly, R94A and R98A resulted in even less relative activity than wt σ^{28} on promoters that were mutant at the −14G and −13C positions (Fig. 5B). R94 and R98 are predicted to be on the same face of an α helix as R91. These residues might interact with promoter DNA nonspecifically to stabilize an interaction between R91 and −14G and −13C positions.

Effects of σ^{28} variants on transcription *in vitro*

Our *in vivo* assay identified D81, R84 and R91 as residues in σ^{28} that might mediate base specific interactions with the promoter. We tested whether we could replicate these results *in vitro*. We carried out single round *in vitro* transcription assays on linear templates with the same promoter sequences used for our *in vivo* assays and compared transcription driven by wt σ^{28} with that driven by σ^{28} variants carrying alanine substitutions for each of the three residues implicated in recognition. Results from *in vitro* studies were consistent with our *in vivo* assays in all respects. First, the *tar* promoter we utilized was absolutely σ^{28} dependent *in vitro* (Fig. 6A), and the D81A, R84A and R91A σ^{28} variants exhibited significantly decreased transcription from this promoter (5- to 10- fold; Figs. 6A and B) as was the case in our *in vivo* assays (Fig. 4A and 5A). Second, transcription of mutated promoters by wt σ^{28} showed the same rank order of transcription as expression assays *in vivo* (compare Fig. 6D with 4A and 5A). We note that there was generally more relative transcription from each mutant promoter *in vitro* than *in vivo*, especially of the −14, −13 AA and TT mutation (~30

and 13 % of the rate of the wt promoter; Fig. 6D) than *in vivo* (~5 and 2 %; Fig. 2B), possibly because the *in vitro* assay does not have wt competitor promoter. Third, and most importantly, we qualitatively reproduced position specific suppression of promoter mutations (Fig. 6C and D). Changes at −11G and −12C significantly decreased transcription with wt σ^{28} but were significantly suppressed (~3 to 5-fold) by D81A and R84A σ^{28} respectively.

Additionally, R91A completely suppressed the single and double A, T mutations tested at −14G and −13C. That we can reproduce position specific suppression in an *in vitro* transcription reaction indicates that suppression is a direct effect of the σ^{28} variant utilized and argues for D81, R84 and R91 mediating base specific interactions with the promoter.

Discussion

σ^{28} is the most widely distributed alternative σ factor and its consensus promoter sequence is also well conserved. Thus, information from the study of *E. coli* σ^{28} might suggest general mechanisms of transcription initiation by its orthologues in a wide range of bacteria. However, there were major uncertainties about the functionally relevant sequences in the −10 region of its promoter and how they were recognized by σ^{28}. In this study, we focused on the GCCG motif in the −10 region. We examined the importance of each base by using a newly developed sensitive *in vivo* assay and by reproducing these results *in vitro*. To identify residues in σ^{28} contributing to base specific interaction of the GCCG motif, we used a validated genetic criterion: loss of the residue interacting with a particular base should suppress the deleterious effects of promoter mutants only at the interacting position(s). Candidates were chosen using our *in vivo* assay and confirmed *in vitro*. Our principal finding was that the GCCG motif is a functionally important composite element: the upstream GC constitutes an extended −10 motif and is recognized by a single residue in Domain 3 of σ^{28}, whereas the downstream CG constitutes the upstream edge of −10 region of the promoter and is recognized by at least two residues in Region 2.4. A schematic comparing the interactions we propose to those in *E. coli* σ^{70} and *T. aquaticus* SigA is presented in Fig. 7A; a model based upon the *T. aquaticus* holoenzyme/fork-junction promoter DNA complex indicates the positions of these amino acids relative to the DNA (Fig. 7B).

The downstream CG is the upstream boundary of the −10 region

We identified D81 as a residue recognizing −11G and R84 as a residue recognizing −12C, based on our finding that an alanine substitution at each position specifically suppressed promoter mutations at the −11 and −12 positions respectively, both *in vivo* and *in vitro*. These residues are located in Region 2.4, which mediates recognition of the −10 promoter region, indicating that this CG motif is the upstream boundary of the −10 promoter region. Although suppression is not complete, our conclusion is supported by several additional pieces of data. First, the location of D81 and R84 is consistent with the function proposed, as a structure based alignment indicates that these amino acids are solvent exposed and are in position to contact the DNA (Fig. 7B). Second, D81 and R84 are universally conserved in σ^{28} orthologues, as expected from residues mediating base specific recognition (Fig. 7C). Additionally, D81 is at the same position in σ^{28} as residues in other Group 3 σs implicated in interacting directly or indirectly with the upstream portion of the −10 regions of their cognate promoters: M124 of *B. subtilis* σE (Tatti et al., 1991) and R96 of *B. subtilis* σH (Daniels et al., 1990). Finally, and provocatively, it has previously been proposed that the DXXR motif is utilized to recognize a CG sequence in the −10 promoter region of Group 4 σs (Wilson & Lamont, 2006). PvdS, a *Pseudomonas aeruginosa* Group 4 σ has a −10 region that starts with CGT. Alanine substitutions at D77 and R80 of PvdS were defective in binding to this promoter. Conversely, if σs have a DXXR motif in Region 2.4, the −10

region of their promoters are likely to have a CG motif. These σs include the Group 4 σs, CarQ of *Myxococcus xanthus*, σ^W and σ^X of *Bacillus subtilis*, and σ^C of *Mycobacterium tuberculosis* (Wilson & Lamont, 2006). Our finding indicates that using DXXR to recognize a CG motif extends to Group 3 σs.

That R84 mediates −10 recognition has important implications for understanding how σ^{28} itself is inhibited from promoter binding. Structural and biochemical studies from Darst and collaborators on *Aquifex aeolicus* σ^{28} suggested that interdomain interactions inhibit free σ^{28} from binding to the promoter and that this conformation is stabilized by a salt bridge between R82 and E145, E146 (Sorenson et al., 2004, Sorenson & Darst, 2006). Relevant here is that R84 of *E. coli* σ^{28} is the homologous residue of R82 in *Aquifex aeolicus*. Thus, it seems that the interactions in free σ^{28} sequester a residue that is critically important in base-specific recognition of the −10 region of the promoter.

All mutations at the −11G and −12C positions of the σ^{28} promoter were exceptionally deleterious, exhibiting only 1–3% of the activity of the wt promoter. In contrast, mutations at comparable positions of the promoter of another Group 3 σ (σ^{32} promoter; −13,−14 CC) exhibited between 10~40% of the activity of the wt promoter (Koo et al., 2009, Wang & deHaseth, 2003). What might account for this distinction? This distinction may reflect the fact that σ^{32} has σRegion 1.2 whereas σ^{28} does not. Both structural data and sequence alignments suggest that all Group 1 and 2 σs and some Group 3 σs have this region (Sorenson et al., 2004, Lonetto et al., 1992, Campbell et al., 2002, Gruber & Gross, 2003). Region 1.2 appears to have several roles in σ^{70}: it is required for open complex formation (Wilson & Dombroski, 1997), it recognizes the discriminator region, just downstream of −10 region of promoter (Haugen et al., 2006, Feklistov et al., 2006), and it may act as a core RNA polymerase (β' subsunit coiled-coil)-dependent allosteric switch that modulates recognition of the −10 promoter element by Region 2 (Zenkin et al., 2007). The role of this region in σ^{32} is unknown, but it may facilitate melting in some way. Because σ^{28} lacks this region, it may be more dependent on the precise composition of the −10 region for function. If this explanation is true, it begs the question of how a −12,−11 CG motif might facilitate melting. One possibility, originally suggested by Losick for σ^H of *B. subtilis* is that nucleotide substitutions at such positions might have an unfavorable effect on the exact conformation of the DNA duplex or its ability to be unwound (Daniels et al., 1990). Alternatively, or in addition, it may serve as a recognition motif for the σ^{28} residues that facilitate melting.

The upstream GC is an extended −10 motif

Our studies indicate that the highly conserved −14,−13 GC motif is functionally important but contains partially redundant information. Changing either−14G or −13C to A or T results in only a very modest (~20%) reduction in promoter strength *in vivo*. In stark contrast, a double AA or TT mutation at −14, −13 leads to a dramatic (20 ~ 50- fold) reduction in promoter strength. The synergistic effect of the double mutant on promoter activity suggests that only one of the two bases is required for function. Satisfyingly, the idea that either −14G or −13C, but not both, is required for high promoter activity resolves a discrepancy in the literature. Single changes at the upstream GC in the context of the *flgKL* promoter, which has both −14G and −13C, caused little or no defect in activity (Wozniak & Hughes, 2008), whereas single changes at −14G in context of the *C. trachomatis hctB* promoter, which has −14G and −13T caused significant defects in transcription (Yu et al., 2006a). In the latter case, mutation at the −14G position eliminates the upstream GC motif. Interestingly, we found that the −14G to C and the −13C to G single changes each reduce promoter activity ~5-fold. This issue is discussed below.

We identified R91 as the residue recognizing both −14G and −13C, based on our finding that an alanine substitution at R91 suppressed promoter mutations at only −14 and −13. Satisfyingly, the ~20-fold defect of R91A σ^{28} is approximately equivalent to the defect in expression observed when both −14G and −13C are removed from the wt promoter and substituted with AA or TT. This equivalence lends credence to the idea that R91 recognizes both positions, and also confirmed the importance of the GC motif for promoter function. Importantly, R91 is universally conserved in σ^{28} orthologues, as expected of a residue that recognizes specific bases in the promoter. Moreover, the hydrogen bond donor properties of arginine are consistent with the phenotype of the substitutions at the −14,−13 positions. Arginine could bond with both the non-template strand G at −14 and the template strand G at −13. Introduction of A or T at either −14 or −13 would still allow binding to the remaining G as well as to the A or T base substitution. There is likely to be sufficient interaction that little or no decrease in expression is observed; hence the requirement for a double mutation. In contrast, a C mutation at either position removes the possibility of hydrogen bonding probably accounting for the fact that single changes of this type exhibit reduced expression (~20% wt) (Luscombe et al., 2001).

R91 is located in the first α-helix in Domain 3 of σ^{28} and a structure based alignment indicates that R91 is solvent exposed and in position to contact the DNA (Fig. 7B). All residues mediating base-specific interactions with the extended −10 motif identified to date reside in this helix: H455/E458 in σ^{70} (Fig. 7A) and analogous residues in other Group 1 σs recognizing −15,−14 TG (Fig. 3B, Sanderson et al., 2003); K173 in σ^{s} recognizing −13C and possibly −14T (Becker & Hengge-Aronis, 2001); and K130 in σ^{32} recognizing −16,−15 CC (Fig. 3B,Koo et al. 2009). By analogy to other σs, this upstream −14,−13 GC constitutes an extended −10 motif. Importantly, the residues implicated in extended −10 recognition in different σs are all predicted to be on the same face of the first α-helix in Domain 3, supporting the proposed function of these residues. We note that the precise position of the residues mediating −10 recognition differs in various σ's, possibly because the length of linker between Domain 2 and 3 in different σs affects the positioning of the first α-helix of Region 3.0 relative to DNA. Alternatively, the somewhat discrepant position of K130 in σ^{32} could result from the fact that this putative helix is immediately adjacent to an insertion, called the RpoH box, which is the most highly conserved region in σ^{32} orthologues (Nakahigashi et al., 1995). At least one residue in this region of σ^{32} interacts with RNA polymerase (Joo et al., 1998) and this interaction might specifically alter the configuration of this helix in σ^{32}.

Group 3 σs have σDomain 3, which encodes the recognition determinants for the extended −10 promoter motif in Group 1 and 2 σs. In addition, these σs utilize promoters that have long −10 regions. These features suggested that the extended −10 motif might be also present in Group 3 σ promoters. However, Domain 3 of the Group 3 σs is the most variable domain in the Group 3 σs, making it dangerous to extrapolate that such recognition occurs. Indeed, two Group 3 σs (σ^{E} and σ^{H} in *B. subtilis*) were previously shown not to utilize an extended −10 motif. We demonstrate here that σ^{28} joins σ^{32} in utilizing an extended −10 motif. Since the σ^{28} branch of Group 3 σs is highly divergent from the σ^{32} branch (Gruber & Gross, 2003, Paget & Helmann, 2003), it is likely that extended −10 recognition will occur in other Group 3 σs In this regard, it is intriguing that promoters recognized by both σ^{B} and σ^{F} in *B. subtilis* have GG as their predicted extended −10 motif (Petersohn *et al.*, 2001, Amaya *et al.*, 2001) and also have an arginine at the position comparable to R91 in σ^{28}. Thus, an arginine residue in the first α–helix of Domain 3 of σ^{B} and σ^{F} might mediate recognition of one or both upstream G's.

Summary and prospects

We establish that the GCCG motif in the −10 region of σ^{28} promoters is a composite recognition element: the upstream GC is an extended −10 region recognized by R91, a residue in Domain 3, whereas the downstream CG initiates the −10 recognition region recognized by (at least) D81 and R84 in Region 2.4 of σ^{28}. Thus, σ^{28} joins σ^{32} as a Group 3 σ that not only uses extended −10 recognition, but also requires three recognition regions, −35, extended −10 and −10 for successful utilization of the core σ^{28} promoter. This study raises the possibility that additional Group 3 σs will have promoters exhibiting these properties and poses several provocative avenues of inquiry. We are interested in the features of Group 3 σs that cause them to have such extensive recognition requirements. We also wonder whether such a requirement might have regulatory advantages. For example, extensive recognition requirements prevent the promiscuous promoter recognition characteristic of housekeeping σs. Alternatively, or in addition, the construction of these promoters may discourage their transcription by the housekeeping σs. In this regard, it is interesting that not only many Group 3 σs but also many Group 4 σs have a G and/or C residue(s) at the upstream end of the −10 region. As G/C residues are never found in this position in housekeeping σ's, this may add to the distinction of promoters recognized by the alternative σs.

Experimental procedures

Strains and plasmids

Strains and plasmids used in this study are listed in Table 1. All strains were grown at 30°C in Luria-Bertani (LB) medium supplemented with appropriate antibiotics if needed.

Construction of *in vivo* assay strain

The *fliA* and *flgM* genes were disrupted using electroporated linear DNA amplified by PCR and *E. coli* DY330 as described previously (Yu et al., 2000). First, CAG57111 (DY330 Δ*flgM::cat*) was constructed by replacing open reading frame (ORF) of *flgM* gene by chloramphenicol resistant cassette (its own promoter and gene). Linear DNA for disruption of *flgM* was generated by amplification of *cat* gene in pACYC184 with the following primers; 5'- CCGATAAATAAGCA ACACATGATA AAAGCGCCCTCAATGAGGAATAAACC ATGGAGAAA AAAATCACTGG -3', and 5'- TAAGCACAGCGGACATCTGGTCGAGGATCTCTGCAAGACGTGTCATACGA TTACGCCCCGCCCTGCCACTC -3' (underlined sequences are complementary to the upstream and downstream non-coding sequences immediately adjacent to the *flgM* ORF). CAG57112 (DY330 Δ*fliA:aadA*) was constructed by replacing the *fliA* ORF with the spectinomycin resistant gene, *aadA*. The *aadA* sequence was amplified from pDH50 using the primers; 5'- CAGAAACGGATAATCATGCCGATAACTCATATAACGCAGGGCTGTTTATC ACCGTGGAAACGGATGAAGGCACG -3', and 5'- ATCATTAAGAACTCCTGGTAGTCAAAGTTAAAGTGCGGCATTTACTGACG TTATTTGCCGACTACCTTGGTG -3' (underlined sequences are complementary to the upstream and downstream non-coding sequences immediately adjacent to the *fliA* ORF).

The assay strain used in this study, CAG57115 (MG1655 Δ*lacX74*, Δ*fliA:aadA*, Δ*flgM::cat*), was constructed by P1 transduction of Δ*flgM::cat* from CAG57111 and Δ*fliA:aadA* CAG57112, and selection for chloramphenicol and spectinomycin resistance, repsectively. The presence of each gene was also confirmed by PCR.

Plasmid construction

The plasmid pSAKT28 encoding wt σ^{28} and pSAKT28 derivatives pBK201-pBK212 encoding σ^{28} variants were used for expression of σ^{28} to determine their activities *in vivo*. The pSAKT28 plasmid derivatives carry the *fliA* gene under control of the isopropyl-β-D-thiogalactopyranoside inducible P_{lac} promoter as well as the *lac* repressor gene under the control of a strong mutant promoter (i^q) (Wang & deHaseth, 2003). pSAKT28 was constructed from pSAKT32, which encodes *rpoH* (σ^{32}) by replacing the *rpoH* ribosome binding site and ORF with the *fliA* ORF and ribosome binding site. In addition, the start codon of *fliA* gene (GTG) was changed to ATG. All σ^{28} variants were constructed by site-directed mutagenesis.

Derivatives of pQF50K (pBK601-pBK618) carrying promoter fragments cloned in the *Bgl*II-*Xba*I sites upstream of *lacZ* were used for measuring promoter strength and activities of σ's (Wang & deHaseth, 2003). Promoter mutant derivatives were constructed using PCR by amplifying the promoter regions using mutagenic primers that encompassed either the *Bgl*II or *Xba*I site.

Derivatives of the pET21a vector (Novagen) encoding σ^{28} and variants were used to overexpress σ^{28} protein for purification. The vectors were constructed using PCR by amplifying from the pSAKT28 series using primers to create flanking *Nde*I and *Hind*III sites and inserted as *Nde*I-*Hind*III fragments into pET21a *Nde*I-*Hind*III vector. The resultant σs contain an N-terminal His_6-tag and PreScission cleavage sit (Patikoglou et al., 2007).

β-galactosidase assay

Assay systems for *in vivo* transcription were constructed by transforming derivatives of pSAKT28 and pQF50K into CAG57115 sequentially. Overnight cultures in LB media supplemented with 100 μg/ml ampicillin and 30 μg/ml kanamycin at 30°C were diluted 1:100 into fresh media and grown for 90 min with aeration. Selected cultures were then induced with 1 mM IPTG and grown for a further 2 hr before harvesting (final ODs at 600nm was approximately 0.7). β-galactosidase assays were performed as described previously and b-galactosidase activity (in Miller Units) was determined as following equation (Miller, 1972): Units = $1000 \times [(OD_{420} - 1.75 \times OD_{550}) / (\text{incubation time in minutes} \times \text{volume of the culture used in the assay in ml} \times OD_{600} \text{ of the culture used in the assay})]$

Overproduction and purification of σ^{28}'s

E. coli BL21 (DE3)/pLysS cells harboring pET21a encoding wt σ^{28} or variants were used to overproduce σ^{28} with an N-terminal His_6-tag and PreScission cleavage site for purification. Overnight cultures were diluted 1:100 into fresh media containing 100 μg/ml ampicillin and 30 μg/ml chloramphenicol, and grown aerobically at 30 °C. When the cultures reached OD_{600} of 0.4, expression of σ^{28} and variants was induced with 1 mM IPTG and growth continued at 30 °C with aeration for 2 hr. 500 ml of each culture was then harvested by centrifugation and the cell pellets resuspended in buffer A1 (20 mM HEPES, pH 8.0, 300 mM KCl, 1 mM β-mercaptoethanol, 5 mM Imidazole, 1 mM AEBSF). The cells were disrupted by sonication and the insoluble fraction was pelleted by centrifugation at 5,000 × g for 10 min and resuspended in 10 ml of buffer A2 (20 mM HEPES, pH 8.0, 300 mM KCl, 1 mM β-mercaptoethanol, 5 mM Imidazole, 6 M guanidine hydrochloride). The σ^{28} proteins were purified from the suspension by metal affinity chromatography using Talon metal affinity resin (Clontech) in denaturing condition but proteins were eluted in native condition by omitting guanidine hydrochloride. The N-terminal His_6-tag was removed using PreScission protease (GE Healthcare) during dialysis into buffer Q (10 mM Tris, pH 7.5, 50mM NaCl, 1mM DTT) at 4 °C for 16 hr. The sample was further purified by a second,

subtractive metal affinity chromatography step to remove uncleaved His_6-σ's and the His_6-tag, followed by ion exchange chromatography (HiTrap Q Sepharose; GE Healthcare). As a final step, protein fractions were further purified by gel filtration chromatography (Superdex 75; GE Healthcare) in order to remove residual contaminants and free σ^{28} fractions were taken and concentrated in buffer B (50 mM Tris, pH 8.0, 200 mM KCl, 1 mM DTT, 0.01% Triton X-100, 50% glycerol). Purified σ^{28}'s were then concentrated in buffer B. Purity of all His6-tag free proteins were >95% judged by SDS-PAGE and native-Protein concentration was determined by the bicinchoninic acid protein assay (Pierce).

In vitro transcription

Run-off single round transcription was performed at 30 °C. Linear DNA templates were generated by amplification of promoter region of pQF50K plasmid series including 220 bp upstream and 200 bp downstream sequence from transcription start site. RNA polymerase core enzyme was purified as described in (Sharp et al., 1999). Holoenzyme was reconstituted by incubation of core RNA polymerase and a 5-fold excess of σ^{28} on ice for 30 min in binding buffer (20 mM Tris pH 8.0, 100 mM K-acetate, 10 mM Mg-acetate, 0.1 mM EDTA, 1mM DTT, 100 μg/ml BSA, 5% glycerol and 0.05% Tween-20). Holoenzyme was diluted in binding buffer and incubated at 30 °C for 5 min. After incubation of holoenzyme and DNA template at 30 °C for 5 min, transcription was initiated by adding transcription mix and incubated at 30 °C for 6 min. Total reaction mixture was given as a 9 μl of 75 nM holoenzyme, 10 nM template DNA, 200 μM ATP, 200 μM GTP, 200 μM UTP, 10 μM CTP, 100 nM [α-^{32}P]CTP (3000 Ci/mmol), 50 μg/ml heparin in binding buffer. Reactions were stopped by the addition of 7 μl of gel loading solution containing 20 mM EDTA, 80% deionized formamide, 0.1% of bromophenol blue and xylene cyanol FF, and 35-nucleotides ^{32}P end-labeled DNA oligomer. After incubation at 90 °C for 2 min, the samples were subjected to electrophoresis in a 6% acrylamide/7M urea/TBE gel. The transcripts and end-labeled oligo were visualized and analyzed using Molecular Dynamics Storm 560 PhosphoImager scanning system and ImageQuant 5.2 densitometry software.

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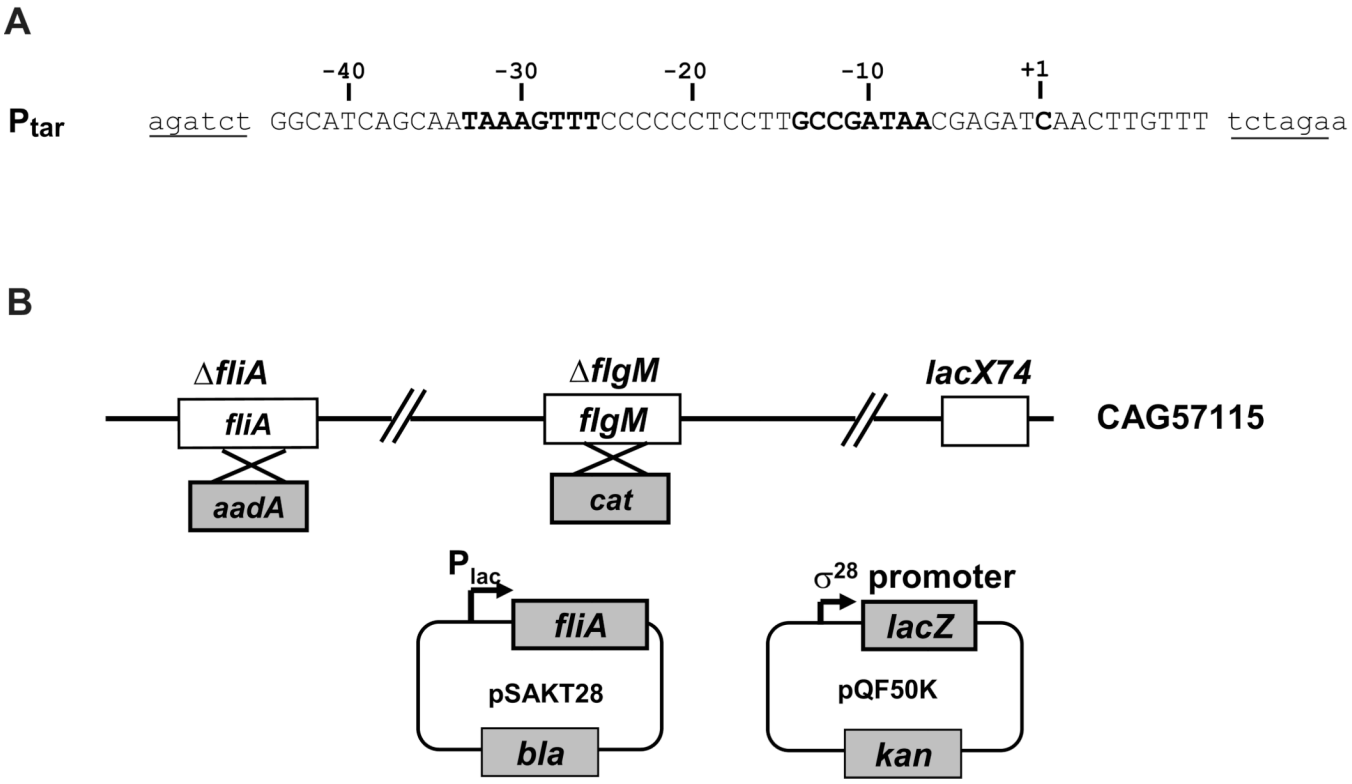


Figure 1. Components of *in vivo* assay systems used in this study

A. Sequence of the P_{tar} σ^{28}-dependent promoter used in this study. Only the wt sequence is shown: single- or double- base changed promoter mutants were constructed from this parent. The native sequence of the *tar* promoter region is shown in capital letters; vector sequences are shown in lowercase; *Bgl*II and *Xba*I cloning sites are underlined; −35 and −10 regions, and the transcription start site are shown in bold.

B. Activity of σ^{28}-dependent promoters in the presence of σ^{28} variants was determined by β-galactosidase assay from the Δ*fliA* and *flgM* strain, CAG57115, carrying the plasmids pSAKT28 and pQF50K. σ^{28}expression was induced from pSAKT28 and derivatives (pBK201-pBK212), which has a p15A replication origin and *fliA* under control of the *lac* promoter. The reporter plasmid, pQF50K and derivatives (pBK601-pBK618) has a pMB1 replication origin and *lacZ* under control of the σ^{28}-dependent *tar* promoter and promoter derivatives.

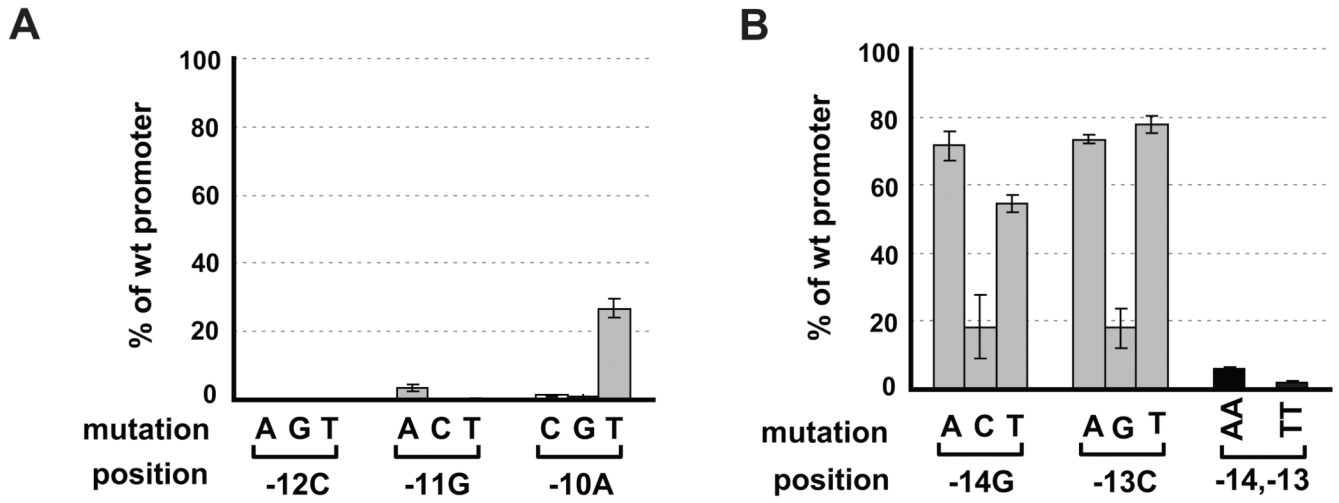


Figure 2. Effects of nucleotide changes in the −10 region on activities of *tar* promoter

A. Effects of single nucleotide changes at −12C, −11G and −10A positions on activity of the *tar* promoter.

B. Effects of single or double nucleotides changes at −14G and −13C positions on activity of the *tar* promoter.

The activity of each single or double nucleotide mutant *tar* promoter with wt σ^{28} is shown as a percentage of the measured β-galactosidase activity of the wt promoter. Assay strains are as described in Fig 1B. The promoter mutations and positions are shown on the x-axis. All values are averages of three independent experiments; error bars indicate 1 standard deviation.

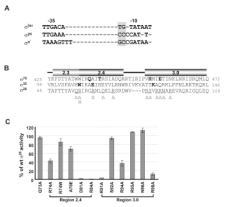


Figure 3. Effects of single amino acid substitutions in Region 2.4 and 3.0 of σ^{28} on activity of the wt *tar* promoter

A. Alignment of consensus core −10 and −35 sequences of *E. coli* σ^{70}, σ^{32} and σ^{28} promoters. The extended −10 motifs of each promoter are denoted by grey shading.

B. Alignment of amino acid sequence of Regions 2.3, 2.4 and 3.0 of σ^{70}, σ^{32} and σ^{28}. Numbers at each end of sequence indicate amino acid position. Amino acid residues of σ^{70} and σ^{32} involved in base specific recognition are shown in bold (Koo et al., 2009, Kourennaia et al., 2005, Sanderson et al., 2003, Siegele et al., 1989). Positions of single amino acid substitutions of σ^{28} used in this study are underlined and the substitutions are shown below the main sequence. The predicted helices at each region was derived from structural data of *Thermus aquaticus* σ^{A} and *Aquifex aeolicus* σ^{28} (Campbell et al., 2002, Sorenson et al., 2004) and are shown above the sequences.

C. β-galactosidase activity driven by each σ^{28} variant on the wt *tar* promoters are shown as a percentage of measured β-galactosidase activity driven by wt σ^{28}. Assay strains are as described in Fig 1B.The different σ^{28} amino acid substitutions and their locations are shown on the x-axis. All values are averages of three independent experiments; error bars indicate 1 standard deviation.

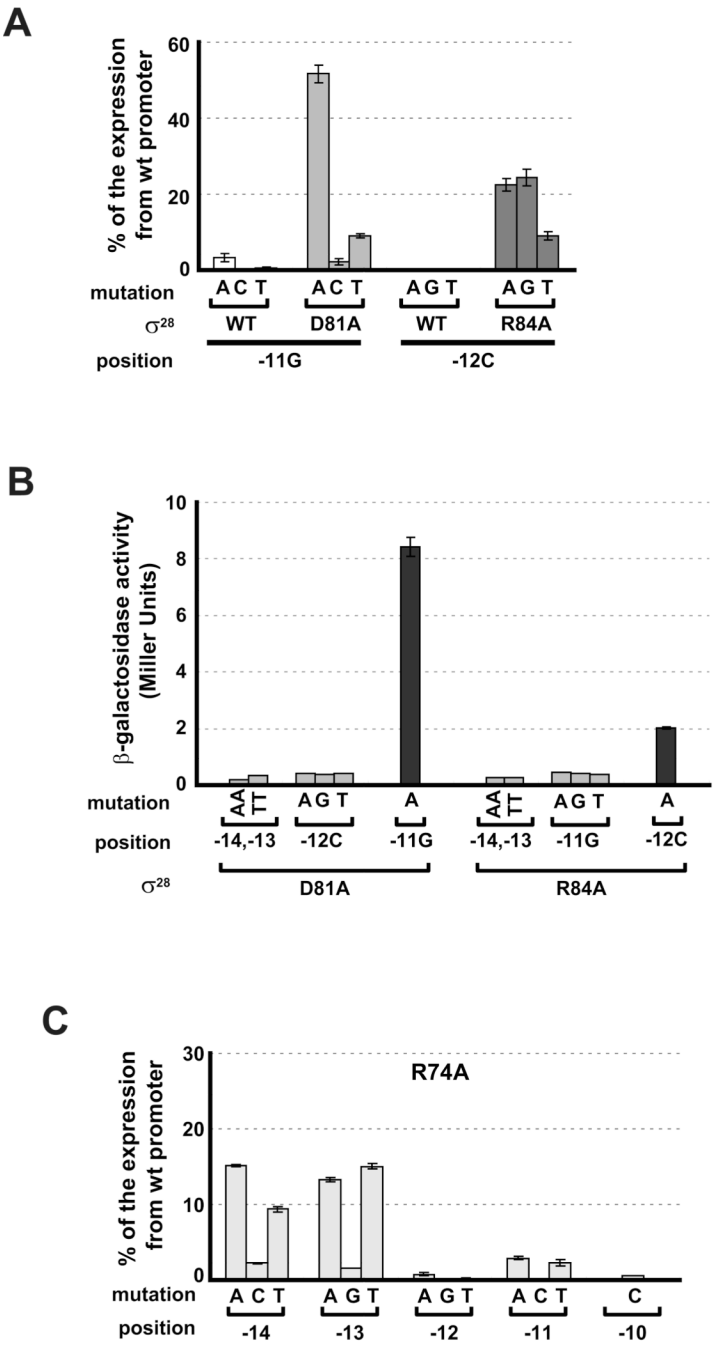


Figure 4. Effect of amino acid substitutions in Region 2.4 of σ^{28} on mutations at −10 promoter region

D81A σ^{28}28 and R84A σ^{28} uniquely suppress single nucleotide changes at positions −11G and −12C of the *tar* promoter respectively (A and B), whereas R74A σ^{28} shows less relative activity than wt σ^{28} on mutant promoters with single nucleotide changes at positions −10 to −14 of the tar promoter (C). For panels A and C, β-galactosidase activity driven by each σ^{28} variant on mutant *tar* promoters is shown as a percentage of the β-galactosidase activities of the same σ^{28} variant on the wt promoter. For panel B, absolute β-galactosidase activity (Miller Units) driven by D81A σ^{28} and R84A σ^{28} on mutant *tar* promoters is indicated. Activities of less than 0.5 Miller Units is at the background level of this assay. Assay strains

are as described in Fig 1B. Below the x-axis, mutation and position indicate the base change at the different promoter positions; σ^{28} indicates the wild type or substituted derivative. All values are averages of three to four independent experiments; error bars indicate 1 standard deviation.

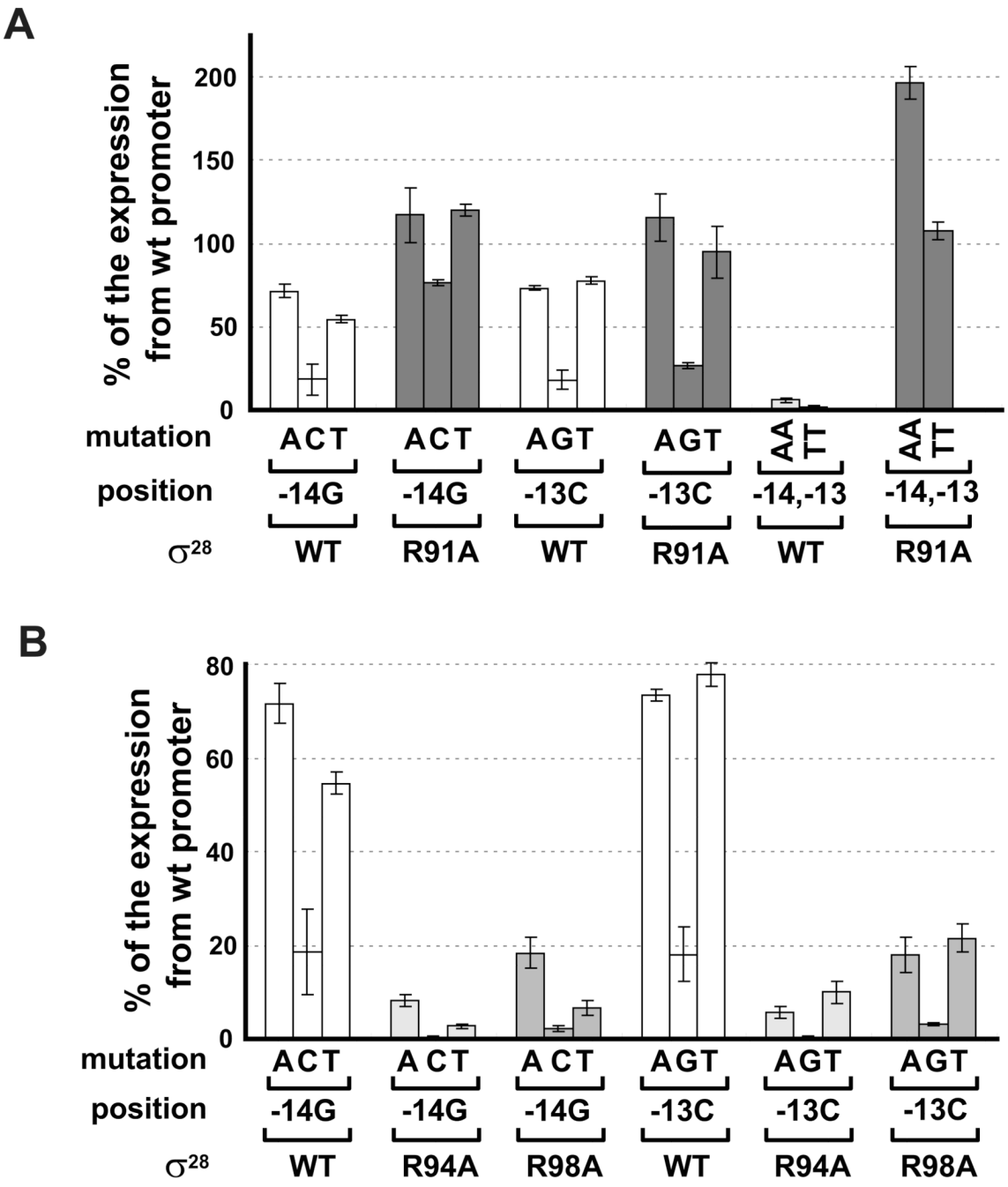


Figure 5. Effect of alanine substitutions in Region 3.0 of σ^{28} on nucleotide changes at positions −14G and −13C of *tar* the promoter

A. Suppression of single or double nucleotides changes at positions −14G and −13C of the *tar* promoter by R91A σ^{28}.

B. Effect of R94A σ^{28} or R98A σ^{28} on changes at positions −14G and −13G of the *tar* promoter.

All data are normalized and presented as shown in Fig. 4.

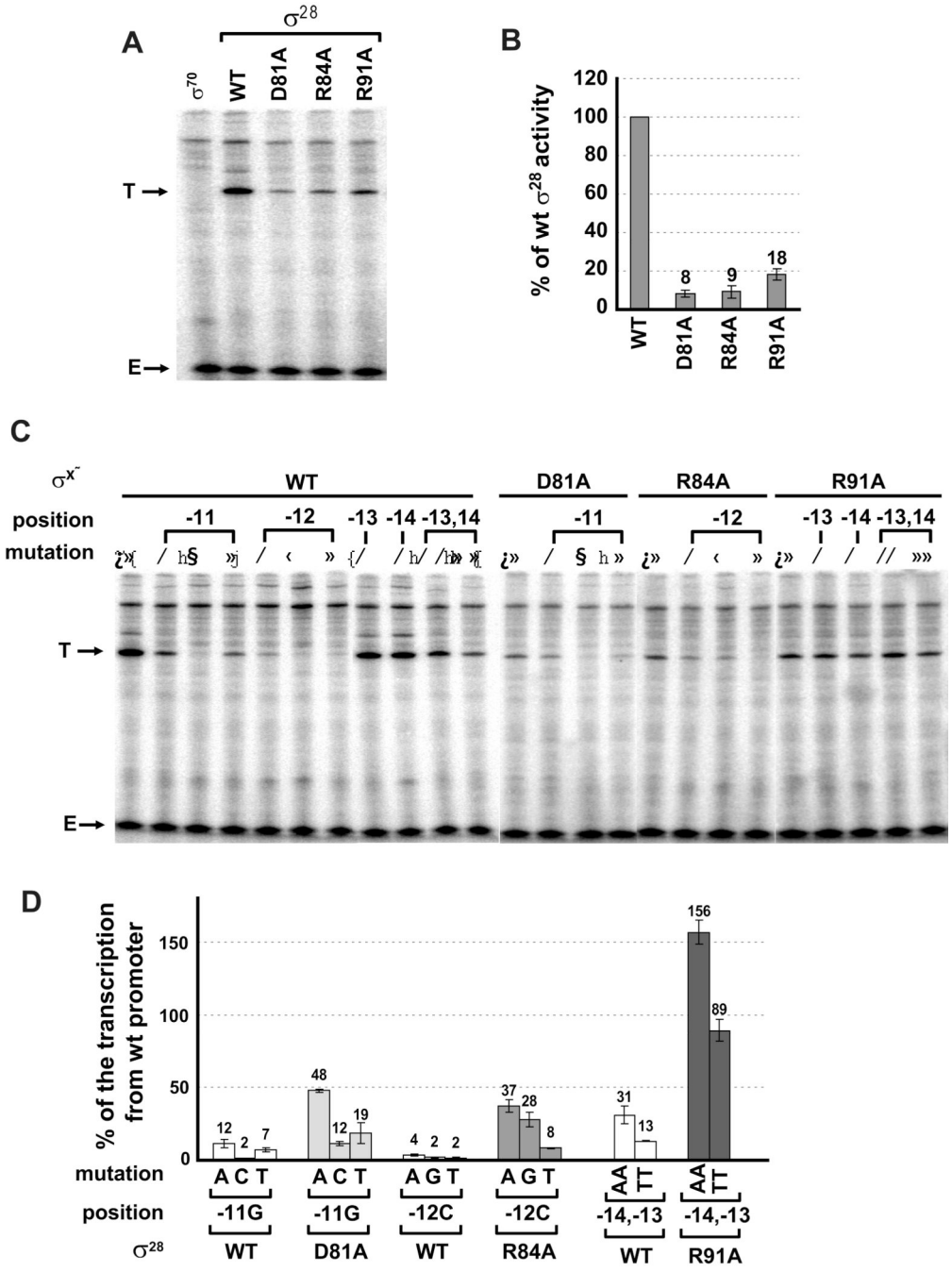


Figure 6. Effect of mutations in the −10 region of the tar promoter and Regions 2.4 and 3.0 in σ^{28} on transcription *in vitro*

Single round *in vitro* transcriptions were performed on linear DNA templates by RNA polymerase containing different σ^{28} variants. DNA templates were prepared as described in the experimental procedures. Each experiment was repeated a minimum of three times; for each experiment a representative gel image is shown and the bar graphs illustrate averaged transcript quantifications from replicate experiments. T; transcript from tar promoter variant driven by different σ^{28} variants. E; end-labeled 35 nucleotides oligomer.

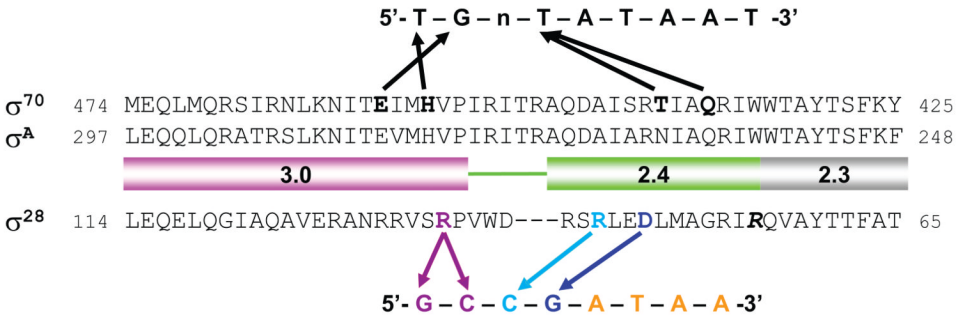
A. *in vitro* transcriptions from the wt *tar* promoter with wt, D81A, R84A and R91A σ^{28}. As a control reaction, transcript driven by σ^{70} is shown.

B. Quantified and normalized data from A. The bars indicate the relative transcription by the different σ^{28} variants as a percentage of total transcription with wt σ^{28}.

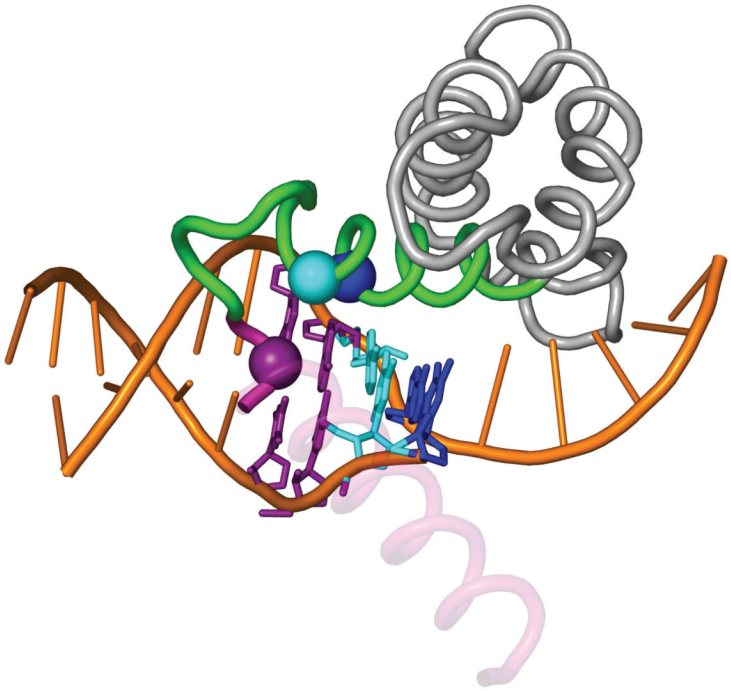
C. *In vitro* transcriptions from different *tar* promoter variants mutated at positions −11 to −14 with wild-type σ^{28} and suppression of promoter substitutions by D81A, R84A and R91A σ^{28}. Above the gel image, σ^{28}indicates wild-type or the different σ^{28} variants, position and mutation indicate the base change at the different promoter positions.

D. Quantified and normalized data from C. The bars indicate the relative transcription by the different σ^{28} variants at the mutant promoters as a percentage of transcription by that variant from the wt promoter. Below the x-axis, mutation and position indicates the base change at the different promoter positions; σ^{28}indicates the wild-type or substituted derivative.

A



B



C

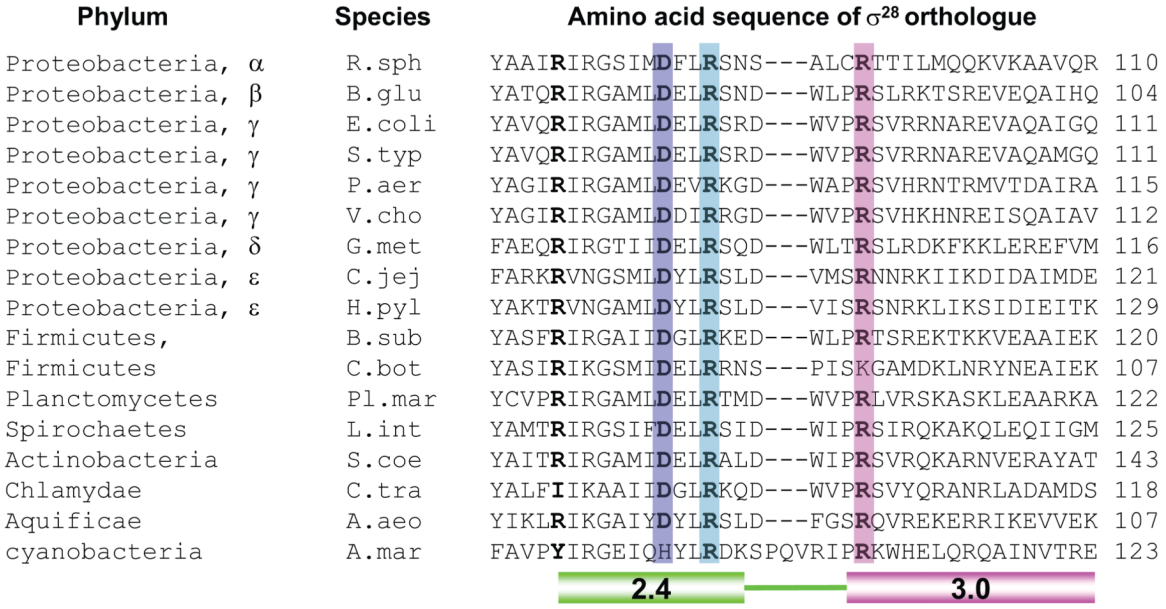


Figure 7. Structural model of recognition of the −10 region of promoters by σ^{28} and conservation of base-specific promoter recognitions among σ^{28} orthologues

A. Comparison of the recognition of the −10 region of promoters by σ^{70} and σ^{28}. Amino acid sequences of Region 2.3, 2.4 and 3.0 of σ^{70}, σ^{A} and σ^{28} are shown. The −10 consensus promoter sequences for each σ is shown above or below the amino acid sequences. Arrows indicate contacts suggested by the previous studies for σ^{70} and in this study for σ^{28}.

B. Shown is the structure of σ^{A} Regions 2.1–3.0 and the −10 element region of the fork junction promoter DNA from the *T. aquaticus* holoenzyme/fork-junction promoter DNA complex (Murakami *et al.*, 2002). The rest of the protein and DNA are not shown. The σ^{A} Region 2.4 is colored green and Region 3.0 is magenta. Most of the 3.0 helix is transparent for clarity. The positions corresponding to residues of σ^{28} that make base-specific contacts with the promoter are shown as α-carbon spheres, and color-coded along with the corresponding DNA base-pair: D81 (R264 in *T. aquaticus* σ^{A})/−11GC, blue; R84 (A267)/−12CG, cyan; and R91 (V277)/−13CG/–14GC, purple. This figure was prepared using PyMol (http://pymol.sourceforge.net/).

C. Conservation of amino acid residues involved in base-specific recognition of the −10 region of σ^{28} promoter among σ^{28} orthologues. Whole amino acid sequences were aligned using ClustalW and sequences from Regions 2.4 to 3.0 are shown. Residues involved in base-specific recognition are denoted by color shading. Residues homologous to R74 of *E. coli* σ^{28} are in bold. *R. sph, Rhodobacter sphaeroides; B. glu, Burkholderia glumae; E. coli, Escherichia coli; S. typ, Salmonella typhimurium; P. aer, Pseudomonas aeruginosa; V. cho, Vibrio cholerae; G. met, Geobacter metallireducens; C. jej, Campylobacter jejuni; H. pyl, Helicobacter pylori; B. sub, Bacillus subtilis; C. bot, Clostridium botulinum; Pl. mar, Planctomyces maris; L. int, Leptospira interrogans; S. coe, Streptomyces coelicolor; C. tra, Chlamydia trachomatis; A. aeo, Aquifex aeolicus; A. mar, Acaryochloris marina*.

Table 1

Strains and plasmids used in this study

Strains/plasmids	Relevant genotype	Source/reference
Strains		
DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Invitrogen
BL21(DE3)pLysS	*dcm ompT hsdS*($r_B^- m_B^-$) *gal λ(DE3)*, pLysS, Cm^R	Invitrogen
DY330	W3110, *ΔlacU169 gal490 λCI857 Δ(cro-bioA)*	(Yu et al., 2000)
CAG22239	MG1655 *ΔlacX74*	Lab collection
CAG57111	DY330, *ΔflgM::cat*, Cm^R	This study
CAG57112	DY330, *ΔfliA::aadA*, Sp^R	This study
CAG57113	MG1655 *ΔlacX74*, *ΔflgM::cat*, Cm^R	This study
CAG57114	MG1655 *ΔlacX74*, *ΔfliA::aadA*, Sp^R	This study
CAG57115	MG1655 *ΔlacX74*, *ΔflgM::cat*, *ΔfliA::aadA*, Cm^R, Sp^R	This study
Plasmids		
pSAKT32	Vector for σ^{32} expression. P15A ori, P_{lac}-*rpoH*, *lacI*^{q}, Ap^R	(Wang & deHaseth, 2003)
pSAKT28	Vector for σ^{28} expression. P15A ori, P_{lac}-*fliA*, *lacI*^{q}, Ap^R	This study
pBK201	σ^{28}-Q73A in pSAKT28, Ap^R	This study
pBK202	σ^{28}-R74A in pSAKT28, Ap^R	This study
pBK203	σ^{28}-R74W in pSAKT28, Ap^R	This study
pBK204	σ^{28}-A78E in pSAKT28, Ap^R	This study
pBK205	σ^{28}-D81A in pSAKT28, Ap^R	This study
pBK206	σ^{28}-R84A in pSAKT28, Ap^R	This study
pBK207	σ^{28}-R91A in pSAKT28, Ap^R	This study
pBK208	σ^{28}-S92A in pSAKT28, Ap^R	This study
pBK209	σ^{28}-R94A in pSAKT28, Ap^R	This study
pBK210	σ^{28}-R95A in pSAKT28, Ap^R	This study
pBK211	σ^{28}-N96A in pSAKT28, Ap^R	This study
pBK212	σ^{28}-R98A in pSAKT28, Ap^R	This study
pBK311	σ^{28} in pET21a*, Ap^R	This study
pBK312	σ^{28}-D81A in pET21a*, Ap^R	This study
pBK313	σ^{28}-R84A in pET21a*, Ap^R	This study
pBK314	σ^{28}-R91A in pET21a*, Ap^R	This study
pQF50K	Vector, pMB1 ori, 1600 ori, promoterless *lacZ*, km^R	(Farinha & Kropinski, 1990)
pBK601	Ptar (−44 to +10, WT) in pQF50K, Km^R	This study
pBK602	Ptar (G-14A) in pQF50K, Km^R	This study
pBK603	Ptar (G-14C) in pQF50K, Km^R	This study

Strains/plasmids	Relevant genotype	Source/reference
pBK604	Ptar (G-14T) in pQF50K, Km^R	This study
pBK605	Ptar (C-13A) in pQF50K, Km^R	This study
pBK606	Ptar (C-13G) in pQF50K, Km^R	This study
pBK607	Ptar (C-13T) in pQF50K, Km^R	This study
pBK608	Ptar (C-12A) in pQF50K, Km^R	This study
pBK609	Ptar (C-12G) in pQF50K, Km^R	This study
pBK610	Ptar (C-12T) in pQF50K, Km^R	This study
pBK611	Ptar (G-11A) in pQF50K, Km^R	This study
pBK612	Ptar (G-11C) in pQF50K, Km^R	This study
pBK613	Ptar (G-11T) in pQF50K, Km^R	This study
pBK614	Ptar (A-10C) in pQF50K, Km^R	This study
pBK615	Ptar (A-10G) in pQF50K, Km^R	This study
pBK616	Ptar (A-10T) in pQF50K, Km^R	This study
pBK617	Ptar (G-14A, C-13A) in pQF50K, Km^R	This study
pBK618	Ptar (G-14T, C-13T) in pQF50K, Km^R	This study
pACYC184	p15A ori, Cm^R, Tet^R	(Chang & Cohen, 1978)
pDH50	*tac* promoter, *lacI*^{q}, Sp^R	(Kleerebezem *et al.*, 1995)

* thrombin cleavage site is modified to PreScission protease cleavage site