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σ^{28} RNA polymerase regulates *hctB*, a late developmental gene in *Chlamydia*

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Summary

Chlamydia is predicted to encode two alternative sigma factors that could provide a mechanism for the regulation of gene expression via alternative forms of RNA polymerase. We have demonstrated that σ^{28}, one of these alternative sigma factors, is transcriptionally active. Chlamydial σ^{28} RNA polymerase was reconstituted from recombinant σ^{28} protein and core enzyme that was biochemically isolated from chlamydiae. In an *in vitro* transcription assay, σ^{28} RNA polymerase transcribed the *hctB* promoter in a σ^{28}-dependent manner. Transcription by σ^{28} RNA polymerase was salt tolerant compared with transcription by σ^{66} RNA polymerase, the major form of chlamydial RNA polymerase. As *hctB* encodes a histone-like protein that is only expressed late in the developmental cycle, our results suggest that σ^{28} RNA polymerase has a role in the regulation of late gene expression in *Chlamydia*.

Introduction

Chlamydiae are pathogenic bacteria and obligate intracellular parasites. *Chlamydia trachomatis* is a leading cause of sexually transmitted disease in the developed world and of preventable blindness in the developing world (reviewed in Schachter, 1999). Chlamydiae have an unusual developmental cycle that requires growth and replication within a eukaryotic cell (reviewed in Hackstadt, 1999). During this cycle, there is conversion between two distinct morphological forms. The elementary body (EB) is a spore-like, infectious form that is metabolically inert. Upon entry into the host cell, the EB differentiates into a reticulate body (RB), which is larger and has de-condensed DNA. The RB is metabolically active and divides repeatedly by binary fission. Late in the developmental cycle, RBs convert back to EBs, which are released to infect new cells.

The mechanism of RB to EB conversion is incompletely defined, but a prominent feature of this process is the condensation of DNA to form a nucleoid. Two histone-like proteins, Hc1 and Hc2 are believed to mediate this change in DNA structure and both are first detectable late in the developmental cycle at the time of RB to EB conversion (Hackstadt *et al*., 1991; Tao *et al*., 1991; Perara *et al*., 1992; Brickman *et al*., 1993). Hc1 and Hc2 have been shown to condense DNA *in vitro*, to condense the *Escherichia coli* chromosome when expressed in *E. coli* and to repress transcription and translation *in vitro* and *in vivo* (Barry *et al*., 1992; 1993; Brickman *et al*., 1993; Pedersen *et al*., 1994; 1996). Hc1 and Hc2 are encoded by *hctA* and *hctB*, respectively, and both genes are only transcribed late in the developmental cycle (Brickman *et al*., 1993; Fahr *et al*., 1995). *hctA*, but not *hctB*, is transcribed by the major (σ^{66}) RNA

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polymerase, as are several other late genes such as *omcA*, *omcB*, *ltuA* and *ltuB* (reviewed in Fahr *et al*., 1995), but the mechanism underlying the transcriptional regulation of late genes is unknown.

Gene expression in bacteria is often regulated at the transcriptional level by transcription factors and by alternative forms of RNA polymerase. Transcription factors modulate RNA polymerase activity while an alternative RNA polymerase contains an alternative sigma factor that recognizes a different promoter structure. The genomes of *Chlamydia* species sequenced to date (Stephens *et al*., 1998; Kalman *et al*., 1999; Read *et al*., 2000; Shirai *et al*., 2000; Read *et al*., 2003), each encode the major sigma factor, σ^{66}, and two predicted alternative sigma factors, σ^{28} and σ^{54}, that are homologous to known bacterial alternative sigma factors (Stephens *et al*., 1998). Transcripts for both predicted alternative sigma factors can be detected by RT-PCR (Mathews *et al*., 1999; Douglas and Hatch, 2000; Shaw *et al*., 2000), but neither sigma factor has been shown to transcribe chlamydial promoters. In fact, the genes that are regulated by σ^{28} or σ^{54} are not known, although some σ^{54} promoters have been predicted based on sequence similarity to bacterial σ^{54} promoters (Mathews and Timms, 2000).

A major impediment to studying gene regulation in *Chlamydia* has been the absence of an experimental genetic system, although *in vitro* assays have been used to define the promoter recognized by the major RNA polymerase (reviewed in Hatch, 1999; Schaumburg and Tan, 2003) and to reconstitute regulated transcription (Wilson and Tan, 2002). In this report, we describe the development of an assay for chlamydial σ^{28} activity that required the reconstitution of active chlamydial σ^{28} RNA polymerase and the identification of a σ^{28}-dependent promoter. σ^{28} RNA polymerase was assembled from recombinant σ^{28} and core enzyme prepared from chlamydiae grown in tissue culture. We identified the *hctB* promoter as a candidate σ^{28} promoter because sequences upstream of its predicted transcription start site are similar to the consensus bacterial σ^{28} promoter structure. Using our *in vitro* assay, we demonstrated that the *hctB* promoter is transcribed by chlamydial σ^{28} RNA polymerase. As *hctB* is only transcribed late in the chlamydial developmental cycle, our results suggest that σ^{28} has a role in the developmental regulation of chlamydial gene expression.

Results

Overexpression and purification of chlamydial σ^{28}

rpsD, the gene encoding a predicted σ^{28} factor, was cloned from *C. trachomatis* serovar L2 and σ^{28} protein was overexpressed in *E. coli* and purified as a six-histidine-tagged recombinant protein. The recombinant σ^{28} protein is highly purified and has a predicted size of 33 kDa (Fig. 1), from addition of a 4 kDa histidine tag to the 29 kDa σ^{28}. To minimize co-purification of *E. coli* proteins that may have formed associations with recombinant chlamydial σ^{28}, soluble protein was precipitated by ammonium sulphate and re-suspended in 6 M guanidine hydrochloride prior to purification with Ni-NTA agarose beads. Antibodies against *E. coli* σ^{28} did not recognize *E. coli* σ^{28} in our purified *C. trachomatis* σ^{28} protein preparation (immunoblot data not shown).

Prediction that the *hctB* promoter is a σ^{28}-regulated promoter

σ^{28} promoter structure is well conserved in bacteria and a consensus promoter structure [TAAAnnnn (n11) GCCGATAA] (reviewed in Helmann, 1991; Gross *et al*., 1992) and a more stringent, extended promoter structure [TAAAGTTT (n11) GCCGATAA] (Ide *et al*., 1999) have been proposed. By inspection, we noted that sequences upstream of the predicted transcription start site of *C. trachomatis* serovar L2 *hctB* (Brickman *et al*., 1993) closely resemble the extended σ^{28} promoter sequence but with a 12 bp spacer between promoter elements rather than the canonical 11 bp (Fig. 2).

Chlamydial σ^{28} RNA polymerase is active and specifically transcribes the hctB promoter

Chlamydial σ^{28} RNA polymerase was reconstituted by mixing recombinant σ^{28} with chlamydial core enzyme. As a source of core enzyme, we used RNA polymerase purified from reticulate bodies by heparin-agarose chromatography. We have previously demonstrated that heparin-agarose-purified RNA polymerase is transcriptionally active and contains core enzyme and σ^{66}, the major sigma factor in *Chlamydia* (Tan and Engel, 1996). Using immunoblots with anti-chlamydial σ^{28} antibodies, we determined that σ^{28} protein was not detectable in the heparin-agarose-purified RNA polymerase (data not shown).

The reconstituted σ^{28} RNA polymerase was used to demonstrate that the *hctB* promoter is a σ^{28}-regulated promoter. Recombinant σ^{28} was pre-incubated with heparin-agarose-purified RNA polymerase and an *in vitro* transcription reaction was performed with plasmid pMT1212, which contains the predicted *hctB* promoter upstream of a synthetic G-less cassette transcription template that does not encode any G residues (Fig. 3). The radiolabelled products of the transcription reaction were electrophoresed on a urea-polyacrylamide gel, which was exposed to a phosphorimaging screen for quantification of the amount of transcript present. There was no transcription with σ^{28} alone (Fig. 4A, lane 1), or with heparin-agarose-purified RNA polymerase alone (Fig. 4A, lane 2), which suggests that σ^{66} RNA polymerase was not able to recognize this *hctB* promoter. A readily detectable transcript of expected size (150 nt) was produced when the reaction was performed with σ^{28} RNA polymerase that had been reconstituted from recombinant σ^{28} and heparin-agarose-purified RNA polymerase (Fig. 4A, lane 3). There is almost a complete lack of background transcription because only the G-less cassette was transcribed in the absence of GTP in the reaction mix (Tan and Engel, 1996).

To confirm that the transcriptional activity of reconstituted σ^{28} RNA polymerase is dependent on chlamydial σ^{28}, we pre-incubated the recombinant σ^{28}-heparin-agarose-purified RNA polymerase mix with anti-chlamydial σ^{28} antibodies prior to *in vitro* transcription. Our results demonstrated that polyclonal antibodies against chlamydial σ^{28} completely inhibited transcription of the *hctB* promoter (Fig. 4A, lane 4). These antibodies had no effect on σ^{66}-dependent transcription of the *dnaK* promoter contained in plasmid pMT1211 (Fig. 4B, compare lanes 2 and 3), which showed that inhibition of *hctB* transcription is σ^{28} specific. These experiments demonstrate that the transcriptional activity of reconstituted σ^{28} RNA polymerase is dependent on chlamydial σ^{28}. Our antibodies against chlamydial σ^{28} do not recognize purified *E. coli* σ^{28} protein by immunoblot (data not shown), which rules out the formal possibility that the activity is due to contaminating *E. coli* σ^{28} in the chlamydial σ^{28} preparation.

σ^{28} associated with free core enzyme in the heparin-agarose-purified RNA polymerase preparations

The preceding results demonstrated that σ^{28} RNA polymerase was successfully reconstituted from recombinant σ^{28} and heparin-agarose-purified RNA polymerase containing core enzyme and σ^{66}. To examine the interactions between σ^{28}, σ^{66} and core enzyme, we performed a titration experiment by adding increasing amounts of σ^{28} to the heparin-agarose-purified RNA polymerase. Each RNA polymerase mix was allowed to pre-incubate and then used in separate reactions containing either the *hctB* promoter or *dnaK* promoter transcription plasmid to assay for σ^{28}-dependent or σ^{66}-dependent activity respectively. There was increased transcription from the *hctB* promoter with increasing concentrations of σ^{28} (Fig. 5A, lanes 3–5) but transcription from the σ^{66}-dependent *dnaK* promoter did not change (Fig. 5B, lanes 3–5). As the reactions were performed with non-saturating amounts of σ^{66} RNA polymerase (C. Schaumburg and M.T., unpubl. results), these results show that addition of σ^{28} did not affect the amount of σ^{66} RNA polymerase activity present. We infer from these results that σ^{28}, in

the concentrations added, associated with free core enzyme in the heparin-agarose-purified RNA polymerase and did not displace σ^{66} from intact holoenzyme.

Effect of salt concentration on transcription of the hctB promoter by σ^{28} RNA polymerase

We performed *in vitro* transcription experiments with different salt concentrations to determine if the chlamydial σ^{28} RNA polymerase was salt tolerant relative to chlamydial σ^{66} RNA polymerase. Kundu *et al*. have shown that σ^{28} (σ^{F})-regulated transcription in *E. coli* is highly salt tolerant compared with σ^{70}-regulated transcription (Kundu *et al*., 1997). We tested potassium acetate concentrations from 0 to 600 mM and found that transcription of the *hctB* promoter by chlamydial σ^{28} RNA polymerase was salt tolerant, with peak transcriptional activity at 400 mM potassium acetate and a significant decrease at 600 mM potassium acetate (Fig. 6). In contrast, transcription of the *dnaK* promoter by σ^{66} RNA polymerase decreased to negligible levels at concentrations above 100 mM (Fig. 6).

Discussion

This is the first report of the reconstitution of a transcriptionally active alternative chlamydial RNA polymerase. Our results indicate that *rpsD* encodes a functional alternative sigma factor, σ^{28}, and that the *hctB* promoter is regulated by σ^{28} RNA polymerase. Shen and Zhang (2002) have presented preliminary work showing that recombinant chlamydial σ^{28} is active in a hybrid RNA polymerase with *E. coli* core enzyme and is able to transcribe a known *E. coli* σ^{28} promoter, *fliC*, *in vitro*. Our assay, which utilizes chlamydial σ^{28} RNA polymerase to transcribe a chlamydial promoter, has the advantage of being a purely chlamydial system.

As we purified recombinant chlamydial σ^{28} protein from *E. coli*, it was important to ensure that any transcriptional activity was not due to contaminating *E. coli* proteins. To prevent the co-purification of *E. coli* core enzyme that could potentially bind to chlamydial σ^{28}, we purified chlamydial σ^{28} under denaturing conditions. In addition, two lines of evidence indicate that our chlamydial σ^{28} does not contain contaminating *E. coli* σ^{28}. First, we did not detect *E. coli* σ^{28} in our chlamydial σ^{28} preparation with anti-*E. coli* σ^{28} antibodies. Second, the transcriptional activity of our reconstituted σ^{28} RNA polymerase was inhibited by anti-chlamydial σ^{28} antibodies, which do not cross-react with *E. coli* σ^{28}.

We reconstituted σ^{28} RNA polymerase by adding recombinant σ^{28} protein to σ^{66} holoenzyme that had been purified from chlamydiae by heparin-agarose chromatography. While it has been noted that *E. coli* σ^{28} has a greater affinity for core enzyme compared with σ^{70} (the *E. coli* homologue of σ^{66}) (Kundu *et al*., 1997), our results suggest that, under our reaction conditions, chlamydial σ^{28} was able to bind to free core enzyme without displacing σ^{66} from intact holoenzyme. The presence of free core enzyme in the heparin-agarose-purified RNA polymerase would not be surprising as *E. coli* RNA polymerase purified by this method is only 30% saturated with σ^{70} (Chamberlin *et al*., 1983).

The high salt tolerance of our reconstituted chlamydial σ^{28} RNA polymerase has also been described for *E. coli* σ^{28} (σ^{F})-regulated transcription (Kundu *et al*., 1997). For *E. coli*, 200–300 mM potassium acetate was required for optimal *in vitro* transcription of two *E. coli* σ^{28}-regulated promoters, *fliC* and *fliD*, while transcription of a σ^{70}-regulated promoter decreased dramatically between 100 mM and 150 mM potassium acetate (Kundu *et al*., 1997). Our reconstituted σ^{28} RNA polymerase showed optimal transcription at 400 mM potassium acetate, a concentration at which σ^{66}-dependent promoter activity is completely inhibited. These results provide further proof that the *hctB* promoter is only transcribed by σ^{28} RNA polymerase and not by σ^{66} holoenzyme from the heparin-agarose-purified RNA polymerase preparation.

While σ^{28} regulates the expression of genes involved in flagellar synthesis, chemotaxis and motility in other bacteria (reviewed in Helmann, 1991; Gross *et al*., 1992; Haldenwang, 1995), the genes that are regulated by σ^{28} in *Chlamydia* spp. are not known. Chlamydiae are non-motile organisms that do not possess flagella. σ^{28} regulates the expression of some type III secretion genes in *Salmonella* spp. (Eichelberg and Galan, 2000) and *Chlamydia* does encode homologues of a few flagellar-like genes that may be structural components of the type III secretion apparatus (reviewed in Kim, 2001). σ^{28} has also been proposed to have a role in developmental regulation. σ^{28}-dependent transcription in *Bacillus* is highest during the transition from log phase to stationary phase growth and is dependent on many of the same *spo0* gene products needed for the initiation of the sporulation cascade (Gilman and Chamberlin, 1983).

We predicted that the *hctB* promoter is a σ^{28}-regulated promoter because sequences upstream of the mapped *in vivo* transcription start site showed strong conservation with the σ^{28} consensus promoter in other bacteria. This transcription start site had been mapped to a site 36 bp upstream of the predicted AUG translation start codon by Brickman *et al*. (1993). Fahr *et al*. (1995) also mapped this site as well as a second *in vivo* transcription start site 79 bp upstream of the predicted AUG. Neither site contains upstream sequences that resemble the optimal promoter structure recognized by the major sigma factor, σ^{66} (Tan *et al*., 1998; Schaumburg and Tan, 2003). In addition, neither predicted promoter produced a radiolabelled transcript in a σ^{66} transcription assay (Fahr *et al*., 1995). We have now shown that the promoter predicted from the transcription start site first mapped by Brickman *et al*. (1993) is recognized by σ^{28} RNA polymerase. The sequences upstream of the second transcription start site mapped by Fahr *et al*. (1995) do not resemble the σ^{28} consensus promoter, suggesting, perhaps, that a different form of RNA polymerase may recognize this putative promoter. Alternatively, this site may not be an actual transcription start site.

The *hctB* promoter strongly resembles the extended σ^{28} consensus promoter proposed by Ide *et al*. (1999). One small difference is that the *hctB* promoter has a 12 bp spacer while the extended promoter sequence and the majority of known bacterial σ^{28} promoters have an 11 bp spacer (reviewed in Helmann, 1991; Gross *et al*., 1992; Ide *et al*., 1999). Slight variability in spacer length of promoters is not without precedence. For example, *E coli* σ^{70} RNA polymerase prefers a 17 bp spacer, but a spacer range of 16–18 bp is not uncommon and σ^{70} promoters with a spacer between 15 and 21 bp have been identified (Mulligan *et al*., 1984).

Transcription of the *hctB* promoter by σ^{28} RNA polymerase suggests that this alternative form of chlamydial RNA polymerase is involved in the regulation of late gene expression. *hctB* is a prototypical late gene that is only transcribed late in the developmental cycle, at the time of RB to EB conversion (Brickman *et al*., 1993; Fahr *et al*., 1995). *hctB* encodes Hc2, a histone-like protein that is believed to be involved in this conversion through its ability to condense DNA. However, other late genes, such as *omcB*, *hctA*, *ltuA*, and *ltuB* have been shown to be transcribed by σ^{66} RNA polymerase (Fahr *et al*., 1995). Thus, it is likely that there is more than one mechanism for the regulation of late gene expression and that these mechanisms involve transcription by σ^{28} RNA polymerase and by regulation of σ^{66} RNA polymerase by an activator or repressor.

If σ^{28} is involved in transcriptional regulation of gene expression during the chlamydial developmental cycle, what regulates σ^{28} activity? The temporal pattern of chlamydial σ^{28} expression has been studied by measuring *rpsD* mRNA and σ^{28} protein expression levels during the developmental cycle. Mathews *et al*. (1999) measured peak *rpsD* mRNA levels early in the developmental cycle and speculated that σ^{28} RNA polymerase may be involved in EB to RB conversion. In contrast, Shaw *et al*. (2000) and Douglas and Hatch (2000) have shown that *rpsD* transcripts were undetectable at early times after infection, but present during

the mid- and late period of infection. σ^{28} protein has been detected early and late in the developmental cycle (A. M. Douglas and T. Hatch, pers. comm.). While these results are not definitive, it does not appear that the presence of σ^{28} protein by itself is sufficient for the regulated transcription of *hctB* at late times in the developmental cycle.

It is known from other bacteria that σ^{28} activity is controlled by a partner switching mechanism that involves an anti-sigma factor (reviewed in Hughes and Mathee, 1998). In *Bacillus* spp., the anti-sigma factor, RsbW, binds to σ^{28} and prevents it from binding with core enzyme to form an active polymerase. RsbW is itself regulated by an anti-anti-sigma factor that is differentially phosphorylated in response to a signalling cascade. The sequenced chlamydial genomes all contain genes that encode homologues of RsbW, RsbV and RsbU, which are proposed members of this partner switching mechanism (Stephens *et al*., 1998; Kalman *et al*., 1999; Read *et al*., 2000; 2003; Shirai *et al*., 2000).

With the reconstitution of active chlamydial σ^{28} RNA polymerase, we now have a powerful assay for studying gene regulation by this alternative RNA polymerase and for identifying other σ^{28}-regulated genes in addition to *hctB*. The *hctB* promoter strongly resembles the consensus bacterial σ^{28} promoter structure but we do not know if the sequences of other chlamydial σ^{28} promoters and the promoter specificity of chlamydial σ^{28} RNA polymerase are similarly conserved. Identification of additional σ^{28} promoters will allow us to gain insight into the role of σ^{28} RNA polymerase in the co-ordinate regulation of genes late in the developmental cycle and under other growth conditions in *Chlamydia*.

Experimental procedures

Reagents

The following products were obtained from the sources given and were used according to the manufacturers' specifications: restriction enzymes, calf intestinal alkaline phosphatase, T4 DNA ligase, rRNasin and *Taq* DNA polymerase, Promega Biotech; T4 polynucleotide kinase and pRSET expression vector, Invitrogen; Sequenase kit, U.S. Biochemicals; *E. coli* BL21 (DE3), Stratagene; nucleoside triphosphates, 3′-*O*-methylguanosine 5′-triphosphate, Amersham; Ni-NTA agarose beads, Qiagen; oligonucleotide primers, Sigma Genosys; ^{32}P-labelled nucleoside triphosphates, ICN; *Pwo* DNA polymerase, Roche Diagnostics; protein assay reagent, Protein A agarose kit, Bio-Rad; *E. coli* σ^{28} protein, monoclonal anti-*E. coli* σ^{28} antibodies, Neoclone.

Cloning of chlamydial *rpsD* (σ^{28} gene)

All *C. trachomatis* sequences are based on information from the Chlamydia Genome Project (http://chlamydia-www.berkeley.edu:4231/). Plasmid pMT1104 contains the *C. trachomatis* serovar L2 σ^{28} gene (*rpsD*) cloned into a His-tagged expression vector pRSET-C. The insert (containing the entire *rpsD* gene with the exclusion of the start codon) was amplified by PCR from *C. trachomatis* L2 genomic DNA by *Pwo* DNA polymerase, using PCR primers T133 (5′ AAGACTCACGATCTCGCAGATACTTG) and T134 (5′ CCCGGTACCCTAAAGCAGACTG). As the sequence of *C. trachomatis* serovar L2 *rpsD* was not available, we compared the sequence of our clone with the sequence of serovar D *rpsD*. There were three nucleotide differences between our amplified L2 *rpsD* gene and the serovar D *rpsD* sequence, but none of them produced a change at the amino acid level. The PCR product was digested with *Kpn*I and cloned into pRSET-C between *Kpn*I and blunted *Bam*HI sites.

Overexpression and purification of chlamydial σ^{28}

σ^{28} was overexpressed in *E. coli* BL21(DE3) freshly transformed with pMT1104. Next, 250 ml of cells were grown at 37°C to an optical density at 600 nm of 0.5 and induced with 1 mM isopropyl-β-D-thiogalactosidase (IPTG). After 3 h, cells were collected by centrifugation, resuspended in 8 ml of Buffer N (10 mM Tris, pH 8.0, 0.3 M NaCl, 10 mM β-mercaptoethanol) containing 20 mM imidazole and disrupted with a Branson Sonifier 450 (30 s × 4) in the presence of 0.2% sarkosyl. Soluble protein was separated from cell debris by centrifugation at 10 000 r.p.m. for 10 min at 4°C (Beckman JA-17 rotor). 2% Triton X-100 was added to sequester the sarkosyl.

σ^{28} protein was then purified under denaturing conditions. The protein lysate was precipitated with 60% ammonium sulphate and the protein pellet was solubilized with 6 M guanidine hydrochloride. Proteins were allowed to bind to Ni-NTA agarose beads at 4°C for 1 h. Bound proteins were washed with Buffer N containing 30 mM imidazole. His-tagged protein was eluted with Buffer N containing 250 mM imidazole. Purified σ^{28} protein was dialysed overnight with two changes of storage buffer (50 mM Tris, pH 8.0, 200 mM KCl, 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 1 mM EDTA, 5 mM 2-β-mercaptoethanol, 20% glycerol). The concentration of the purified σ^{28} protein was approximately 350 μg ml^{-1}.

Production of polyclonal anti-σ^{28} antibodies

Recombinant σ^{28} was gel purified by SDS–PAGE and used to generate rabbit polyclonal antibodies (Harlan Bio-products for Science). Antibodies were purified by Protein-A agarose column according to the manufacturer's instructions (Bio-Rad).

Purification of *C. trachomatis* RNA polymerase from chlamydiae grown in tissue culture

Chlamydia trachomatis LGV serovar L2 was grown in mouse L929 cells and harvested at 18 h post-infection. RNA polymerase was partially purified by heparin-agarose chromatography as previously described (Tan and Engel, 1996).

Construction of transcription plasmids

Plasmid pMT1212 contains the promoter region of *C. trachomatis hctB* (−135 to +5), which was amplified by PCR from *C. trachomatis* serovar L2 genomic DNA with primers T12 (5′-ATTTATTTGATCTATCGAC) and T13 (5′-GCCGAAT-TCAGGATTTGGTGTGC). Transcription from this plasmid produced a 150 nt transcript. Plasmid pMT1211 contains the *C. trachomatis dnaK* promoter (−180 to +5) amplified from *C. trachomatis* MoPn strain genomic DNA with primers dnaK2 (5′-AAGTTGGTGTCATTATAGGAAAACC) and dnaK180 (5′-AGGGAATTCGATCGAATCTCGATCTGG). Transcription from this plasmid produced a 130 nt transcript. For each promoter, the insert was cloned upstream of a promoter-less, G-less cassette transcription template in plasmid pMT1125 (Wilson and Tan, 2002).

In vitro transcription

The general transcription reaction was performed in a 10 μl final volume with the following components: 50 mM potassium acetate, 8.1 mM magnesium acetate, 50 mM Tris acetate pH 8.0, 27 mM ammonium acetate, 2 mM DTT, 400 μM ATP, 400 μM UTP, 1.2 μM CTP, 0.06 μM [α-^{32}P]-CTP (3000 Ci $mmol^{-1}$), 100 μM 3′-O-methylguanosine 5′-triphosphate Na salt, 18 units rRNasin, 10% glycerol and 25 nM supercoiled DNA template. In some experiments, the potassium acetate concentration was varied over a range from 0 to 600 mM. The transcription reaction was incubated at 37°C for 15 min and terminated by the addition of 10 μl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). Next, 7 μl of the sample was electrophoresed on an 8 M urea/6% polyacrylamide gel

and quantified by phosphorimager analysis with a Bio-Rad Personal FX scanner and analysis with Bio-Rad Quantity One software, as previously described (Wilson and Tan, 2002).

In general, σ^{28} transcription reactions were performed with chlamydial σ^{28} RNA polymerase that had been reconstituted by mixing 0.5 μl purified His_6-σ^{28} (final concentration of 480 nM) with 1 μl heparin-agarose-purified RNA polymerase (Schaumburg and Tan, 2000) at 4°C for 15 min, immediately prior to the transcription reaction. For the σ^{28} titration experiments, a range of σ^{28} concentrations (30, 120 or 480 nM final concentration) was used for the reconstitution. Next, 1 μl heparin-agarose-purified *C. trachomatis* RNA polymerase was used for the σ^{66} transcription reactions. For the antibody-inhibition reactions, anti-chlamydial σ^{28} antibodies were pre-incubated with each RNA polymerase for 20 min at room temperature prior to transcription.

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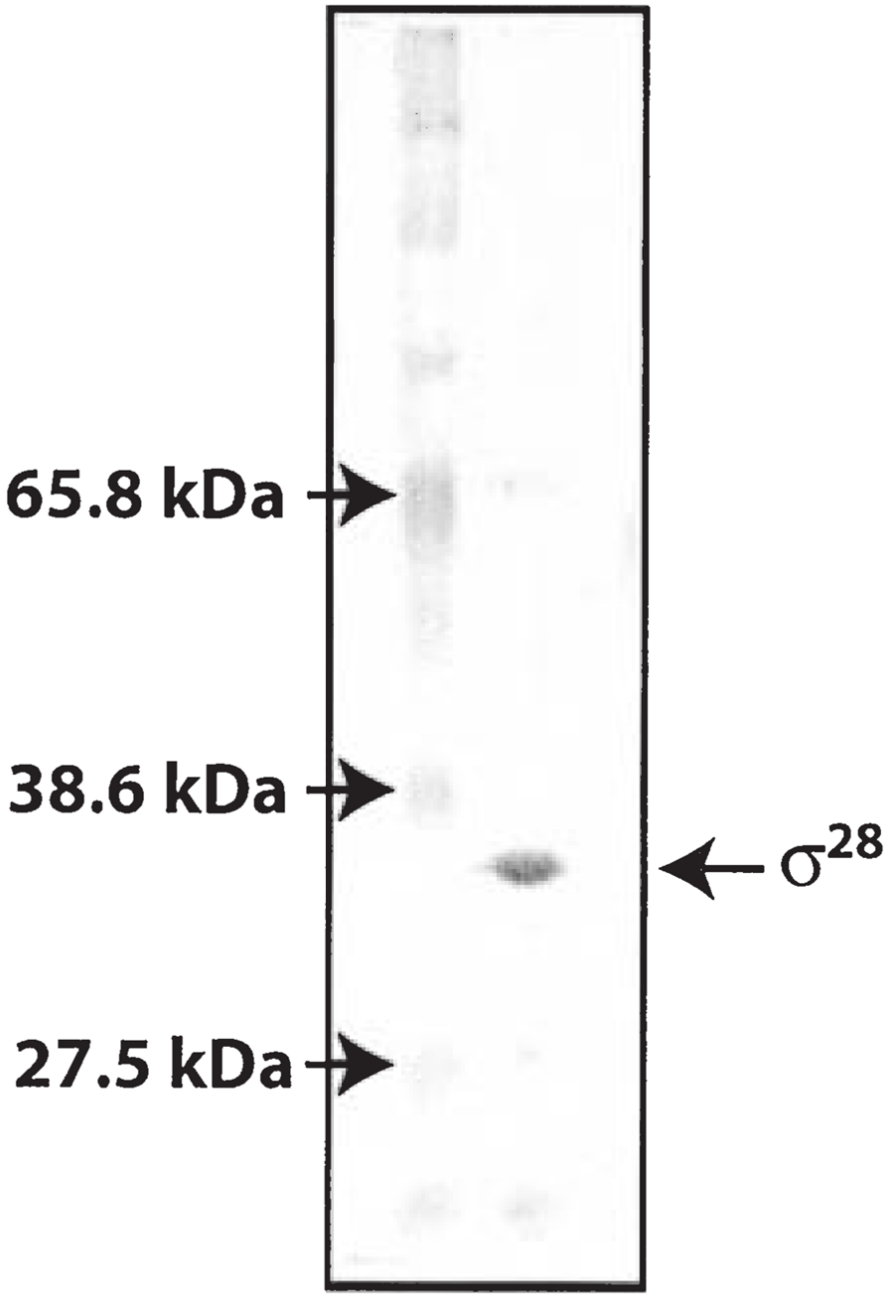

Fig. 1.
Silver stain of SDS–PAGE showing purified recombinant histidine-tagged chlamydial σ^{28} protein. Lane 1, marker; Lane 2, σ^{28} after purification with Ni-NTA beads.

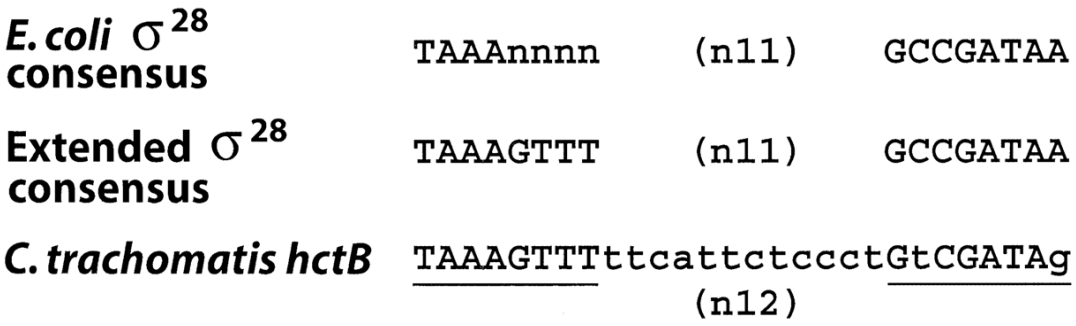

Fig. 2.

Alignment of *Chlamydia trachomatis* serovar L2 *hctB* promoter (Brickman *et al*., 1993) with the σ^{28} consensus promoter structure (reviewed in Helmann, 1991; Gross *et al*., 1992) and σ^{28} extended promoter structure (Ide *et al*., 1999) derived from *Escherichia coli* and *Salmonella* sequences. Predicted promoter elements are underlined with the sequence differences from the extended promoter structure shown in lowercase.

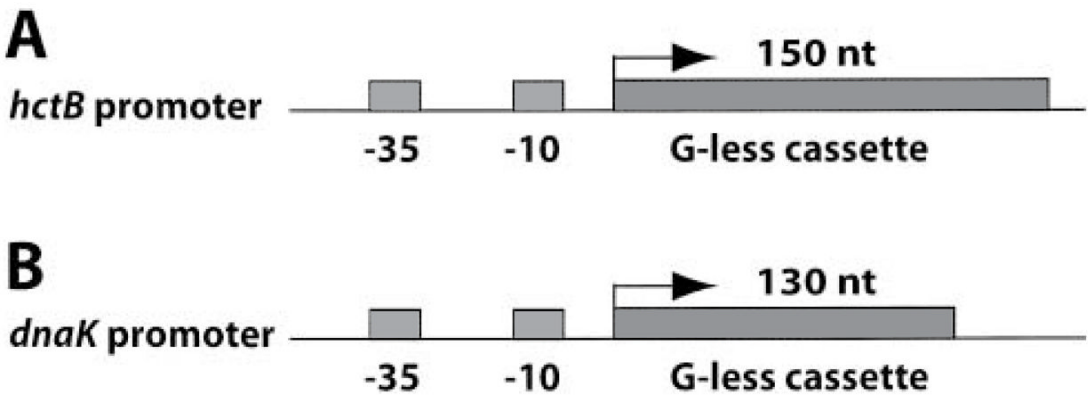

Fig. 3. Schematic diagram of the transcription plasmids

A. The *C. trachomatis hctB* promoter precedes a 150 nt G-less cassette transcription template in pMT1212.

B. The *C. trachomatis dnaK* promoter precedes a 130 nt G-less cassette in pMT1211.

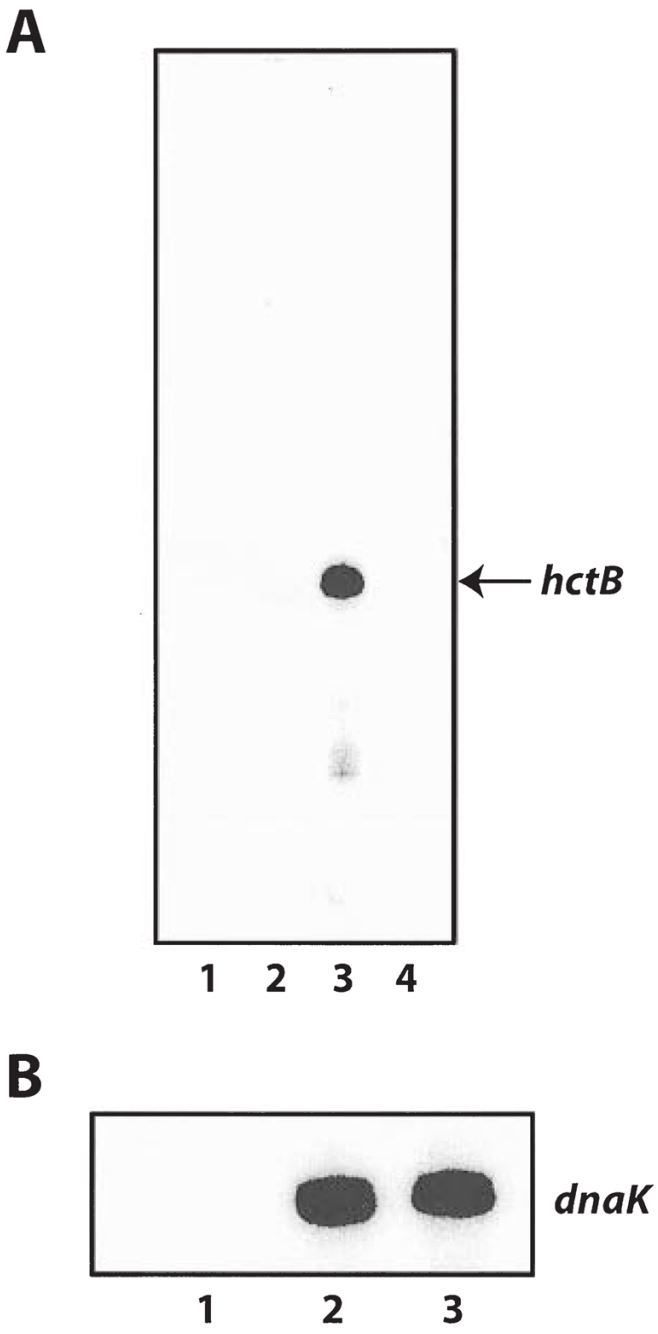

Fig. 4. *In vitro* transcription reactions with chlamydial σ^{28} RNA poly-merase

A. Transcription from the *hctB* promoter with the following components present in the reaction: Lane 1, σ^{28} alone; Lane 2, heparin-agarose-purified RNA polymerase alone; Lane 3, chlamydial σ^{28} RNA polymerase reconstituted from σ^{28} and heparin-agarose-purified RNA polymerase; Lane 4, chlamydial σ^{28} RNA polymerase in the presence of anti-chlamydial σ^{28} antibodies.

B. σ^{66}-dependent transcription from the *dnaK* promoter with the following components present in the reaction: Lane 1, σ^{28} alone; Lane 2, heparin-agarose-purified RNA polymerase alone; Lane 3, heparin-agarose-purified RNA polymerase in the presence of anti-chlamydial σ^{28} antibodies.

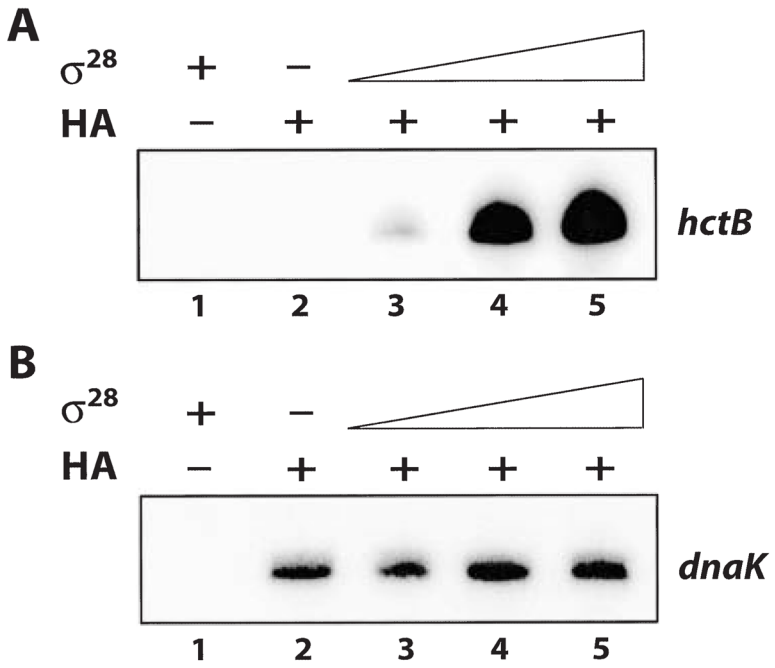

Fig. 5. *In vitro* transcription reactions showing the effect of increasing amounts of σ^{28} on the reconstitution of σ^{28} RNA polymerase

A. Transcription from the *hctB* promoter. Lane 1, σ^{28} alone; Lane 2, heparin-agarose-purified RNA polymerase (HA) alone; Lane 3–5, heparin-agarose-purified RNA polymerase (HA) with increasing amounts of σ^{28} (30, 120 and 480 nM final concentration).

B. Transcription from the *dnaK* promoter. Lane 1, σ^{28} alone; Lane 2, heparin-agarose-purified RNA polymerase (HA) alone; Lane 3–5, heparin-agarose-purified RNA polymerase (HA) with increasing amounts of σ^{28} (30, 120 and 480 nM final concentration).

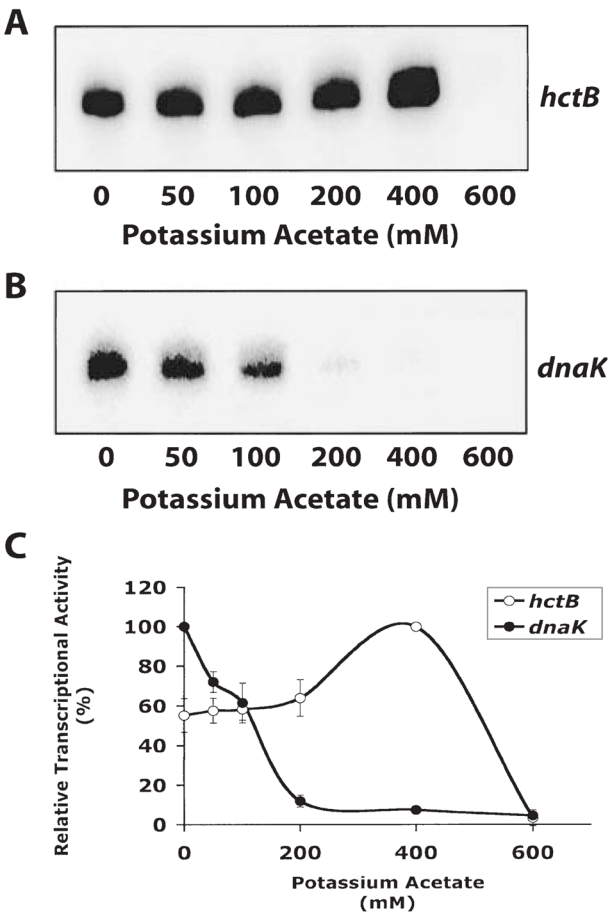

Fig. 6. ***In vitro*** **transcription reactions showing the effect of varying salt concentration**

A. Transcription from the *hctB* promoter by chlamydial σ^{28} RNA polymerase. Lane 1–6, increasing concentrations of potassium acetate (0, 50, 100, 200, 400 and 600 mM).

B. Transcription from the *dnaK* promoter by heparin-agarose-purified RNA polymerase. Lanes 1–6, increasing concentrations of potassium acetate (0, 50, 100, 200, 400 and 600 mM).

C. Quantification of transcriptions. Reactions were performed in triplicate and quantified by phosphorimager analysis. Results were normalized to 100% for the highest level of transcription for each promoter. Error bars are SD from the mean.