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The Transcriptional Repressor EUO Regulates Both Subsets of *Chlamydia* Late Genes

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SUMMARY

The pathogenic bacterium *Chlamydia* replicates in a eukaryotic host cell via a developmental cycle marked by temporal waves of gene expression. We have previously shown that late genes transcribed by the major chlamydial RNA polymerase, σ^{66} RNA polymerase, are regulated by a transcriptional repressor EUO. We now report that EUO also represses promoters for a second subset of late genes that are transcribed by an alternative polymerase called σ^{28} RNA polymerase. EUO bound in the vicinity of six σ^{28}-dependent promoters and inhibited transcription of each promoter. We used a mutational analysis to demonstrate that the EUO binding site functions as an operator that is necessary and sufficient for EUO-mediated repression of σ^{28}-dependent transcription. We also verified specific binding of EUO to σ^{66}-dependent and σ^{28}-dependent promoters with a DNA immunoprecipitation assay. These findings support a model in which EUO represses expression of both σ^{66}-dependent and σ^{28}-dependent late genes. We thus propose that EUO is the master regulator of late gene expression in the chlamydial developmental cycle.

Keywords

sigma 28; transcription factor; promoter; temporal regulation; DNA immunoprecipitation

INTRODUCTION

The human pathogen *Chlamydia* replicates within an infected host cell via an unusual developmental cycle in which there is conversion between two specialized forms of the bacterium (Abdelrahman & Belland, 2005). The elementary body (EB), which is the infectious form, initiates the intracellular infection by binding and entering the host cell. Early in the infection, within the first few hours of entry, the EB converts into a second morphologic form called a reticulate body (RB). The RB is metabolically active and replicates through multiple rounds of binary fission during midcycle of the infection. At about 18–24 hours post infection (hpi), the intracellular infection enters its late stage when individual RBs convert into an EB, prior to exit from the host cell to infect new cells.

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Chlamydial genes are expressed in three main temporal classes that correspond to these three stages of the developmental cycle (Belland *et al.*, 2003, Shaw *et al.*, 2000, Nicholson *et al.*, 2003). Early genes are transcribed within three hours of EB entry, and are believed to be important for establishing the intracellular infection. Midcycle genes are involved in RB growth and replication and make up the large majority of chlamydial genes. Late genes are a small group of specialized genes that are first transcribed or upregulated towards the end of the developmental cycle. Early, midcycle and late genes can all be transcribed by the major chlamydial RNA polymerase, σ^{66} RNA polymerase (Tan, 2012).

Many late genes are involved in RB-to-EB conversion and EB function. For example, the late operon *omcAB* encodes two cysteine-rich outer membrane proteins that are highly abundant in EBs (Mukhopadhyay *et al.*, 2006). *hctA* and *hctB* are late genes that encode the histone-like proteins HctA (Hc1) and HctB (Hc2), which bind and compact DNA, mediating the DNA condensation that is characteristic of an EB (Barry *et al.*, 1992) (Brickman *et al.*, 1993). Other late genes include *tsp*, which encodes a chlamydial protease, *tlyC_1*, which encodes a putative hemolysin, and several genes for the type III secretion apparatus, such as *scc2*, *cdsJ*, and *cdsU* (Yu *et al.*, 2006b, Case *et al.*, 2010). The expression of late genes must be delayed until RBs have replicated in order to prevent premature RB-to-EB conversion.

Late genes transcribed by σ^{66} RNA polymerase are regulated by a transcription factor called EUO (Rosario & Tan, 2012). EUO provides a mechanism to differentially regulate σ^{66}-dependent late genes from early and midcycle genes that are transcribed by the same form of RNA polymerase. EUO, which stands for early upstream operon, was first identified because its transcript is expressed at very early times in the developmental cycle (Wichlan & Hatch, 1993). It is a DNA binding protein that recognizes a 15 bp A/T-rich consensus sequence (Zhang *et al.*, 1998, Zhang *et al.*, 2000). We have shown that EUO selectively binds to this operator sequence in the vicinity of σ^{66}-dependent late promoters and inhibits their transcription (Rosario & Tan, 2012). These findings support a model in which the early expression of EUO represses transcription of σ^{66}-dependent late genes until they are derepressed at late times by an undefined mechanism.

Not all late genes are controlled by σ^{66} RNA polymerase, however, for a subset is regulated by an alternative chlamydial RNA polymerase called σ^{28} RNA polymerase (Yu & Tan, 2003). σ^{28} RNA polymerase transcribes promoters for three *C. trachomatis* late genes, *hctB*, *tsp*, and *tlyC_1* (Yu et al., 2006b). However, σ^{28} is transcribed from a midcycle gene *rpsD* (Douglas & Hatch, 2000, Shen *et al.*, 2004), raising the question of whether the activity of this alternative RNA polymerase is temporally regulated.

From these published observations, we hypothesized that the transcription of σ^{28}-dependent late genes is regulated to control their temporal expression. We were particularly interested in EUO as a well-characterized chlamydial transcription factor that controls late gene expression. We found that EUO bound and repressed promoters for all known σ^{28}-dependent genes, including the three late genes and three additional σ^{28}-dependent genes that are expressed prior to late times. Our results demonstrate that EUO regulates both σ^{66}-dependent and σ^{28}-dependent late promoters, supporting a role for this transcriptional

repressor as a master regulator of late gene expression in the chlamydial developmental cycle.

RESULTS

EUO binds in the vicinity of chlamydial σ^{28}-dependent promoters

To investigate if EUO has a broader role in regulating late chlamydial gene expression, we examined if this transcriptional repressor of σ^{66}-dependent late genes also binds *C. trachomatis* promoters transcribed by σ^{28} RNA polymerase. In an electrophoretic mobility shift assay (EMSA), recombinant *C. trachomatis* EUO (rEUO) produced a gel shift of two bands with the σ^{28}-dependent *hctB* promoter (−55 to +5, relative to the transcription start site, +1) (Fig. 1) (Yu & Tan, 2003). This EMSA pattern was similar to the shift produced with the *omcAB* promoter, which is a σ^{66}-dependent EUO target (Rosario & Tan, 2012). The binding was sequence-specific because there was no binding to the *dnaK* P1 promoter, which is an early σ^{66}-dependent chlamydial gene that is not regulated by EUO (Fig. 1). EUO also bound to DNA fragments for the *tsp*, *tlyC_1*, *bioY*, *dnaK* P2 and *pgk* promoters, which are five other *C. trachomatis* σ^{28}-regulated promoters that have been experimentally verified (Fig. 1 and Table 1) (Yu et al., 2006b).

In these *in vitro* binding studies, EUO bound the six σ^{28}-dependent promoters in different locations relative to their respective −35 and −10 promoter elements (Table 1). For example, EUO bound within −55 to +5 of the *hctB* promoter, which encompasses the −35 and −10 elements. However, EUO appears to bind upstream of the −35 element of the *tsp* promoter because it bound a DNA fragment containing sequences from −90 to +5 (Fig. 1) but not −55 to +5 (data not shown). In contrast, EUO appears to bind downstream of the −10 element of the *tlyC_1* promoter because it bound a DNA fragment from −30 to +30 (Fig. 1) but not −55 to +5 (data not shown).

EUO represses transcription of chlamydial σ^{28}-dependent promoters

We next measured the functional effect of EUO binding on these six σ^{28}-dependent promoters with an *in vitro* transcription assay. These studies were performed with σ^{28} RNA polymerase reconstituted from recombinant *C. trachomatis* σ^{28} and *E. coli* core enzyme (Yu & Tan, 2003). This heterologous σ^{28} RNA polymerase has a similar promoter recognition as σ^{28} RNA polymerase reconstituted from recombinant *C. trachomatis* σ^{28} and partially purified *C. trachomatis* RNA polymerase but has the advantage of lacking other co-purified chlamydial proteins (Yu & Tan, 2003).

For five of the six σ^{28} promoters, rEUO decreased transcription to 31–35% relative to transcription in the absence of EUO (Fig. 2). This level of inhibition was similar to repression of the *omcAB* promoter that was measured in parallel experiments with σ^{66} RNA polymerase (Fig. 2). In a negative control experiment, EUO did not inhibit the *dnaK* P1 promoter, which is not regulated by EUO. In contrast to the five other σ^{28} promoters, EUO only decreased transcription of the *hctB* promoter to 75% of baseline levels, which was a statistically significant ($p < 0.005$), but small effect.

We performed additional studies on the *hctB* promoter to determine if it is regulated by EUO. This promoter is highly transcribed by chlamydial σ^{28} RNA polymerase *in vitro* and its sequence closely resembles the bacterial consensus σ^{28} promoter sequence (Yu & Tan, 2003). In a first approach, we tested the effect of EUO at a higher EUO:DNA ratio, which we achieved by using less transcription template. At a plasmid DNA concentration of 3 nM, instead of 13 nM, EUO decreased transcription of the *hctB* promoter to 25% of baseline, providing evidence that it is an EUO target promoter and is repressed similarly to the other σ^{28}-dependent promoters (Fig. 3A).

In a complementary approach, we tested the effect of EUO on three mutant *hctB* promoters that have reduced promoter activity because of a point substitution at position −12 (Fig. 3B, 3C) (Yu *et al.*, 2006a). EUO caused inhibition, though modest, of these weaker promoters, reducing transcription to 61%, 43%, and 50% of baseline for a C-to-T, C-to-A and C-to-G substitution, respectively (Fig. 3E). Together these results provide evidence that *hctB* is regulated by EUO. The differences among the six promoters, in the extent of repression by EUO, are consistent with previous observations of promoter-specific effects on the efficiency of repressor action (Lanzer & Bujard, 1988).

Mapping the location of the EUO operator for a σ^{28}-dependent promoter

We used a mutational approach to verify that an EUO binding site is necessary for repression. We chose to map the operator for *tsp* because its location appeared, from our EMSA studies, to be farthest from the σ^{28} promoter. We identified a candidate site from −73 to −59 (Fig. 4A) based on its resemblance to the EUO consensus binding sequence (Zhang et al., 2000). In our mutational studies, we were guided by previous observations that showed that nine, but not three, nucleotide substitutions were necessary to greatly reduce EUO binding to the *C. trachomatis omcAB* promoter (Rosario & Tan, 2012). Therefore, we introduced nucleotide substitutions in 10 of the 15 bp of the predicted *tsp* operator, replacing adenine or thymine residues with guanine or cytosine, respectively (Fig. 4A). EUO was unable to bind a *tsp* promoter template containing this mutant operator (Fig. 4B) nor inhibit its transcription by σ^{28} RNA polymerase (Fig. 4C). These results provide evidence for an operator upstream of the −35 element of the *tsp* promoter that is necessary for repression by EUO.

The EUO operator is sufficient for EUO-mediated repression

We next examined if a promoter could be converted into an EUO-regulated promoter by addition of an EUO operator. We placed the *omcAB* EUO operator into the EUO-independent *dnaK* P1 promoter and tested for binding by EMSA (Fig. 4A). The core 15 bp of the *omcAB* EUO operator was not sufficient for EUO binding (data not shown), which is consistent with published observations (Zhang et al., 2000). Instead EUO binding required the 15 bp core EUO operator to be present in the middle of a 30 bp *omcAB* fragment (data not shown), suggesting that additional sequences flanking the core operator are also important for binding (Fig. 4A). EUO reduced transcription of this operator-containing *dnaK* P1 promoter to 26% of transcription in the absence of EUO, but did not inhibit transcription of the wild type *dnaK* P1 promoter (Fig. 4D). These results demonstrate that a 30 bp nucleotide sequence containing the EUO operator is sufficient for EUO-mediated repression.

EUO selectively binds both σ^{28}-dependent and σ^{66}-dependent late promoters

We used DNA immunoprecipitation to provide further evidence that EUO selectively regulates late genes. *C. trachomatis* genomic DNA was incubated in the presence or absence of rEUO, and EUO-bound DNA fragments were isolated by immunoprecipitation and quantified by qPCR. In control experiments without EUO, we recovered < 0.15% of input DNA for all of the six promoters tested, providing a measure of background DNA immunoprecipitation. EUO binding to specific promoters was detected by DNA recovery above this background threshold and by enrichment when comparing recovery in the presence and absence of EUO.

EUO bound to two σ^{28}-dependent late promoters with a 7.3-fold enrichment for *hctB* (0.95% of input in the presence of EUO versus 0.13% in the absence of EUO), and a 21-fold enrichment for *tlyC1* (1.04% versus 0.05% input) (Fig. 5). EUO also bound to two σ^{66}-dependent late promoters, with a 65-fold enrichment for *omcAB* (2.6% versus 0.04% input) and a 46-fold enrichment for *ltuB* (0.91% versus 0.02% input) (Fig. 5). EUO addition produced a modest enrichment for two non-late promoters, with a 2-fold enrichment for *ompA* (0.1% versus 0.05% input), and a 2.8-fold enrichment for *fliF* (0.14% versus 0.05% input) (Fig. 5). However, this DNA recovery, even with the addition of EUO, was below the background threshold, consistent with a lack of specific binding to these non-late promoters. These immunoprecipitation studies demonstrate that EUO specifically binds both σ^{28}-dependent and σ^{66}-dependent late genes.

DISCUSSION

This study provides evidence that the chlamydial transcriptional repressor EUO regulates promoters transcribed by σ^{28} RNA polymerase. EUO bound and repressed the six known chlamydial σ^{28}-dependent promoters (Yu et al., 2006b), and we identified a putative EUO binding site in the vicinity of each promoter (Fig. 6). In addition, we demonstrated that the EUO binding site functions as an operator that is necessary and sufficient for EUO-mediated repression. Binding and transcriptional inhibition of σ^{28} promoters by EUO, as well as the location of the operator near the promoter, were similar to EUO-mediated repression of σ^{66} late promoters (Rosario & Tan, 2012).

The mechanism of EUO-mediated repression is not known. The location of many EUO operators in the close vicinity of their promoter, and often overlapping the −35 and −10 elements (Rosario & Tan, 2012 and Fig. 6), suggests that EUO may repress these promoters throught the classic mechanism of steric hindrance. A noteable exception is the *tsp* operator, which is located relatively far upstream of its promoter. The 15 bp core *tsp* operator from −73 to −59 has a limited predicted overlap with the region of σ^{28} RNA polymerase-promoter binding, which has been mapped in *E. coli* from −62 to +14 by DNase I footprinting (Kundu *et al.*, 1997, Payankaulam *et al.*, 2010). However, the region of EUO binding may be larger because the EUO DNAse I footprint covers 27–37 bp (Zhang et al., 2000), and we found that additional flanking sequences were important for EUO-mediated binding and repression. Alternatively, it is possible that EUO represses the *tsp* promoter via mechanisms subsequent to RNA polymerase-promoter binding. For example, EUO may inhibit promoter

melting or clearance, which are more likely to be rate-limiting steps in transcription initiation for the highly-transcribed *tsp* promoter (Rojo, 2001).

These findings support a role for EUO as the master regulator of late gene expression in the chlamydial developmental cycle. Late genes can be divided into two subsets that are transcribed by either σ^{66} RNA polymerase, which is the major chlamydial RNA polymerase, or σ^{28} RNA polymerase (Tan, 2012). We previously showed that EUO regulates σ^{66}-dependent late genes because it repressed transcription of six late promoters by σ^{66} RNA polymerase and bound the promoter regions of four additional σ^{66}-dependent late genes (Rosario & Tan, 2012). By showing that EUO also controls six σ^{28}-dependent promoters, we now provide evidence that EUO regulates both subsets of late chlamydial genes. Our data indicate that the EUO regulon consists of at least 16 operons encoding 17 late genes in *C. trachomatis*. The total number of EUO target genes is not known, however, and it is difficult to accurately identify EUO operators in the genome because of their degenerate A/T-rich sequence. It is also not yet known whether EUO is solely responsible for late gene expression during the developmental cycle.

EUO provides an elegant mechanism to coordinately regulate the temporal expression of multiple chlamydial late genes. EUO is expressed from very early times in the chlamydial developmental cycle with transcripts and protein detected within 1 hour post infection (Zhang et al., 2000). We have proposed that this early expression of EUO inhibits late promoters until its transcriptional repression is relieved at late times by an as-yet-undefined mechanism (Rosario & Tan, 2012). As a transcriptional repressor, EUO can regulate multiple promoters provided that each target gene has an operator in the vicinity of its promoter for EUO binding. As we have shown in the current study, this mechanism of promoter-specific regulation does not depend on the form of RNA polymerase that transcribes each target late gene. There is precedent for a transcription factor to regulate two forms of RNA polymerase because *Klebsiella pneumoniae* NAC (nitrogen assimilation control) represses both σ^{54}- and σ^{70}-dependent transcription (Rosario *et al.*, 2010).

We propose that EUO-mediated repression may be calibrated so that individual promoters are repressed to different extents. In this study, and in our previous studies with σ^{66}-dependent promoters (Rosario & Tan, 2012), we observed that the level of binding and transcriptional inhibition by EUO varied by promoter. Thus it is likely that target genes within the EUO regulon will not have an identical temporal profile even though they are all late genes that are regulated by EUO. For example, strongly repressed genes may have an off-on expression pattern, while partially repressed genes may be transcribed at baseline levels and then upregulated when EUO-mediated repression is relieved. These predictions are consistent with the observed expression patterns of late genes, which include genes that are first transcribed at late times, as well as genes that are upregulated to higher expression levels at late times (Belland et al., 2003).

As the master regulator of late gene expression, EUO is likely to play a critical role in the chlamydial developmental cycle. Proteins encoded by late genes are important for RB-to-EB conversion, which is a terminal differentation step. For example, the σ^{28}-dependent late gene *hctB* encodes the histone-like protein HctB which is proposed to mediate the compaction of

chlamydial DNA into the condensed chromatin of an EB (Brickman et al., 1993). σ^{66}-dependent late genes include *omcAB*, which are the genes for two EB-specific outer membrane proteins (Liu *et al.*, 2010, Clarke *et al.*, 1988), and *scc2* and *cdsU*, which encode components of the Type III secretion system that is utilized for EB entry into a new host cell (Betts-Hampikian & Fields, 2010). It is not known why late genes are transcribed by two forms of RNA polymerase, but the timing of all late genes must be regulated to prevent premature RB-to-EB conversion before RBs have undergone multiple rounds of replication. We propose that EUO coordinates the expression of the two subsets of late genes, and in doing so controls the balance between chlamydial replication and the production of infectious progeny. This important regulatory role could be used as the basis for a novel anti-chlamydial antibiotics strategy in which EUO is targeted to limit chlamydial replication by promoting premature RB-to-EB conversion.

EXPERIMENTAL PROCEDURES

Construction of *in vitro* transcription plasmids

Promoter sequences were amplified by PCR from *C. trachomatis* serovar D UW-3/Cx genomic DNA or produced by annealing complementary oligonucleotides. Mutant *hctB* promoter mutants were generated as previously described (Yu et al., 2006a). The mutant *tsp* promoter, lacking its operator, was generated by PCR using an oligo containing substitutions of ten nucleotides in the predicted EUO operator (Fig. 4). The mutant *dnaK* P1 promoter, containing the *omcAB* operator, was generated by annealing complimentary 60 bp oligonucletoides containing the nucleotide substitutions shown in Fig. 4. DNA fragments was collected through a mini Quick Spin DNA column (Roche). Each promoter sequence was cloned upstream of the promoterless G-less cassette transcription template pMT1125 as previously described (Wilson & Tan, 2002). All constructs were verified by sequencing (Genewiz). Plasmids used in this study are listed in Table 2.

Purification of recombinant EUO

Purification of recombinant His-tagged EUO (rEUO) was previously described (Rosario & Tan, 2012). *E. coli* strain BL21 was transformed with pMT1181, which is an expression plasmid encoding *C. trachomatis* EUO with a 6×His tag at the C-terminus. Transformed cells were grown in 1 L LB containing 100 μg/ml ampicillin to mid-logarithmic growth and induced with 1 mM IPTG for 2 h at 37°C. Pelleted cells were resuspended in buffer N [10 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 10 mM 2-mercaptoethanol] containing 20 mM imidazole, and sonicated twice with a Branson digital sonifier 250D for 30 s at 22% output. The material was centrifuged and the supernatant was incubated with a 1 ml slurry of Ni-NTA beads (Qiagen) for 1 h at 4°C. The beads were then washed with 500 ml of buffer N containing 20 mM imidazole, and protein was eluted with 6 bed volumes of buffer N containing 250 mM imidazole. The eluted protein was dialyzed overnight against 1 L of storage buffer [10 mM Tris HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 100 mM NaCl, 10 mM 2-mercaptoethanol, 30% (v/v) glycerol]. Dialyzed protein was aliquoted and stored at −70°C.

Electrophoretic mobility shift assays (EMSA)

Annealed 60 bp complementary primers were labelled by T4 polynucleotide kinase (New England Biolabs) with approximately 30 μCi [γ-^{32}P]-ATP (10 mCi mmol^{-1}; MP Biomedicals). Free nucleotides were removed with a mini Quick Spin DNA column (Roche). Approximately 0.5 nM labelled DNA was incubated with 320 nM rEUO in binding buffer [40 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 70 mM KCl, 125 μM EDTA, 100 μM dithiothreitol, 7.5% glycerol, 10 ng of salmon sperm DNA] at room temperature for 20 min. Samples were loaded onto a 6% polyacrylamide EMSA gel at 150 V in 0.5× Tris-borate-EDTA (TBE) buffer (Read, 1996). After electrophoresis, the gel was dried on Whatman paper and exposed to a phosphorimager screen, which was scanned with a Bio-Rad Personal FX scanner.

In vitro transcription assays

In vitro transcription of σ^{28}-dependent promoters was performed as previously described (Yu et al., 2006b) with slight modifications. Approximately 13 nM (or 3 nM in Fig. 3) plasmid DNA containing the transcription template was incubated with 2.5 μM rEUO at room temperature for 15 minutes, and then transcription was initiated with σ^{28} RNA polymerase consisting of 0.4 U *E. coli* core enzyme (Epicentre) and 1 μl *C. trachomatis* recombinant His-tagged σ^{28}. σ^{66}-dependent promoters were transcribed with 0.4 U *E. coli* RNA polymerase holoenzyme (Epicentre). The transcripts were resolved on an 8 M urea-6% polyacrylamide gel. The amount of transcripts loaded onto the gel was adjusted to give similar intensities among the different promoter constructs. After electrophoresis, the gel was fixed, dried and exposed to a phosphorimager screen. The screen was scanned with a Bio-Rad Personal FX scanner, and the amount of each transcript was quantified using Quantity One software (Bio-Rad). For each promoter, the relative transcription was calculated by measuring transcript levels in the presence of EUO and normalizing to levels in the absence of EUO. Values are reported as the mean of the relative transcript levels with standard deviation from at least three individual experiments.

Micrococcal Nuclease digestion

C. trachomatis RBs (serovar L2 434/Bu) were purified on a renografin gradient as previously described (Zhang et al., 1998). Genomic DNA was isolated using a DNeasy Blood and Tissue Kit (Qiagen). 2 μg genomic DNA was digested to 300–1200 bp fragments by incubating with 1.5 units of Micrococcal Nuclease (NEB) at 37°C for 5 minutes.

DNA Immunoprecipitation

For each immunoprecipitation, 30 μl of Protein-G beads (GE Healthcare) were prepared at 4°C by preincubating with 5 μg of anti-His antibody (GE Healthcare) for 30 minutes, blocking with 500 μl of 5% BSA containing 200 μg/ml sheared salmon sperm DNA for 30 minutes, and then washing twice with 250 μl of Wash Buffer (40 mM Tris pH 8, 4 mM $MgCl_2$, 70 mM KCl, and 7.5% glycerol).

Digested *C. trachomatis* serovar L2 434/Bu genomic DNA (10 pM) was incubated in the presence or absence of rEUO (20 nM) on ice for 30 minutes and then incubated with an

aliquot of prepared Protein-G beads for a further 30 minutes. The supernatant was discarded and the beads were washed four times with 250 µl of Wash Buffer. Protein/DNA complexes were eluted from the beads with 100 µl of 0.1 M glycine pH 2.5, followed by addition of 12 µl of 1 M Tris pH 8.5 to neutralize the pH. Samples were heated to 95°C for 10 minutes to denature the protein. The DNA immunoprecipitation was performed as three independent experiments.

Quantitative PCR

DNA immunoprecipitation products were analyzed with quantitative PCR on a BioRad iCycler using the iQ SYBR Green Supermix (BioRad) with promoter-specific primers (Table S1). For each target promoter, the Ct value for 100% of input DNA (10 pM) was determined. Using the same primer pair, the Ct value for the immunoprecipitated DNA was then measured and normalized to the input DNA and reported as a percentage. The fold enrichment for each promoter was calculated from the ratio of DNA immunoprecipitated in the presence of EUO: DNA immunoprecipitated in the absence of EUO. Results are reported as the mean of three independent DNA immunoprecipitation experiments and the calculated standard deviation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Zhang L, Howe MM, Hatch TP. Characterization of *in vitro* DNA binding sites of the EUO protein of *Chlamydia psittaci*. Infect Immun. 2000; 68:1337–1349. [PubMed: 10678946]

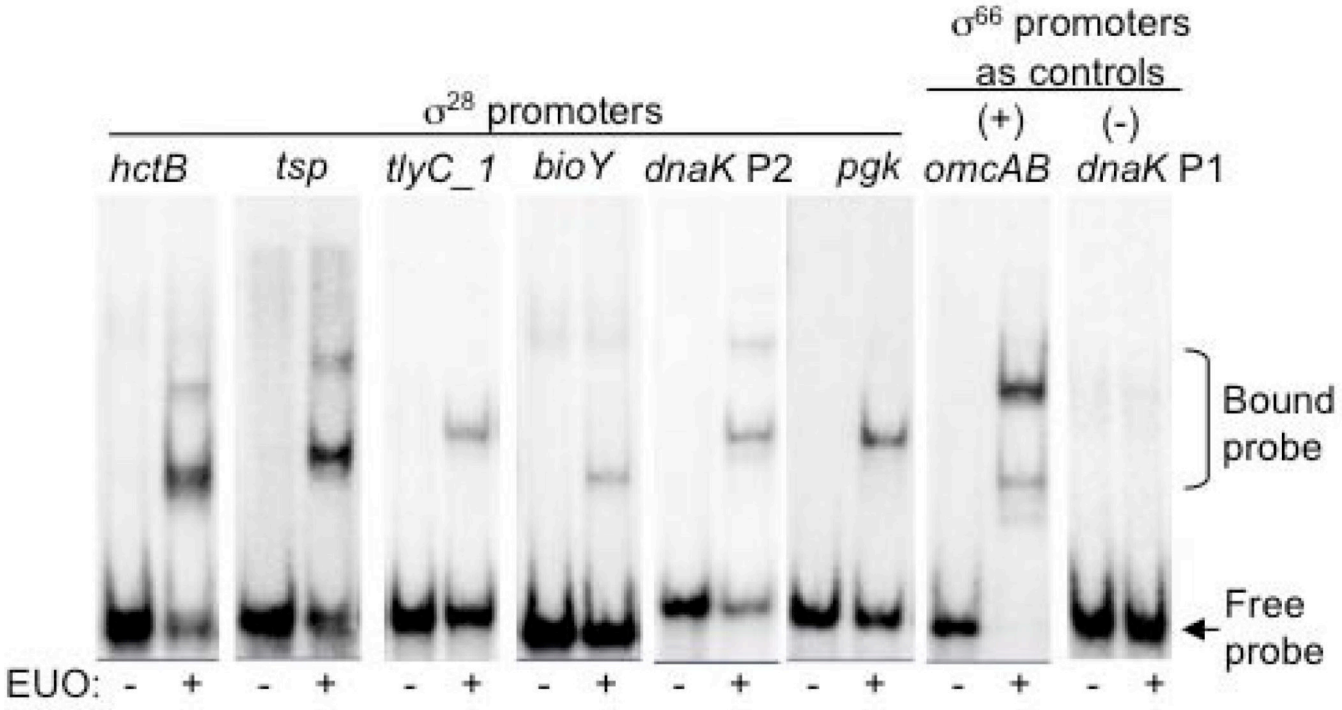

Figure 1. EUO binds to *C. trachomatis* σ28-dependent promoters

EMSA reactions were performed in the absence or presence of 320 nM rEUO. Each promoter region was contained on a 60 bp DNA probe,with the exception of *tsp* which was a 90 bp DNA probe (Table 1). Bands corresponding to the bound and free probes are indicated to the right.

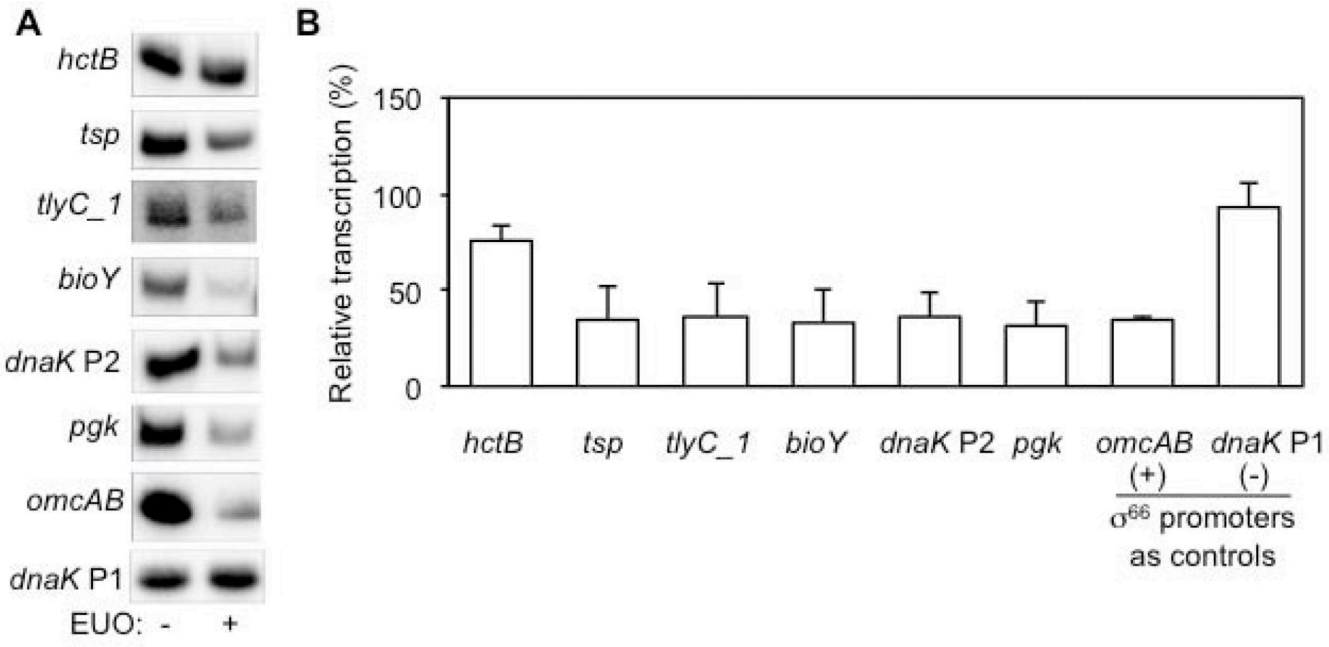

Figure 2. EUO represses transcription of σ^{28}-dependent promoters

A. Representative *in vitro* transcription assay for each promoter, which was present on a supercoiled transcription plasmid at a concentration of 13 nM. Transcription reactions were performed with σ^{28} RNA polymerase in the absence or presence of 2.5 μM rEUO. σ^{66}-dependent control promoters were transcribed with *E. coli* holoenzyme. These gels do not reflect the relative strength of the promoters because different amounts of transcription reaction were used for each promoter in order to have a similar baseline transcription in the absence of EUO. However for a given promoter, the same amount of transcription reaction in the absence or presence of EUO was analyzed. B. Graph of the effect of EUO on transcriptional activity. For each promoter, transcription in the presence of EUO was normalized to baseline transcription in the absence of EUO. Values are from the average of at least three independent experiments with standard deviation indicated by the error bar.

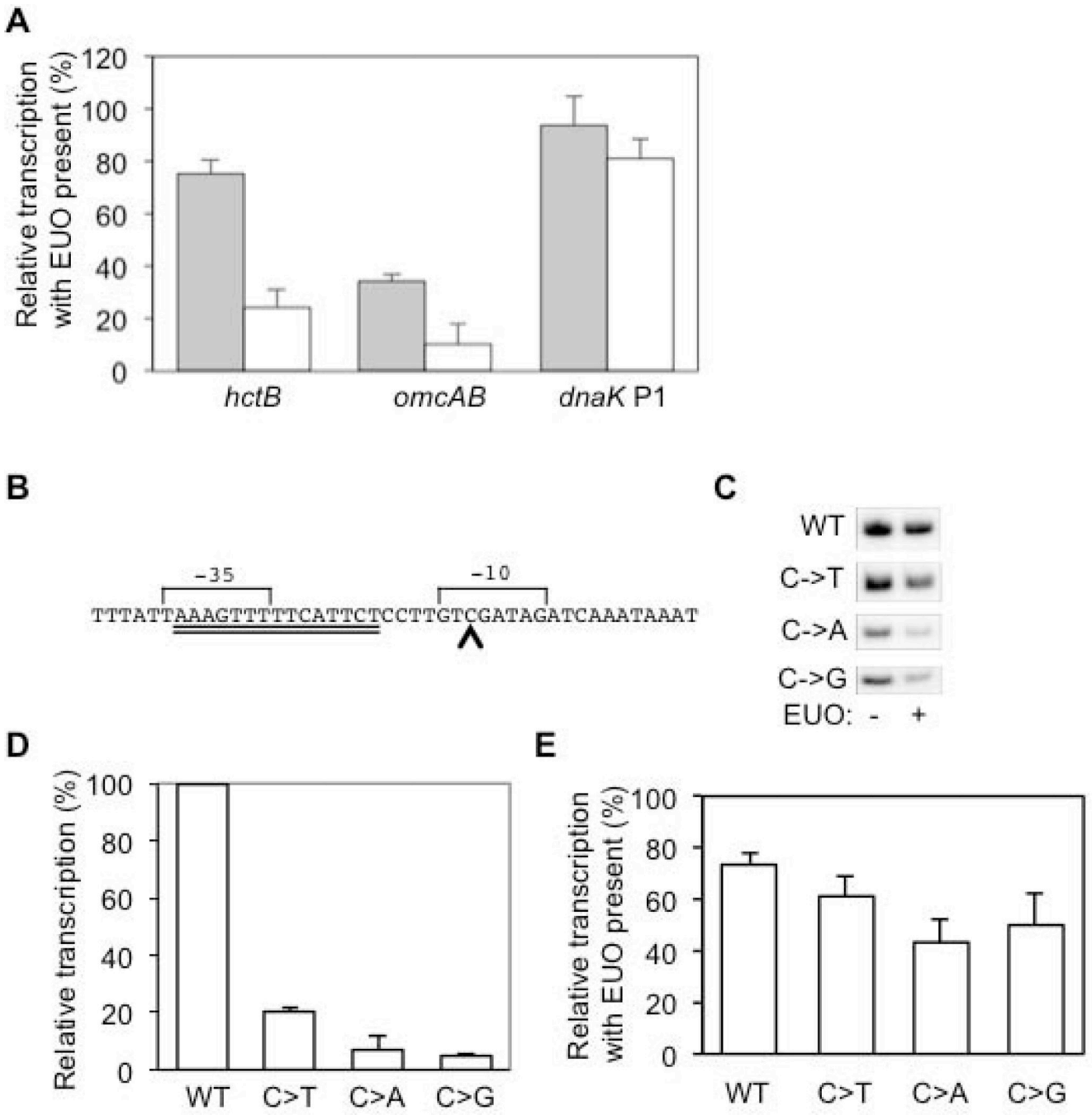

Figure 3. Regulation of the *hctB* promoter by EUO

A. The *hctB* promoter is repressed at a higher EUO:DNA ratio. Graph showing the effect of 2.5 μM rEUO on transcription of the *hctB* promoter at a plasmid DNA concentration of 13 nM (gray bars) or 3 nM (white bars). The *hctB* promoter was transcribed by σ^{28} RNA polymerase, while σ^{66}-dependent control promoters (*omcAB* and *dnaK* P1) were transcribed with *E. coli* holoenzyme. For each promoter, transcription in the presence of EUO was normalized to baseline transcription in the absence of EUO. Values are from the average of at least three independent experiments with standard deviations indicated by error bars. B.

DNA sequence of the *C. trachomatis hctB* promoter, with the putative 15 bp core EUO-binding site indicated with a double underline, and the C residue at position −12 marked with a carat. The −35 and −10 promoter elements are labeled. C. Representative gels showing *in vitro* transcription of the wild type (WT) *hctB* promoter and mutant promoters containing a single nucleotide substitution of the C at position −12. Transcriptions were performed with σ^{28} RNA polymerase in the absence or presence of 2.5 μM rEUO. Only 1/4 of the transcription reactions with the WT promoter were loaded on the gel because this promoter was transcribed at much higher levels than the mutant promoters. D. Graph showing quantification of these transcription results. Transcript levels from the WT promoter were defined as 100%, and transcript levels from each mutant *hctB* promoter were normalized to the WT promoter. E. Graph showing effect of EUO on transcription of WT and mutant *hctB* promoters. For each promoter, transcription in the presence of EUO was normalized to baseline transcription in the absence of EUO. Values are from the average of at least three independent experiments with standard deviation indicated by the error bar.

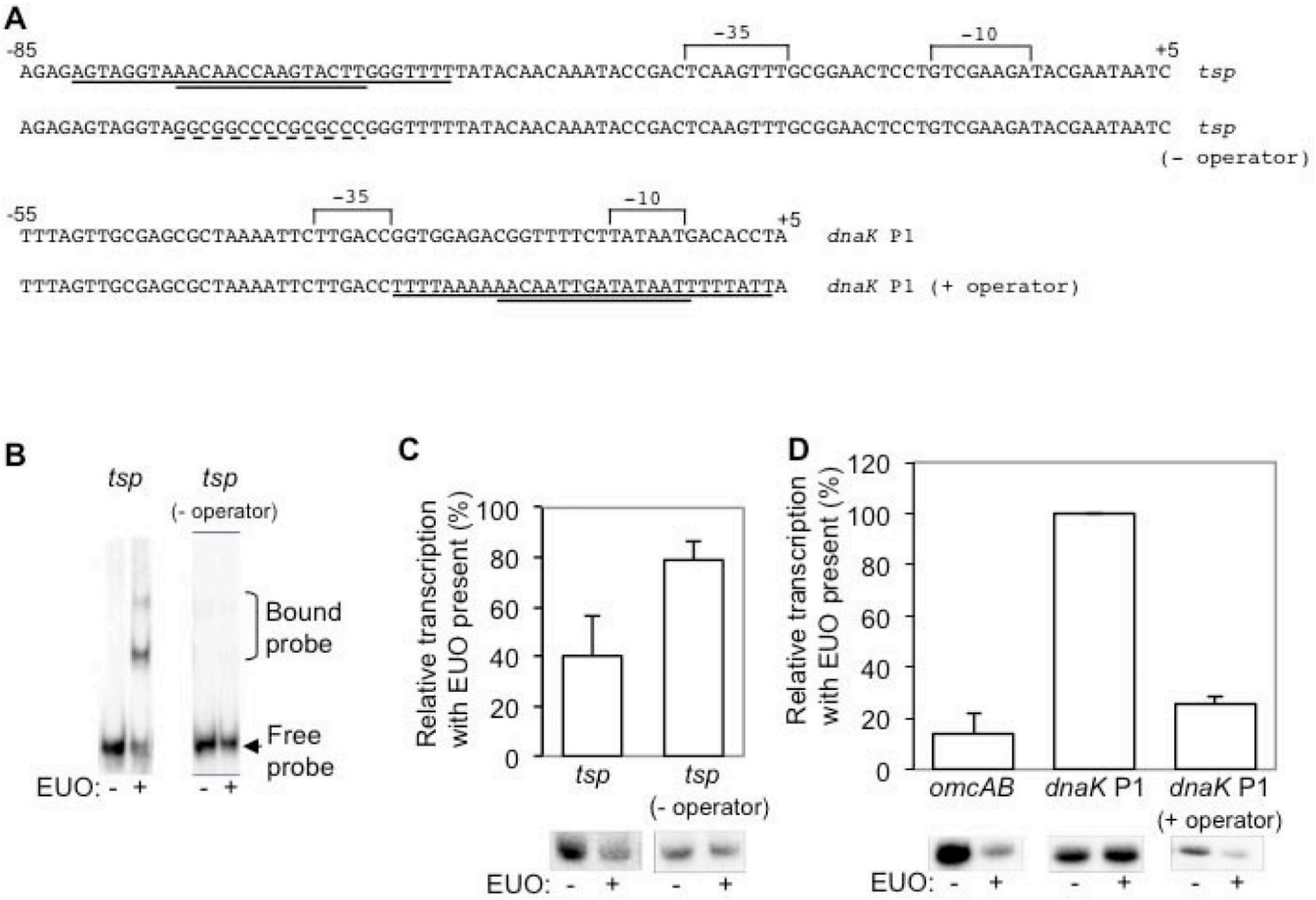

Figure 4. The EUO operator is necessary and sufficient for repression

A. DNA sequence of wild type and mutant *tsp* and *dnaK* P1 promoters used in the mutational analyses of the EUO operator. The putative 15 bp core EUO-binding site (double underline) with flanking sequence (single underline) is indicated, and the mutated sequence is indicated by a dashed underline. The −35 and −10 promoter elements are labeled, and nucleotide positions relative to the transcription start site (+1) are indicated. The *tsp* (− operator) mutant promoter has nucleotide substitutions in 10/15 bp of the core EUO-binding site. The *dnaK* P1 (+ operator) mutant promoter contains the EUO binding site from the *omcAB* promoter on a 30 bp insert. This insert preserved both the sequence of the −10 promoter element and its spacing relative to the −35 promoter element. B. EMSA experiments performed with 90 bp DNA fragments containing either the wild type *tsp* promoter, with its operator located from −76 to −59, or a mutant promoter [labeled as *tsp* promoter (− operator)] with substitutions in 10 of 15 nucleotides of the operator. Experiments were performed in the absence or presence of 320 nM EUO. Bands corresponding to the bound and free probes are indicated to the right. C. Graph and representative gels showing effect of EUO on transcription of the wild type or mutant *tsp* promoter templates by σ^{28} RNA polymerase. D. Graph and representative gels showing effect of EUO on transcription by σ^{66} RNA polymerase for the positive control *omcAB* promoter, the negative control wild type *dnaK* P1 promoter, which lacks an EUO operator, and a mutant *dnaK* P1 promoter containing the EUO operator of the *omcAB* promoter

located in the center of a 30 nucleotide sequence (*dnaK* P1 promoter + operator). For each promoter, transcription by RNA polymerase in the presence of 2.5 μM EUO was normalized to baseline transcription in the absence of EUO. Values are reported as the average of at least three independent experiments with standard deviation indicated by the error bar.

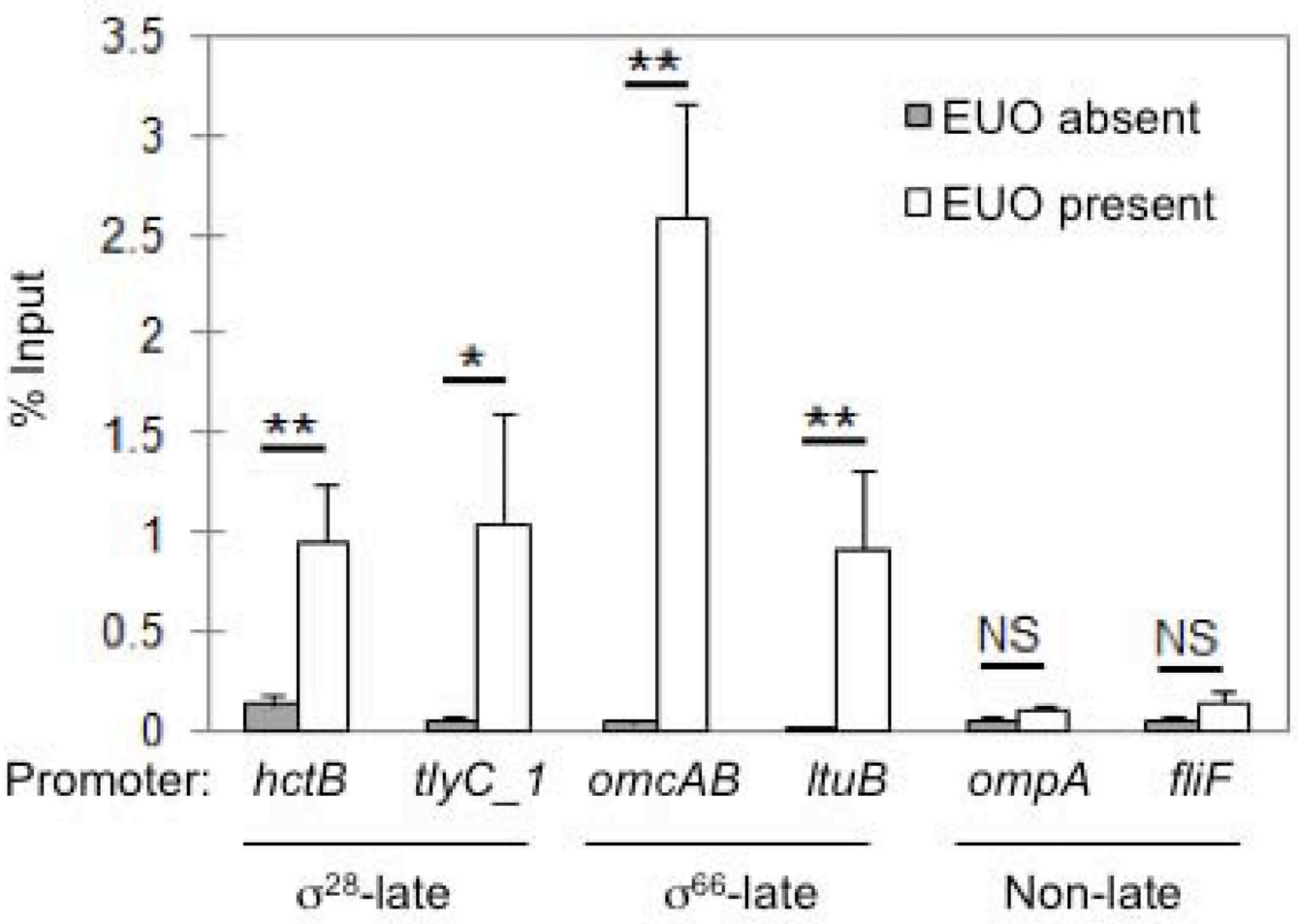

Figure 5. EUO selectively binds to both σ^{28}-dependent and σ^{66}-dependent late promoters

DNA immunoprecipitation was performed in the absence (grey bars) or presence of 20 nM rEUO (white bars). Immunoprecipitated DNA was amplified with promoter-specific primers and calculated as a percentage of input DNA as described in the Experimental Procedures. Values are reported as the average of three independent experiments with standard deviation indicated by the error bars. Statistical analysis: * indicates $p < 0.01$, ** indicates $p < 0.005$, and NS indicates no statistically significant difference.

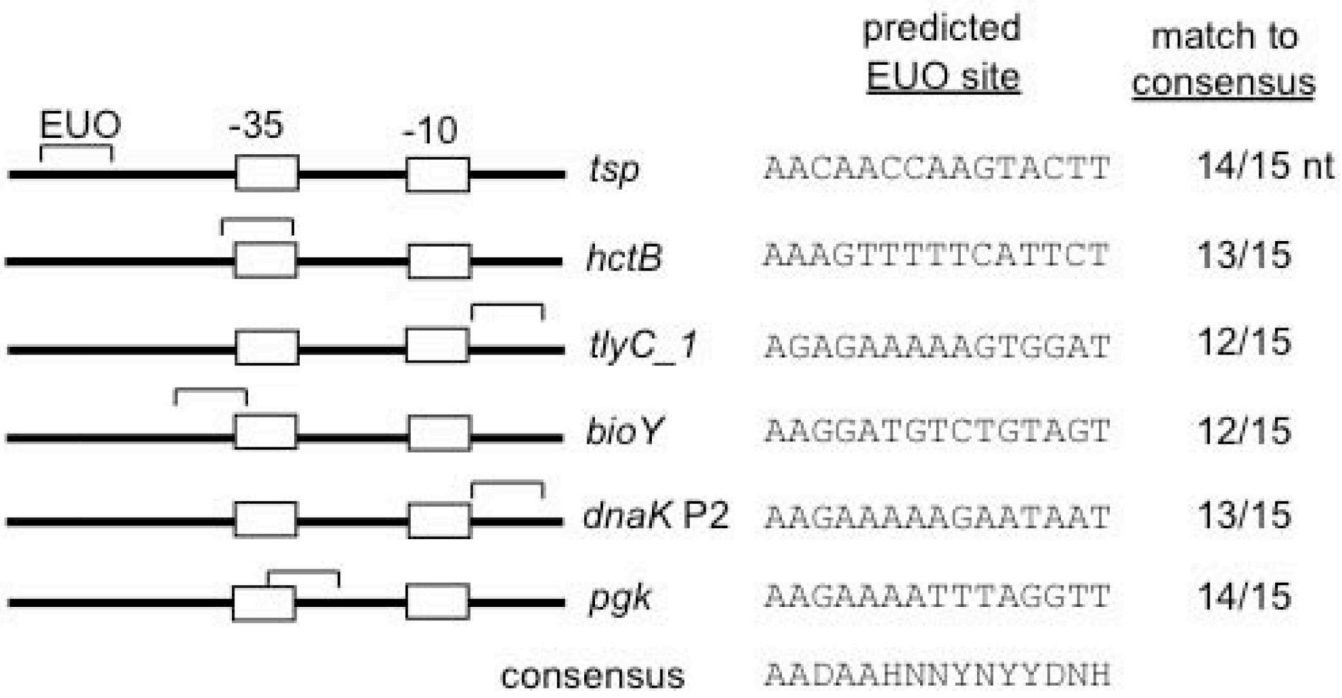

Figure 6. Identification of putative EUO-binding sites for σ^{28}-dependent promoters

Diagram showing the location of each EUO-binding site (marked by a bracket) relative to the −35 and −10 promoter elements (marked by boxes). For comparison, the consensus EUO-binding site is shown below (D = not C, H = not G, N = G, A, T, C, Y = C or T) (Zhang *et al*., 2000). For each promoter, the DNA sequence of the predicted EUO-binding site and the number of nucleotides that match the 15 nt consensus EUO binding sequence are shown.

Table 1

C. trachomatis σ^{28}-dependent promoter regions tested in EMSA reactions for EUO binding

Promoter	Region tested (relative to transcription start site, +1)
hctB	−55 to +5
tsp	−55 to +5
	−85 to +5
tly_1	−55 to +5
	−30 to +30
bioY	−55 to +5
dnaK P2	−55 to +5
pgk	−55 to +5

Table 2

C. trachomatis transcription templates used in this study.

Plasmid	Promoter (nucleotides relative to transcription start site, +1)	Reference
pMT1150	*omcAB* promoter region from −122 to +5	(Wilson & Tan, 2002)
pMT1212	*hctB* promoter region from −164 to +5	(Yu & Tan, 2003)
pMT1228	*hctB* promoter region from −39 to +6	(Yu et al., 2006a)
pMT1232	*tlyC_1* promoter region from −230 to +5	(Yu et al., 2006b)
pMT1234	*pgk* promoter region from −266 to +5	Hilda Yu (unpublished)
pMT1236	*tsp* promoter region from −273 to +5	(Yu et al., 2006b)
pMT1272	*hctB* promoter region from −39 to +6; nucleotide substitution at position −12 from C to A	(Yu et al., 2006a)
pMT1273	*hctB* promoter region from −39 to +6; nucleotide substitution at position −12 from C to T	(Yu et al., 2006a)
pMT1274	*hctB* promoter region from −39 to +6; nucleotide substitution at position −12 from C to G	(Yu et al., 2006a)
pMT1456	*bioY* promoter region from −219 to +5	(Yu et al., 2006b)
pMT1457	*dnaK* P2 promoter region from −269 to +5	(Yu et al., 2006b)
pMT1647	*tsp* promoter region from −85 to +5	This work
pMT1648	*tsp* promoter region from −85 to +5 containing a 10 bp nucleotide substitution in the EUO operator site	This work
pMT1662	*dnaK* P1 promoter region from −55 to +5	This work
pMT1663	*dnaK* P1 promoter region from −55 to +5 containing a 30 bp nucleotide substitution with *omcAB* EUO-binding operator at positions −26 to +4 in *dnaK*	This work