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Regulation of Chlamydia Gene Expression by Tandem Promoters with Different Temporal Patterns

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ABSTRACT
Chlamydia is a genus of pathogenic bacteria with an unusual intracellular developmental cycle marked by temporal waves of gene expression. The three main temporal groups of chlamydial genes are proposed to be controlled by separate mechanisms of transcriptional regulation. However, we have noted genes with discrepancies, such as the early gene dnaK and the midcycle genes bioY and pgk, which have promoters controlled by the late transcriptional regulators EUO and σ28. To resolve this issue, we analyzed the promoters of these three genes in vitro and in Chlamydia trachomatis bacteria grown in cell culture. Transcripts from the σ28-dependent promoter of each gene were detected only at late times in the intracellular infection, bolstering the role of σ28 RNA polymerase in late gene expression. In each case, however, expression prior to late times was due to a second promoter that was transcribed by σ66 RNA polymerase, which is the major form of chlamydial polymerase. These results demonstrate that chlamydial genes can be transcribed from tandem promoters with different temporal profiles, leading to a composite expression pattern that differs from the expression profile of a single promoter. In addition, tandem promoters allow a gene to be regulated by multiple mechanisms of transcriptional regulation, such as DNA supercoiling or late regulation by EUO and σ28. We discuss how tandem promoters broaden the repertoire of temporal gene expression patterns in the chlamydial developmental cycle and can be used to fine-tune the expression of specific genes.

IMPORTANCE
Chlamydia is a pathogenic bacterium that is responsible for the majority of infectious disease cases reported to the CDC each year. It causes an intracellular infection that is characterized by coordinated expression of chlamydial genes in temporal waves. Chlamydial transcription has been shown to be regulated by DNA supercoiling, alternative forms of RNA polymerase, and transcription factors, but the number of transcription factors found in Chlamydia is far fewer than the number found in most bacteria. This report describes the use of tandem promoters that allow the temporal expression of a gene or operon to be controlled by more than one regulatory mechanism. This combinatorial strategy expands the range of expression patterns that are available to regulate chlamydial genes.

A defining feature of the pathogenic bacterium Chlamydia is an unusual intracellular developmental cycle with three main stages (1). During the early stage, an extracellular form of chlamydiae, called the elementary body (EB), enters the host eukaryotic cell and differentiates into a reticulate body (RB), which is the metabolically active but noninfectious form. During the midstage, the RB replicates via multiple rounds of binary fission. Finally, in the late stage, RBs convert back into infectious EBs. This developmental cycle lasts 48 to 72 h and ends with the release of EBs to infect a new host cell. These fundamental steps of the developmental cycle are conserved among species of the genus Chlamydia, even though members of this genus cause different infections ranging from sexually transmitted disease to infectious blindness and pneumonia (2).

Another characteristic feature of the intracellular Chlamydia infection is the temporal expression of chlamydial genes in three main classes that correspond to these three stages of the developmental cycle (3–5). Early genes are transcribed within 1 to 3 h of chlamydial entry, when the EB is beginning to convert into an RB. Midcycle genes, which make up the large majority of chlamydial genes, are first expressed during RB replication. Late genes, many of which have important roles in RB-to-EB conversion or EB function, are first transcribed or upregulated at late times.

The temporal classes of chlamydial genes are differentially regulated by specific mechanisms (6). DNA supercoiling, which peaks in midcycle, is proposed to regulate genes with supercoiling-responsive promoters, which include midcycle genes and a subset of early genes (7, 8). Late genes consist of two subsets that are transcribed by either the major chlamydial RNA polymerase, which contains the sigma factor σ66, or an alternative RNA polymerase containing σ28 (7, 9–11). Both subsets of late genes, however, are negatively regulated by the same transcription factor, EUO, which appears to be the master regulator of late gene expression (12, 13). It is hypothesized that EUO prevents the premature expression of late genes, thereby delaying RB-to-EB conversion until after there has been sufficient RB replication (12).

Even though σ28 regulates a subset of late genes, there are questions about its temporal role in the developmental cycle. Three of the six known σ28-dependent promoters in Chlamydia trachoma-
tis control late genes (hctB, tsp, and tlyC_1). However, $\sigma^{28}$ RNA polymerase also transcribes promoters for an early gene (dnaK) and two midcycle genes (bioY and pgl) (4, 10). Unexpectedly, we recently found that all six $\sigma^{28}$ promoters are bound and repressed by EUO in vitro (13). Thus, $\sigma^{28}$-regulated genes appear to share a potential mechanism of late gene regulation, even though they have different temporal expression patterns.

To resolve this issue, we examined if the three genes with a $\sigma^{28}$-dependent promoter but a non-late expression profile can be regulated by additional mechanisms. In each case, the $\sigma^{28}$-dependent promoter was transcribed only at late times, but the gene was transcribed at earlier times from a second promoter. These tandem promoters allow the gene to be differentially regulated by two forms of chlamydial RNA polymerase and to have an overall expression pattern that differs from the temporal expression pattern of a single promoter.

MATERIALS AND METHODS

Construction of in vitro transcription plasmids. Promoter sequences were amplified from C. trachomatis serovar D UW-3/Cx genomic DNA by PCR or produced by annealing complementary oligonucleotides. Each promoter sequence was cloned upstream of the promoterless G-less cassette transcription template pMT1125 as previously described (14). All constructs were verified by sequencing (Genewiz). The plasmids used in this study are listed in Table 1.

Table 1. C. trachomatis transcription templates used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Promoter*</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMT1150</td>
<td>omcAB promoter region from −122 to +5</td>
<td>14</td>
</tr>
<tr>
<td>pMT1234</td>
<td>pgl promoter region from −266 to +5</td>
<td>Hilda Yu (unpublished data)</td>
</tr>
<tr>
<td>pMT1456</td>
<td>bioY P2 promoter region from −219 to +5</td>
<td>10</td>
</tr>
<tr>
<td>pMT1457</td>
<td>dnaK P2 promoter region from −269 to +5</td>
<td>10</td>
</tr>
<tr>
<td>pMT1662</td>
<td>dnaK P1 promoter region from −55 to +5</td>
<td>This work</td>
</tr>
<tr>
<td>pMT1663</td>
<td>bioY P1 promoter region from −55 to +5</td>
<td>This work</td>
</tr>
</tbody>
</table>

* Nucleotide positions relative to the transcription start site at position +1.

Preparation of supercoiling and relaxed plasmid DNA templates. Plasmid DNA was first isolated from Escherichia coli using a Macherey-Nagel NucleoBond Xtra midi prep kit. Plasmid DNA was further purified on a CsCl gradient by ultracentrifugation in TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) containing 5.9 M CsCl and 0.9 mg/ml ethidium bromide in a Beckman NVT80 rotor at 58,000 rpm for 18 h at 20°C. The band corresponding to the supercoiled plasmid DNA was removed, cleaned with isopropanol to remove the ethidium bromide, and recovered by ethanol precipitation.

To relax the plasmid DNA, 10 μg of CsCl gradient-purified plasmid DNA was treated with wheat germ topoisomerase I (Promega) as previously described (7). DNA relaxation was verified by electrophoresis on a 1% agarose gel in 1 X TAE buffer (0.01 M Tris-acetate, 1 mM EDTA). The DNA concentration was measured using a NanoDrop ND1000 spectrophotometer.

In vitro transcription assays. In vitro transcription of $\sigma^{28}$-dependent and $\sigma^{46}$-dependent promoters was performed as previously described (12, 13). Supercoiled or relaxed plasmid DNA (13 nM) containing the transcription template was transcribed by $\sigma^{28}$ RNA polymerase, reconstituted from 1 μl C. trachomatis recombinant His-tagged $\sigma^{28}$ and 0.4 U E. coli RNA polymerase core enzyme (Epitrends), or 0.4 U E. coli $\sigma^{40}$ RNA polymerase holoenzyme (Epitrends). rEUO (2.5 μM) was added to some action mixtures where indicated. Transcripts were resolved on an 8 M urea–6% polyacrylamide gel and quantified with a Bio-Rad Personal FX scanner and Quantity One software (Bio-Rad). The effect of EUO was measured by normalizing the transcript levels in the presence of EUO to the levels in the absence of EUO, and the results are reported as a percentage. For each plasmid, transcription assays were performed as a minimum of three independent experiments, and values are reported as the mean of the repression + standard deviation.

RNA preparation. Mouse fibroblast L929 cells were infected with C. trachomatis lymphogranuloma venereum serovar L2 EBs at a multiplicity of infection of 3 in a 6-well plate. Total RNA was harvested from the infected cells with RNA STAT-60 (Tel Test) according to the manufacturer’s directions. In brief, cells were resuspended in 1 ml RNA STAT, and the RNA in the aqueous layer was precipitated with isopropanol and resuspended in diethyl pyrocarbonate-treated water. DNA-free RNA was prepared by treating approximately 10 μg RNA with 10 U RQI DNase (Promega) at 37°C for 2 h, and the absence of genomic DNA was verified by PCR.

5’ RACE. Approximately 5 μg DNA-free RNA was used for 5’ rapid amplification of cDNA end (RACE) reactions with a First Choice RLM-RACE kit (Ambion) per the manufacturer’s directions. RNA was modified at the 5’ end by removal of the pyrophosphate with tobacco acid pyrophosphatase, followed by ligation of a DNA-specific sequence (Ambion). Reverse transcription (RT) was performed with Moloney murine leukemia virus reverse transcriptase in the presence of 250 ng random primers. Approximately 2 μl of cDNA preparation was used for PCR with a primer specific for the 5’ ligated DNA sequence and a gene-specific primer. A second round of PCR was performed using the products from the first PCR together with a primer specific for a sequence on the 5’ ligated DNA fragment and a gene-specific primer. PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. We verified that the PCR products corresponded to the chlamydial genes (promoters) under study by excising each PCR product from the gel using a Macherey-Nagel gel extraction kit, cloning it into the pGEM-T vector (Promega), and determining its DNA sequence (Genewiz).
RT-PCR. RT was performed with approximately 2 μg DNA-free RNA with 20 U avian myeloblastosis virus (AMV) reverse transcriptase (Promega) in the presence of 250 ng random primers (Invitrogen), 1 mM deoxynucleoside triphosphates, and 40 U RNasin (Promega) at 42°C for 30 min. The reactions were terminated by incubation at 70°C for 10 min. Approximately 2 μl of the RT reaction mixture was used for PCR with gene-specific primers. PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

qRT-PCR. Quantitative RT-PCR (qRT-PCR) was used to quantify the bioY transcripts at selected times in the chlamydial developmental cycle. cDNA was first generated using approximately 2 μl DNA-free RNA (which is equivalent to 0.04% of the total amount of RNA in the sample), 20 U AMV reverse transcriptase (Promega), and a primer specific for the bioY gene. Real-time quantitative PCR (qPCR) was performed with a Bio-Rad iCycler iQ instrument, using the Bio-Rad iQ SYBR green master mix. Transcripts solely from bioY P1 were measured with one set of primers, while a second set of primers was used to measure the total transcripts from bioY P1 and P2. To normalize the results, genomic DNA was extracted from Chlamydia-infected L929 cells at each time point using a Qagen DNeasy blood and tissue kit, and the number of genome copies was determined by qPCR using the same primer pairs. Expression at each time point was reported as the number of transcripts per genome. The standard deviation was determined for triplicate samples.

RESULTS

To understand the role of σ^28 in Chlamydia temporal regulation, we first examined two chlamydial genes that are transcribed prior to late times, even though they each have a σ^28-dependent promoter controlled by the late regulator EUO (13). C. trachomatis dnaK and bioY have been classified as early and midcycle genes, respectively, in Chlamydia transcriptional profiling studies (4). However, this expression profiling was based on transcript levels for each gene and did not examine promoter-specific transcription. Using 5’ RACE to examine promoter-specific transcripts from C. trachomatis-infected cells, we detected transcripts only from the σ^28-dependent promoters of dnaK and bioY at 24 h postinfection (hpi), which is late in the chlamydial developmental cycle, but not at 14 hpi, which corresponds to midcycle (Fig. 1A).

Thus, these σ^28-dependent promoters are late promoters that cannot account for the expression of dnaK as an early gene and bioY as a midcycle gene.

We examined if the early onset of dnaK transcription (4) was due to a second dnaK promoter. In addition to its own σ^28-dependent promoter, dnaK is transcribed as part of an operon from a σ^36-dependent promoter (dnaK P1) upstream of hrcA (Fig. 2) (15). We detected transcription from dnaK P1 at both 14 and 24 hpi, consistent with expression from midcycle or earlier (Fig. 1B). Thus, dnaK is transcribed from two promoters, with initial transcription from σ^66-dependent dnaK P1 taking place at early times and additional transcription from σ^28-dependent dnaK P2 taking place at late times.

These findings prompted us to examine if the biotin transporter gene bioY (16) also has a second promoter to account for its expression as a midcycle gene (4). Only a single σ^28-dependent bioY promoter has been reported to date (10). However, we noted that another transcription start site was mapped upstream of this bioY promoter in a C. trachomatis genome-wide deep sequencing study (17). By inspection, we identified sequences resembling the sequence of the optimal chlamydial σ^66 promoter (18, 19) immediately upstream of this transcription start site (Fig. 3A). We tested

![FIG 1 Temporal regulation of the σ^28- and σ^66-dependent promoters of dnaK and bioY. 5’ RACE analysis of transcription from the dnaK promoter (A) and bioY promoter (B) of dnaK and bioY performed on RNA extracted from C. trachomatis-infected cells at 14 and 24 hpi. Promoter-specific PCR products were resolved on a 2% agarose gel. Each band was excised and sequenced to confirm that the PCR product originated from the chlamydial promoter.](http://jb.asm.org/)

![FIG 2 Diagram of tandem promoters for the dnaK operon, bioY, and pgg of C. trachomatis. The relative positions of the σ^66-dependent P1 promoters and σ^28-dependent P2 promoters are marked (the diagram is not to scale). Transcripts are indicated by arrows.](http://jb.asm.org/)

![FIG 3 The bioY P1 promoter is transcriptionally active and supercoiling dependent. (A) Sequence of bioY P1. The predicted −35 and −10 elements are underlined, and the preferred C. trachomatis σ^66 promoter sequences are shown below for comparison. (B) In vitro transcription of bioY P1 by E. coli RNA polymerase σ^35 holoenzyme and partially purified C. trachomatis σ^66 RNA polymerase, as indicated. bioY P1 was present on a supercoiled transcription template. (C) Comparison of bioY P1 transcription from supercoiled and relaxed plasmid templates. As controls, a supercoiling-independent promoter (omcAB) and a supercoiling-dependent promoter (dnaK P1) were also tested. Transcription reactions were performed with E. coli σ^35 RNA polymerase.](http://jb.asm.org/)
this candidate bioY promoter in an in vitro transcription assay and found that it was transcribed by σ66 RNA polymerase (Fig. 3B) but not by σ28 RNA polymerase (data not shown). The σ66-dependent bioY promoter was transcribed at a higher level from a supercoiled DNA template than from a relaxed template (Fig. 3C), demonstrating supercoiling-dependent promoter activity that is characteristic of chlamydial midcycle genes (7, 11). We propose to call this new σ66 promoter bioY P1 and to call the original σ28 promoter bioY P2, reflecting the location of P1 upstream of P2 (Fig. 2).

We used a promoter-specific 5′ RACE analysis of C. trachomatis-infected cells to examine the temporal expression of bioY P1 and P2. σ66-dependent bioY P1 was detected at 14 hpi (midcycle) and 24 hpi (late in the cycle), but σ28-dependent bioY P2 was detected only at 24 hpi (Fig. 1). bioY thus provides another example of a gene that is first transcribed from a σ66 promoter before being expressed from a σ28-dependent promoter at late times.

We next investigated if this differential control of tandem promoters is due to the temporal regulator EUO. EUO represses σ66-dependent promoters of late genes (12), as well as all six known σ28-dependent promoters (13). In EMSAs, recombinant C. trachomatis EUO produced a gel shift with DNA fragments containing dnaK P2 but not dnaK P1 (Fig. 4A). EUO also inhibited dnaK P2 but not dnaK P1 in an in vitro transcription assay (Fig. 4B and C), verifying that only σ28-dependent dnaK P2 is an EUO target. EUO bound both bioY promoters (Fig. 4A), and it repressed σ28-dependent bioY P2 and caused modest inhibition of σ66-dependent bioY P1 (Fig. 4B and C).

We then examined a third chlamydial gene that is transcribed prior to late times, even though it has a σ28-dependent promoter. The phosphoglycerate kinase gene pgk is a midcycle gene (4) that has overlapping σ66 and σ28 promoters which initiate from the same transcription start site (Fig. 2) (10). In EMSAs, EUO bound to pgk (Fig. 4A), but we could not distinguish between binding to its σ66 and σ28 promoters since they overlap. Intriguingly, however, EUO inhibited transcription only of σ28-dependent pgk P2 and not of σ66-dependent pgk P1 (Fig. 4B and C). Thus, we found a consistent pattern in which EUO regulated the σ28 promoter of three tandem promoter pairs. EUO may also regulate bioY P1, although the modest inhibition suggests that EUO may cause only partial repression of this promoter.

We used qRT-PCR to determine the relative contributions of the tandem bioY promoters to overall expression of this gene over the course of the chlamydial developmental cycle. Using specific primers, we measured transcripts from bioY P1 alone and total transcripts from bioY P1 and bioY P2 (Fig. 5A). We could not directly measure P2-only transcript levels, because this promoter is downstream of P1 and there is no P2-specific miRNA sequence, but we were able to calculate P2 transcript levels by subtraction. At 8 and 16 hpi, the levels of total transcripts (P1 plus P2) were similar to P1 transcript levels (Fig. 5B). At 24 hpi, total transcript levels were modestly higher than those at 16 hpi, but levels from bioY P1 were only 34% of the total levels, implying that the majority of transcripts were now from P2. Total transcript levels were much lower at 32 hpi, but P1-only transcript levels were 42% of the total, again indicating more transcription from P2. The transcript levels measured from σ66-dependent bioY P1 and the transcript levels calculated from σ28-dependent bioY P2 are shown in Fig. 5C. These results indicate that P1 by itself can account for bioY transcription during midcycle, when there is no significant transcription from bioY P2. However, at late times in the developmental cycle, σ28-dependent bioY P2 becomes active, and there is an additive effect of transcription from the tandem bioY promoters.

To identify additional temporally regulated tandem promoters, we used a bioinformatics approach to identify chlamydial genes with more than one promoter and then analyzed the expression pattern of each promoter during an intracellular chlamydial infection. We first examined the results from a genome-wide analysis of C. trachomatis transcripts for genes with more than one transcription start site (17). We next predicted the promoters for each transcription start site, based on sequence similarity to the optimal chlamydial σ66 and σ28 promoters (10, 18, 19). We then analyzed promoter-specific transcripts in C. trachomatis-infected cells at 12 hpi (midcycle) and 24 hpi (late in the cycle). With this approach, we identified a candidate σ28 promoter (P1) upstream of a predicted σ66 promoter (P2) for cdt415, a gene of unknown function (Fig. 6A). Using RT-PCR, we detected a cdt415 P1-specific transcript only at 24 hpi, while a region of the transcript common to cdt415 P1 and P2 was detected at both 12 and 24 hpi (Fig. 6B). We then used 5′ RACE to detect promoter-specific transcripts and found that σ66-dependent P2 was transcribed only at 12 hpi, while σ28-dependent P1 was expressed only at 24 hpi (Fig. 6C). cdt415 thus provides another example of a chlamydial gene with tandem promoters that have different temporal expression patterns and different mechanisms of regulation. The σ28-dependent promoter
DISCUSSION

This study describes a new mechanism of temporal regulation in Chlamydia in which genes are transcribed from tandem promoters that have different temporal expression profiles. Prior to this study, tandem promoters had been noted for a few chlamydial genes (19, 20), but they were not known to have a role in temporal gene regulation. We identified four C. trachomatis genes that each has a \( \sigma^{66} \) promoter which is transcribed prior to late times and a \( \sigma^{28} \) promoter that is expressed only late in the developmental cycle.

The four tandem promoter pairs that we studied showed differences in promoter organization (Fig. 2 and 6). bioY and ct415 have a straightforward promoter arrangement in which a single gene is transcribed from two promoters, although with a different order of \( \sigma^{66} \) and \( \sigma^{28} \) promoters upstream of the gene. The tandem promoters of pgk overlap and initiate transcription from the same start site. DNAK has a more complicated configuration in which the gene is transcribed as part of an operon, while it also has its own internal promoter. In each case, however, one promoter was expressed prior to late times and the second promoter was detected during late development, suggesting that the order of the tandem promoters relative to the gene is not critical.

These findings highlight the key role of the promoter in allowing a chlamydial gene to be temporally regulated by a specific form of RNA polymerase and by different mechanisms of transcriptional control (6). For example, chlamydial genes with supercoiling-responsive promoters have been proposed to be upregulated in midcycle by increased chlamydial DNA supercoiling levels at this stage of the developmental cycle (7, 8, 11). In contrast, late genes have been proposed to be repressed during early times and midcycle by the transcription factor EUO, until this repression is relieved at late times by an as-yet-undefined mechanism (12, 13). This study makes a conceptual advance by demonstrating that a chlamydial gene can be regulated by more than one of these temporal mechanisms by having more than one promoter. However, it also shows the limitations of trying to predict the mechanism of transcriptional regulation from the temporal expression pattern.

FIG 6 The tandem promoters of ct415 have different temporal expression patterns. (A) Diagram showing the relative locations of the predicted P1 and P2 promoters of ct415 (the diagram is not to scale). The primers (arrows) used for RT-PCR and 5' RACE studies and the predicted RT-PCR products (dashed lines) are shown. The sequences of the predicted \( \sigma^{66} \)-dependent P1 promoter and \( \sigma^{28} \)-dependent P2 promoter are shown, with promoter elements underlined. For comparison, the preferred (consensus) sequences for the C. trachomatis \( \sigma^{66} \) and \( \sigma^{28} \) promoters are shown. (B) RT-PCR analysis of ct415 transcription in Chlamydia-infected cells. Primers were designed to amplify a P1-specific RT-PCR product and a RT-PCR product common to both P1 and P2. (C) 5' RACE analysis of ct415 expression at 12 and 24 hpi showing P1- and P2-specific transcription (marked by arrows).
DNA supercoiling in bacteria (21). This approach has shown that which is a commonly used surrogate marker of chromosomal amined by measuring the superhelical density of the plasmid, coiling during the chlamydial developmental cycle have been ex-

in weak transcriptional profile that we determined for supercoiling-de-

creasing in midcycle and then decreases at late times, which is the promoter paired with a late promoter. We anticipate that there are likely to be additional combinations of temporal promoters in Chlamydia, leading to a wider repertoire of transcriptional profiles beyond the three main temporal classes of early, midcycle, and late genes that have been described.

Tandem promoters also make it more difficult to study chlamydial transcriptional regulators because they obscure the contribution of an individual promoter to the temporal expression pattern of the gene. For example, the temporal role of $\sigma^{28}$ has been ambiguous because $\sigma^{28}$ RNA polymerase transcribes genes with different temporal patterns, ranging from early to midcycle to late expression (10). The late expression pattern of three of these $\sigma^{28}$-dependent genes ($\text{hctB}$, $\text{tsp}$, and $\text{tlyC}_{1,1}$) was apparent because these genes are transcribed only from a single $\sigma^{28}$-dependent promoter (9, 10). However, we now know that late expression from the $\sigma^{28}$-dependent promoters of $\text{dnaK}$, $\text{bioY}$, $\text{pgk}$, and $\text{ct415}$ was not obvious from transcriptional profiling studies (4) because each of these genes is transcribed from earlier times by a second promoter. Analyses at the promoter level have been necessary to reveal that these $\sigma^{28}$-dependent promoters share a consistent pattern of late temporal expression (13; this study). These findings clarify the role of $\sigma^{28}$ RNA polymerase as an alternative chlamydial RNA polymerase that transcribes late promoters.

Tandem promoters also provide an explanation for why the late regulator EUO is able to bind and repress promoters for the non-late genes $\text{dnaK}$, $\text{bioY}$, and $\text{pgk}$ (13). We showed that each of these three genes has a late promoter that is regulated by $\sigma^{28}$ and EUO, but the late transcriptional pattern was not apparent because of a second promoter. We have also made an interesting observation that EUO appears to repress the $\sigma^{28}$ promoter and not the $\sigma^{28}$ promoter of $\text{pgk}$, even though these promoters overlap. How EUO selectively regulates the $\sigma^{28}$ promoter is not known, but we speculate that EUO-operator binding may cause greater steric hindrance of $\sigma^{28}$ RNA polymerase-promoter binding than $\sigma^{28}$ RNA polymerase-promoter binding. Alternatively, EUO-operator binding may selectively affect other steps in $\sigma^{28}$ RNA polymerase-dependent transcription initiation, such as isomerization or promoter clearance. These findings affirm the role of EUO as a critical regulator of late chlamydial genes. They also emphasize that a transcriptional regulator controls the temporal expression of its target promoter but not necessarily that of its gene.

In summary, we describe how a Chlamydia gene can be controlled by two promoters, each with its own temporal pattern due to transcription by a specific form of RNA polymerase and regulation by different mechanisms. This strategy allows a chlamydial gene to be controlled by multiple regulatory signals and to have a hybrid temporal expression pattern that may not be possible with a single promoter. Chlamydia has a limited means to differentially regulate its genes because it has only about a dozen transcription factors (6, 19, 22–24). Tandem promoters provide a relatively simple approach to fine-tune the expression of a chlamydial gene by using existing mechanisms of temporal regula-
tion. This combinatorial approach also broadens the repertoire of temporal expression patterns that are available to regulate chlamydial genes.

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