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 chlamydia genetic functions.

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, σ^{28} RNA polymerase also transcribes promoters for an early gene (dnhK) and two midcycle genes (bioY and pgk) (4, 10). Unexpectedly, we recently found that all six σ^{28} promoters are bound and repressed by EUO in vitro (13). Thus, σ^{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 σ^{28}-dependent promoter but a non-late expression profile can be regulated by additional mechanisms. In each case, the σ^{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 PCR or produced by annealing complementary oligonucleotides. Each were amplified from pMT1456/H9268 and stored at −70°C. This work

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Promoter^a</th>
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<tr>
<td>pMT1150</td>
<td>omcAB promoter region from −122 to +5</td>
<td>14</td>
</tr>
<tr>
<td>pMT1234</td>
<td>pgk 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>
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<tr>
<td>pMT1457</td>
<td>dnaK P2 promoter region from −269 to +5</td>
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<tr>
<td>pMT1662</td>
<td>dnaK P1 promoter region from −55 to +5</td>
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<tr>
<td>pMT1663</td>
<td>bioY P1 promoter region from −55 to +5</td>
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^a Nucleotide positions relative to the transcription start site at position +1.

In vitro transcription assays. In vitro transcription of σ^{28}-dependent and σ^{rL}-dependent promoters was performed as previously described (12, 13). Supercoiled or relaxed plasmid DNA (13 nM) containing the transcription template was transcribed by σ^{28} RNA polymerase, reconstituted from 1 μl *C. trachomatis* recombinant His-tagged σ^{28} and 0.4 U *E. coli* RNA polymerase core enzyme (Epipcrentic), or 0.4 U *E. coli* σ^{rL} RNA polymerase holoenzyme (Epipcrentic). 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 RQ1 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 a 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).
We detected transcription from \( \text{dnaK} \) from \( C. \text{trachomatis} \) using 5’ RACE to examine promoter-specific transcripts. 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.

To understand the role of \( \sigma^{28} \) in temporal regulation, we examined two chlamydial genes that are transcribed prior to late times, even though they each have a \( \sigma^{28} \)-dependent promoter controlled by the late regulator EUO (13). \( C. \text{trachomatis} \) \( \text{dnaK} \) and \( \text{bioY} \) have been classified as early and midcycle genes, respectively, in \( C. \text{trachomatis} \) 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 \( C. \text{trachomatis} \) cells, we detected transcripts only from the \( \sigma^{28} \)-dependent promoters of \( \text{dnaK} \) and \( \text{bioY} \) 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, transcription from \( \text{dnaK} \) as an early gene and \( \text{bioY} \) as a midcycle gene occurred.

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

These findings prompted us to examine if the biotin transporter gene \( \text{bioY} \) (16) also has a second promoter to account for its expression as a midcycle gene (4). Only a single \( \sigma^{28} \)-dependent \( \text{bioY} \) promoter has been reported to date (10). However, we noted that another transcription start site was mapped upstream of this \( \text{bioY} \) promoter in a \( C. \text{trachomatis} \) genome-wide deep sequencing study (17). By inspection, we identified sequences resembling the sequence of the optimal chlamydial \( \sigma^{66} \) promoter, which is not shown for comparison. (B) In vitro transcription of \( \text{bioY} \) P1 by \( E. \text{coli} \) RNA polymerase \( \sigma^{30} \) holoenzyme and partially purified \( C. \text{trachomatis} \) \( \sigma^{36} \) RNA polymerase, as indicated. \( \text{bioY} \) P1 was present on a supercoiled transcription template. (C) Comparison of \( \text{bioY} \) P1 transcription from supercoiled and relaxed plasmid templates. As controls, a supercoiling-independent promoter (\( \text{omcAB} \)) and a supercoiling-dependent promoter (\( \text{dnaK} \) P1) were also tested. Transcription reactions were performed with \( E. \text{coli} \) \( \sigma^{30} \) RNA polymerase.
this candidate bioY promoter in an in vitro transcription assay and found that it was transcribed by σ^66 RNA polymerase (Fig. 3B) but not by σ^28 RNA polymerase (data not shown). The σ^66-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 σ^66 promoter bioY P1 and to call the original σ^28 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. σ^66-dependent bioY P1 was detected at 14 hpi (midcycle) and 24 hpi (late in the cycle), but σ^28-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 σ^28 promoter before being expressed from a σ^66-dependent promoter at late times.

We next investigated if this differential control of tandem promoters is due to the temporal regulator EUO. EUO represses σ^66-dependent promoters of late genes (12), as well as all six known σ^28-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 σ^28-dependent dnaK P2 is an EUO target. EUO bound both bioY promoters (Fig. 4A), and it repressed σ^66-dependent bioY P2 and caused modest inhibition of σ^28-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 σ^28-dependent promoter. The phosphoglycerate kinase gene pgk is a midcycle gene (4) that has overlapping σ^66 and σ^28 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 σ^66 and σ^28 promoters since they overlap. Intriguingly, however, EUO inhibited transcription only of σ^28-dependent pgk P2 and not of σ^66-dependent pgk P1 (Fig. 4B and C). Thus, we found a consistent pattern in which EUO regulated the σ^28 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 mRNA 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 σ^66-dependent bioY P1 and the transcript levels calculated from σ^28-dependent bioY P2 are shown in Fig. 5C. These results indicate that P1 by itself can account for bioY transcription during midcyte, when there is no significant transcription from bioY P2. However, at late times in the developmental cycle, σ^28-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 σ^66 and σ^28 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 σ^28 promoter (P1) upstream of a predicted σ^66 promoter (P2) for ct415, a gene of unknown function (Fig. 6A). Using RT-PCR, we detected a ct415 P1-specific transcript only at 24 hpi, while a region of the transcript common to ct415 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 σ^66-dependent P2 was transcribed only at 12 hpi, while σ^28-dependent P1 was expressed only at 24 hpi (Fig. 6C). ct415 thus provides another example of a chlamydial gene with tandem promoters that have different temporal expression patterns and different mechanisms of regulation. The σ^28-dependent promoter
of *ct415* was similar to the other \( \sigma^{28} \) promoters in having a late transcriptional pattern.

**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 \( \sigma^{66} \) 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.
which is a commonly used surrogate marker of chromosomal supercoiling. By measuring the superhelical density of the plasmid, we observed that transcription increases in midcycle and then decreases at late times, which is the temporal pattern predicted to result from lower supercoiling levels at late times (Fig. 7A). This temporal pattern predicts that supercoiling responsiveness of chlamydial genes may be modulated 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 regulation.
tion. This combinatorial approach also broadens the repertoire of temporal expression patterns that are available to regulate chlamydial genes.

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