The intracellular pathogen *Chlamydia* has an unusual developmental cycle marked by temporal expression patterns whose mechanisms of regulation are largely unknown. To examine if DNA topology can regulate chlamydial gene expression, we tested the in vitro activity of five chlamydial promoters at different superhelical densities. We demonstrated for the first time that individual chlamydial promoters show a differential response to changes in DNA supercoiling that correlates with the temporal expression pattern. The promoters for two midcycle genes, *ompA* and *pgk*, were responsive to alterations in supercoiling, and promoter activity could be regulated more than eightfold. In contrast, the promoters for three late transcripts, *omcAB*, *hctA*, and *ltaB*, were relatively insensitive to supercoiling, with promoter activity varying by no more than 2.2-fold over a range of superhelicities. To obtain a measure of how DNA supercoiling levels vary during the chlamydial developmental cycle, we recovered the cryptic chlamydial plasmid at different times after infection and assayed its superhelical density. The chlamydial plasmid was most negatively supercoiled at midcycle, with an approximate superhelical density of $-0.07$. At early and late times, the plasmid was more relaxed, with an approximate superhelicity of $-0.03$. Thus, we found a correlation between the responsiveness to supercoiling shown by the two midcycle promoters and the increased level of negative supercoiling during mid time points in the developmental cycle. Our results support a model in which the response of individual promoters to alterations in DNA supercoiling can provide a mechanism for global patterns of temporal gene expression in *Chlamydia*.

*Chlamydia* is an important human pathogen that causes infections of the reproductive tract, the eyes, and the respiratory tree (44). *Chlamydia trachomatis* genital infections are the most common notifiable sexually transmitted disease, with more than one million newly diagnosed cases each year (8). Other serovars of *C. trachomatis* cause trachoma, one of the leading causes of preventable blindness in the world. Another species, *Chlamydia pneumoniae*, causes community-acquired pneumonia and has been associated with atherosclerotic heart disease (23). While different *Chlamydia* spp. and strains can produce such different disease manifestations, the essential elements of chlamydial growth and replication inside an infected cell are remarkably similar.

All *Chlamydia* spp. are obligate intracellular bacteria that replicate inside a eukaryotic cell via an unusual developmental cycle in which there is conversion between two stage-specific forms (18). The infectious form is the elementary body (EB), which is metabolically inert and characterized by a highly disulfide-linked protein coat and condensed chromatin. After entry into a eukaryotic host cell, the EB differentiates into a larger, metabolically active reticulate body (RB), which is able to express RNA and proteins and replicate its DNA. After many rounds of binary fission, RBs redifferentiate into EBs before release to infect new cells (22). The whole developmental cycle takes about 48 to 72 h, depending on the species.

Gene expression is temporally regulated during this chlamydial developmental cycle. Regulation appears to be at the transcriptional level with early, mid, and late classes of genes (4, 14, 34, 41, 42). For example, *ompA* belongs to the class of midcycle genes, which can be detected by about 12 h postinfection (hpi) for *C. trachomatis* (41). *ompA* encodes the abundant major outer membrane protein, which is an important immunogenic determinant for different serovars and subspecies (7). A number of late genes have functions related to the morphologically dramatic events that begin at about 24 hpi, when RBs are in the process of converting back to EBs (14). For instance, *omcA* and *omcB* encode two abundant cysteine-rich proteins that are found only in the outer membrane of EBs and not on RBs (19). In addition, *hctA* and *hctB* encode the *Chlamydia*-specific histonelike proteins Hc1 and Hc2, respectively, which mediate the condensation of DNA observed when RBs convert into EBs (2, 6). This regulated expression of late genes demonstrates the principle that gene products appear to be transcribed only at a time in the developmental cycle when they are needed.

Although this temporal pattern of gene expression appears to be well coordinated in *Chlamydia*, the regulatory mechanisms are incompletely understood. We have shown that a subset of late genes is transcribed by the alternative sigma factor $\sigma^{26}$ (53, 54). However, not all late genes are regulated by $\sigma^{26}$ RNA polymerase. Some of these genes are known to be regulated by the major form of RNA polymerase, which contains $\sigma^{66}$ (14). As representative early, mid, and late genes have all been shown to be transcribed by $\sigma^{66}$ RNA polymerase (4, 14, 34, 41, 42), there clearly must be additional mechanisms for the temporal regulation of global gene expression in *Chlamydia*.

DNA supercoiling has been shown to be a global mechanism of gene regulation in *Escherichia coli* and other bacteria (9, 20, 36, 47). Changes in negative supercoiling can modulate promoter activity directly by altering DNA structure and melting energy or indirectly by affecting the binding of transcription...
factors. In addition to studies on the supercoiling sensitivity of individual bacterial promoters, microarray studies using gyrase inhibitors have demonstrated that a large number of genes in a bacterium can be coordinately regulated by changes in negative supercoiling (17, 35).

In this report, we show that chlamydial promoters respond differently to changes in DNA supercoiling in a manner that correlates with their temporal class and that intrachlamydial DNA supercoiling varies during the developmental cycle. We found that the midcycle promoters tested were more responsive to supercoiling than late promoters. In addition, by isolating the native chlamydial plasmid and determining its superhelical density at different times during infection, we obtained evidence that the highest levels of negative supercoiling occur at mid time points in the developmental cycle. From these findings, we propose a mechanism for temporal gene regulation in Chlamydia based on the differential response of promoters to changes in DNA supercoiling during the developmental cycle.

MATERIALS AND METHODS

Construction of in vitro transcription plasmids. Each promoter insert was cloned upstream of a promoterless, G-less cassette transcription template, as previously described (46). Transcription plasmids pMT1103, pMT1150, and pMT1234 with promoter sequences for C. trachomatis LGV serovar L2 but (positions −132 to +1) and omcAB (positions −122 to +1) and C. trachomatis serovar D ppp (positions −267 to +1) have been described elsewhere (40, 49, 54). Plasmid pMT1185 contains the promoter sequence of hct4 (positions −137 to +1) (14) amplified from C. trachomatis serovar D genomic DNA with primers T284 (5′-ACCGAATTCTTTTCTATTAACAGAGGAAAAATAACCTA-3′) and T288 (5′-TTTTATTTTTAATTAGTTTGTTCAAA-3′) and cloned into pMT1125 (49). Plasmid pMT1187 contains the P2 promoter sequence of omp1 (positions −137 to +1) (11) amplified from C. trachomatis serovar D genomic DNA with primers T282 (5′-CCGAATTCCTTTTCTATTAACAGAGGAAAAATAACCTA-3′) and T290 (5′-TTTTATTTTTAATTAGTTTGTTCAAA-3′) and cloned into pMT1125 (49). For some experiments, transcription plasmid DNA was linearized by digestion with EcoRI and was purified by extraction with phenol-chloroform (1:1) and with chloroform, and the DNA was recovered by ethanol precipitation.

Generation of transcription plasmid topoisomers. For each transcription plasmid, a series of topoisomers were generated by using the method of Rhee et al. (38). Ten micrograms of CsCl gradient-purified plasmid DNA was treated for 3 h at 27°C with 5 U of wild-type topoisomerase I (Promega) in 40-μl mixtures containing 50 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, 10% glycerol, and concentrations of ethidium bromide ranging from 0 to 40 μM. The ethidium bromide was removed by two extractions with phenol-chloroform (1:1) and one extraction with chloroform, and the DNA was recovered by ethanol precipitation and resuspended in 30 μl of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA). The DNA concentration and purity were determined using a NanoDrop ND1000 spectrophotometer. The plasmid topoisomers were resolved on 1.4% agarose gels in 1× TAE buffer (0.04 M Tris-acetate, 1 mM EDTA) with 0.02 to 0.16 μg/ml ethidium bromide at concentrations ranging from 0 to 80 μM. The topoisomers were resolved on one- and two-dimensional chloroquine agarose gels (see below) and used as reference standards for the migration of native chlamydial plasmid topoisomers.

Purification of chlamydial RNA polymerase. RNA polymerase was partially purified from C. trachomatis LGV serovar L2 at 24 hpi by heparin-agarose chromatography as previously described (46).

In vitro transcription. Transcription reaction experiments were performed as described previously (46), using either 2 μl of heparin-agarose-purified C. trachomatis RNA polymerase or 0.05 μl of E. coli σ20-holoenzyme (1 U/μl; Epicentre) together with 25 nM plasmid topoisomer in a 10-μl reaction mixture. The relative promoter activity was calculated by defining the maximal promoter activity for the range of superhelicities tested as 100% and normalizing the promoter activity obtained for each topoisomer. Three measurements of relative promoter activity were obtained for each topoisomer, and a mean and a standard deviation were calculated.

FIG. 1. Determination of the superhelical density of topoisomers by electrophoresis. Topoisomers of a 3.1-kb transcription plasmid were resolved on two 1.4% agarose gels with different ethidium bromide (EtBr) concentrations; the higher concentration of ethidium bromide was used for resolution of more supercoiled topoisomers. The negative images of the gels shown make it easier to visualize the topoisomer bands. The concentration of ethidium bromide used in the topoisomer conditions was ΔLK, and the calculated superhelical density of the plasmid (σ) are indicated above each lane. ΔLK was determined by counting the topoisomer bands between the predominant band (arrow) of the population of topoisomers in each lane. Lane 1 contained a topoisomer population containing a completely relaxed plasmid with a superhelical density of zero, which was used as the reference band. The superhelical density was determined based on ΔLK, as described in Materials and Methods.

Plasmid extraction. Chlamydial plasmid pCT-L2 was extracted from 3 × 108 to 1.8 × 109 C. trachomatis LGV serovar L2-infected murine L929 host cells grown in suspension. A multiplicity of infection of 100 was used for plasmid isolation at 2 and 6 hpi, and a multiplicity of infection of 3 was used for all other time points. Chlamydial RBs were recovered and lysed as previously described (46). Chlamydial EBs were recovered and lysed in the same way as RBs, except that L929 host cells were lysed by sonication (three 30-s pulses; 0.125-in. microtip; Branson 250D digital Sonifier). The plasmid was extracted from the chlamydial lysate using a Qiagen plasmid midi kit or by CsCl gradient extraction.

Generation of the chlamydial plasmid topoisomer standard. Topoisomers of the chlamydial plasmid were constructed by using the method that was used for the transcription plasmid topoisomers. Five micrograms of CsCl-purified pCT-L2 plasmid DNA was used per reaction mixture together with ethidium bromide at concentrations ranging from 0 to 80 μM. The topoisomers were resolved on one- and two-dimensional chloroquine agarose gels (see below) and used as reference standards for the migration of native chlamydial plasmid topoisomers.

One- and two-dimensional chloroquine agarose gels. For topoisomer separation, 250-ng samples of the native chlamydial plasmid were resolved on a 0.7% agarose gel in 1× Tris-borate-EDTA with concentrations of chloroquine ranging from 0 to 50 μg/ml at 3.5 V cm⁻¹ for 20 to 24 h at room temperature with buffer circulation. For the two-dimensional gels, two samples were loaded 8 to 10 cm apart and electrophoresed in the first dimension using a chloroquine concentration of 0, 0.25, 1.0, or 2.5 μg/ml. Electrophoresis in the second dimension was performed perpendicular to the electrophoresis in the first dimension with 1.0, 2.5, or 10 μg/ml chloroquine for 16 h at 2.5 V cm⁻¹; the other electrophoresis conditions were unchanged. Prior to the second electrophoresis step, the gel was soaked for 5 h in the new concentration of chloroquine for equilibration.

Southern blotting. Agarose gels were first soaked in distilled H₂O for 1 h, stained in 3 μg/ml ethidium bromide for 1 h, destained in distilled H₂O to up to 2 h, and examined under UV light. Each gel was then incubated in denaturing solution (1.5 M NaCl, 0.5 M NaOH) and in renaturing solution (1.5 M NaCl, 1 M Tris) for 30 min each and transferred to a nylon membrane in 6× SSC by capillary blotting for 16 h (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The membrane was rinsed in 2× SSC-0.1% sodium dodecyl sulfate (SDS) and hybridized with γ-32P-labeled oligonucleotides specific for the chlamydial plasmid (T1129 [5′-AGGGAAGGCTT GACAGTGCC-3′] and T1130 [5′-GAAAAAGACTCGGAAAGGT-3′]) in hybridization solution (0.5 M NaPO₄ [pH 7.2], 7% SDS) at 42°C for 16 h. The membrane
was washed with 0.04 M NaPO₄ (pH 7.2)–1% SDS for 5 min room temperature, for 15 min 42°C, and for 2 min room temperature and then exposed to a phosphorimager plate. The plates were scanned with a Bio-Rad personal FX scanner, and the data were analyzed with Bio-Rad Quantity One software. Densitometric traces of the topoisomer populations were generated with ImageJ 1.38x (http://rsb.info.nih.gov/ij; developed by Wayne Rasband, NIH, Bethesda, MD) to determine the predominant topoisomer bands.

RESULTS

Construction of transcription plasmid topoisomers with various negative superhelicities.

To assess the effect of DNA supercoiling on the activities of five chlamydial promoters, we cloned each promoter into a transcription plasmid and generated a series of topoisomers for each plasmid. We produced these topoisomers with different superhelical densities by performing in vitro topoisomerase reactions using different concentrations of ethidium bromide and the method of Rhee et al. (38). Figure 1 shows the results for a representative series of topoisomers for one of the transcription plasmids. Each reaction produced a small number of topoisomers that differed from each other by one linking number (one turn of the DNA helix) that could be resolved on an ethidium bromide gel as a ladder of distinct bands. The first reaction mixture contained a totally relaxed plasmid with a superhelical density of zero and served as a reference. There was enough overlap between the ladders so that the linking number of the predominant band for each topoisomer could be easily determined and the average superhelical density could be calculated. For each promoter, the average superhelical density of the topoisomer series ranged from 0 to approximately −0.074 (Table 1). Thus, our topoisomer superhelical densities encompassed and exceeded the physiologic range of global superhelical densities in *E. coli*, which vary from −0.03 to −0.06 depending on the growth state (20).

Chlamydial promoters respond differentially to changes in superhelical density. In initial experiments to examine if DNA supercoiling affects chlamydial promoter activity, we tested five chlamydial promoters, comparing transcription of a supercoiled template and transcription of a linearized template. The supercoiled plasmids, which were isolated from *E. coli* during logarithmic growth, each had a superhelical density of about −0.06, as determined by agarose gel electrophoresis along with in vitro-generated topoisomer standards (data not shown). Using an in vitro transcription assay with partially purified *C. trachomatis* RNA polymerase (46), we found that DNA supercoiling had a significant effect on the level of transcription from two midcycle promoters but not on the level of transcription from three late promoters. For the midcycle promoters, transcription from a supercoiled template was 57-fold higher for *ompA* and 26-fold higher for *pgk* than transcription from the same template in linearized form (data not shown). In contrast, the promoters for the late transcripts *omcAB*, *hctA*, and *ltuB* showed 0.7-, 1.9-, and 1.9-fold changes in activity, respectively, for a supercoiled template compared with a linearized template (data not shown).

To obtain a more detailed view of the effect of negative supercoiling on chlamydial promoter activity, we tested a series of topoisomers for each of the five promoters in individual in vitro transcription reactions using heparin-agarose-purified chlamydial RNA polymerase (A) or *E. coli* RNA polymerase (B). For each promoter, a series of topoisomers with increasing negative superhelical densities ranging from 0 to approximately −0.1 were tested, and an individual topoisomer was used in each transcription reaction. The temporal class (4, 14, 34, 41) and the maximum difference in transcriptional activity over the range of superhelical densities tested are shown for each promoter.

<table>
<thead>
<tr>
<th>C. trachomatis promoter</th>
<th>Temporal class</th>
<th>Range of superhelical densities of topoisomers</th>
<th>Plasmid (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ompA</td>
<td>Mid</td>
<td>0 to −0.097</td>
<td>pMT1187</td>
</tr>
<tr>
<td>pgk</td>
<td>Mid</td>
<td>0 to −0.097</td>
<td>pMT1234 (54)</td>
</tr>
<tr>
<td>omcAB</td>
<td>Late</td>
<td>0 to −0.097</td>
<td>pMT1150 (49)</td>
</tr>
<tr>
<td>hctA</td>
<td>Late</td>
<td>0 to −0.074</td>
<td>pMT1185</td>
</tr>
<tr>
<td>ltuB</td>
<td>Late</td>
<td>0 to −0.091</td>
<td>pMT1013 (40)</td>
</tr>
</tbody>
</table>

* Data from references 4, 14, 34, and 41.

a Superhelical densities were determined as described in Materials and Methods.

FIG. 2. In vitro transcription of chlamydial promoters, using heparin-agarose-purified chlamydial RNA polymerase (A) or *E. coli* RNA polymerase (B). For each promoter, a series of topoisomers with increasing negative superhelical densities ranging from 0 to approximately −0.1 were tested, and an individual topoisomer was used in each transcription reaction. The temporal class (4, 14, 34, 41) and the maximum difference in transcriptional activity over the range of superhelical densities tested are shown for each promoter.
vitro transcription reactions. The difference in responsiveness to supercoiling between the two midcycle genes and the three late genes again was apparent (Fig. 2A). For both midcycle genes, the promoter activity was lowest for a totally relaxed transcription template; with increased negative supercoiling, transcription increased over a 50-fold range for the \textit{ompA} promoter and by up to 8-fold for the \textit{pgk} promoter. In comparison, the activities of the \textit{omcAB}, \textit{hctA}, and \textit{ltuB} promoters showed only modest changes in activity, with maximum differences of 1.6-, 2.2-, and 1.7-fold, respectively, over the entire range of superhelical densities tested. This difference in supercoiling responsiveness between the midcycle and late promoters was confirmed when we repeated these transcription assays with separately prepared sets of topoisomers (data not shown).

To see how promoter activity varied with increasing negative supercoiling, we calculated the relative promoter activity by defining the maximum promoter activity for the range of superhelicities tested as 100% and normalizing the promoter activity obtained for each topoisomer. Figure 3A shows that for the midcycle genes, \textit{pgk} and \textit{ompA}, the promoter activity from a relaxed template was low, and a minimal superhelical density (–0.01 to –0.02) was required for a relative promoter activity of 50%. In contrast, the relative activity for the late promoters, \textit{omcAB}, \textit{hctA}, and \textit{ltuB}, was ≥50% over the entire range of superhelical densities. Thus, in contrast to the midcycle promoters, the late promoters were generally insensitive to changes in DNA supercoiling and could be transcribed at high levels even if the template was completely relaxed.

The relative responsiveness of the chlamydial promoters to supercoiling is the same with chlamydial and \textit{E. coli} RNA polymerases. To determine if the difference in responsiveness to supercoiling is intrinsic to the individual promoters or due to...
chlamydial RNA polymerase, we also transcribed the promoter topoisomers with *E. coli* RNA polymerase. We performed in vitro transcription reactions with *E. coli* σ^70^ polymerase as previously described (50) but were unable to test the *ompA* promoter because it is not transcribed by this heterologous RNA polymerase (12). Transcription of supercoiled and linearized templates by *E. coli* RNA polymerase showed the same promoter-specific differences that we observed with chlamydial RNA polymerase. DNA topology had a large effect on the activity of the *pgk* midcycle promoter (26-fold range) but made little difference to the activities of the *ompA* (1.3-fold), *hctA* (0.7-fold), and *ltuB* (1.2-fold) promoters (data not shown).

We observed the same differential response to supercoiling for a midcycle chlamydial promoter versus a late chlamydial promoter when we transcribed a topoisomer series with *E. coli* polymerase. The *pgk* promoter showed a 17-fold range in promoter activity (Fig. 2B), and a minimal superhelicity of approximately −0.025 was required for ≥50% relative promoter activity (Fig. 3B). In contrast, the late *ompA* promoter showed little variation in promoter activity (1.4-fold) (Fig. 2B), and the relative promoter activity was ≥50% over the entire range of superhelical densities tested (Fig. 3B).

*Chlamydia* supercoiling-responsive promoters have a high G or C content in the −10 element and the spacer region.

To identify features of chlamydial promoters that might account for the observed differences in supercoiling responsiveness, we aligned the five chlamydial promoters that were tested in this study (Fig. 4). The *ompA* promoter has an unusual −10 element with a G or C at three of the six positions (11), which is quite different from the A- or T-rich sequence of the preferred supercoiling-responsive promoters (39, 45) and consensus *E. coli* σ^70^ −10 promoter element (21). In fact, *E. coli* RNA polymerase is unable to transcribe this promoter (12), even when it is tested over a wide range of different superhelical densities (data not shown). Our two supercoiling-sensitive midcycle promoters both have an unusually high G or C content in the spacer (41 and 47% for *ompA* and *pgk*, respectively), although a high G or C content was not found in the discriminator region that is between the −10 element and the transcription start site. In contrast, the G or C content in the spacer of the late promoters for *ompA* and *ltuB* are 0 and 6%, respectively, although the G or C content is 28% for the third late promoter, *hctA*. Thus, at least for the promoters tested, there was a correlation between high G or C content in the promoter spacer and the −10 element and responsiveness to supercoiling.

**The superhelical density of the chlamydial plasmid is higher in RBs than in EBs.** To determine how the supercoiling state changes during chlamydial growth, we isolated the cryptic chlamydial plasmid pCT-L2 (28) at 24 hpi, when chlamydiae are mostly in the RB stage, and at 46 hpi, when most RBs have converted to EBs. We then compared the migration of the native plasmid to the migration of topoisomer standards of the chlamydial plasmid on chloroquine agarose gels. Chloroquine gels were used rather than ethidium bromide gels because they gave better resolution of the 7.5-kb chlamydial plasmid (data not shown). For these studies, we compared the plasmids on gels at different chloroquine concentrations, because no single concentration resolved all the topoisomers. Plasmid bands were then detected by Southern blotting.

At 10 μg/ml chloroquine, the topoisomer standards showed that a completely relaxed plasmid topoisomer migrated considerably faster than a more supercoiled species (Fig. 5A, left panel). Compared to these standards, the native plasmid isolated at 24 hpi was highly supercoiled and the plasmid isolated at 46 hpi was more relaxed (Fig. 5A, right panel).

At 1 μg/ml chloroquine, the migration pattern of the topoisomer standards was a little more complicated, but it allowed better resolution of moderately and highly negatively supercoiled topoisomers (Fig. 5B, left panel). On the gels, totally relaxed plasmid (lane 1) still migrated faster, but so did highly supercoiled plasmids (lanes 5 and 6). In contrast, topoisomers with low and intermediate superhelical densities migrated slower (lanes 2 to 4). Compared to these standards alone, the fast migration of the 24-h native plasmid was consistent with either a highly relaxed form or a supercoiled form (Fig. 5B, right panel), but based on this finding, together with the migration pattern at 10 μg/ml chloroquine (Fig. 5A, right panel), we concluded that the 24-h chlamydial plasmid had a highly negatively supercoiled topology. The 46-h native plasmid produced a pattern with a predominant band that migrated slowly, consistent with a low to intermediate superhelical density (Fig. 5B, right panel).

In the absence of chloroquine, the completely relaxed plasmid (Fig. 5C, left panel, lane 1) could be clearly resolved as a more slowly migrating band that was different from topoisomers with any level of negative supercoiling (lanes 2 to 6). The

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**FIG. 4.** Alignment of promoter sequences for the promoters tested. Sequences are aligned at position 1, and upstream sequences to position −50 are shown for each promoter. The −35 and −10 elements of an optimal chlamydial σ^66^ promoter (39, 45) and the tested promoters are indicated by bold uppercase letters. The *ompA* promoter is *ompA* P2 (11). A promoter was considered supercoiling responsive if its relative promoter activity dropped below 50% over the range of superhelical densities tested.
migration of both the 24- and 46-h native plasmids was fast, which indicates that neither plasmid had a completely relaxed state (Fig. 5C, right panel).

To calculate the level of supercoiling, we resolved the native plasmids and a completely relaxed topoisomer of the chlamydial plasmid using two-dimensional topoisomer gels. Figure 6 shows representative two-dimensional gels in which electrophoresis in the first dimension occurred in the presence of 1 μg/ml chloroquine and electrophoresis in the second dimension occurred in the presence of 10 μg/ml chloroquine. With these results and the results for additional one- and two-dimensional topoisomer gels with different chloroquine concentrations (data not shown), we were able to count the differences in the numbers of topoisomer bands for the 24-h, 46-h, and completely relaxed plasmid populations (i.e., the ΔLK).

This information allowed us to calculate the superhelical densities of the two native plasmid populations. Compared to the relaxed plasmid, which had a superhelical density of zero, the 24-h plasmid consisted of a population of topoisomers with superhelical densities ranging from 0.063 to 0.077 and the 46-h plasmid had superhelical densities ranging from -0.028 to -0.035. These values correspond well to the superhelical densities in E. coli (-0.06 during mid-log growth and -0.03 in stationary phase).

The supercoiling level of the Chlamydia plasmid varies with the developmental cycle. As the global supercoiling state of the chlamydial plasmid changed more than twofold between 24 and 46 hpi, we isolated the native plasmid at additional time points during the developmental cycle to determine the temporal pattern of DNA supercoiling in Chlamydia. We electrophoresed the plasmids on agarose gels at different chloroquine concentrations to best resolve the topoisomer bands. Figure 7 shows representative gels. At 1.5 μg/ml chloroquine, we were able to show that the plasmid isolated at 18 h and the plasmid isolated at 24 h were more supercoiled than the plasmid isolated at an earlier time point (2 or 6 h) and the plasmid isolated at a later time point (46 h), both of which were less supercoiled (Fig. 7A). Using an agarose gel with 5 μg/ml chloroquine, we were able to look at the plasmids isolated at mid to late time points and demonstrate that supercoiling was greatest at 18, 24, and 28 h and that the plasmid was relatively relaxed at 40 and 46 h (Fig. 7B).

DISCUSSION

Although there is ample evidence that transcription is temporally regulated during the chlamydial developmental cycle (4, 14, 34, 41, 42), the mechanisms for this temporal regulation have not been well defined (29, 45). The alternative sigma factor σ^38 appears to have a specific role in regulating a subset of late genes, including hctB, which encodes the histonelike protein Hc2 (53, 54), and CT441, which encodes a tail-specific protease (26). A role for a second alternative sigma factor, σ^54, in temporal gene regulation has not been established, although this factor has been proposed to function in midcycle gene expression, based on its likely regulation of two midcycle genes (31). As the major RNA polymerase, containing σ^66, transcribes early, mid, and late genes (4), there must be additional mechanisms for the selective transcription of temporal classes of σ^66-dependent genes, such as regulation by specific transcription factors. The transcriptional activator ChxR has been proposed to be a chlamydial midcycle regulator based on its own temporal expression pattern (25). IHF is another potential regulator in Chlamydia, as it has been shown to bind upstream
of the omcAB promoter, inducing a sharp bend and modestly increasing promoter activity (56). These proposed regulators, however, do not appear to sufficiently account for the patterns of temporal gene expression in Chlamydia.

In this report, we provide evidence that the response of individual promoters to changes in DNA supercoiling can be utilized as a mechanism of gene regulation in Chlamydia during its unusual developmental cycle. Mathews and Stephens previously noted that the C. trachomatis ompA promoter was sensitive to supercoiling changes induced by coumermycin treatment when it was transcribed in vivo by E. coli expressing C. trachomatis σ26 (30). We demonstrate here that representative promoters belonging to two different temporal classes of chlamydial promoters respond differently to changes in DNA supercoiling. Furthermore, our results show that there is a correlation between the supercoiling responsiveness of promoters for the midcycle genes, ompA and pgk, and the higher levels of intrachlamydial DNA supercoiling at mid time points in the developmental cycle. The role of DNA supercoiling in the regulation of early genes has not been defined yet as the supercoiling responsiveness of these promoters has not been determined.

DNA supercoiling is a global mechanism for regulating gene expression in other bacteria, and in fact, it has been proposed to be the highest level in the hierarchy of prokaryotic gene regulation (20). Global studies of E. coli and Haemophilus influenzae have shown that changes in supercoiling can alter the expression of hundreds of genes (17, 35). Some bacterial promoters, such as E. coli topA (48), gyrA and gyrB (33), and ibY/C (38) and Salmonella enterica serovar Typhimurium leu-500 (37), are very responsive to supercoiling, while others are relatively insensitive.

We propose that the differential sensitivity to supercoiling displayed by chlamydial promoters is due to differences in the promoter sequence or structure. In support of this hypothesis, we found that individual chlamydial promoters showed the same differential response to supercoiling whether they were transcribed by C. trachomatis or E. coli RNA polymerase. In other bacteria, promoter features, such as nonconserved sequences in the promoter elements, the G or C content of the discriminator region, or the length and sequence of the spacer, have been associated with sensitivity to supercoiling (9, 20, 47). Spacer length did not appear to be a major determinant in our study as it was 17 bp for the two midcycle promoters and 17 or 18 bp for the nonresponsive late promoters. Our observations suggest that a high overall G or C content in the −10 element and spacer may be a feature of a supercoiling-sensitive promoter in Chlamydia. This hypothesis is testable, although it would require identification of additional active chlamydial promoters (45). For example, we attempted to transcribe additional midcycle promoters, including plasmid promoters, but we were not able to obtain sufficient levels of transcription to perform the topoisomer studies.

Our studies show that the chlamydial cryptic plasmid had the highest levels of negative DNA supercoiling at mid and mid-to-late times in the chlamydial developmental cycle and was relatively relaxed at early and late time points. These results are consistent with the results of Barry et al., who found that the chlamydial plasmid isolated late in an infection (36 hpi) was more relaxed than the plasmid purified from an early-to-mid time point (12 hpi) (3). However, Solbrig et al. detected additional plasmid topoisomers, including what appeared to be a higher supercoiled species in EBs, but not RBs, which may have been due to the presence of copurified proteins (43). In these studies the superhelical density of the chlamydial plasmid was not determined.

We propose that the superhelical density of the chlamydial plasmid at different time points can be used as an estimate of global superhelical density during the developmental cycle. At the least, we believe that the changes in plasmid supercoiling that we measured reflect the direction and trends of global supercoiling. Based on our measurements we propose a model in which the global supercoiling level changes more than twofold during the chlamydial developmental cycle and is highest in midcycle and lowest at early and late time points. The average superhelical density of the chlamydial plasmid was
A number of factors known to affect global DNA superhelicity in other bacteria may regulate DNA supercoiling in chlamydiae. Global supercoiling homeostasis in a bacterial cell is regulated by DNA topoisomerases (13). In all available genome sequences for Chlamydia spp., there are homologues for the main antagonistic topoisomerases, topoisomerase I and gyrase, which remove and introduce negative supercoils, respectively. As gyrase activity depends on the [ATP]/[ADP] ratio (16), DNA supercoiling can be altered by intracellular energy charge variation during bacterial growth. In addition, specific environmental stimuli, such as temperature, osmotic stress, anaerobiosis, or nutrient availability, have been shown to affect DNA superhelicity (10, 13). Barry et al. have shown that low-level expression of the chlamydial histonelike protein Hc1 can decrease superhelical density and modulate transcription of heterologous supercoiling-sensitive promoters (3). Our studies did not measure the effect of Hc1, Hc2, or other DNA-binding proteins on DNA topology and supercoiling as we had removed proteins from our chlamydial plasmid DNA in order to resolve the topoisomers on chloroquine gels.

In summary, our results support the hypothesis that there is a mechanism of temporal regulation during the developmental cycle of Chlamydia based on the differential response of promoters to alterations in DNA supercoiling. DNA supercoiling may be particularly important for genes that are upregulated during midcycle, as we found that two midcycle genes showed the greatest response to supercoiling and we also measured the highest levels of intrachlamydial negative superhelicity at mid time points. In contrast, DNA supercoiling may not play a large role in the activation of late genes as we found that three σ6-dependent late promoters were largely insensitive to changes in superhelicity. Thus, while a subset of late genes is regulated by σ28, additional mechanisms are necessary to account for the temporal regulation of σ6-dependent late genes. Nonetheless, the different response to superhelicity of late genes may still be important as the insensitivity of these genes to supercoiling may ensure that they are transcribed at low superhelical densities when supercoiling-sensitive promoters are relatively inactive. It remains to be seen whether DNA supercoiling is a general mechanism for temporal gene regulation in Chlamydia or one of an expanding repertoire of mechanisms for the regulation of specific subsets of genes.

ACKNOWLEDGMENTS

We thank Kinrin Yamanaka for excellent technical assistance and Elizabeth DiRusso Case, Johnny Akers, Allan Chen, Ayako Hasegawa, and Kirsten Johnson for critical reading of the manuscript and helpful advice.

This work was supported by grant AI 44198 from the NIH. M.T. was supported by NIH Independent Scientist Award AI 057563.

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DNA Supercoiling-Dependent Gene Regulation in *Chlamydia*

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Volume 190, no. 19, p. 6419–6427, 2008. Page 6425: Fig. 6 should appear as shown below.