Nucleic Acids Research, 2003, Vol. 31, No. 2 **551–555**
DOI: 10.1093/nar/gkg150

Mutational analysis of the *Chlamydia trachomatis dnaK* promoter defines the optimal –35 promoter element

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Received October 21, 2002; Revised and Accepted November 14, 2002

ABSTRACT

A long-standing question in the biology of the intracellular bacterium, *Chlamydia*, has been the structure of the promoter recognized by its RNA polymerase. The 'RNA polymerase sigma subunit paradox' refers to the difficulty reconciling the conservation between the RNA polymerases of *Chlamydia* and *Escherichia coli*, especially at the level of the promoter-recognition sigma subunit, with the general lack of homology between chlamydial promoters and the *E.coli* σ^{70} consensus promoter. While the –10 promoter element appears to be conserved between *Chlamydia* and *E.coli*, the structure of the chlamydial –35 promoter element has not been defined. We have investigated the structure of the –35 element of the *Chlamydia trachomatis dnaK* promoter by measuring the effects of single base pair substitutions on *in vitro* promoter activity. Most substitutions produced large decreases in promoter activity, which allowed us to define the optimal –35 sequence in the context of the *dnaK* promoter. We found that the optimal chlamydial –35 promoter sequence is identical to the *E.coli* σ^{70} consensus –35 promoter element (TTGACA). These results indicate that the optimal promoter specificities of the major form of chlamydial RNA polymerase and *E.coli* σ^{70} RNA polymerase are in fact highly conserved. A further implication of our results is that many chlamydial promoters have a suboptimal promoter structure. We hypothesize that these chlamydial promoters are intrinsically weak promoters that can be regulated during the chlamydial developmental cycle by additional transcription factors.

INTRODUCTION

Chlamydia is a Gram-negative pathogenic bacterium and an obligate intracellular parasite of eukaryotic cells. It has an unusual developmental cycle characterized by two morphologic forms (1–3). The elementary body is an infectious, but metabolically inactive, form. Upon infection of a host cell, it converts into the reticulate body, which is the intracellular, metabolically active form that divides by binary fission as part of the replicative phase of the developmental cycle.

The chlamydial developmental cycle is characterized by the coordinate transcriptional regulation of gene expression (1–3). A major unresolved issue in the field of chlamydial transcription is the structure of the chlamydial promoter. Chlamydial promoters show variability in the –35 element, which has made it difficult to deduce a consensus chlamydial promoter sequence (reviewed in 4,5), and few chlamydial promoters resemble the *Escherichia coli* σ^{70} consensus promoter structure. In contrast, the RNA polymerases of *Chlamydia* and *E.coli* are highly conserved, especially in the sigma subunit, which recognizes promoter sequences. Stephens has described the lack of conservation of chlamydial promoters in the face of RNA polymerase conservation as the 'RNA polymerase sigma subunit paradox' (6). There has also been speculation that chlamydial RNA polymerase may have an altered promoter specificity (7,8).

In the absence of an experimental chlamydial genetics system, promoter structure in *Chlamydia* has been studied with *in vitro* transcription methods (7–11) and a heterologous *in vivo* system in *E.coli* (12). We previously used an *in vitro* saturation mutagenesis approach to define the optimal –10 promoter sequence of the *Chlamydia trachomatis* rRNA promoter (10). This optimal sequence resembles the *E.coli* σ^{70} consensus –10 promoter element. However, we were unable to define the optimal –35 promoter sequence of the rRNA promoter because single base substitutions in this region had little effect on promoter activity. Examination of the predicted sequence of other chlamydial promoters also shows a well conserved –10 promoter element but poor sequence conservation in the –35 region (4,10).

To address the 'RNA polymerase sigma subunit paradox', and to determine the promoter specificity of chlamydial RNA polymerase, we have defined the optimal –35 sequence of the *dnaK* promoter. The *C.trachomatis dnaK* promoter is a heat shock promoter that has recently been shown to be negatively regulated by a heat shock repressor, HrcA (13). We chose to study the *dnaK* promoter because it is one of the few predicted chlamydial promoters that resembles the *E.coli* σ^{70} consensus

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promoter structure, and it has a 5/6 match in the –35 region. In this report, we demonstrate that substitutions in the –35 region of the *dnaK* promoter produced large decreases in promoter activity. This mutational approach has allowed us to define for the first time the optimal –35 promoter sequence for a specific class of chlamydial promoters. This optimal chlamydial promoter structure strongly resembles the *E.coli* σ^{70} consensus promoter, and suggests that the optimal promoter specificity of chlamydial and *E.coli* RNA polymerases is highly conserved. These results raise the question of why many chlamydial promoters, including the normally highly transcribed rRNA promoter, do not resemble the optimal promoter structure. We discuss the possibility that many chlamydial promoters are intrinsically weak because of their suboptimal promoter structure, thereby allowing for regulation during the chlamydial developmental cycle by additional transcription factors.

MATERIALS AND METHODS

Reagents

Products were obtained from the following sources and were used according to the manufacturer's specifications. Restriction enzymes, calf alkaline phosphatase, T4 DNA ligase, rRNasin and *Thermus aquaticus* DNA polymerase from Promega Biotech (Madison, WI); T4 polynucleotide kinase from New England Biolabs (Beverly, MA); T7 Sequenase DNA polymerase and dideoxynucleotide kit from United States Biochemical Corp. (Cleveland, OH); nucleoside triphosphates, 3′-*O*-methylguanosine 5′-triphosphate from Amersham Pharmacia Biotech (Arlington Heights, IL); ^{32}P-containing nucleoside triphosphates from ICN Pharmaceuticals, Inc. (Costa Mesa, CA); ampicillin from Fisher Scientific (Pittsburgh, PA); and purified *E.coli* RNA polymerase from Epicentre (Madison, WI).

DNA manipulation

Nucleic acid preparation and analysis were performed according to standard recombinant DNA protocols, as described previously (10).

Construction of the wild-type *dnaK* transcription template

The *dnaK* promoter region (–39 to +6) from *C.trachomatis*, mouse pneumonitis strain, was amplified by PCR. The promoter insert was cloned upstream of a promoterless G-less cassette transcription template in plasmid pMT504 (10). pMT504 also contains an internal control transcription template consisting of the *C.trachomatis* rRNA promoter (–53 to +5) upstream of a shorter G-less cassette. Transcription of the plasmid produced a 159 nt test transcript and a 130 nt control transcript.

Construction of *dnaK* transcription templates containing mutations

Individual mutant promoters were produced by PCR, with the desired mutation introduced on an oligonucleotide primer. Each template contained the *dnaK* promoter region from –39 to +6. A 5 bp mutation of the –35 region was produced by altering the sequence from –34 to –30 (TTGAC to GGTCA). Eighteen mutant *dnaK* promoters (–39 to +6), with single base pair substitutions in the –35 region, were produced, so that the effect of all possible single substitutions from –34 to –29 could be tested. The mutant *dnaK* promoters were cloned upstream of a promoterless G-less cassette transcription template in plasmid pMT504 as previously described (10).

Purification of *C.trachomatis* RNA polymerase

RNA polymerase was partially purified from *C.trachomatis* serovar LGV L2 at 20 h post-infection by heparin–agarose chromatography as previously described (9).

In vitro transcription

The following reaction mixture was assembled: 50 mM potassium acetate, 8.1 mM magnesium acetate, 50 mM Tris acetate pH 8.0, 27 mM ammonium acetate, 2 mM DTT, 400 µM ATP, 400 µM UTP, 1.2 µM CTP, 0.06 µM [α-^{32}P]CTP (3000 Ci/mmol), 100 µM 3′-*O*-methylguanosine 5′-triphosphate Na salt, 18 U rRNasin, 10% glycerol and 1.0 µl heparin–agarose-purified *C.trachomatis* RNA polymerase or 0.03 U of *E.coli* σ^{70} RNA polymerase. Supercoiled DNA template (final concentration 25 nM) was added and the reaction was incubated at 37°C for 15 min. The final reaction volume was 10 µl. The reaction was terminated by the addition of 10 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). An aliquot of 7 µl of the sample was electrophoresed on an 8 M urea/6% polyacrylamide gel. Transcripts were visualized by autoradiography and quantified with a BioRad Personal Molecular Imager FX (Hercules, CA).

Calculation of promoter activity

Each transcription reaction produced a test transcript from the *dnaK* promoter, and a control transcript from the *C.trachomatis* rRNA promoter. Promoter activity was calculated as the ratio of the test transcript to the control transcript. Relative promoter activity was determined by normalizing the promoter activity of each mutant promoter to that of the wild-type *dnaK* promoter defined as 100%. Three measurements of relative promoter activity were obtained for each promoter, and a mean and a standard deviation were calculated. Fold changes in promoter activity were obtained by comparing the relative promoter activity of each mutant promoter to that of the wild-type *dnaK* promoter.

RESULTS

Substitution of the –35 region of the *dnaK* promoter decreased transcription by *C.trachomatis* RNA polymerase

We have previously shown that the *C.trachomatis dnaK* promoter is transcribed by chlamydial RNA polymerase *in vitro* (14). To determine if the –35 promoter element is important for transcription, we made a 5 bp substitution of the predicted –35 region, changing the sequence from TTGAC to GGTCA at positions –34 to –30 (Fig. 1). This substitution of the –35 region produced a 22-fold decrease in transcription by *C.trachomatis* RNA polymerase (Fig. 2A, lanes 1 and 2). The *C.trachomatis dnaK* promoter is also transcribed by *E.coli* RNA polymerase (14). The same 5 bp substitution of the –35

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E. coli consensus:  TTGACA                  TATAAT
                       -30       -20       -10     . +1
dnaK (wt):          TCTTGACCAGAGGCTCCGGTTTTCCTATAATGACACC
dnaK (sub-35):      TCggtcaCAGAGGCTCCGGTTTTCCTATAATGACACC
dnaK (C-29T):       TCTTGACtAGAGGCTCCGGTTTTCCTATAATGACACC
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Figure 1. Sequence of the *C.trachomatis dnaK* promoter region. A 5 bp substitution in the –35 region (sub-35), and an example of a single base pair substitution (C to T change at –29, C-29T) are shown beneath the wild-type (wt) promoter sequence. The *E.coli* σ^{70} consensus sequence is shown for comparison. Predicted –35 and –10 promoter sequences are underlined. Substitutions are shown in lower case type. Dots above the sequence indicate *in vivo* transcription initiation sites with the numbering shown relative to the position marked +1.

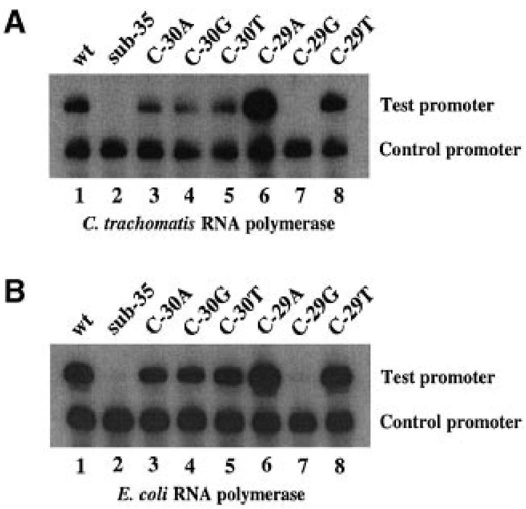

Figure 2. *In vitro* transcription of *C.trachomatis dnaK* promoter templates by *C.trachomatis* RNA polymerase (**A**) or *E.coli* RNA polymerase (**B**). The *dnaK* templates contained the wild-type (wt) sequence (lane 1), a 5 bp substitution of the –35 region (sub-35) (lane 2), a single base pair substitution of the wild-type C at position –30 with A (lane 3), G (lane 4) or T (lane 5), or a single base pair substitution of the wild-type C at position –29 with A (lane 6), G (lane 7) or T (lane 8). The *C.trachomatis* rRNA promoter was used as the control promoter.

region produced a 54-fold decrease in transcription by *E.coli* RNA polymerase (Fig. 2B, lanes 1 and 2).

Single base substitutions in the –35 region of the *dnaK* promoter also had an effect on promoter activity

We tested the effect on *in vitro* transcription of single base substitutions in the predicted –35 region from positions –34 to –29. At each position, we tested each of the three possible substitutions separately. Most of the single base substitutions produced a large effect on transcription by *C.trachomatis* RNA polymerase. For example, each of the substitutions at positions –34, –33, –32 and –31 decreased promoter activity from 3- to >20-fold (Fig. 3). Point substitutions at –30 had less effect, with substitution of the wild-type C by an A, G or T producing a 2.3-, 2.5- or 1.6-fold decrease in transcription, respectively. Of all the positions in the –35 promoter region, only substitutions at –29 produced an increase in transcription. At this position, substitution of the wild-type C with an A or T produced a 3.1- and 1.2-fold increase, respectively.

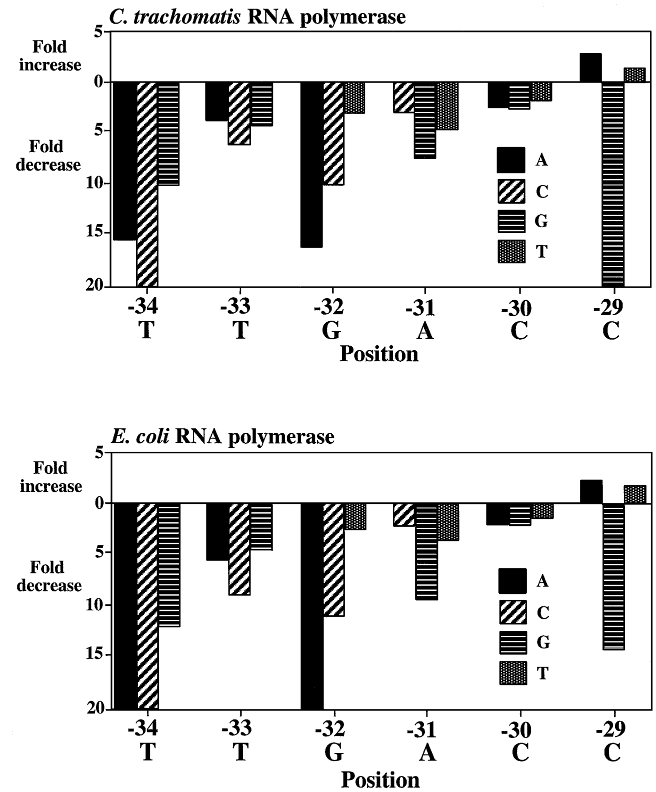

Figure 3. Effects of single base pair substitutions on the *C.trachomatis dnaK* promoter by *C.trachomatis* or *E.coli* RNA polymerases. All three possible substitutions were tested at each position from –34 to –29. The wild-type sequence at each position is shown below each graph. Changes in promoter activity are shown as fold increases or decreases relative to wild-type promoter activity. Decreases >20-fold are not illustrated as extending beyond the bottom axis. Each experiment is the mean of three separate experiments.

Examples of transcription of promoters containing single base substitutions are shown in Figure 2A.

Single base substitutions in the –35 region of the *dnaK* promoter also affected transcription by *E.coli* RNA polymerase

We also assayed the promoter activity of this series of *C.trachomatis dnaK* promoter mutations when transcribed by *E.coli* RNA polymerase. Most of the single base substitutions produced a large decrease in transcription with a pattern similar to that obtained with *C.trachomatis* RNA polymerase (Figs 2B and 3). The smallest differences were also seen at –30 and –29. At –30, substitution of the wild-type C with an A, G or T, produced a 1.8-, 1.9- or 1.2-fold decrease in transcription, respectively (Fig. 2B). As with *C.trachomatis* RNA polymerase substitutions of wild-type C at –29 with an A or T produced a slight increase in promoter activity (up 2.4- and 1.7-fold, respectively).

Derivation of an optimal promoter sequence

To determine if there is a sequence preference in the –35 promoter element, we examined each position in the –35 promoter region, comparing the effect of each of the four possible nucleotides on promoter activity. To facilitate this

Table 1. Promoter activities of *dnaK* promoter templates with single base pair substitutions

Position	Wild-type sequence	Relative promoter activity ± SD[a] *Chlamydia trachomatis* RNA polymerase				*Escherichia coli* RNA polymerase			
		A	C	G	T	A	C	G	T
–34	T	6.5 ± 2.3	3.0 ± 0.5	10.0 ± 2.0	100	4.0 ± 0.4	3.0 ± 0.3	8.4 ± 1.4	100
–33	T	28.8 ± 1.3	16.6 ± 3.0	24.4 ± 2.3	100	18.6 ± 1.6	11.4 ± 2.3	22.6 ± 1.3	100
–32	G	6.2 ± 1.0	9.9 ± 1.6	100	34.4 ± 4.7	4.0 ± 0.2	9.1 ± 1.2	100	41.0 ± 2.3
–31	A	100	35.2 ± 8.8	13.7 ± 3.4	22.1 ± 8.7	100	46.6 ± 3.2	10.9 ± 0.7	29.2 ± 1.6
–30	C	43.0 ± 6.3	100	40.7 ± 4.3	63.8 ± 10.1	57.0 ± 4.5	100	53.2 ± 1.8	80.8 ± 7.3
–29	C	312.6 ± 12.3	100	2.1 ± 0.6	118.3 ± 26.4	242.0 ± 37.7	100	7.1 ± 1.9	168.9 ± 13.0

[a]The relative promoter activity was determined by normalizing to that of the wild-type *dnaK* promoter defined as 100%.
Each value is the mean of three separate experiments.

analysis, we determined a relative promoter activity for each mutant promoter by normalizing the promoter activity to that of the wild-type *dnaK* promoter, which was defined as 100% (Table 1). For example, at position –34, the relative promoter activity when transcribed by *C.trachomatis* RNA polymerase was 6.5, 3.0, 10.0 or 100% for an A, C, G or T at this position, respectively. Thus, at –34 there was a marked sequence preference for a T and all other substitutions decreased promoter activity significantly. These results are best displayed in Figure 4 where, for each position, the height of the letter representing each nucleotide is proportional to its relative promoter activity. In this manner, the optimal –35 promoter sequence for *C.trachomatis* RNA polymerase was determined to be TTGACA in the context of the *dnaK* promoter. The optimal –35 promoter sequence for *E.coli* RNA polymerase in the context of the *C.trachomatis dnaK* promoter was TTGA(C/T)A which compares very favorably with the *E.coli* σ^{70} consensus –35 promoter element (15).

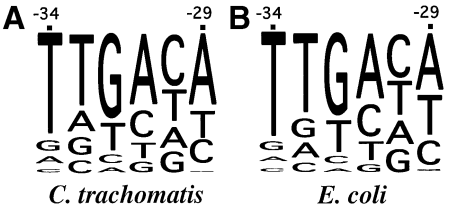

Figure 4. Base preference for *C.trachomatis* RNA polymerase (**A**) or *E.coli* RNA polymerase (**B**), based on a mutational analysis of the *C.trachomatis dnaK* promoter from positions –34 to –29. For each position the height of each letter is proportional to its relative promoter activity. The position relative to the start site of transcription is indicated above the letters.

DISCUSSION

We have defined the optimal –35 promoter sequence of the *dnaK* promoter by measuring the effect of single base pair substitutions on *in vitro* transcription by *C.trachomatis* RNA polymerase. This RNA polymerase contains σ^{66} (9), the major chlamydial sigma subunit, which is the homolog of *E.coli* σ^{70} (16,17). In a previous study of the rRNA promoter we were able to use this approach to define the optimal chlamydial –10 promoter element, but not the –35 element (10). We hypothesize that the mutational approach in the current study was successful because the –35 element of the *dnaK* promoter resembles the optimal chlamydial promoter structure more closely than the rRNA promoter.

The optimal chlamydial –35 promoter sequence, TTGACA, is identical to the *E.coli* σ^{70} consensus –35 promoter element. The strongest sequence preference lies in the first three positions, while the fifth position showed the least sequence specificity (Fig. 4). Our results obtained with *E.coli* RNA polymerase were very similar, although not identical. Both the optimal chlamydial and *E.coli* –35 promoter sequences that we derived strongly resemble the *E.coli* σ^{70} consensus –35 promoter element (15). Furthermore, the *E.coli* consensus –35 promoter element also shows greatest sequence conservation in the first three positions. These results, together with our previous definition of the –10 promoter element, demonstrate that there is strong conservation between the –35 and –10 promoter elements of *C.trachomatis* and other bacteria such as *E.coli* (15) and *Bacillus subtilis* (18).

Prior to this study, a major unresolved issue in the field of chlamydial transcription was the 'RNA polymerase sigma subunit paradox', as noted by Stephens (6). This paradox referred to the difficulty reconciling the strong sequence conservation between the RNA polymerases of *Chlamydia* and *E.coli*, and the lack of consensus homology between chlamydial and *E.coli* promoters. In fact, it has not been possible to determine a consensus chlamydial –35 promoter sequence (reviewed in 4,5). Our studies show that the optimal promoter sequence recognized by chlamydial and *E.coli* RNA polymerases is actually very similar, which agrees with the amino acid conservation in the subregions of the RNA polymerase σ subunit that have been shown to recognize promoter elements (16,17). It has been proposed that additional regions of chlamydial σ^{66} outside these promoter recognition domains may have a role in promoter recognition (12). Our results support the idea that the paradox can be explained at the promoter level, and that there is a lack of sequence conservation among chlamydial promoters because many have a suboptimal promoter sequence, particularly in the –35 element (4,10).

One possible explanation for a suboptimal promoter structure is that these promoters are weak by design so that they can be regulated by transcription factors acting via *cis*-acting regulatory sequences (4,10,19). Douglas and Hatch have raised the possibility that sequences located in the spacer region between the promoter elements of the *C.trachomatis* MOMP P2 promoter are required for high level transcription (4,8). We have defined a *cis*-acting element in the spacer region of the *C.trachomatis* rRNA promoter that we have

called the spacer A/T region because of its location and a sequence preference for A and T residues (10). Spacer A/T regions have also been identified in other chlamydial promoters (19) that we now recognize as having suboptimal –35 promoter elements. We do not know if the Spacer A/T region has an intrinsic ability to increase promoter activity, or whether it serves as a binding site for a positive-acting factor.

An intrinsically weak rRNA promoter with a suboptimal promoter structure is unusual among bacteria. In *E.coli*, the rRNA promoter closely resembles the consensus promoter sequence and is a strong promoter (20). rRNA is transcribed at high levels so that the ribosomal translational machinery is available for bacterial growth. A weak rRNA promoter that can be upregulated would be more consistent with an organism such as *Chlamydia*, where there are distinct phases in the developmental cycle, with a metabolically inactive elementary body and a metabolically active reticulate body. Chlamydial rRNA transcription is first detectable at 7 h after infection of a host cell, at about the time the elementary body is converting into a reticulate body (21).

With the definition of the optimal *C.trachomatis* promoter sequence, we are also able to identify chlamydial promoters that are likely to be strong promoters because they are predicted to have optimal promoter sequences. The promoter for the *omcB* gene (22), which encodes the 60 kDa cysteine-rich protein, is predicted to be a strong promoter, with good –35 and –10 promoter elements. It also contains a spacer A/T region that has been shown to increase its transcription (19). The *dnaK* and PCT promoters (23) are also predicted to be strong promoters, but they do not contain an identifiable spacer A/T region. The –35 and –10 elements of the *dnaK* promoter are well conserved between chlamydial strains, including *C.trachomatis* (mouse pneumonitis strain, and serovars D and L2) and *Chlamydia pneumoniae* (C. S. Schaumburg and M. Tan, unpublished observations).

We have defined the optimal –35 promoter sequence recognized by the main form of *C.trachomatis* RNA polymerase without having had to derive a consensus promoter sequence, which would have required the definition of multiple promoter structures. Using similar methods, we have also derived the optimal –35 promoter sequence that is recognized by *E.coli* RNA polymerase, and the strong resemblance to the *E.coli* σ^{70} consensus promoter provides validation for this approach.

ACKNOWLEDGEMENTS

We would like to thank Dr Bert Semler, Allen Chen, Adam Wilson and Hilda Hiu Yin Yu for their valued suggestions and support. This work was supported by a grant from the NIH (AI 44198).

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