DNA structure and novel amino and carboxyl termini of the *Chlamydia* σ^{70} analogue modulate promoter recognition

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Genes from the eubacterium *Chlamydia* typically do not share promoter consensus sequences with those of *Escherichia coli* and are not expressed when cloned in *E. coli*; nevertheless, the major σ -subunit identified from *Chlamydia trachomatis* has nearly identical amino acid sequence to *E. coli* σ^{70} in regions that contact DNA. Following expression of the chlamydial σ -subunit gene in *E. coli*, expression was specifically initiated from chlamydial promoter regions. Selective recognition of chlamydial promoters by holoenzyme was dependent upon the structure of the promoter DNA coupled with novel amino- and carboxyl-terminal extensions of the chlamydial σ -subunit.

Keywords: *Chlamydia*, transcription, sigma factors, gene regulation

INTRODUCTION

DNA transcription in prokaryotes is initiated by RNA polymerase core enzyme with subunit structure $\alpha 2\beta\beta'$ and one of the multiple species of σ subunit (Burgess, 1976; Yura & Ishihama, 1979). The interaction of different σ subunits with core enzyme provides RNA polymerase with the ability to selectively recognize different promoter sequences (Ishihama, 1988). The majority of known σ factors belong to the family whose members are evolutionarily related to the *Escherichia coli* major σ factor, σ^{70} . The σ^{70} family is comprised of proteins with strong sequence identity and has been subdivided into two groups: (1) the major σ factors required for vegetative growth of the bacterial cell, and (2) the minor σ factors which act in transcriptional response to changing environmental conditions or physiological states (Stragier *et al.*, 1985; Gribskov & Burgess, 1986; Helmann & Chamberlin, 1988; Lonetto *et al.*, 1992). The *Chlamydia trachomatis* σ^{70} analogue, σ^{66} , shows high sequence identity with the major σ factors of other eubacteria, especially in the regions responsible for interacting with the -10 and -35 hexamers of the consensus promoter (Engel & Ganem,

1990; Koehler *et al.*, 1990; Lonetto *et al.*, 1992), yet DNA sequences upstream of chlamydial transcription start sites rarely contain consensus promoters (Engel & Ganem, 1987; Stephens *et al.*, 1988). These evaluations are consistent with the general inability of chlamydial sequences to efficiently complement *E. coli* genes, suggesting that chlamydial transcription mechanisms have significantly diverged from those of other eubacteria (Stephens *et al.*, 1988).

Chlamydiae are phylogenetically unique eubacteria because of their unusual developmental cycle, and because of their obligate intracellular growth they have had limited opportunities to exchange information with other bacterial genera (Wagar *et al.*, 1995). Understanding the activities of σ^{70} -analogues from atypical micro-organisms offers insights into σ function as these represent natural mutants of functional subunits that have diverged by evolutionary design. The intracellular developmental cycle of chlamydiae involves the inter-conversion between the infectious spore-like elementary body (EB) and the larger vegetative reticulate body (RB). The EB enters the host eukaryotic cell and converts into the RB, which multiplies by binary fission until the latter stages of infection when RBs differentiate to reform EBs (Moulder, 1991). Chlamydial differentiation is transcriptionally regulated primarily by the expression of a family of genes late in the developmental cycle (Stephens *et al.*, 1988).

Using an *in vitro* transcription system developed for

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Abbreviations: EB, elementary body; RB, reticulate body; CAT, chloramphenicol acetyltransferase.

Chlamydia it has been shown that chlamydial RNA polymerase containing σ^{66} is capable of initiating transcription from both *E. coli* consensus and non-consensus promoter sequences (Mathews *et al.*, 1993; Douglas *et al.*, 1994). The ability of chlamydial RNA polymerase containing σ^{66} to accommodate promoter sequence variation (Mathews & Sriprakash, 1994) suggests that cognate promoter sequences may not play dominating roles in chlamydial gene transcription and regulation, as is the case for most other bacteria. Investigation of chlamydial gene expression by *in vitro* transcription is technically demanding and has some application limits since the RNA polymerase has not been purified from other chlamydial regulatory proteins. In addition, *in vitro* investigations of gene transcription may not provide similar data when studied *in vivo* (Dorman, 1995).

A complementary approach to *in vitro* transcription was sought to investigate chlamydial transcription mechanisms and several considerations led us to use *E. coli* as a host to test the feasibility of generating a functional hybrid holoenzyme to study chlamydial gene expression. These factors included: (1) the inability to undertake direct genetic investigations in *C. trachomatis*, (2) the high amino acid identity of the α , β and β' RNA polymerase subunits of *C. trachomatis* with other eubacterial core RNA polymerase subunits (Engel *et al.*, 1990; Gu *et al.*, 1995), (3) the significant sequence similarity σ^{66} shows with other σ factors in regions of common function (Lonetto *et al.*, 1992), and (4) studies have demonstrated functional hybrid holoenzyme reconstitution *in vivo* involving complementation of σ factors from different species of bacteria with *E. coli* core enzyme (Shorenstein & Losick, 1973; Achberger & Whiteley, 1980; Chen & Helmann, 1992; Schurr *et al.*, 1994).

A heterologous gene expression system was developed based upon expression of *C. trachomatis* σ^{66} in *E. coli* and measuring activity initiated by σ^{66} hybrid holoenzyme from chlamydial DNA sequences cloned upstream of a reporter chloramphenicol acetyltransferase (CAT) gene. The upstream sequence of the *C. trachomatis* major outer-membrane protein gene (*ompA*) was chosen as a candidate for the heterologous gene expression system because it has two developmentally regulated transcripts potentially mediated by tandemly arranged promoters, P1 and P2, upstream from the structural gene (Stephens *et al.*, 1988). The expression of OmpA from transcript P1 is constitutive, and expression from transcript P2 only occurs late in chlamydial development. Alternatively, it has been proposed that transcription is mediated solely from the P2 promoter and the P1 transcript results from processing of the P2 transcript (Douglas & Hatch, 1995). Results from this investigation provide evidence that both *ompA* promoters, P1 and P2, are templates for RNA polymerase containing σ^{66} and that the context of DNA flanking these promoters, as well as the topology of the region, regulate transcriptional activity. Significantly, the ability of this σ subunit to initiate tran-

scription is dependent upon the unique amino- and carboxyl-terminal extensions of *C. trachomatis* σ^{66} .

METHODS

Plasmid constructions. Restriction endonucleases, DNA ligase and *Taq* polymerase were purchased from Boehringer Mannheim. PCR mixtures (100 μ l) contained 10 pmol each of forward and reverse primers (listed in Table 1), 10 nmol of *C. trachomatis* 434/L2 DNA, 200 μ mol of each dNTP and 2 U *Taq* polymerase. DNA was amplified in 35 cycles of 1 min at 94 °C, 2 min at 56 °C and 2 min at 72 °C, followed by a final 10 min extension at 72 °C. The PCR products were extracted using phenol/chloroform (1:1, v/v) and precipitated in ethanol (100%) before restriction endonuclease digestion. pS66, pS66 Δ 5 and pS66 Δ 3 were generated by cloning *Nde*I/*Bam*HI-restricted PCR products (primer pairs listed in Table 1) into pET3a (Novagen) similarly restricted. pS66 was sequenced, and pS66 Δ 5 and pS66 Δ 3 confirmed, by restriction endonuclease mapping using 4 bp restriction enzymes *Sau*3A1, *Alu*I and *Hae*III.

pPCR-L was constructed by annealing oligonucleotides MBTA-5 and MBTA-3 and cloning the resultant synthetic linker into *Bam*HI and *Sall* sites of pPCR1000 (Stratagene). pPCR-P1P2 was generated by amplifying the 381 bp of the upstream region of *ompA* with primers P2R5' and P1R3', restricting with *Bam*HI and *Sall*, and ligating into pPCR1000. Plasmids pACYC-L and pACYC-P1P2 were produced by replacing the 635 bp *Eco*RI/*Xmn*I fragment of pACYC184 with the 240 bp and 601bp *Eco*RI/*Xmn*I restriction endonuclease fragments from pPCR-L and pPCR-P1P2, respectively. pACYC-L cleaved with *Xho*I and *Kpn*I was used as a vector to construct all other pACYC-derived recombinant plasmids. To produce negative (pACYC-LR) and positive (pACYC-ECP) control plasmids, linkers containing *Xho*I and *Kpn*I restriction endonuclease sites were annealed with the oligonucleotide pairs listed in Table 1 as previously described (Lathe *et al.*, 1984). pACYC-LR lacks an *E. coli* promoter adjacent to the CAT gene and pACYC-ECP was designed to contain an *E. coli* promoter to optimally express CAT. The remaining pACYC-based clones were constructed using insert DNA generated by PCR amplification using oligonucleotide primers shown in Table 1. All pACYC-derived clones were confirmed by sequencing 1 μ g plasmid DNA using Sequenase version 2.0 (United States Biochemical) and [³⁵S]dATP α S (NEN Dupont).

Bacterial strains. *E. coli* DH10 α (Promega) was used for recombinant cloning. Bacterial strains for CAT extracts used *E. coli* host BL21(DE3) (Studier *et al.*, 1990) transformed with different combinations of plasmids. BL21(DE3) was initially transformed with the pET3a-derived plasmid, expression of the recombinant sequences was confirmed, and cells were subsequently transformed with the pACYC-derived plasmid. *E. coli* strain BL21 lacking the DE3 prophage was used in control experiments.

***E. coli* CAT extracts and assays.** Extracts of *E. coli* were made to measure CAT activity by inoculating 5.0 ml of media containing antibiotics (50 μ g carbenicillin ml⁻¹ for pET3a-derived plasmids and 12.5 μ g tetracycline ml⁻¹ for pACYC-derived plasmids) with overnight cultures of recombinant *E. coli*. Cultures were incubated with shaking for 2 h at 37 °C until the OD₆₀₀ reached 0.5–0.7, then IPTG was added to 1 mM and bacterial growth continued for 1 h. Chloramphenicol was then added to a final concentration of 30 μ g ml⁻¹, and growth continued for a final 30 min. In experiments where

Table 1. Oligonucleotides used for plasmid constructions

Oligonucleotide designation	Sequence*	Plasmid constructions
S66E5†	5'GGTggatccGGCCATATGCGCATGGATACGCTA§	pS66, pS66Δ5
S66L3‡	5'GCAggatccATTTTTATAACTTTTAATCTTAC§	pS66, pS66Δ3
5-S66/11†	5'GCTGgatcccatatgCAAGAAGAAGAAATCCAA§	pS66Δ3
3-S66/555‡	5'TTCggatccTAGATCTAGGAACGCTCG§	pS66Δ5
P2R5'†	5'aaggggtaccgaagggtttctcgagCATTCTCAACAATCAACATA	pACYC-P1P2, pACYC-P2X, pACYC-P2b
P2R3'‡	5'aaggggtaccCCATAACAAAATTTTC	pACYC-P2X, pACYC-P270X, pACYC-P250X, pACYC-P230X, pACYC-P210X
P1R5'†	5'aaggggtaccgaagggtttctcgagCATTGAACGACATGTTC	pACYC-P1
P1R3'‡	5'aaggggtaccTCTTACCTCTAAATTGTA	pACYC-P1P2, pACYC-P1, pACYC-P170, pACYC-P150, pACYC-P130, pACYC-P110
5'P2-70†	5'AACActcgagCAATATGGCTTTT	pACYC-P270X, pACYC-P270
5'P2-50†	5'TTTGctcgagGTTTCAGAGCGATTTT	pACYC-P250X, pACYC-P250
5'P2-30†	5'GATTctcgagGCAAAAACAAGAACAT	pACYC-P230X, pACYC-P230
5'P2-10†	5'GAACctcgagCATAAAAAGATATACA	pACYC-P210X, pACYC-P210
5'P1-70†	5'TAAGctcgagTTTACTTGCAAGACAT	pACYC-P170
5'P1-50†	5'AGACctcgagCAGGCCATTAATTGCT	pACYC-P150
5'P1-30†	5'ATTGctcgagGACATCTTGTCTGGCT	pACYC-P130
5'P1-10†	5'CTGGctcgagCTAGGACGCGAGTGCCG	pACYC-P110
3'P2-25‡	5'CGTTgtgaccAGGAAGAAGCCCTTAA	pACYC-P2b, pACYC-P270, pACYC-P250, pACYC-P230, pACYC-P210
MBTA-5	5'gatccggtacccccctcgaggaattaattcg¶	pACYC-L
MBTA-3	5'tcgacgaattaattcctcgaggggggtaccg¶	pACYC-L
5-KX/1	5'tcgactgcagggatccccctcgagtgtac	pACYC-LR
3-KX/1	5'actcgagggggatccctcgac	pACYC-LR
MB25-5	5'tcgagttgacaattagttactattgttataatgtattcgatac	pACYC-ECP
MB25-3	5'cggaatacatataacaatagttaactaattgtcaac	pACYC-ECP

* Upper case letters indicate *C. trachomatis* sequences and lower case letters represent restriction sites used for cloning and generating the polylinker or synthetic consensus promoter.

† and ‡ indicate forward and reverse primers, respectively, used to generate PCR products for cloning.

§ *C. trachomatis* σ^{66} nucleotide sequence given by Koehler *et al.* (1990).

|| *C. trachomatis* *omp1* upstream sequence given by Stephens *et al.* (1988).

¶ Insert cloned into pPCR1000 and subsequently shuttled to produce pACYC-L.

coumermycin A₁ (Sigma) was included, either 1.0 μ g or 25 μ g coumermycin A₁ ml⁻¹ was added prior to IPTG induction. Aliquots (2.0 ml) of induced *E. coli* cultures were chilled on ice and centrifuged at 10000 *g* at 4 °C for 5 min. The pelleted cells were washed with 2.0 ml ice-cold 0.1 M Tris/HCl (pH 7.8) and the cells resuspended in 350 μ l 0.1 M Tris/HCl (pH 7.8) before sonication for 20 s using a probe sonicator. CAT extracts were either used immediately or stored at -20 °C. Protein concentration of CAT extracts was determined by a protein mini-assay (Bio-Rad) and 2 μ g protein was used for CAT assays as previously described (Neumann *et al.*, 1987; Sankaran, 1992) with 0.5 μ Ci (18.5 kBq) [³H]acetyl-CoA (CAT assay grade, NEN Dupont).

Immunoblot analysis. Cell lysates for protein gels and immunoblot analyses were prepared by centrifugation (10000 *g*, 2 min) of 1.0 ml induced cells and resuspending the pellet in 200 μ l 1 × SDS-PAGE sample buffer containing 5% β -mercaptoethanol and 2% SDS. Samples were boiled for 5 min before 15 μ l aliquots were loaded and proteins separated on

10% SDS-polyacrylamide gels. Following electrophoresis, the proteins were transferred to nitrocellulose membrane (Schleicher & Schuell), incubated with mAb 2G10 (Strickland *et al.*, 1988) and immune reactions detected as previously described (Koehler *et al.*, 1990).

RESULTS

Chlamydial-specific gene expression in *E. coli*

A system to study the gene expression of *C. trachomatis* promoter sequences was designed by co-transforming *E. coli* strain BL21(DE3) with pET3a (Studier *et al.*, 1990) containing the *C. trachomatis* σ^{66} gene (pS66) and a second plasmid containing putative promoter sequences cloned upstream of a CAT reporter gene. The pET3a vector contains the ColE1 origin of replication and ampicillin resistance gene whilst the CAT reporter plasmid, pACYC184 (Chang & Cohen, 1978), carries

Fig. 1. (a) Schematic representation of the *C. trachomatis ompA* upstream sequence including -35 and -10 hexamers with spacing (bp) of promoters P1 and P2, transcription initiation sites (+), initiation codon (ATG), *XhoI* site and inverted repeat (arrows) (sequence numbers according to Stephens *et al.*, 1988). Below the upstream sequence is a representation of the sequences cloned into pACYC-L to produce pP1P2 (P1P2), pP1 (P1) and pP2X (P2X). (b) Immunoblot of protein extracts from IPTG-induced *E. coli* detected with mAb 2G10. Lanes 1–10: extracts of *E. coli* BL21(DE3) containing no plasmids (lane 1), or plasmids pET3a (lane 2), pS66 (lane 3), p184 (lane 4), pLR (lane 5), pP2X (lane 6), pET3a and pLR (lane 7), pS66 and pLR (lane 8), pET3a and pP2X (lane 9), or pS66 and pP2X (lane 10). Lane 11 contains an extract from *E. coli* BL21 containing plasmids pS66 and pP2X in the absence of T7 polymerase. (c) CAT activity from the IPTG-induced *E. coli* extracts (bacterial strains in same order as numbers 1–11 in b). Values for specific activity of CAT are from duplicate assays (less than 10% deviation) and represent at least three assays performed on separate occasions. (d) CAT activity from BL21(DE3) containing either pP1P2, pP1 or pP2X and either pET3a (open bars) or pS66 (solid bars). Values are from duplicate assays (less than 10% deviation) and represent the results of at least three assays performed on separate occasions.

genes for both chloramphenicol and tetracycline resistance and the origin of replication from p15A, enabling it to co-exist with vectors carrying the ColE1 origin. The *E. coli* promoter, which controls the CAT gene of pACYC184, was replaced by restriction endonuclease site linker sequences, resulting in the plasmids pACYC-L and pACYC-LR (see Table 1). pACYC-L was used as the cloning vector to deliver promoter sequences upstream of the CAT gene. pACYC-LR contains a short sequence that disrupts the pACYC-L cloning site and was used in most experiments as the negative control. *E. coli* strain BL21(DE3) contains a copy of the T7 RNA polymerase gene under *lac* control on a defective λ prophage (DE3), where induction of T7 RNA polymerase by IPTG initiates σ^{66} expression from pS66. If σ^{66} forms a functional hybrid holoenzyme with *E. coli* core RNA polymerase then the result is gene expression from *C. trachomatis*-specific promoter sequences, resulting in increased CAT activity.

E. coli BL21(DE3) was transformed with either pET3a without the σ^{66} gene or pET3a containing the σ^{66} gene (pS66), and each were then transformed with pACYC containing the following modifications: p184 (original *E. coli* promoter-CAT expression plasmid), pLR (promoterless CAT reporter vector) or pP2X [*C. trachomatis ompA* P2 promoter region (-353 bp to -138 bp; Fig. 1a)]. In addition, plasmids pS66 and pP2X co-transformed into *E. coli* BL21 (DE3⁻ background) were used as controls for the system in the absence of T7 RNA polymerase expression of σ^{66} . Fig. 1(b) shows immunoblot detection of *C. trachomatis* σ^{66} present in extracts of IPTG-induced cultures using an anti- σ^{70} mAb 2G10 (Strickland *et al.*, 1988) that reacts with σ^{66} (Koehler *et al.*, 1990). σ^{66} was expressed in BL21(DE3) strains containing pS66 (lanes 3, 8 and 10) and not produced when either the σ^{66} gene was absent (lanes 1, 2, 4, 5, 6, 7 and 9) or the T7 RNA-polymerase-containing DE3 prophage was absent (lane 11). The

Fig. 2. (a) Schematic representation of the *C. trachomatis ompA* upstream sequence surrounding promoter P1 with the segment of DNA cloned to produce promoter plasmids pP110, pP130, pP150, pP170 and pP1 indicated underneath (abbreviations as in Fig. 1). (b) σ^{66} -induced CAT activity from BL21(DE3) containing *C. trachomatis ompA* P1 promoter plasmids as described in (a). Net σ^{66} -induced CAT activity was determined by subtracting the CAT activity of BL21(DE3) containing the promoter plasmid with pET3a from the CAT activity of BL21(DE3) containing the promoter plasmid with pS66. Values are from duplicate assays (less than 10% deviation) and represent the results of at least three assays performed on separate occasions.

CAT activity from the induced *E. coli* strains shown in Fig. 1(b) was determined (Fig. 1c). CAT was expressed from p184 (containing an *E. coli* σ^{70} promoter cognate sequence) by *E. coli* RNA polymerase, but not from the control plasmid pLR. CAT expression from p184 was not affected following expression of σ^{66} (data not shown). Thus expression of σ^{66} did not significantly interfere with plasmid gene expression by σ^{70} . σ^{66} (pS66) was required for CAT expression from pP2X (lane 10) whereas CAT was not expressed in *E. coli* containing pP2X without T7 polymerase (lane 11) nor in *E. coli* containing T7 polymerase (pET3a) without pS66 (lanes 6 and 9). Moreover, σ^{66} did not direct CAT expression from pLR, which lacks a *C. trachomatis* promoter (lane 8). These results show that *C. trachomatis* σ^{66} is capable of forming a functional hybrid holoenzyme with *E. coli* core RNA polymerase and directing *C. trachomatis*-specific transcription from the *ompA* promoter, P2.

The *ompA* putative constitutive promoter region, P1 (sequence -138 to +28; Fig. 1a) in plasmid pACYC (pP1) and the entire upstream sequence containing both promoters P1 and P2 of *ompA* (-353 to +28 in plasmid pP1P2) were tested in the heterologous expression system and compared to pP2X expression. Fig. 1(d) shows that pP1 produced expression preferentially by *E. coli* expressing σ^{66} (pS66), although a background of CAT expression without σ^{66} was observed. In contrast, CAT expression was observed from pP1P2 regardless of σ^{66} induction. No obvious *E. coli* consensus promoter was identified in the sequence generated by cloning the *ompA* P1P2 sequence into pACYC, but the AT-rich character may account for fortuitous expression. T7-polymerase-induced expression from pP1P2 was ruled out by testing this construct in a DE3⁻ background and no significant change in CAT activity was found (data not shown). Although endogenous *E. coli* RNA polymerase holoenzyme initiated some limited transcription from the *ompA* sequence contained within pP1, the

heterologous system resulted in apparently *Chlamydia*-specific transcription from both *ompA* promoter regions P1 and P2 in *E. coli* expressing σ^{66} .

P1 promoter region length-dependent transcription by σ^{66}

Having determined that the *ompA* promoter P1 region served as a template for σ^{66} , the promoter region sequence was truncated to identify the minimal *C. trachomatis* sequence required for σ^{66} -mediated transcription. Recombinant pACYC clones containing 20 bp incremental length differences of *C. trachomatis* P1 sequence (Fig. 2a) were tested in the heterologous expression system. Following induction of σ^{66} , the net expression of CAT from P1 region clones was incrementally reduced with step-wise removal of P1 region DNA (Fig. 2b). A minimum of 50 bp upstream of the putative -35 hexamer was required to detect σ^{66} -specific CAT expression (Fig. 2a; P150). A net negative effect on CAT expression from plasmids pP110 and pP130 was observed in the presence of σ^{66} perhaps due to endogenous *E. coli* σ factor(s) competing with σ^{66} for P1 region DNA.

Expression from *ompA* P2 promoter is sensitive to DNA context

Analogous experiments to those designed for the *ompA* P1 promoter region were engineered for the *ompA* P2 transcript region. P2 clones identical in context to the truncated P1 sequences were produced (Fig. 3a) and tested for expression initiated by the presence of σ^{66} (Fig. 3b). The clone containing the smallest *ompA* P2 promoter sequence, pP210, was consistently responsive to σ^{66} , which suggests that only 10 bp upstream from the putative -35 hexamer was required for σ^{66} -containing holoenzyme recognition of *ompA* promoter P2. When an additional 20 or 40 bp were included upstream of the

Fig. 3. (a) Schematic representation of the *C. trachomatis ompA* upstream sequence surrounding promoter P2 with the segment of DNA cloned to produce promoter plasmids pP210, pP230, pP250, pP270, pP2b, pP210X, pP230X, pP250X, pP270X and pP2X indicated underneath (abbreviations as in Fig. 1). (b, c) σ^{66} -induced CAT activity from BL21(DE3) harbouring *C. trachomatis ompA* P2 promoter plasmids (b) without the inverted repeat and (c) with the inverted repeat, as shown in (a). σ^{66} -induced CAT activity was determined and represented as described in the legend to Fig. 2.

P2 promoter (clones pP230 and pP250) the ability of σ^{66} to promote transcription was lost. However, the addition of another 20 bp (clone pP270) completely restored σ^{66} -specific transcription, which was then lost when a final 20 bp was added (clone pP2b). The marked on/off expression dependent upon incremental changes in the size of P2, suggests that, unlike P1, σ^{66} recognition of P2 was strongly influenced by the 5'-proximal DNA sequence.

Given the effect of proximal DNA sequence on recognition of P2, the addition of sequence distal to P2 was evaluated as it appeared likely that the conserved inverted repeat found downstream from the *ompA* P2 promoter (Stephens *et al.*, 1988) could function to alter the context or topology of the P2 promoter. Clones containing the same *ompA* P2 5' sequences described above, but including the inverted repeat (sequences -198 to -144; Fig. 3a) were produced and evaluated. The resultant plasmids, pP210X, pP230X, pP250X, pP270X and pP2X, showed a notably different pattern of σ^{66} -initiated expression compared to the *ompA* P2 clones lacking the inverted repeat (Fig. 3c). Expression

from the σ^{66} -responsive *ompA* P2 promoter (pP210) was increased with the addition of the inverted repeat (pP210X), and the sequences contained within pP250 and pP2b, which were not responsive to σ^{66} , became strongly responsive following the addition of the inverted repeat region (clones pP250X and pP2X). In contrast, the addition of the inverted repeat region to the *ompA* P2 sequence in clone pP270 (pP270X) resulted in a loss of recognition by RNA polymerase containing σ^{66} . The presence of the inverted repeat downstream from the *ompA* P2 transcription start site changed the pattern of expression observed among the *ompA* P2 promoter clones. The results of transcription from the different *ompA* P2 promoters demonstrate that the context of the sequence surrounding the promoter affects the ability of σ^{66} to specifically initiate transcription. The formation of secondary structure by the inverted repeat could alter the architecture of the *ompA* promoter P2, thus affecting recognition by the σ^{66} RNA polymerase holoenzyme.

Amino- and carboxyl-terminal σ^{66} extensions alter expression from *ompA*

To determine if there is a role for the σ^{66} amino- and carboxyl-terminal extensions in transcription, pET3a recombinant plasmids containing the σ^{66} genes were truncated to encode the analogous amino- and carboxyl-terminal amino acids found in *E. coli* σ^{70} . Plasmid pS66 Δ 5 lacks the coding sequence for the first 14 amino acids of σ^{66} and contains a methionine start codon produced by cloning with *Nde*I. pS66 Δ 3 was designed by removing the last 16 codons of the σ^{66} ORF. Expression of the truncated σ^{66} isoforms from pS66 Δ 5 (Δ N- σ^{66}) and pS66 Δ 3 (Δ C- σ^{66}) was determined by immunoblot with mAb 2G10. The Δ N- σ^{66} and Δ C- σ^{66} proteins were smaller than the wild-type σ^{66} and their level of expression was the same as observed with σ^{66} relative to the amount of *E. coli* σ^{70} (Fig. 4a).

σ^{66} -responsive clones pP1 and pP2X were investigated for recognition by RNA polymerase containing the truncated σ^{66} proteins. Removal of the σ^{66} amino-terminal extension (Δ N- σ^{66}) did not affect CAT expression from clone pP1 whereas Δ C- σ^{66} partially decreased CAT expression from pP1 (Fig. 4b). In contrast, Δ N- σ^{66} and Δ C- σ^{66} were unable to initiate transcription from P2X. These data demonstrate that both the pS66 Δ 5 and pS66 Δ 3 gene products form functional hybrid holoenzymes with *E. coli* core RNA polymerase because of transcription from P1, but the changes in σ^{66} structure ablated recognition of the *ompA* promoter P2.

Given the exceptional role of the amino- and carboxyl-terminal extensions on σ^{66} recognition of the promoter region P2X, other σ^{66} -responsive P2 promoter region constructs were tested. Although Δ N- σ^{66} RNA polymerase holoenzyme failed to permit transcription from the chlamydial sequences in pP2X, pP270 and pP210, it mediated transcription from the sequences in pP250X and pP210X. In addition, Δ C- σ^{66} RNA polymerase did not function from any *ompA* P2 sequences, suggesting

Fig. 4. (a) Immunoblot of protein extracts from IPTG-induced *E. coli* detected with mAb 2G10. Lanes 1–4: extracts of *E. coli* BL21(DE3) containing plasmids pET3a (lane 1), pS66Δ5 (lane 2), pS66 (lane 3) and pS66Δ3 (lane 4). Arrow indicates the migration of σ^{70} and bracket includes ΔN - σ^{66} (lane 2) σ^{66} (lane 3) and ΔC - σ^{66} (lane 4) proteins. (b) σ^{66} - (black bars), ΔN - σ^{66} - (hatched bars) and ΔC - σ^{66} - (open bars) induced CAT activity from BL21(DE3) harbouring *C. trachomatis ompA* promoter plasmids. Values were determined by subtracting the CAT activity of BL21(DE3) containing the promoter plasmid with pET3a from the CAT activity of BL21(DE3) containing the promoter plasmid with the σ plasmid pS66, pS66D5 or pS66D3. Values are from duplicate assays (less than 10% deviation) and represent the results of at least three assays performed on separate occasions.

that the carboxyl-terminal extension of σ^{66} is essential for recognition or function from *ompA* promoter P2. It is interesting to note that ΔN - σ^{66} RNA polymerase functioned differently with the *ompA* P2 sequences contained within plasmids pP210X and pP210. Since the only difference between these plasmids was the presence (pP210X) or absence (pP210) of the inverted repeat, it appeared that the inverted repeat compensates for the absence of the σ^{66} amino-terminal extension. The failure of the inverted repeat to compensate for the deletion of the σ^{66} amino terminus in initiating transcription from pP2X could be due to A-tracts in the *C. trachomatis* upstream sequence (not found in pP210X) altering the promoter context. Results of CAT expression from bacterial strains harbouring either σ factor ΔN - σ^{66} or ΔC - σ^{66} and different σ^{66} -responsive *ompA* P2 clones, pP270, pP250X, pP210X and pP210, demonstrated that the σ^{66} amino- and carboxyl-terminal extensions are required for transcription and that they have independent functional roles in promoter recognition.

Superhelical density of DNA is important for σ^{66} -induced expression from *ompA* P2

The results presented above suggest that a combination of DNA sequence, DNA context and the σ^{66} amino- and carboxyl-terminal extensions direct σ^{66} -mediated ex-

pression from chlamydial *ompA* P2 sequences in *E. coli*. Since the DNA context could be altered by DNA topology that is introduced by the greater DNA superhelical density as chlamydiae differentiate into late-stage EBs (Solbrig *et al.*, 1990), this hypothesis was tested by evaluating the effect of changes in DNA topology from several *ompA* P2 sequences on CAT expression initiated by σ^{66} , ΔN - σ^{66} and ΔC - σ^{66} RNA polymerase holo-enzymes.

The eubacterial DNA gyrase inhibitor coumermycin A₁ was used to alter DNA superhelical density *in vivo* (Drlica & Franco, 1988). The drug was added to induce changes in DNA topology prior to σ^{66} induction. Initially, the effect of coumermycin A₁ treatment on *E. coli* RNA polymerase transcription was determined by assaying plasmids p184 and pECP, which contain different *E. coli* promoters upstream of the CAT reporter gene. Coumermycin A₁ treatment had little effect on CAT expression (Fig. 5a) indicating that *E. coli* RNA polymerase was not functionally impaired by the drug treatment. The effect of coumermycin A₁ treatment on plasmid topology and σ^{66} expression was assessed by Southern blot and immunoblot analysis. A slight reduction in supercoiled plasmid DNA was observed following treatment with 1 μ g coumermycin A₁ ml⁻¹, whereas 25 μ g coumermycin A₁ ml⁻¹ resulted in near complete relaxation of all supercoiled plasmid (data not shown). The level of expression of σ^{66} in coumermycin-A₁-treated cells was significantly lower than the amounts expressed in untreated cells; however, the amounts were nevertheless detectable by immunoblot and similar to the amount of σ^{70} detected (data not shown). The lower expression of σ^{66} may be the result of poor T7 RNA polymerase induction or inhibition of T7-polymerase-mediated transcription of σ^{66} . Regardless of the lower level of expression, σ^{66} -specific transcription was nevertheless detected (Fig. 5).

The effect of coumermycin A₁ treatment on expression from σ^{66} -responsive (pP2X, pP270) and non-responsive plasmids (pP2b and pP270X) was determined following induction of σ^{66} (Fig. 5b). Partial relaxation (1 μ g coumermycin A₁ ml⁻¹) of plasmid DNA halved σ^{66} -induced expression from pP2X, had no significant effect on expression from pP270, and had no effect on expression from pP2b or from pP270X. Total relaxation (25 μ g coumermycin A₁ ml⁻¹) of plasmid DNA eliminated σ^{66} -induced expression from plasmids pP2X and pP270 but marginally increased σ^{66} -induced expression from plasmids pP270X and pP2b. These results suggest that local DNA topology plays a role in σ^{66} -initiated expression from *ompA* promoter P2 upstream sequences.

The effect of different superhelical densities using either ΔN - σ^{66} and ΔC - σ^{66} was determined in *E. coli* containing σ^{66} -responsive plasmids pP2X or pP270 (Fig. 5c). ΔN - σ^{66} and ΔC - σ^{66} , which did not function from the *ompA* P2 promoter pP2X in a native supercoiled state, mediated transcription when the template was totally relaxed in the presence of 25 μ g coumermycin A₁ ml⁻¹ (Fig. 5c). A similar response was identified for ΔN - σ^{66} and ΔC - σ^{66}

Fig. 5. Effect of DNA relaxation on transcription. DNA relaxation was achieved in BL21(DE3) cultures by treating with either 0 μg (black bars), 1.0 μg (hatched bars) or 25 μg (open bars) coumermycin A₁ ml⁻¹. (a) Effect of DNA relaxation on transcription from *E. coli* consensus promoters in BL21(DE3) containing promoter pET3a and either pECp or p184. CAT activity is represented as described in the legend to Fig. 2(b). (b) Effect of DNA relaxation on σ^{66} -induced activity from promoter plasmids pP2X, pP270X, pP2b and pP270. σ^{66} -induced CAT activity is represented as described in the legend to Fig. 3. (c) Effect of DNA relaxation on σ^{66} , $\Delta\text{N-}\sigma^{66}$ and $\Delta\text{C-}\sigma^{66}$ activity from promoter plasmids pP2X and pP270. σ^{66} , $\Delta\text{N-}\sigma^{66}$ and $\Delta\text{C-}\sigma^{66}$ -induced CAT activity was determined by subtracting the CAT activity of BL21(DE3) containing the promoter plasmid with pET3a from the CAT activity of BL21(DE3) containing the promoter plasmid with the σ plasmid pS66, pS66D5 or pS66D3.

with *ompA* P2 promoter plasmid pP270 where $\Delta\text{N-}\sigma^{66}$ was more sensitive than $\Delta\text{C-}\sigma^{66}$ to relaxed template (Fig. 5c). In contrast, native σ^{66} did not transcribe from totally relaxed template. These results implicate the amino- and carboxyl-terminal extensions with recognition of DNA topology as their removal reduced the ability of the resultant σ factor to discriminate between supercoiled and relaxed template. Since plasmid pP2X contains the inverted repeat and pP270 does not, it can be concluded that the DNA structure caused by the inverted repeat alters the recognition of *ompA* P2 promoter sequences by $\Delta\text{N-}\sigma^{66}$.

DISCUSSION

Understanding the molecular basis of how chlamydiae transcriptionally regulate constitutive and developmental-stage-specific gene families has remained enigmatic. The upstream sequences of chlamydial genes share little homology with the prototypical consensus promoter and alignment of these sequences has failed to produce an alternative consensus promoter (Engel & Ganem, 1987; Stephens *et al.*, 1988). Based on the homology of the chlamydial RNA polymerase subunits α , β and β' to other eubacterial subunits (Engel *et al.*, 1990; Gu *et al.*, 1995), we hypothesized that the chlamydial σ^{70} analogue, σ^{66} , would be able to interact with the *E. coli* core RNA polymerase and initiate *Chlamydia*-specific transcription. Thus, an *in vivo* heterologous transcription system was developed in *E. coli* by expressing σ^{66} and assaying σ^{66} -dependent expression from chlamydial sequences cloned into a CAT reporter vector. Two classes of chlamydial promoters were transcriptionally active for the *C. trachomatis* major outer-membrane protein (MOMP) gene *ompA*. This is not unexpected because differential developmental-stage expression from *ompA* promoters

P1 and P2 in *C. trachomatis* is observed *in vivo* (Stephens *et al.*, 1988). However, only P2 has been shown to be functional in *in vitro* assays (Mathews *et al.*, 1993; Douglas *et al.*, 1994; Douglas & Hatch, 1995), and the developmentally earlier P1 transcript has been reported to be derived by processing and capping of the nascent P2 transcript (Douglas & Hatch, 1995). Why chlamydiae would process this transcript is unknown, and the molecular basis for complete processing of this transcript early in development and the production of equimolar amounts of each transcript coincident with the transcription of late-developmental stage-specific genes is unclear.

Expression from *ompA* promoter P1 was detected in this system and was found to be sensitive to sequence constraints but not structural constraints. Optimal σ^{66} -mediated transcription from promoter P1 in *E. coli* required sequences greater than 50 bp upstream of the -35 hexamer (85 bp from the transcription start site). The unusually long 5' sequence for P1 transcription by σ^{66} and the observation of a wide tolerance of σ^{66} for specific promoter sequences determined by *in vitro* transcription assays (Mathews & Sriprakash, 1994) suggest that σ^{66} can interact with this chlamydial promoter region DNA at multiple sites. The lack of clear fidelity related to cognate promoter sequence recognition of the P1 region, especially with background expression without σ^{66} , makes it difficult to confidently conclude that the P1 region is an authentic promoter.

The pattern of expression from the *ompA* promoter P2 clones, containing different amounts of upstream sequence, contrasted to that observed for promoter P1 wherein promoter P2 was not progressively sensitive to sequence limitations but sensitive to structural constraints. σ^{66} -induced expression from *ompA* promoter P2 was differentially affected by the content of upstream

and downstream sequence. For example, since pP210 was responsive to σ^{66} it was surprising that the inclusion of either 20 bp or 40 bp of chlamydial sequence upstream of *ompA* promoter P2 prevented σ^{66} -mediated transcription. To add to the intrigue, σ^{66} responsiveness was re-established with the addition of a further 20 bp and lost when a final 20 bp of chlamydial sequence was included upstream of *ompA* promoter P2. The observed periodic effect that permits or limits transcription is reminiscent of phase-dependent transcription initiation. A different pattern of σ^{66} -induced expression emerged when the inverted repeat was present downstream of the *ompA* P2 transcription start site. The secondary structure caused by the inverted repeat has the potential to alter the DNA context surrounding the promoter and affect σ^{66} recognition from the same sequences. Whilst the inverted repeat was not necessary for transcription from promoter P2, it may play a role in modulating transcription from the *ompA* promoter P2 as promoters were differentially recognized by σ^{66} by inclusion of the inverted repeat (compare promoters P250, P270, P2b versus P250X, P270X and P2X).

The effects of DNA context on σ^{66} promoter recognition can be considered by examining the DNA surrounding the *ompA* promoter P2 sequence. The 130 bp sequence immediately upstream of the *ompA* P2 transcription initiation site is 66% (A+T)-rich and contains five tracts of at least four nucleotides of A or T residues, including a seven nucleotide T-tract. A-tracts are capable of inducing intrinsic conformational changes in the DNA that can substitute the requirement of specific DNA-binding proteins, such as the integration host factor of *E. coli* (Molina-Lopez *et al.*, 1994; Perez-Martin *et al.*, 1994) and even act as transcriptional activators when found in helical array (Nickerson & Achberger, 1995). Thus, it seems reasonable to consider that the sequence upstream of *ompA* promoter P2 may facilitate a specific DNA conformation which provides an alternative to activator-induced gene expression for *Chlamydia*. Consequently, removal of upstream sequences that change the (A+T)-rich character and specific alignment of A- or T-tracts within the upstream region of *ompA* promoter P2 may affect the DNA structure required for σ^{66} to recognize the promoter resulting in the 'on/off' pattern of expression observed for the 20 bp truncated *ompA* P2 clones.

Amino- and carboxyl-terminal structural motifs of σ^{66} displayed different roles in transcription since the amino-terminal truncation had no effect on expression from promoter P1 (clone pP1) but destroyed the ability of σ^{66} to recognize promoter P2 (clone pP2X). Similarly, the carboxyl-terminal truncation only partially reduced the ability of σ^{66} to promote transcription from P1 but abolished activity from P2. Moreover, evaluation of transcription from some of the truncated P2 promoter constructs suggested that the amino- and carboxyl-terminal extensions operate independently. These results can be interpreted as indicating that the truncated σ^{66} proteins are able to form a functional holoenzyme with *E. coli* core RNA polymerase. The differential

effect observed between the *ompA* P2 promoter constructs and the truncated σ^{66} holoenzymes is probably due to the DNA context within which the chlamydial promoter is embedded.

Since the *ompA* P2 promoter was sensitive to changes in DNA context, the effect of changing the superhelical density of the template *in vivo* was investigated. The assays using a DNA gyrase inhibitor suggested that the topology of the *ompA* P2 template DNA defined the ability of σ^{66} to initiate transcription. The carboxyl-truncated σ^{66} was able to initiate transcription when the template was completely relaxed by coumermycin A₁ treatment suggesting that the σ^{66} carboxyl-terminal extension functions to restrict RNA polymerase recognition of supercoiled templates. In contrast, the amino-terminal-truncated σ^{66} recognition of *ompA* P2 promoters with different superhelical density relied upon the presence of the inverted repeat. Thus it can be concluded that the σ^{66} amino-terminal extension has a role in controlling promoter recognition from DNA with specific topological architecture. Another bacterium whose major σ subunit contains a conspicuous additional 20 amino acids at the amino terminus is *Caulobacter crescentus* (Malakooti & Ely, 1995). Like chlamydiae, *Caulobacter crescentus* promoters are not recognized by *E. coli* RNA polymerase and do not share consensus promoter sequences with *E. coli* (Malakooti *et al.*, 1995). Also like *Chlamydia* (Mathews & Sriprakash, 1994), the *Caulobacter* σ factor also has a broader specificity for promoter recognition than σ^{70} (Malakooti & Ely, 1995).

Our findings suggest chlamydiae have adapted novel sequence modifications of the σ subunit as a mechanism of regulated gene transcription coupled to their differentiation. Such a mechanism of promoter recognition and altered transcription based upon the superhelical structure of the target DNA is consistent with another attribute unique to chlamydiae – the extraordinary superhelical density of chlamydial DNA that is induced late in the developmental cycle (Solbrig *et al.*, 1990). The hallmark of chlamydial developmental differentiation is marked by progressive condensation of the chromosome (Costerton *et al.*, 1976). This has been shown to be mediated by specific late-developmental-stage-specific proteins (Wagar & Stephens, 1988). It has also been shown that late-stage chlamydial DNA is unusually highly supercoiled and this is amplified and stabilized by these DNA-binding proteins (Solbrig *et al.*, 1990). The proteins that mediate these unusual interactions are apparently homologous to eukaryotic histone H1 (Tao *et al.*, 1991; Barry *et al.*, 1992; Perara *et al.*, 1992). It may be more than coincidental that *Caulobacter crescentus* also has a developmental cycle punctuated by significant changes in nucleoid structure mediated by M_r 26000 proteins (Evinger & Agabian, 1979).

We speculate that the induction of increased supercoiling of chlamydial DNA late in the developmental cycle and the fundamental property of progressive DNA

compaction provide a substrate for σ^{66} to additionally recognize late-developmental-stage-specific promoter regions that are locally and differentially affected by the superhelical state of the DNA. Thus a single σ subunit has the capacity to initiate transcription as a primary molecular mechanism of global gene regulation independent of additional *Chlamydia*-specific transcription factors. Consistent with this proposal is the fact that late in the developmental cycle approximately equal amounts of each of the two *ompA* mRNAs are present, suggesting simultaneous transcription from both P2 and P1 (Stephens *et al.*, 1988). Using *in vitro* assays, Douglas & Hatch (1995) did not detect transcription from the P1 promoter and provided evidence that the constitutive P1 transcript may arise following RNA processing of the P2 transcript. Nevertheless, P1 region DNA was capable of initiating transcription by σ^{66} in this *E. coli* model. If these *in vivo*-modelled analyses reflect the natural biology of chlamydiae, constitutive genes are transcribed by initiation with σ^{66} , and coordinate linkage of developmental-stage-specific gene transcription and condensation of the chromosome may represent a novel strategy for developmental-cycle-dependent gene regulation. Alternatively, if P1 transcripts solely arise by RNA processing in chlamydiae, the P2 promoter region may be representative of a constitutive promoter. As transcription can significantly contribute to an increased supercoiled state (Wang & Lynch, 1993), the cumulative effects of transcription itself may be the primary developmental clock that signals chlamydial differentiation. The ability of σ^{66} to initiate *Chlamydia*-specific transcription in *E. coli* provides a new, complementary approach in the study of chlamydial gene expression since the inability to stably transform *Chlamydia* and difficulty in isolating and purifying chlamydial RNA polymerase has greatly hindered genetic investigations of the organism. This model should have application for evaluating the regulatory roles of alternative sigma subunits represented by gene orthologues for σ^{28} and σ^{54} recently identified in the *C. trachomatis* genome <http://chlamydia-www.berkeley.edu:4231>.

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