The CrP operon of *Chlamydia psittaci* and *Chlamydia pneumoniae*

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One of the critical developmental events during the unique intracellular life cycle of Chlamydiae is their differentiation from a metabolically active, replicative form or reticulate body (RB) to an infectious extracellular form of the organism (elementary body or EB). This process is characterized by the expression of two extraordinarily cysteine-rich envelope proteins of molecular masses 9 kDa and 60 kDa. We describe the molecular cloning and sequence determination of the 9 kDa cysteine-rich proteins (CrPs) of *C. pneumoniae* and *C. psittaci*. Comparison of these 9 kDa CrP amino acid sequences with those of *C. trachomatis* showed regions of structural variation and conservation. Transcription of the 9 kDa CrP genes occurred as both a monocistronic message and as a bicistronic message which included the 60 kDa CrP gene. Transcription of the 9 kDa and 60 kDa CrP genes was tightly linked to the chlamydial growth cycle with synthesis of their mRNAs and consequent translation of the 60 kDa CrPs occurring as RBs differentiated to form EBs. The maximal rate of transcription occurred late in the growth cycle from a single but highly conserved promoter which had close similarity with the *Escherichia coli* consensus promoter sequences. A stem and loop structure which could be involved in regulating translation of mRNA occurred in all three species between the transcriptional start point and the ribosome binding site. Although transcription is initiated from a single promoter in all three chlamydial species, transcriptional termination points for the monocistronic and bicistronic mRNAs differ in both number and position.

Keywords: Chlamydia, transcription, cysteine-rich proteins

INTRODUCTION

Chlamydia are obligate intracellular bacteria with a wide range of hosts. The genus is currently divided into four species: *Chlamydia trachomatis*, *Chlamydia psittaci*, *Chlamydia pneumoniae* and the recently defined *Chlamydia pecorum* (Fukushi & Hirai, 1992). *C. trachomatis* is a major human pathogen, responsible for sexually transmitted disease and the complex blinding disease, trachoma. In contrast, *C. psittaci* has a diverse range of animal hosts covering almost all domestic and feral mammals, birds and marsupials. Humans do not appear to be the primary host for *C. psittaci* although opportunistic infection with ovine abortion and avian strains are well documented (Storz, 1990). *C. pneumoniae* is a respiratory tract pathogen and like *C. trachomatis* it is associated with human disease

although isolates from both horses and koalas have been described (Storey *et al.*, 1993).

The Chlamydia have a unique developmental life cycle involving two distinct morphological forms. The elementary body (EB) is a small spore-like stable structure which is the infectious form of the organism. Following endocytosis by a susceptible host cell, EBs develop rapidly into the metabolically active, osmotically fragile reticulate bodies (RBs) which then undergo several rounds of rapid division in membrane-bound structures within the host cell. Late in the growth cycle, the chlamydial DNA condenses within RBs as a prelude to individual RBs re-forming as EBs which are then released on cell lysis to recommence the infectious cycle (Ward, 1988).

EBs possess a well characterized group of cysteine-rich proteins (CrPs; Hatch *et al.*, 1984). These proteins include the major outer-membrane protein (MOMP), a 60 kDa protein and a smaller protein of approximately 12 kDa (Hatch *et al.*, 1986; Newhall, 1987; Zhang *et al.*, 1987).

Abbreviations: CrP, cysteine-rich protein; EB, elementary body; MOMP, major outer-membrane protein; RB, reticulate body.

The 12 kDa protein is acylated with an unprocessed protein moiety of predicted mass 9 kDa (Everett & Hatch, 1991). CrPs can be extracted together in EB outer-envelope preparations and form a highly cross-linked disulphide-bonded matrix which, in the absence of a defined peptidoglycan layer, gives structural rigidity to the EB. MOMP constitutes the major proportion of protein in the outer envelope (60%), is surface-exposed and considered to be a membrane porin which carries neutralizing epitopes. The 9 kDa and 60 kDa CrPs are associated with the outer envelope but do not appear to be exposed at the EB surface as assayed by immunological techniques (Collett *et al.*, 1989; Watson *et al.*, 1994) and detergent-based fractionation studies (Everett & Hatch, 1995). The 9 kDa and 60 kDa CrPs are expressed late in the growth cycle and, unlike MOMP, are found almost exclusively in EBs; interestingly cysteine deprivation in the cell culture medium retards chlamydial differentiation from RBs to EBs (Allan *et al.*, 1985).

Preliminary transcriptional studies have demonstrated that the 9 kDa and 60 kDa CrP genes of *C. trachomatis* are closely linked on the chlamydial chromosome and are transcribed as a single mRNA species and as a bicistronic mRNA from tandem promoters (Lambden *et al.*, 1990). In contrast, the *C. psittaci* 9 kDa CrP has been suggested to have a single transcriptional start point both *in vivo* and in cell-free extracts (Mathews *et al.*, 1993). Transcriptional studies of the MOMP gene show that it may possess multiple promoters which have significantly different specificities in different isolates of *C. trachomatis* and *C. psittaci* (Stephens *et al.*, 1988; Yuan *et al.*, 1990). Because of the difficulties of extracting intact developmentally regulated mRNA for outer-envelope proteins from this intracellular parasite, none of these studies was able to complement primer extension analysis with S1 nuclease protection assays to define fully the mRNA termini. Thus the purpose of this work was to characterize the temporally regulated 9 kDa CrP genes from *C. psittaci* and *C. pneumoniae*. We have focused our interest on the 9/60 kDa CrP 'operon' because detailed analysis of a critical, temporally regulated promoter system for these important EB structural proteins may provide insights into the control of chlamydial development and also may ultimately provide useful resources for the future manipulation of all Chlamydia.

METHODS

Growth of Chlamydia. *C. pneumoniae* (IOL-207) and *C. psittaci* (EAE/A22/M) were propagated in mouse L929 cells (Flow Laboratories) grown in suspension culture. The host cells were grown in DMEM (Flow Laboratories) supplemented with 10% (v/v) foetal calf serum, 1% non-essential amino acids and 20 µg gentamicin ml⁻¹ (Sigma). The cells were grown as 500 ml suspension cultures in 1 litre glass culture vessels (Techne) on a microcarrier stirrer at 25 r.p.m., at 37 °C in the presence of 5% (v/v) CO₂. Cell growth was started at 2 × 10⁶ cells ml⁻¹ and continued until the cell density reached 1 × 10⁶ cells ml⁻¹ at which point 300 ml was harvested by centrifugation at 1000 g for 5 min. Cells were resuspended in 100 ml medium containing either 1 µg cycloheximide ml⁻¹ (*C. psittaci*) or 1 µg emetine ml⁻¹

(*C. pneumoniae*) and incubated at 37 °C for 10 min, then centrifuged as before and resuspended in 80 ml DMEM, 1% non-essential amino acids, 1% foetal calf serum, 40 µg gentamicin ml⁻¹ (growth medium). The chlamydial inoculum was added at an m.o.i. of 10 and was sedimented on to the host cells by centrifugation at 700 g for 1 h. Infected cells were resuspended and chlamydial growth was continued at 37 °C with stirring (25 r.p.m.) in the presence of 5% CO₂. Samples were taken at 0, 16, then at 8 h intervals up to 72 h post-infection.

Chlamydial RNA. A 40 ml aliquot of the Chlamydia-infected cell suspension was removed from the culture flask and centrifuged at 900 g for 10 min. Total RNA from the cell pellet was extracted using 2 ml RNazol according to the manufacturer's instructions (Biogenesis) (Chomczynski & Sacchi, 1987). RNA pellets were stored at -20 °C under 80% (v/v) ethanol until required when they were dried briefly and resuspended in RNase-free water. Northern blot analysis was performed as described by Lambden *et al.* (1990).

Chlamydial protein. Aliquots (2 ml) of Chlamydia-infected cells in suspension culture were pelleted at 8000 g for 20 s. The cell pellet was resuspended in 0.5 ml SDS-PAGE gel loading buffer. SDS-PAGE and immunodetection of proteins was performed as described by Watson *et al.* (1994).

Molecular cloning. Genomic DNA from *C. pneumoniae* IOL-207 was digested with *NheI* and the fragments separated on a 1% agarose gel. A narrow gel slice around a single band (8.5 kb) known to hybridize with a 60 kDa CrP gene probe (Clarke *et al.*, 1994) was excised and purified using GeneClean (Bio101). This DNA was ligated into *SpeI*-cut arms of the λ Zap II vector (Stratagene). Recombinants were screened using a gene probe prepared from a *C. trachomatis* 60 kDa CrP clone (Watson *et al.*, 1989). Recombinants carrying the insert in both orientations were identified and the plasmid from one of these recombinants (pMWPU1A) was used for sequencing. A complete *EcoRI* library was also created in λ Zap II using *C. psittaci* (EAE/A22/M) DNA and was screened using a PCR-generated probe to the 9 kDa CrP gene of *C. trachomatis* serovar L1 (Lambden *et al.*, 1990). Primer pair P1 and P2 were used to generate the probe using *C. trachomatis* serovar L1 genomic DNA as template and are shown in Table 1. Several recombinants were selected for sequence analysis.

Sequencing. All sequencing reactions were performed using the dideoxy-nucleotide method and modified T7 DNA polymerase, Sequenase (USB). Both strands of each recombinant were sequenced to give an unambiguous consensus sequence.

Generation of labelled probes. ³²P-labelled probes were generated by random primer labelling using [α -³²P]dCTP with a Multiprime kit (Amersham). Unreacted nucleotides were removed using Sephadex G-25 gel filtration. Oligonucleotides were labelled at the 5' end with [γ -³²P]ATP (50 µCi, 1.85 MBq) (Amersham) using T₄ polynucleotide kinase. The labelling reactions were allowed to proceed at 37 °C for 2 h. The labelled products were purified from unincorporated nucleotides using a NENsorb 20 Cartridge (NEN Research), according to the manufacturer's instructions.

Oligonucleotides. Oligonucleotides, including 5' amino-link oligonucleotides, for use as sequencing or PCR primers were synthesized on a model 381A automated DNA synthesizer (Applied Biosystems) using β -cyanoethyl phosphoramidite chemistry. Amino-link oligonucleotides were biotinylated using D-biotinyl-aminocaproic acid-N-hydroxy succinimide ester (BCL) at 2 mg ml⁻¹ in 0.1 M potassium carbonate buffer at

Table 1. Oligonucleotide PCR primers for generation of DNA probes

The co-ordinates of the primers refer to their position on the GenBank entries for these sequences. Accession numbers: M35148 (*C. trachomatis*), X53512 (*C. psittaci*) and X53511 (*C. pneumoniae*).

DNA source	Forward primers (5' → 3')	Reverse primers (5' → 3')	Product use
<i>C. trachomatis</i>	P1: 183TCTAGAAAGGAGTCTGGA ¹⁸⁰	P2: 481TGTGTGGGTAGGCTCTTA ⁴⁸³	9 kDa CrP probe for Southern analysis of λ recombinants
<i>C. psittaci</i>	P3: 33TTTCTACCGGAAAAACAA ⁵⁰	P4: 594TGTGAACCTTACCTCA ⁵⁷⁷	9 kDa CrP Northern blot probe (Fig. 2a)
<i>C. pneumoniae</i>	P5: 128AAGAGTTTAAATACAA ¹⁴⁴	P6: 627CCTAACTACCTGATCAA ⁶¹¹	9 kDa CrP Northern blot probe (Fig. 2b)
<i>C. psittaci</i>	P7: 450GTACATCTCTAGACGGTA ⁴⁶⁷	P8: biotin 678ACCGTAACCTACTCGTCTGAT ⁶⁵⁹	S1 mapping terminus of 9 kDa CrP mRNA (Fig. 5b)
<i>C. psittaci</i>	P9: 2277TAACGGTACCTGTTGCTGAT ²²⁹⁸	P10: biotin 2539GTTCTCACCAGACATAC ²⁵³³	S1 mapping terminus of 60 kDa CrP mRNA (Fig. 5c)
<i>C. pneumoniae</i>	P11: 501TCAACAGAGTGTAACCT ⁵¹⁷	P12: biotin 883GCACTAGCGACGATCTTA ⁸⁴⁶	S1 mapping terminus of 9 kDa CrP mRNA (Fig. 6b)
<i>C. pneumoniae</i>	P13: 2356TCTGATACACTGACTTACCC ²³⁷⁵	P14: biotin 2660AAGCAGGTTACTAGG ²⁶⁴⁴	S1 mapping terminus of 60 kDa CrP mRNA (Fig. 6c)

pH 9.0 for 16 h at 37 °C. Oligonucleotides were separated from unreacted biotin by gel filtration on Sephadex G-25 spin columns.

Probe generation. The primers for PCR-generated probes are summarized in Table 1. Probes used for S1 nuclease protection assays were radioactively labelled during the PCR reaction. The only adjustments made to the PCR conditions were to decrease the amount of non-radioactive dCTP to 100 μ M and add [α -³²P]dCTP (50 μ Ci, 1.85 MBq).

S1 nuclease protection assays. The complementary DNA strand required for hybridization (the non-coding strand) was generated using a biotinylated oligonucleotide in the 'radioactive' PCR reaction. The PCR product was coupled to Dynabeads (80 μ l M-280; Dynal) and single-stranded solid phase DNA produced according to the manufacturer's instructions. The magnetic beads were resuspended in formamide/PIPES buffer (PIPES, 40 mM; NaCl, 400 mM; EDTA, 1 mM, pH 6.5; deionized formamide, 20%, v/v) to give 50 ng DNA in 30 μ l. When required, the single-stranded DNA was released from the bead by heating to 90 °C for 5 min in formamide/PIPES. Single-stranded DNA was hybridized in solution with mRNA in formamide/PIPES buffer by heating to 95 °C for 2 min and cooling to 37 °C. Annealing was continued at 37 °C for 16 h.

Following hybridization, 300 μ l of S1 nuclease buffer (NaCl, 0.25 M; sodium acetate, 0.05 M, pH 4.5; ZnSO₄, 4.5 mM) containing 1000 U S1 nuclease (BCL) was added to the hybridization mixture. S1 nuclease digestion was performed at 37 °C for 1.5 h and terminated with 80 μ l S1 nuclease stop mixture (ammonium acetate, 4 M; EDTA, 50 mM, pH 8.0). The supernatant containing the remaining RNA/DNA hybrids was extracted with phenol/chloroform (Sambrook *et al.*, 1989), precipitated and dried. The hybrid solution was resuspended in 10 μ l water and 20 μ l formamide dyes (Sambrook *et al.*, 1989). An aliquot was then run on a sequencing gel adjacent to a set of sequencing reactions performed with the coding strand primer (3' end mapping) or the non-coding strand primer (5' end mapping) used in the initial PCR.

Primer extension. Primers used for reverse transcription primer extension were chosen so that the extension products would be between 50 and 200 bp based on the predicted positions of the RNA 5' ends. Oligonucleotides were annealed to RNA for reverse transcription primer extension studies using the conditions described above. After hybridization of the RNA sample with the 5' ³²P-labelled oligonucleotide primer, the RNA/primer hybrids were precipitated with 2.5 vols ethanol. The RNA/primer hybrid was centrifuged at 9000 g for 15 min, the supernatant discarded and the pellet resuspended in 40 μ l TE. The RNA/primer hybrid was re-precipitated and washed.

RNA/primer hybrids were finally resuspended in water and the primer extension reaction was performed using Superscript (Gibco-BRL) as recommended by the manufacturers. Placental ribonuclease inhibitor (Amersham) was added to the reactions at 1 U μ l⁻¹. Reactions were allowed to proceed for 1 h before phenol/chloroform extraction (Sambrook *et al.*, 1989) and ethanol precipitation. The extension products were finally resuspended in 10 μ l water and analysed on a sequencing gel as described above.

RESULTS AND DISCUSSION

Molecular cloning and sequence analysis of the 9 kDa CrP genes

Purified chromosomal DNA from *C. pneumoniae* and *C. psittaci* was digested to completion with a series of restriction endonucleases and used in Southern blotting experiments with a plasmid probe carrying the complete nucleotide sequence of the *C. trachomatis* B/Jali-20/OT 60 kDa CrP (Watson *et al.*, 1989; Clarke *et al.*, 1994). In *C. pneumoniae* DNA, a single cross-hybridizing 8.5 kb *NheI* fragment was observed; this was purified and ligated into pre-prepared λ Zap II arms digested with *SpeI*. In *C. psittaci* DNA, a single cross-hybridizing 7.6 kb *EcoRI* fragment was similarly chosen for cloning in λ Zap II. The large size of both these fragments relative to the 9 kDa CrP ORF suggested that each fragment carried a complete 9 kDa CrP gene sequence. The 8.5 kb *NheI* fragment from *C. pneumoniae* cloned into the λ Zap II vector was released as an intact recombinant pBluescript plasmid. In contrast, *in vivo* excision of the *C. psittaci* 7.6 kb *EcoRI* fragment from the λ Zap II vector generated only truncated inserts in the recombinant pBluescript plasmid. However, further Southern hybridization (data not shown) revealed the complete 9 kDa CrP gene was present in these truncated recombinants. Difficulties cloning a similar fragment from *C. psittaci* have also been reported by Everett & Hatch (1991), presumably because the promoter system is highly active in *Escherichia coli* and the complete 60 kDa CrP gene is unstable on high copy number vectors (Clarke & Lambden, 1988). It was expected that the 9 kDa CrP gene sequences would be closely linked to the 60 kDa CrP gene sequences as had been shown in *C. trachomatis* (Lambden *et al.*, 1990). Therefore, sequence analysis was started using upstream facing oligonucleotide primers from the two 60 kDa CrP genes. This confirmed the presence of 9 kDa CrP genes

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Fig. 3. Thin section transmission electron micrographs of mouse L929 cells grown in suspension culture and infected with *C. psittaci* and *C. pneumoniae*. (a) Inclusions of *C. psittaci* at 32 h post-infection. (b) An inclusion of *C. pneumoniae* at 64 h post-infection. Samples were prepared and examined in a Hitachi H7000 transmission EM as previously described (Carter *et al.*, 1991). Bars (marked with asterisk), 2 µm.

pneumoniae Northern blots were re-probed using 60 kDa CrP PCR-generated probes. The results (data not shown) showed that the large transcript of 2.4 kb was a bicistronic message and that both RNAs are transcribed concurrently during the growth cycle reaching peak rates of synthesis at 32 h for *C. psittaci* and 64 h for *C. pneumoniae*. A thin section transmission EM of chlamydia-infected cells (Fig. 3) confirmed that *C. psittaci* has a faster growth cycle than *C. pneumoniae*, with more compact inclusion formation. Fig. 3 shows that RB to EB differentiation has begun to occur at 32 h post-infection for *C. psittaci* and at 64 h post-infection for *C. pneumoniae*. This process is coincident with a maximal rate of 9 kDa CrP and 9/60 kDa CrP mRNA synthesis for the two species (Fig. 2). Immunoblotting of the 60 kDa CrPs from both species at the selected time points during the growth cycle (Fig. 4) also shows that 60 kDa CrP translation occurs at peak rates at 32 and 64 h post-infection.

Primer extension studies

RNA extracted at 32 h for *C. psittaci* and 64 h for *C. pneumoniae*, the times of maximal 9 kDa and 60 kDa CrP mRNA synthesis, was used for primer extension studies. The primer extension results for *C. psittaci* and *C. pneumoniae* can be seen in Figs 5(a) and 6(a), respectively. The first base transcribed in both species is an adenine residue 96 bp upstream of the start codon of the 9 kDa CrP gene. However, Lambden *et al.* (1990) described a tandem promoter system for the 9 kDa and 60 kDa CrP genes of *C. trachomatis* serovar L1. These transcriptional analyses were repeated using two different reverse transcriptases and when Superscript RNase H⁻ was substituted for RAV-2, the reverse transcriptase used by Lambden *et al.* (1990), a single transcript was observed (data not shown). Premature termination in primer extension reactions giving rise to such heterogeneous products occur because of the tendency for reverse transcriptase to stop or pause in regions of high secondary structure in the template mRNA (Sambrook *et al.*, 1989). For the *C. trachomatis* 9/60 kDa CrP mRNA, the shorter cDNA terminates close to the 3' base of a predicted stem

Fig. 4. Immunodetection of *C. psittaci* (a) and *C. pneumoniae* (b) 60 kDa CrPs expressed during the chlamydial growth cycle. Protein samples were extracted at the times (h) indicated above each sample track and the 60 kDa CrPs were detected using a pool of monoclonal antibodies raised against *C. trachomatis* 60 kDa CrPs (Newhall & Basinski, 1986). The two processed forms of the 60 kDa CrP are indicated with arrowheads.

Fig. 5. Definition of *C. psittaci* 9 kDa and 9/60 kDa CrP mRNA termini by primer extension and S1 nuclease mapping. (a) Primer extension reaction (E) and sequencing ladder (SEQ) defining the 5' terminus of the mRNA. Oligonucleotide 243 GTAGCTAGTAAAACAGC 227 was used in the primer extension and sequencing reactions. Sequencing reaction tracks were loaded in the order AGCT. The single shared transcriptional start point for the various mRNAs at the adenine residue is indicated by an arrowhead. (b) S1 nuclease mapping of the 3' terminus of the 9 kDa CrP mRNA. The mRNA was hybridized to the biotinylated radiolabelled DNA template amplified with primers P7 and P8. Track S1 contains nuclease-protected DNA and the two transcriptional termination points are marked by brackets and adjacent arrowheads. The sequencing ladder (SEQ) was generated using P7 and the tracks are loaded in the order TCGA. (c) S1 nuclease mapping of the 3' terminus of the 9/60 kDa CrP bicistronic mRNA. The mRNA was hybridized to the biotinylated radiolabelled DNA template amplified with primers P9 and P10. Track S1 contains nuclease-protected DNA showing the two separate transcriptional termination points for this mRNA which are indicated by brackets and arrowheads. The sequencing ladder (SEQ) was generated using P9 and the tracks are loaded in the order TCGA.

and loop structure (Fig. 7). Such a secondary structure in the mRNA may be involved in modulating translation. In this study, the primer extension results using Superscript reverse transcriptase were supported by S1 nuclease analysis of the 5' termini of the mRNAs (data not shown) confirming the existence of a single promoter for the 9 kDa CrP gene in agreement with Mathews *et al.* (1993).

Mapping the mRNA termini with S1 nuclease

To ensure accurate definition of the mRNA termini, it is essential to match the primer extension studies with S1 nuclease analysis. Because of the relatively low abundance of developmentally regulated chlamydial mRNA in

Fig. 6. Definition of *C. pneumoniae* 9 kDa and 9/60 kDa CrP mRNA termini by primer extension and S1 nuclease mapping. (a) Primer extension reaction (E) and sequencing ladder (SEQ) defining the 5' terminus of the mRNA. Oligonucleotide 377 ACAATCTACAATGCGGC 361 was used in the primer extension and sequencing reactions. Sequencing reaction tracks were loaded in the order TGCA. The single shared transcriptional start point for the various mRNAs at the adenine residue is indicated by an arrowhead. (b) S1 nuclease mapping of the 3' terminus of the 9 kDa CrP mRNA. The mRNA was hybridized to the biotinylated radiolabelled DNA template amplified with primers P11 and P12. Track S1 contains nuclease-protected DNA and the transcriptional termination point is marked by the three adjacent arrowheads. The sequencing ladder (SEQ) was generated using P11 and the tracks are loaded in the order TCGA. (c) S1 nuclease mapping of the 3' terminus of the 9/60 kDa CrP bicistronic mRNA. The mRNA was hybridized to the biotinylated radiolabelled DNA template amplified with primers P13 and P14. Track S1 contains nuclease-protected DNA showing three widely separated transcriptional termination points which are indicated by brackets and arrowheads. The sequencing ladder (SEQ) was generated using P13 and the tracks are loaded in the order TCGA.

extracts of whole cells, we developed a simple, sensitive solid-phase S1 nuclease protection procedure using biotinylated oligonucleotide primers and streptavidin-coated magnetic beads.

The results of the 3' S1 terminal mapping for *C. psittaci* and *C. pneumoniae* 9 kDa CrP mRNAs can be seen in Figs 5 and 6, respectively. For the small 0.45 kb transcript in *C. psittaci* there were two main sites of termination, at 73–75 bp and 111–112 bp downstream from the 9 kDa CrP ORF stop codon. Two termination points were also observed at 69–71 bp and 119–121 bp downstream from

Fig. 7. Nucleotide sequence alignment summarizing the regulatory features of the 9/60 kDa CrP operon. Identical nucleotides are indicated by dots. Dashes indicate sequence gaps for optimal alignment. The -35 and -10 regions of the promoter are indicated in italics and are shaded. The unique single transcriptional start point for all the mRNAs is highlighted by a shaded box and vertical arrow. Horizontal arrows above the sequences denote predicted stem-loop structures at the 5' termini of the mRNA. Potential ribosome bindings sites (RBS) are indicated as open boxes. The coding sequences for the 9 and 60 kDa CrP structural genes are represented by open boxes between the start and stop codons. Transcriptional termination points for the *C. psittaci* mRNAs are marked by vertical arrows above shaded boxes. Transcriptional termination points for the *C. pneumoniae* mRNAs are marked by vertical arrows below shaded boxes.

the *C. psittaci* 60 kDa CrP ORF stop codon (Fig. 7). In contrast, the S1 nuclease results for *C. pneumoniae* showed three transcriptional termination points at 98 bp, 162 bp and 229 bp downstream from the stop codon for the 60 kDa CrP ORF and a single transcriptional termination point 71 bp downstream from the 9 kDa CrP ORF stop codon. The transcriptional termination points occur following computer-predicted stem and loop structures (data not shown) which have some similarity to *p*-independent termination signals (Rosenberg & Court, 1979).

Conserved developmentally regulated promoters

This study shows that Chlamydia synthesize a complex set of mRNAs for the 9 kDa and 60 kDa CrP genes. All the mRNAs share a highly conserved promoter and their synthesis is subject to a temporal switch late in the growth cycle, coincident with RB to EB differentiation. However, the obligate intracellular life cycle remains a major obstacle towards an understanding of genetic regulatory mechanisms in Chlamydia. Therefore at present, it is possible only to make comparisons with other prokaryotic systems. Although the upstream sequences of the

9/60 kDa CrP promoter contain some short inverted repeat sequences, these do not resemble the global control features such as the cAMP receptor protein binding sites (Kolb *et al.*, 1993) or the DNA looping structures (Matthews, 1992) seen in *E. coli*.

It has been suggested that developmentally regulated promoter activity in Chlamydia may be governed by specific σ factors (Ward, 1988). $\sigma 70$ -like factors have been demonstrated in Chlamydia (Hatch *et al.*, 1990; Koehler *et al.*, 1990; Engel & Ganem, 1990). Two groups of Chlamydial promoters have now been described which exhibit different levels of similarity to the $\sigma 70$ consensus of *E. coli* (Mathews & Sriprakash, 1994). The 9 kDa CrP promoters are grouped together with the well characterized antisense promoters from the cryptic plasmid of *C. trachomatis* (Ricci *et al.*, 1993) as they show the most homology with the *E. coli* consensus. The properties of this antisense promoter are difficult to equate with a critical role in the chlamydial life cycle as the function of these mRNAs are unknown and many Chlamydia, including human *C. pneumoniae* isolates, do not possess a cryptic plasmid. *In vitro* transcription/translation studies using a chlamydial cell-free extract with a *C. psittaci* 9 kDa

CrP promoter have demonstrated that transcription is dependent on the activity of the chlamydial $\sigma 70$ equivalent. Transcriptional activity of this promoter was inhibited by the addition of a monoclonal antibody to *E. coli* $\sigma 70$ (Mathews *et al.*, 1993), suggesting multiple σ factors are not involved in the regulation of the 9 kDa CrP promoter.

As RBs differentiate to become EBs, the chlamydial chromosomal DNA undergoes a dramatic reorganization and condenses to form a compact nucleoid structure. This event is induced by histone-like proteins expressed late in the growth cycle (Barry *et al.*, 1992). It has been suggested that such histone-like proteins have a role in inactivating the expression of chlamydial genes (Christiansen *et al.*, 1993), especially the inhibition of DNA replication and the shutdown of transcription and translation (Barry *et al.*, 1993; Pedersen *et al.*, 1994). However, the 9 kDa CrP promoters become most active at this time, thus it seems unlikely that DNA condensation has a major role in regulating the transcription of these promoters.

Northern blotting experiments were not able to resolve the exact sizes and complexities of the mRNAs produced from this operon. However, the overall sizes of the two groups of late-expressed 9 kDa and 9/60 kDa mRNA transcripts determined by reverse transcription primer extension and S1 nuclease assay were in general agreement with the values predicted from the Northern blots. The transcriptional start point of these mRNA populations in all three species was at an adenine residue, 96 bp (*C. psittaci* and *C. pneumoniae*) or 91 bp (*C. trachomatis*) upstream of the start codon for the 9 kDa CrP gene. The 9 kDa CrP gene promoter region is well conserved to 44 bp upstream from the first residue transcribed. Temporal control of these proteins is regulated by mRNA synthesis, however, the long stem (22 bp) and short loop structure in the mRNA of all three species (Fig. 7) between the transcriptional start point and the ribosome binding site is a prominent shared feature which suggests chlamydial 9 kDa and 60 kDa CrP expression is also subject to translational modulation.

The genus-conserved temporally regulated promoter structures described here will be useful in future studies of the control of chlamydial gene expression. Such tightly controlled promoters may ultimately help in the development of shuttle vectors as part of a much needed chlamydial gene transfer system.

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