Expression of a plasmid gene of *Chlamydia trachomatis* encoding a novel 28 kDa antigen

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Plasmid pCT is present in essentially all isolates of *Chlamydia trachomatis* and may encode factors important for survival in the natural environment. However, no pCT-associated phenotype has been described so far. With the purpose of investigating the possibility of a role of pCT in *C. trachomatis* pathogenicity, we examined the expression of an ORF (ORF3), potentially encoding a 28 kDa polypeptide (pgp3). Analysis of RNA extracted from chlamydia-infected Vero cells detected ORF3-specific transcripts, from 20 h post-infection onwards, mainly as discrete RNA species of 1390 nucleotides comprising the downstream ORF4 sequence. ORF3 DNA was cloned and expressed in *Escherichia coli* as a 39 kDa fusion protein (MS2/pgp3). Antibodies raised against purified MS2/pgp3, specifically recognized a 28 kDa protein on Western blots of protein from purified chlamydial elementary bodies (EBs). The same antibodies detected chlamydial inclusions in methanol-fixed infected cells by immunofluorescence. Western blot analysis of EBs extracted with 2% Sarkosyl, showed that a large proportion of the 28 kDa antigen is associated with the detergent-insoluble ('membrane') fraction. Antibodies recognizing pgp3 epitopes were detected in sera from patients with chlamydial infections, but not in sero-negative control sera. The findings support the hypothesis that pCT may provide a function related to chlamydial cell physiology.

Introduction

pCT is a plasmid of 7.5 kb described previously as pCHL1, pCTT1 and pLGV440 (Palmer & Falkow, 1986; Sriprakash & MacAvoy, 1987; Hatt *et al.*, 1988; Comanducci *et al.*, 1988, 1990b) commonly found in all isolates of the human pathogen *Chlamydia trachomatis*, an obligate intracellular parasite of eukaryotic cells (Moulder *et al.*, 1984; Moulder, 1985). A recent report of a plasmid-less strain indicates that pCT may be non-essential for growth and propagation of *C. trachomatis* in tissue culture (Peterson *et al.*, 1990). However, in human infections there is clearly a strong selection in favour of this plasmid, since, except for the above-mentioned case, it has been found in all serotypes and

clinical isolates examined so far (Palmer & Falkow, 1986; Sriprakash & MacAvoy, 1987; Hatt *et al.*, 1988; Comanducci *et al.*, 1990b; Peterson *et al.*, 1990). Accordingly, studies in which the plasmid was used as a diagnostic target showed a good correlation between plasmid detection and tissue culture isolation (Horn *et al.*, 1986; Dean *et al.*, 1989; Ostergaard *et al.*, 1990; Ratti *et al.*, 1991). No specific phenotype associated with pCT has been described so far; however, bacterial pathogenicity factors are often encoded on plasmids (Eberhard, 1989) and a similar situation could be true for *C. trachomatis*.

Comparative sequence analysis of four pCT variants (Comanducci *et al.*, 1990b) present in strains with different pathogenic properties (B and D serotypes, belonging to the 'trachoma' biovar, and L1 and L2 of the 'lymphogranuloma' biovar) showed that the nucleotide sequence of pCT is strongly conserved, with < 1% variation, and that a number of point mutations correlate with the biovar from which the plasmid is isolated. As a consequence, some of the polypeptides deducible from the eight major ORFs of pCT showed amino acid residues which are conserved in isolates of the same biovar, but differ between isolates belonging to different

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Abbreviations: COMC, chlamydial outer-membrane complex; EB, elementary body; mAb, monoclonal antibody; MOMP, major outer-membrane protein; nt, nucleotide; PID, pelvic inflammatory disease.

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biovars. Interestingly, more than half of such biovar-linked substitutions (8/15) were found in the amino acid sequence specified by ORF3 (nomenclature according to Comanducci *et al.*, 1990b). The frequency of such mutations in ORF3 was significantly higher (> seven-fold) when compared to the average value for all the other ORFs. Since this observation suggested a possible relationship between ORF3 and a chlamydial pathogenicity phenotype, we investigated the expression of this putative gene in the chlamydial cell.

In this paper we report on the detection and characterization of ORF3-specific transcripts in *C. trachomatis* and the identification of the ORF3-encoded product as a novel 28 kDa chlamydial antigen.

A preliminary report containing some data presented in this paper has been published (Comanducci *et al.*, 1990a).

Methods

Bacterial strains and biological materials. Go/86 is a serotype D *C. trachomatis* strain, isolated in 1986 in Bologna, Italy, from a patient with non-gonococcal urethritis; the serotype was confirmed by sequencing of the cloned *omp1* gene (M. Comanducci, unpublished data). The 7.5 kb plasmid cloned from Go/86 was described previously as a variant of pCHL1 (Comanducci *et al.*, 1990b) and here is more conveniently indicated as pCT-D. Chlamydial infection of cultures of LLCMK2 (Rhesus monkey kidney), Vero or McCoy cells, and purification of elementary bodies (EBs) were performed by standard techniques (Caldwell *et al.*, 1981). pUC8-pCTD is a recombinant pUC8 plasmid (Vieira & Messing, 1982), containing the whole pCT-D sequence as a *Bam*HI insert (Comanducci *et al.*, 1990b). Plasmid expression vector pEX34A, a derivative of pEX29 and pEX31 (Strebel *et al.*, 1986), and host *Escherichia coli* K12 Δ H1 Δ trp (Remaut *et al.*, 1983) were obtained from Dr R. Rappuoli (IRIS, Siena, Italy) and were originally gifts from Mo-Quen Klinkert (Centre for Molecular Biology, Heidelberg, Germany) and W. Fiers (State University of Ghent, Ghent, Belgium), respectively. The monoclonal antibody (mAb) specific for the N-terminal portion of bacteriophage MS2 replicase was a gift from Dr M. Bigio (IRIS, Siena, Italy). The anti-major outer-membrane protein (MOMP) mAb E7-12 has been described previously (Cevenini *et al.*, 1987).

DNA techniques. Standard techniques were used throughout (Sambrook *et al.*, 1989). DNA sequencing (Sanger *et al.*, 1977) was performed using the Sequenase kit (United States Biochemicals). Synthetic oligonucleotides were prepared with an automatic DNA synthesizer (Applied Biosystems, model 380B). Analysis of nucleotide and amino acid sequences was performed with the computer programs of the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

Analysis of ORF3 transcripts. Chlamydia-infected LLCMK2 or Vero cells were grown in 150 cm² flasks and after rinsing with PBS, directly solubilized in 10 ml of 4 M-guanidinium isothiocyanate, 25 mM-sodium citrate, pH 7.0, 0.5% Sarkosyl, and 0.1 M-2-mercaptoethanol. RNA extraction, electrophoresis on formaldehyde-agarose (1.5%) gels and Northern blots were performed as described by Chomczynski & Sacchi (1987) and Sambrook *et al.* (1989). Probe A (Fig. 1a) was prepared from the ORF3 DNA segment described below for pEX34A cloning and was ³²P-labelled by random priming (Sambrook *et al.*, 1989). Probe B (Fig. 1a) was similarly prepared by PCR (Saiki *et al.*, 1988) on the

plasmid pUC8-pCTD, using the following primers: (4918) 5' ATGCAAAATAAAAGAAAAG 3' (4936) and (5226) 5' CTATTC-AGCCTTGGAAAAC 3' (5208). The coordinates (indicated in parentheses) are expressed as the number of nucleotides from the unique *Bam*HI site of pCT-D (Comanducci *et al.*, 1990b). Primer extensions on ORF3 and ORF4 transcripts were performed as described by Boorstein & Craig (1989) and Scarlato *et al.* (1989), with primers (4136) 5' GGCTCTGTCATTGCCCCAAC 3' (4117) and (5056) 5' GGTAAG-TTCTAAGGGAGAA 3' (5037) for ORF3 and ORF4, respectively. The same primers were used for the sequencing reactions (Sanger *et al.*, 1977) serving as size markers for fine mapping. S1-nuclease mapping experiments were performed as described by Berk (1989). Probes C and D in Fig. 1(a) were prepared by PCR followed by digestion with appropriate restriction endonucleases. For probe C, the primers C1 and C2 (Fig. 1b) were used. The PCR product was digested with *Hind*III, to create a precise 5' end internal to the ORF sequence, electrophoretically purified and end-labelled with T4 polynucleotide kinase and [γ -³²P]ATP (Sambrook *et al.*, 1989). Probe C covered the DNA segment from the *Dde*I site at 113 bp upstream of the first ATG codon of ORF3 to the internal *Hind*III site (Fig. 1b). For probe D we used the primers D1 (Fig. 1b), and D2: (5551) 5' GAATATCgAATTCTCTAAACTG 3' (the lower case letter indicates a mismatched nucleotide introduced to create an *Eco*RI site, underlined). The PCR product was digested with *Cl*aI and *Eco*RI, purified and 3'-labelled with Klenow enzyme and [α -³²P]dATP (Sambrook *et al.*, 1989). Probe D corresponded to a 913 base DNA segment from 219 bp upstream from the stop codon of ORF3 to 226 bp inside the ORF5 sequence.

Cloning of ORF3. pUC8-pCTD (1 ng) was used as a template for PCR with the primers 3-1 and 3-2 (Fig. 1b). *Taq* DNA polymerase and the GeneAmp kit (Perkin-Elmer Cetus) were used. Twenty-five PCR cycles (1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C) were performed. The amplified DNA fragment, cleaved with *Bam*HI and *Pst*I was purified by agarose gel electrophoresis and ligated to pEX34A. Competent cells of *E. coli* K12 Δ H1 Δ trp (Remaut *et al.*, 1983) were transformed with the above ligation mixture, and grown at 30 °C in the presence of ampicillin (30 μ g ml⁻¹). One of the ampicillin-resistant (pEX34A phenotype) colonies was shown, by DNA sequencing, to contain a recombinant pEX34A plasmid with an in-frame DNA insert identical to ORF3 in pCT-D (Comanducci *et al.*, 1990b). This clone (*E. coli* PO3/Go/MC1) was selected for further work.

Expression of ORF3 fusion gene in E. coli and purification of the recombinant protein. The pEX34 expression vector/host system puts the cloned gene under the control of the λ _{PL} promoter of λ bacteriophage and a *ts* mutant of its repressor, which is active at 30 °C but is inactivated at 42 °C. Exponential phase cultures of *E. coli* PO3/Go/MC1 were grown at 30 °C in LB medium containing 30 μ g ampicillin ml⁻¹, and induced by a temperature shift to 42 °C. Urea (7 M) protein extracts of induced and non-induced cultures were prepared and analysed as described by Nicosia *et al.* (1987). The 7 M-urea extracts were dialysed against PBS (10 mM-sodium phosphate, 15 mM-NaCl, pH 7.4), at 4 °C overnight. This caused precipitation of several *E. coli* proteins, but left most of the MS2/ppg3 protein in solution. After dialysis, MS2/ppg3 was further purified by SDS-PAGE (Sambrook *et al.*, 1989). The protein eluted from the gels was precipitated with acetone (Harlow & Lane, 1988), and resuspended in sterile PBS for antibody preparation. Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce).

Preparation of anti-gpg3 antibodies. Three New Zealand White rabbits were injected in several lymph nodes with 20, 50 and 100 μ g purified MS2/ppg3 protein (dissolved in 1 ml 0.15 M-NaCl and emulsified with 1 ml Freund's complete adjuvant), respectively. The same amounts were injected, together with Freund's incomplete

Fig. 1. (a) Graphic representation of the ORF3-ORF4 DNA region. Arrowed open boxes indicate direction of transcription of the respective ORFs. The thin arrows indicate RNA transcripts described in the text. The DNA segments used as probes are indicated as bars A, B, C and D; pCT-specific and PCR-created (in parentheses) restriction sites are shown at the ends of probes C and D. The short black bars within ORF3 and ORF4 indicate the positions of oligonucleotides used in primer extension experiments. Two RNA 5' ends and the major RNA 3' end are indicated by diamonds and a triangle, respectively. (b) Detailed map of ORF3 and adjacent regions. Nucleotide coordinates refer to the distance from the unique *Bam*HI site of pCT-D. Deduced peptide sequences are shown in one-letter code; the polypeptide sequence encoded by the pCT-D ORF3 variant is indicated as *pgp3* (264 amino acids, 27802 Da); amino acid substitutions, found in the LGV (serotypes L1 or L2) variants are shown below the main translation line and the biovar-specific substitutions are indicated in bold. Synthetic oligonucleotide primers and the *Dde*I and *Hind*III restriction sites (see Methods) are indicated. Positions of RNA 5' ends upstream of ORF3 and ORF4 are marked with arrows. Potential -10 and -35 sequences and a ribosome binding site, upstream of ORF3, are underlined.

adjuvant, 25, 40 and 55 days after the first injection. During the immunization, the increase in titre of anti-MS2/*pgp3* antibodies was followed by Western blot analysis (Towbin *et al.*, 1979) using a peroxidase-labelled mouse serum to rabbit immunoglobulins. The three sera, collected 15 days after the last injection, had similar titres (> 30 000 in immunoblot assays) and were pooled for further use.

Immunofluorescence. Assays were performed as described by Cevenini *et al.* (1987) using monolayers of McCoy or LLCMK2 cell cultures (on glass coverslips). Thirty hours after infection with chlamydial EBs, cells were washed with sterile PBS and fixed with methanol at -20 °C. Incubations with anti-MS2/*pgp3* rabbit serum (dilution 1:100-1:500 in PBS) were performed at room temperature for 30 min, and were followed by incubation with fluorescein-labelled mouse serum to rabbit immunoglobulins (Boehringer). In some cases the cells were counterstained with Evans Blue. The inclusions were observed at 400× and

1000× magnifications, in ultra-violet light, with an Axiophot microscope (Leitz).

Immunoblotting analyses. Electrophoresis of *E. coli* or *C. trachomatis* protein extracts was performed on SDS-polyacrylamide gels using the Laemmli system (Sambrook *et al.*, 1989). Transfer onto nitrocellulose filters and immunodetection were performed as described previously (Cevenini *et al.*, 1987, 1991; Towbin *et al.*, 1979). In the experiments with the anti-MS2/*pgp3* rabbit serum the blots were developed with goat anti-rabbit IgGs conjugated with horseradish peroxidase (Cappel; dilution 1:2500), followed by colorimetric detection with 4-chloro-1-naphthol (Sambrook *et al.*, 1989). Analysis with human sera was performed with 32 sera containing antibodies against *C. trachomatis* (obtained from patients referred to the Microbiology Institute, St Orsola Hospital, Bologna, Italy, for diagnosis of chlamydial infection) and 20 control sera from sero-negative healthy blood donors. Anti-

Fig. 2. Northern blots of total RNA extracted from Vero cells before infection (lanes 1), and from cells infected with *C. trachomatis* at 6, 17, 20, 30 and 48 h p.i. (lanes 2, 3, 4, 5 and 6, respectively). Each lane was loaded with 5 µg total RNA. Blots were hybridized with ORF3-specific probe A (a) or with ORF4-specific probe B (b). Sizes (nt $\times 10^{-3}$) of rRNA markers are shown on the left, and of major pCT transcript species, calculated from electrophoretic mobilities, on the right.

chlamydia IgG titres were determined by single antigen (LGV-2) immunofluorescence. Western blot strips with the 39 kDa fusion protein were incubated overnight with the serum under test, diluted 1:100 in PBS containing 0.05% Tween 20. After washing, antigen-antibody complexes were detected with a peroxidase-labelled rabbit antiserum to human immunoglobulin (Dako) as described previously (Cevenini *et al.*, 1991).

Detergent treatments. Samples of purified EBs, containing about 1 mg protein in 1 ml PBS, were centrifuged in an Eppendorf centrifuge at 14000 r.p.m. at 4 °C. The EB pellets were resuspended twice in PBS containing 1% (w/v) disodium *N*-lauryl-sarkosine (Sarkosyl), briefly sonicated, incubated for 1 h at 37 °C and centrifuged at 100000 *g* for 1 h. The insoluble pellets so obtained were then resuspended in (i) 2% Sarkosyl in PBS, (ii) 2% Sarkosyl in PBS with 10 mM-DTT, (iii) 2% (w/v) SDS in PBS, or (iv) 2% SDS in PBS with 10 mM-DTT. These suspensions were incubated for 1 h at 37 °C and again centrifuged at 100000 *g*. Supernatants and pellets were collected and analysed by Western blotting.

Protease treatments. Samples of purified EBs corresponding to about 150 µg protein were pelleted and resuspended in 1 ml 0.25 M-sucrose–10 mM-sodium phosphate–5 mM-L-glutamic acid (pH 7.2) containing either 50 µg trypsin (Type XIII, Sigma), chymotrypsin A4 (Sigma) or thermolysin (Boehringer, Mannheim, Germany) ml⁻¹. These suspensions were incubated for 2 h at 37 °C in a shaking water bath. PMSF (Boehringer) was then added and the samples were chilled at 4 °C. After centrifugation, the pellets were dissolved in Laemmli's sample buffer and aliquots were analysed by Western blotting.

Results

ORF3 transcripts

RNA transcripts from the ORF3 region of pCT (Fig. 1) were analysed on Northern blots of RNA extracted from Vero cells before infection and at 6, 17, 20, 30 and 48 h post-infection (p.i.) with *C. trachomatis*. Fig. 2(a) shows a blot hybridized with probe A (Fig. 1a), corresponding

to the whole ORF3 (792 bp). A major RNA band, with a mobility corresponding to about 1300 nucleotides (nt), was detected in the 20, 30 and 48 h samples, whereas no bands were detectable in the earlier samples up to 17 h p.i. Two minor bands of about 1000 and 800 nt were also detected. The intensity of the signals increased at 30 h and decreased in the 48 h sample, when loss of intracellular chlamydiae, due to host-cell lysis, started to occur. ORF3 transcripts were further analysed by S1-mapping and primer extension experiments, performed on the RNA sample collected 30 h p.i., and using the pre-infection sample as a control. S1-nuclease protection assays (Fig. 3a) with probe C (see Fig. 1a) showed that a major RNA species in the 30 h sample had a 5' end mapping at about 19 nt from the ATG start codon of ORF3. This result was confirmed by primer extension analysis (not shown) with an oligonucleotide complementary to the 5'-terminal region of ORF3 mRNA (see Fig. 1b). We concluded that the major 1300 nt band detected on Northern blots by probe A represents a transcript starting immediately upstream of ORF3. In this case, the transcript would extend for > 500 nt after the stop codon of ORF3, including therefore the whole ORF4 sequence. This hypothesis was investigated by Northern blot analysis with probe B (Fig. 1a), corresponding to ORF4. This probe detected a 1300 nt band, as with probe A, and two other bands at about 500 and 330 nt (Fig. 2b). Interestingly, the 1000 and 800 nt bands hybridizing to probe A were not detected by probe B.

Further data were obtained by S1-nuclease protection experiments designed to map RNA 3' ends in the region between 600 and 1500 nt from the start of ORF3 (probe

Fig. 3. Mapping of RNA ends in the ORF3-ORF4 region. (a) Identification of an RNA 5' end upstream of ORF3. S1 nuclease protection experiment with the 426 bp probe C on RNA extracted from Vero cells before (lane 4), and 30 h after chlamydial infection (lane 5). Lanes: 1, molecular mass markers (1 kb DNA ladder, Gibco-BRL); 2 and 3, control samples containing no RNA, prepared and processed as for samples in lanes 4 and 5, before (2) and after (3) S1-nuclease treatment. The S1-nuclease-protected segment given by the ORF3-specific transcript is indicated as P₃. (b) Identification of an RNA 5' end upstream of ORF4 by primer extension. An end-labelled primer complementary to the ORF4 mRNA 5' end region (see Methods) was hybridized to total RNA extracted from Vero cells before (lane 1) and 30 h after chlamydial infection (lane 2), and extended with reverse transcriptase in the presence of cold dNTPs. The extension product is indicated as P₄. Lanes T, C, G and A show results obtained by sequencing pCT-D DNA using the same primer: these served as markers to allow correct sizing of the P₄ electrophoretic band. (c) Mapping of the ORF3-ORF4 transcript 3' end by S1-nuclease protection with the 913 bp probe D. Lanes: 1 and 6, molecular mass markers; 2 and 3, control samples containing no RNA before (2) and after (3) S1-nuclease treatment; 4 and 5, results obtained with RNA from Vero cells before (4) and 30 h after chlamydial infection (5). The major S1-nuclease-protected segment in lane 5, given by transcripts starting upstream, and ending within the probe D sequence (see Fig. 1a), is indicated as T_{3/4}.

D, Fig. 1a). The probe was 3'-end-labelled with ³²P, hybridized to the same 30 h p.i. RNA sample used previously, and digested with S1-nuclease. The results (Fig. 3c) showed a major protected DNA band of about 800 bases, which corresponded to an RNA transcript originating upstream of probe D, and ending about 113 nt within the ORF5 sequence (Fig. 1a). Therefore both 5' and 3' end mapping data are consistent with the presence of a 1390 nt long ORF3-ORF4 transcript, in good agreement with the 1300 nt value deduced from Northern blot analysis.

Since Northern blot analysis indicated the presence of RNA species hybridizing to ORF4 probes but not to ORF3 probes, a primer complementary to a sequence downstream of the ATG start codon of ORF4 was hybridized to chlamydial mRNA and extended to detect possible RNA 5' ends immediately upstream of ORF4. The result (Fig. 3b) showed the presence of an RNA 5' end mapping at 29 nt upstream of the ATG start codon

of ORF4. A transcript starting at this site and ending at the major RNA 3' endpoint would be 539 nt long, a value which is consistent with the presence of the band at 500 nt on the Northern blots. The minor RNA bands seen on Northern blots, and minor S1-nuclease-protected DNA bands observed in the 3' end mapping experiment, could be due to RNA degradation or processing; these molecular species, being outside the scope of the present study, were not investigated further.

The ORF3-encoded protein

The segment of pCT-D DNA corresponding to ORF3 (Fig. 1b) was cloned into the inducible expression vector pEX34A. *E. coli* cells, transformed with the recombinant vector, overexpressed, on induction, a 39 kDa hybrid protein (MS2/pgp3) which, according to the DNA construct, should comprise an 11 kDa N-terminal segment of the MS2 bacteriophage replicase fused to the

Fig. 4. Detection of the 28 kDa chlamydial antigen by antibodies raised against the 39 kDa pgp3 fusion protein. Protein samples were electrophoretically fractionated on 12.5% SDS-polyacrylamide gels, blotted on nitrocellulose and reacted with the rabbit serum described in the text. Lane 1: partially purified protein extract (Nicosia *et al.*, 1987) of *E. coli* over-expressing the 39 kDa fusion protein; the equivalent of 40 μ l of an overnight culture was loaded on the gel. Lane 2: purified Go/86 EBs (equivalent of 50 μ g of EB protein) were solubilized by boiling in Laemmli's sample buffer, containing 1% SDS, 10 mM-2-mercaptoethanol, and loaded directly on the gel.

28 kDa chlamydial polypeptide, pgp3 (Fig. 1b). Rabbits were immunized with electrophoretically purified MS2/pgp3 and the serum so obtained was used to detect the putative ORF3 product in the chlamydial cell. Fig. 4 shows an immunoblot analysis of partially purified *E. coli* extracts containing MS2/pgp3 (lane 1), and protein extracted from purified EBs of *C. trachomatis* Go/86 (lane 2). The serum recognizes the 39 kDa band used for immunization in the *E. coli* extract (smaller additional bands are possibly degradation products), and only one protein band, of 28 kDa in the chlamydial extract. Similar blots (not shown) developed with a mAb specific for the MS2-replicase segment, showed the 39 kDa band in the *E. coli* extract but no bands in the chlamydial extract. The rabbit serum could specifically detect, by indirect immunofluorescence, typical chlamydial inclusions in infected cell cultures, which were fixed with methanol 30 h p.i. (Fig. 5). The fluorescence within the chlamydial inclusions appeared both as a diffused cloud and as discrete, more brilliant dots of variable size. Attempts to detect a reaction of the anti-MS2/pgp3 serum with unfixed EB preparations by immunofluorescence gave negative results.

Membrane association of the 28 kDa antigen

Purified EBs were treated with 2% Sarkosyl or 2% SDS in the presence or absence of a reducing agent (Bavoil *et al.*, 1984). Sarkosyl treatment is commonly assumed to discriminate the proteins of Gram-negative bacteria into 'outer-membrane' (insoluble) and 'cytoplasmic' (sol-

uble) fractions. Sarkosyl-soluble and -insoluble fractions were analysed by Western blotting, using a mixture of anti-pgp3 rabbit serum and, as a control, a mAb against the MOMP. The results showed that, whilst some of the 28 kDa antigen was solubilized by 2% Sarkosyl, a greater proportion of it remained associated with the insoluble, 'membrane' fraction (Fig. 6). The control treatments with 2% SDS, in contrast, solubilized most of the MOMP and all of the 28 kDa antigen. The behaviour of the 28 kDa antigen essentially paralleled that observed for the MOMP, with the exception that Sarkosyl treatment under reducing conditions, which did not alter MOMP solubility, appeared to release a certain amount of the 28 kDa antigen into the supernatant (Fig. 6, lanes 6 and 7). To investigate whether parts of the 28 kDa antigen could be exposed on the chlamydial surface, purified EB samples were treated with proteases (α -chymotrypsin, thermolysin and trypsin) and analysed by immunoblotting. Protease-digested EB protein extracts were analysed by Western blotting. The results (Fig. 7) showed that, whilst the surface-exposed MOMP was sensitive to protease digestion as expected (Hackstadt & Caldwell, 1985), the 28 kDa band was not affected.

Recognition of pgp3 by human immune sera

The possibility that antigenic determinants of pgp3 may be immunogenic in human chlamydial infections was investigated by immunoblot analysis with sera from 32 patients positive for anti-chlamydia antibodies, and 20 sero-negative blood donor controls. The patient sera were obtained either from females with pelvic inflammatory disease (PID; 10 cases) or from males with isolation-positive chlamydial urethritis (22 cases). The group selected had anti-chlamydia IgG titres of ≥ 64 (male urethritis cases) or 128–520 (PID cases). Screening with chlamydial EB extracts was found to be unsuitable because for immunoblot detection of bands in the 28 kDa range, the SDS-polyacrylamide gels had to be overloaded with a consequent loss of resolution. Furthermore, other chlamydial antigens in the 27–29 kDa range have been described and assignment of signals to the plasmid-encoded 28 kDa antigen would have been unreliable. Analysis was therefore performed with a partially purified protein extract from *E. coli* over-expressing the MS2/pgp3 protein. Controls were performed with similar extracts from *E. coli* transformed with the pEX34A vector, and therefore overexpressing the MS2-replicase peptide only. All sera were used at a standard 1:100 dilution, and none of them reacted with the MS2 peptide encoded by the pEX34A vector. All 20 chlamydia-negative control sera reacted with one or more bands of the *E. coli* extracts, but none of them reacted with the recombinant 39 kDa antigen. A typical

Fig. 5. Indirect immunofluorescence results obtained with rabbit anti MS2/pgp3 antibodies reacting with (a) McCoy cell cultures (bar, 2.4 μ m) or (b) LLCMK2 cells (bar, 4.5 μ m) infected with *C. trachomatis* and fixed with methanol. Similar results were obtained using either *C. trachomatis* Go/86 or L2/434/Bu.

Fig. 6. Immunoblot analysis of soluble and insoluble fractions of Go/86 EBs, after detergent treatment, probed with a mixture of E7-12 (anti-MOMP) and anti-MS2/pgp3 antibodies. The positions of the MOMP and 28 kDa bands are indicated. Lanes: 1, untreated EBs; 2 and 3, pellet (2) and supernatant (3), after treatment with 1% Sarkosyl; 4 and 5, pellet (4) and supernatant (5) after treatment of the 1% Sarkosyl pellet with 2% Sarkosyl; 6 and 7, as for lanes 4 and 5, in the presence of 10 mM-DTT; 8 and 9, pellet (8) and supernatant (9) after treatment with 2% SDS; 10 and 11, as for lanes 8 and 9, in the presence of 10 mM-DTT.

Fig. 7. Immunoblot analysis of Go/86 EBs after protease treatment. Lanes 1 to 4, untreated EBs (1), and EBs treated with α -chymotrypsin (2), thermolysin (3) and trypsin (4). (a) Blot probed with an immune serum detecting MOMP peptides. (b) Duplicate blot probed with anti-MS2/pgp3 antibodies. Arrows show the position of the intact MOMP band. Molecular mass markers are indicated on the left.

result is shown in Fig. 8, strip 1. In contrast 15 (68%) of the 22 urethritis sera, and all the PID sera showed, besides the variable pattern of *E. coli* bands observed in the control sera, a clear reaction with the MS2/pgp3 protein band. In Fig. 8, strips 2–16, typical immunoblots obtained from patients from both patient groups are

Fig. 8. Screening of human sera. The strips shown are from a Western blot prepared with a partially purified extract of *E. coli*, over-expressing the MS2/pgp3 antigen. The arrow on the right shows the position of the 39 kDa band identified by staining the gel with Coomassie blue, and by testing a portion of the blot with the anti-MS2/pgp3 serum. All sera were diluted 1:100. Strips: 1, typical result obtained with sero-negative control sera; 2 to 6, results obtained with urethritis sera; 7 to 16, results obtained with PID sera. Molecular mass markers are indicated on the left.

shown. It can be seen that the intensity of signal from the overexpressed MS2/pgp3 antigen was variable, but did not seem to correlate with the general intensity of response to *E. coli* antigens.

Discussion

ORF3 transcripts were detected in the late phase of the chlamydial infection cycle mostly as dicistronic RNA molecules together with ORF4; however, transcripts of ORF3 or ORF4 alone were also detected. Two RNA 5' ends were mapped at 19 and 29 nt upstream from the ATG start codons of ORF3 and ORF4, respectively, and a single major RNA 3' end was mapped at 1390 and 536 nt from the two 5' ends, respectively. No transcription termination signals could be identified near the 3' end map positions from nucleotide sequence analysis.

Sequences at 10 and 35 nt upstream of the startpoint of ORF3 transcripts (Fig. 1b), TTATAA and TTGAAA, respectively, show similarity to the -10 (TATAAT) and -35 (TTGACA) consensus sequences for σ^{70} -selected promoters in *E. coli*. The chlamydial -35 sequence T...CTTGA may also be considered as similar to -35 sequences found in σ^{32} -selected heat-shock promoters of *E. coli* (TNtCNCcCTTGAA; Cowing *et al.*, 1985). A putative chlamydial promoter with similar characteristics (i.e. similarity to *E. coli* heat-shock promoters limited to the -35 region only) has been described (Birkelund *et al.*, 1990) for a gene encoding a chlamydial protein homologous to the heat-shock DnaK protein of *E. coli*. However, whilst the putative *dnaK* promoter (Birkelund *et al.*, 1990) and other pCT

promoters (Ricci *et al.*, 1993) have been reported to function in *E. coli*, attempts to detect ORF3 transcripts in pCT-transformed *E. coli* (grown at 27, 37 or 42 °C) gave negative results (unpublished data). Similarly, we could not identify promoter sequences for ORF4 or detect ORF4 transcription in *E. coli* (unpublished data). Therefore, whether ORF3–ORF4 transcription in *C. trachomatis* is controlled by sequences immediately upstream of these ORFs remains to be proven.

ORF3 was expressed in *E. coli* as a 39 kDa fusion protein. Antibodies against this protein were mono-specific for a 28 kDa antigen present in protein extracts of chlamydial EBs and recognized chlamydial inclusions in methanol-fixed infected cell cultures. These data suggest that the 28 kDa chlamydial antigen is probably identical to the ORF3-encoded polypeptide, pgp3. Furthermore, the isoelectric point of the 28 kDa EB antigen deducible from bidimensional electrophoretic maps (pI = 4.3–4.6; L. Bini & V. Pallini, personal communication) is in agreement with the value which can be calculated for the pgp3 amino acid sequence.

The 28 kDa antigen remained essentially insoluble upon treatment with Sarkosyl, a property which is typical of outer-membrane proteins in Gram-negative bacteria. Although *C. trachomatis* is an atypical Gram-negative bacterium lacking peptidoglycan, treatment of EBs with Sarkosyl was shown to generate cytoplasm-deprived cell 'ghosts' (Caldwell *et al.*, 1981) called chlamydial outer-membrane complexes (COMCs). This solubility behaviour would not have been predicted from the amino acid sequence of pgp3, since it does not show a recognizable N-terminal signal peptide for secretion, nor a hydrophobicity profile (Devereux *et al.*, 1984; Kyte & Doolittle, 1982) indicative of a membrane protein. Also, the stability of the 28 kDa protein towards protease digestion of intact EBs, and the fact that for immunofluorescence detection, methanol fixation of the inclusions was required for antibody accessibility, argue against a surface location of this antigen. Therefore, Sarkosyl insolubility seems more likely to be due to some kind of interaction of the 28 kDa protein with membrane structures, e.g. through a specific domain or a side-chain modification providing a membrane-anchoring moiety. Alternatively, the protein could be transported into the periplasmic space by a secretion system not requiring a classical N-terminal signal peptide, and could remain in this way entrapped in the Sarkosyl-insoluble COMCs.

Specific IgG's present in the sera of some patients, with proven or probable *C. trachomatis* infection, recognized ORF3-encoded peptides. The implication is that these antibodies may be generated by an immune response elicited by the plasmid-encoded 28 kDa antigen in the course of chlamydial infections. This observation adds interest for further investigations on the significance

of this new antigen for chlamydial pathogenicity. On the whole, the data support the initial hypothesis (Comanducci *et al.*, 1990b) that plasmid gene ORF3 has some function in the chlamydial cell rather than being required just for plasmid maintenance.

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