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Sequence Analysis of the Gene Encoding the *Chlamydia pneumoniae* DnaK Protein Homolog

JODI M. KORNAK, CHO-CHOU KUO, AND LEE ANN CAMPBELL*
Department of Pathobiology, University of Washington, Seattle, Washington 98195

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The antigen-coding region of a 4.2-kb *Pst*I fragment of *Chlamydia pneumoniae* (pLC3), which encodes a 75-kDa immunoreactive protein recognized during human *C. pneumoniae* infection, was localized to a 2.0-kb *Eco*RI fragment. This subclone expressed an immunoreactive fusion protein of ca. 82 kDa. Nucleotide sequence analysis of the *C. pneumoniae* gene revealed that it consisted of a 1,980-base open reading frame with an inferred 71,550-Da protein of 660 amino acids. Putative *Escherichia coli*-like promoters and a ribosomal binding site were located in the 5′ upstream region, and an 11-base dyad forming a stable stem-loop structure following two in-frame stop codons was identified. The *C. pneumoniae* 75- kDa protein is a member of the hsp70 family of heat shock proteins and has 87% amino acid similarity with the *Chlamydia trachomatis* protein.

Chlamydia pneumoniae, formerly known as *Chlamydia* sp. strain TWAR, is a newly established species of the genus *Chlamydia* (9). The pathogen has been shown to be an important cause of acute respiratory disease in humans (8). It has been associated with bronchitis and pharyngitis in addition to endemic and epidemic pneumonia (8). Because of the difficulty associated with isolating and growing large amounts of the organism for antigenic and biochemical characterization, we have used recombinant DNA techniques to identify genes encoding immunoreactive proteins. Previously, a 4.2-kb *Pst*I fragment of *C. pneumoniae* was found to encode a 75-kDa protein. This gene was shown to contain a genus-reactive determinant that was recognized during *C. pneumoniae* infection (6). Separately, Maclean et al. (16) described a 75-kDa protein in *Chlamydia trachomatis* that was genus reactive. Monospecific polyclonal rabbit antisera to this protein neutralized infectivity. Sequence analysis of the gene encoding the *C. trachomatis* 75-kDa protein revealed sequence homology to the hsp70 family of heat shock proteins (2, 7).

The purposes of our studies with the gene encoding a 75-kDa protein of *C. pneumoniae* were to identify the region encoding the genus-reactive determinant, to precisely define the coding sequence of the gene, to investigate the relationship between the *C. pneumoniae* gene and the *C. trachomatis* gene, and to perform DNA and amino acid sequence analyses.

The *C. pneumoniae* isolate AR-39, which had been adapted to grow in HeLa 229 cells, was harvested and purified in a linear gradient of meglumine diatrizoate (Hypaque-76; Winthrop-Breon Laboratories, New York, N.Y.) (13). The final products usually contained 1.0×10^8 to 5.0×10^8 inclusion-forming units per ml of organisms.

The 4.2-kb *Pst*I fragment of *C. pneumoniae* AR-39 (pLC3) (6) was digested with *Eco*RI, and the resulting fragments were ligated into similarly digested pATH1, pATH3, and pATH11 vectors (25) by standard protocol (17). These vectors permit cloning in all possible reading frames and result in the overexpression of a fusion polypeptide linked to the amino terminus of the *Escherichia coli trpE* operon. The *trpE* portion is a 37-kDa polypeptide. After transformation into *E. coli* HB101 by the method of Hanahan (10), transformants were plated onto Luria-Bertani agar containing 20 μg of tryptophan per ml and 50 μg of ampicillin per ml. Transformants were screened for inserts by DNA hybridization, and plasmid inserts were analyzed by restriction endonuclease digestion. The plates were overlaid with nitrocellulose disks (Schleicher & Schuell, Inc., Keene, N.H.), colonies were lysed, and adsorbed DNA was probed with the gel-purified ^{32}P-labeled 4.2-kb *Pst*I fragment from pLC3 at 42°C as previously described (5). Plasmid DNA was isolated from the recombinant clones by standard techniques (17) and then was digested with *Eco*RI.

HB101 strains containing recombinant plasmids were induced for the expression of recombinant fusion protein by using previously described methods to prepare whole cell lysates and insoluble fractions (12, 25). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels with a 5% stacking gel by the method of Laemmli (14). After protein separation by SDS-PAGE, proteins were transferred electrophoretically to nitrocellulose by the method of Towbin et al. (27). Immunoblotting was performed as described previously by using rabbit immune sera prepared against *C. pneumoniae* AR-39 (6).

The Sanger dideoxy-chain termination method of DNA sequencing (22) was carried out on single-stranded fragments cloned into M13mp18 and M13mp19 (28) by using the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio). Exonuclease III digestions were done to generate nested deletions by using the Erase-a-Base kit (Promega, Madison, Wis.). Sequence analyses were performed by the Pustell sequence analysis program (IBI) and the University of Wisconsin Genetics Computer Group programs.

In order to localize the region encoding the genus-reactive determinant in the *C. pneumoniae* clone pLC3, *Eco*RI restriction fragments making up the 4.2-kb *Pst*I fragment (Fig. 1) were subcloned into the pATH expression vectors. Protein profiles demonstrated the induction of a ca. 82-kDa fusion protein in a pATH11 recombinant clone containing the 2.0-kb *Eco*RI fragment (Fig. 2A). Immunoblots revealed that this protein was recognized by anti-*C. pneumoniae* rabbit immune sera (Fig. 2B). Recombinant clones containing the 1.1-kb *Eco*RI fragment did not express any novel or immunoreactive fusion proteins (data not shown).

* Corresponding author.

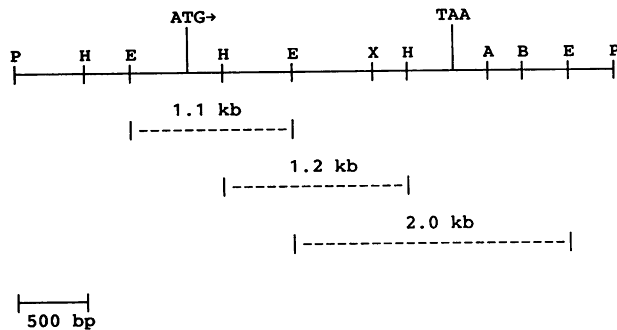

FIG. 1. Restriction map of and sequencing strategy for the 4.2-kb *Pst*I *C. pneumoniae* fragment. The 2.0-kb *Eco*RI and the adjacent 1.1-kb *Eco*RI fragments were sequenced in addition to the overlapping 1.2-kb *Hin*dIII fragment. The resulting ATG start and TAA stop codons for the gene encoding the *C. pneumoniae* 75-kDa protein are indicated. Restriction sites: A, *Ava*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; X, XhoI.

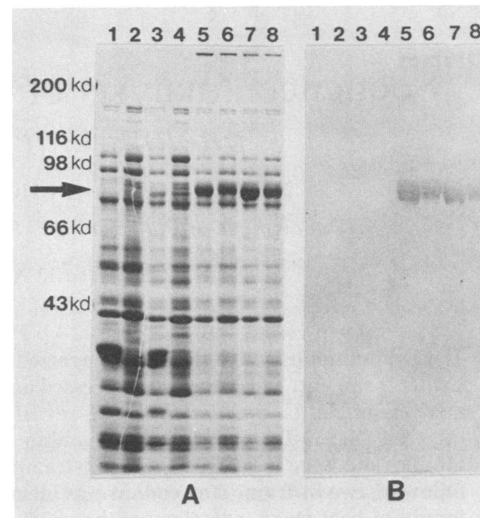

FIG. 2. SDS–10% polyacrylamide gel stained with Coomassie blue (A) and corresponding immunoblot (B). Shown are whole-cell lysates and the insoluble fraction, respectively, of *E. coli* HB101 containing induced pATH11 (lanes 1 and 3), uninduced pATH11 (lanes 2 and 4), induced pATH11 with 2.0-kb *Eco*RI insert (lanes 5 and 7), and uninduced pATH11 with 2.0-kb *Eco*RI insert (lanes 6 and 8).

Southern blot analyses comparing the *C. pneumoniae* gene (pLC3) to the *C. trachomatis dnaK* homolog (7) showed that the 2.0- and 1.1-kb *Eco*RI fragments and an overlapping 1.2-kb *Hin*dIII fragment of *C. pneumoniae* had sequence homology with the *C. trachomatis* gene (this clone was kindly provided by Robert C. Brunham, Department of Medical Microbiology, University of Manitoba) (data not shown). Subsequently, these three fragments were cloned into M13mp18 and M13mp19 and sequenced by generating nested deletions. The complete nucleotide sequence encoding the 75-kDa *C. pneumoniae* protein and the inferred amino acid sequence are shown in Fig. 3. The open reading frame, beginning with a codon for methionine, consists of 1,980 nucleotides and ends with two in-frame stop codons. Comparison of this open reading frame with the *C. trachomatis* serovar D sequence reported by Danilition et al. (7) revealed that the two genes are 73% homologous at the DNA level. Thirty bases downstream from the second stop codon, an 11-base dyad, beginning at base 2011 and ending at base 2036, was identified.

Upstream from the translational start site, the Shine-Dalgarno sequence (24) is indicated by the boxed 5-bp sequence in Fig. 3 centered at −11. This sequence was identified by searching for homology with the 3′ end of the *E. coli* 16S rRNA. Further upstream, potential transcription promoters were identified by their homologies to typical *E. coli* promoters (11). The −35 and −10 consensus regions are centered around −129 and −108, respectively.

The *C. pneumoniae* open reading frame encodes a protein of 660 amino acids with a calculated molecular weight of 71,550. The protein is 40% hydrophobic with areas of intervening hydrophilicity. Comparison of the inferred amino acid sequence of the *C. pneumoniae* 75-kDa protein with that of the *C. trachomatis* serovar D protein indicated 87% similarity. In addition, regions that are heterogeneous among the hsp70 proteins but conserved between the 75-kDa proteins of *C. trachomatis* and *C. pneumoniae* have been identified (amino acids 23 to 36, 69 to 140, 281 to 326, 529 to 559, and 603 to 647) (Fig. 4). There is also a region which is relatively conserved among the hsp70 proteins but unique to the 75-kDa protein of *C. trachomatis* (amino acids 198 to 215).

A comparison of the nucleotide sequence of the 75-kDa gene of *C. pneumoniae* with sequences of the GenBank nucleic acid sequence data base (release 63) revealed a large degree of similarity between the 75-kDa gene and genes encoding members of the hsp70 family (Fig. 4). As previously reported with the *C. trachomatis* protein (2, 7), the hsp70 amino acid sequences of *Bacillus megaterium* DnaK and *E. coli* DnaK showed the greatest degrees of similarity (74 and 72%, respectively) to that of the *C. pneumoniae* 75-kDa protein.

In general, the molecular genetic characterization of *Chlamydia* spp. has been difficult because of the lack of good mutagenesis techniques and the absence of known mechanisms of genetic exchange. Although recombinant DNA techniques have proved invaluable in chlamydial genetic studies, very few chlamydial genes are expressed well in *E. coli* (20). The problems encountered with *C. trachomatis* and *Chlamydia psittaci* gene expression in *E. coli* have also been found with *C. pneumoniae* (4). However, the genes encoding the 75-kDa proteins of both *C. trachomatis* and *C. pneumoniae* are readily expressed in the *E. coli* host (2, 6, 7), and the *C. pneumoniae* gene is expressed in an *E. coli* in vitro transcription-translation system (6). These observations suggest that this chlamydial promoter is recognized by the *E. coli* transcription and translation machinery. Nucleotide sequence analysis of the *C. trachomatis* gene revealed *E. coli*-like promoter sequences (7, 21). Similarly, putative promoters homologous to typical *E. coli* promoters were identified in the *C. pneumoniae* gene. In contrast, Birkelund et al. (2) described a mixed promoter for the *C. trachomatis* L2 *dnaK* gene in which the −35 region is similar to a heat shock promoter and the −10 region is a classical TATA box. However, unlike most heat shock genes, which have highly conserved promoters, no classical heat shock promoters were identified upstream of the *C. pneumoniae* gene. Pollack et al. (21) also reported the absence of typical heat shock promoter sequences in the *C. trachomatis* mouse biovar gene encoding a DnaK homolog. Whether or not the *C. pneumoniae* gene is being transcribed from the *E. coli*-like promoters will remain unclear until S1 nuclease experiments

FIG. 3. Nucleotide sequence and inferred amino acid sequence of the gene encoding a 75-kDa protein of *C. pneumoniae*. The nucleotide and amino acid sequences of the corresponding *C. trachomatis dnaK* gene are also shown (above and below, respectively) where they differ from those of the *C. pneumoniae* gene. A dot (·) indicates a gap in the sequence. The gene contains an ATG start codon followed by a 1,980-nucleotide open reading frame and ends with two in-frame TAA stop codons. The putative promoter is shown as a boxed region upstream centered at −108 and −129. The ribosomal binding site is shown as a boxed region centered at −11. The 11-base transcription terminator dyad is indicated by the inverted arrows from 2011 to 2036.

are done. In *C. trachomatis*, the promoter recognized by the chlamydial RNA polymerase during heat shock-induced transcription of the chlamydial *dnaK* gene appears to be different from the *E. coli*-like promoter sequence upstream from the gene (21).

While putative promoter and terminator regulatory regions in other chlamydial genes have been identified (7, 23, 26), no chlamydial consensus promoter sequences have been identified. The terminator sequences reported for other chlamydial genes demonstrate typical rho-independent stem-loop structures (7, 26). In contrast, the region downstream of the translational stop signal in the *C. pneumoniae* gene revealed an 11-base dyad which forms a stem-loop structure, but there are no thymidine residues characteristic of the rho-independent terminator. The terminator sequence for the *C. pneumoniae* major outer membrane protein gene also contains a stem-loop structure which lacks a poly(T) tail (20a). However, there is another predicted stem-loop structure formed from bases 2036 to 2090. Transcription of the 11-base dyad would result in the folding of the mRNA into a stem-and-loop structure with a calculated least free energy of −15.0 kcal. If the transcription of the second stem-loop structure is included in this calculation, a lower calculated least free energy of −22.7 kcal results. Perhaps the second stem-loop structure acts in conjunction with the 11-base dyad to terminate transcription.

```
                  1                                                 50
C. pneumoniae     MSEHKKSSKIIGIDLGTTNSCVSVMEGGQAKVITSSEGTRTTPSIVAF.K
C. trachomatis    ---KR--N----------------------P---A--------------.-
E. coli           ......MG--------------AI-D-TTPR-LENA--D------I-YTQ
B. megaterium     ......M---------------A-L---EP---PNP--N------V--.-
51                                                                   120
GNEKLVGIPAKRQAVTNPEKTLGSTKRFIGRKY..SEVASEIQTVPYTVTSGSKGDAVFEVDGKQYTPEE
-G-T------------------A---------F..---E---K----K-APN-------D-EQ-L-----
DG-T---Q----------QN--FAI--L---RFQDE--QRDVSIMF-KIIAADN---WV--K-QKMA-PQ
NG-RQ--EV-----I---N.-II-V--HM-........................T-HKV-AE------Q-
121                                                                  190
IGAQILMKMKETAEAYLGETVTEAVITVPAYFNDSQRASTKDAGRIAGLDVKRIIPEPTAAALAYGIDK.
---------------------------------------------------------------------.
-S-EV-K---K---D----P--------------A--QA----------E-----N----------L--G
MS-I--QHL-GY--E----P--K-----------AE-QA-----K----E-E---N----------LL-T
191                                                                  260
VGDKKIAVFDLGGGTFDISILEI....GDGVFEVLSTNGDTLLGGDDFDEVIIKWMIEEFKKQEGIDLSK
E------SLR-RRRN-RYFY-GNRW-...------------H-------........-------------
T-NRT---Y-----------I--DEVD-EKT----A-----H---E---SRL-NYLL-----DQ----RN
DE-QTVL-Y--------V----L....-------RA-A--NR-------Q---DYLVA----EN-K----
261                                                                  330
DNMALQRLKDAAEKAKIELSGVSSTEINQPFITMDAQGPKHLALTLTRAQFEKLAASLIERTKSPCIKAL
----------------------------------I--N---------------H--S------Q--AQ--
-PL-M----E----------SAQQ-DV-L-Y--A--T----MNIKV---KL-S-VED-VN-SIE-LKV--
-K--------------KD----T--Q-SL----AGEA--L--EVS-S--K-DE-S-G-V---MA-VRQ--
331                                                                  400
SDAKLSAKDIDDVLLVGGMSRMPAVQETVKELFGKEPNKGVNPDEVVAIGAAIQGGVLGGEVKDVLLLDV
K------S------------------AV--RSLV-SLI-A------------------------------
Q--G--VS-----I----QT---M--KK-A-F-----R-D-----A------V-----T-D---------
K--G---SEL-K-I----ST-I----DAL-KET-QD-H----------L---------T-D----V----
401                                                                  470
IPLSLGIETLGGVMTTLVERNTTIPTQKKQIFSTAADNQPAVTIVVLQGERPMAKDNKEIGRFDLTDIPP
---------------P------------------------------------------------------
T--------M-------IAK------KHS-V----E---S----H------KR-A---SL-Q-N-DG-N-
T--------M---F-K-I--------S-S-V------S-T--D-H--------SA---TL---Q------
471                                                                  540
APRGHPQIEVSFDIDANGIFHVSAKDVASGKEQKIRIEASSGLQEDEIQRMVRDAEINKEEDKKRREASD
----------T--------L------A---R------------K-----Q-I----LH-----Q-K----
----M-----T-----D--L------KN-------T-K-----N-----K------A-A-A-R-FE-LVQ
----V----------K---VN-R---LGTN---A-T-KS-T--SD---D---KE--E-ADA--Q-K-EVE
541                                                                  610
AKNEADSMIFRAEKAIKDYKEQIPETLVKEIEERIENVRNALK..DDAPIEKIKEVTEDLSKHMQKIGES
V-----G--------V---HDK--AE-------H--K--Q-I-..E--STTA--AASDE--T-------A
TR-QG-HLLHSTR-QVEEAGDKL-ADDKTA--SALTALET---GE-K-A--AKMQELAQV-QKLME-AQQ
LR----QLV-TT--TL--LEGKVE-AE-TKAN-AKDALKA-IEKN-...L-E--AKKDE-....-E-VQA
611                                                         671
MQSQSASAAASSAANAKGGPNINTEDLKKHSFSTKPPSNNGSSEDHIEEADVEIIDNDDK-
--A-------------Q------S----------R--AGGSA-STDNI-DADVE-VDKPE.
QHA-QQT-G-DAS-.........................-NAKD-DVVD-EF-EVKDKK..
LTVK-YEQ-Q......................QAQQAGEQ-AQN-DVVD-EF-EVND-K-.
```

FIG. 4. Comparison of the amino acid sequence of the 75-kDa protein of *C. pneumoniae* with those of hsp70 proteins of *C. trachomatis* (75-kDa protein), *E. coli* (DnaK), and *B. megaterium* (DnaK). A dash indicates the same amino acid as in the *C. pneumoniae* 75-kDa protein. A dot represents a gap in the sequence.

Antigenic properties of the 75-kDa chlamydial protein suggest that it is important in the elicitation of antichlamydial responses and that it may provide a broad-based target for intervention in chlamydial infection (1, 3, 16). The 75-kDa proteins of *C. pneumoniae* and *C. trachomatis* were compared to identify shared regions that might be potential targets of such a response, and they were found to have a degree of amino acid similarity higher than that of other hsp70 proteins (15). In comparing amino acid sequences of the *C. trachomatis* 75-kDa protein to other bacterial hsp70 proteins, Danilition et al. (7) identified conserved regions and variable regions. Comparison of the inferred amino acid sequences of the *C. pneumoniae* and *C. trachomatis* 75-kDa proteins identified conserved sequences shared by the two *Chlamydia* species that are found in generally divergent regions of the hsp70 proteins. Interestingly, these regions are also identified as regions of high antigenic index when a computer algorithm that compiles data which predicts secondary structure, flexibility, mobility, hydrophilicity, and surface probability is used. For other intracellular parasites, heat shock proteins have been shown to be important in the elicitation of the immune response. T-lymphocyte responses and B-cell responses against heat shock proteins have been reported for *Mycobacterium tuberculosis* (30) and *Plasmodium falciparum* (29), respectively. In the *C. trachomatis* immune response, the hsp60 homolog has been associated with the delayed-hypersensitivity immunopathology of chlamydial infection (18, 19).

The heat shock response is universal, and the stress proteins are among the most highly conserved genetic elements known (15). In addition to their immunogenic roles, a multitude of biologic functions has been ascribed to the proteins of the hsp70 family. They have been associated with a role in the developmental cycles of various organisms, including leishmanias, trypanosomes, plasmodia, histoplasmas, and chlamydiae (15, 29, 30). The hsp70 protein has also been implicated in a variety of other processes, including DNA replication, protein transport, protein binding, and the uncoating of coated vesicles (15). Perhaps the *Chlamydia* 75-kDa protein utilizes one of these mechanisms in order to perpetuate or maintain its pathogenicity.

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