Analysis and Detection of Chlamydial DNA

By TIMO HYYPIÄ,¹* STEVEN H. LARSEN,² TOM STÅHLBERG¹ AND
PERTTI TERHO^{1,3}

¹Department of Virology, University of Turku, Kiinamyllynkatu 13, SF-20520 Turku, Finland

²Department of Microbiology and Immunology and ³Department of Medicine, Indiana University
School of Medicine, Indianapolis, Indiana 46223, USA

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Elementary bodies of lymphogranuloma venereum (LGV) strains of *Chlamydia trachomatis* contain, in addition to the genomic DNA, a 6.7 kb plasmid. The plasmid from serovar L2 (434-B) was cloned at the *Bam*HI site of pBR327 into *Escherichia coli* and a restriction cleavage map of this pLGV125 recombinant plasmid determined. All 15 *C. trachomatis* serovars contained DNA sequences that hybridized with pLGV125. When total DNA from L2 elementary bodies was used as a probe in Southern blotting and spot hybridization, serovars L1, L2 and L3 exhibited significant homology. The detection level of homologous DNA was 100 pg and LGV DNA was detectable in infected cells when total L2 probe was used in the nucleic acid hybridization test. These DNA probes may be useful as investigative and diagnostic reagents for *C. trachomatis*.

INTRODUCTION

Chlamydia trachomatis strains cause ocular diseases (trachoma, conjunctivitis) and genital infections (urethritis, epididymitis, cervicitis, salpingitis, lymphogranuloma venereum) in humans (Schachter & Dawson, 1978). During the unique developmental cycle of this obligatory intracellular parasite, the infectious elementary body (EB) is transformed into a larger reticulate body (RB). After multiplication by binary fission RBs condense back to infectious EBs (Ward, 1983).

Current knowledge of the chlamydial nucleic acids is relatively limited. Both EBs and RBs contain DNA and RNA; EBs contain less RNA than DNA whereas RBs contain three to four times more RNA than DNA (Becker, 1978). Sarov & Becker (1969) isolated DNA from *C. trachomatis* TE 55 and using electron microscopy and sucrose density gradients showed that the DNA is circular with an estimated molecular mass of 660 MDal. The intraspecies DNA reassociation homology between different *C. trachomatis* strains was estimated to be 96–97% by Kingsbury & Weiss (1968). *C. trachomatis* has been reported to contain a plasmid with a molecular mass of 4.4 MDal (Lovett *et al.*, 1980; Peterson & de la Maza, 1983).

There is an obvious need for clinical diagnostic methods in chlamydial infections. Current tests, such as the demonstration of chlamydial growth in cell cultures, may be sensitive and specific, but they are laborious and relatively time consuming, and require infectious material. Diagnostic detection of specific DNA sequences by nucleic acid hybridization has recently been introduced for several micro-organisms (Brandsma & Miller, 1980; Moseley *et al.*, 1982; Stålhandske *et al.*, 1983). This method is based on hybridization between DNA of the specimen, bound onto a nitrocellulose filter, and the radioactively labelled probe. The technique is rapid, specific and sensitive.

Abbreviations: LGV, lymphogranuloma venereum; EB, elementary body; RB, reticulate body.

In the present study we have further characterized chlamydial DNA, including cloning of a plasmid, and applied the hybridization method to detect chlamydial DNA sequences in infected cells.

METHODS

Purification of chlamydial DNA. *C. trachomatis* LGV strains L1 (440-L), L2 (434-B) and L3 (404-L) were grown in McCoy cells in the presence of 1 µg cycloheximide ml⁻¹. EBs were purified by differential centrifugation in Renografin (E. R. Squibb, Princeton, USA) gradients (Terho & Matikainen, 1982).

The purified EBs were treated with 1.5% (w/v) sarkosyl, 1% (w/v) sodium dodecyl sulphate (SDS) and 20 mM-EDTA for 1 h at 37 °C, followed by digestion with 0.1 mg proteinase K ml⁻¹ (Merck) for 2 h at 37 °C. The nucleic acids were isolated by successive phenol/chloroform extractions and ethanol precipitation (Maniatis *et al.*, 1982). RNA was removed by treatment with 0.1 mg ribonuclease A ml⁻¹ (Sigma) for 30 min at 37 °C. The density of chlamydial DNA was determined by CsCl equilibrium density centrifugation.

Gel electrophoresis and Southern blotting. DNA from EBs was analysed by electrophoresis in 0.7% (w/v) or 1% (w/v) agarose gels in TEA-buffer (40 mM-Tris pH 8.1, 2 mM-EDTA, 20 mM-sodium acetate). Gels were run for 8 h at 30 mA, stained with ethidium bromide and photographed under UV light. For Southern blotting, DNA in the gel was denatured, neutralized, and transferred onto a nitrocellulose filter (BA 85, Schleicher & Schüll) (Southern, 1975). The filter was hybridized with a total L2 DNA probe as described below.

Restriction enzyme (BRL) digestions were performed as previously described (Larsen & Nathans, 1977; Larsen *et al.*, 1979).

DNA fragments were recovered from agarose gels by transfer onto DEAE-cellulose membranes (Schleicher & Schüll) by electrophoresis, elution from the membrane in 1 M-NaCl, 0.1 mM-EDTA, 20 mM-Tris/HCl pH 8.0, for 30 min at 60 °C, and precipitation with ethanol.

Cloning of the plasmid. Plasmid DNA was isolated from L2 elementary bodies by the alkaline extraction procedure of Birnboim & Doly (1979). The L2 plasmid DNA was digested with restriction endonuclease *Bam*HI and ligated to the *Bam*HI cleaved plasmid pBR327. Ligation conditions were: 66 mM-Tris/HCl pH 7.6, 10 mM-MgCl₂, 3 mM-dithiothreitol, 1 mM-ATP and T4 DNA ligase (BRL) at 16 °C for 16 h. The recombinant plasmid was transformed into *Escherichia coli* C600 strain SL1 (Rao & Rogers, 1978). Plasmid DNA from ampicillin resistant (Amp^r) and tetracycline sensitive (Tet^s) colonies was extracted and analysed by electrophoresis in 1% agarose/ethidium bromide gels (Larsen & Nathans, 1977).

Spot hybridization detection of chlamydial DNA. The spot hybridization technique was a modification of a method described by Stålhandske *et al.* (1983). McCoy cells (1.5 × 10⁵) on a circular 12 mm diameter cover glass were infected with the LGV strains and collected 48 h after infection when typical intracytoplasmic inclusions were visible in more than 80% of the cells. Infected and control cells were treated with 0.1 mg proteinase K ml⁻¹ for 1 h at 37 °C. The cell lysate, as well as the purified DNA from LGV strains, was boiled in 0.3 M-NaOH for 10 min, chilled on ice and neutralized with HCl. The specimens were then adjusted to contain 6 × SSC (0.9 M-NaCl, 0.09 M-sodium citrate; 1 × SSC = 0.15 M-NaCl, 0.015 M-sodium citrate) and spotted onto a nitrocellulose filter in a microsample filtration manifold (Schleicher & Schüll). The filter was air dried, baked for 2 h at 80 °C and pre-hybridized for 2 h at 65 °C in 6 × SSC supplemented with 5 × Denhardt's solution (Denhardt, 1966) and 0.1 mg salmon sperm DNA ml⁻¹. Total L2 DNA was labelled with ³²P, by nick translation (Rigby *et al.*, 1977), to a specific activity of approximately 1 × 10⁷ c.p.m. µg⁻¹.

The denatured DNA probe was added into the hybridization bag in 6 × SSC supplemented with 5 × Denhardt's solution and 0.5% (w/v) SDS. The filter was hybridized overnight at 65 °C, given three 45 min washes in 2 × SSC, 0.1% SDS, and then dried. The radioactivity was revealed by autoradiography, after overnight exposure, or detected by scintillation counting.

RESULTS

Characterization of the chromosomal DNA

Chromosomal DNA from strain L2 appeared as a single band after CsCl equilibrium density centrifugation and was 2 mg ml⁻¹ less dense than *E. coli* chromosomal DNA.

DNA from each of the LGV strains L1, L2 and L3 migrated as four observable bands in agarose gel electrophoresis (Fig. 1*a*). All the fragments were also detectable by hybridization with total L2 DNA probe after Southern transfer (Fig. 1*b*). The three bands with lower molecular weights were also detected by pLGV125 probe (see below), indicating that they represent three conformations of a plasmid. pLGV125 did not react with the chromosomal DNA band and no reaction appeared with chlamydial DNA when vector DNA was used as a probe in Southern

Fig. 1. Analysis of LGV DNA by Southern blotting. Purified DNA from strains L1, L2 and L3 was run in a 0.7% agarose gel and stained with ethidium bromide (a). The DNA was then denatured and transferred onto nitrocellulose, and the filter was hybridized with total L2 DNA probe. The radioactivity on the filter was detected by autoradiography after overnight exposure (b).

blotting experiments (data not shown). The size of the plasmid DNA was identical in the three strains tested.

Characterization of the plasmid DNA

A single, small, covalently closed circular DNA species was isolated from L2 EBs by the alkaline extraction procedure. This DNA (pLGV434) was cleaved with several restriction endonucleases and analysed by agarose gel electrophoresis. When compared to the *Hind*III digestion products of phage λ DNA (Sanger *et al.*, 1982) by gel electrophoresis, the pLGV434 plasmid was found to be about 6.7 kb in length. *Bam*HI and *Pst*I had single cleavage sites on the plasmid and were therefore appropriate for cloning the entire plasmid. pLGV434 and pBR327 (Soberon *et al.*, 1980) were digested with *Bam*HI and ligated together, and Amp^r Tet^s transformants were isolated. Proper insertion was confirmed by comparing restriction digests to digests of the original pLGV434 and of whole isolated DNA. One recombinant plasmid, containing the *Bam*HI insertion, was called pLGV125.

Plasmid pLGV125 was mapped by single and double enzyme digestions (Fig. 2). The location of the *Eco*RI D fragment (1.7 to 2.1 kb on the map) was identified by *Eco*RI digestion of a gel purified *Cla*I B fragment (0.4 to 4 kb). Likewise, the *Cla*I D fragment (0 to 0.4 kb) position was confirmed by *Cla*I digestion of the purified *Eco*RI C fragment (0 to 1.7 kb).

A total of 16 restriction sites for 8 different endonucleases were identified and mapped for pLGV125 (Fig. 2). Eleven of these sites were in the inserted pLGV434. The accuracy of positioning was about ± 200 bp as judged by internal consistency of double-digestion fragment lengths.

pLGV125 was labelled with ³²P by nick translation and used to hybridize to spots of all 15 *C. trachomatis* serovars (Wang & Grayston, 1982). Every serotype reacted, indicating that the pLGV434 plasmid probably contained DNA sequences which were common to all *C. trachomatis* strains (data not shown).

Fig. 2. Restriction endonuclease cleavage map of pLGV125. The single lined part of the circle represents pBR327 sequences; the double line represents the pLGV434 sequences in pLGV125. The size of the total recombinant plasmid is 10 kb as indicated inside the circle.

Fig. 3. Spot hybridization detection of L2 DNA. Various amounts of purified DNA and Herpes simplex virus (HSV) DNA (non-related control) were spotted onto a nitrocellulose filter and detected by a homologous probe. The radioactivity bound to the filter was detected either by autoradiography after overnight exposure (a) or by scintillation counting (b: ●, L2 DNA; ○, HSV DNA).

Detection of LGV DNA by spot hybridization

The sensitivity of the spot hybridization assay was evaluated by reacting the nick-translated total L2 DNA probe with various amounts of homologous DNA on a nitrocellulose filter; 100 pg DNA was detectable, giving visible spots after autoradiography (Fig. 3a). The sensitivity was similar when scintillation counting was used to demonstrate the radioactivity incorporated (Fig. 3b). Herpes simplex DNA was used as a non-related control DNA. No background reaction was

Fig. 4. Spot hybridization detection of LGV DNA. Purified DNA (100 ng) from strains L1, L2 and L3 as well as DNA from 1.5×10^5 McCoy cells, either L2 infected or uninfected, was spotted in duplicate on a nitrocellulose filter and detected with an L2 DNA probe. The radioactivity bound onto the filter was revealed by autoradiography after overnight exposure.

noticed in the test except that very large amounts of DNA on the filter caused non-specific signals (Fig. 3a). The total L2 DNA probe reacted with both L1 and L3 DNA in the hybridization test (Fig. 4), as would be expected on the basis of the Southern blotting results. McCoy cells infected with strain L2 gave a positive signal in the assay, while control cells did not (Fig. 4).

DISCUSSION

Purified DNA from LGV strains of *C. trachomatis* was analysed in CsCl gradients and by agarose gel electrophoresis. The density of L2 chromosomal DNA was 2 mg ml^{-1} less than that of *E. coli* DNA. Thus, assuming that *E. coli* DNA has a GC content of 50% (Szybalski, 1968), then chlamydial DNA has a GC content of about 47%. This result is similar to that found for other strains of *Chlamydia* (Weiss *et al.*, 1970).

Representatives of each of the three LGV serovars were examined and found to contain a plasmid of 6.7 kb. All the plasmids appeared similar by restriction enzyme analysis and hybridization. The plasmid from L2 was cloned in a pBR327 vector into *E. coli*, and a restriction cleavage map generated. The plasmid DNA was also used to probe DNA from all 15 *C. trachomatis* serovars. Every serovar had homologous sequences, suggesting that this particular DNA is present in all known *C. trachomatis* serotypes. Our results extend the previous studies of Lovett *et al.* (1980) and Peterson & de la Maza (1983). However, the function of the plasmid is not known.

The LGV strains are closely related in antigenic and biological properties and therefore it is not surprising that all the DNA fragments of the three serovars studied share sufficient homology to be cross detectable both in spot hybridization and in Southern blotting. More detailed analysis with restriction enzymes is needed to determine the amount of homology between the different strains.

Nucleic acid hybridization with the total L2 DNA probe was used to detect chlamydial sequences in infected cells. This technique also allows detection of chlamydial DNA in clinical specimens. Our preliminary results indicate that some of the isolation-positive specimens give a signal in the hybridization test when total L2 DNA or pLGV125 probes with high specific activity are used.

The specificity of the hybridization reaction must be carefully evaluated because chlamydial DNA may share homology with DNA of various other prokaryotic organisms. Kingsbury & Weiss (1968), however, could detect little if any DNA homology with bacteria of other genera, including *Neisseria gonorrhoeae*, a micro-organism likely to be found in specimens assayed for the presence of chlamydiae. Any cross reactions shown may be obviated by cloning parts of the genome or the plasmid and using these clones as probes.

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