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THE CONSTRUCTION OF GENOMIC LIBRARIES OF *COWDRIA RUMINANTIUM* IN AN EXPRESSION VECTOR, λ *gt*11*

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ABSTRACT

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Genomic libraries of the Welgevonden and Kwanyanga isolates of *Cowdria ruminantium* have been constructed in an expression vector. These libraries contain approximately 4×10^5 and 3×10^5 recombinants respectively.

INTRODUCTION

The study of gene structure and expression relies on the availability of cloned genes as probes. To analyse the molecular basis of immunity to heartwater it is essential that purified *Cowdria* antigens become available. Antigens can be purified directly from parasites by using either conventional purification techniques or affinity chromatography with monoclonal antibodies. However, in the case of heartwater these methods are severely restricted by limitations on the propagation of *Cowdria ruminantium in vitro*. Because of this, recombinant DNA techniques could be applied to eliminate these difficulties and facilitate the identification of protective antigens, the development of vaccination protocols and the analysis of the immune response.

Recombinant DNA techniques have been applied successfully in a large number of cases to isolate and identify immunologically important epitopes, as well as in the development of diagnostic DNA probes (Barker, Suebsaeng, Rooney, Alecrim, Dourado & Wirth, 1986; Knight, Simpson, Bickle, Hagan, Moloney, Wilkins & Smithers, 1986; McLaughlin, Edlind & Ihler, 1986; Owens & Diener, 1981) for the rapid identification of parasites.

With the objective of producing large amounts of *C. ruminantium* protein antigens and epitopes, and the further objective of developing DNA probes for the rapid identification of these organisms *in vivo*, we have constructed 2 expression libraries of *C. ruminantium* DNA in *Escherichia coli*. This system is based on the expression vector, bacteriophage λ *gt*11 which expresses *C. ruminantium* sequences as β-galactosidase fusion proteins.

MATERIALS AND METHODS

DNA isolation and construction of libraries

Two libraries were constructed in λ *gt*11 (Huynh, Young & Davis, 1985). In the one case (Library A) *C. ruminantium* DNA of the Welgevonden isolate (Du Plessis, 1985) was isolated from the red blood cell fraction of a sheep infected with the parasite and bled at peak parasitaemia. The blood was centrifuged 3 times and the buffy coat removed. The red cells were then lysed by adding an equal volume of distilled water and passed through a Whatman CF-11 cellulose column (Ambrosio, Potgieter & Nel, 1986).

Parasites collected from the column were lysed overnight at 37 °C in 10 % sodium dodecyl sulfate, 2 mg $m\ell^{-1}$ proteinase K, 25 mM EDTA (pH 8,0). Lysates were extracted with phenol, phenol-chloroform, chloroform and then ethanol precipitated. DNA was resuspended in TE buffer (10 mM Tris, pH 8,0, 1 mM EDTA) and used for restriction endonuclease digestion.

A second library (Library B) was constructed in λ *gt*11 using the Kwanyanga isolate of *C. ruminantium* (MacKenzie & Van Rooyen, 1981). DNA was extracted from parasites grown *in vitro* in E5 bovine endothelial cells (Bezuidenhout, 1987).

Cells were harvested and digested overnight at 37 °C in 10 % SDS, 10 mg $m\ell^{-1}$ proteinase K, 100 mM NaCl, 25 mM EDTA (pH 8,0). Digested cells were then extracted with phenol, phenol-chloroform and chloroform, ethanol precipitated and the DNA resuspended in TE buffer.

C. ruminantium DNA (for both Library A and Library B) was digested with *Eco*R1 to generate fragments suitable for cloning into λ*gt*11. Restricted DNA was ligated into the vector (Maniatis, Fritsch & Sambrook, 1982) and *in vitro* packaged with commercial packaging reactions according to the manufacturers instructions (Amersham, UK)[1]. Packaged phage were plated on *E. coli* strain Y1090 (Huynh *et al.*, 1985).

The non-recombinant background in each library was determined by growth on isopropyl-β-D-thiogalactopyranoside (IPTG)-5-bromo-4-chloro-3-indolyl-β-D-galactopiranoside (X-gal) plates (Huynh *et al.*, 1985).

Screening of libraries

Plaque hybridisations

Total DNA preparations from *C. ruminantium* (Welgevonden) and *C. ruminantium* (Kwanyanga) infected material were radiolabelled with (α–P) CTP (Amersham, 3000 Ci $mmol^{-1}$) by nick-translation (Rigby, Dieckmann, Rhodes & Berg, 1977). Labelled DNA (8×10^6 cpm per 100 ng DNA) was then boiled for 5 min and added directly to the filters for hybridisation. Hybridisation was at 65 °C for 16 h. Filters were washed under conditions of moderate stringency (2X SSC, 37 °C) followed by one high stringency wash (1X SSC, 65 °C). The hybridisations were analysed by routine autoradiography.

Immune-blotting of libraries

The libraries were screened as phage plaques on *E. coli* host strain Y1090 at a density of approximately 1 000 plaques per 82 mm plate. After plating, the plates were incubated for 3 h at 42 °C to initiate plaque formation without expression from the *lac*Z gene promoter of λ *gt*11. Plates were then overlaid with nitrocellulose disk saturated with IPTG (10 mM) and incubated for a further 3 h at 37 °C to induce *lac*Z-directed expression. The filters were then rinsed in TBS (50 mM tris-HCl, pH 8,0, 150 mM NaCl) and screened with antibody probes for antigen produced by recombinant clones (Huynh *et al.*,

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1985). Positive clones were identified by using peroxidase conjugated second antibody and detected with 4-chloro-1-naphtol as substrate (Adams, Smith & Kuhlenschmidt, 1986). Primary antibody was from an hyperimmune bovine and was used diluted in 50 % horse serum.

Fusion proteins were produced in *E. coli* strain Y1089 essentially as described by Huynh *et al.* (1985) and identified by western blot analysis using monoclonal anti-β-galactosidase antibodies followed by rabbit anti-cow IgG conjugate.

Results and Discussion

Using DNA derived from *C. ruminantium* (Welgevonden isolate) infected blood a DNA library was constructed in λ *gt*11. To minimise the possibility of host DNA contamination in the library, the red blood cell fraction was passed through a Whatman CF-11 cellulose column which has been shown to remove most of the host's DNA (Ambrosio *et al.*, 1986). The library was constructed after *Eco*R1 digestion of the *Cowdria* DNA to yield fragments of up to 6 kb for cloning into the unique *Eco*R1 site of λ *gt*11. This library (Library A) contained approximately 2×10^5 to 4×10^5 recombinant clones.

A second library was constructed as described above from bovine endothelial cell culture infected with the Kwanyanga isolate of *C. ruminantium*. As reported elsewhere (Bezuidenhout, 1987), these cells produce large numbers of parasites and are thus suitable for the isolation of *Cowdria* DNA. This library contains approximately 3×10^5 recombinant clones of which a significant number should be bovine DNA recombinants as it was not possible to differentially lyse the E5 cells to isolate unlysed *Cowdria* organisms. Plating both libraries on IPTG-Xgal demonstrated that less than 8 % of the clones did not contain a DNA insert.

Library A was screened with a high titer polyclonal serum from an hyperimmune bovine as described under the previous heading "Materials and methods" in conjunction with a peroxidase-conjugated second antibody.

Approximately 50 000 clones were screened and 6 putative positive clones were isolated. Normal serum always produced background levels of antibody binding. Library B was screened by plaque hybridization (Maniatis *et al.*, 1982) of filters probed with nick-translated DNA from infected E5 cells.

The library was also probed with *C. ruminantium* DNA depleted of bovine DNA contamination by first hybridising total nick-translated *C. ruminantium* DNA to 200 μg of nitrocellulose bound bovine DNA. Neither of these approaches have so far yielded positive clones. This could possibly be due to the high bovine DNA background in this library reducing the probability of a *Cowdria* sequence being detected.

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