USE OF CONVERSION ADAPTORS TO CLONE ANTIGEN GENES IN \textit{Ag}t11

C. K. STOVER, M. G. VODKIN, AND E. V. OAKS

MANUSCRIPT

DTIC ELECTED

OCT 7 1988

Charles R. Stover

DD FORM 1473, 84 MAR

UNCLASSIFIED
Use of Conversion Adaptors to Clone Antigen Genes in $\lambda$gt11

CHARLES K. STOVER,* MICHAEL H. VODKIN,† AND EDWIN V. OAKS*

*Department of Rickettsial Diseases, Walter Reed Army Institute of Research, Washington, D.C. 20307-5100, and
†U.S. Army Medical Research Institute of Infectious Diseases, Airborne Diseases Division, Rickettsial Diseases Laboratory, Fort Detrick, Frederick, Maryland 21701-5011

Received September 26, 1986

A strategy has been devised and tested to employ EcoRI conversion adaptors for cloning cohesive-ended restriction fragments into the unique EcoRI site of the $\lambda$ gt11 expression vector. Five $\lambda$gt11 chromosomal libraries were constructed with Rickettsia tsutsugamushi genomic DNA digested with restriction enzymes generating five different cohesive ends. Recombinant phage yields as high as 10$^9$ plaque forming units were achieved without amplification of the five libraries. Sequences encoding epitopes of all eight R. tsutsugamushi polypeptide antigens, previously identified by Western blot analysis, were obtained in the five $\lambda$gt11 expression libraries. Recombinant antigen expression was dependent on $\lambda$gt11 lac promoter induction in 39% of the recombinants assayed. This method significantly improves the efficiency of genomic $\lambda$gt11 library construction by eliminating blunt-ended ligation and simplifying the removal of unligated EcoRI-ended oligonucleotides.

KEY WORDS: recombinant DNA; restriction fragments; antigens; expression vector; Rickettsia tsutsugamushi; $\lambda$gt11 adaptors.

The $\lambda$gt11 expression vector system was developed for the cloning and expression of foreign gene products in Escherichia coli (1-4). In this system, genes can be expressed either as 8-galactosidase fusion proteins, dependent on the lac promoter, or as polypeptides not regulated by the lac promoter. Foreign antigens produced in a recombinant $\lambda$gt11 library are detected by immunoscreening with polyclonal or monoclonal antisera. Cloning into the unique EcoRI site of the $\lambda$acZ gene in $\lambda$gt11 requires that the insert DNA fragments be less than 8.3 kb (1,2) and have EcoRI cohesive ends. Random fragments of genomic DNA are usually generated for $\lambda$gt11 cloning by mechanical shearing, which necessitates the repair of damaged DNA ends before the blunt-end ligation to EcoRI linkers can be accomplished. The ligation of EcoRI linkers to blunt-ended insert DNA must be driven by a large molar excess of linker. Following linker ligation, two cycles of EcoRI digestion and size fractionation (by gel filtration or gel electrophoresis) are usually required to completely remove excess linkers. Quantitative removal is necessary to prevent the oligonucleotide linkers from competing with insert DNA for ligation to the EcoRI site of the $\lambda$gt11 vector. A significant loss of genomic insert DNA usually occurs when excess linkers are removed.

To improve the efficiency of $\lambda$gt11 cloning, duplex conversion adaptor oligonucleotides (5) were designed, synthesized, and used to construct $\lambda$gt11 expression libraries with genomic insert DNA generated by digestion with restriction endonucleases which leave protruding 5' cohesive termini (Fig. 1). Conversion adaptors are short duplex oligonucleotides that have two different cohesive ends (5). An EcoRI conversion adaptor carries an EcoRI cohesive end at one terminus (for $\lambda$gt11 ligation) and a specified cohesive end at the opposite terminus (for genomic DNA ligation). EcoRI conversion adaptors of three different lengths were
constructed so that gene fusion in all three reading frames was possible. Because ligation between cohesive-ended DNA is much more efficient than blunt-end ligation, it was not necessary to use the large molar excess of oligonucleotide required to drive linker ligations. Consequently, excess adaptors were easily removed by PEG2 precipitation with minimal losses of insert DNA.

The use of EcoRI conversion adaptors allows the rapid (1 day) and efficient construction of genomic λgt11 expression libraries by eliminating the most inefficient and troublesome steps (blunt-end ligation and removal of linker excess) normally encountered when constructing λgt11 libraries with sheared or blunt-ended DNA. The use of these EcoRI adaptors is demonstrated with the cloning of eight polypeptide genes of R. tsutsugamushi (an obligate intracellular bacterium which causes scrub typhus in humans) in λgt11 using genomic DNA inserts generated by restriction enzymes recognizing and cutting at conserved tetrancleotide or degenerate pentanucleotide sequences.

MATERIALS AND METHODS

Synthesis of duplex oligonucleotide adaptors. Most of the single-stranded oligonucleotides used to form the duplex adaptors were custom-synthesized by New England Biolabs. Two oligonucleotides were commercially available from New England Biolabs. One strand (A strand) of each duplex adaptor contains the EcoRI cohesive end (ATTAT) at the 5' terminus attached to the 12-mer core annealing sequence (Fig. 1a). Three lengths of the “A strand” (A1, A2, A3) were synthesized by the addition of single cytosine residues between the 5' end of the core sequence and the 3' end of the EcoRI cohesive end. Oligonucleotides complementary to each length of the A strand core an-
nealing sequences (13-mer = B1, 14-mer = B2, 15-mer = B3) were synthesized with one of five cohesive termini (Fig. 1a) added to the 5' end of the "B strand."

The duplex conversion adaptors were formed by separately annealing A strands and B strands with matching lengths of complementary core sequences. Complementary oligonucleotides were mixed in equimolar portions (A1 + B1, A2 + B2, A3 + B3) with the duplex DNA concentration adjusted to 20 μM in 25 mM Tris–HCl, 8.0, 10 mM MgCl2. These mixtures were heated to 95°C and slowly cooled (approximately 1 h) to room temperature. At this point the three lengths of each duplex conversion adaptor with identical cohesive ends were mixed in a 1:1:1 ratio and stored at −80°C for future use.

DNA purification and manipulation. R. tsutsugamushi was cultured in mouse L929 cells and purified by density gradient centrifugation on Renografin (Squibb Diagnostics) discontinuous gradients of 19, 30, and 50%. Chromosomal DNA was prepared by dodecyl sulfate lysis, proteinase K digestion, RNase A treatment (all from Sigma Chemical Co. St. Louis MO), phenol/chloroform extraction, and ethanol precipitation as described by Silhavy et al. (6). Bacteriophage DNA was purified as described by Silhavy et al. (6). Restriction endonucleases and DNA modification enzymes were obtained from New England Biolabs and used as per manufacturer’s specifications. Purified chromosomal DNA was partially digested with restriction enzymes recognizing four base pair sequences in the following manner. Single-enzyme partial digestions were performed on genomic DNA with the restriction enzymes Ddel, Hinfl, Sau3A, and TaqI. Complete digestions with equimolar restriction enzyme pairs generating identical cohesive ends (AvrII, Sau96I, and HinP1, HpaI) were necessary to result in restriction fragments suitably sized (upper limit 8 kb) for insertion into the λgt11 vector DNA. All chromosomal DNA digestions were monitored by gel electrophoresis on 0.8% agarose gels. Restricted chromosomal DNA was extracted with phenol:chloroform:isoamyl alcohol (24:23:1), ethanol precipitated, and washed sequentially with 70 and 100% ethanol in preparation for cloning. All recombinant λgt11 DNA was analyzed for cloned insert DNA by EcoRI digestion and electrophoresis on 1% agarose gels.

**Ligation of adaptors to restriction fragment DNA.** Conversion adaptor mixtures (20 μM) were added to 2 μg of restriction fragment DNA at a molar ratio of 10 to 1, respectively. This was a fivefold molar excess of duplex adaptor molecules to insert cohesive ends. These ratios were based on the average size of the insert DNA which was estimated by comparison to molecular weight standards on ethidium bromide-stained agarose gels (0.8%). The adaptor-insert DNA mixtures were ligated in a volume of 50 μl (50 mM Tris–HCl, pH 7.4, 10 mM MgCl2, 10 mM dithiothreitol, 0.5 mM ATP) with 4 units of T4 DNA ligase for 60 min at 16°C. Because the adaptor-insert molecule has a single-stranded nick, temperatures are maintained below the melting temperature of the duplex oligonucleotide (~50°C). Therefore, heat inactivation of enzymes is avoided in all subsequent steps. The ligation mixtures were extracted once with a mixture of phenol:chloroform:isoamyl alcohol (24:23:1) and extracted once more with chloroform:isoamyl alcohol (24:1). (Phenol extraction is necessary to inactivate the ligase activity, and the final chloroform extraction removes enough residual phenol to allow the following PEG precipitations.) The volume of the extracted ligation mixture was adjusted to 100 μl with water.

**Removal of excess adaptors.** At this stage adaptor oligonucleotides are either ligated to insert DNA or present in the monomeric form because adaptors were not phosphorylated prior to ligation. High-molecular-weight insert DNA was selectively precipitated with PEG (7), leaving monomeric adaptor in solution. A 100-μl solution of 1.5
CONVERSION ADAPTORS IN λgt11

M NaCl, 13% PEG (PEG 8000, Sigma) solution was added to the extracted ligation mix to adjust the NaCl and PEG concentrations to 0.75 M and 6.5%, respectively. After chilling on ice for 1 h, the mixture was centrifuged in an Eppendorf microfuge for 10 min. The supernatant was removed with a drawn-out glass Pasteur pipet, and 200 μl of 0.75 M NaCl, 6.5% PEG was added. The invisible DNA precipitate was washed in this solution by pulse vortexing and microfuge centrifugation for 5 min. The supernatant was removed as above, and the invisible pellet was dissolved in 50 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). An equal volume of the 1.5 M NaCl, 13% PEG solution was added to this mixture, and the precipitation, centrifugation, and washing steps were repeated as described above. Two additional 70% ethanol rinses were then performed on the invisible DNA pellet to remove any excess salt and residual phenol or chloroform.

Phosphorylation of adaptor-modified insert DNA. Adaptor-modified insert DNA was prepared for ligation into λgt11 by phosphorylation of adaptor 5' ends with T4 polynucleotide kinase. Following PEG precipitation and washing, the DNA was resuspended in 50 μl of ligation buffer. T4 polynucleotide kinase (1 unit) was added, and the mixture was incubated at 37°C for 30 min. The kinase reaction was terminated by a single extraction with a phenol:chloroform:isoamylalcohol (24:23:1) mixture. (It is important to inactivate all kinase activity because dephosphorylated λgt11 vector is used. Residual kinase activity will result in a large nonrecombinant background.) The DNA was concentrated by ethanol precipitation in the presence of 2 M ammonium acetate. Residual phenol, chloroform, and ammonium acetate was removed from the DNA precipitate with two ethanol washes (70 and 100%). The DNA pellet was desiccated briefly under a vacuum and resuspended in 3–5 μl of ligation buffer. All or portions of this DNA can be utilized in the following ligation to λgt11.

Ligation of insert DNA to λgt11 vector. All ligations of insert DNA and λgt11 vector DNA were performed with 1 μg of dephosphorylated λgt11 vector DNA (Promega Biotec, Madison, WI) and 0.5 unit of T4 DNA ligase, in 5 μl of ligation buffer at 16°C for 2 to 18 h. In an effort to achieve optimal ratios of insert-to-vector DNA in the ligation reaction, the amount of insert DNA was varied by performing three separate ligations using 1/50, 1/10, and 1/2 of the adaptor-modified insert.

Each ligation was packaged using commercially prepared packaging mixes at 22°C for 2 h (Vector Cloning Systems, San Diego, CA). The packaging reactions were diluted with 500 μl of SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM MgSO4, 0.01% gelatin). Dilutions of these libraries were titered directly on E. coli Y1090 (r-m' derivative, Promega Biotec) lawns in soft agar containing IPTG (160 μg/ml) and Xgal (40 μg/ml, Bethesda Research Laboratory). The recombinant libraries were screened directly without amplification.

Immunoscreening of λgt11 recombinant libraries. All recombinant libraries were screened for antigen-positive recombinants as described by Young and Davis (4) with sera from rabbits hyperimmunized with the Karp strain of R. tsutsugamushi. Before screening, these sera were absorbed with E. coli Y1090 and PEG-precipitated λgt11 to remove background antibodies that react with E. coli and λ antigens. Fractions of sonicated Y1090, detergent-extracted membrane, and concentrated phage were prepared as the absorbents. Sera absorbed with these fractions had a significantly reduced amount of reactivity with E. coli proteins when monitored by Western blot analysis and λ plaque-screening procedures. Details of this procedure will be described elsewhere (manuscript in preparation).

Recombinant antigen-selected antibody. The antibody directed against each recombinant R. tsutsugamushi antigen epitope was affinity-purified from hyperimmune serum using the antigen-selected antibody proce-
An overnight culture of *E. coli* Y1090 (0.4 ml) was infected with 0.5-3 × 10^7 plaque-purified recombinant phage and plated in Luria broth soft agar (0.7%) overlays on 150-mm petri dishes. The plates were incubated for 3 h at 42°C or until phage plaques were evident. Nitrocellulose disks (139 mm, Schleicher & Schuell BA85) saturated with IPTG (10 mg/ml) were overlaid onto the recombinant lawn. The nitrocellulose filters were incubated on the plates at 37°C for at least 2 h before the filters were inverted and incubated for at least 2 more h. The filters were removed and rinsed for 10 min with Tris-saline (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) and blocked with 2% casein in Tris-saline. Rabbit anti-*R. tsutsugamushi*, diluted 100-fold, was incubated with the filters for 3 h at room temperature. Nonspecifically bound antibody was removed by 10-min washes with Tris-saline, twice with Tris-saline-Triton (0.05% Triton X-100), once with Tris-saline, and once with saline. Antibody directed to recombinant antigens was eluted from the filters by washing the filters with 10 ml of 0.2 M glycine, pH 2.8, 0.15 M NaCl buffer and was subsequently adjusted to pH 7.0 with solid Tris (8 mg/ml solution). This eluate was diluted 3-fold with 2% casein and employed in Western blot analysis of *R. tsutsugamushi* lysates for identification of the rickettsial polypeptide corresponding to the cloned epitopes. Antibody affinity purified in this manner characteristically reacted with only one of the antigens of *R. tsutsugamushi*.

**Polyacrylamide gel electrophoresis and Western blot analysis.** A discontinuous polyacrylamide gel system was used for the separation of both rickettsial and *E. coli* polypeptides (10). The gels consisted of 9% acrylamide crosslinked with N,N'-diallyltartardiamide. Electrophoresis was performed overnight at a constant voltage of 60 V.

Western blot analysis was performed as previously described (11) but modified by using casein (2% in Tris-saline) as a filler and diluent of antisera (12). Goat anti-rabbit IgG conjugated with alkaline phosphatase (Coo-per Biomedicals, Malvern, PA) was used for the detection of antibody bound to rickettsial antigens. Rabbit anti-β-galactosidase (Coo-per Biomedicals) was utilized to detect fusion polypeptides.

**RESULTS**

**Construction of λgt11 recombinant libraries utilizing adaptors.** The chromosomal DNA used to test the λgt11 cloning protocol developed in this study is derived from the causative agent of scrub typhus in humans, *R. tsutsugamushi*. A diagram summarizing the protocol utilizing conversion adaptors to construct λgt11 recombinant expression libraries in this study is shown in Fig. 2. Insert DNA was generated for three of the five libraries listed in Table I by partial restriction enzyme digestion. Complete digestion was used to generate the insert DNA for two libraries (HpaII + HincII, Avall + Sau96I), because complete digestion of the AT-rich rickettsial DNA (GC content of 35%) was necessary to obtain restriction fragment populations significantly smaller than 8 kb.

After ligation to insert DNA restriction fragments, excess EcoRI adaptor oligonucleotides were removed by PEG size fractionation. The removal of 5'-α-32P-radiolabeled adaptor oligonucleotides from the insert-adaptor ligation mix was monitored before and after PEG precipitation. It was found that two PEG precipitations were necessary to exhaustively remove labeled adaptors, while adaptor-labeled restriction fragments above 300 base pairs were not significantly depleted (data not shown).

The unamplified yields of recombinant phage (Table 1) obtained in the λgt11 libraries constructed with conversion adaptors compare very favorably with those obtained using linkers and blunt-ended DNA. Only 50-200 ng of insert DNA was necessary to obtain these yields (3 × 10^6-1 × 10^7) when ligated to 1 μg of λgt11 DNA. The recombinant phage yields obtained with Avall
Fig. 2. Summary of method using conversion adaptors to construct genomic libraries in λgt11. A small excess of unphosphorylated conversion adaptors (one length for each of three reading frames) is ligated to restriction fragments (e.g., Sau3A fragments). Excess (unligated) adaptors are removed from the ligation mix by PEG precipitation. The EcoRI adaptor-ended restriction fragments are phosphorylated at their 5' terminus (EcoRI cohesive end) and ligated to EcoRI-cleaved, alkaline phosphatase-treated λgt11 vector. The concatamerized vector/insert ligation mix is packaged and plated for immunoscreening with the appropriate antisera.
TABLE 1
SUMMARY OF R. TSUTSUGAMUSHI RESTRICTION FRAGMENT LIBRARIES IN λgt11

<table>
<thead>
<tr>
<th>Restriction enzyme libraries</th>
<th>Total recombinant phage (% recombinant)</th>
<th>Antigen-positive frequency per 10⁶</th>
<th>IPTG-dependent antigen expression (%)</th>
<th>Epitopes identified in libraries (antigen, kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hpall + HinPl</td>
<td>1 × 10⁶ (&gt;99%)</td>
<td>30</td>
<td>61</td>
<td>58, 110</td>
</tr>
<tr>
<td>Avdl + Sau96I</td>
<td>7 × 10⁶ (94%)</td>
<td>27</td>
<td>8</td>
<td>49, 56, 58, 110, 138, 150, 165</td>
</tr>
<tr>
<td>Ddel</td>
<td>3 × 10⁶ (96%)</td>
<td>31</td>
<td>48</td>
<td>49, 58, 110</td>
</tr>
<tr>
<td>HinPl</td>
<td>4 × 10⁶ (87%)</td>
<td>21</td>
<td>24</td>
<td>58, 110, 150</td>
</tr>
<tr>
<td>Sau3A/Taq1</td>
<td>3 × 10⁶ (85%)</td>
<td>26</td>
<td>71</td>
<td>56, 58, 72, 110</td>
</tr>
</tbody>
</table>

Note: Insert DNA was generated for each library by digesting R. tsutsugamushi DNA with the enzymes indicated in column 1. Complete digests of R. tsutsugamushi genomic DNA with equal unit mixtures of Avdl + Sau96I and Hpall + HinPl were necessary to generate restriction fragments smaller than 8 kb. Digestions with each of these combinations result in complementary ends (Fig. 1). Partial digests of R. tsutsugamushi genomic DNA were performed with the enzymes Sau3A, TaqI, Ddel, and HinPl. Complementary conversion adaptors (Fig. 1) were ligated to all resulting restriction fragments as described above. The Sau3A/TaqI library was constructed by mixing adaptor-ended Sau3A and TaqI restriction fragments prior to ligation with λgt11. The recombinant phage totals listed in column 2 were obtained directly from the in vitro packaging of the vector insert ligation mix, without amplification. The recombinant percentage is the proportion of clear plaques (lacZ-) to blue plaques (lacZ+) on Xgal plates. Antigen-positive plaques per 10⁶ recombinants are indicated in column 3. Lysates of Y1089::recombinant Agt11 lysogens grown with and without IPTG were assayed by Western blot analysis for lac-dependent expression of recombinant R. tsutsugamushi antigen epitopes (column 4). A minimum of 15 lysogens were assayed in this manner for each library. Recombinant antigen-selected antibody was used to identify the cross-reacting rickettsial antigens by Western blot analysis of R. tsutsugamushi lysates (Fig. 3). The corresponding R. tsutsugamushi antigen epitopes identified in each library are listed in column 5 (antigen molecular mass in kDa).

+ Sau96I, Ddel, and HinPl libraries are comparable to those obtained with the Hpall + HinPl and Sau3A/TaqI libraries, even though the libraries in the former group were constructed using restriction enzymes leaving degenerate ends.

Identification and evaluation of antigen-positive recombinants. Rickettsial antigen-positive recombinants were immunoscreened using polyclonal antiserum preabsorbed with lysates of E. coli and λgt11. The frequency of antigen-positive recombinants was similar for each library (Table 1, column 3). Antigen-positive clones were isolated and plaque purified twice for further analysis. Antigen-positive recombinant phages were used to prepare recombinant antigen-selected antibody reagents from the screening sera. The recombinant antigen affinity-purified sera were then used as probes in Western blot analysis of R. tsutsugamushi lysates (Fig. 3) to identify the cross-reacting rickettsial antigens. Western blot analysis of R. tsutsugamushi antigens had revealed at least eight polypeptide antigens with this antiserum (manuscript in preparation). Gene epitopes from each of these antigens were represented in the five libraries constructed. The recombinant antigen epitopes identified in each library are summarized in Table 1. At least portions of the 110- and 58-kDa antigen genes are represented in each of five recombinant libraries, while a 72-kDa antigen gene recombinant was observed only in the Sau3A/TaqI library.

Expression of antigen genes cloned in λgt11 can be independent of or dependent on lac promoter induction. In the latter case,
were expressed in the \(HpaII/HinPl\), \(Ddel\), and \(Sau3A/TaqI\) libraries.

Size analysis of cloned insert DNAs in antigen-positive recombinants. Phage DNA was prepared from 45 antigen-positive recombinants and digested with \(EcoRI\) for analysis of cloned insert size by agarose gel electrophoresis. The sizes of the cloned insert DNAs ranged from 0.3 to 7 kb pairs. Restriction fragments analysis of inserts for each clone type (e.g., 56, 110 kDa) did not reveal repetition of cloned inserts except in the case of three 72-kDa antigen-positive recombinants from the \(Sau3A/TaqI\) library (data not shown). All three of the 72-kDa antigen-positive recombinants carried an identical 1-kb restriction fragment.

DISCUSSION

The use of conversion adaptors for the \(\lambda gt11\) system has facilitated the construction of expression libraries from \(R. tsutsugamushi\). The yield of recombinant phage and percentage of recombinants were quite high even without amplification. The libraries appear to be representative of the rickettsial genome, because epitopes have been cloned from all eight \(R. tsutsugamushi\) antigens previously identified by Western blot analysis. This is thought to result from an operon fusion. The majority of the recombinant \(\beta\)-galactosidase-antigen fusion proteins

antigen genes are either fused to \(lacZ\) (gene fusion) and expressed as \(\beta\)-galactosidase fusion proteins or a 5' proximal gene cotranscribed with the antigen gene is fused to \(lacZ\) (operon fusion), resulting in the expression of the antigen as a complete or truncated protein (13). One hundred recombinant lysogens (in \(E. coli\) Y1089) (3,4) were evaluated by Western blot analysis for the expression of polypeptides which were induced by IPTG (\(lac\)-dependent). Thirty-nine of these recombinant lysogens were found to express antigenic polypeptides when grown in the presence of IPTG (data not shown). All but one of these \(lac\)-dependent antigenic polypeptides were found to be fused with a \(\beta\)-galactosidase protein as indicated by reaction with anti-\(\beta\)-galactosidase with Western blot analysis. This is thought to result from an operon fusion. The majority of the recombinant \(\beta\)-galactosidase-antigen fusion proteins
remainder of the recombinant E. coli expressed rickettsial antigens independent of IPTG (lac) induction.

The size of the R. tsutsugamushi genome was previously determined to be 54% of the E. coli genome or approximately 2200 kb (unpublished data). DNA encoding the eight recognized antigen genes is estimated to represent 1% of the genome. Therefore, 1% of all recombinants obtained in this study should carry at least a portion of an antigen gene, but only a fraction of these will actually express antigen epitopes. In the five libraries constructed in this study, an average of 27 antigen-positive clones (expressing epitopes) were obtained per 10,000 recombinants (0.27%). Gene fusion to lacZ at any restriction site within a gene should lead to lac-dependent expression in one out of six instances, because 50% of restriction fragments are inserted in lacZ in the correct orientation and one-third of these are ligated in the correct reading frame. More than one-third (39%) of the antigen-positive clones obtained in this study was lacZ gene fusions, dependent on IPTG induction for expression of R. tsutsugamushi epitopes. These results (frequency of antigen-positive recombinants and percentage lacZ gene fusions) compare favorably with the theoretically predicted frequencies.

Because these λgt11 libraries were not amplified prior to immunoscreening, the presence of sibling recombinants was not likely. However, it was thought that the generation of insert DNA for expression vector cloning by nonrandom restriction endonuclease digestion might result in the inadvertent selection and repetitious cloning of identical restriction fragments within each library. This was not observed within the group of 45 antigen-positive recombinants analyzed for insert size, except in the case of the 72-kDa antigen clones in the Sau3A/TaqI library.

The nonrandom generation of fragments by restriction endonuclease digestion for "shotgun" cloning into λgt11 made it necessary to use a battery of restriction endonucleases to construct multiple R. tsutsugamushi genomic libraries. As the procedure used in this study is extremely efficient and easily performed, the construction of multiple libraries with a battery of restriction endonucleases and conversion adaptors was practical. Furthermore oligonucleotides of three lengths (Fig. 1) were synthesized for each conversion adaptor to ensure that gene fusion in any reading frame was a possibility at any restriction site. Five groups of conversion adaptors were used in this study to clone restriction fragments generated with eight restriction enzymes. This laboratory now uses seven groups of conversion adaptors designed for use with restriction enzymes recognizing 13 unique 4-bp or degenerate 5-bp sequences (Fig. 3). In theory, this would allow the restriction of genomic DNA at approximately every 20 bp (assuming 50% GC content). With potential sites so closely spaced, the nonrandom generation of fragments, inherent to restriction enzymes, is minimized. The most versatile group of EcoRI conversion adaptors, CG, can ligate to cohesive ends generated by at least five restriction enzymes which recognize four unique 4-bp or degenerate 5-bp sequences (Fig. 1). When possible, partial digestion was employed to generate restriction fragments for cloning. Partial digestion was preferred because of the possibility that any restriction enzyme can cut within DNA sequences encoding desired antigen epitopes. However, antigen-positive cloned inserts resulting from complete digestions of genomic DNA may be more easily aligned with DNA sequence data and could facilitate the process of epitope mapping.

The core sequences of the EcoRI conversion adaptors synthesized and used in this study were based on the core sequence of the XmnI series "long adaptor" oligonucleotides designed by New England Biolabs. This design was chosen for the following reasons: (i) Termination codons are not encountered in any of the three reading frames. This is an important feature if gene fusions to β-galac-
Conversion adaptors could be used to rapidly clone and assay specified restriction fragment templates for the ability to express epitopes in the context of the Agt11 system. While the EcoRI conversion adaptors used in this study were designed and intended for the construction of Agt11 expression libraries, they are also useful for routine subcloning into the EcoRI sites of many other vectors (e.g., M13). The conversion adaptors could also be useful as primers for the sequencing of restriction fragments cloned with the adaptors.

ACKNOWLEDGMENTS

We thank James Weber, Ruth Gellers-Wolff, Jerry Buyse, and Malabi Venkatesan for their excellent comments and advice. We also thank David Marana, Fernand Brown, and David Taragin for their excellent technical assistance.

REFERENCES