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λ ZAP: a bacteriophage λ expression vector with *in vivo* excision properties

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ABSTRACT

A lambda insertion type cDNA cloning vector, Lambda ZAP, has been constructed. In *E. coli* a phagemid, pBluescript SK(-), contained within the vector, can be excised by f1 or M13 helper phage. The excision process eliminates the need to subclone DNA inserts from the lambda phage into a plasmid by restriction digestion and ligation. This is possible because Lambda ZAP incorporates the signals for both initiation and termination of DNA synthesis from the f1 bacteriophage origin of replication (1). Six of 21 restriction sites in the excised pBluescript SK polylinker, contained within the NH_2-portion of the *lacZ* gene, are unique in lambda ZAP. Coding sequences inserted into these restriction sites, in the appropriate reading frame, can be expressed from the *lacZ* promoter as fusion proteins. The features of this vector significantly increase the rate at which clones can be isolated and analyzed.

The lambda ZAP vector was tested by the preparation of a chicken liver cDNA library and the isolation of actin clones by screening with oligonucleotide probes. Putative actin clones were excised from the lambda vector and identified by DNA sequencing. The ability of lambda ZAP to serve as a vector for the construction of cDNA expression libraries was determined by detecting fusion proteins from clones containing glucocerbrosidase cDNA's using rabbit IgG anti-glucocerbrosidase antibodies.

INTRODUCTION

Bacteriophage lambda has been used extensively as a vector to clone both complementary DNA (cDNA) and genomic DNA. The efficiency of lambda phage packaging and subsequent infection of *E. coli* exceeds the efficiency of plasmid transformation 100-fold. This increased efficiency greatly facilitates the construction of primary libraries containing greater than $1x10^6$ independent clones and, thereby, improves the likelihood of isolating rare clones. Lambda phage plaques can be screened when plated at high plaque density and produce little background when screened with DNA or antibody probes. However, subsequent characterization of these cloned genes is normally a lengthy process. The large size of lambda phage vectors complicates the restriction mapping and the sequence analysis of genes after cloning. Therefore, after initial isolation in a lambda phage vector, DNA inserts are usually subcloned into plasmid vectors. This process can be time consuming.

We sought to develop a rapid and efficient *in vivo* system for the transfer of DNA from lambda to plasmid cloning vectors. A few potential excision mechanisms were considered for the development of such a lambda phage vector, each with certain advantages and disadvantages. The lambda excision/integrase system has been well studied and is capable of excising inserted DNA (2). Lambda vectors containing a colE1 origin of replication and an antibiotic resistance gene flanked by *att* sequences which are recognized by the lambda genes *xis* and *int*, can be excised to generate plasmid vectors containing inserted DNA. However, the *att* signals required for integration and excision serve no useful function in the excised plasmids and their use within the lambda vector prevents the formation of lambda

phage lysogens. This host recombination system can also excise DNA from a lambda phage that is flanked by DNA sequences containing direct repeats. However, this system is relatively inefficient and requires the use of *RecA+* hosts for efficient excision. These *RecA+* strains are undesirable for the construction of DNA libraries that contain an abundance of repeated sequences (3).

The DNA replication signals of f1 phage have been studied extensively (4-13). Based on these studies, we have constructed a novel vector, lambda ZAP, that allows the *in vivo* excision of a phagemid containing the DNA insert from the lambda vector. The excision is accomplished by utilizing two distinct, but overlapping sequences that make up the f1 phage origin of replication (1). One is the site where DNA synthesis of the viral plus strand is initiated and the other is the signal for termination of the DNA replication process. In f1 bacteriophage the **initiator** signal is recognized by the f1 phage gene II protein which nicks the DNA strand. This nick is then used as an initiation site for DNA synthesis (4). Replication then proceeds unidirectionally from this origin, displacing the f1 plus strand. The plus strand is then cleaved at the second signal, the **terminator**, which contains the same nicking site that was used for initiation. Following nicking at the termination site by the gene II protein, the two ends of the single stranded DNA are ligated to form a circular single stranded copy of the f1 plus-strand genome. The plus strand DNA is converted to a double-stranded RF molecule by synthesis of the (-) strand from the minus strand origin utilizing host derived proteins (14).

Initiator and terminator signals were identified previously by deletion mapping and functional analysis within plasmid vectors (1). The functions of the terminator signal sequences were assayed by their ability, when positioned downstream of an intact f1 origin within a plasmid vector, to produce circular molecules containing an intact f1 origin and only the sequences positioned between the two domains without replication of sequences between the terminator and intact origin. We have constructed subclones of both the initiator and terminator domains of the f1 origin, as defined by these experiments, and positioned them separately within a lambda cloning vector. The DNA lying between the two signals within the lambda phage is the Bluescript SK(-) phagemid sequence. This sequence is excised in the presence of f1 or M13 phage gene II protein, proceeding as follows: after initiation (nicking), one strand of the Bluescript SK(-) phagemid DNA sequence is displaced as DNA synthesis proceeds from the initiator signal toward the terminator signal within the lambda vector. At the terminator site, the displaced strand is nicked and circularized by gene II protein, after which the strand is packaged by helper phage proteins and released from the cell.

The Bluescript SK(-) phagemid sequences in lambda ZAP were positioned between the two partial origin domains so that the excision of the plasmid DNA from the lambda ZAP vector would generate a complete pBluescript SK(-) phagemid, including an intact, reconstituted f1 origin of replication. Entire DNA inserts within the lambda ZAP vector can be recovered within the Bluescript SK(-) phagemid, positioned within the pBluescript polylinker in a manner suitable for further analysis.

In this paper we describe the construction of the insertion vector, lambda ZAP, the preparation of a lambda ZAP cDNA library, the identification of actin and glucocerebrosidase clones in lambda ZAP using DNA or antibody probes, respectively, and the characterization these clones following excision of the Bluescript phagemids containing the cDNA inserts using helper phage.

MATERIALS AND METHODS

Supplies

DNA modifying enzymes, buffers, salts, and antibiotics were obtained from Stratagene Cloning Systems, Sigma, and Calbiochem. [α-^{32}P]dATP (400-600 Ci/mmol) and [γ-^{32}P]dATP (7000 Ci/mmol)

were obtained from New England Nuclear. Gigapack Gold *in vitro* lambda packaging extract and competent *E. coli* cells (JM109 and AG1) were prepared at Stratagene Cloning Systems. Oligonucleotides were synthesized on an Applied Biosystems Inc. (Foster City, California) DNA 381A synthesizer using chemicals supplied by the manufacturer.

Strains

The *E. coli* strain, DP50 (15), was used for the plating and replication of lambda phage genomes during vector construction. The strains XL1-Blue (16) and BB4 (William Bullock, Stratagene) were used as lambda ZAP hosts. (Genotype of **XL1-Blue**: *endA1, hsdR17(rk-,mk+), supE44, thi-1, lambda$^-$, recA1, gyrA96, relA1, lac$^-$, [F', proAB, lacI^{q}ZΔM15, Tn10(tetR)]*. Genotype of **BB4**: *supF58, supE44, hsdR514 (rk-, mk+), galK2, galT22, trpR55, metB1, tonA, lambda$^-$, Δ(arg-lac)U169 [F', proAB, lacI^{q}ZΔM15, Tn10(tet^R)]*.)

The helper phage R408 was a generous gift of Dr. M. Kelly rV1 (17), M13K07 was provided by Dr. J. Viera, and IR1 (18) was provided by Dr. D. Mead.

The genotype of the lambda vectors are listed below:

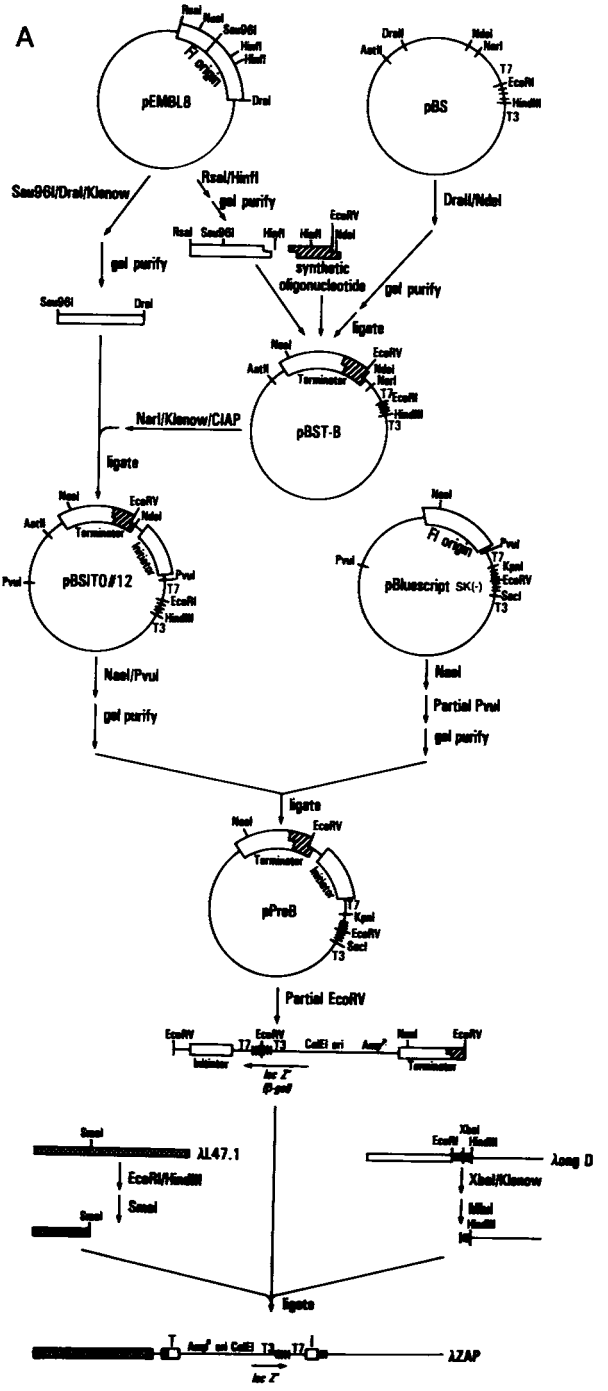
Name	Genotype
Lambda L47.1	- *λchiA131 sbhIλ1^O Δ(srIλ1-2) srIλ3^O imm434cI srIλ4^O nin5 shnλ6^O srIλ5^O Δ(sbhIλ3-sbhIλ4)*
Lambda cI857,S100	- *lac5 Δ(shnλ2-3) srIλ3^O Δ(sscIλ1-2) cI857 srIλ4^O nin5 srIλ5^O Sam100*
Lambda cI857,ind1,S7	- *cI857 ind1 Sam7*
Lambda Long C	- *{Δ(srIλ1-shnλ3)::(pUC19 polylinker EcoRI-HindIII)} cI857 srIλ4^O nin5 srIλ5^O Sam100*
Lambda Long D	- *{Δ(srIλ1-shnλ3)::(pUC19 polylinker EcoRI-HindIII)} sxhIλ cI857 srIλ4^O nin5 srIλ5^O Sam100*
Lambda ZAP	- *λchiA131 sbhIλ1^O {Δ(smIλ1-shnλ3):pPreB and pUC19 polylinker XbaI to HindIII} cI857 srIλ4^O nin5 srIλ5^O Sam100*
Lambda ZAPII	- *Lambda ZAP; S+ gene from λgt10*

Construction of Bluescribe and Bluescript Phagemids

The plasmid pUC19 (19) was digested with EcoRI, dephosphorylated, and ligated to complimentary oligonucleotides, having EcoRI-compatible ends, coding for a T7 RNA polymerase promoter (20). A clone, pJF3, was identified which contained the T7 promoter oriented to direct RNA synthesis towards the multiple cloning site of the pUC19 plasmid. pJF3 was digested to completion with HindIII, dephosphorylated and ligated to complimentary oligonucleotides, having HindIII-compatible ends, coding for a T3 RNA polymerase promoter (21,22). A plasmid was identified that contained the T3 promoter oriented to direct RNA synthesis towards the multiple cloning site of pJF3. This plasmid was designated Bluescribe (pBS).

pBS was digested with AatII and NarI, treated with mung bean nuclease and alkaline phosphatase, then ligated to a 456 base pair (b.p.) RsaI/DraII blunt-end fragment isolated from pEMBL8 (23). The 456 b.p. fragment contained the intergenic region of f1 phage, but did not contain the f1 gene II promoter sequence. Phagemid clones were isolated containing both orientations of the intergenic region [pBS(+), pBS(-)]. pBS(+) contains the intergenic region in the same orientation as the *lacZ* gene, therefore in vectors designated with (+), the *lacZ* sense strand is packaged.

To produce pBluescript SK(-) and SK(+) (Figure 2), the pUC19 polylinker was removed from both pBS(-) and pBS(+) by digestion with EcoRI and HindIII. The resulting phagemid was blunt-ended with the


A
pEMBL8
pBS
Sau96I/DraI/Klenow
RsaI/HinfI
gel purify
DraI/NdeI
synthetic oligonucleotide
ligate
pBST-B
NarI/Klenow/CIAP
pBSITO/#12
pBluescript SK(-)
NaeI/PvuI
NaeI
Partial PvuI
pPreB
Partial EcoRV
λL47.1
EcoRI/HindIII
SmaI
XbaI/Klenow
λseq D
λZAP

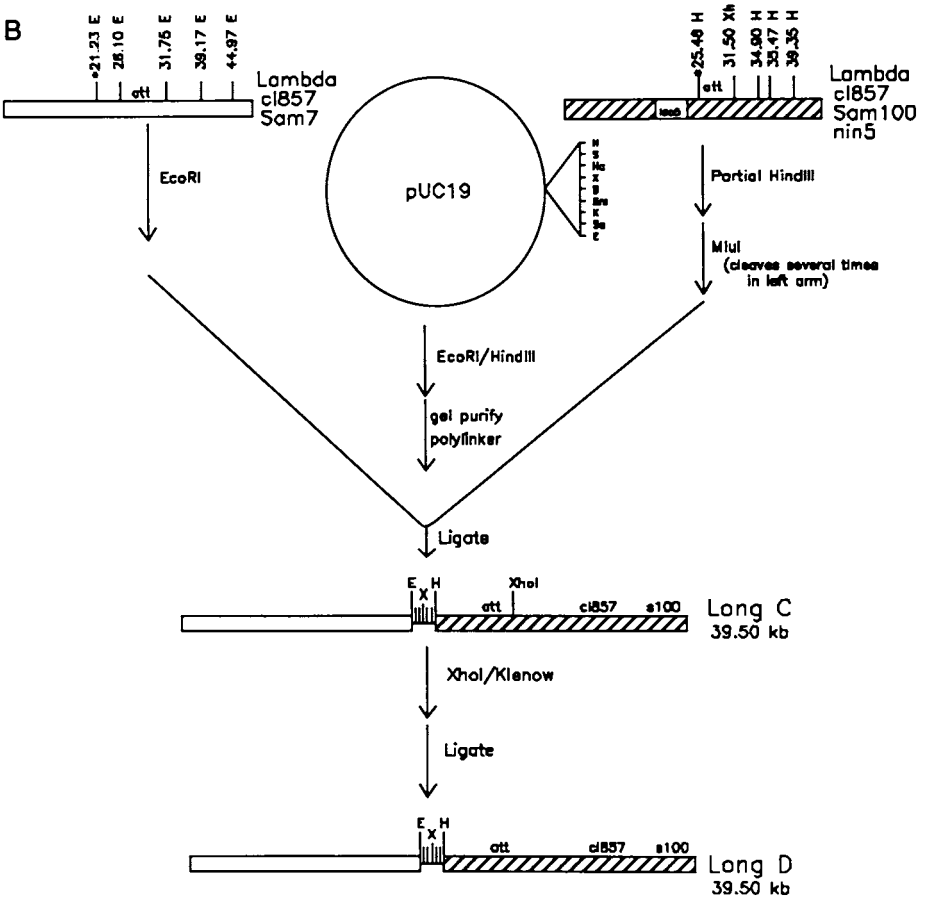


Figure 1A. Construction of Lambda ZAP Vector.

The lambda ZAP vector construction is outlined above and described in the text. The circular maps denote plasmid or phagemid vectors; linear maps denote lambda genomes or DNA fragments obtained from plasmids, as indicated in the drawing. Long D construction is outlined in Figure 1B. (I=Initiator domain; T=Terminator domain; T3= T3 Phage RNA Polymerase Promoter; T7= T7 Phage RNA Polymerase Promoter; CIAP=Calf Intestinal Alkaline Phosphatase; |-|-|-| = multiple cloning site; box= DNA from f1 origin; hatched box indicates synthetic oligonucleotide (Figure 3); arrow= direction of *lacZ* gene transcription).

Figure 1B. Construction of Long D.

The construction of the lambda vector Long D is described in the text. The right arm of Long D was used in the construction of lambda ZAP as depicted in Figure 1A. The circular map is the plasmid pUC19 (27). The linear maps are lambda genomes and are identified in the figure. (X= XbaI, E= EcoRI, Xh= XhoI, S= SphI, Hc= HincII, B= BamHI, Sm= SmaI, K= KpnI, Sa= SacI, *= site used in cloning).

Klenow fragment of DNA polymerase I and ligated to a blunt-ended synthetic polylinker containing 21 unique restriction sites (Figure 2). The 21 restriction sites were positioned with 3' flanking 5' sites to permit construction of unidirectional deletions with exonucleaseIII and single strand nuclease as

described by Roychoudhury and Wu (24) and modified by Henikoff (25). The SK (SacI - KpnI) designation indicates the orientation of the polylinker with respect to the synthesis of *lacZ* mRNA. The first letter indicates the restriction site closest to the *lacZ* promoter.

Vector Construction

For construction of the lambda ZAP vector, digestion with restriction enzymes, transformation of *E. coli*, and purification of plasmid and lambda phage DNA were carried out as described (15).

Prior to restriction digestion of lambda DNA, all lambda *cos* ends were ligated at a DNA concentration greater than 200ng/ul. Mixtures of left and right lambda arms were used in constructing lambda Long C and lambda ZAP (Figure 1A & 1B). Ligations were incubated overnight at room temperature in 20mM Tris-HCl (pH 7.5), 2mM $MgCl_2$, 0.5mM ATP, 2mM dithiothreitol with 1 Weiss unit of T4 DNA ligase. After ligation, lambda vectors were packaged for 2 hours at room temperature in Gigapack Gold packaging extract (efficiency = 2 - $4x10^9$ pfu/ug of lambda arms).

Construction of a Chicken cDNA Library

A cDNA library was constructed to verify the ability of lambda ZAP to function as a cDNA cloning vector. To construct this library 10 micrograms of chicken liver poly A+ mRNA was used to prepare double-stranded cDNA as previously described (15). The DNA was ligated overnight at 5°C at a 1:1 molar ratio of cDNA to EcoRI digested, phosphatased lambda ZAP arms. A cDNA library containing 2.0 X 10^6 pfu/ug with less than 5% non-recombinant background plaques was obtained following plating with BB4 host cells on NZY plates. The percentage of non-recombinant background plaques was determined by plating an aliquot of the library on NZY plates containing 10mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 5mg/ml of X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) and determining the ratio of blue to white plaques. Under these conditions non-recombinant, background plaques were blue and recombinant plaques were white.

These high concentrations of IPTG and X-Gal are required for the blue-white color assay using lambda ZAP since the vector contains the large pBluescript SK polylinker which interrupts the *lacZ* gene (Figure 2). The light blue color generated by the β-galactosidase (β-gal) protein coded by the *lacZ* gene within lambda ZAP is not an indication of decreased *lac* promoter strength. A darker blue is achieved through the use of the bacterial strain XL1-Blue. XL1-Blue supports vigorous growth of lambda ZAPII, but not lambda ZAPI which contains the *Sam100* mutation.

The cDNA library was amplified by plating at confluency on forty 150 mm NZY plates with BB4 cells. After six hours of growth at 37°C, the phage were eluted overnight from the agar plates with 5 mls of SM buffer per plate (100mM NaCl, 8mM $MgSO_4$, 50mM Tris-HCl, pH7.5, 0.01% gelatin) (15). The library was stored in 0.3% chloroform at 4°C.

Library Screening with Oligonucleotide Probe

To verify that the chicken cDNA library contained cDNA synthesized from chicken mRNA, it was screened for the presence of actin clones using an oligonucleotide probe. To accomplish this, the amplified cDNA library was plated at a density of 50,000 plaques per 150mm plate on BB4 cells and incubated overnight at 37°C. Phage from the plaques were transferred to nitrocellulose filters in duplicate. Phage DNA was released from the phage head and denatured by standard procedures (15). Filters were prehybridized in 0.4% SDS, 6xSSC, 5xDenhardts, 20mM NaH_2PO_4, and 500ug/ml sonicated salmon sperm DNA for 2 hours. A 17 base synthetic oligonucleotide (5' CTTCACCACCACAGCCG 3') corresponding to amino acids #199 to #205 of the chicken β-actin sequence (26), was end-labeled

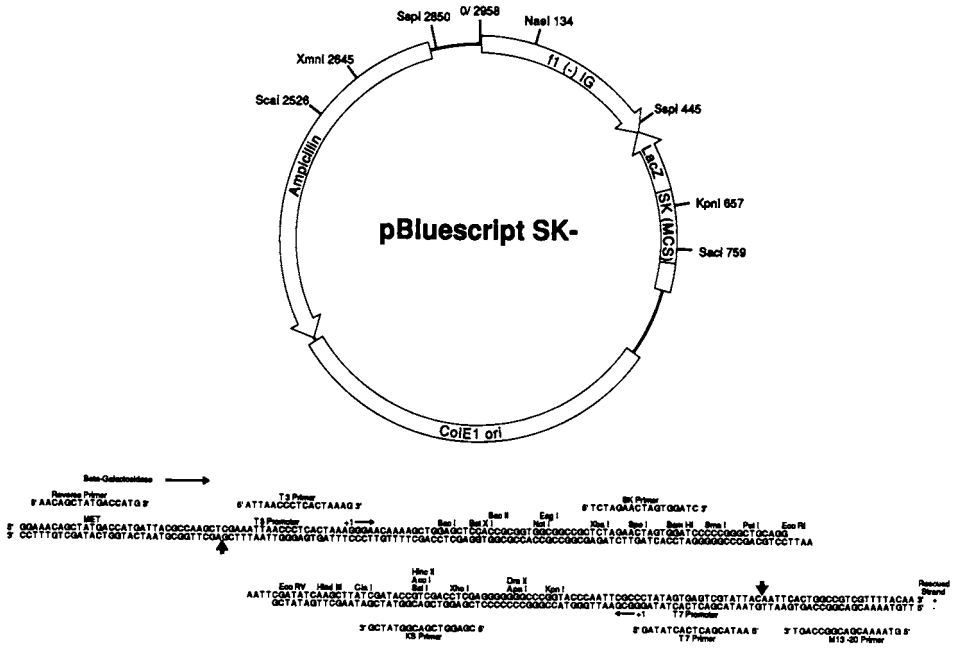


Figure 2. Bluescript SK(-) Phagemid and Polylinker

The restriction map of pBluescript SK(-) is shown with the SK polylinker split at the EcoRI restriction site. The end points of the synthetic oligonucleotides used to construct the vector are indicated by the veritical arrows. The sequence of pBluescript SK(+) is identical to SK(-) with the exception that the f1 origin sequence (position 6 to 462) is inverted. The transcriptional start sites from T3 and T7 polymerase occur at the guanine residues indicated by the +1. The arrow indicates the direction of transcription. Also included are commonly used primer binding sites for DNA sequence analysis. Met marks the initiation codon for the alpha-complementing portion of the β-galactosidase gene.

with ^{32}P using polynucleotide kinase and ^{32}P γ-ATP, to a specific activity of $1x10^8$ dpm/ug (15). The probe was added directly to the prehybridization mix to give a final concentration of $2x10^6$ dpm/ml and then allowed to hybridize overnight at 37°C. Following hybridization, the filters were washed at 46°C in 6xSSC, 0.1% SDS and then exposed to XAR film (KODAK).

Excision of Plasmids from the Lambda ZAP Vector (Figure 3)

For further analysis of the lambda phage clones, selected phage plaques were cored from agar plates and transferred to a sterile microfuge tube containing 500 ul of SM buffer and 20 ul of chloroform. The tube was vortexed to release lambda phage particles from the agar plug into the SM buffer. To perform an excision of the pBluescript phagemid, 200ul of OD_{600}=1.0 XL1-Blue cells, 200ul of lambda ZAP phage stock (containing approximately 1 X 10^5 phage particles), and 20ul of R408 helper phage (1 X 10^{11} pfu/ml) were placed in a 50 ml conical tube. This mixture was incubated at 37°C for 15 minutes. 5 ml of 2xYT medium (10g NaCl, 10g yeast extract, 16g bactotryptone per 1 liter of H_20) was then added to the mixture and incubation was continued at 37°C for two to six hours with shaking. After incubation, the mixture was heated to 70°C for 20 minutes to inactivate the parent lambda phage and to kill bacteria.

Figure 3. Sequence of the f1 Origin

Sequence of the f1 origin of replication is shown as it is found in the bacteriophage f1 and pEMBL8. The nicking site of gene II protein is indicated {*} and all map numbers are derived from the f1 phage sequence determined by Beck & Zink (12). The upper box outlines the sequences utilized for terminator activity, while the lower box depicts those sequences utilized for initiator activity (2).

The tube was then centrifuged for 5 min. at 1000g and the supernatant was decanted into a sterile tube and stored at 4°C for up to 1 month. This stock contained the Bluescript phagemid packaged in the f1 phage particles, as well as the f1 helper phage. To recover the excised phagemid from this stock, 200ul of OD_{600}=1.0 XL1-Blue host cells and 200ul of phage stock containing the phagemid were combined and incubated at 37°C for 15 minutes. 100 ul of samples diluted up to 10^{-3} were plated on LB/ampicillin plates. Ampicillin resistant colonies containing the phagemid were visible following a 12 hour incubation at 37°C.

Rescue and Purification of Single Stranded DNA and Dideoxy Sequencing

During the excision of pBluescript SK(-) from lambda ZAP, the partial origins are joined to form a single functional f1 origin. To check the fidelity of this origin, XL1-Blue cells containing rescued phagemid

carrying putative actin cDNA's were grown to O.D.$_{600}$ = 0.3, then inoculated with R408 helper phage at a multiplicity of infection of 10 to 1 (phage:bacteria). The inoculated culture was incubated for 8 hours at 37°C with vigorous shaking. The culture, containing both the rescued recombinant phagemid and helper phage particles, was centrifuged at 12,000 g for 5 min. to remove the cells. Phage particles were precipitated with PEG and DNA was isolated as previously described (27). The rescued single stranded DNA served as a template for DNA sequencing that was performed according to Sanger, *et al.* (28).

Construction of a Lambda ZAP cDNA Clone Expressing a Human Glucocerebrosidase Gene

The ability of lambda ZAP to serve as an expression vector was verified by comparing the expression of fusion proteins synthesized in lambda ZAP to that of the commonly used cloning vector lambda gt11 (29). A human glucocerebrosidase (GCS) cDNA clone was obtained from a lambda gt11 library using an anti-GCS antibody (30). For direct comparison of antibody screening of fusion proteins from lambda gt11 and lambda ZAP, we removed the GCS clone from lambda gt11 and transferred it to lambda ZAP. Since the EcoRI site of lambda ZAP is in a different reading frame than the EcoRI site of lambda gt11, the GCS EcoRI fragment could not be directly subcloned into lambda ZAP without a shift in the protein reading frame and loss of GCS protein expression. In order to obtain the correct reading frame, a coding fragment of GCS was removed from the lambda gt11 clone by a complete BamHI digestion. The fragment contained greater than 90% of the GCS coding region. The overhangs were then "filled-in" with the Klenow fragment of DNA Polymerase I as previously described (15). Two micrograms of phosphorylated EcoRI linkers were ligated to one microgram of the Klenow-treated GCS DNA for 30 min. at room temperature, followed by overnight ligation at 5°C. Following digestion with EcoRI nuclease to generate the cloning ends, the insert DNA was size fractionated over a Sepharose4B-CL column. The modified fragment was ligated to EcoRI digested, phosphatased lambda ZAP arms and then packaged *in vitro*. The packaged DNA was plated with BB4 cells and plaques were isolated following overnight incubation at 37°C.

The Bluescript phagemid was excised from several of these plaques and DNA was prepared from the clones as described elsewhere in the Methods. The clones were restriction mapped to confirm the presence and proper orientation of the GCS clone within the lambda ZAP vector. The lambda ZAP clone GCS#4 was used for subsequent antibody screening tests.

Antibody Screening of Lambda ZAP Clones

The GCS clone in the lambda ZAP vector was plated with 200ul of OD$_{600}$=1.0 BB4 cells and then grown at 42°C for 3 hours. Nitrocellulose filters, previously soaked in 10mM IPTG, were then placed onto the BB4 lawn and the plates were returned to 42°C for an additional six hours. Following the incubation period, the filters were removed from the plates and washed in TBST (50mM Tris-HCl, pH8, 150mM NaCl, 0.5% Tween 20), wrapped in saran wrap, and stored at 5°C overnight. To make a duplicate set of the original filters, a second set of filters were placed onto the lawn and incubated overnight at 42°C. The filters were removed from the BB4 lawn and the duplicate sets of filters were then washed in TBST containing 1% BSA. All subsequent steps were performed as described in Stratagene's Picoblue antibody screening kit. Rabbit IgG anti-GCS primary antibody was detected by binding an alkaline phosphatase conjugated goat anti-rabbit IgG secondary antibody. The alkaline phosphatase accumulated at the site of the primary antibody was exposed to BCIP (5-Bromo-4-Chloro-Indol-Phosphate) and NBT (Nitro Blue Tetrozolium) for 15 min. and then terminated with stop buffer (20 mM Tris-Cl, pH2.9; 0.1 mM EDTA). Blots were photographed with polaroid PN55 film.

RESULTS

Construction of Lambda Zap

A vector, lambda ZAP, that excises the pBluescript phagemid was constructed by separating two overlapping domains of the f1 origin of replication (Figure 1). The **Initiator** domain contains the site for nicking of double stranded DNA by gene II protein and the initiator site of DNA synthesis for the plus strand. The **Terminator** domain, contains the site for cleavage of single stranded DNA by gene II protein and circularization of the single stranded DNA. The presence of this intergenic region in phagemid vectors [e.g. pEMBL8 (23), the pBS series (Stratagene, 1985), pTZ (31)] is sufficient to allow these vectors to replicate in the presence of gene II product of the f1, fd or M13 phage, independent of the ColE1 origin. The intergenic origin region also contains the sequences required to package single stranded DNA into phage particles and the origin for minus strand DNA synthesis. The sequence in Figure 3 shows the f1 456 base pair intergenic region located between two naturally occurring restriction sites, RsaI (position 5587) and DraI (position 5942) (All position numbers refer to the bacteriophage f1 (11)). The overlapping initiator and terminator portions of the intergenic region are marked in the figure.

As outlined in Figure 1A the f1 intergenic region was obtained from the phagemid vector pEMBL8. The majority of the sequences contained in the terminator portion of the f1 origin which lacks initiator activity were contained in the RsaI (position 5587) to HinfI (position 5767) restriction fragment. To provide the remainder of the terminator sequences, an oligonucleotide was synthesized. The sequence of the oligonucleotide constructed for this purpose extended from the naturally occurring HinfI site at position 5767 to position 5809. Position 5809 corresponds to the end of the termination signal as defined by Dotto, *et al.* (1) and is 28 b.p. downstream of the gene II cleavage site (Figure 4). Eight base pairs were added to base 5809 to make EcoRV and NdeI restriction sites at the end of the oligonucleotide. This synthetic oligonucleotide, the RsaI - HinfI restriction fragment from the f1 origin, and an 3009 b.p. DraII/NdeI fragment from pBluescribe (pBS) were ligated together to construct the plasmid, pBST-B, which contained the f1 terminator domain (Figure 1A).

The initiator domain of the intergenic region was isolated from pEMBL8 by cutting with the restriction enzymes Sau96I and DraI. This generated a fragment of DNA extending from 5725 to 5942 of the f1 phage origin. The ends of this DNA fragment were made blunt by filling-in with the Klenow fragment of DNA polymerase I. The DNA fragment was then subcloned into the NarI site of pBST-B. The resulting vector, pBSITO#12, contained the initiator and terminator domains of the f1 origin separated by the unique restriction sites EcoRV and NdeI, as well as 52 b.p. of DNA sequence from the vector pBS (Figure 1A). pBSITO#12 was then modified to include the pBluescript polylinker, resulting in the plasmid pPreB (Figure 1A).

The pPreB plasmid, which contains the initiator and terminator sequences, was inserted into a

```
      5767
      HinfI                                          EcoRV NdeI
5'  AGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAAGATATCCA  3'

3'     GGTGCAAGAAATTATCACCTGAGAACAAGGTTTGACCTTCTATAGGTAT  5'
```

Figure 4. Sequence of the Synthetic Oligonucleotide.

Sequence of the synthetic oligonucleotide used to construct the terminator domain lacking initiator activity, as illustrated in Figure 1A and described in the text. The site of nicking by gene II is indicated by {*}. All sequence, excluding that creating the EcoRV and NdeI sites, was derived from the f1 origin as illustrated in Figure 2.

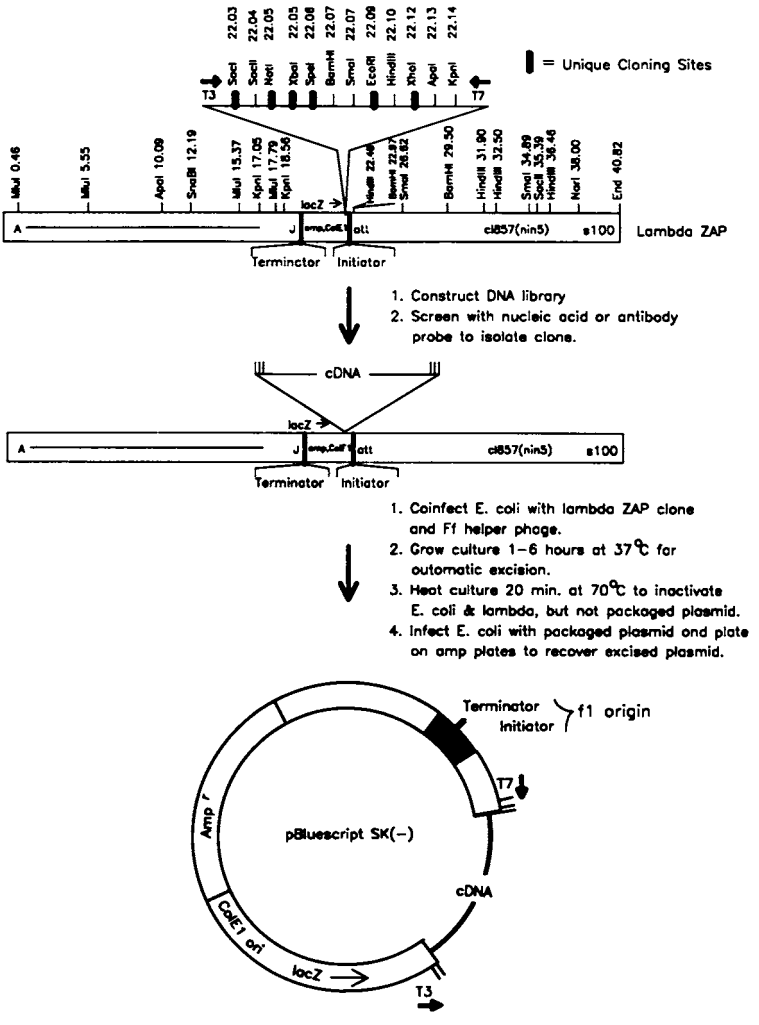


Figure 5. Outline of the *In Vivo* Excision Process.

The *in vivo* excision process is outlined in the above diagram and detailed in the Methods section: Libraries prepared in lambda ZAP can be screened with either nucleic acid or antibody probes. Lambda plaques recognized by the probe are isolated and allowed to infect cells which are co-infected with fd, f1, or M13 helper phage. Inside the cell trans-acting proteins from the helper phage recognize two separate domains (Initiator and Terminator) positioned within the lambda ZAP arms. Both of these signals are recognized by the helper phage gene II protein and a new DNA strand is synthesized, displacing the existing strand. The displaced strand is circularized and packaged as a filamentous phage by the helper phage proteins, then secreted from the cell. Bluescript plasmids are recovered by infecting an F' strain and plating on ampicillin plates, giving bacterial colonies.

The lambda ZAP map is shown in the top half of the figure. Restriction sites unique in lambda ZAP are shown by the bold hatch marks. The polylinker containing the six unique restriction sites is identical to the pBluescript SK polylinker (1). (Ff= Filamentous phage, T7;T3= Phage RNA promoters, black box= F1 origin sequences).

Table I. **Excision Efficiencies**

Helper phage	Lambda phage		Time of growth at 37°C # of colonies excised		
	(pfu)		(2hr.)	(4hr.)	(6hr.)
M13K07	---	0	0	0	0
IR1	---	0	0	0	0
R408	---	0	0	0	0
---	10^8	ZAP#1	0	0	0
M13K07	10^8	ZAP#1	$1x10^6$	$>1x10^6$	$>1x10^6$
IR1	10^8	ZAP#1	$>1x10^6$	$>1x10^6$	$>1x10^6$
R408	10^8	ZAP#1	$>1x10^6$	$>1x10^6$	$>1x10^6$
M13K07	10^7	ZAP#1	$2.5x10^5$	$>1x10^6$	$>1x10^6$
IR1	10^7	ZAP#1	$>1x10^6$	$>1x10^6$	$>1x10^6$
R408	10^7	ZAP#1	$2.5x10^5$	$>1x10^6$	$>1x10^6$
M13K07	10^6	ZAP#1	$1.8x10^4$	$8.6x10^4$	$1.2x10^5$
IR1	10^6	ZAP#1	$4.5x10^4$	$3.0x10^5$	$6.0x10^5$
R408	10^6	ZAP#1	$4.0x10^4$	$1.1x10^5$	$1.3x10^5$
M13K07	10^5	ZAP#1	$<5x10^2$	$6.5x10^3$	$1.2x10^4$
IR1	10^5	ZAP#1	$6.5x10^3$	$1.2x10^5$	$3.0x10^5$
R408	10^5	ZAP#1	$3.0x10^3$	$8.0x10^3$	$9.5x10^3$
M13K07	10^4	ZAP#1	$<5x10^2$	$1.0x10^3$	$1.0x10^3$
IR1	10^4	ZAP#1	$1.5x10^3$	$1.1x10^4$	$3.8x10^4$
R408	10^4	ZAP#1	$<5x10^2$	$<5x10^2$	$1.0x10^3$

The results in the above table were taken from one representative excision experiment of at least four independent experiments. 200ul of OD_{600}=1.0 XL1-Blue cells were infected with lambda phage for each sample. The excision efficiency of the lambda ZAP clone, ZAP#1 (2000 b.p. insert), was evaluated varying the following parameters: (1) incubation time during rescue, (2) titer of the lambda phage, and (3) the strain of the helper phage. The total colony number was determined by extrapolation from plating 10ul of the 5ml excision culture prepared as described in the Methods section.

lambda phage to construct Lambda ZAP. The plasmid sequences were inserted into a nonessential region of a lambda phage such that the plasmid sequences were flanked by the initiator and terminator domains. The lambda arms were constructed to contain few restriction sites in order to maintain a number of unique restriction sites within the polylinker region for the subcloning of desired genes. Long D was used to prepare the right arm since it contains the *nin5* (2.5 kilobases (k.b.)) deletion (32) and has no XhoI restriction site (Figure 1B). The left arm was taken from lambda L47.1 (33) using the SmaI site that flanks the essential gene, J, of lambda (Figure 1A). The PreB plasmid was linearized at the EcoRV restriction site positioned between the initiator and terminator regions (Figure 1A), and ligated to the lambda arms. The resulting lambda phage is 40.8 k.b. long, which permits the phage to be packaged with 0 to 10 kilobase inserts. There remain 6 six unique restriction sites in the polylinker region (Figure 5). The initiator and terminator domains are positioned on opposite ends of the 3.0 kilobases of plasmid DNA, which confers ampicillin resistance, and has a ColE1 origin of replication, a pBluescript SK polylinker, the alpha portion of the *lacZ* gene required for alpha-complementation (19), and T7 and T3 RNA polymerase promoters. Excision of the plasmid sequences by helper phage protein(s) will generate a phagemid that is identical to pBluescript SK(-) (Figure 2). The version of lambda ZAP that lacks the *Sam100* mutation, lambda ZAPII, was constructed by replacing the lambda S gene contained in a 4254

Table II. **Excision of Clones Selected with β-actin Oligonucleotide Probe**

Clone #	Lambda phage titer	Total Colony # (4 hr)	Insert size (bp)
1	$1x10^7$	$7.71x10^6$	1400
2	"	$4.92x10^6$	2000
3	"	$3.32x10^6$	3150
4	"	$4.95x10^6$	2200
5	"	$3.19x10^6$	1800
6	"	$8.14x10^6$	2200
7	"	$5.53x10^6$	1800
8	"	$5.00x10^6$	1900
13	"	$3.53x10^6$	1000
16	"	$3.62x10^6$	1400
35	"	$3.19x10^6$	1650
49	"	$3.51x10^6$	2500

Excision was performed as described in the Methods section with an incubation time of four hours using the R408 helper phage. The colony number is represented as the total number of colonies recovered in the 5 ml excision culture. Insert size was estimated by agarose gel electrophoresis. Lambda phage concentrations were diluted to give equivalent starting titers. These titers were confirmed by consistent plaque numbers (+/- 10%) obtained in duplicate platings.

b.p. NcoI DNA fragment of lambda ZAP with the 4254 b.p. NcoI DNA fragment from lambda gt10 (34).

Lambda ZAP Excision of cDNA Clones

The efficiency of the automatic excision process was tested on a randomly selected cDNA clone (insert size 2 k.b.) isolated from a chicken liver cDNA library. This experiment was carried out utilizing the excision protocol diagrammed in Figure 5 and described in the Methods section. Five parameters were evaluated to determine the conditions for maximum recovery of phagemid-containing colonies: (1) titer of the lambda ZAP used in the excision experiment, (2) time of incubation, (3) effects of different M13 and f1 helper phage strains, (4) titer of the M13 and f1 helper phage strains, and (5) incubation temperature of the excision reaction. The results of the excision experiments are presented in Table I.

The titer of the lambda phage is important to obtain high efficiency of phagemid recovery. Excision of the phagemid from lambda ZAP as described requires at least $1X10^2$ lambda phage particles. Increasing the phage titer resulted in a linear increase in the number of excised phagemid vectors.

Although the titer of the helper phage used in the excision experiments was tested over a range of $2x10^5$ to $2x10^9$ pfu, this caused no appreciable difference in the number of ampicillin-resistant colonies obtained (data not shown). $2x10^5$ pfu of helper phage gives a multiplicity of infection (m.o.i.) of 10^{-3}. This indicates that the helper phage replicates and, therefore compensates for its lower initial titer. Interestingly, a difference in the excision efficiencies was observed with the different helper phage strains. IR1 (18) consistently gave 5 to 10-fold higher excision efficiencies than either M13K07 (J. Viera, unpublished) or R408 (17). M13K07 and R408 showed no significant differences in repeated experiments. In a separate experiment the helper phage RV1 (35) gave only 20% as many colonies as either M13K07 or R408 (data not shown). However, the high efficiency of this procedure permits the effective use of each helper phage.

Figure 6. Restriction Digestion of Excised cDNA Clones.
The DNA of the twelve putative actin clones was digested with EcoRI and electrophoresed on a 1% agarose gel. Hind III digested lambda *cI857, ind1, Sam7* DNA was used as a molecular weight marker (M). The two lambda ZAP lanes (Z) show the EcoRI-digested, excised vector, i.e. pBluescript SK(-), without an insert. This pBluescript SK(-) band is present in all of the twelve rescued clones (duplicates are indicated by {'}). Additional bands in EcoRI digested cDNA clones represent insert DNA. Several clones (numbers 3,4,6 and 49) contain more than two DNA bands, indicating that these clones contain an internal EcoRI restriction site. (UN= undigested pBluescript, pBT= linearized pBluescript).

Lambda ZAP with cloned inserts ranging in size from 1000 b.p. to 3200 b.p. showed no correlation between excision efficiency and the size of the DNA insert (see Table II and next section).

Identification of β-actin Clones

A lambda ZAP chicken liver cDNA library was screened with a 17 base oligonucleotide derived from the published chicken β-actin sequence (26). Twelve clones were titered at a low density and rescreened with the oligonucleotide probe. Isolated plaques were cored from agar plates and resuspended in SM buffer. These clones were then excised from the lambda ZAP vector as described in the Methods section. All phage containing putative actin clones gave rise to ampicillin-resistant colonies following excision from the lambda vector. Up to a 10-fold variation in the number of excised phagemids leading to colonies was observed (data not shown), and although this number did not correlate with the size of the DNA insert, as shown in the results presented in Table II., it did correlate with the starting lambda ZAP phage titer.

The size of the insert was determined by restriction mapping double stranded DNA prepared from the colonies obtained after excision. Two colonies, for each putative actin clone obtained from separate excision experiments, were selected and digested with EcoRI and analyzed on a 1% agarose gel (Figure 6). The size of the twelve inserts ranged from 1000 b.p. to 3200 b.p. with no variation between like clones. Clone number 6 both contained one of the largest inserts (2200 b.p.) and gave the highest excision efficiency (Table 1). Independent excisions of the same twelve clones showed equivalent results and no deletion or rearrangement of the insert was observed (data not shown).

Single-stranded templates were prepared for sequence analysis of the twelve putative actin clones by helper phage rescue. Six of the rescued clones (numbers 1,2,3,4,5 and 7) were sequenced to 200 b.p. downstream of the oligonucleotide binding site, using the screening probe as a sequencing primer. Based on 100% homology with the published chicken β-actin sequence, clones #2 and #7 were positively identified as chicken β-actin (26). Both β-actin clones #2 and #7 were essentially full-length based on the published 1900 b.p. size of the β-actin cDNA. The other four clones either represented other actin sub-types or related genes (36).

It should be noted that the use of the partial f1 origins in the lambda ZAP vector permitted the excision of intact clones regardless of the presence of internal EcoRI restriction sites (numbers 3,4,6 and

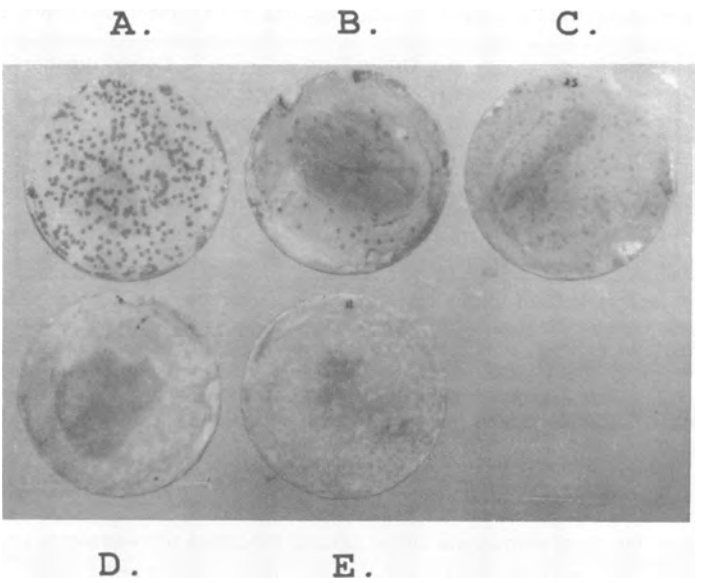


Figure 7. Antibody Screening of Fusion Proteins Produced in Lambda ZAP and Lambda gt11.
Lambda plaques previously grown on a BB4 bacterial lawn were transferred to nitrocellulose filters and screened with the GCS antibody and an alkaline phosphatase conjugated secondary antibody. The screening conditions are outlined in the Methods section. Filters: **A.** Lambda ZAP GCS#4, **B.** Lambda gt11 GCS#4, **C.** Mixture of Lambda ZAP GCS#4 with Lambda ZAP, **D.** Lambda ZAP alone, & **E.** Lambda gt11 alone.

49). These results demonstrate the utility of the *in vivo* excision process and advantage of the Bluescript SK(-) phagemid for rapid sequence analysis.

Antibody Screening of Lambda ZAP Clones

Lambda gt11 and lambda ZAP clones containing the GCS insert were screened with GCS antiserum. These phage were first plated on a lawn of BB4 cells to form plaques for screening. In addition, separate control plates were prepared containing lambda gt11 and lambda ZAP plaques without inserts, as well as plates containing a mixture of lambda ZAP phage with and without insert and lambda gt11 with and without insert. Duplicate nitrocellulose filter lifts were prepared from each of these plates. The filters were screened with the GCS primary antibody and the alkaline phosphatase conjugated secondary antibody as outlined in the Methods section.

As shown in Figure 7(A-E), the antibodies did not bind to either lambda gt11 or lambda ZAP lacking inserts, as expected (Figure 7D & E). Filters containing either lambda gt11/GCS or lambda ZAP/GCS gave distinct, positive signals. In addition, the anticipated number of clones were positive on nitrocellulose filters containing a mixture of lambda ZAP/GCS and lambda ZAP phage (Figure 7C). Equivalent signal intensities were obtained from lambda gt11 and lambda ZAP were equivalent (Figure 7A & B). In subsequent experiments we have compared the signal intensity from lambda gt11 fusion proteins on the recommended host strain, Y1090 (*RecA+*), to that of lambda ZAP on BB4 (*RecA+*). In

Table III. **Transformation Efficiency of Various Plasmids into a Gene II Expressing *E. coli* Strain**

Plasmid	Origin Type		Colony number
	f1	ColE1	
pBR322	-	+	1000
pBS	-	+	400
pBS(+)	+	+	0
pBSITO#12	+*	+	0
pPreB	+*	+	1

Gene II expressing BHB(lambdaCH616) cells (36) were made competent by the $CaCl_2$ treatment (19). These cells were transformed with 100 ng. of each plasmid and 1/10 of the transformation reaction was plated onto LB/ampicillin plates. Plasmids pBS and pBR322 contain only the Col E1 origin of replication. Plasmids pBS(+), pBSITO#12, and pPreB all contain f1 origins of replication and were derived from pBS. This is a representative result from at least 4 independent transformation experiments. (* = pPreB and pBSITO#12 contains the separated initiator and terminator domains). The kanamycin resistant plasmids, pfdA2 & pfdB2, contain only the f1 origin and were stably maintained in BHB(lambdaCH616) cells, indicating that gene II was functional in these cells.

these experiments the signal intensity was similar although the plaque size was slightly larger with the BB4 cells. Lambda gt11 and Lambda ZAP yield plaques of equivalent size on BB4 cells.

DISCUSSION

The *in vivo* excision mechanism utilizing the initiator and terminator signals of the f1 origin offers several advantages over excision utilizing the host recombination system (*recA*). The host recombination system relies on the existence of two homologous sequences flanking the region to be excised. The efficiency of this procedure is directly related to the length of the repeats (37,38). However, even with large repeats, this efficiency is several orders of magnitude lower than that utilizing the f1 partial origins.

The Bluescript SK(-) phagemid, excised from the lambda ZAP vector with helper phage, is a 2958 b.p. multipurpose phagemid vector designed for rapid DNA sequence analysis. Since the f1 origin is regenerated by excision from the lambda ZAP vector, single stranded DNA can be rescued from the phagemid by helper phage. However, the most efficient helper phage strain for excision (IR1, Table I) of the Bluescript SK(-) phagemid from lambda ZAP is different from the helper phage strains that are best for single stranded DNA rescue (M13K07 and R408) as judged by the phagemid to helper phage ratios in single stranded rescue (JMF, unpublished observation). Potential explanations for this observation include the difference in the gene II proteins of the different helper phage, which are essential for the nicking at the initiator and terminator sites and religation to reform the intact f1 origin. The gene II protein may also interact differently with the partial origins within the lambda vector than with the intact origin in pBluescript. Differences in helper phage packaging efficiency could also account for these variations. It is likely that the variation in excision efficiency of the helper phage strains is a result of the complex interaction of the rate of lambda phage, helper phage, and cell replication.

Attempts to excise the pBluescript SK (-) phagemid from lambda ZAP using strains expressing f1 gene II protein from a gene integrated in the host chromosome (39) were unsuccessful. As indicated in Table III, the presence of two functioning origins (f1 and ColE1) within the same vector is detrimental to efficient maintenance replication of these phagemids. There is less than a 10-fold difference in the

number of ampicillin resistant colonies appearing with these phagemids after transformation into XL1-Blue cells, which do not express gene II (data not shown). These observations must be considered in any attempts to stably maintain these dual-origin vectors in cell lines expressing gene II protein.

Although the excision process utilized by the lambda ZAP vector is an attractive feature, the vector also offers several other advantages. The polylinker within lambda ZAP contains twenty one restriction sites, six of which are unique in the lambda vector. The NotI restriction site is particularly significant since it cuts only once in every 250,000 b.p. in most eukaryotic DNA. The presence of this and other restriction sites permits the use of "forced cloning" strategies for expression of cDNA inserts from the *lac* promoter. In addition, the Not I site within lambda ZAP should prove useful in the construction of jumping or junction libraries which depend upon infrequently cutting restriction enzymes (40) or in cloning strategies utilizing NotI linkers to avoid methylation of the DNA insert to prevent cleavage. The vector also has an insert capacity of up to 10 k.b. without exceeding the packaging limit, which is 105% of wild type lambda phage (51 k.b.). This large capacity, in addition to the choice of cloning sites and *in vivo* excision process, makes the lambda ZAP vector attractive for the construction of genomic libraries. In addition, lambda phage preparations are no longer required for obtaining large quantities of DNA inserts, since DNA can be prepared from plasmid preparations. Finally, our results demonstrate that the lambda ZAP vector can be used efficiently as a cDNA expression vector.

The ability to rapidly and efficiently convert lambda phage clones to phagemids reduces time for analysis of cloned genes. We are currently expanding the utility of this vector by introducing phagemids suitable for expression in eukaryotic cells between the initiator and terminator signals of lambda ZAP. These vectors will allow the construction of high efficiency lambda libraries instead of the standard plasmid libraries utilized for eukaryotic expression. Clones could then be screened by standard procedures or the entire library could be excised and transfected into eukaryotic cells, then screened for expression of the cloned gene.

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