EK2 Derivatives of Bacteriophage Lambda Useful in the Cloning of DNA from Higher Organisms: The λgt*WES* System

Abstract. *A derivative of bacteriophage λ has been modified and tested together with an appropriate host system to meet the criteria of EK2 biologic containment for cloning DNA from higher organisms. In this report certain of the safety features are summarized and some of the tests carried out to confirm the containment properties of the vector are described. The cloning efficiency of this system, together with available gene purification and hybrid screening technology, indicate that it can be used to clone DNA fragments carrying specific, unique mammalian genes.*

Thomas *et al.* (*1*) have constructed a mutant strain of coliphage λ which is especially suited for cloning fragments of foreign DNA. The phage, λgt·λC, carries two Eco RI restriction sites between which Eco RI–generated DNA fragments of 1,000 to 14,000 base pairs (1 to 14 kilobase pairs) can be inserted. Because incorporation of a DNA fragment of approximately 1 kbp or more is required for phage growth, this system also provides a strong positive selection for fragment-bearing recombinants.

Recently we introduced three amber mutations—*W*am403, *E*am1100, and *S*am100—into this phage with the aim of reducing its likelihood of encountering a susceptible host and surviving in nature (*2*). We have further modified the resulting phage, λgt*WES*·λC, by substituting the phenotypically inert Eco RI λ fragment, λB, for λC (Fig. 1). The λC fragment contains the genes for specialized recombination and, according to its EK2 requirements, must be biochemically separated from the two larger λ fragments before in vitro recombination. The new derivative λgt*WES*·λB, can be used in recombination experiments directly (*3*). For testing purposes we have also constructed two model recombinants, one of which contains the genes for galactose metabolism derived from the λp*gal*8 recombinant described in Enquist *et al.* (*2*) (Fig. 1). The other contains a 7.6-kbp Eco RI fragment of *Escherichia coli* DNA.

These phage contain amber mutations in genes corresponding to (i) the major capsid protein of the phage, which is also required for cleavage of replicated DNA (the *E* gene), (ii) a protein needed for the joining of phage heads and tails (the *W* gene), and (iii) a protein required for host lysis (the *S* gene). While the *W* and *E* genes are suppressed by several amber suppressors including *sup*E, the S mutation is not efficiently suppressed by *sup*E and requires a specific suppressor, *sup*F. In lysates, the frequency of wild-type revertants of each of these mutations is approximately 10^{-5} to 10^{-6} (Table 1). Wild-type revertants could not be detected in lysates of phage carrying double or triple mutations. λgt*WES*·λC may be propagated conveniently through lytic culture or as a lysogen since it carries the λ integration-excision system and the temperature-sensitive repressor *c*I857. λgt*WES*·λB can be prepared as a high-titer stock through lytic infection in liquid culture. Both phage can accept Eco RI fragments of foreign DNA as noted above, but λgt*WES*·λB can also accept Sst I fragments 0.01 to 10 kbp in length (Fig. 2). The presence of these Sst I sites also provides a selection against parental type Eco RI recombinants since the vector may be digested with both Sst I and Eco RI, destroying the parental λB fragment.

Bacteriophage λ has a number of features that recommend it as a safe and easily contained vector. This phage has a fastidious host range and is apparently restricted to growth in a limited number of *E. coli* strains. In fact, we have surveyed more than 300 separately isolated, human hospital strains of *E. coli* and were unable to find any capable of supporting growth of either λ wild-type or λgt*WES* phage (Table 2). In addition, λ and the λgt*WES* derivatives are readily inactivated by passage through the mouse alimentary tract

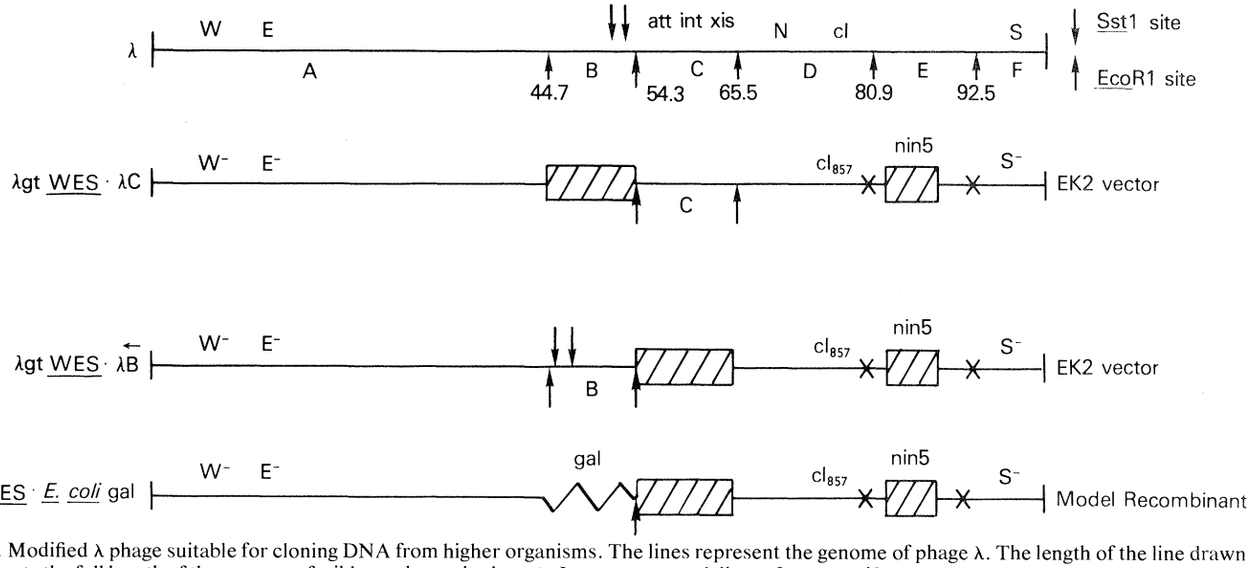

Fig. 1. Modified λ phage suitable for cloning DNA from higher organisms. The lines represent the genome of phage λ. The length of the line drawn represents the full length of the genome of wild-type bacteriophage λ. Letters over each line refer to specific λ genes. Letters under each line refer to Eco RI restriction fragments of λ with each arrow indicating an Eco RI site. The numbers under each arrow represent the position of the site as a percentage of the λ genome. Arrows over each line indicate the position of an Sst I site (note inversion as compared to wild type). Scored boxes represent deleted portions of the λ genome. X represents the point at which an Eco RI site has been eliminated by mutation. The broken line represents the location of *E. coli* DNA containing the galactose operon. Details of the construction of the two vectors have been described by Enquist *et al.* (*2*) and Tiemeier *et al.* (*3*). The model recombinant was constructed by crossing λgt*S*am100·λC with λ*W*am403 *E*am1100 *gal*8 *bio*256 *imm*434 *c*Its1 as described by Enquist *et al.* (*2*). The resulting phage eliminated the RI site at 54.3 percent λ and substituted an RI site within and close to the right end of the *gal*8 insertion. The internal Eco RI fragment containing a small portion of the *gal*8 insertion and the λ genes *int*, *xis*, and a portion of the *red* gene were deleted by in vitro recombination, yielding the phage depicted in the diagram. The position of the left end of the *gal*8 substitution is at 44.7 percent on the λ map (*13*), very close to the original λ Eco RI site.

Table 1. Ability of λ derivatives to grow on nonsuppressor-containing hosts. All stock lysates had titers of 1 to 2 × 10^{10} per milliliter. Where possible, each mutation was tested individually in the strain used in its construction (*2*).

Derivative	Frequency of amber$^+$ revertants
λ*W*am403 *E*am1100	< 10^{-8}*
λ*W*am403 *E*am1100 *S*am100 · λC	< 10^{-8}*
λ*S*am100 · λC	~ 10^{-5}†
λ*W*am403	~ 5 × 10^{-6}†

*A 0.01-ml portion of undiluted stock plated on *Escherichia coli* strain 594 (*sup*0). †A 0.01-ml portion of 1000-fold diluted stock plated on *E. coli* strain 594 (*sup*0).

Table 2. Ability of phage λ, safer vector, and model recombinant to grow on environmentally encountered *E. coli* strains. Experiments on hospital samples obtained from the NIH Clinical Center Microbiology Diagnostic Laboratory were tested by plating 0.02-ml droplets of phage containing ~ 10^8 plaque-forming units on lawns of each strain, using standard procedures (*1*). λgt*WES* · *E. coli* contains a 7.6 kilobase pair Eco RI fragment of *E. coli* DNA.

Strain source	Number tested	λ	Number of strains permitting plaque formation	
			λgt-*WES* · λC	λgt-*WES* · λC
Waterborne*	> 1000	0	NT	NT
Hospital samples	> 300	0	0	0

*Data supplied by R. Davis.

Table 3. Ability of model recombinant to survive in mouse alimentary tract. Three BALB/c mice were fed drinking water containing 10^7 plaque-forming units of λgt*WES* · *E. coli*, a model recombinant, per milliliter. Approximately 100 ml was consumed during the experiment. Stool samples (~ five droppings) were collected on sterile paper on the days indicated and were suspended in TMG (0.01*M* tris, 0.01*M* $MgSO_4$, 0.01 percent gelatin, *p*H 7.4) containing chloroform. The suspension was tested for plaques on *E. coli* Ymel (a *sup*F host).

Sample	Phage recovered (per milliliter)
Water supply, day 1	~ 10^7
Stool, day 1	0
day 2	0
day 4	0
day 6	0
Water supply, day 7	~ 10^7

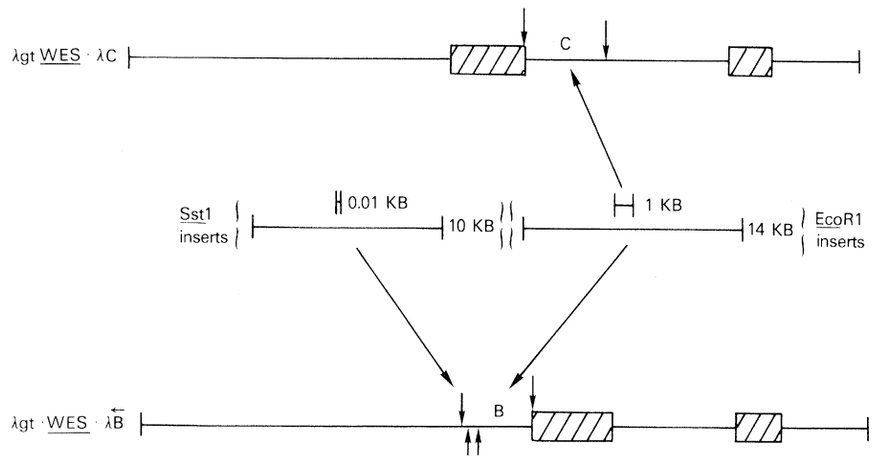

Fig. 2. Lengths of Eco RI and Sst I restriction fragments which may be inserted into λgt*WES*·λC and λgt*WES*·λB. Symbols are as indicated in the legend to Fig. 1. The arrows above the line represent Eco RI sites, and those below the line represent Sst I sites. Abbreviation: *KB*, 1000 base pairs.

Table 4. Safety features of λgt*WES* · λC or λgt*WES* · λB.

1. Three conditional lethal mutations (amber)
   *E*am1100; no phage heads and no cleavage of DNA to form cohesive ends
   *W*am403; no joining of phage heads and tails
   *S*am100; no lysis or phage release
   *sup*F suppresses all three mutations
   sup E does not suppress *S*am 100 efficiently
2. *c*Its857
   Temperature-sensitive repressor; at 37°C, λ*c*Its 857 will not form a stable lysogen
3. *nin*5 deletion
   Removes a transcription stop signal; greatly reduces probability of plasmid formation
4. Recombinants are recombination defective (*red*$^-$)
   λ *red*$^-$ phage grow less well than *red*$^+$ phage, conferring a selective disadvantage
5. Under permissive conditions, recombinants are capable of high-titer growth in small, easily manipulated volumes
6. Restriction barrier
   Phage can be propagated in nonmodifying hosts; unmodified DNA is destroyed on infection of a restricting host

Table 5. Test for the persistent association of cloned fragment with the permissive host used in its propagation. Routine preparative procedure: *E. coli* DP50 [*sup*F, *sup*E, *dap*D8, *lac*Y, Δ (*gal-uvr*B) *thy*A, *nal*A^r, *hsd*S] was grown overnight in tryptone broth supplemented with diaminopimelic acid (DAP, 100 μg/ml) and thymidine (50 μg/ml), and washed and resuspended in an equal volume of 0.01*M* $MgSO_4$. Approximately 1.5 × 10^8 bacteria were incubated with 10^6 λ *W*am403 *E*am 1100 *gal*$^+$ (*att-red*)$^\Delta$ *c*Its857 *nin*5 *S*am100 (multiplicity of infection ≅ 10^{-2}) for 10 minutes at 32°C in 0.2 ml of 0.01*M* $MgSO_4$, then diluted with 5 ml of L broth (supplemented with DAP and thymidine as above) and incubated at 38°C with vigorous shaking. Lysis occurred after 8 to 9 hours and incubation was continued for 24 hours. The phage titer was determined at the time of lysis (9 hours). Viable bacteria were determined by removing 1-ml portions, diluting and washing with 5 ml of 0.01*M* $MgSO_4$, centrifuging and washing again, and resuspending in 1 ml of 0.01*M* $MgSO_4$. Incubation was at 32°C on minimal-salts plates supplemented with DAP (100 μg/ml), thymidine (40 μg/ml), biotin (0.5 μg/ml), lysine (4 μg/ml), and glucose (0.5 percent). The *gal*$^+$ bacteria were determined from portions of cells treated as just described, except that plating was on medium containing 0.5 percent galactose in place of glucose. In control experiments, supplemented minimal-salts plates were checked for their ability to grow *gal*$^-$ and *gal*$^+$ derivatives of DP50 *sup*F in the presence of glucose or galactose. In the sample immediately after lysis, 4.5 × 10^5 cells were plated on minimal-galactose plates incubated at 32°C. No *gal*$^+$ colonies were detected. In the sample 24 hours after lysis, 7.4 × 10^4 cells were plated on minimal-galactose plates. No *gal*$^+$ colonies were detected. The validity of the detection assay was assessed by adding *gal*$^+$ DP50 *sup*F (λ*pgal*8 *c*Its857) cells to overgrown cells before spreading on minimal-galactose plates. Recovery of *gal*$^+$ cells was approximately 100 percent.

Time	Phage titer (ml^{-1})	Bacterial titer (ml^{-1})	*gal*$^+$ transductants (ml^{-1})
At lysis	2.3 × 10^{10}	4.8 × 10^5	< 1
24 hours after lysis		7.4 × 10^4	< 10

[Table 3 and Geier, Trigg, and Merril (*4*)].

While λgt*WES* phage are at an obvious survival disadvantage because of their suppressor requirements, they have a further disadvantage in that fragment-containing phage lack the λ general recombination functions (*red*) required for normal phage growth (*5*). The yield of such *red*-deficient phage is one-half to one-tenth that of the λ wild type. Nevertheless, sufficient recombinant phage may be prepared from 1 to 2 ml of lysates to permit detailed enzymatic, hybridization, and electron microscopic analyses (*6*). The specific safety features of the λgt*WES* system are summarized in Table 4 (*2*). The presence of each genetic feature, including the amber mutations, the temperature-sensitive repressor gene, the *nin*5 deletion, and the absence of the specialized recombination genes, can be determined by simple genetic tests (*2, 3*).

In addition to other detailed tests required for EK2 certification (*7*), we have tested the possibility that a cloned fragment could form a persistent association with a bacterial host used in its propagation. The experiments described in Table 5 represent a typical preparative phage growth using the model gal^+ phage recombinant and the attenuated host strain *E. coli* K12 DP50/*sup*F (*8*). The gal^+ genes carried by the model recombinant represent a cloned DNA fragment. Any persistent association with the DP50/*sup*F host (in which the entire galactose operon is deleted) during lytic growth of the λ*WES* gal^+ phage will give rise to gal^+ colonies among the bacterial survivors in the lysate. As shown in Table 5, no gal^+-containing bacteria were detected among the bacteria surviving either at the time of lysis or 24 hours thereafter.

λgt*WES*·λC was certified as an EK2 vector by the NIH Advisory Committee on Recombinant DNA Research at its January 1976 meeting, but has been superceded by λgt*WES*·λB, which was certified in February 1977. The Advisory Committee has further required that the phage be grown preparatively in the attenuated host strain *E. coli* K12 DP50/*sup*F (*8*).

We have considerable experience in the use of λgt*WES* derivatives for cloning of partially purified DNA fragments derived from total mouse genomic DNA. We generally obtain about 4500 hybrid phage per microgram of Eco RI DNA fragment (*6*). This efficiency suggests that any unique sequence purified about 1000-fold could be identified by available screening techniques (*9*). We recently cloned an Eco RI fragment containing a segment of the mouse ribosomal RNA sequences (*6*). This fragment occurs about once per 5000 mouse Eco RI fragments and is approximately 200-fold reiterated with respect to unique sequences. McClements and Skalka (*10*) cloned a similar ribosomal fragment from genomic chicken DNA using the λgt*WES*·λC vector. While the requirements of EK2 biological containment clearly reduce the flexibility of the cloning technology, the λgt*WES* system should permit cloning of unique sequences of genomic DNA from higher organisms. Other attenuated strains of λ, such as those constructed by Blattner and his associates (*11*) and Donoghue and Sharp (*12*) should be useful in this respect as well.

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8. This strain, derived by R. Curtis, D. Pereira, and their associates at the University of Alabama (personal communication) has a number of convenient genetic features that make it useful for testing and reduce its ability to grow outside the laboratory (see Table 5). To propagate the λgt*WES* phages, we have introduced *sup*F into this strain.
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14. We are again most grateful to C. Kunkle for her expert editorial assistance in the preparation of this manuscript.

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Chemical Synthesis of Restriction Enzyme Recognition Sites Useful for Cloning

Abstract. *By a triester chemical synthesis method, three decameric DNA's have been made; these act as substrates for several restriction endonucleases, including Eco RI, Bam I, and Hind III. These homogeneous decamers form duplexes that can be efficiently blunt-end ligated to themselves or to other DNA molecules by the action of T4 DNA ligase and thus are useful tools for molecular cloning experiments.*

Restriction endonucleases recognize specific sequences in double-stranded DNA. Many of these endonucleases cleave their recognition sites to produce blunt ends, while others such as Eco RI, Bam I, and Hind III, leave cohesive or "sticky" ends (*1–3*). DNA fragments with these cohesive ends can be inserted easily into, and subsequently excised from, a suitable cloning vehicle such as the plasmid pMB9 (*4*), which has one site each for Eco RI, Bam I, and Hind III.

Unfortunately, cohesive ends are lacking in many DNA fragments of interest, such as randomly cleaved DNA, complementary DNA (cDNA), and many restriction endonuclease fragments. We have recently introduced a new general procedure suitable for cloning almost any DNA molecule (*5*). This method (Fig. 1) involves the addition by blunt-end ligation of short DNA segments to both ends of the DNA to be cloned. The added segments contain restriction endonuclease cleavage sites, and treatment with the corresponding endonuclease leaves cohesive ends on the subject DNA. This DNA then can be cloned by incorporating it into a plasmid or other vector that has been opened with the same restriction enzyme. The feasibility of this new method was demonstrated by adding an octadeoxyribonucleotide containing the Eco RI recognition sequence to a 21-base-pair duplex bearing the *lac* operator sequence. A clonable, excisable DNA fragment was obtained, and was shown to function as *lac* operator should, both in vitro and in vivo (*5*).

We report here the chemical synthesis by the improved triester method of Itakura *et al.* (*6*) of three new decameric "linker" molecules (see Fig. 1), which together contain seven different recognition sites cleavable by 23 different restriction endonucleases including Eco RI, Bam I, and Hind III (*2*). These linker molecules should add great flexibility to cloning methodology, and also provide substrates for physicochemical studies on restriction enzyme recognition, methylation, and cleavage mechanisms.

The synthetic scheme in Fig. 2 outlines the strategy used in the convergent synthesis of the Bam I decamer by the triester method, using 2,4,5-triisopro-