
Purification of genomic sequences from bacteriophage libraries by recombination and selection *in vivo*

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ABSTRACT

Cloned genes have been purified from recombinant DNA bacteriophage libraries by a method exploiting homologous reciprocal recombination *in vivo*. In this method 'probe' sequences are inserted in a very small plasmid vector and introduced into recombination-proficient bacterial cells. Genomic bacteriophage libraries are propagated on the cells, and phage bearing sequences homologous to the probe acquire an integrated copy of the plasmid by reciprocal recombination. Phage bearing integrated plasmids can be purified from the larger pool of phage lacking plasmid integrates by growth under the appropriate selective conditions.

INTRODUCTION

The in-situ plaque hybridization assay of Benton and Davis (1) allows the recovery of bacteriophage clones bearing specific gene sequences from large random 'libraries' of phage-borne DNA. In a typical application of this assay as many as 10^6 clones may be simultaneously screened for the presence of a sequence of interest.

In this paper we demonstrate an alternative method for the recovery of recombinant phage, applied here to phage bearing human β -globin gene sequences. The method relies on homologous reciprocal recombination between a very small 'probe' plasmid, and globin gene-containing phage bearing two amber mutations in essential phage genes. The probe plasmid contains a suppressor tRNA gene, and a short probe segment derived from genomic single-copy DNA flanking the β -globin gene. Recombination between phage and plasmid yields phage bearing an integrated copy of the probe plasmid. The suppressor tRNA gene of the integrated plasmid then allows the amber-mutated globin phage to grow in

suppressor-free hosts. The efficiency of the recombination process allows the direct recovery of single-copy sequences from bacteriophage libraries of mammalian DNA.

MATERIALS AND METHODS

Strains and Reagents. *P. phaseolicola* strain HB10Y bearing plasmid pLM2 (2) was obtained from L. Mindich, phage PRD1 (3) from B. Stitt. *E. coli* strain W3110 $r^{-m}+$ was a spontaneous thy^+ revertant of a strain donated by J. Campbell (4). Strains bearing the $lac Z_{1000}$ am mutation (5) were provided by D. Zipser. A 590 bp pMB1-derived replicon was obtained by excising the appropriate fragment from plasmid pKB413 (6), supplied by K. Backman. Plasmid pGA46 (7) was donated by G. An. A 203 bp synthetic tyrosine tRNA suppressor gene (8) was excised from the plasmid pRD69, provided by R. Dunn. Bam H1 and Eco R1 linkers were the gifts of R. Scheller and H. Drew, respectively. Enzymes were purchased from New England Biolabs, or were the gift of M. Alonso.

Constructions. The amber-mutated RP1 plasmid pLM2 was transferred between strains by mating on plates containing 50 $\mu\text{g/ml}$ of kanamycin sulfate. pLM2 was introduced into an intermediate strain, AB1157, by thermal selection against the donor, HB10Y (2). From AB1157 the plasmid was transferred to W3110 $r^{-m}+$ thy^- selecting against the multiple auxotrophy of AB1157. A spontaneous revertant to thymidine prototrophy was then selected on minimal plates. The tra^- pLM2 derivative p3 was selected by spotting a concentrated suspension of phage PRD1 on a lawn of W3110 $r^{-m}+$ /pLM2 cells plated in top agar on a kanamycin plate. Suppressor plasmids were selected in the resulting strain, W3110 $r^{-m}+$ /p3 either on plates containing 15 and 25 $\mu\text{g/ml}$ of tetracycline and ampicillin, respectively, or in liquid media containing half these concentrations. The polylinker segment of the πVX plasmid was assembled in several steps as follows. Plasmid pGA46 was partially digested with Mst I, and a Bam H1 linker joined to the Mst I flush end. The monomer-length linear fragment was isolated by electroelution and cleaved with Hind.III and Bam H1 to give a mixture of fragments which were ligated to the large Bam H1 to Hind III fragment of plasmid pBR322. Plasmids which had incorporated sequences containing the pGA46

Bgl II and Pst I sites were isolated, digested with Sal I, and the ends made flush by treatment with *E. coli* DNA polymerase large fragment in the presence of all four deoxynucleotide triphosphates. A Bam HI linker was ligated to the flush end, the linear fragment isolated by electroelution, digested with Bam HI, and ligated at high dilution to give plasmid circles which were introduced into *E. coli* by transformation. Plasmid recovered from transformed cells was treated with Eco RI methylase in the presence of S-adenosyl methionine, digested with Sal I, treated with DNA polymerase large fragment as before, and ligated to Eco RI linkers. Linear fragments were recovered by electroelution, digested with Eco RI, and recircularized by ligation. The Sal I site was inadvertently destroyed during this manipulation. The resulting polylinker contained restriction enzyme cleavage sites for, in order, Eco RI Cla I Hind III Bgl II Pst I Bam HI and Eco RI. An equimolar mixture of the polylinker plasmid and plasmid pACYC 177 (which lacks an Eco RI site) was digested with Bam HI and ligated. A composite plasmid was isolated, cleaved with Eco RI and recircularized, selecting for the pACYC kanamycin resistance. The product was a plasmid containing the permuted sequence Bam HI Eco RI Cla I Hind III Bgl II Pst I Bam HI inserted in the Bam HI site of pACYC 177. The plasmid p793 was linearized by partial Eco RI digestion and inserted in the sole Eco RI site of the pACYC polylinker plasmid. The miniplasmid was then freed of the pACYC plasmid by digestion with Bam HI, and recircularized. The resulting microvector, π V1, was the progenitor of the π BP and π BHg plasmids described in the text. Approximately 100bp of sequence between the Hind III and Bgl II sites were subsequently removed from the polylinker by digestion with Hind III and Bgl II, treatment with DNA polymerase, ligation to an Xba I site linker having sequence TCTAGA, cleaving with Xba I, and ligating to form circles at high dilution. The resulting sequence is shown in the polylinker segment of the π VX sequence shown in Fig. 1.

Transformation and Preparation of DNAs. Plasmid transformation of W3110 $r^{-m+}/p3$ cells was performed by the method of Dagert and Ehrlich (9), except that transformed cells were plated in top agar without drug immediately after heat shock. Plasmid intermediates for the construction of polylinkers were transformed

into x1974, a spontaneous thy⁺ derivative of x1776, by an unpublished procedure of D. Hanahan. Plasmid DNAs were prepared from saturated 10 ml cultures in M9CA or xM9CA containing 1mg/ml uridine (10) by the method of Klein et al. (11), or, more recently, by a modification (12) of the procedure of Birnboim and Doly (13). For most of the constructions small amounts of vector were digested with two or more enzymes and the vector fragment purified by electroelution from minigels. Because the p3 plasmid has unique sites for Eco R1, Bam H1, Bgl II and Hind III restriction enzymes, few Sma I and Pst I sites (14), and is present in low copy number, there is little contamination of miniplasmid fragments with p3 DNA. Minigel-purified insert fragments were mixed with vector in ~5:1 ratio, ligated, and transformed into competent cells prepared as described above. Phage DNAs were prepared from small scale liquid cultures or plate stocks by digestion with RNase and DNase (ca. 1µg/ml each) for 1 hr, followed by incubation with 50-100µg/ml Proteinase K in the presence of SDS and EDTA for one to two hours at 70°. DNAs were precipitated directly from the digested culture fluids by addition of 1/2 volume of 20% (w/v) LiCl, (M. Mitchell, personal communication) and 1 volume isopropyl alcohol. Resuspended precipitates were either phenol extracted and digested with restriction enzymes, or additionally precipitated with 10mM spermine (15), resuspended in a small volume of 5 M NaCl at 70°, diluted and reprecipitated with ethanol before digestion.

Phage-plasmid Recombination and Selection for Suppressor-Transducing Phage. Between 2.5×10^5 and 10^6 phage were preadsorbed for 10 minutes at 37° with 0.1 to 0.2 ml of a fresh overnight culture of miniplasmid strain grown in L broth containing maltose (0.2%), tetracycline (7.5µg/ml), and ampicillin (12.5µg/ml). The phage were then plated in soft agar on L plates containing tetracycline (15µg/ml) either with or without ampicillin (25µg/ml). Plate stocks were harvested, titered, and plated on nonpermissive bacteria. Up to 5×10^9 of these phage were plated on a 10 cm plate with 0.25 ml of a fresh overnight culture of nonpermissive cells. When the vector lacked amber mutations, selection could be accomplished on cells containing the rec A am 99 allele (16) for target phage having the fec⁻ phenotype (D. Di-

Maio and D. Goldberg, personal communication), or on immunity 21 lysogens (17) for phage bearing immunity 21. The immunity 21 virulence selection yielded spontaneous revertant phage at a frequency of about 10^{-7} , and the rec A am selection was leaky and gave poor titers of suppressor phage. Most of the experiments reported below were performed with the Human Hae III/Alu I library constructed by Lawn et al. (18), which has a very low proportion of am⁺ phage. Other libraries have been found to yield higher titers of am⁺ phage, perhaps because the NIH Guideline-mandated UV treatment of the packaging bacteria was omitted. A more practically oriented description of the use of π VX in library screening can be found in the Maniatis, Fritsch and Sambrook manual (44).

RESULTS

Two features are desirable in a plasmid designed for insertion in library phage DNA: (i) the plasmid should be as small as possible, so that insertion rarely gives rise to DNA molecules too large to be packaged into viable phage; and (ii) the plasmid should carry at least one marker allowing phage bearing inserted plasmids to be easily selected from phage lacking plasmids during lytic growth. To meet these objectives a plasmid of 793 basepairs (p793) was assembled from a 590 bp pMB1-derived replicon excised from the plasmid pKB413 (6), and a 203 bp synthetic tyrosine tRNA suppressor gene (8) excised from the plasmid pRD69.

Cells bearing p793 were selected by transformation of suppressor-free bacteria harboring the RP1 plasmid derivative pLM2 (2). The 57 kbp (19) pLM2 plasmid contains an intact kanamycin resistance gene, and amber mutated ampicillin and tetracycline resistance elements. Introduction of p793 into cells containing pLM2 causes the host bacteria to simultaneously express ampicillin and tetracycline resistances. However p793 has little utility as a vector for cloned sequences because the individual fragments that comprise the plasmid each terminate in Eco RI cohesive ends, and contain few or no internal restriction sites suitable for the insertion of foreign sequences (6).

To convert p793 into a cloning vehicle, short 'polylinker' segments ranging in size from approximately 200 to 109 bp were

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      10      20      30      40      50      60      70      80      90
1  GAATTCATGTTTGACAGCTTATCATCGATAAAGCTTCTAGAGATCTTCCATACCTACCAAGTTCTCCGCTGCAGCAATGGCAAGAACGTTGCCCGGATC
101 CGGTCGCGCGAAATCTTTCGGACTTTTGAAGTGATGGTGGTGGGGGAAGGATTGCAACCTTCGAAGTCGATGACGGCAGATTTAGAGTCTGCTCCCTTT
201 GGCGCTCGGGAAACCCACCA CGGGTAATGCTTTTACTGGCCCTGCCCTTATCGGGAAAGCGGGGCGCATCATATCAAATGACGCGCCGCTGTAAAGTGT
301 TACGTTGAGAAAGAAATTCGGCGTTGCTGGCGTTTTTTCATAGGCTCCGCCCCCTGACGAGCATCAAAAAATCGACGCTCAAGTCAGAGGTGGCGAAAC
401 CCGACAGGACTATAAAGATACCAAGGCGTTTCCCCCTGGAAAGCTCCCTCGTGGCGCTCTCTGTTCCGACCTGCGCGCTTACCGGATACCTGTCGCGCTTTC
501 TCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTGCTCCAAGCTGGGCTGTGTGCACGAACCCCC
601 CGTTCAGCCCGAACCGCTGCGCTTATCGGTAACATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCA CTGGTAACAGG
701 ATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTGGTATCTGCGCTCTGC
801 TGAAGCCAGTTCCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAAACAAACCCGCTGGTAGCGGTGTTTTTTTGTGCAAGCAGCAGATTACG
901 cc

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Figure 1. Nucleotide sequence of plasmid π VX. The polylinker segment occupies positions 1-109, the suppressor tRNA gene positions 110-212, and the origin of replication positions 213-902. The sequence of the origin is taken from preexisting data (6), and was not redetermined here.

created which contained multiple restriction enzyme recognition sequences suitable for cloning. The polylinker segments were flanked by Eco R1 sites, and were inserted between the suppressor and replicon segments of p793. The smallest of the resulting plasmids was the 902 bp vector π VX, which contains unique cleavage sites for the enzymes Cla I, Hind III, Xba I, Bgl II, Pst I, and Bam H1. The sequence of π VX, and an abbreviated restriction site map, are given in Figures 1 and 2 respectively.

The DNA sequences of the polylinkers derive from a mixture of natural and synthetic sources. A detailed account of their construction is given in the Materials and Methods. Briefly, the nucleotides between the Eco R1 site at position 1 and the Hind III site at position 32 are also found in the plasmid pBR322, as are the nucleotides between the Pst I site at 70 and the Hpa II site at 94 (20). The nucleotides between the Bgl II and Pst I sites derive from the plasmid pGA46 (7), and essentially all remaining nucleotides are derived from chemically synthesized DNA.

Because the pLM2 suppressor-selection plasmid descended from the conjugation proficient plasmid RP1 (2), the cloning system consisting of π VX and pLM2 may not conform to the NIH regulations for EK1 host vector systems. Accordingly, the tra^- (conju-

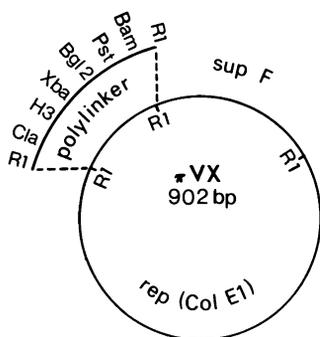


Figure 2. Gross structure of the π VX plasmid. The sequence presented in Figure 1 precedes clockwise from the EcoRI site joining the replication origin and polylinker segments.

gation deficient) plasmid p3 was derived from pLM2 by simultaneous selection for kanamycin resistance, and resistance to the phage PRD1 (3). The phage PRD1 host range is confined to bacteria bearing plasmids of the N, P and W incompatibility groups (3), and resistance to phage of the PRD1 family impairs the conjugation proficiency of RP1 and other plasmids in this group (21). p3 exhibits mating frequencies of less than 10^{-7} per donor in liquid culture, and less than 5×10^{-6} per donor on plates.

To test the microplasmid recombination system, a 700 bp Pst I to Bgl II fragment lying 2 kbp upstream of the β -globin gene (22) was introduced into the π V1 microvector. The resulting plasmid, π BP, was introduced into a bacterial host harboring the p3 selection plasmid. Plate stocks of different amber-mutated phage bearing or lacking globin genes were prepared on π BP-containing cells, and the proportion of \underline{am}^+ phage measured among the resulting progeny. The globin-containing phage H β G1 (18) yielded \underline{am}^+ phage at a frequency of 10^{-3} , while the vector Charon 4A (23) yielded no revertants ($<10^{-9}$). Globin-bearing and control \underline{am}^+ phage were plated on lac Z \underline{am} bacteria in the presence of IPTG and XGal, and in all cases the globin-bearing \underline{am}^+ phage gave blue plaques, while the control phage showed no color reaction. The presence of the integrated suppressor plasmid was then directly verified by restriction enzyme digestion of the phage DNA.

Approximately 5×10^6 phage from the amplified Charon 4A/Human Hae III/Alu I library of Lawn et al. (18) were next plated on 6 10 cm plates seeded with the π BP host bacteria

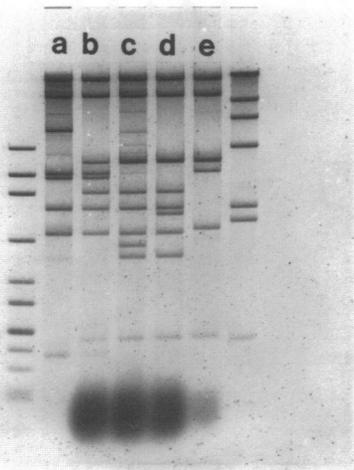


Figure 3. EcoRI fragment patterns of library phage recovered by recombination with a plasmid containing sequences flanking the human β -globin gene. Lane a is the fragment pattern of the previously isolated phage H β G1; lanes b through e are fragment patterns of phage isolated by recombination. Unlabeled lanes are standard length markers.

described above. A plate stock having a titer of 6×10^{10} phage/ml was recovered, and found to contain \underline{am}^+ phage at a frequency of 1.4×10^{-8} . Because the expected frequency of globin phage in the library is $\sim 5 \times 10^{-6}$, and the frequency of recombination per phage is $\sim 10^{-3}$, we would expect to see recombinant phage at a frequency of $\sim 5 \times 10^{-9}$. A similar library stock prepared from host bacteria harboring π VX alone yielded no \underline{am}^+ phage in a sample of 7.8×10^9 phage (a frequency $< 1.3 \times 10^{-10}$). 87 of the \underline{am}^+ phage were transferred to plates containing lac Z \underline{am} indicator bacteria with IPTG and XGal, and all but one gave a blue plaque. 10 of the phage yielding a blue plaque were chosen at random, and their DNA prepared from small cultures and digested with restriction enzymes to verify the presence of the probe plasmid. Four distinct Eco RI fragment patterns were found among the ten phage chosen. The four patterns are shown in Figure 3. One pattern results from plasmid integration in the phage H β G1 previously characterized by Lawn et al. (18), and the remainder reflect plasmid integration in new globin clones not previously recovered from this library (18,22). Further digestion with the restriction enzymes Bam HI, Hind III, and Kpn I allowed the identification of the insert fragments shown in Figure 4, as well as the absolute orientation of the inserts with respect to the phage vector arms. The novel

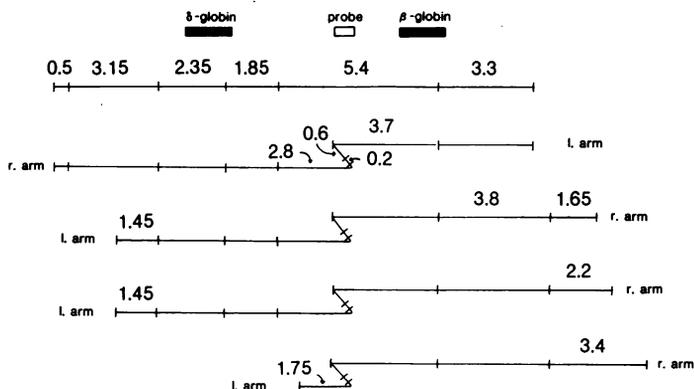


Figure 4. Eco RI restriction map of sequences inserted in the phage analyzed in Fig. 3. The location of the δ - and β -globin genes is shown, as well as the location of the segment chosen for insertion in π BP. Plasmid insertion results in a duplication of the probe segment, and the interruption of the native genomic sequences with plasmid DNA.

clones selected by recombination bear inserts in which the globin gene sequences have the same transcriptional orientation as the lambda late genes. The opposite orientation is observed in the three β -globin-bearing phage previously isolated from this library. The recovery of novel clones here is significant, since the Hae III/Alu I library was screened repeatedly for β -globin genomic clones (E. Fritsch, pers. communication). However at present no significance can be attached to the apparent failure of hybridization screening to yield phage bearing globin genes in the orientation uncovered by recombination.

To examine the dependence of recombination frequency on sequence divergence between plasmid probe and phage target sequences, a 427 bp segment containing the β -globin genomic coding sequences between the Hgi AI site at position 158 of the Lawn et al. sequence (24) and the Bam HI site of position 585 was inserted into a microvector. The frequencies of integration of this plasmid (π BHg) into phage bearing various members of the human and rabbit β -globin gene families were then measured by the plate recombination procedure described in the Materials and Methods. Because π BHg contains the rapidly diverging globin intron I, for most of the target phage the effective homology covers only 297

TABLE I
Recombination Frequency as a Function of Sequence Divergence

| Target Phage | Globin Genes | % Divergence | Log ₁₀ Recombination |
|--------------|---------------------------------------|----------------|---------------------------------|
| HβG1 | Human δ, β | 6.3, 0(25, 24) | -2.78 ± .07 |
| HβG3 | Human β | 0(24) | -2.11 ± .15 |
| HγG5 | Human γ ^A , γ ^G | 24(26) | -5.20 ± .06 |
| HγG2 | Human γ ^A , γ ^G | 24(26) | -5.37 |
| HεG1 | Human ε | 21.3(27) | <-7 |
| RβG1 | Rabbit β1 | 10.7(28) | -5.05 ± .28 |
| RβG3 | Rabbit β1 | 10.7(28) | -4.88 ± .45 |
| Rβ'G7 | Rabbit β3, β4 | 29, 25(29, *) | -4.84 ± .02 |
| Rβ'G9 | Rabbit β3, β4 | 29, 25(29, *) | -4.89 ± .07 |

Phage bearing human (18,22) and rabbit (28,30) β-globin genes were recombined with the plasmid πBHg. Divergence percentages were calculated from data taken from the references indicated in the % divergence column. *, Hardison and Butler, personal communication.

bp of coding sequence, subdivided into an 88 bp exon I fragment and a 209 bp exon II fragment. The 130 bp β-globin intron I is 12.3% divergent from the δ-globin intron, but has no significant homology with any of the other globin gene introns (31). The divergence figures exhibited in Table I were determined from the coding sequences alone, because the introns are in general highly divergent; but if the δ-globin intron segment is taken into account, the net homology between β- and δ-globin sequences is 8.2%. The frequency of recombination for the perfectly homologous target is two orders of magnitude greater than the frequency for the 11% divergent rabbit β1 target. However phage bearing targets as divergent as 24 to 29 % recombine nearly as well as those bearing 11% divergent targets. Table I also shows that the πBHg plasmid does not integrate into λHεG1, although the homology appears sufficient to allow recombination. In this case the large size of the genomic insert (18kbp) may severely reduce the viability of the plasmid recombinant. The πBHg plasmid does not recombine with the α-globin phage λHαG1 (32) (frequency <10⁻⁹), which contains no significant homology to the probe outside of the first exon segment (33). The homology with the α-globin gene in exon I is 41%, and the largest run of perfect homology is 10 bp (33). The longest runs of perfect homology for the human γ, rabbit β1, β3, and β4 genes are 20, 29, 22 and 23 nucleotides respectively.

Genomic globin clones were also recovered from the Hae

III/Alu I library with the π BHg plasmid. Eco R1 fragment patterns resulting from plasmid integration into the previously isolated H β G1, H β G2, and H β G3 phage DNAs (18,22) were observed, as well as patterns resulting from plasmid integration in the novel phage species isolated by π BP recombination. In addition, a seventh independent clone was observed, and several clones in which the δ to β intergenic segment was deleted. In at least one case the deletion event appeared to have taken place after recombination, since faint bands corresponding to the undeleted forms were observed in the Eco R1 fragment pattern (data not shown). Experience with other probes and genes has not shown deletion after recombination to be commonplace (unpublished results; D. DiMaio, pers. comm.; K. Zinn, pers. comm.), but the potential for deletion has not been studied in great detail.

The loss of approximately 130 bp of homology due to intron I divergence does not appear to be a significant factor in the decline in recombination frequency of the divergent target phage, since plasmids bearing less than 100 contiguous bp of perfect homology recombine with appropriate target phage with a frequency of greater than 10^{-3} (Table II). The plasmids π lac and π 14 (P. Little, pers. comm.; unpublished) contain 57 bp of homology to lac operator DNA. When tested for recombination with the target phage λ Sep 6A (see below), these plasmids exhibited a high frequency of integration (Table II and unpublished results). The λ Sep 6A phage was created (D. Goldberg, pers. comm.) by crossing the lambda A and B gene amber mutations from λ Charon 16A (23) into the vector λ Sep 6 (34). The resulting phage is a $red^- gam^+$ double amber vector bearing a duplication of part of the lac 5 insertion. The phage also bears the immunity region from phage 21, which allows selection for plasmid integration based on replicon function. In this selection phage bearing plasmid integrates become capable of growth on imm 21 lysogens (17), presumably because plasmid replication allows the titration of repressor from phage operator sequences. Insertion of the plasmid π lac in λ Sep 6A occurs with a frequency between 10^{-2} and 10^{-3} when measured either by amber suppression or by pseudovirulence (Table II). Because the phage is fec^+ , recombination can also be measured in an otherwise isogenic rec A background (35). The p3

TABLE II
 Variations on the Recombination of a 57bp lac Segment

| <u>Plasmid</u> | <u>rec A</u> | <u>selection</u> | <u>log₁₀ recombination</u> |
|----------------|--------------|------------------------|---------------------------------------|
| πlac | + | <u>am</u> ⁺ | -2.72 |
| | + | <u>vir</u> | -2.33 |
| | - | <u>am</u> ⁺ | -6.52 |
| | - | <u>vir</u> | -6.37 |
| π14 | + | <u>am</u> ⁺ | -0.33 |
| | + | <u>vir</u> | -0.57 |

Column 2 describes the rec A status of the cell in which recombination took place; column 3 describes the phenotype scored after recombination.

plasmid was introduced into HMS174 (W3110r⁻m⁺ rec A, (4)) by mating, and the resulting strain transformed with πlac DNA. The recombination frequency with λSep 6A was ~10⁴ fold lower than observed in the comparable rec⁺ background (Table II). Recombination can not be detected by plaque formation of λSep 6A on an imm 21 lysogen harboring πlac, which suggests that the recombinant does not replicate well in competition with unintegrated πlac. An elevated recombination frequency was observed following infection of bacteria harboring the high copy number plasmid π14, which has a deleted and rearranged origin of replication.

Because recombination between plasmid and phage results in the interruption of genomic sequences, it would be desirable to have a marker or selection for plasmid excision. Experiments designed to generate such a marker revealed an unexpected alteration in suppressor tRNA function. Strains containing the lac Z₁₀₀₀ amber mutation require a glutamine-inserting suppressor tRNA gene for β-galactosidase activity (5,36). The tyrosine-inserting sup F gene of πVX should not suppress this mutation. Phage φ80 psuIII (φ80 sup F) makes white plaques on bacterial plates seeded with Z₁₀₀₀, IPTG and XGal. On other lac Z am strains φ80 psuIII makes a blue plaque which is considerably deeper in color than the plaques formed by the suppressor plasmid integrates constructed in this study. λHβG1 bearing an integrated πBP plasmid makes a blue plaque on Z₁₀₀₀, however, indicating that the suppressor tRNA gene has acquired a mutation allowing

the tRNA to charge with glutamine. Similar mutations have been selected in phage-borne sup F genes, and were shown to cause sequence alterations in the stem region of the tRNA (36,37). The p3 plasmid was transferred into Z₁₀₀₀ and the ability of πVX and other vectors to suppress the Z₁₀₀₀ mutation in the resulting strain examined by color reaction on plates containing IPTG and XGal. The light blue reaction observed did not allow unambiguous identification of the amino acyl charging specificity of the suppressors, so the suppressor tRNA gene and flanking regions of πVX were sequenced by chemical cleavage. No alterations from the published sequence were observed, indicating that the πBP suppressor mutation arose sometime after the insertion of the BP segment. Although λHBG1/πBP makes a blue plaque on Z₁₀₀₀ strains, it forms white plaques on Z₁₀₀₀ lysogenized with φ80 psuIII, suggesting that competitive insertion of tyrosine reduces the ability of the phage-borne suppressor to make glutamine-containing lac Z polypeptide.

Although the 590 bp replication origin fragment of πVX has been reported to contain all of the sequences necessary for relaxed Col E1-like replication (6), suppressor plasmids exhibit atypical replication behavior. The πVX plasmid copy number is approximately 1/5 the copy number of plasmid pBR322, based on agarose gel analysis and CsCl density gradient purification of alkaline lysates of stationary phase cells. The πVX replicon does not amplify appreciably in the presence of chloramphenicol, and in this and several related constructions (unpublished), no plasmids have been obtained in which the direction of transcription of the suppressor tRNA gene opposes that of the Col E1 replication primer RNA. Because the πVX origin fragment does not contain the -35 sequences of the primer RNA promoter, it appears that suppressor gene transcription may play a role in maintaining plasmid copy number by readthrough into the origin. This possibility is supported by the observation that introduction of promoter sequences upstream from the origin results in substantially improved copy number (P. Little, pers. comm.; unpublished results). Because the polylinker segment falls between the origin and the suppressor tRNA gene, it is also possible that insertion of sequences which cause transcription termination might result

in plasmids with reduced copy number, or in failure to recover the desired plasmid. Further, in the present configuration, integration of the plasmid in phage interrupts the suppressor-origin linkage, and may compromise the replication selection in imm 21 phage. The experiments demonstrating the replicon selection in Table II were carried out with plasmids whose construction assured that origin function could be observed in the integrated form of the plasmid (data not shown). Several derivatives have now been made in which the polylinker lies upstream of the suppressor tRNA gene ($\pi 14$, unpublished; πAlac , P. Little, pers. comm.; πAN7 , H. Huang, pers. comm.).

DISCUSSION

We have assembled a very small plasmid vector from an amber mutant suppressor gene, an origin of replication, and a short 'polylinker' segment containing multiple restriction enzyme sites suitable for the insertion of foreign DNA fragments. The plasmid can be maintained in host bacteria bearing amber mutations in genes coding for selectable functions. In this work a convenient bacterial host was created by introducing a 57 kbp kanamycin resistance plasmid bearing amber mutated ampicillin and tetracycline resistance elements into a nonsuppressing bacterial strain having high transformability and a restriction-deficient, modification competent phenotype. Insertion of the suppressor plasmid in the resulting strain was marked by the simultaneous appearance of amp and tet resistance mediated by the large drug-selection plasmid. This approach was chosen over a more conventional selection with auxotrophic or other metabolic amber mutations for two reasons: (i) in the present selection transformed cells can be grown rapidly in rich broth so that cloning experiments can be carried out with the same rapidity possible with conventional plasmids, and (ii) the selection plasmid can be easily introduced into a variety of different chromosomal backgrounds by exploiting the weak self-transmissibility of the plasmid when mated on agar plates. Although selection for suppressor function may be compromised by the spontaneous appearance of suppressor tRNA genes in the *E. coli* chromosome, existing transformation protocols (9) allow plasmid uptake at sufficiently high frequency that

the action of chromosomal suppressors is rarely observed.

Because of their small size, the suppressor plasmids presented here contain few recognition sequences for restriction enzymes which cleave prokaryotic DNAs with high frequency. This feature simplifies the manipulation of foreign segments inserted in the plasmids by reducing the number of obstructing fragments that arise from the vector segments. The plasmids may also be useful whenever prokaryotic sequences are suspected of interfering with the expression of genes reintroduced in eukaryotic cells (38).

Very small suppressor plasmids can also be used to purify genomic sequences from recombinant DNA bacteriophage libraries by in vivo recombination. In this application a short probe segment homologous to the genomic sequence of interest is inserted in the suppressor plasmid to form a 'probe plasmid.' The probe plasmid is maintained in *E. coli* by selection for suppressor function as described above. Bacteriophage libraries can be propagated with good efficiency on bacteria harboring the probe plasmid. During the course of growth on cells containing the probe plasmid, phage bearing DNA segments homologous to the probe may acquire an integrated copy of the plasmid. The frequency of this recombination event is high; one in 10^3 of the progeny resulting from the growth of purified phage bearing probe homology will have acquired an inserted probe plasmid. The frequency of recombination declines either when the probe region is very small, or when the target and probe are imperfectly homologous.

In our experiments the shortest probe segment giving high recombination was ~60 bp long. Phage bearing a 10% divergent target homology recombined with a frequency about two orders of magnitude lower than the rate for the perfectly homologous target. However virtually the same rate was observed for phage sequences 25 to 30% divergent from the probe. Although the details of the sharp decline in recombination frequency between 0 and 10% divergence are not known, the phenomena reported here suggest that the recombination process might offer a valuable selectivity not obtainable by conventional hybridization screening. For example the identification of the genomic sequences giving rise to a particular cDNA segment is occasionally made difficult by the presence

in the genome of closely similar members of the same gene family. In our experiments a probe plasmid containing human β -globin coding sequences was found inserted into genomic β -globin sequences almost exclusively, despite the presence of an 8.2% divergent δ -globin genomic sequence both in the same library, and, frequently, in the same clones (data not shown).

The lack of selectivity observed over the 10 to 30% divergence range may also have useful applications if it proves general. The data of Table I indicate that all rabbit β -like globin phage recombine with a human coding sequence probe plasmid with about the same frequency. Recombination and selection of the rabbit library with this plasmid should yield a roughly equally represented population of all β -like globin clones.

Recombination between plasmid and phage results in phage bearing a (possibly inexact) duplication of the probe segment flanking the inserted plasmid sequences. In some cases this unnatural interruption of genomic sequences is undesirable. When probe and target are perfectly homologous, reciprocal recombination can allow excision of the plasmid and reconstitution of the original gene segment. At present there is no efficient selection against the presence of the plasmid, although the spontaneous excision frequency is occasionally high enough to allow plasmidless phage to reach high titers in a population grown for many generations under nonselective conditions.

Two types of selection have been applied to purify phage bearing plasmid integrates from the larger pool of phage lacking integrates: selection for suppressor function, and selection for plasmid replication function. At present neither type of selection is universally applicable.

Suppressor selections are effective when amber mutations in bacterial or phage-encoded genes essential for phage maturation can be sufficiently suppressed by the transient expression of the phage-borne suppressor to allow phage growth. Because the frequency of reversion of an amber mutation is generally 10^{-5} to 10^{-6} , at least two amber mutations in essential phage genes are presently needed to impose a selection stringent enough to allow the direct recovery of genomic sequences from mammalian bacteriophage libraries. The amber mutations in the phage lambda A

and B genes (23) can be effectively suppressed in this way, although apparent recombination frequencies measured with target phage bearing W, E and S gene mutations (39) are several orders of magnitude lower than frequencies measured with phage bearing A and B gene mutations (M. Tainsky, pers. comm.) It may be that large amounts of E gene product, the major capsid protein (40,41), are required for efficient λ morphogenesis. The bacterial rec A am 99 allele (16) can be suppressed well enough to allow fec⁻ suppressor phage to make plaques on rec A am bacterial lawns, although the viral yield is low and it is sometimes difficult to recover phage from the plaques.

Recently several groups have reported selections for the introduction of plasmid replication origins into lambdoid phage (17,42,43). At present only one of these selections has been demonstrated with a suppressor plasmid. Windass and Brammar (17) have shown that phage bearing immunity 21 acquire the ability to form plaques on imm 21 lysogens when Col E1-type plasmids are inserted in the phage. We have observed a similar pseudovirulent phenotype among imm 21 phage containing a miniplasmid copy inserted by recombination. Selections for growth on the gro P mutant K802 (43), or growth on imm λ lysogens having low repressor titer have not been successful.

In this article we have shown that recombination and selection can be applied to recover previously unrecognized isolates from a library repeatedly screened for globin genes by plaque hybridization. We have also found that the recombination process may have a potentially useful selectivity for phage bearing perfect homology to probe DNA, and may show little sensitivity to nucleotide divergence over a range of 10 to 30% sequence nonhomology. The technique should be particularly useful for the repetitive isolation of mutant alleles of existing cloned genes, and has applications in the manipulation of sequences on existing cloned phage.

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