(R. Rodriguez, F. Bolivar, H. Goodman, H. Boyer, and M. Betlach, personal communication). Hence, incorporation of DNA into a Bam I site can be monitored by looking for ampicillin resistance and tetracycline sensitivity in these two plasmid strains.

The linkers reported here have been used to clone eukaryotic DNA and complementary DNA (cDNA). As described (*15*), the Eco I decamer has been ligated to repetitive DNA from sea urchin sperm, and the resultant cohesive-ended fragments were cloned in RSF2124 plasmids. These clones will provide a source of repetitive DNA in quantity, helping to determine new features of the eukaryotic genome. The Bam I decamer has been added to cDNA's and cloned to the tetracycline gene of the pB313 plasmid (J. Shine and H. Goodman, personal communication). The decamers have also been shown to be useful for constructing new cloning vehicles by converting one restriction site to another, for example, Hind III to Eco RI (H. Heyneker, personal communication), and Eco RI to Bam I (A. Riggs, unpublished data). Therefore, the chemically synthesized decameric linkers reported here are generally applicable tools for molecular cloning experiments.

The three decameric linkers themselves were constructed with CC . . . . . GG at the ends for stability (T_m = 48°C), resulting in efficient blunt-end ligation, and so that polymerized decamers could be cloned and cut apart again by Hae III restriction enzyme. Cloning would provide a means of obtaining a steady supply of more linkers. However, "polylinker" clones have not yet been obtained, perhaps because of in vivo instabilities. Therefore, triester chemical synthesis may prove easier than cloning for moderate scale production of DNA molecules as small as decamers. At present, 25-mg amounts of greater than 90 percent pure material can be made in a relatively short time.

Note added in proof: The linker synthesis has also been pursued in parallel with this work by Bahl *et al.* (*16*).

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Screening λgt Recombinant Clones by Hybridization to Single Plaques in situ

Abstract. *A rapid, direct method for screening single plaques of λgt recombinant phage is described. The method allows at least 10^6 clones to be screened per day and simplifies physical containment of recombinants.*

Recombinant DNA technology has made it possible, in principle, to isolate large quantities of DNA corresponding to any single gene. This isolation can be accomplished in any of three ways: (i) purification of the gene or its transcript before insertion into a vector for cloning (*1*); (ii) genetic selection of the desired gene after "shotgun" (*2*) cloning the genome of the donor organism (*3, 4*); or (iii) the use of complementary RNA or DNA probes to screen clones produced through a shotgun experiment. The first method is limited by the practical difficulty of physically purifying the complete nucleic acid sequence of any gene. Application of the second is limited to genes for which appropriate mutations are available in hosts which can express foreign genetic information. The third method is limited only by the availability of complementary nucleic acid probes and has been the most widely applied.

Screening methods have been developed for both recombinant plasmid (*5*) and bacteriophage (*6, 7*) cloning systems. The most rapid of these, the methods of Grunstein and Kramer, are based on a technology developed by Olivera and Bonhoeffer (*8*) for growing and lysing cells on membrane filters. The speed and simplicity of these methods is limited by the need to pick and spot plaques or colonies from plates onto filters for further growth. We have found that a single plaque of a λgt recombinant phage contains enough phage DNA for detectable hybridization to complementary labeled nucleic acid, and that this DNA can be fixed to a nitrocellulose filter by making direct contact between the plaque and the filter. These findings have allowed us to develop a method for screening recombinant phage which has significant advantages over previous methods: (i) physical containment of recombinant molecules (and organisms carrying them) is made simpler because this method requires fewer manipulations of viable phage, requires the growth and handling of much smaller quantities of recombinant DNA-containing phage and cells, and produces no contaminated materials other than the petri dishes on which the plaques are grown. (ii) At least 2×10^4 plaques can be screened per hour (*9*), which compares with about 100 per hour by the fastest previous method (*7*). (iii) Because up to 2×10^4 plaques can be screened on a single petri dish and nitrocellulose filter, the method requires smaller amounts of materials, including radioactively labeled probe. (iv) Because screening can be done directly from the transfection plate, all the independently constructed phages containing similar or identical inserts can be isolated and individually

characterized. This can give information on the heterogeneity of repeated genes or of genes from different somatic tissues.

We believe that this method will be widely applicable and, therefore, present it here in detail.

Cells (*10*) are infected with phage, plated in L-soft agar on L plates (*11*), and grown for at least 12 hours at 37°C. Large plaques are usually desirable, and 0.1 ml of late log phase cells per plate has been found to give large plaques of many λgt phages. The number of phages which can be plated on a single petri dish depends on the relative growth rates of the desired recombinant and the background phages. After growth, the plates are placed at 4°C for about 15 minutes to harden the agar. Phage and DNA are transferred to a nitrocellulose filter by placing the dry filter (88 mm, Millipore HA or Schleicher and Schuell, BA 85) on the lawn of cells, no air bubbles being allowed to form between the soft agar and the filter. Phages are allowed to adsorb to the filters for 1 to 20 minutes. If duplicate filters are desired, several filters can be adsorbed sequentially for 30 to 60 seconds each, or filters can be stacked and adsorbed at once. If filters are stacked, they must be allowed to adsorb for at least 20 minutes. During adsorption, it is convenient to mark the filters and plates for orientation. After adsorption, the filter is carefully lifted from the plate to avoid removing the soft agar layer which occasionally adheres to the filter. Should this occur, the agar can be removed by gently shaking the filter during the denaturation and neutralization steps (see Fig. 1, plate 2a and replicas b to d, for examples). Both the DNA and phage are denatured and fixed in situ by dipping the filters in 0.1*N* NaOH and 1.5*M* NaCl for 20 seconds; the filters are then neutralized by dipping in 0.2*M* tris, *p*H 7.5, and 2 × SSCP (*12*) for 20 seconds. Filters are blotted and baked at 80°C in a vacuum for 1.5 to 2 hours.

Hybridization to the nitrocellulose replicas is carried out in a siliconized glass petri dish. The ^{32}P-labeled probe (*13*) (10^5 to 10^6 counts per minute per filter) is placed in enough 5 × SSCP and 50 percent formamide to cover all filters. The hybridization mixtures were usually incubated for 12 to 18 hours. Buffer and probe can be recovered and reused for at least 2 weeks. After hybridization, the filters are washed in a large (10 to 15 ml per filter) volume of 5 × SSCP and 50 percent formamide at 42°C for 30 minutes and then for 20 to 30 minutes in 2 × SSCP at room temperature. The filters are then covered with plastic wrapping

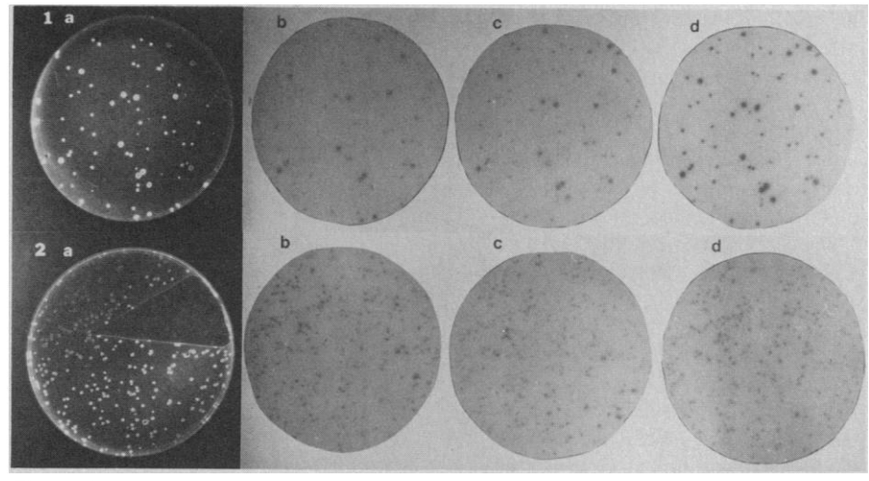

Fig. 1. Plaques 1a and 2a of λgt-Sc1109 phage and autoradiographic replicas (1b to 1d and 2b to 2d). Replicas were produced (as described in the text) by sequential adsorption to filters, beginning with 1d and 2d. The probe was ^{32}P-labeled ribosomal RNA and Chronex 4 x-ray film was exposed for 20 hours for autoradiography. A sector of the top agar from plate 2a was removed with the last filter adsorbed (2b). Note that plaques are visible on the bottom agar and that the autoradiographic replica is not changed in the sector. Plaques 1a and 2a are visualized by the fluorescence of ethidium bromide which is sprayed onto the plates.

film and placed against x-ray film for autoradiography. From 24 to 48 hours of exposure is usually sufficient with a homogeneous probe of specific activity of 10^5 to 10^6 count min^{-1} μg^{-1}. X-ray intensifying screens (Kodak X-Omatic Regular) have been used with flash-activated film at −70°C to shorten exposure time.

The method described above should be generally applicable to all recombinants of λgt phages. In the experiments described below we used the phage λgt-Sc1109 (*7, 14*) in model systems to optimize the method. Phage grown on *E. coli* C600 for less than 9 hours give negligible autoradiographic signals; 12 to 14 hours of growth is optimal. Although T plates generally yield larger plaques, these apparently contain fewer phage particles and less DNA than comparably sized plaques grown on L plates because, at all growth times, nitrocellulose replicas of T plates gave lower signals than those of L plates (*11*).

To determine the maximum number of plaques per plate which can be screened under various relative growth conditions, two experiments were performed. In the first, to model a situation in which the background grows much better than the desired recombinant phage, a small number of λgt-Sc1109 phage were grown together with increasing numbers of λgt2 (*15*) phage. No effect on the signals was apparent up to about 2000 plaque-forming units (pfu) per plate, but the signals were weak and variable with more than 4000 pfu per plate. In the second competition experiment, λgt-Sc1109 was plated with increasing numbers of λgt-λB, which has approximately the same plaque size as the recombinant phage. In this case, sufficient signal was obtained with more than 2×10^4 pfu per plate. A practical rule seems to be that the method will accommodate the greatest number of plaques that does not produce confluent lysis.

Time of phage and DNA adsorption to the nitrocellulose replica filter does not seem to be critical; adsorption times of 1 to 60 minutes have been used and little difference noticed. Very long adsorptions resulted in slightly more diffuse plaque spots on the nitrocellulose replicas and the autoradiograms. Stacks of at least five filters can be adsorbed at one time to produce replicates (for hybridization to several different probes, for example). For most applications, better replicates are produced by placing the filters sequentially on the plate for a brief adsorption to each (Fig. 1).

Dipping the nitrocellulose replica directly into a beaker of denaturing or neutralizing solution does not result in loss of signal or in distortion of the shapes or relative positions of the plaque spots. Denaturation and neutralization times of 0 to 60 seconds all give acceptable results; 15 to 20 seconds of each is optimal, giving an approximately twofold increase in signal over either no denaturation or no neutralization. Baking the filters before hybridization is not necessary, but in this model system the signal was increased approximately twofold by baking for 90 to 120 minutes.

After autoradiography, it is necessary to align the autoradiogram of the nitrocellulose replica with the original plaque

plate to allow the plaques corresponding to positive signals to be picked. The most reliable system we have used for this alignment involves labeling the filters and plates during adsorption by puncturing the filter at three points around its circumference with a hypodermic needle and injecting 0.1 to 0.2 μl of India ink into the agar at each point. The ink diffuses very little during the 2 days normally required for hybridization and autoradiography, and it allows absolute orientation of the filters, autoradiograms, and plates. From a plate with more than 1000 plaques, we normally pick about 20 plaques from the area of each positive signal for rescreening and plaque purification. Once pure clones are obtained, the sizes and restriction spectra of the inserted DNA's can be rapidly determined by the method of Cameron *et al.* (*16*).

The large capacity and speed of this method make practical, we believe, the isolation of eukaryotic unique genes by screening all the recombinants produced through shotgun cloning the entire genome. This should make possible isolation of a large number of genes for which physical or genetic purification schemes are not available.

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12. Standard saline citrate phosphate buffer (SSCP) is (1×) 120 m*M* NaCl, 15 m*M* sodium citrate, 13 m*M* KH_2PO_4, 1 m*M* EDTA, titrated to *p*H 7.2 with NaOH.
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Use of Phage Immunity in Molecular Cloning Experiments

Abstract. *Immunity to phage superinfection is a useful selective marker in molecular cloning experiments. Plasmids which have unique sites for several different restriction endonucleases and which specify immunity to bacteriophage* λ *are described.*

Antibiotic resistance genes carried on plasmids are useful as selective markers in molecular cloning experiments (*1*). We show here that phage λ immunity may be used as an alternative selective marker on plasmids that are carried in *Escherichia coli*. Plasmids bearing the *c*I (repressor) gene of bacteriophage λ direct the synthesis of λ repressor and render carrier cells immune to superinfection by λ (*2*).

In a model experiment, we have compared selection for immunity or antibiotic resistance, using a plasmid (pKB158) (*2*) that carries both the λ *c*I gene and a gene specifying tetracycline resistance (*tet*). Cells were transformed (*3*) with either no DNA, 1 μg of pKB158 DNA, 1 μg of pKB158 DNA digested with Eco RI, or 1 μg of pKB158 DNA digested with Eco RI and subsequently treated with T4 polynucleotide ligase. Transformants were selected on either tryptone plates plus 20 μg of tetracycline per milliliter or tryptone plates seeded with 10^9 λKH54h_λ and 10^9 λKH54h_{80} (*2*, *4*). The results are given in Table 1. Selection for λ immunity yielded about half as many transformants as did selection for tetracycline resistance. A small background of mucoid bacteria survived the selection for immunity even when no DNA was present in the transformation; however, all (36 of 36 tested) of the nonmucoid λ immune clones carried pKB158, as judged by the fact that they were tetracycline resistant. In summary, clones carrying plasmids which bear the λ *c*I gene are easily selected and distinguishable from the small background of mucoid bacteria which survive the selection procedure. A possible disadvantage of the use of phage immunity in molecular cloning experiments is that selection for immunity is not possible with phage-resistant strains (such as χ1776) and is not easily maintained during growth in liquid media with standard *E. coli* strains.

We briefly describe two well-charac-

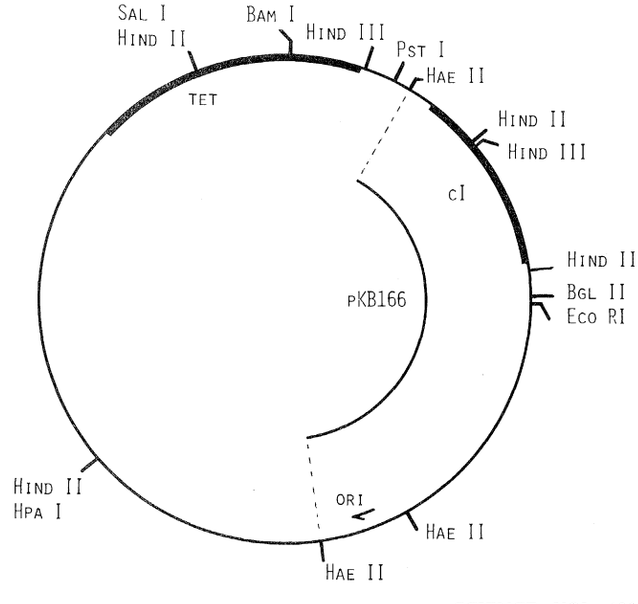

Fig. 1. Structure of pKB158. Map showing the locations of various restriction endonuclease cleavage sites on pKB158. Also shown are genes *c*I and *tet* (as heavy lines) and the Col E1 origin of replication *ori* (by an arrow). Only three of the more than ten Hae II sites are indicated. The material contained in pKB166 is indicated by an arc inside the map of pKB158. pKB158 has four Hind II sites, one of which is also recognized by Sal I and another of which is also recognized by Hpa I.