terized plasmids which carry the λ *c*I gene and which may be useful as cloning vectors. Plasmid pKB158, used in the experiments described above, contains unique cleavage sites for the restriction endonucleases Eco RI, Bgl II, Pst I, Bam I, Sal I, and Hpa I (see Fig. 1). Hpa I cleavage of pKB158 produces a flush-ended linear molecule, which may be used to clone flush-ended DNA fragments generated by a variety of restriction endonucleases (*5*). Joining of flush-ended DNA molecules to each other is mediated by high concentrations of T4 polynucleotide ligase (*2*).

We have prepared a smaller derivative of pKB158 which lacks the *tet* gene, in the following manner: pKB158 was digested with Bam I and Hpa I. The larger fragment produced (see Fig. 1) contained the λ *c*I gene and the Col E1 origin of replication. This fragment was partially digested with Hae II, diluted, and treated with T4 polynucleotide ligase. Cells were transformed with this DNA and λ immune transformants were selected as described above. About 5000 immune colonies were washed from their plates with tryptone broth, and a drop of this pool was used as an inoculum for a culture from which plasmid DNA was prepared. The plasmid DNA was separated by polyacrylamide gel electrophoresis and the smallest plasmid present on the gel was eluted and used to transform cells; this plasmid was named pKB166 (see Fig. 1).

Plasmid pKB166 contains only two Hae II fragments (2000 and 350 base pairs long), including the one reported to contain the Col E1 origin of replication (*6*). It is one of the smallest plasmids described to date, about 2400 base pairs long (1.6×10^6 daltons). Of those 2400 base pairs, 1100 are derived from the λ immunity region (*c*I and adjacent sequences) and 1300 are derived from Col E1. Since *E. coli* strains stably maintain pKB166 and are immune to colicin E1 when they harbor pKB166, the 1300 base pairs of pKB166 which are derived from Col E1 [corresponding to the region from 0.79 to 1.00 on the map in (*6*)] contain sufficient genetic information to direct replication and to express immunity to colicin E1.

pKB166 has unique recognition sites for Eco RI, Bgl II, and Hind III, and has only two recognition sites each for Hae II and Hind II. DNA fragments generated by Bam I or Mbo I as well as Bgl II may be cloned in the Bgl II site in pKB166, because all three endonucleases produce identical sticky ends. DNA fragments generated by Hae II may be cloned in partially digested pKB166. DNA fragments cloned in the Hind III or Hind II sites in pKB166 will destroy *c*I or its promoter and eliminate the expression of λ immunity. pKB166 is comparable with the so-called mini Col E1 plasmids (*7*) in that it is extremely small, can be amplified by treatment with chloramphenicol, determines two selectable phenotypes (immunity to phage λ and to colicin E1), and has several restriction endonuclease cleavage sites in which DNA fragments may be cloned.

Table 1. Selection of pKB158-bearing clones by λ immunity or by tetracycline resistance. Cells (5 to 6 × 10^7) from a transformed culture were spread on selective plates (described in the text) and incubated overnight at 37°C. The number of colonies which grew are tabulated.

DNA	Selection for	
	λ Immunity	Tetracycline resistance
None	6	0
pKB158	248	451
pKB158 + Eco RI	9	1
pKB158 + Eco RI + T4 ligase	28	51

The polypeptides encoded by pKB166 are few and with the exception of λ repressor are small. This we infer from experiments involving a plasmid (pKB266) which is nearly identical to pKB166, differing principally in that pKB266 has *lac* promoter fragments which transcribe *c*I [compare pKB158 and pKB252 in (*2*)]. A minicell-producing strain, χ1274 (*8*), was transformed with pKB266 and minicells were purified as described by Inselberg (*9*). The minicells were labeled with [^{35}S]methionine and extracts of the labeled minicells were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The principal (> 95 percent) ^{35}S-labeled polypeptide greater than 8000 daltons present in such extracts was λ repressor (molecular weight, 26,000).

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Hybridization in situ of SV40 Plaques: Detection of Recombinant SV40 Virus Carrying Specific Sequences of Nonviral DNA

Abstract. *The detection and recovery of SV40 genomes containing foreign DNA sequences can be facilitated, and the risk of accidental dispersal reduced, by in situ hybridization and radioautography.*

Specific nucleic acid sequences contained in the cells of bacterial colonies can be detected without prior isolation of the cell's DNA by in situ hybridization of radioactive nucleic acid probes (RNA or DNA) to imprints of the bacterial colonies immobilized on nitrocellulose disks (*1*). Similarly, λ phage plaques, formed on lawns of sensitive bacteria, can be screened for the presence of recombinant phages carrying specific DNA segments (*2*). Beside facilitating detection and isolation of bacterial or phage clones carrying recombinant DNA molecules, such methods decrease the risk of dispersion of these organisms by reducing the scale and simplifying the number of mechanical and chemical manipulations generally used to identify organisms carrying specific recombinant DNA molecules.

SV40 DNA has been used as a vector for cloning and propagating foreign DNA segments in animal cells (*3–5*). Where the desired recombinants do not possess a distinguishable phenotype, the recombinant virus genomes are detected by hybridization of the viral DNA isolated from cells infected with each of the putative recombinant virus stocks (*3–5*). But this procedure is cumbersome and time-consuming; furthermore, because the number of plaques that can be screened is limited, the ability to detect

recombinants that occur at low frequency is compromised.

To circumvent this problem we have developed a simple procedure that distinguishes plaques containing SV40 genomes carrying specific segments of nonviral DNA from those caused by infection with SV40 alone. This is achieved by (i) transferring the cell monolayers and their plaques to nitrocellulose disks, (ii) treatment of the imprinted nitrocellulose with alkali to denature and immobilize the cell and viral DNA to the disk, (iii) hybridization with appropriate highly labeled nucleic acid probes, and (iv) radioautography to identify the plaques containing DNA homologous to the probes. Virus contained in the plaques can be recovered from the corresponding region of the agar overlay which was removed from the cell layer prior to the imprinting step. By this procedure λ-SV40 hybrid viral genomes (*4*) can be detected and recovered even when they are present in an SV40 preparation at a frequency of about 10^{-5}.

Cell and plaque imprints on nitrocellulose. Infection of CV-1P monolayer cultures with SV40 virus or DNA produces plaques that are discernible after staining of the cell monolayer with 0.01 percent neutral red. Before transfer of the cell monolayers and virus plaques to nitrocellulose disks, the agar overlays were removed by reaming the agar–petri dish boundary and gently prying the agar layer into a sterile petri dish. Alignment marks on the nitrocellulose disk, petri plate, and agar overlay permit subsequent recovery of virus from plaques that contain the desired DNA sequences. A simple method for marking plaques on the agar is to lay another nitrocellulose membrane over the agar and record the position of the plaques with a suitable marker prior to removing the agar overlay from the petri dish. The agar, wrapped in plastic wrap, can be stored at 4°C until the radioautograms are developed (about 3 days).

To make an imprint of the cell monolayer and plaque contents suitable for in situ hybridization, a dry nitrocellulose disk (Millipore, 47 mm) was laid carefully on top of the moist, but not wet, cell monolayer and patted down with a rubber policeman. The filter was then moistened by blotting with wet paper toweling (with a solution of 50 m*M* tris at *p*H 7.5 and 0.15*M* NaCl) and peeled off the plate with forceps. No dry spots should remain on the nitrocellulose disk because it may stick to the dish and be torn during removal. Transfer of the cell monolayer to the nitrocellulose is quantitative, and, where the neutral red stain has been applied, the plaques are readily visible on the filter (Fig. 1A). Although faded, the plaques remain visible even after the denaturation and annealing procedure.

Cells and virus adsorbed to the nitrocellulose disk were lysed by placing the disk on a blotter saturated with 0.5*N* NaOH for about a minute after which the disk was dried on a piece of blotting paper for about a minute. This procedure was repeated (once or twice) more, and the membrane was then neutralized by repeating the above procedure with 1.0*M* tris-HCl, *p*H 7.6. Finally, the filters were immersed in a solution of 0.1*M* tris-HCl, *p*H 7.6, and 0.15*M* NaCl, air-dried, and baked for 2 hours at 80°C at reduced pressure.

Hybridization of plaque imprints. The nitrocellulose disks were incubated prior to use in a solution (PM) of triple-strength SSC (saline sodium citrate) containing 0.02 percent each of Ficoll, polyvinylpyrrolidine, and bovine serum albumin for 6 hours at 65°C to reduce the background due to nonspecific DNA binding (*6*). Then, such pretreated disks were annealed in PM solution (3.0 ml) containing 6 × SSC, salmon sperm DNA (100 μg/ml) and ^{32}P-labeled nick-translated DNA (*7*) in a chamber constructed from two glass plates (8.5 by 8.5 cm) separated by a rubber gasket. The particular radioactive probes, their specific activities, and the amounts used in the various annealing experiments are indicated below. After annealing, the filters were rinsed three times at 65°C for 1 hour in the following buffers: (i) PM with 6 × SSC; (ii) 0.1*M* phosphate buffer, *p*H 7.0 with 2 × SSC, and (iii) 2 × SSC. Autoradiograms of the nitrocellulose disks were made with Kodak X-Omat XR/5 film and a Kodak X-Omatic in-

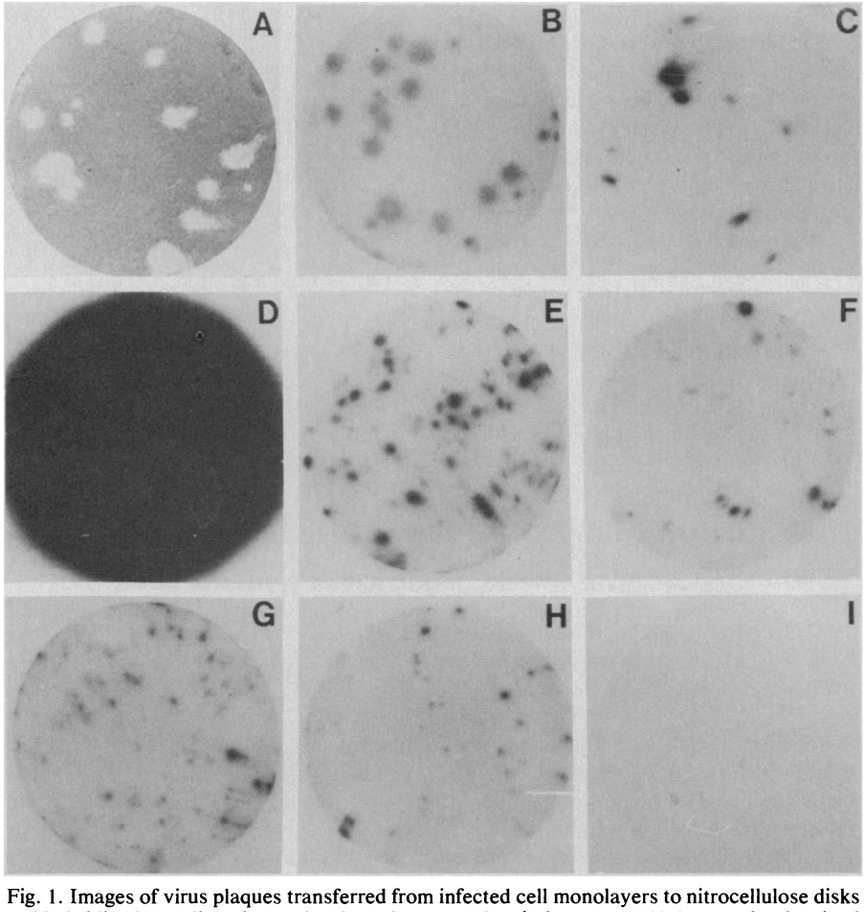

Fig. 1. Images of virus plaques transferred from infected cell monolayers to nitrocellulose disks and hybridized to radioactive probes homologous to the viral genome. (A) A neutral red stained monolayer of SV40 infected CV-1P cells after transfer to a nitrocellulose disk; (B) autoradiogram of a nitrocellulose imprint of SV40 plaques after annealing with ^{32}P-labeled nick-translated SV40 DNA; (C) autoradiogram of a nitrocellulose imprint of λ-SVGT plus tsA58 induced plaques after annealing with ^{32}P-labeled nick-translated λ DNA; (D) autoradiogram of an imprint of a monolayer infected with 10^5 pfu of wild-type SV40 4 days earlier and annealed with labeled SV40 DNA; (E and F) autoradiogram of a monolayer infected with 10^4 pfu of wild-type SV40 plus 50 and 10 pfu, respectively, of a λ-SVGT, 4 days earlier and annealed with labeled λ DNA; (G and H) autoradiogram of monolayer infected with 10^5 pfu of wild-type SV40 plus 50 and 10 pfu, respectively, of λ-SVGT 4 days earlier and annealed with labeled λ DNA; (I) autoradiogram of monolayer infected with 10^5 pfu of wild-type SV40 alone and annealed with labeled λ DNA. Autoradiograms were exposed for 14 or 48 hours after annealing with labeled SV40 or λ DNA, respectively.

tensifying screen to increase the sensitivity and decrease the exposure times.

Figure 1A shows a nitrocellulose disk after transfer of the cell monolayer and its plaques; the infection had occurred about 6 days earlier, and the monolayer had been stained a day before. After hybridization with ^{32}P-labeled nick-translated SV40 DNA (5×10^7 count/min per microgram, 10^5 count/min per disk) and radioautography, the location of the plaques is clearly discernible (Fig. 1B) and coincides with the position of the visible plaques in the corresponding disk. If transfer of the monolayer and plaques to the nitrocellulose disks is delayed beyond 7 days, viral DNA appears to be lost from the plaques (presumably, because of lysis or detachment of the virus-bearing cells from the dish), and the autoradiographed images of the plaques display hollow centers ("halos"). With the same ^{32}P-labeled SV40 DNA probe annealed to nitrocellulose disks imprinted with uninfected cell monolayers, there are no plaques detectable on the radioautogram (not shown). To recover the virus from the plaques, agar plugs were removed from the agar overlay with the use of the radioautographic images to locate the plaques; about 10^4 plaque-forming units (pfu) per plug were recovered after dispersion and removal of the agar.

Detection of recombinant viral genome. An SV40 variant containing a recombinant genome of λ and SV40 DNA (λ-SVGT-1) has been constructed, cloned, and propagated in monkey kidney cell cultures (*4*). Because the λ DNA segment replaces the genes coding for the virion capsid proteins, propagation of the recombinant virions requires a helper virus to supply these missing functions; those functions are provided by coinfection of the cells with ts*A*58, a mutant of SV40 defective in an early gene, at 41°C. Thus, monkey kidney cell monolayers infected with λ-SVGT-1 and ts*A*58 DNA's or their virions yield plaques that contain the helper virus and the defective recombinant (*3, 8*). When such mixedly infected monolayers are transferred to nitrocellulose each plaque registers on the radioautogram after hybridization with either ^{32}P-labeled nick-translated SV40 DNA (Fig. 1B) or λ DNA (Fig. 1C); the latter probe, which had a specific activity of 2×10^7 count/min per microgram, was made with a recombinant plasmid DNA carrying the λ DNA segment included in the λ-SV40 hybrid genome (D. Charney and P. Berg, unpublished). No radioautographic plaques were detected when the labeled λ DNA was annealed to imprints of monolayers infected with SV40 alone (see Fig. 1I).

One of our purposes in developing this method was to simplify the search for a rare recombinant genome. Since the ordinary plaque assay accommodates not more than 100 to 200 plaques per plate, the detectability of specific recombinants would be limited to frequencies of not less than 10^{-3}, although greater sensitivity would be achieved if the number of plates screened is increased. To determine whether specific recombinant genomes could be detected in the presence of a great excess of normal genomes, the following mixed infections were performed.

One series of CV-1P monolayer cultures (5×10^6 cells per culture) was infected with 10^5 pfu of wild-type SV40 plus 50 or 10 pfu of λ-SVGT; the titer of the λ-SVGT was determined independently with excess of ts*A*58 in the usual way (*8*). Another series of similar CV-1P monolayer cultures was infected with 10^4 pfu of wild-type SV40 plus 50 or 10 pfu of λ-SVGT. Cultures were overlayed with agar and incubated at 37°C; imprints were made 3, 4, 5, and 6 days after infection, and these were subsequently annealed with either ^{32}P-labeled SV40 or λ DNA. No significant hybridization was detected on any of the radioautograms after 3 days of infection (data not shown). Four days after infection, hybridization of each of the nitrocellulose imprints with labeled SV40 DNA yielded intense and uniform blackening of the film (Fig. 1D) indicating that SV40 DNA had replicated throughout the cultures, a not too surprising finding in view of the input multiplicity of wild-type virus. But, at the same time, in the cultures infected with 10^4 pfu of wild-type virus, areas that hybridize with the λ DNA probe could readily be detected (Fig. 1, E and F). Even in the cultures infected with 10^5 pfu of wild-type virus, foci containing λ DNA sequences could be seen (Fig. 1, G and H). After 5 days, the foci that contain λ DNA were larger and easily counted; the titer of the λ-SVGT-1 agreed within 30 percent of the value obtained in the standard way with ts*A*58 as the helper. Six days after the infection there was extensive degeneration of the cell monolayer, and it was too difficult to make imprints on the nitrocellulose disks.

This experiment suggests that, given the appropriate radioactively labeled probe, which can be RNA or DNA complementary to the sequence being sought, a recombinant SV40 genome can be detected if it is present with a frequency of about 10^{-5} or greater. Very likely by examining a larger number of cultures an even rarer recombinant could be found.

After a region in the culture containing a clone of recombinant genomes is detected, it is also important to recover it for further purification and characterization. When cultures were used that contained only one or two λ-SVGT-1 foci per infected culture (10^5 pfu), the agar layers were cored at the positions indicated by the radioautogram and the extracted material was assayed for the ratio of λ-SVGT to wild-type virus. Whereas the λ-SVGT-1 constituted only about 10^{-4} of the input virus, it was present at 10^{-2} frequency in the recovered virus. Quite likely one or two repetitions of this procedure would yield isolated plaques containing the recombinant and helper virions.

The procedure described here is applicable, in principle, to any animal virus that can be used for molecular cloning, whether it produces plaques or foci of infected cells, provided that a suitably labeled nucleic acid probe for the DNA segment being sought is available. It simplifies the task of detecting rare recombinant genomes but it is especially helpful where the recombinant genome cannot contribute any genetic functions, and, therefore, requires a nondefective virus helper for its propagation—as, for example, in (*3*). This method of screening recombinants reduces the probability of dispersing potentially hazardous recombinants. Instead of having to pick innumerable plaques and to isolate viral DNA from cultures infected with each of them (operations which require considerable handling, centrifugations, and the like), the desired recombinants can be detected and isolated within the more secure confines of a petri plate. Thus, the adoption of in situ hybridization provides "physical containment" over and above that provided by the laboratory design.

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