This paper was submitted for the yearly Univ. Florida Research Competition. This was also the first "paper" written on AAV's first use as a gene therapy vector and while unpublished it was distributed .

1984 University of Florida Medical Guild, 1st Place Research Award

Use of Adeno-Associated Virus as a Mammalian Transducing Vector:

Transformation of Human and Murine Cells to G-418 Resistance

Paul L. Hermonat

Department of Immunology and Medical Microbiology

Box J-266 JHMHC

College of Medicine

University of Florida

Gainesville, Florida 32610

Abstract

There is considerable interest in the development of viral transducing vectors for use in the introduction of foreign genes into mammalian cells. Because the helper-dependent parvovirus adeno-associated virus (AAV) latently infects cells readily, integrates specifically, and has a broad host range it was apparent that AAV could be a useful mammalian transducing vector. To determine this we have replaced AAV's capsid gene region with the G-418 (Neo) antibiotic resistance gene in an infectious AAV/pBR322 recombinant plasmid. A non-packagable complementor plasmid has also been constructed to provide missing capsid functions in "trans". A G-418^R transducing virus stock can be derived by co-transfection of these two plasmids into adenovirus (AAV's helper virus) infected cells. We confirm here AAV's utility as a mammalian vector by using it to transform human and murine tissue culture cells to G-418 resistance at a maximum efficiency of 10%. We further demonstrate that the AAV/G-418^R transducing DNA can be rescued and amplified from transformed cells by superinfection with adenovirus.

Introduction

The present state of knowledge of gene regulation and expression has been in great part attributable to the ability to introduce foreign genes into eukaryotic cells. The ability to introduce new genetic material into cells also allows for the possibility of treating genetic disease by augmenting defective genes with functional ones. Several techniques have been used to transform cells. The calcium phosphate precipitation technique is inefficient and allows for cell transformation at a frequency of only 10^{-4} at best (1). Microinjection is efficient but only small numbers of cells can be transformed (2). Viruses are also being used to transduce foreign genes into cells. SV40, a papovavirus, has frequently been used but has a limited host range and integrates chromosomally in a nonspecific manner (3). Bovine Papilloma virus also has a limited host range and exists in a nonintegrated state with variable copy numbers (4). Retroviruses are useful transducing vectors as they have a wide host range, integrate in a specific manner, and their proviral long terminal repeats (LTRs) contain sequences required for efficient transciption initiation and termination (5). They have been used to transform cells in tissue culture of a patient with Lesch-Nyhan syndrome from HPRT negative to positive (6). Retroviruses do, however, have drawbacks. They are associated with disease in animals and man and their LTRs alone are capable of causing cancer (7).

Our goal is to develop an adeno-associated virus (AAV) vector system, analogous to the retrovirus vector system, for basic research and possible gene replacement therapy. AAV is a naturally defective parvovirus requiring co-infection with adenovirus or herpes simplex virus for productive infection (8). It has many of the same desirable features that retroviruses have for use as a vector. AAV readily sets up a latent infection by integrating chromosomally in a stable and specific manner when no helper virus is present (9). It also has an

extremely broad host range. Additionally, no disease has been associated with AAV. In fact, it is known to inhibit the replication and oncogenicity of adenovirus and the oncogenicity of herpes simplex (10). AAV is transcriptionally silent in the latent state, unlike most retroviruses. AAV can also be rescued from its latent state and its copy number amplified by superinfecting the latent cell with a helper virus (11). This is in contrast to many retroviruses in which latently infected cells continually shed virus (12).

In a previous paper we have mapped the functional regions of the AAV genome (13) by mutating the infectious AAV/pBR322 recombinant plasmid pSM620 (14). In this paper we report the construction and usage of an AAV transducing vector plasmid containing the resistance gene to the antibiotic G-418 (15), and the construction of an AAV complementor plasmid to provide missing functions of the vector in "trans". An AAV/G-418^R transducing virus stock can be prepared by co-transfecting these two plasmids into adenovirus infected cells. This viral stock is capable of transforming human and murine tissue culture cells to G-418 resistance.

Material and Methods

Cells

All cell lines were maintained in MEM containing 10% fetal calf serum, 1% glutamine, penicillin, and streptomycin.

Enzymes

Restriction enzymes, T4 ligase, T4 DNA polymerase, T4 polynucleotide kinase, E. coli DNA polymerase I and the Klenow fragment were purchased from New England Biolabs and Bethesda Research Laboratories and the enzyme reactions were performed according to the suppliers specifications. BglII linkers were purchasaed from New England Biolabs. BamHI linkers were purchased from Collaborative Research.

DNA Transfection, Virus Infection and Virus Stock Preparation

Cells were transfected using the DEAE-dextran method (16) as described except that 0.5 to 11 g form I plasmid DNA was used per 10 cm dish. After transfection the cells infected with adenovirus at a multiplicity of infection (MOI) of 5. Five g of d152-91/G-418^R and 0.5 g of ins96- -M plasmid DNA were transfected into KB cells to obtain transducing virus stocks. This stock was prepared by freezing and thawing the transfected plates twice at 48 hours post-infection and heating the media for 2 hours at 56°C.

DNA Extraction and Hybridization Procedure

Low molecular weight DNA was isolated from tissue culture cells at 24 to 48 hours post-transfection or infection by the method of Hirt (17) as previously described. The DNA was fractionated by electrophoresis on 1.4% agarose gels, transferred to nitrocellulose (18) and annealled to ^{32}P-labelled nick-translated d152-91/G-418^R plasmid DNA as previously described. Plasmid DNA was isolated as described previously (19).

Vector and Complementor Plasmid Construction

d152-91/G-418^R was constructed as shown in Figure 1. The G-418 resistance gene also confers resistance to Neomycin in prokaryotes. Restricted fragments were isolated by electrophoresing on a 1.0% agarose gel and concentrated by Elutip-d (Schleicher and Schuell). Insert 96-λ-M was constructed by digesting pSM620-XbaI (kindly provided by J. Samulski) with XbaI. This plasmid has an XbaI linker inserted at map unit 96, a non-essential region in AAV. An 8 bp BglII linker was then ligated into this site giving ins96 (BglII). Bacteriophage DNA was digested with Sau3A and ligated with ins96 (BglII) which had been digested with BglII. Mutants were screened by restriction analysis and one was found to contain an 1,100 bp insert and designated ins96-λ-M.

Virus Titer Determination

AAV wild type virus titer was determined by immunofluorescence using anti-AAV capsid antibodies (20). Transducing virus titer was determined by quantitating the amount of transducing AAV DNA replication relative to known quantities of titered wild type AAV replication, Figure 4.

Transformation Determination

Titered transducing virus stock was used to infect various numbers, 10^2 to 10^5, of KB, D3405 or L tk$^-$ cells at various MOI's. Cells were infected 12 - 24 hours post-seeding and selected with G-418 (Gibco) at 12 - 24 hours or 7 days post-infection. D3405 and KB cells were selected with 1 mg/ml G-418 and L tk$^-$ with 0.4 mg/ml G-418. Heat shocked plates were heated to 42°C for 20 minutes at 0 hours, 4 hours, and 24 hours post-infections. Cells were fed every 3 to 4 days. At 8 - 14 days post-selection cells were fixed with ethanol, stained with Giemsa stain and colonies counted.

Results

Construction of AAV/G-418^R Vector and Complementor Plasmids

In a previous paper (13) we have mapped three functional, phenotypically distinct, regions to the AAV genome (shown in Figure 2). These are: the rep region, required for replication (mu [map units] 11-42); the cap region (mu 63-91), coding for the main AAV capsid protein; and the lip region (mu 48-55), which appears to code for part of at least one of AAVs two minor capsid proteins. For the initial attempt at using AAV as a vector it was decided to leave the vector with the ability to replicate without complementation. This would allow for easier analysis of transformed cells by simply infecting with adenovirus and looking for vector rescue and replication by Hirt extraction and Southern blot analysis (Fig. 6). We therefore had to leave the rep region intact, delete out the lip and cap region, and replace with a selectable marker. We therefore constructed a large lip-cap AAV deletion (pHM 1320) by using two already existing AAV mutants as was previously described (13) shown in Figure 2. This mutant is a lip-cap deletion of 1842 bp (or 40% of AAVs genome) which was replication positive. The vector d152-91/G-418^R was constructed by using the G-418 resistance gene from pBR-NEO (G-418^R) (15) and the constitutive SV40 early promoter (SV40EPR) from pSV2-dhfr (21) as outlined in Figure 1. The construction resulted in a wild type size

vector (4.7 kb), shown in Figures 1 and 2, with the G-418^R gene under SV40 EPR control and utilizing AAV's poly-adenylation sequence located at mu 95.

Preparation of G-418^R Transducing Virus Stock

Initial experiments with this vector plasmid to produce transducing virus using the wild type pSM620 plasmid as a complementor were, for the most part, negative. d152-91/G-418^R plasmid DNA was co-transfected with the wild type plasmid into tissue culture cells infected with adenovirus. Forty-eight hours later the media was assayed for the presence of virus by transferring an aliquot to a second tissue culture plate infected with adenovirus and looking for AAV replicating species by Hirt extract and Southern blot analysis. d152-91/G-418^R can be distinguished by a unique BglII restriction site (at mu 66) which is not present in the wild type genome. It was found that the major species of virus present was wild type. Only minute amounts of d152-91/G-418^R could be detected, results not shown. Therefore, although d152-91/G-418^R was of wild type size there appeared to be a strong packaging bias toward the wild type genome.

In order to circumvent this problem we constructed a complementor AAV plasmid which would be too large to be packaged, thus eliminating the packaging bias. This complementor could provide all functions in "trans" as the wild type plasmid does. This was done by inserting an 1,100 bp bacteriophage DNA fragment into a non-essential region of AAV (mu 96) resulting in ins96-λ-M, Figure 2. When 5 μg of d152-91/G-418^R was transfected into KB cells with 0.5 μg of ins96-λ-M, a 10:1 ratio, a viral stock resulted, approximately 10 ml of media, contained a d152-91/G-418^R to wild type virus ratio of about 5:1 (Figure 4). The appearance of a wild type genome was the result of recombination between the replicating vector and complementor. This recombination phenomenon between two replicating AAV species has been observed before (13) and was expected. If lower transfection ratios of vector/complementor were used the ratio of d152-91/G-418^R virus obtained compared to wild type was dramatically reduced.

Titering the Transducing Virus Stock

Our method of titering this d152-91/G-418^R transducing virus stock was to assess the amount of replicating AAV species of an aliquot of the stock in adenovirus infected cells relative to that of an aliquot of a wild type AAV stock of known titer. The wild type stock titer was determined by immunofluorescence with anti-AAV capsid antibody as described previously (20). This technique would only be valid if wild type AAV and d152-91/G-418^R replicated with equal efficiency. This was determined by transfecting equal amounts of these two plasmids separately into adenovirus infected tissue culture cells and comparing the replicating species by Hirt extract and Southern blot analysis at 24 hours. When this was done it was found that wild type AAV and the vector d152-91/G-418^R did indeed replicate equally. This is shown in Figure 3 by comparing the monomer duplex species of the wild type transfection with that of the 3.1 kb large BglII fragment of the d152-91/G-418^R transfection. Both of these species have

approximately the same homology with the labeled probe which was the d152-91/G-418^R plasmid.

To titer the stock we infected approximately 10^7 tissue culture cells with known quantities of wild type AAV or an 100 1 aliquot of the d152-91/G-418^R transducing virus. We compared the replicating species by Hirt extract and Southern blot analysis at 48 hours, shown in Figure 4. From this technique the d152-91/G-418^R titer was determined to be approximately 10^6 infectious virus per ml accompanied by approximately 2 x 10^5 wild type AAV virus per ml.

Transformation of Tissue Culture Cells to G-418 Resistance

With the titered G-418^R transducing virus stock we infected various cell types in tissue culture and selected with the antibiotic G-418 to assay transformation. Three cell lines were tested. D3405 cells and KB cells are human cell lines and were selected with 1 mg/ml G-418 in the media. Ltk$^-$ cells are a murine cell line and were selected with 0.4 mg/ml G-418. Control plates of 10^5 cells uninfected with the transducing virus stock were dead 6 to 10 days post-selection. 10^5 D3405 cells were infected with an MOI (multiplicity of infection) of 0.1 or 1 transducing virus and 10^2 D3405 cells were infected with an MOI of 10^2 or 10^3 and then selected with 1 mg/ml G-418 in the media at 12 - 24 hours post-infection, shown in Figure 5 and Table 1. The transformation efficiency of D3405 cells plateaued rapidly above an MOI of 1, shown in Table 1. An MOI of 1,000 yield 3% transformation while an MOI of 1 yield 0.7%. Because 1,000x more infectious virus per cell gave only four times more transformants there appears to be a limited subpopulation of cells which were competent for transformation. Human KB cells and murine Ltk$^-$ cells could both be transformed to G-418 resistance but at a lower efficiency, shown in Table 1.

In an attempt to improve transformation efficiency two additional techniques were tried. First, the time between transducing virus infection and G-418 selection was increased from 12-24 hours to 7 days. This change resulted in a threefold increase in transformation, from 3% to 10%, at an MOI of 1,000, shown in Table 1. Second, preliminary results indicate that heat shocking the cells during infection resulted in an almost threefold increase in transformation of D3405 cells (data not shown). We will try both of these techniques, delayed selection and heat shock, together.

Rescue of Transducing Virus DNA from G-418^R Transformed Cells

AAV latently infects cells by integrating chromosomally and can be rescued and amplified by superinfecting with adenovirus (11). We analyzed our G-418^R transformed cells to determined if we could rescue the transducing virus DNA, d152-91/G-418^R, from them. A plate of 10^5 D3405 cells were infected with an MOI of 10, selected with G-418, allowed to grow up and split twice. The resulting population of transformed cells probably represented approximately 10^3 clones. Plates of 10^7 transformed cells were either infected by adenovirus or not infected and Hirt DNA extract, Southern blot analysis performed, shown in Figure 6. For comparison the same procedure was done on a cloned known latently wild type AAV infected cell line 7374 (9).

When the G-418^R transformed cells are infected with adenovirus an AAV species is rescued and replicated. The fact that this species is digestable with BglII (4.7 kb → 3.1+1.6) indicates that this is the transducing virus d152-91/G-418^R DNA being rescued and not wild type AAV as there is no BglII site in the wild type genome. There is a small amount of uncut monomer (4.7 kb) AAV species remaining suggesting that there may be some wild type AAV also latent in this transformed population. This is not unexpected since the transducing virus stock did contain almost 20% wild type virus. This uncut species may also be due to incomplete BglII digestion. No AAV species was present in the Hirt extract from the transformed cells not infected with adenovirus. This data is consistent with that obtained from the known latent cell line and suggests that the transducing virus in the transformed cells is present in an integrated state (provirus) and not present episomally. It is noteworthy that the amount of rescued species from the transformed cells is much higher than from the latent 7374 cell line. The reason for this is unknown but may be a function of cell line passage number and resulting non-rescuability of the latent AAV species.

Discussion

We are interested in utilizing adeno-associated virus as a vector for efficient and stable introduction of foreign genes into mammalian cells. As a prototype system we have constructed a plasmid containing AAV sequences essential for replication and integration linked to a lethal selectable marker conferring resistance to the antibiotic G-418. We have also constructed a complementor plasmid containing the entire functional AAV genome but which is too large, due to a 1,100 bp insert, to be efficiently packaged. Co-transfection of these two plasmids into adenovirus infected cells results in a G-418^R transducing virus stock. A titer of 10^6 d152-91/G-418^R transducing virus per ml has been obtained. Using this virus stock we have been able to transform both human and murine tissue-culture cells to G-418 resistance. The maximum transformation efficiency obtained was 10% under conditions of high MOI and delayed selection. Thus, we have shown that AAV can be used as an efficient transducing vector. We have also shown that the transducing virus DNA can be rescued and amplified by superinfecting the transformed cells with adenovirus. Our data is consistent with the transducing virus DNA being in an integrated state (provirus) in these transformed cells and not present extra-chromosomally. Genomic DNA isolation and Southern blot analysis is being performed on several transformed cell clones to verify this.

The d152-91/G-418^R transducing virus is of wild type size (4.7 kb), yet when complemented with wild type AAV the transducing vector is not packaged (not shown). We do not know the reason for this packaging bias but it may be due to the deleted sequences. A complementor with a 550 bp insert was also constructed but was found to be packageable (not shown). Therefore, the size of the d152-91/G-418^R vector does not appear to be the cause of this negative packaging bias. The ability to package larger than wild type genomes suggests that we can use our present capsid deletion system (lip and cap region are 2.1 kb in size)

with an inserted gene up to 2.6 kb in size. Further studies are being carried out on more extensively deleted AAV vectors.

All our attempts to increase transformation efficiency by different techniques (high MOI, delayed selection, heat shock) were successful. This suggests that there may be many manipulations to the system we have developed that could increase the transformation efficiency above the 10% level which we have obtained presently.

In its latent state AAV is transcriptionally silent (11). Our data suggests that there is no strong inhibition by the remaining AAV sequences of the transducing vector on the transduced foreign gene. The G-418 resistance gene appears to be expressed using its own SV40 early promoter.

AAV has advantages over several techniques for introducing foreign genes. It can transform efficiently, integrate in a specific manner, and can be rescued by super-infection with adenovirus helper. AAV has potential advantages over retroviruses as a general transducing vector as it is transcriptionally silent and appears to have an extremely broad host range. The additional fact that no disease is associated with AAV makes it appealing as a possible vector for introducing genes into whole animals.

References

1. Graham, F.L. & van der Eb, A.J. (1973) Virology 52, 456-467.
2. Capecchi, M. (1980) Cell 22, 479-488.
3. Gluzman, Y. (1982) in Eukaryotic Viral Vectors, ed. Gluzman, Y. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) pp 1-5.
4. Howley, P.M., Sarver, N., Law, M.Y. (1983) Methods Enzymol. 101, 387-402.
5. Varmus, H.E. & Swanstrom, R. (1982) in RNA Tumor Viruses, Molecular Biology of Tumor Viruses, eds. Weiss, R., Teich, N., Varmus, H.E. & Coffin. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) pp. 369-512.
6. Miller, A.D., Jolly, D.J., Friedmann, T. & Verma, J.M. (1983) Proc. Natl. Acad. Sci. USA 80, 4709-4713.
7. Hayward, W.S., Neel, B.G., Astrin, S.M. (1981) Nature 290, 475.
8. Carter, B.J., Laughlin, C.A. (1984) in The Parvoviruses, ed. Berns, K.I. (Plenum Press) pp. 67-128.
9. Cheung, A.K., Hoggom, M.D., Hauswirth, W.W. & Berns, K.I. (1980) J. Virol. 33, 739-748.
10. Cukor, G., Blacklow, N.R., Hoggan, M.D. & Berns, K.I. (1984) in The Parvoviruses, ed. Berns, K.I. (Plenum Press) pp. 48-60.
11. Berns, K.I., Cheung, A.K., Ostrove, J.M. & Lewis, M. (1982) in Virus Persistence, eds. Mahy, B.W., Mirson, A.C. & Darby, G.K. (Cambridge University Press) p. 249.
12. Varmus, H.E. (1982) Science 216, 812-820.
13. Hermonat, P.L., Labow, M.A., Wright, R., Berns, K.I. & Muzyczka, N. (1984) submitted J. Virol.
14. Samulski, R.J., Berns, K.I., Tan, M. & Muzyczka, N. (1982) Proc. Natl. Acad. Sci. USA 79, 2077-2080.

15. Southern, P.J. & Berg, P. (1982) Molec. Appl. Genet. 1, 327-341.
16. Muzyczka, N. (1980) Gene 11, 63-77.
17. Hirt, B. (1967) J. Mol. Biol. 26, 365-369.
18. Southern, E.M. (1975) J. Mol. Biol. 98, 503-518.
19. Rawlins, D.R. & Muzyczka, N. (1980) J. Virol. 36, 611-616.
20. Carter, B.J., Laughlin, C.A., de la Maza, L.M. & Meyers, M. (1979) Virology 92, 449-461.
21. Subramani, S., Mulligan, R.S. & Berg, P. (1981) Mol. Cell Biol. 1, 854-864,
22. Maniatis, I., Fritsch, E.F. & Sanbrook, J. (1982) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Figure 1: d152-91/G-418^R (Neo) plasmid construction. Plasmid construction was made using standard techniques (22). Abbreviations for restriction enzymes are B, BamHI; E, EcoRI; H, HindIII; P, PvuII. In all cases fragments were ligated with T4 ligase. Thin lines indicate AAV sequences, small box indicates AAV's terminal repeats, dotted lines indicate pBR322 sequences, thick stippled line indicates SV40 sequences, thick empty line indicates dhfr sequences, and thick cross-hatched line indicates G-418^R (Neo) sequences.

Figure 2: Physical organization of AAV phenotypes and AAV derived mutants and constructs. Above the map unit marker line, the thick solid line indicates the regions of the genome responsible for the three presently known AAV phenotypes discussed in Results. Below the map unit marker line are maps of constructed AAV plasmids. Sequences are represented as in Figure 1. Thick criss-cross indicates Bacteriophage sequences. d152-91/neo indicates d152-91/G-418^R. Arrow indicates BglII site. d152-91/dhfr is not discussed.

Figure 3: Comparison of wild type and transducing virus replication. One microgram of form I supercoiled plasmid DNA was transfected into KB cells which had been infected with adenovirus at an MOI of 5. At 24 hours, cells were lysed, treated with pronase and low molecular weight DNA was isolated by Hirt extraction (17). One tenth of each DNA extract was then fractionated on a 1.4% agarose gel, transferred to nitrocellulose by the method of Southern (15) and hybridized to nick translated d152-91/G-418^R (Neo) plasmid DNA. Where indicated the extract was digested with BglII. The large BglII fragment, 3.1 kb, of d152-91/G-418^R (Neo) has the same approximate homology to the probe as does the full size, 4.7 kb, wild type AAV band. D152-91/neo indicates d152-91/G-418^R. d152-91/dhfr is not discussed.

Figure 4: Transducing virus titer determination. 10^7 D3405 cells in 10 cm dishes were either infected with known quantities of wild type AAV or 100 1 of transducing virus stock derived from transfected KB cells (see Materials and Methods) and also infected with adenovirus at an MOI of 5. At 48 hours DNA was harvested from the dishes and analyzed as in Figure 3. Each lane represents 10% of the DNA recovered from one dish. d152-91/neo indicates d152-91/G-418^R. (KB) denotes KB derived virus stock. (293) derived stocks are not discussed.

Table 1

Cell Line	$G\text{-}418^R$ Transducing Virus MOI	Selection Time	Transformation Efficiency
D3405 (human)	0.1	12-24 hours	.04%
D3405 (human)	1	12-24 hours	.7%
D3405 (human)	100	12-24 hours	1.3%
D3405 (human)	1000	12-24 hours	3%
D3405 (human	1000	7 days	10%
KB (human)	1	12-24 hours	.07%
L tk^- (murine)	0.4	12-24 hours	.02%

Figure 1.

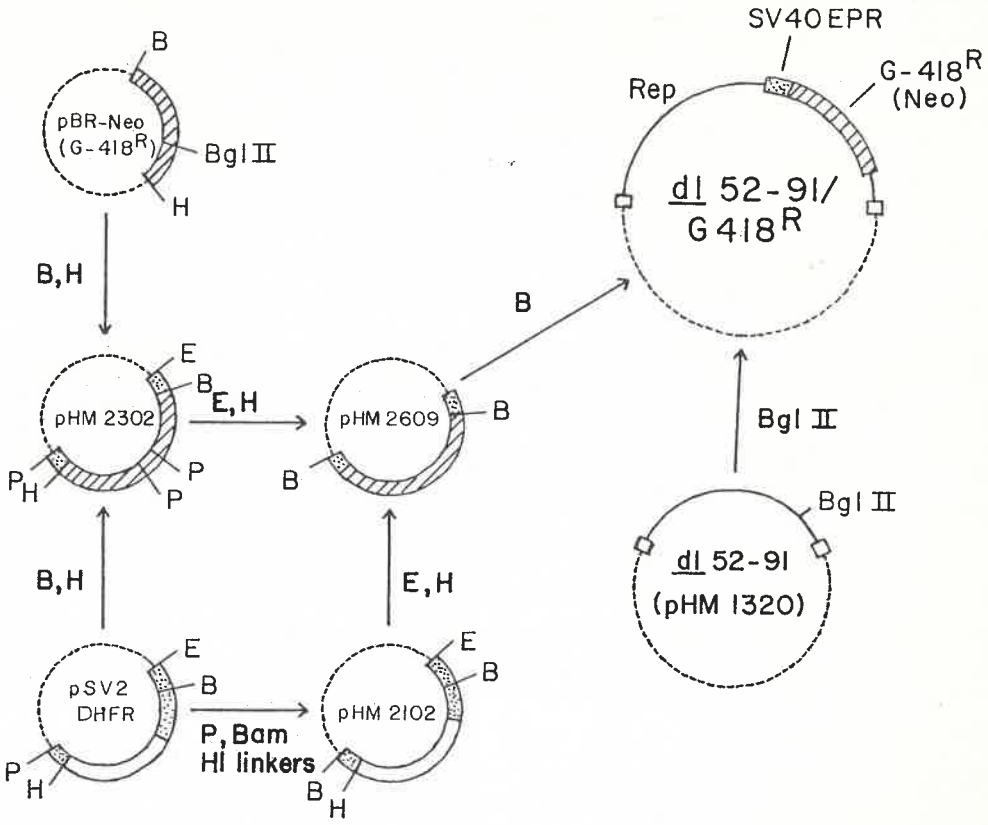

Figure 2.

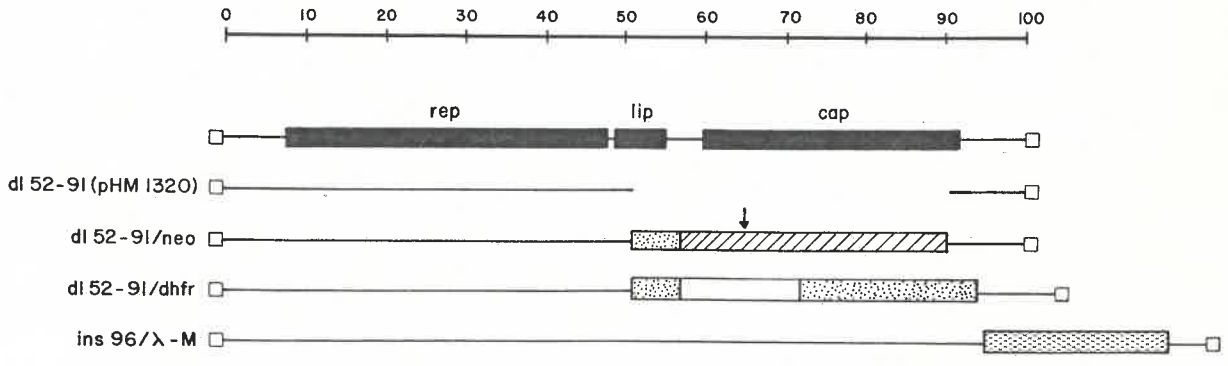

Figure 3.

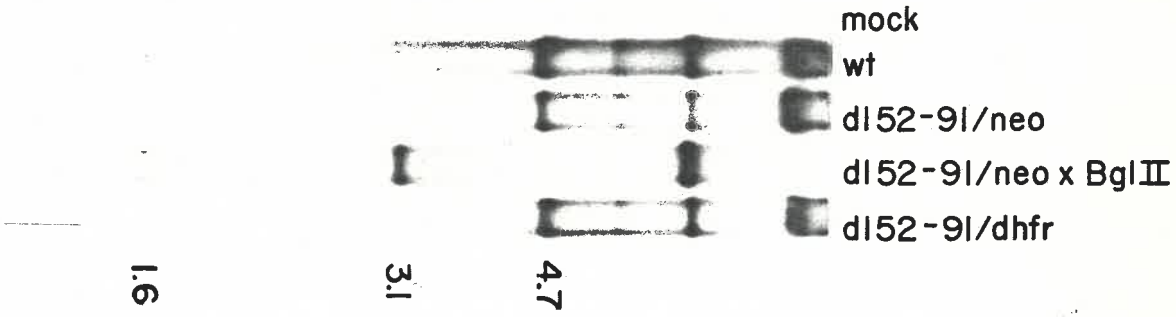

Figure 4.

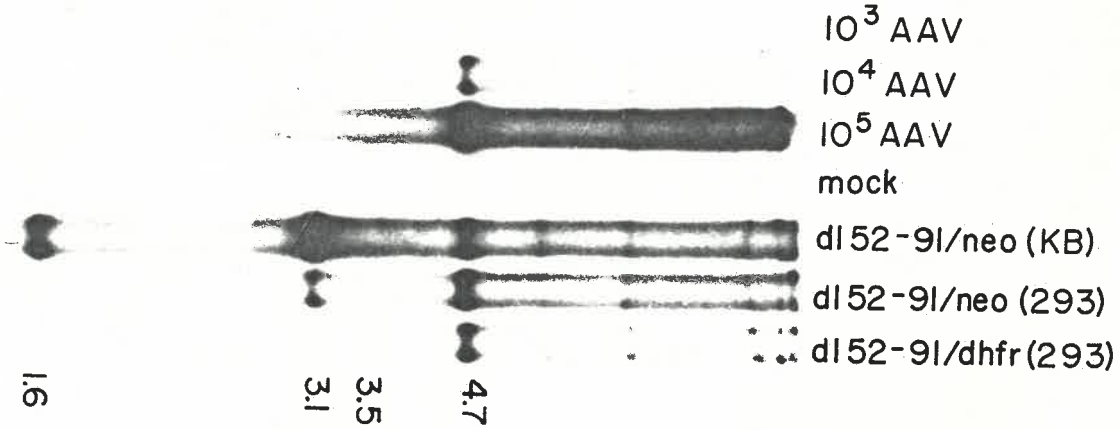

Figure 5.

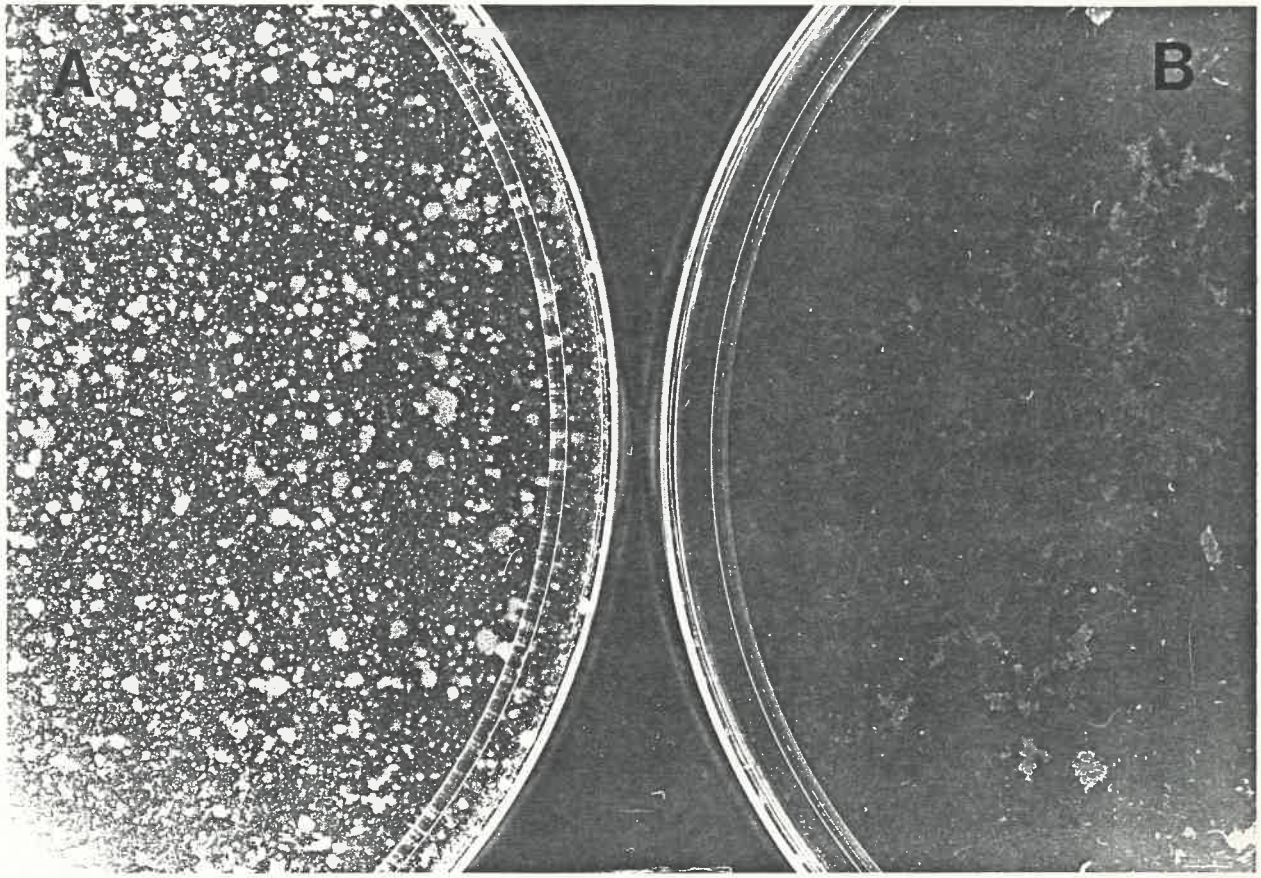

Figure 6.