Plaque Size Phenotype as a Selectable Marker To Generate Vaccinia Virus Recombinants

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In this report, we provide a new method for selection of vaccinia virus recombinants expressing foreign genes. The method is based on the use of the gene encoding the viral 14,000-molecular-weight envelope protein that rescues the small-plaque-size phenotype of a vaccinia virus variant to large-plaque-size virus. Selection of recombinants is easily obtained after visual inspection of large viral plaques.

The possibility of genetically manipulating the vaccinia virus genome has provided the means to use this virus as a eucaryotic viral vector. Foreign genes can easily be inserted into the vaccinia virus genome by homologous recombination (9). A number of insertion vectors have been constructed which allow introduction of foreign DNA sequences into the thymidine kinase (tk) region of the viral genome (9). Selection of recombinant viruses is carried out by isolation of a tk− virus (7, 12) and by the ability of recombinant viruses to express the gene markers β-galactosidase (3) and neomycin (6). Although coexpression of procaryotic markers is a highly efficient approach for the selection of recombinants, their use is not desirable when recombinants are designed for vaccination purposes. Thus far, the only viral genetic marker used for selection of recombinants has been the tk gene (7, 12). We have previously observed that alterations in the coding sequence of the 14,000-molecular-weight (14K) gene are responsible for the small-plaque-size phenotype of variants of vaccinia virus (4, 5). This observation suggested that the 14K gene could be used as a selectable marker and the flanking sequence could be used as a site of insertion of a foreign gene. To study this possibility, we cloned the neomycin resistance gene (neo) fused to the vaccinia virus promoter pl11 from the plasmid pVV:NEO (6) into the plasmid pDel 13 (Fig. 1). This plasmid contains a 1.2-kilobase-pair fragment of vaccinia virus DNA with the entire coding sequence of the 14K gene (5, 13). A Cla–HindIII fragment of 1.25 kilobase pairs, corresponding to upstream sequences of the 14K gene, was isolated from plasmid pE17 (5, 13). This fragment was cloned between the SalI and HindIII restriction sites of pD13NEO to generate p14K:NEO (Fig. 1). This vector contains the classic features of previously described vaccinia virus insertion vectors, i.e., a chimeric gene fused to a viral promoter and flanked by vaccinia virus DNA sequences.

Selection of vaccinia virus recombinants by plaque size. To assess whether the p14K:NEO could efficiently rescue the small-plaque-size phenotype of vaccinia virus variants, DNA transfection experiments were carried out. Cultures of BSC-40 cells were infected (0.01 PFU per cell) with variant 48-7, which produces small plaques, and 4 h later cells were transfected with DNA of p14K:NEO. Viral progeny was collected at 48 h postinfection and titrated. The presence of viruses producing large plaques was determined after staining, with crystal violet, monolayers of BSC-40 cells infected with about 250 PFU per 100-mm dish. Figure 2A shows the size of virus plaques from cells infected with wild-type virus (panel 1), mutant 48-7 (panel 2), mutant 48-7 and transfected with p14K:NEO (panel 3), and a plaque isolate after one round of virus growth (panel 4). As determined from three independent marker rescue experiments, 0.07 ± 0.02% of viruses had the large-plaque-size phenotype. The size of virus plaques obtained after marker rescue and when measured 7 days after virus infection was about five times the size of the parental mutant virus.

Since marker rescue could be the result of homologous recombination with the 14K gene or with the p14K:NEO flanking sequences, it was of interest to establish to what extent viruses that have rescued the large plaque size also contain the neo gene. Thus, dot blot hybridization analysis was carried out. Several plaque-purified viruses with the large-plaque-size phenotype were used to infect cultures of BSC-40 cells. Total DNA was extracted at the time of extensive cytopathic effect, and the DNA was dot blotted and hybridized with either the agarose-purified 32P-labeled viral 14K gene or the labeled neo gene. While DNA from cells infected with the virus isolates hybridized with the 14K gene (data not shown), about 60% of the infected cultures contained sequences from the neo gene (Fig. 2B). These findings showed that 40% of large-plaque-size viruses were generated by homologous recombination with the left flanking sequence of the vector (containing the wild-type 14K gene) while 60% of the recombinants were generated by homologous recombination with the left and right flanking sequences of the p14K:NEO vector. We conclude that rescue of large-plaque-size vaccinia virus can be used as a visual marker for the selection of viral recombinants.

Genetic stability of large-plaque-size recombinants. We have previously described that besides the small-plaque-size phenotype of variant 48-7, this virus has two other well-defined genetic markers: alterations in the electrophoretic mobility of a 14K envelope protein and an 8-megadalton (MDa) deletion on the left terminus of the viral genome (5, 10, 11). These markers were then used to assess the identity and stability of the large-plaque-size recombinants. As shown in Fig. 3A, immunobLOTS reacted with monoclonal antibody C3, which is specific for the 14K protein (14, 15), revealed a single polypeptide of 14K in cells infected with wild-type virus (lane 1), a 15K protein in cells infected with variant 48-7 (lane 2), two polypeptides of 15.5K and 14K in cells infected with a mixed population of small- and large-plaque-size viruses (lane 3), and a 14K protein in cells

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infected with a large-plaque-size recombinant virus containing the neo gene (lane 4). The same pattern was maintained after multiple passages of plaque-purified viral recombinants in cells in culture. Southern blot hybridization analysis established that the large-plaque-size recombinants maintained the 8-MDa deletion at the left end of the viral genome (Fig. 3B), because hybridization was only observed with the right end (HindIII-B) of vaccinia virus DNA. By DNA hybridization analysis, we also established that the neo gene was inserted into the 14K region of the viral DNA, because digestion with various restriction enzymes gave fragments of expected sizes (Fig. 3C).

We conclude that the 14K region of vaccinia virus DNA can be used to insert foreign genes and that recombinants derived from variant 48-7 have well-defined and stable genetic markers.

Expression of NEO gene during vaccinia virus infection. It was of importance, for the future use of the 14K gene as an insertion vector, to establish whether the neo gene was expressed in our recombinants. This was documented after examination of single-step growth curves in the presence of the antibiotic G-418 (Fig. 4). While replication of wild-type virus was markedly inhibited by the antibiotic G-418, as previously described (6), a representative large-plaque-size recombinant, 19, grows as efficiently in the presence of the drug as in its absence. Growth of this recombinant was found to be more efficient than the parental variant 48-7, which normally yields about 10⁶ PFU/ml at 24 h postinfection (4).

The findings shown in Fig. 4 demonstrate that the NEO gene is expressed in the vaccinia virus recombinant when placed upstream of the 14K gene of vaccinia virus DNA. Moreover, the recombinant replicates as efficiently in cells in culture as the wild-type virus.

In this investigation, we provide a new viral genetic marker. We have shown that recombinants are easily produced by transfecting, with the vector p14K:NEO, cell cultures infected with a small-plaque-size variant of vaccinia virus. These recombinants are detected as early as 48 h after infection by acquisition of a large-plaque-size phenotype (Fig. 2). The small-plaque-size variant of vaccinia virus has a frequency of reversion to large-plaque-size viruses of less than 1 per 10⁶. The novelty of this new insertion vector is that the 14K gene can be used as an efficient viral marker and that the upstream sequences of this gene are suitable for
FIG. 2. Identification of large-plaque-size vaccinia virus recombinants containing the neomycin gene into the viral genome. (A) Monolayers of BSC-40 cells were infected with wild-type virus, Mut 48-7 virus, or rescued viruses, and after virus adsorption cells were covered with Dulbecco medium containing 0.9% agarose and 2.5% newborn calf serum. After 7 days, the overlay was removed and monolayers were stained with 1% crystal violet in 2% ethanol. The plaques shown were formed by wild-type virus (panel 1), Mut 48-7 (panel 2), lysates of BSC-40 cells infected with Mut 48-7 and transfected with p14K:NEO (panel 3), and a large plaque isolate after one round of virus growth (panel 4). (B) Dot blots to show neomycin gene insertion into the viral genome. Several large-plaque-purified viruses were used to infect cultures of BSC-40 cells. When cytopathic effects developed in approximately 50% of each culture, cells were recovered by trypsinization, DNA extracted, and used for dot blot hybridization with 32P-labeled neo gene as a probe. Numbers indicate different isolates. WR, Wild-type vaccinia virus; M7, mutant virus; U, uninfected cells; U+NEO, uninfected cells plus 100 pg of pVV:NEO plasmid.

foreign gene insertion. The main advantage of this selection vector is that there is no requirement for metabolic selection (i.e., tk−), there is no need for screening large number of virus plaques by DNA hybridization, and there is no need for the use of foreign gene markers (i.e., β-galactosidase and neo). The 14K gene can also be used in the isolation of recombinants when inserted into a nonessential region (i.e. tK) of the vaccinia virus variant (Gong et al., manuscript in preparation).

The new viral marker is specially designed for the con-
The construction of highly attenuated recombinants of vaccinia virus. This is because the small-plaque-size variant has a large 8-MDa deletion on the left terminus of the viral genome which markedly decreases virulence in both normal and immunosuppressed animals and has a mutation in the 14K gene (4, 11; manuscript submitted). In vaccinated animals, this deletion mutant elicits a protective immune response against wild-type virus (4). Moreover, using luciferase as a reported gene, we have found that the tissue tropism of this attenuated variant is the same as that of wild-type virus (16; D. Rodriguez, J. R. Rodriguez, D. Trauber, and M. Esteban, Proc. Natl. Acad. Sci. USA, in press). Since inactivation of the vaccinia virus tk locus results in viruses with reduced virulence (2), combining the acquisition of an 8-MDa deletion with a tk phenotype should generate recombinants further decreased in virulence. Thus, it is now possible to generate safe and stable nonvirulent recombinants of vaccinia virus with defined genetic markers for vaccination purposes. This minimizes the chances of producing the kinds of serious complications found in the past with the practice of human vaccination (1).

FIG. 3. Genetic markers of recombinant viruses. (A) Immunoblots showing alterations on the electrophoretic mobility of the 14K polypeptide as identified by monoclonal antibody C3 and made with extracts from cells infected with wild-type vaccinia virus (lane 1), mutant 48-7 (lane 2), semipurified neomycin-resistant recombinants (lane 3); and plaque-purified recombinant 19 (lane 4). (B) Deletion on the left end of the viral genome. Cells were infected with wild-type virus (lane 1), mutant 48-7 virus (lane 2), or purified neomycin-resistant recombinant 19 virus (lane 3). The DNA was isolated and digested with HindIII, and fragments were separated in a 0.7% agarose gel, blotted, and hybridized with the 1-kilobase-pair 32p-labeled SalI terminal fragment of vaccinia virus DNA that maps within the 8-MDa deletion (10). The HindIII fragments corresponding to the left (HindIII-C) and right (HindIII-B) ends of the viral genome are indicated (B and C, respectively). (C) DNA from wild-type vaccinia virus-infected cells (lanes 1 to 4) and neomycin-resistant recombinant 19 virus-infected cells (lanes 5 to 8) was digested with various restriction enzymes, and Southern blots were hybridized with the labeled 14K gene (lanes 1 to 4) or with the labeled neo gene (lanes 5 to 8). Digestions were with EcoRI (lanes 1 and 5), ClaI (lanes 2 and 6), HindIII (lanes 3 and 7), and BamHI (lanes 4 and 8). The DNA fragment sizes are indicated in kilobases.

FIG. 4. Expression of neomycin resistance by vaccinia virus recombinants. BSC-40 cells grown in 60-mm dishes either untreated or treated with 1 mg of G-418 (GIBCO Laboratories) per ml were infected with 2 PFU of wild-type vaccinia virus per cell or with the neomycin-resistant recombinant 19. Virus yields (PFU per milliliter) at different times postinfection were determined by plaque assays in BSC-40 cells.

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LITERATURE CITED