JOURNAL OF VIROLOGY, June 1990, p. 3108–3111
0022-538X/90/063108-04$02.00/0

Vol. 64, No. 6

Transient Dominant Selection of Recombinant Vaccinia Viruses

FALKO G. FALKNER† AND BERNARD MOSS*

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892

Received 23 January 1990/Accepted 28 February 1990

A general method for constructing and selecting recombinant vaccinia viruses with insertions, deletions, or mutations in any gene that is similar in principle to one originally devised for *Saccharomyces cerevisiae* (S. Scherer and R. W. Davis, Proc. Natl. Acad. Sci. USA 76:4951–4955, 1979) is described. The selectable marker used, *Escherichia coli* guanine phosphoribosyltransferase, is not retained within the final recombinant virus, and hence, this procedure may be used serially to introduce several foreign genes or to make multiple site-directed mutations.

Homologous recombination may be used to introduce new genes or mutations into DNA viruses. Unless there is some phenotypic marker, however, it is difficult or tedious to distinguish rare plaques containing recombinant virus from the much larger number of plaques composed of parental virus. Because of the increasing importance of recombinant vaccinia viruses as tools for gene expression studies in animals and cultured cells, several different selection and screening methods have been developed. These include selection for thymidine kinase (TK)-negative and -positive phenotypes (9, 14, 23), β-galactosidase screening (4, 13), dominant selective markers for neomycin (8) or mycophenolic acid (3, 7) resistance, screening for hemagglutinin-negative phenotype (19), and reversal of plaque size (16) and host range (15) mutations. While each of these methods is useful for certain purposes, each has one or more limitations such as the requirement of special cell lines or mutagenic agents, the production of a virus that either retains an extra marker gene or is attenuated due to a concomitant gene disruption, or the inability to be used in a serial fashion for introducing multiple genes without intermediate steps. Moreover, none of the above methods provide a general selection procedure applicable to the construction of site-directed mutations.

We have employed a strategy called transient dominant selection (TDS) that may be used repeatedly to make insertions, deletions, or mutations at any site in the vaccinia virus genome. To implement this procedure, a plasmid is needed that contains the desired segment of mutated vaccinia virus DNA as well as a dominant selectable marker, such as the *Escherichia coli* guanine phosphoribosyltransferase (*gpt*) gene (12), under the control of a vaccinia virus promoter. Such a construct differs from previous ones (3, 7) in that the marker should not be flanked by vaccinia virus DNA and hence will not be stably integrated into the vaccinia virus genome by gene conversion or double crossover events. We demonstrate the TDS method by constructing a new insertion vector, pTK53-gpt, that has *gpt* under the control of the vaccinia virus P7.5 early and late promoter (7) and the vaccinia virus *tk* gene within which is the strong vaccinia virus late P11 promoter (2) with its translation initiation codon followed by a set of unique restriction endonuclease sites (Fig. 1). Although the recombinant virus made with this vector will be TK^-, this property was not used for selection and is not a requirement of the method.

To further simplify the final characterization of isolated recombinant virus plaques in these pilot experiments, the *E. coli lacZ* gene is present in the *Bam*HI site of pTK53gpt-lacZ and the latter was transfected into vaccinia virus-infected cells as previously described (7). Ten different virus isolates were plaque purified five times. The first two rounds of plaque isolation were in the presence of mycophenolic acid, xanthine, and hypoxanthine, which only allows the growth of virus that expresses *E. coli gpt* (7). The next two rounds were without selection. In the final plaque assay, the virus isolates were checked for expression of *gpt* and *tk* by use of selective media (10) and for *lacZ* expression by color screening with X-gal (4). The results are summarized in Table 1. None of the final isolates could grow in mycophenolic acid, indicating rapid loss of the *gpt* gene after plaque purification under nonselective conditions. The virus isolates 1, 5, 6, 7, and 10 were recombinants with a TK^- phenotype, as indicated by their ability to plaque in the presence of 5-bromodeoxyuridine, and β-galactosidase$^+$, as indicated by their ability to hydrolyze X-gal and produce a blue color. In contrast, virus isolates 2, 4, 8, 9, and 11 were parental TK^+, β-galactosidase$^-$.

The genotypes of the virus isolates were determined by restriction endonuclease digestion of DNA and probing of Southern blots with ^{32}P-labeled DNA fragments. Restriction endonuclease *Eco*RI was expected to excise the 3-kilobase-pair *lacZ* fragment from the *tk* gene of recombinant viruses. We found 3-kilobase-pair *Eco*RI fragments that hybridized to *lacZ* DNA from isolates 1, 5, 6, 7, and 10 but not from isolates 2, 4, 8, 9, and 11 (Fig. 2). A control hybridization with a vaccinia virus *tk* DNA probe, in addition to the *lacZ* probe, revealed the expected *Eco*RI fragments of about 7 and 9 kilobase pairs from both β-galactosidase$^+$ and β-galactosidase$^-$ virus-infected cells (data not shown), confirming that there were comparable amounts of vaccinia virus DNA in each case. Hybridization to *gpt* DNA did not occur, indicating that the gene had been deleted from the final recombinant viruses (data not shown). Thus, all of the isolated viruses had undergone a second crossover event; half were parental or wild type, and half were recombinants.

Two of the viruses (v53#1 and v53#6) were plaque isolated three additional times under nonselective conditions and were then purified by sucrose gradient centrifugation. The DNA was extracted, and the retention of *lacZ* DNA was confirmed by hybridization. In addition, the site of *lacZ*

* Corresponding author.
† Present address: IMMUNO AG Research Center, Uferstr. 15, A-2304 Orth-Donau, Austria.

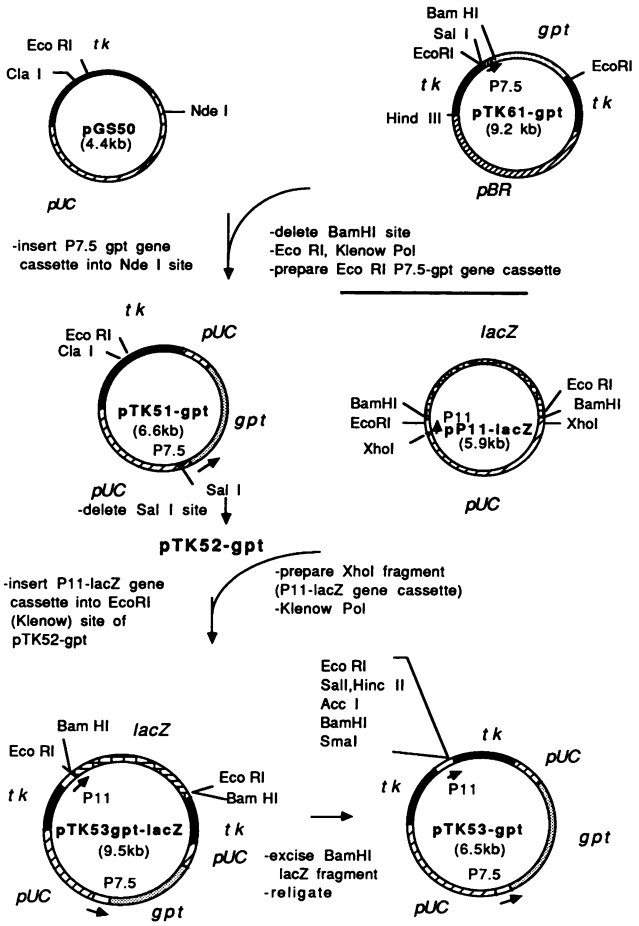

FIG. 1. Construction of the vaccinia virus insertion vector pTK53-gpt. Vaccinia virus DNA sequences including the *tk* gene (■), the *E. coli gpt* gene (▨), and the plasmid vector (□) are indicated. Arrows show the direction of transcription of the P11 (2) and P7.5 (5) promoters derived from the 11-kilodalton protein and the 7.5-kilodalton protein genes, respectively. The pUC sequences contain the ampicillin resistance gene and the origin of plasmid replication. The sequence downstream of the P11 promoter is 5′-ATG AAT TCC TGC AGG TCG ACT CTA GAG GAT CCC CGG GTA CCG-3′ and contains a translation initiation codon and the indicated restriction endonuclease cleavage sites. ▭, *E. coli lacZ* gene.

integration was verified by digesting the DNA with *Hin*dIII, which cleaves outside of the *tk* gene, and probing Southern blots with ^{32}P-labeled *tk* DNA. Major bands of 5 and 8 kilobases were detected with DNA from wild-type and v53#1 viruses, respectively (Fig. 3). The 8-kilobase band also hybridized to *lacZ* DNA (data not shown), consistent with the insertion of the 3-kilobase *lacZ* fragment.

The simplified recombination scheme (Fig. 4) can explain the TDS method. A single, rare crossover event results in the integration of the circular plasmid into a homologous segment of the vaccinia virus genome. The resulting recombinant contains the uninterrupted *tk* gene of the parental virus separated by the *gpt* gene from the *lacZ*-interrupted *tk* gene of the plasmid. Because the *gpt* gene is sandwiched between direct repeats, it is readily lost. Until the selective pressure is removed, however, plaques containing virus with the *gpt* gene can be isolated repeatedly. Rapid deletion of *gpt*

TABLE 1. Phenotypes of isolated virus plaques[a]

Plaque no.	Phenotype after plaque purification round:			
	1 and 2	5		
	gpt	gpt	β-gal	TK
1	+	−	+	−
2	+	−	−	+
4	+	−	−	+
5	+	−	+	−
6	+	−	+	−
7	+	−	+	−
8	+	−	−	+
9	+	−	−	+
10	+	−	+	−
11	+	−	−	+

[a] CV-1 cells were infected with vaccinia virus strain WR and were transfected with plasmid pTK53gpt-lacZ. After 48 h, the cells were harvested and lysed. The virus was applied to BSC-1 cell monolayers in the presence of mycophenolic acid, hypoxanthine, and xanthine, and plaques were detected by staining with neutral red (7). Ten plaques of normal size and shape were picked and then reassayed a second time under selective conditions. Three more plaque purifications were carried out under nonselective conditions. The TK and β-galactosidase (β-gal) phenotypes were determined by plaque assay in TK$^-$ cells in the presence of 5-bromodeoxyuridine and in BSC-1 cells with an X-gal overlay as described previously (4, 10).

occurs upon removal of selection, consistent with the known high frequency of intramolecular recombination in vaccinia virus-infected cells (1, 6, 11, 21). Depending on the position of the crossover with respect to the insertion or mutation, either the desired recombinant or the parental DNA segment is retained. The relative frequency of the two types of crossovers will depend on the amount of sequence homology on either side of the mutation.

The TDS procedure is similar in principle to the transplacement method, described by Scherer and Davis (18), for

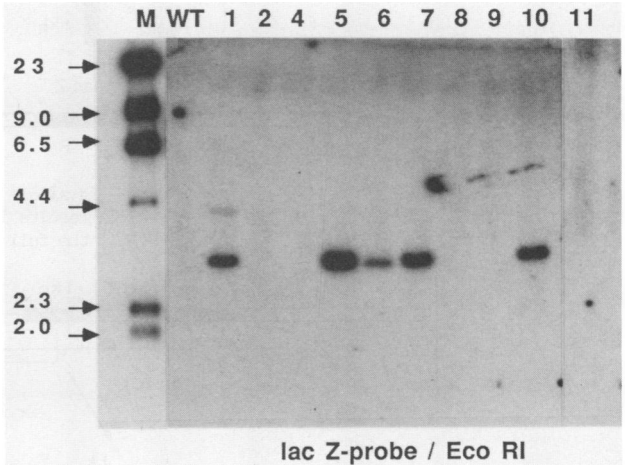

FIG. 2. Genomic analysis of 10 virus isolates. Total DNA was purified from CV-1 cells infected with virus isolates corresponding in number to those shown in Table 1 and with wild-type (WT) virus. The DNAs were cut with restriction endonuclease *Eco*RI, separated on a 1% agarose gel, and transferred to a nitrocellulose membrane. The immobilized DNAs were hybridized with a ^{32}P-labeled *lacZ* probe (plasmid pMC1871 from Pharmacia). The markers (M) consisted of a *Hin*dIII digest of phage lambda DNA that was analyzed on the same gel and hybridized to ^{32}P-labeled lambda DNA. The sizes of the marker DNAs in kilobases are indicated to the left of the autoradiograph.

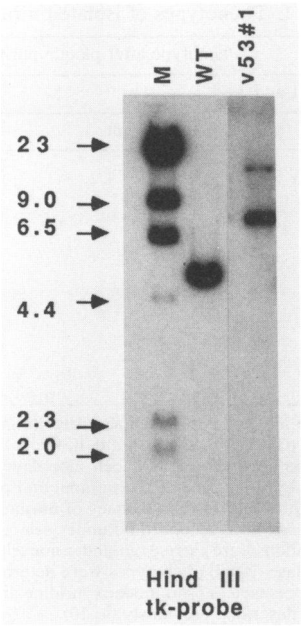

FIG. 3. Genomic analysis of DNA from purified recombinant virus v53#1. DNAs from purified wild-type virus (WT) and from recombinant v53#1 were digested with restriction endonuclease *Hin*dIII, analyzed by agarose gel electrophoresis, transferred to a nitrocellulose membrane, and hybridized to a ^{32}P-labeled vaccinia virus *tk* probe. Markers (M) are the same as those described in the legend to Fig. 2.

the replacement of *S. cerevisiae* chromosomal segments with altered DNA sequences. In that study, the yeast transformant was grown permissively for 10 generations and then only 7 out of 900 colonies lost the selectable marker; of these, 3 were the desired recombinants. Because of the relatively low rate of intramolecular recombination in *S. cerevisiae* cells, specific counterselection methods are advantageous (17, 22). In this study, we found that 10 out of 10 vaccinia virus plaques lost the selectable marker after only 3 cycles of plaque picking; of these, half were the desired recombinants, making counterselection methods less important. Screening of the relatively small number of plaques is easily accomplished by either DNA hybridization or polymerase chain reaction. The latter reaction is very convenient and can be used with only a small portion of the DNA recovered from a single plaque (Y. Zhang, personal communication).

TDS has some advantages over previous methods of isolating recombinant vaccinia viruses. For example, the absence of the selection marker from the final recombinant virus may be desirable if the latter is to be used for vaccine purposes or when gene disruptions are made in order to determine the effects on the pathogenicity of a virus. The lack of mutagenicity of the selective agent, mycophenolic acid, is also of advantage for the above uses. Moreover, the automatic loss of the selection marker gene greatly simplifies the task of inserting multiple genes in succession, since the same selection procedure can be reused each time. In addition, TDS provides a general procedure for introducing site-directed mutations into the vaccinia virus genome, since selection does not depend on the resulting phenotype. The high percentage (up to 50%) of recombinant plaques obtained by the TDS procedure might also facilitate the screen-

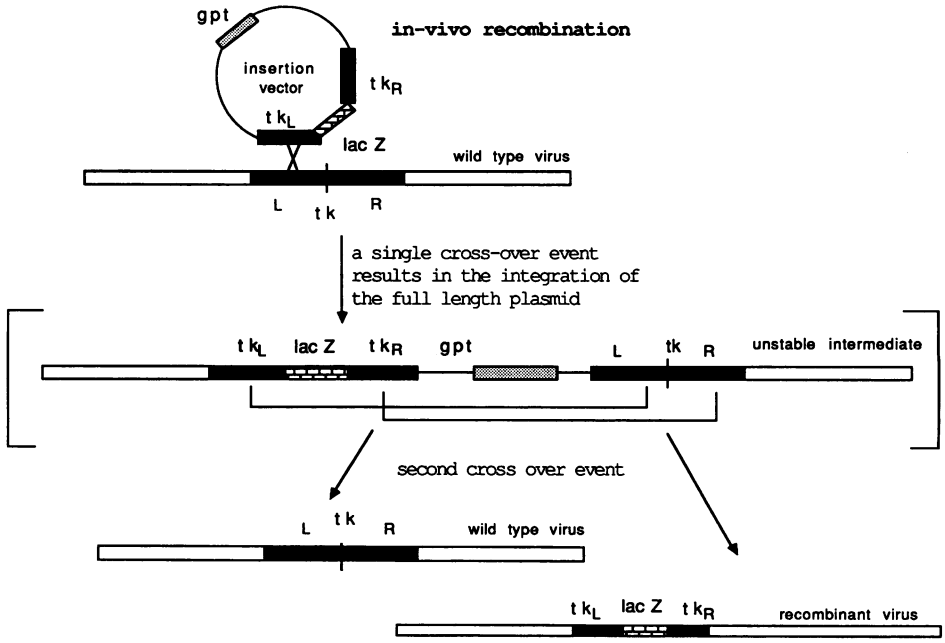

FIG. 4. Schematic outline of homologous recombination events. The circular plasmid contains the vaccinia virus *tk* gene and flanking sequences (■), interrupted by the *E. coli lacZ* gene (▤), into left (tk$_L$) and right (tk$_R$) halves, and by the *E. coli gpt* gene (▥). When the plasmid is transfected into a cell that has been infected with vaccinia virus, recombination occurs. The first step is a single crossover event that results in the integration of the full-length plasmid into the genome of vaccinia virus. Because of the presence of direct repeats, a second crossover event occurs with the formation of either wild-type virus or recombinant virus containing *lacZ*. All three types of genomes can be packaged separately into particles and are infectious, but only virus containing the *gpt* gene can form plaques under selective conditions.

ing of conditionally lethal (e.g., temperature-sensitive) mutants derived by in vitro chemical mutagenesis, a procedure that has not been reported to date.

TDS may be useful for other virus systems in addition to vaccinia virus. Two requirements are high rates of recombination and a relatively large DNA capacity, such that genomes incorporating the entire plasmid may be packaged.

We thank J. Eibl and F. Dorner for supporting the completion of this work and S. Kandels for expert technical assistance.

ADDENDUM

After the manuscript was submitted, Spehner et al. (20) reported another way of achieving transient expression of a marker gene, in their case, β-galactosidase, which depends on flanking the marker with direct repeats.

LITERATURE CITED

1. **Ball, L. A.** 1987. High frequency recombination in vaccinia virus DNA. J. Virol. **61**:1788–1795.
2. **Bertholet, C., R. Drillien, and R. Wittek.** 1985. One hundred base pairs of 5′ flanking sequence of a vaccinia virus late gene are sufficient to temporally regulate late transcription. Proc. Natl. Acad. Sci. USA **82**:2096–2100.
3. **Boyle, D. B., and B. E. H. Coupar.** 1988. A dominant selectable marker for the construction of recombinant poxviruses. Gene **65**:123–128.
4. **Chakrabarti, S., K. Brechling, and B. Moss.** 1985. Vaccinia virus expression vector: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. **5**:3403–3409.
5. **Cochran, M. A., C. Puckett, and B. Moss.** 1985. In vitro mutagenesis of the promoter region for a vaccinia virus gene: evidence for tandem early and late regulatory signals. J. Virol. **54**:30–37.
6. **Evans, D. H., D. Stuart, and G. McFadden.** 1988. High levels of genetic recombination among cotransfected plasmid DNAs in poxvirus-infected mammalian cells. J. Virol. **62**:367–375.
7. **Falkner, F. G., and B. Moss.** 1988. *Escherichia coli gpt* gene provides dominant selection for vaccinia virus open reading frame expression vectors. J. Virol. **62**:1849–1854.
8. **Franke, C. A., C. M. Rice, J. H. Strauss, and D. E. Hruby.** 1985. Neomycin resistance as a dominant selectable marker for selection and isolation of vaccinia virus recombinants. Mol. Cell. Biol. **5**:1918–1924.
9. **Mackett, M., G. L. Smith, and B. Moss.** 1982. Vaccinia virus: a selectable eukaryotic cloning and expression vector. Proc. Natl. Acad. Sci. USA **79**:7415–7419.
10. **Mackett, M., G. L. Smith, and B. Moss.** 1984. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. J. Virol. **49**:857–864.
11. **Merchlinsky, M.** 1989. Intramolecular homologous recombination in cells infected with temperature-sensitive mutants of vaccinia virus. J. Virol. **63**:2030–2035.
12. **Mulligan, R., and P. Berg.** 1981. Selection for animal cells that express the E. coli gene coding for xanthine-guanine phosphoribosyl transferase. Proc. Natl. Acad. Sci. USA **78**:2072–2076.
13. **Panicali, D., A. Grzelecki, and C. Huang.** 1986. Vaccinia virus vectors utilizing the β-galactosidase assay for rapid selection of recombinant viruses and measurement of gene expression. Gene **47**:193–199.
14. **Panicali, D., and E. Paoletti.** 1982. Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc. Natl. Acad. Sci. USA **79**:4927–4931.
15. **Perkus, M. E., K. Limbach, and E. Paoletti.** 1989. Cloning and expression of foreign genes in vaccinia virus, using a host range selection system. J. Virol. **63**:3829–3836.
16. **Rodriguez, J. F., and M. Esteban.** 1989. Plaque size recombinants as a selectable marker to generate vaccinia virus recombinants. J. Virol. **63**:997–1001.
17. **Rykowski, M. C., J. W. Wallis, J. Choe, and M. Grunstein.** 1981. Histone H2B subtypes are dispensable during the yeast cell cycle. Cell **25**:477–487.
18. **Scherer, S., and R. W. Davis.** 1979. Replacement of chromosome segments with altered DNA sequences constructed in vitro. Proc. Natl. Acad. Sci. USA **76**:4951–4955.
19. **Shida, H.** 1986. Nucleotide sequence of the vaccinia virus hemagglutinin gene. Virology **150**:451–462.
20. **Spehner, D., R. Drillien, and J.-P. Lecocq.** 1990. Construction of fowlpox virus vectors with intergenic insertions: expression of the β-galactosidase gene and the measles virus fusion gene. J. Virol. **64**:527–533.
21. **Spyropoulos, D. D., B. E. Roberts, D. L. Panicali, and L. K. Cohen.** 1988. Delineation of the viral products of recombination in vaccinia virus-infected cells. J. Virol. **62**:1046–1054.
22. **St. John, T. P., S. Scherer, M. W. McDonnell, and R. W. Davis.** 1981. Deletion analysis of the Saccharomyces GAL gene cluster. Transcription from three promoters. J. Mol. Biol. **152**: 317–334.
23. **Weir, J. P., G. Bajszar, and B. Moss.** 1982. Mapping of the vaccinia virus thymidine kinase gene by marker rescue and by cell-free translation of selected mRNA. Proc. Natl. Acad. Sci. USA **79**:1210–1214.