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Dominant negative selection of vaccinia virus using a thymidine kinase/thymidylate kinase fusion gene and the prodrug azidothymidine

Georg W. Holzer, Josef Mayrhofer, Werner Gritschenberger, Falko G. Falkner*

Baxter BioScience/Vaccines, Biomedical Research Center, Uferstrasse 15, A-2304 Orth/Donau, Austria

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Abstract

The *Escherichia coli* thymidine kinase/thymidylate kinase (tk/tmk) fusion gene encodes an enzyme that efficiently converts the prodrug 3′-azido-2′,3′-dideoxythymidine (AZT) into its toxic triphosphate derivative, a substance which stops DNA chain elongation. Integration of this marker gene into vaccinia virus that normally is not inhibited by AZT allowed the establishment of a powerful selection procedure for recombinant viruses. In contrast to the conventional vaccinia thymidine kinase (tk) selection that is performed in tk-negative cell lines, AZT selection can be performed in normal (tk-positive) cell lines. The technique is especially useful for the generation of replication-deficient vaccinia viruses and may also be used for gene knock-out studies of essential vaccinia genes.
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Keywords: Dominant selection; Vaccinia virus; Thymidine kinase; Thymidylate kinase; Viral vectors

Introduction

Vaccinia virus is a useful tool in biomedical research and recombinant viruses are normally generated by homologous recombination using a broad number of selection markers (Moss, 1996). At present, the most frequently used procedures are tk-negative selection by insertional inactivation of the endogenous vaccinia tk-gene (Mackett et al., 1982) color screening using the *Escherichia coli* β-galactosidase or β-glucuronidase genes (Carroll and Moss, 1995; Chakrabarti et al., 1985) and dominant positive selection using the *E. coli* hypoxanthine-guanine phosphoribosyltransferase (gpt) marker gene (Boyle and Coupar, 1988; Falkner and Moss, 1988). Finally, rescue techniques of functionally defective viruses are fast ways to obtain replicating recombinants (Blasco and Moss, 1995; Holzer et al., 1998). Defective vaccinia viruses growing only in engineered complementing cell lines are particularly difficult to select and, in order to obtain marker-free viruses, are usually purified by transient selection procedures requiring 6–8 rounds of plaque purification (Holzer and Falkner, 1997). Therefore, in order to more stringently select for recombinant defective viruses, a dominant negative selection procedure is desirable. Moreover, a clear identification of essential genes still depends on conditionally lethal mutations (Condit et al., 1983; Ensinger, 1982) that can be mapped to the respective locus. Therefore, the dominant selection fusion marker gene tk/tmk, that is independent of tk-negative cell lines, was studied to overcome these restrictions.

Results

The tk/tmk fusion gene inhibits growth of vaccinia in the presence of AZT

Wild-type vaccinia virus replicating in a host cell cannot be inhibited by AZT (Shneider et al., 1987), implying that the endogenous vaccinia tk-gene and also the host cell enzymes do not use the prodrug efficiently. In contrast, the *E. coli* tk/tmk fusion gene efficiently converts AZT to AZT-

* Corresponding author. Fax: +43 1 20100 4000.
E-mail address: falknef@baxter.com (F.G. Falkner).

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TP suggesting that it might work as a selection system for vaccinia recombinants in wild-type cells. In order to test this assumption a recombinant virus was constructed containing the tk/tmk 'suicide' gene under control of the vaccinia P7.5 early/late promoter. The insertion plasmid ptk-D4-suci, used in the recombination experiment, directs the P7.5 tk/tmk gene cassette into the vaccinia tk-gene inactivating it. The resulting virus, obtained by rescue of a defective virus with the D4R marker was termed vtk-suci2 (Fig. 1B) and is based on the WR laboratory strain. This prototype virus was tested together with the wild-type control virus in inhibition experiments with the prodrug AZT in the monkey kidney cell line CV-1. Without AZT, both viruses grew normally (Figs. 2A, C). In the presence of the prodrug, however, the tk/tmk expressing virus vtk-suci2 was strongly inhibited (Fig. 2D), while the wild-type virus formed large plaques (Fig. 2B) demonstrating proof-of-principle of vaccinia AZT selection.

Determination of the minimal inhibitory dose of AZT

The prototype recombinant virus vtk-suci2 has the tk/tmk fusion gene integrated into the vaccinia tk-locus. To prove that the selection procedure does not interfere with vaccinia tk activity and that also strains other than WR can be used, an AZT-sensitive virus based on the Lister strain was constructed, having the marker integrated in an intergenic region (the region located between the D4R and the D5R open reading frames) leaving the endogenous vaccinia tk gene intact. The plasmid pDD4.4-suzi2, in which the tk/tmk gene is located in the intergenic region downstream of D4R, was used to construct this novel drug-sensitive vaccinia recombinant. The virus was termed vsuci2-L (Fig. 1C).

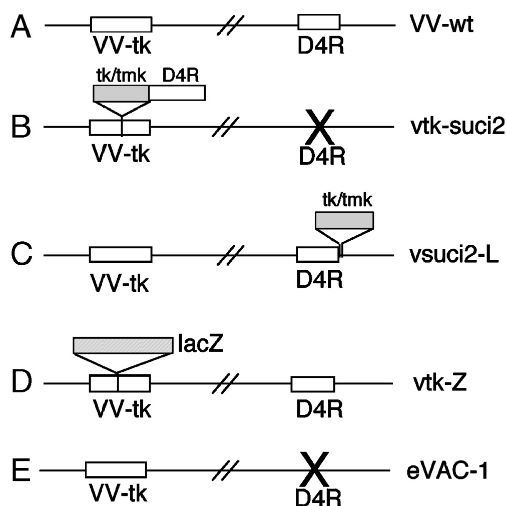

Fig. 1. Schematic representation of the viruses. The wild-type virus (VV-wt) is shown in (A). The virus vtk-suci2 has the tk/tmk fusion gene integrated in the vaccinia tk-locus (B). In the virus vsuci2-L (C), the suicide gene is inserted in the intergenic region of ORFs D4R and D5R. The virus vtk-Z has the lacZ gene integrated into the vaccinia tk-locus (D). The virus eVAC-1 is a defective having the D4R gene deleted (E).

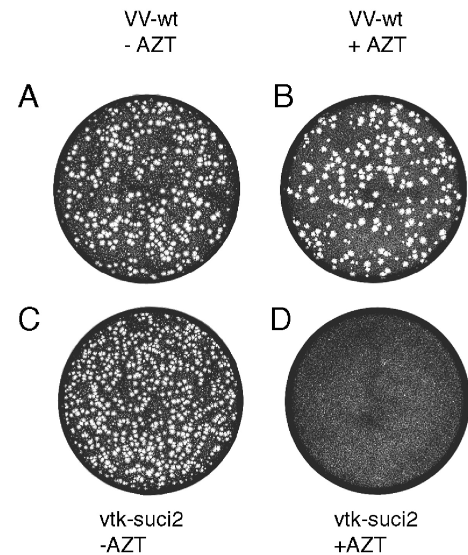

Fig. 2. Inhibition of the virus vtk-suci2 and of wild-type virus (VV-wt) by AZT. In the absence of the drug (A, C) both viruses grow normally. In the presence of AZT, wild-type vaccinia is not inhibited (B), while tk/tmk-positive virus is totally suppressed.

We next performed more detailed studies on the minimal inhibiting prodrug dose using the two tk/tmk-positive viruses vtk-suci2 and vsuci2-L and three control viruses, vtk-Z, eVAC-1 and wild-type vaccinia (for genetic structures see Fig. 1; for genotypes see Table 1). The four viruses were titered at different concentrations of AZT in CV-1 cells and also in the rabbit kidney cell line RK44.20 used for complementation of defective viruses (Table 1). Six well plates of cells were infected with 50 pfu/well of the indicated viruses and were then incubated at increasing AZT concentrations. Plaques were visualized 3 days after infection by crystal violet staining. The tk/tmk expressing viruses could be completely inhibited at AZT concentrations >0.25 mg/ml in CV-1 cells. Viruses without the tk/tmk gene formed clearly visible plaques up to 1 mg/ml AZT. However, a gradual reduction in plaque size and number was observed, being detectable at 0.5 mg/ml of the prodrug. The AZT sensitivity of eVAC-1, a defective virus bearing no suicide gene, was also tested. Plaques were still detectable at 1 mg/ml of the drug although at moderately reduced size. The toxic effect on CV-1 and RK44.20 cells was observed at AZT concentrations >1.5 mg/ml. The vaccinia tk-gene had no inhibiting effects on virus growth in the presence of AZT. However, double tk-positive viruses (vsuci2-L) were slightly more sensitive to AZT inhibition (Table 1). As a conclusion, a concentration of 0.5 mg/ml AZT suppressed all types of suicide gene bearing viruses, without significant interference with recombinants that are lacking the marker. The only exception was the virus vtk-suci2 grown in RK44.20 cells, which needed 0.75 mg/ml for full inhibition. The dose of 0.75 mg/ml was therefore used in the following selection experiments.

Table 1
Inhibition of VV recombinants by AZT

Virus	Genotype		Vaccinia strain	Cell line	% growth at AZT concentration (mg/ml)				
	Suci	VVtk			0	0.25	0.5	0.75	1.0
vsuci2-L	+	+	Lister	CV1	100	–	–	–	–
VV Wt	–	+	Lister	CV1	100	100	100	100*	80*
vtk-suci2	+	–	WR	CV1	100	–**	–**	–	–
vtk-Z	–	–	WR	CV1	100	100	90	30*	–**
vsuci2-L	+	+	Lister	44.20	100	–**	–	–	–
VV Wt	–	+	Lister	44.20	100	100	100	90*	50*
vtk-suci2	+	–	WR	44.20	100	50	20*	–**	–
vtk-Z	–	–	WR	44.20	100	100	100*	100*	100*
evac-1	–	+	WR	44.20	100	100	100	100	100

–, total inhibition.
* Plaques 50% of normal size.
** Minute lesions detectable.

Construction of a defective recombinant virus using tk/tmk selection

Based on the inhibition studies, a new selection protocol using a pre-made tk/tmk positive virus, a recombination plasmid and AZT was outlined (Fig. 3). To construct a desired recombinant virus, the tk/tmk marker is substituted by the gene of interest (transgene, TG) by homologous recombination using a recombination plasmid having homologous flanking regions (in this example D3R and D5R sequences). Since in the initial selection round with AZT most recombinant viruses were killed by the by-stander effect (not shown), a positive first selection step preceding the negative selection is required (Fig. 3, positive selection). This step is performed using the gpt and, optionally, the β-galactosidase marker located between short repeats. The gpt- and β-galactosidase positive plaques are selected and purified for a second round. Then, the plaques are purified twice in the presence of AZT. Since located between repeats, the gpt/lacZ marker gene is lost as soon as the gpt selective agents are removed (Fig. 3, step 2). During AZT selection, the tk/tmk marker gene together with the adjacent vaccinia gene are deleted according to the architecture of the recombination plasmid. The so enforced loss of an essential viral function is provided by the appropriate complementing cell line. By this strategy, defective viruses are more rapidly obtained while parental virus or intermediate forms that still contain the tk/tmk gene are actively killed.

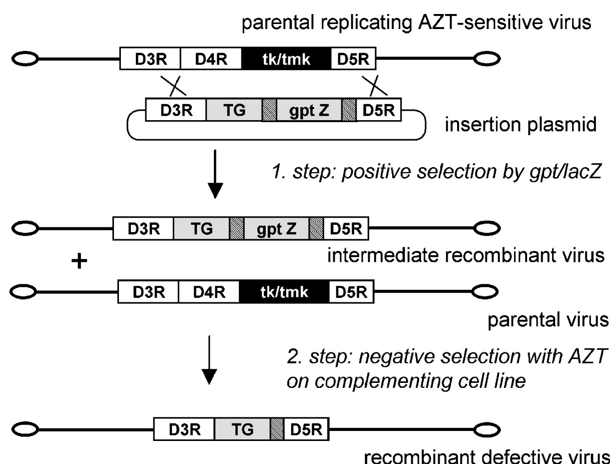

Fig. 3. Schematic representation of selecting defective viruses. The parental replicating AZT-sensitive virus has the marker gene inserted in an intergenic region (in this example between ORFs D4R and D5R). Homologous recombination with an insertion plasmid carrying the transgene (TG) and a gpt/lacZ (gptZ) marker flanked by sequences of the D3R and the D5R ORF results, using the positive and negative selection steps, in the rapid generation of defective viruses having the essential gene D4R deleted. If the marker gene is flanked by direct repeats, the finally obtained virus is marker-free.

To prove this protocol, the empirically difficult selection of a defective virus (with transgene integration between the D3R and D5R genes deleting D4R) based on the more attenuated vaccinia strain Lister was attempted. For this purpose, the virus vsuci2-L that carries the suicide gene in the D4/D5 intergenic region, and the plasmid was pDW-mH5-hTrp2 (in which the transgene and the gpt/lacZ marker are located between ORFs D3R and D5R) were delivered into the complementing host cells. The transgene in the plasmid is an expression cassette for the melanocyte differentiation antigen TRP-2 (human tyrosinase-related protein 2) (Bouchard et al., 1994) the adjacent gpt/lacZ marker cassette is flanked by repeats to allow for transient selection. Infection and transfection steps were performed in the complementing cell line RK44.20. As outlined above, the recombinant viruses were identified by two rounds of gpt selection including lacZ staining in RK44.20 cells. To evaluate the efficiency of the negative selection procedure, the plaque purifications were done in parallel with 0.75 mg/ml AZT and without AZT selection. In the first AZT selection steps, a markedly reduced plaque count was observed, compared to total gpt-positive plaques. This may reflect the inhibition of mixed plaques that cannot be excluded by the positive selection procedure. After a total of four rounds of plaque purification, only one wild-type free isolate out of 12 examined was obtained by the positive gpt selection protocol alone, whereas all 11 primary isolates

could be successfully purified by AZT selection (Table 2). Since many rounds of plaque purification are required to obtain a sufficient number of isolates of pure defective virus (using the Lister strain as parental virus) without negative selection pressure, dominant negative selection is a powerful tool to counterselect against replicating parental virus. This counterselection actively kills residual tk/tmk positive virus still present in recombinant virus stocks. Using positive selection alone obviously requires more purification steps.

The replacement of the suicide marker by the correctly inserted gene of interest was demonstrated for the isolates purified under AZT by Southern blotting using a tk/tmk and a TRP-2 gene probe (Fig. 4). Ten of 11 virus isolates grown in the complementing cell line RK44.20 had the expected structure (lanes 2, 3, 5–12). One isolate did not contain the foreign gene and also not the suicide gene (lane 4). Since this isolate did not grow in CV-1 cells, it is an illegitimate defective recombinant virus and not a contaminating parental virus. As expected, the Lister wild-type virus did not react with both gene probes (lane 14), whereas the parental virus vsuci2-L showed the correct 3.0 kb signal with the tk/tmk probe but not with the foreign gene (Trp2) probe (lane 13). The blot was negative for a gpt/lacZ probe (not shown). The results therefore confirm the structures of the recombinants and validate the selection procedure.

The components and the application of the system are summarized in Fig. 5. The plasmid used to construct the virus vsuci2-L, pDD4.4-suci2, may also serve as the source of the p7.5-tk/tmk gene cassette. The plasmids pDRMa (Ober et al., 2002) can be used to insert foreign gene cassettes into the multiple cloning site (MCS) and further to construct replicating recombinants together with the parental virus vsuci2-L (or similar vectors based on other vaccinia strains) by AZT selection. Plasmids such as pDW (Holzer et al., 1998) allow the construction of marker-free defective viruses by the new procedure.

Mutation rate of BudR versus AZT

High frequencies of tk-negative point mutants (20–90%, sometimes more) are a known to occur with classical vaccinia tk-negative selection in recombination experiments requiring the use of an additional positive selection or screening step. Usually, between 10 and 80% of tk-negative plaques are formed by recombinants, although the percentage may be significantly lower (Chakrabarti et al., 1985). A high frequency of reversions would also prevent the use of the AZT selection procedure as a tool to characterize

Table 2
Plaque purifications of isolates on CV-1 and RK44.20 cells

Method	Round 1	Round 2	Round 3	Round 4
gpt/color screening	0/12[a]	0/12	1/12	1/12
AZT-negative selection	0/11	2/11	11/11	11/11

[a] Ratio of pure isolates (growing only in RK44.20 cells)/total isolates.

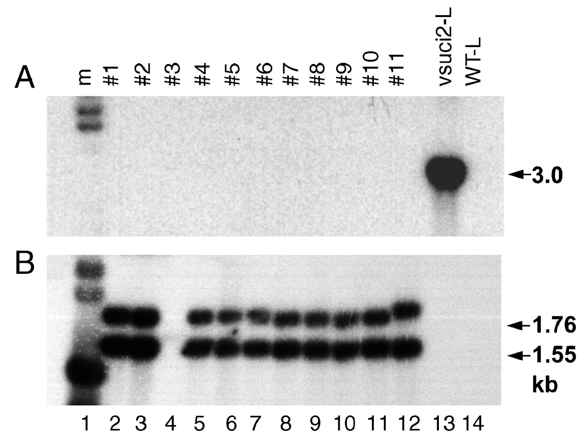

Fig. 4. Characterization of the recombinant viruses by Southern blotting. DNA fragments of viral isolates was separated by agarose gel electrophoresis and blotted according to standard techniques. (A) Fragments were hybridized to the tk/tmk probe (isolated as an *Nco*I–*Sal*I fragment from plasmid pDD4.4-suci2). The tk/tmk-positive parental virus vsuci2-L is the only virus showing a signal (lane 13); no tk/tmk gene is present after AZT selection. (B) Hybridization with the TRP2 gene probe (plasmid pPCR-hTrp2) shows, that all but one isolate had the transgene integrated resulting in the expected sized fragments.

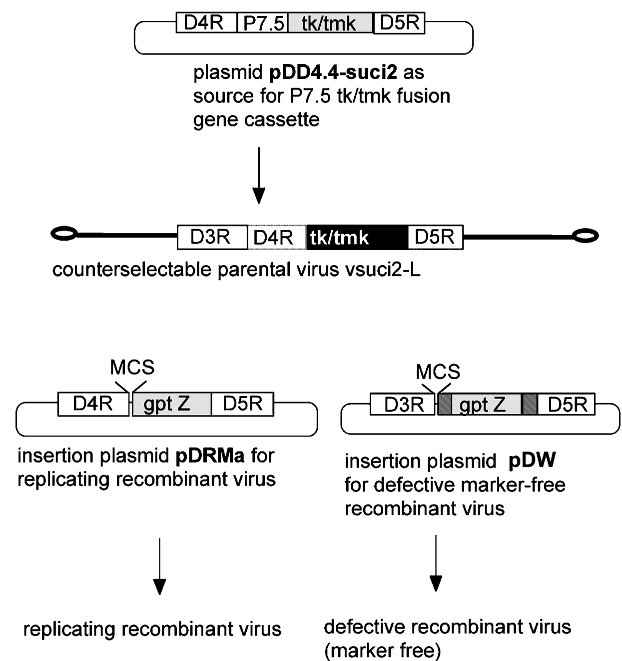

Fig. 5. Components of the AZT selection system. The plasmid pDD4.4-suci2 which contains the P7.5-tk/tmk gene cassette was used to construct the virus vsuci2-L, the parental virus for insertions into an intergenic region (D4/5 region) derived from the vaccinia Lister strain. The plasmid DRMa may serve to take up foreign gene cassettes (the multiple cloning site, MCS, has the unique restriction sites *Not*I, *Xho*I, *Hin*dIII) and is used to construct replicating recombinant viruses. The multiple cloning site of the plasmid pDW has the unique restriction sites *Sma*I, *Not*I, *Xho*I, *Hin*dIII for insertion of genes; the plasmid may be used to construct defective recombinants without marker genes. P7.5, vaccinia promoter driving the 7.5-kDa gene; tk/tmk, fusion gene consisting of the *E. coli* genes thymidine kinase and thymidylate kinase; D3R, D4R, D5R, vaccinia open reading frames; gpt, *E. coli* gene encoding hypoxanthine-guanine phosphoribosyltransferase; Z, *E. coli* β-galactosidase gene.

essential genes. We were therefore interested in the reversion rate of tk/tmk expressing viruses. In inhibition experiments, 10,000 pfu of drug-sensitive virus, plated under AZT onto two Roux bottles, did not result in visible escape mutants suggesting reversion rates $<1:10^4$. Spiking experiments with 100 pfu wild-type virus per flask done in parallel showed that tk/tmk-negative virus can be detected in the presence of drug-sensitive virus under the chosen experimental conditions. Using higher infectivities per cell of drug-sensitive virus did not permit the detection of spiked wild-type viruses.

In order to further address the question of the mutagenic potentials of BudR and AZT, five typical isolates of wild-type and of tk/tmk virus were isolated and then titered with and without selective pressure. First, both the wt and tk/tmk viruses were plated out on CV-1 cells without selection, five typical plaques of each parent were picked and grown to small lysates. The titers of the WR wt lysates (obtained from one 175 cm^2 Roux bottle) were in the range of $0.32–4.8 \times 10^8$ pfu/ml and the ones obtained from the vSuzi2-L virus in the range of $2–9 \times 10^7$ pfu/ml (Table 3), reflecting the better growth of WR wild-type compared the Lister recombinant virus. Each of these lysates were now grown in the presence of selection, the WR plaques under BudR in tk-negative 143B cells and the tk/tmk plaques under AZT in standard CV-1 cells.

In case of BudR selection, cells were infected at a MOI of 0.05 pfu/cell and the lysates were grown and selected in 175 cm^2 flasks to increase the detection limits. As shown in Table 3, the mutation rate of wild-type virus in 143B cells under BudR was in the expected range of 1: $1.6–7.7 \times 10^4$ pfu. Despite background cpe, the mutant plaques were clearly visible. Unfortunately, in case of AZT selection (0.75 mg/ml) in CV-1 cells (infected with a MOI of 0.05 pfu/cell) mutants could not be identified unambiguously. At the same MOI (0.05 pfu/cell) or even at a lower one (0.01 pfu/cell), background cpe and the previously observed bystander killing prevented good plaque formation of spiked wild-type virus and therefore did not allow clear identification of tk/tmk negative mutants. A higher AZT concentration of 1.25 mg/ml reduced background cpe but also prevented plaque formation of the wild-type spike virus. Therefore, a precise mutation rate could not be determined for the AZT selection procedure by the chosen procedures.

Table 3
Estimation of mutation rates of vaccinia clones in tk-negative 143B cells after BrdU selection and in CV-1 cells after AZT selection

Virus clone	Titer of lysate ($\times 10^8$ pfu/ml)	Number of mutants	Mutation rate ($\times 10^4$)[a]
WR#1	1.0	48	2.1
WR#2	4.8	31	3.2
WR#3	1.5	33	3.0
WR#4	2.1	62	1.6
WR#5	0.32	13	7.7
vSuzi2#1	0.66	–	–[b]
vSuzi2#2	0.90	–	–
vSuzi2#3	0.50	–	–
vSuzi2#4	0.22	–	–
vSuzi2#5	0.45	–	–

[a] 143B cells were infected at a MOI of 0.05 and CV-1 cells at MOIs‘ of 0.05 and 0.01.
[b] Mutation rates could not be determined due to background cpe and/or bystander killing.

Identification of essential genes by AZT selection

Vaccinia genomes encode approximately 200 genes, many of which are still uncharacterized. Gene knock-out experiments may further identify a gene as essential, however, attempts to delete or substitute an essential gene by recombination techniques usually fail because potent counterselection procedures are not available. In a model experiment, we therefore attempted to co-delete the known essential D4R open reading frame (Upton et al., 1993) together with the tk/tmk suicide gene. For this experiment, we performed the same recombination as described above using the plasmid pDW-mH5-hTrp2, however, using non-complementing CV-1 cells in all steps. Twelve plaques that were isolated under gpt selection/lacZ screening were purified a second time either by gpt/lacZ or by AZT selection. Clearly visible large plaques were obtained by gpt/lacZ selection, indicating a mixed phenotype with helper viruses. When gpt/lacZ-positive plaques from round 1 were further selected with AZT, only minor lesions with weak lacZ expression were visible (data not shown). The material from these lesions could not be propagated in a subsequent round of selection in CV-1 cells indicating that co-deletion of the essential gene together with a dominant negative marker was not possible. Co-deletion experiments combining the positive gpt/lacZ procedure followed by negative AZT-selection may therefore be used as a tool to discern essential from nonessential vaccinia genes.

Discussion

It is usually easy to obtain recombinant viruses with the Western Reserve strain because of the large plaques this strain induces in many host cell lines. Recombinants with more attenuated vaccine strains (such as Lister or MVA) are more difficult to construct and therefore need improved selection systems. With the AZT selection method, an efficient negative selection protocol could now be established even for vaccinia recombinants that are otherwise difficult to isolate and where classical vaccinia tk selection is not possible. The novel *E. coli* tk/tmk fusion gene provides stringent selection allowing isolation of recombinants starting with a tk/tmk-positive parental virus. The fact that a marker gene containing parental virus has to be initially constructed limits the usefulness of the method. However, for the generation of vaccinia Lister recombinants, the virus vsuci-L is now available as parental virus enabling negative counterselection with AZT together with the recombination plasmids pDRMa for the construction of

replicating viruses or the plasmid pDW for defective recombinant viruses. In addition, since the positive selection step is performed with the transient gpt/lacZ marker gene, the procedure results in a marker-free recombinant virus by a series of simple plaque purification steps. The procedure is particularly useful for the rapid construction of defective viruses reducing the numbers of plaque purifications substantially and should be considered as further selection principle when complex vaccinia recombinants with multiple inserts are constructed.

Further, in a model knock-out experiment with a known essential gene, the *E. coli* tk/tmk fusion gene served as a tool for the characterization of an essential viral open reading frame. Due to the empirically apparent low spontaneous reversion to the wild-type (AZT resistant) phenotype, the marker provides sufficient selection pressure to make a decisive statement on the role of the gene to be deleted. Thus, this fusion marker gene is the basis for a stringent negative selection procedure for vaccinia recombinants in a standard cell line and may also be a useful marker gene in other viral systems, such as herpes viruses.

Materials and methods

Viruses and cell lines

The African green monkey kidney cell line CV-1 (ATCC CCL-70), the vaccinia virus strains Western Reserve (WR; ATCC VR119) and Lister/Elstree (ATCC VR-862) were obtained from the American Type Culture Collection. The cell line RK44.20 and the defective vaccinia virus eVAC-1 are described elsewhere (Holzer and Falkner, 1997; Holzer et al., 1998).

Selective media

AZT was purchased from Invivogen Inc, CA. For dose finding studies, infections were done in DMEM (Gibco/BRL), 10% fetal calf serum, containing the indicated concentration of the selective agent. Since the inhibitory potential of AZT is cell line dependent and seems to vary from batch to batch it is recommended to determine the inhibitory dose with each new batch of AZT. For plaque purifications, DMEM/1% low gelling agarose overlays with the drug were prepared.

Construction of plasmids and viruses

ptk-D4-suci and vtk-suci2

The tk/tmk fusion gene was PCR-amplified from the plasmid pGT63-LacZ (Invivogen Inc, CA) using the primers o-mut-tdk-1 5′-GTC CTT GCC ACC ATG GCA CAG CTA TAT TTC-3′ and 5′-CGG GCC GCG TCG GAA GCT TAC GCG TCC AAC TCC-3′ and placed under control of the vaccinia promoter P7.5. This expression cassette was cloned between sequences of the vaccinia tk-locus for homologous recombination together with the vaccinia D4R gene as a rescue marker. Twenty micrograms of plasmid ptk-D4-suci were transfected into CV-1 cells infected with the defective virus eVAC-1 and recombinants were obtained by rescue with the D4R marker (Holzer et al., 1998). The replicating tk-negative virus vtk-Z has been described previously (Holzer et al., 1999).

pDD4.4-suzi2 and v-suci2-L

The tk/tmk fusion gene was excised from the plasmid pGT63-lacZ as a *Nco*I/*Hin*dIII fragment and inserted into *Nco*I/*Hin*dIII digested plasmid pDD4-P7.5, a derivative of pER (Holzer et al., 1998), which contains the P7.5 promoter followed by a multiple cloning site in the intergenic region between the vaccinia D4R and D5R genes. It was used for the construction of the virus v-suci2-L, pDD4.4-suzi2 was transfected into CV1 cells previously infected with the defective parental virus dVV-L according to standard procedures (the defective virus dVV-L is based on the Lister strain and the D4R gene is deleted (Ober et al., 2002). Plaques were purified in three passages in CV1 cells. During these plaque purifications, the recombinant v-suci2-L isolates are selected by their ability to grow normally, while the parental defective virus cannot propagate outside the complementing RK44 cell line. After three rounds of plaque purification, the isolates were grown to large scale, curde stocks were prepared. Crude stocks were titered in the absence of AZT and tested for inhibition in the presence of AZT (1.25 mg/ml) to verify the purity of the isolate.

pDW-mH5-hTrp2

The human tyrosinase-related protein 2 (TRP-2) coding sequence was PCR amplified from a human melanoma cDNA library (Clontech cat. #7440-1) with the oligonucleotides 5′-ATC ATG AGC CCC CTT TGG TGG-3′ and 5′-CAA TTG TTA ACC CTA GGC TTC TTC TGT GTA TC-3′ as primers. The Trp2 cDNA was placed under control of the modified VV H5 promoter mH5 (Wyatt et al., 1996) and inserted into the multiple cloning site of the plasmid pDW-2, a derivative of pDW with more cloning sites (Holzer et al., 1998).

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