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Evaluation of the thymidine kinase (tk) locus as an insertion site in the highly attenuated vaccinia MVA strain

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Summary. The highly attenuated 'modified vaccinia Ankara' (MVA) strain is a potential live vaccine vector. Insertional inactivation of the tk-gene resulted in viruses difficult to purify. Co-integration of a functional fowlpox virus tk-gene allowed easy generation of recombinants, indicating that the genetically stable tk-gene region is a suitable insertion site, if tk-gene activity is substituted.

Introduction

Recombinant vaccinia viruses are currently widely used as vectors for gene expression and their potential as live vaccines has been evaluated [1]. Since the previously used vaccine strains and, in particular, laboratory strains remain virulent, recombinants derived therefrom would be hazardous for use in humans and in animals. Vaccinia vaccine strains, though attenuated, can cause infections with severe outcome in immunocompromized hosts [2]. Vaccines based on vaccinia strains are, however, desirable because of the unique properties of vaccinia based live vaccines [1]. Highly attenuated vaccinia strains have been developed, such as the MVA strain [3], the CV-1 strain [4] and the LC16m0 strain [5]. In addition to the classically attenuated strains, genetically engineered strains have been described [6]. The vaccinia MVA strain was passaged over 570 times in chicken embryo fibroblasts [7] resulting in six major deletions, including 31 kb of genomic material [8, 9]. The virus is therefore severely restricted in its host range and cannot grow in human and most mammalian cells. The MVA strain was used in large vaccine trials and clinical experience for primary vaccination of over 120 000 humans exists. No side effects were associated with its use, even when high risk patients received primary vaccination [10]. Recently, viral recombinants have been described in which the site of a deletion has been used for the insertion of a foreign gene into the MVA genome [11, 12].

We have now investigated the use of a classical insertion site, the thymidine kinase gene locus and report the conditions under which this locus can be used to insert foreign genes.

Materials and methods

Virus and cells

The vaccinia virus strain MVA was kindly provided by Prof. A. Mayr, Munich, Germany. Primary chicken embryo fibroblasts (CEF) were prepared as described in [13]. The cells were grown in tissue culture medium 199 (TCM 199; Gibco-BRL) supplemented with 5% fetal calf serum, glutamine and antibiotics.

Cell infections and plaque assays

Plaque assays were performed on confluent CEF monolayers cells in 60 cm^2 tissue culture dishes or in 6 well plates. The virus suspension was allowed to adsorb to the cells in a volume of 2.5 ml (dishes) or 0.6 ml (wells) TCM 199 with occassional rocking for 1 h. The suspension was removed by aspiration and replaced by an overlay consisting of serum free Dulbecco's MEM (DMEM, Gibco-BRL), antibiotics and 1% low melting agarose (LMA; Gibco-BRL). Plaques were stained with 30 µg/ml neutral red (Sigma) on the fifth or sixth day of infection.

In vivo recombination

CEF cells were grown in 60 cm^2 tissue culture dishes and infected with 0.5 plaque forming units (pfu) per cell of vaccinia MVA. The virus was adsorbed for 1 h at 37 °C in 2.5 ml TCM 199. Subsequently, the medium was removed and the infected monolayers were overlayed with a DNA-Ca-phosphate precipitate, consisting of 20 µg of plasmid DNA in Hepes buffered saline in a final volume of 1 ml as described earlier [14]. After a 30 min incubation period at room temperature, 9 ml of TCM 199 were added and the incubation was continued for another 4 h at 37 °C. The medium was replaced with 10 ml of fresh TCM 199 and the plates were incubated for two days. The cells were then scraped into the medium, centrifuged and the pellets were lysed by three successive cycles of freezing and thawing. Progeny virus was then assayed for the presence of recombinants.

Selection and plaque purification of the recombinants

Virus with lacZ gene inserts were identified by blue plaque screening as described in [15] with the following modifications: confluent CEF cells were infected with viral crude stocks derived from recombination experiments and overlayed with serum free DMEM containing 1% LMA. After 5–6 days, the monolayers were stained with a second overlay consisting of 1% LMA in phosphate buffered saline (PBS) and 600 µg/ml of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). Blue plaques could be detected after 6–12 h. Recombinant viruses with gpt-gene inserts were identified on the basis of their resistence to the drug mycophenolic acid (MPA) essentially as described in [14] with the following modifications: monolayers of CEF cells were infected with recombinant virus and overlayed with DMEM supplemented with 125 µg/ml xanthine, 5–25 µg/ml MPA and 1% LMA. After 5–6 days the plaques were visualized by staining with a second overlay consisting of 1% LMA in PBS containing 30 µg/ml neutral red. In the case of gpt- and lacZ positive recombinants, the overlay also contained 600 µg/ml of X-Gal. The plaques were subjected to several rounds of plaque purification.

Construction of plasmids

The plasmid pTZgpt-TK + lacZa was prepared by cleaving the *Sma*I-*Stu*I fragment containing the *lacZ* gene from the plasmid pFP-Zsart and inserting the fragment into pTZgpt-TK + 1 [13].

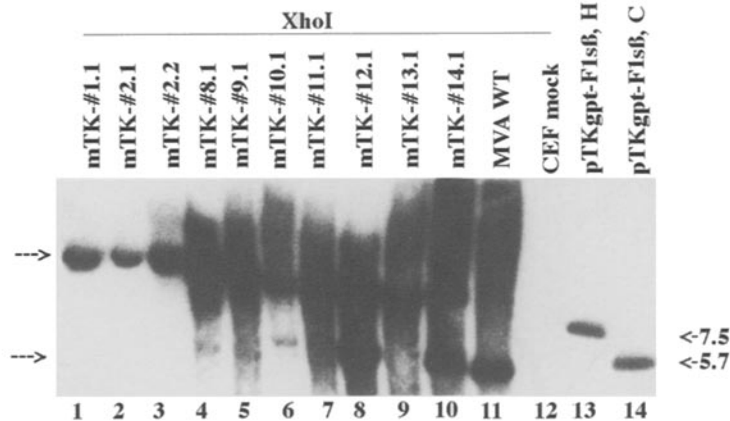

Fig. 1. Southern blot analysis of tk-negative MVA vaccinia recombinants. The genomic XhoI fragments were separated by polyacrylamide gel electrophoresis, blotted and hybridized to a tk-gene probe. *1–10* MVA mTK-clones; *11* MVA wild-type virus; *12* mock infected chicken embryo fibroblasts; *13, 14* pTKgpt-F1sβ digested with HindIII (*H*) and ClaI (*C*) as size markers. The upper arrow (left) indicates the recombinant genomic fragment; the lower arrow points to the wild-type tk-gene

Results

The tk-locus is a well characterized insertion site that allows genetically stable integration of foreign genes in many vaccinia strains. The vaccinia tk-gene is not essential for growth of the virus in cell culture [16]. Viruses with inactivated tk-genes do, however, show decreased virulence and are highly attenuated in a mouse challenge model [17]. To investigate the behavior of tk-negative MVA derived viruses, the tk-gene was inactivated by gene insertions. The plasmid pTKgpt-F1sβ [14] was inserted into the tk-locus of the MVA strain by in-vivo recombination techniques in chicken embryo fibroblasts (CEF). The plasmid pTKgpt-F1sβ contains the *E. coli* gpt and the lacZ gene between tk-gene flanking regions. Recombinant viruses were identified by gpt-selection [14] and screening for lacZ expression [15]. Eighteen blue gpt-positive plaques were purified four times, and small virus stocks were grown and further characterized. Only ten plaques survived and could be fully purified. For further characterization, CEFs were infected with the small virus stocks, and after 72h total DNA was prepared, digested with XhoI and further analyzed by Southern blotting (Fig. 1). The immobilized fragments were hybridized to a vaccinia tk-gene probe. As a size marker, the plasmid pTKgpt-Flsβ was digested with HindIII (Fig. 1, lane 13) or with ClaI (Fig. 1, lane 14). The expected fragment of about 10.5 kb containing the foreign gene and the flanking tk-gene was detected (upper arrow). Most of the tk-negative MVA viruses, however, still contained the 5.3 kb wild-type tk-gene fragment (Fig. 1, lower arrow) indicating contamination by wild-type virus. Longer exposures of the blot showed that only isolates 11.1 and 13.1 were free of wild-type virus. Unexpectedly, the tk-negative MVA was difficult to handle, beginning with identification of plaques and plaque purification. Thus tk-

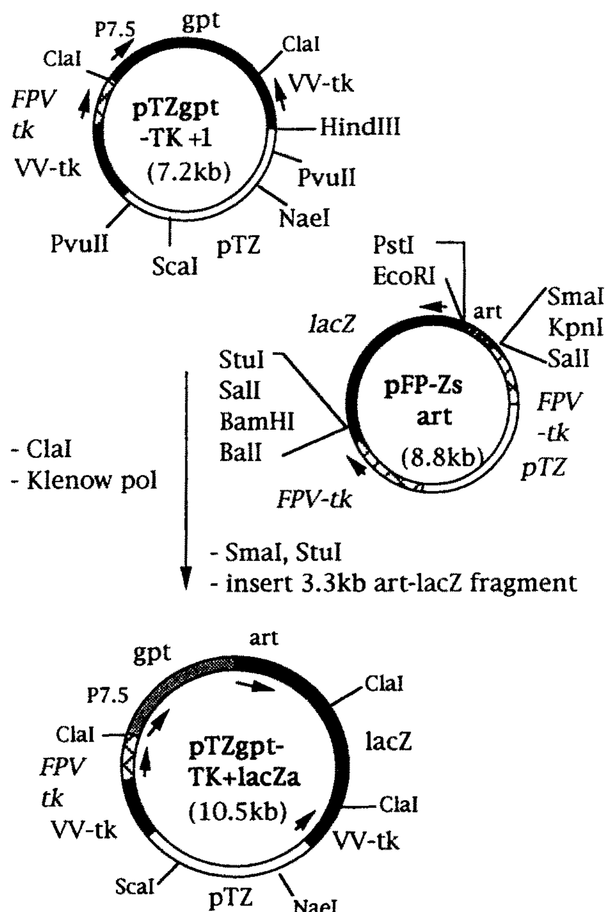

Fig. 2. Schematic representation of the plasmid constructs. *FPV-tk* Fowlpox virus thymidine kinase sequences; *VV*-tk vaccinia tk-sequences; *gpt E. coli* xanthine guanine phosphoribosyltransferase gene; *lacZ E. coli* β-galactosidase gene; *art* artificial promoter; arrows indicate the orientation of transcription

negative MVA probably needs a wild-type helper virus for efficient growth. Since tk-negative vaccinia, in general, is highly attenuated in animals [17], tk-negative MVA must be further attenuated, which is not desirable in the light of the already high degree of attenuation of MVA.

To overcome the drawbacks observed after using insertional inactivation of the tk-locus, the fowlpox virus (FPV) tk-gene was co-integrated into the vaccinia MVA tk-locus. Although the genes are related, they show little conservation of sequence on the nucleotide level [18]. To generate the FPV tk-positive viruses, the plasmid pTZgpt-TK + lacZa was constructed (Fig. 2) which contains three foreign genes located between vaccinia tk-gene flanking regions, an intact FPV tk-gene and the E. coli genes gpt and lacZ. The latter two allow for gpt-selection and lacZ screening. pTZgpt-TK + lacZa was inserted into the tk-locus of the MVA strain by marker rescue. From ten initially isolated plaques, nine survived four rounds of purification without difficulties. Viruses were characterized by Southern analyses using a vaccinia tk-gene probe (Fig. 3). As a size marker, the plasmid pTKgpt-F1sβ was digested with HindIII (lane 12) and with ClaI (lane 13). The viruses showed the expected restriction and hybridization patterns of

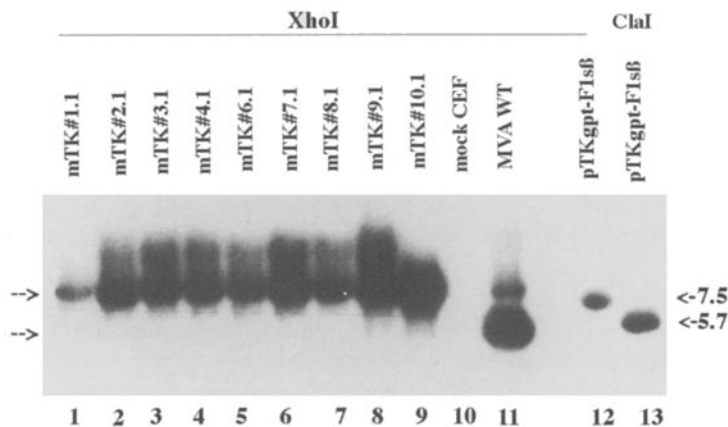

Fig. 3. Southern blot analysis of tk-positive MVA vaccinia recombinants. The XhoI fragments were separated by polyacrylamide gel electrophoresis, blotted and hybridized to a tk-gene probe. *1–9* MVA isolates mTK#1.1, mTK#2.1, mTK#3.1, mTK#4.1, mTK#6.1, mTK#7.1, mTK#8.1, mTK#9.1 and mTK#10.1; *10* mock infected chicken embryo fibroblasts; *11* MVA wild-type virus; *12, 13* pTKgpt-F1sβ digested with XhoI and ClaI as size markers. The upper arrow (left side) indicates the recombinant genomic DNA fragment; the lower arrow points to the wild-type tk-gene

one fragment of about 8 kb. The wild-type XhoI fragment containing the tk-gene (Fig. 3, lane 11) was not visible even after long exposure times in the lanes of the mtk-viruses (lower arrow), indicating that the isolates were free of wild-type virus after four rounds of plaque purification. This was in contrast to the experience made with the tk-negative MVA viruses.

Discussion

The tk-locus is a desirable insertion site also for attenuated vaccinia strains because it is located in a genetically stable region of the genome. Further attenuation of an already poorly replicating virus by inactivation of the tk-gene, however, should be avoided in order not to alter the growth properties of an evaluated vaccine strain. To solve this problem, several approaches to circumvent insertional inactivation seem practicable. One approach would be the use of intergenic regions [19] around the MVA tk-gene as insertion sites. We have explored an alternative approach, the substitution of the inactivated endogenous tk-gene by the fowlpox virus tk-gene. Control experiments indicated that tk-negative MVA virus was difficult to purify while the tk-positive MVA recombinants could be handled like, and grew to titers similar to, the wild-type virus. Cointegration of the fowlpox virus tk-gene, therefore, provides a strategy for using the MVA tk-locus as an integration site for foreign genes minimizing further attenuation. In addition, co-insertion of the FPV tk-gene into already existing vaccinia vectors with tk-gene flanking regions will allow the further use of these vector constructs.

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