Non-replicating vaccinia vector efficiently expresses bacteriophage T7 RNA polymerase

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Abstract **Modified vaccinia virus Ankara (MVA), a host range restricted and highly attenuated vaccinia virus strain, is unable to multiply in human and most other mammalian cell lines. Since viral gene expression is unimpaired in non-permissive cells recombinant MVA viruses are efficient as well as exceptionally safe expression vectors. We constructed a recombinant MVA that expresses the bacteriophage T7 RNA polymerase and tested its usefulness for transient expression of recombinant genes under the control of a T7 promoter. Using the chloramphenicol acetyltransferase (CAT) gene as a reporter gene, infection with MVA-T7pol allowed efficient synthesis of recombinant enzyme in mammalian cells. Despite the severe host restriction of MVA, enzyme activities induced by infection with MVA-T7pol were similar to those determined after infection with a replication-competent vaccinia-T7pol recombinant virus. Thus, MVA-T7pol may be used as a novel vaccinia vector to achieve T7 RNA polymerase-specific recombinant gene expression in the absence of productive vaccinia virus replication.**

Key words: Poxvirus; Host restriction; Attenuation; Expression vector; T7 RNA polymerase

1. Introduction

Recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase gene allowed the establishment of widely applicable vector systems for the synthesis of recombinant proteins in mammalian cells [1]. In all protocols, recombinant gene expression relies on the synthesis of the T7 RNA polymerase in the cytoplasm of vaccinia virus infected eukaryotic cells. Most popular became a protocol for transient gene expression [2]. First, a foreign gene of interest is inserted into a plasmid under the control of the T7 RNA polymerase promoter. In the following, this plasmid is introduced into the cytoplasm of cells infected with the recombinant vaccinia virus producing T7 RNA polymerase using standard transfection procedures. The transient expression system is simple because no new recombinant viruses need to be made and very efficient with usually greater than 80% of the cells expressing the gene of interest [3]. The advantage over other transient expression systems is very likely its independence on the transport of plasmids to the cellular nucleus. The vaccinia virus/T7 RNA polymerase transient expression system has already been proven extremely successful to analysis of structure and function of viral proteins [4–6], rescue of mutant viruses [7], virus assembly and synthesis of defective viral pseudo-particles [8], and expression of eukaryotic polypeptides such as ion channels [9] or neurotransmitter receptors [10].

However, vaccinia virus, despite its historical use as a vaccine, is infectious for humans and for its use as expression vector in the laboratory precautions were taken and safety regulations have been established [11]. To overcome this limitation of the use of vaccinia virus with the T7 system, adoption of highly attenuated viruses would be desirable. Furthermore, important future applications of the system, e.g. the generation of recombinant proteins or recombinant viral particles for therapeutic or prophylactic use in humans, could be impeded by the productive replication of the recombinant vaccinia virus.

Recently, a novel vaccinia vector system was established on the basis of the host range restricted and highly attenuated modified vaccinia Ankara (MVA) strain of vaccinia virus [12]. Derived by longterm serial passage in chicken embryo fibroblasts, MVA lost its capacity to grow productively in human and most other mammalian cells [13–15]. Viral replication in human cells is blocked late in infection preventing the assembly to mature infectious virions. Nevertheless, MVA is able to express viral and recombinant genes at high levels even in non-permissive cells and can serve as an efficient and exceptionally safe expression vector [16]. To further exploit the use of MVA we constructed recombinant viruses that allow expression of the bacteriophage T7 RNA polymerase gene under the control of the vaccinia virus early/late promoter P7.5. The usefulness of MVA-T7pol recombinant viruses as expression system was tested by the co-transfection of plasmid DNA containing the *E. coli* CAT gene under the control of a T7 RNA polymerase promoter. We demonstrate here that highly attenuated MVA vectors can be used in the vaccinia virus-T7 RNA polymerase expression system as efficiently as a fully replication-competent recombinant vaccinia virus.

2. Materials and methods

2.1. Cells and viruses

Cells were maintained in a humidified air–5% CO_2 atmosphere at 37°C. Human HeLa, monkey CV-1 and chicken embryo fibroblast cells (CEF) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Vaccinia virus strain MVA was routinely propagated and titered by endpoint dilution in CEF to obtain the 50% tissue culture infectious dose ($TCID_{50}$). Recombinant vaccinia virus (strain Western Reserve) vTF7-3 was kindly provided by B. Moss (Laboratory of Viral Diseases, NIAID, NIH, Bethesda, USA) and propagated in CV-1 cells and titered in CEF ($TCID_{50}$).

2.2. Plasmid constructions

Sequences of MVA DNA flanking the site of a 2500-bp deletion in the *Hin*dIII N fragment of the MVA genome [17] were amplified by PCR and cloned into pUC18. The primers for the left 600-bp DNA flank were 5′-CAG CAG GGT ACC CTC ATC GTA CAG GAC GTT CTC-3′ and 5′-CAG CAG CCC GGG TAT TCG ATG ATT ATT TTT AAC AAA ATA ACA-3′ (sites for restriction enzymes *Kpn*I and

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*Sma*I are underlined). The primers for the right 550-bp DNA flank were 5′-CAG CAG CTG CAG GAA TCA TCC ATT CCA CTG AAT AGC-3′ and 5′-CAG CAG GCA TGC CGA CGA ACA AGG AAC TGT AGC AGA-3′ (sites for restriction enzymes *Pst*I and *Sph*I are underlined). Between these flanks of MVA DNA, the *Escherichia coli lacZ* gene under control of the vaccinia virus late promoter P11 was inserted to generate the plasmid pUCII LZ. In the following, a 3.1 kbp DNA fragment containing the entire gene of bacteriophage T7 RNA polymerase under control of the vaccinia virus early/late promoter P7.5 was excised with *Eco*RI from plasmid pTF7-3 [2], and cloned into a unique *Sma*I restriction site of pUCII LZ to make the plasmid transfer vector pUCII LZ T7pol.

2.3. Generation of recombinant viruses

CEF infected with MVA at a multiplicity of 0.05 $TCID_{50}$ per cell were transfected with plasmid as described previously [18]. Recombinant MVA virus expressing the T7 RNA polymerase and co-expressing β-galactosidase (MVA P7.5-T7pol) was selected by five consecutive rounds of plaque purification in CEF stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (300 μg/ml) [19]. Subsequently, recombinant viruses were amplified by infection of CEF monolayers, and the DNA was analyzed by PCR.

2.4. Analysis of [^{35}S]methionine-labeled polypeptides

Transfected or untransfected cell monolayers grown in 12-well plates were infected with virus at a multiplicity of 20 $TCID_{50}$ per cell. At 2–5 h after infection, the medium was removed, and the cultures were washed once with 1 ml of methionine-free medium. To each well, 0.2 ml of methionine-free medium supplemented with 50 μCi of [^{35}S]methionine was added and incubated for 30 min or overnight at 37°C. Cytoplasmic extracts of infected cells were prepared by incubating each well in 0.2 ml of 0.5% Nonidet P-40 lysis buffer for 10 min at 37°C and samples were analyzed by SDS-PAGE.

2.5. Chloramphenicol acetyltransferase (CAT) assay

CV-1 cells in 12-well tissue-culture plates were transfected with 10 μg pOS6 (derived from pTM1 [1] containing the *E. coli* CAT gene under control of T7 RNA polymerase promoter and kindly provided by B. Moss, NIH, Bethesda, USA) and infected with 20 $TCID_{50}$ recombinant vaccinia virus per cell. At 12 h after infection cells were suspended in 0.2 ml of 0.25 M Tris-HCl (pH 7.5). After three freeze–thaw cycles, the lysates were cleared by centrifugation, the protein content of the supernatants was determined [20], and samples containing 0.5, 0.25, 0.1 μg total protein were assayed for enzyme activity as described by Mackett et al [21]. After autoradiography, labeled spots were quantitated using an imaging analyzer (Fujix BAS 1000, Fuji, Japan).

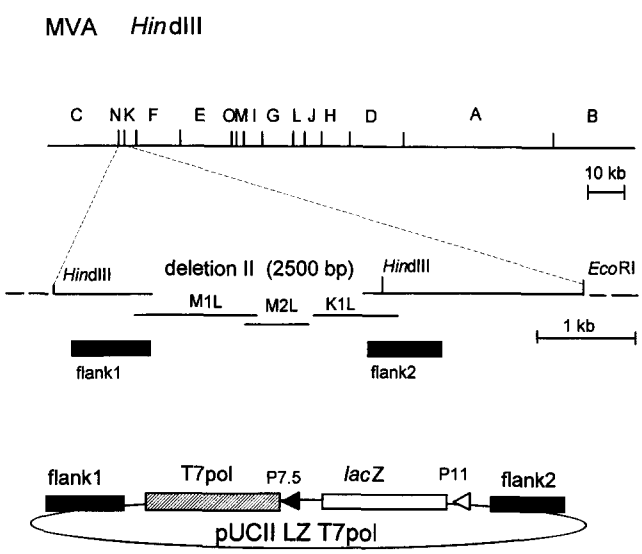

Fig. 1. Schematic map of the genome of MVA and plasmid pUCII LZ T7pol designed for insertion of the bacteriophage T7 RNA polymerase gene by homologous recombination. *Hin*dIII restriction endonuclease sites within the genome of MVA are indicated at the top (kb, kilobase pairs). DNA sequences (flank 1 and flank 2) adjacent to deletion II within the *Hin*dIII N fragment [17] (affecting the three open-reading frames M1L, M2L, and K1L present in the Western Reserve strain of vaccinia virus) were cloned to allow recombination into the MVA genome. Cassettes for the expression of the genes of bacteriophage T7 RNA polymerase and *E. coli lacZ* were inserted between the MVA DNA flanks. P11 and P7.5 refer to well-characterized late and early/late vaccinia virus promoters, respectively.

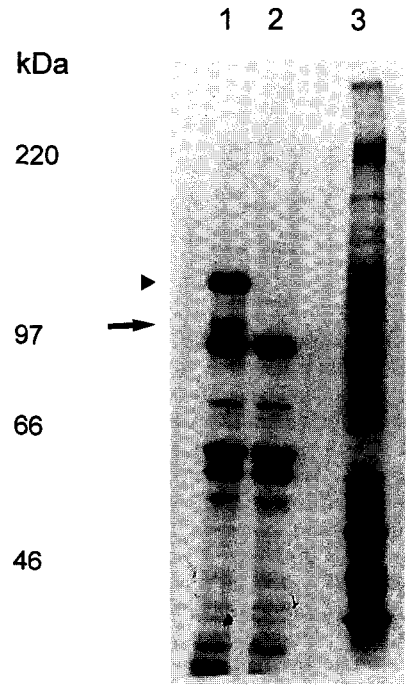

Fig. 2. Expression of the bacteriophage T7 RNA polymerase gene by MVA recombinant virus. SDS/PAGE of CV-1 cells infected with MVA LZ T7pol (lane 1), or MVA (lane 2). The infected cultures were labeled with [^{35}S]methionine at 5 h postinfection. Cell lysates were analyzed by electrophoresis on a 10% polyacrylamide gel. Lane 3 = uninfected CV-1 cell extract. The numbers on the left indicate the positions and molecular masses (in kDa) of protein standards. The protein band representing the enzyme T7 RNA polymerase is marked by an arrow, the position of the marker protein β-galactosidase is indicated by an arrowhead.

3. Results and discussion

3.1. Construction and isolation of MVA recombinants expressing the T7 RNA polymerase gene

Previous work demonstrated that MVA may serve as efficient expression vector in human and other mammalian cells where it is unable to produce infectious progeny [16]. To test the usefulness of MVA recombinants in a hybrid expression system relying on the bacteriophage T7 RNA polymerase, an enzyme with exceptional transcription activity [22,23], we constructed the MVA plasmid transfer vector pUC II LZ T7pol (Fig. 1). The vector plasmid contains MVA DNA sequences adjacent to a deletion of 2500 bp affecting the three open reading frames M1L, M2L, and K1L present in the Western Reserve strain of vaccinia virus [17]. These flanking sequences target the insertion of the foreign genes precisely to the site of this naturally occuring deletion in the MVA genome. For expression of the bacteriophage T7 RNA polymerase gene we chose the well-characterized vaccinia virus early/late promoter

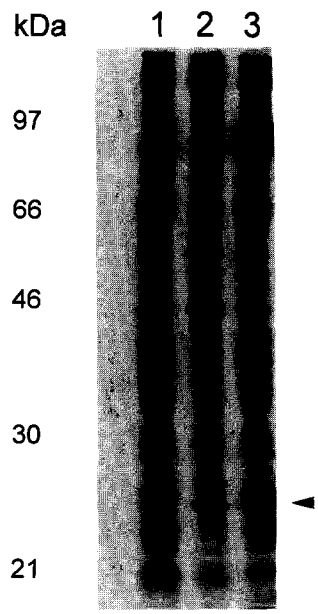

Fig. 3. Transient expression of CAT using vaccinia-T7 RNA polymerase recombinant viruses. SDS/PAGE of human HeLa cells transfected with pOS6 and infected with MVA (lane 1), vTF7-3 (lane 2), or MVA-T7pol (lane 3). The infected cultures were labeled overnight with [^{35}S]methionine at 6 h after transfection. Cell lysates were analyzed by electrophoresis on a 10% polyacrylamide gel. The numbers on the left indicate the positions and molecular masses (in kDa) of protein standards. The protein band representing the enzyme CAT is marked by an arrowhead.

P7.5 as transcriptional regulator. The identical promoter has been used to construct the replication-competent WR recombinant virus vTF7-3 [2] that is commonly used in established vaccinia virus/T7 polymerase expression systems.

Recombinant vaccinia virus MVA-T7pol was formed in CEF that were infected with MVA and transfected with pUCII LZ T7pol. Recombinant virus plaques were identified by screening for β-galactosidase production with a chromogenic substrate [19]. Multiple plaque isolations were performed, and the correct insertion of the foreign DNA and absence of parental virus was ascertained by PCR analysis.

3.2. Characterization of expressed recombinant proteins

A basic protocol [3] describing the vaccinia virus/T7 RNA polymerase system for transient expression of recombinant genes recommends transfection and infection of monkey CV-1 cells. When we characterized viral gene expression in this cell line metabolic labeling with [^{35}S]methionine of cells infected with MVA T7pol revealed the synthesis of two additional polypeptides (Fig. 2). One protein band of about 116,000 Da (marked by an arrowhead) was likely to represent the *E. coli* β-galactosidase that is co-expressed as a marker enzyme to allow screening for recombinant virus. The strong expression activity mediated by the vaccinia virus late promoter P11 is nicely seen in the large amount of β-galactosidase made. Another additional band (marked by an arrow) migrated as a polypeptide of about 98,000 Da, the expected size of the bacteriophage T7 RNA polymerase [24]. This single-subunit enzyme has an extremely high transcriptase activity. Therefore, overexpression of the catalytic polypeptide is not needed and even might not be desirable. We chose the vaccinia virus early/late promoter P7.5 to control expression of the T7 RNA polymerase. The advantage of a promoter system suitable for both vaccinia virus early and late transcription is that synthesis of recombinant T7-RNA polymerase is induced immediately after viral entry and maintained during the complete cycle of vaccinia viral gene expression.

3.3. Transient expression of CAT in MVA T7pol infected cells

To determine whether bacteriophage T7 RNA polymerase made by recombinant MVA can function in mammalian cells, we used the *E. coli* CAT gene with a T7 promoter as target gene in transient transfection assays. Additionally, we chose for comparative purposes the well-characterized Western Reserve strain recombinant virus vTF7-3 that contains the identical P7.5 promoter-T7pol expression cassette but that multiplies well in human cells. In a first experiment human HeLa cells were transfected with plasmid pOS6 containing the CAT gene and infected with MVA-T7pol, vTF7-3 or MVA. Protein synthesis was monitored by SDS/PAGE of cytoplasmic proteins from cells in vivo-labeled with [^{35}S]methionine at two hours after infection (Fig. 3). A band corresponding in size to the CAT polypeptide, was clearly seen among the proteins from cells infected with MVA-T7pol (lane 3) or vTF7-3 (lane 2). The extract of cells infected with MVA alone (lane 1) contained a minor amount of a polypeptide that migrated with almost the same molecular weights as CAT representing, most likely, a vaccinia viral or cellular protein of similar size. To our surprise and despite the inability of MVA to replicate productively in human HeLa cells, the amount of recombinant CAT produced in MVA-T7pol infected cells seemed to be higher than that made in cells infected with the replicating recombinant vaccinia virus vTF7-3. For further comparison in a more quantitative manner, a second series of experiments was performed in CV-1 cells. Again transfection of pOS6 was followed by infection with the recombinant vaccinia viruses expressing the T7 RNA polymerase. It is noteworthy that MVA-T7pol, in sharp contrast to vTF7-3, produced no visible cytopathogenic effects in the mammalian cell lines HeLa and CV-1 even at high multiplicities of infection (MOI). At 24 hours after infection cell extracts were made, diluted, and tested for CAT activity by

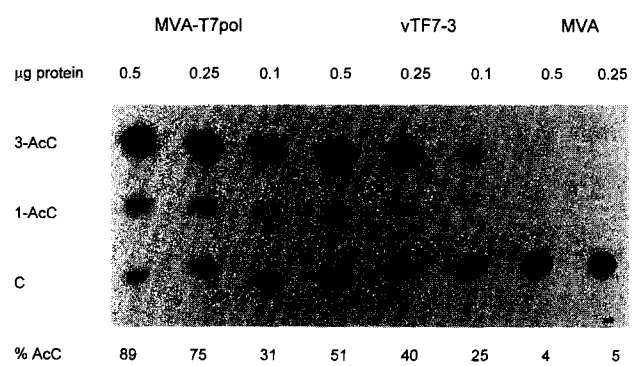

Fig. 4. CAT activity in lysates from CV-1 cells transfected with pOS6 and infected with MVA T7pol, vTF7-3, or MVA. Cell lysates were prepared 12 h after infection and aliquots containing 0.1, 0.25 and 0.5 μg total protein were assayed for CAT. An autoradiogram is shown, with the positions of chloramphenicol (C) and acetylated forms of chloramphenicol (AcC). CAT activity is expressed as percentage of acetylated product formed in 60 min.

measuring the conversion of [^{14}C]chloramphenicol to acetylated derivates separable by thin-layer silica gel chromatography. As illustrated by the autoradiogram in Fig. 4, only background acetylation activity was detectable in extracts of MVA infected CV-1 cells that had been transfected with pOS6. In contrast, [^{14}C]chloramphenicol was acetylated efficiently when added to extracts of cells infected with recombinant viruses MVA-T7pol or vTF7-3. Interestingly, enzyme activities detected in extracts of MVA-T7pol infected cells were at least equal to those found in extracts from CV-1 cells infected with vTF7-3. These results indicate that the recombinant vector MVA-T7pol can be used very efficiently for the expression of T7-ptomoter controlled target genes in mammalian cell lines. The level of gene expression achieved with MVA-T7pol in transient assays was in the same range as obtained with a replication competent and presumedly much more virulent recombinant vaccinia virus.

In conclusion, the replication-deficient virus MVA-T7pol represents an important addition to already vaccinia/T7 polymerase expression systems and its adoption should drastically reduce the risk of infecting laboratory workers. Besides of this safety aspect, lack of viral replication and low cytopathogenicity in mammalian cells, characteristic features of the parent MVA virus, may significantly expand the power of the hybrid vaccinia virus/T7 RNA polymerase expression system. Promising future applications of MVA-T7pol vectors should include both functional analysis of recombinant genes in eukaryotic environment and production of recombinant proteins for the development of new therapeutics or vaccines.

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References

[1] Moss, B., Elroy-Stein, O., Mizukami, T., Alexander, W.A. and Fuerst, T.R. (1990) Nature 348, 91–92.
[2] Fuerst, T.R., Niles, E.G., Studier, F.W. and Moss, B. (1986) Proc. Natl. Acad. Sci. USA 83, 8122–8126.
[3] Elroy-Stein, O. and Moss, B. (1994) in: Current Protocols in Molecular Biology (Ausubel, F.M. et al. eds.) vol. 16, IV 16.19., Wiley, New York.
[4] Buonocore, L. and Rose, J.K. (1990) Nature 345, 625–628.
[5] Barrett, N., Mitterer, A., Mundt, W., Eibl, J., Eibl, M., Gallo, R.C., Moss, B. and Dorner, F. (1990) AIDS Res. Hum. Retroviruses 5, 159–171.
[6] Buchholz, C.J., Retzel, C., Homann, H.E. and Neubert, W.J. (1994) Virology 204, 770–776.
[7] Li, Y., Luo, L., Snyder, R.M. and Wagner, R.R. (1988) J. Virol. 62, 776–782.
[8] Pattnaik, A.K. and Wertz, G.W. (1991) Proc. Natl. Acad. Sci. USA 88, 1379–1383.
[9] Karschin, A., Aiyar, J., Gouin, A., Davidson, N. and Lester, H.A. (1991) FEBS Lett. 278, 229–233.
[10] Ho, B.Y., Karschin, A., Raymond, J., Branchek, T., Lester, H.A. and Davidson, N. (1992) FEBS Lett. 301, 303–306.
[11] Advisory Committee on Dangerous Pathogens and Advisory Committee on Genetic Modifications (1990) Vaccination of Laboratory Workers Handling Vaccinia and Related Poxviruses Infectious for Humans (HMSO, London) pp. 1–16.
[12] Sutter, G. and Moss, B. (1995) Dev. Biol. Stand. Karger 84, 195–200.
[13] Mayr, A., Hochstein-Mintzel, V. and Stickl, H. (1975) Infection 3, 6–14.
[14] Meyer, H., Sutter, G. and Mayr, A. (1991) J. Gen. Virol. 72, 1031–1038.
[15] Sutter, G., Ramsey-Ewing, A., Rosales, R. and Moss, B. (1994) J. Virol. 68, 4109–4116.
[16] Sutter, G. and Moss, B. (1992) Proc. Natl. Acad. Sci. USA 89, 10847–10851.
[17] Altenburger, W., Suter, C.P. and Altenburger, J. (1989) Arch. Virol. 105, 15–27.
[18] Sutter, G., Wyatt, L.S., Foley, P.L., Bennink, J.R. and Moss, B. (1994) Vaccine 12, 1032–1040.
[19] Chakrabarti, S., Brechling, K. and Moss, B. (1985) Mol. Cell. Biol. 5, 3403–3409.
[20] Smith, P.K., Krohn, R.J., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[21] Mackett, M., Smith, G.L. and Moss, B. (1984) J. Virol. 49, 857–864.
[22] Chamberlin, M. and Ryan, T. (1982) in: The Enzymes (Boyer, P.D. ed.) vol. 15, pp. 82–108, Academic, New York.
[23] Dunn, J. and Studier, F.W. (1983) J. Mol. Biol. 166, 477–535.
[24] Davanloo, P., Rosenberg, A.H., Dunn, J.J. and Studier, F.W. (1984) Proc. Natl. Acad. Sci. USA 81, 2035–2039.