EARLY GENE EXPRESSION OF VACCINIA VIRUS STRAINS REPLICATING (PRAHA) AND NON-REPLICATING (MODIFIED VACCINIA VIRUS STRAIN ANKARA, MVA) IN MAMMALIAN CELLS

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Summary. – Modified vaccinia virus Ankara strain (MVA) is a safe highly attenuated non-pathogenic virus suitable as a vector for developing various vaccines. Study of expression of a reporter ß-galactosidase gene under the control of an early vaccinia virus (VV) promoter in MVA and non-modified vaccinia virus Praha strain showed that early transcription in MVA is elevated in comparison with non modified VV. This property was demonstrated in various cell cultures including CV1 cells, human lung diploid cells, chicken primary fibroblasts but not in bone marrow-derived mouse dendritic cells. There the relationship between the elevated early transcription and the permisivity of cells for MVA was not observed.

Key words: vaccinia virus; poxviruses; MVA; gene expression; dendritic cells; early promoter

MVA has been derived from VV Ankara strain by multiple passaging in primary chick embryo fibroblast (CEF) cultures and used as safe, attenuated, live vaccine against smallpox (Mayr and Danner, 1978). MVA has limited capability of replication in various human cell lines and is non-pathogenic for animals (Mayr *et al.*, 1978).

In comparison with parental VV Ankara strain MVA genome comprises at least six major deletions representing 15% of the original DNA (Antoine *et al.*, 1998). DNA synthesis is normal and both early and late viral proteins are synthesized in non-permissive cells. It was shown that replication block which occurs at the late stage of replication cycle is manifested by inhibition of proteolytic processing of viral structural proteins and by occurrence of immature particles in infected cells (Sutter and Moss, 1992). Because MVA is exceptionally safe virus (Sutter and Moss,1995; Moss,1996) it is supposed to be used as recombinant virus vector for developing various prophylactic and therapeutic cancer vaccines.

Recently we reported that the 3-ß-hydroxysteroid dehydrogenase (3ß-HSD) activity in CV-1 cells infected with MVA is higher than that with VV strains WR, Wyeth, LIVP and Praha (Sroller *et al.*, 1998).

As 3ß-HSD is an early gene product we decided to examine, whether the elevated 3ß-HSD activity was due to an extended expression of early genes in MVA infected CV-1 cells. We compared the expression of reporter ß-galactosidase gene under the control of the early VV promoter in MVA and in VV Praha strain in various cell cultures. The respective recombinant viruses were derived from MVA and the clone 13 of VV Praha strain (P13) (Kutinova *et al.*, 1995). Recombinant viruses expressing lacZ gene were generated using the pMJ601-early vector. The latter was prepared by

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Abbreviations: 3ß-HSD = 3-ß-hydroxysteroid dehydrogenase; araC = cytosine arabinoside; BrUdR = 5-bromo-2′-deoxyuridine; CEF = chick embryo fibroblast; GM-CSF = granulocyte macrophage colony stimulation factor; MOI = multiplicity of infection; MVA = modified vaccinia virus Ankara strain; P13 = vaccinia virus Praha strain clone 13; p.i. = post infection; TK = thymidine kinase; VV = vaccinia virus; X-gal = 5-bromo-4-chloro-3-indolyl-ß-D-galactoside

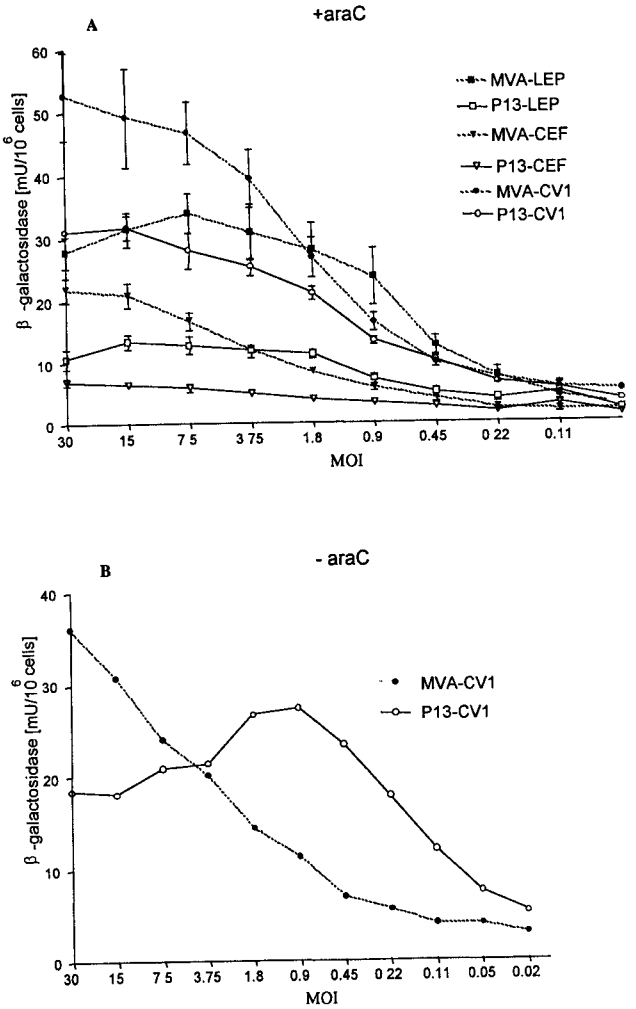

Fig. 1
ß-galactosidase synthesis by recombinant vaccinia viruses P13-E and MVA-E in LEP, CEF and CV1 cells

For the experimental procedure see the text. AraC present (A) or absent (B) in the cultivation medium. For the abbreviations see their list on the front page of the article.

excision of late synthetic promoter from the pMJ601 vector (Davison and Moss, 1990) by cleaving with *Nar*I and *Cla*I and subsequent recircularization by DNA ligase. Recombinant viruses were isolated as described previously (Kutinova *et al.*, 1990). We found that MVA TK$^-$ recombinant viruses could be easily selected and plaque purified on monolayers of RAT 2 TK$^-$ cells in the presence of 5-bromo-2'-deoxyuridine (BrUdR). Resulting recombinant viruses were denoted MVA-E and P13-E. P13-E was grown on CV1 cells and MVA-E was propagated in primary CEF cultures. The viruses were purified by sucrose density gradient centrifugation (Joklik, 1962).

The expression of lacZ gene was followed in CV1 cells, human diploid fibroblast LEP cells and CEF cells infected with the recombinant viruses. Also infectious virus titers were determined from the number of blue stained cells. The cell monolayers (3 x 10^5 cells per well) in 24-well plates were infected with serial 10-fold dilutions of virus stock. After 1 hr at 37°C the cells were rinsed twice, a fresh medium containing 40 μg/ml araC was added and cultivation was continued. After 24 hrs the cells were washed with PBS twice, fixed with 0.05% glutaraldehyde in PBS for 10 mins, washed again with PBS and stained overnight with 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X gal) solution (Nielsen *et al.*, 1983). ß-galactosidase activity in infected cultures was assayed as follows. The cultures of infected cells were frozen 1hr or 24 hrs post infection (p.i.). The cells were lyzed by three freeze/thaw cycles and the lyzates were centrifuged to remove cellular debris. The ß-galactosidase activity of cell extracts was determined by spectrophotometry using o-nitrophenyl ß-D-galactopyranoside (Sambrook *et al.*, 1989). The enzymatic activity of cultures frozen 1 hr p.i. represented the background value, which was subtracted from each sample value. Because of the use of purified virus stocks, the background values were very low.

The early lacZ gene expression in MVA and P13 viruses was assayed in CV1, LEP and CEF cells. The ß-galactosidase activity in cells infected at various multiplicity of infection (MOI) in the absence of DNA synthesis (araC present) is shown in Fig. 1A. The lacZ expression in MVA-E infected cells was higher than that in P13-E-infected cells. This difference was found in all cell types tested and within a wide range of MOI. A higher lac Z expression in MVA was observed also in the absence of araC, but only for MOI above 4 (Fig. 1B). In the cells infected at lower MOI, the lacZ expression in P13 prevailed over that in MVA, probably due to higher multiplication of P13 virus.

The difference between the MVA and P13 early expression could not be found at the level of gene transcription as demonstrated by Northern blot analysis of lacZ transcripts performed as follows. Total RNA was isolated from infected CV1 cells at various intervals p.i. (Chomczynski, 1992), fractionated on formaldehyde-1% agarose gel in 40 mmol/l MOPS buffer. RNA was blotted onto Hybond N$^+$ membrane (Amersham) and lacZ transcripts on the blot were detected by hybridization using ECL direct nucleic acid labeling and detection system (Amersham) with DNA probe containing the entire lacZ gene. The lacZ mRNA transcribed from early promoter is shown in Fig. 2. This result suggests that the higher level of ß-galactosidase activity found in MVA-E infected cells could be caused by higher rate of translation or posttranslation rather than by higher mRNA synthesis

Further we examined whether the higher early expression in MVA-E occurs also in dendritic cells. Briefly, seven-day-old dendritic cell cultures, derived from mouse bone marrow

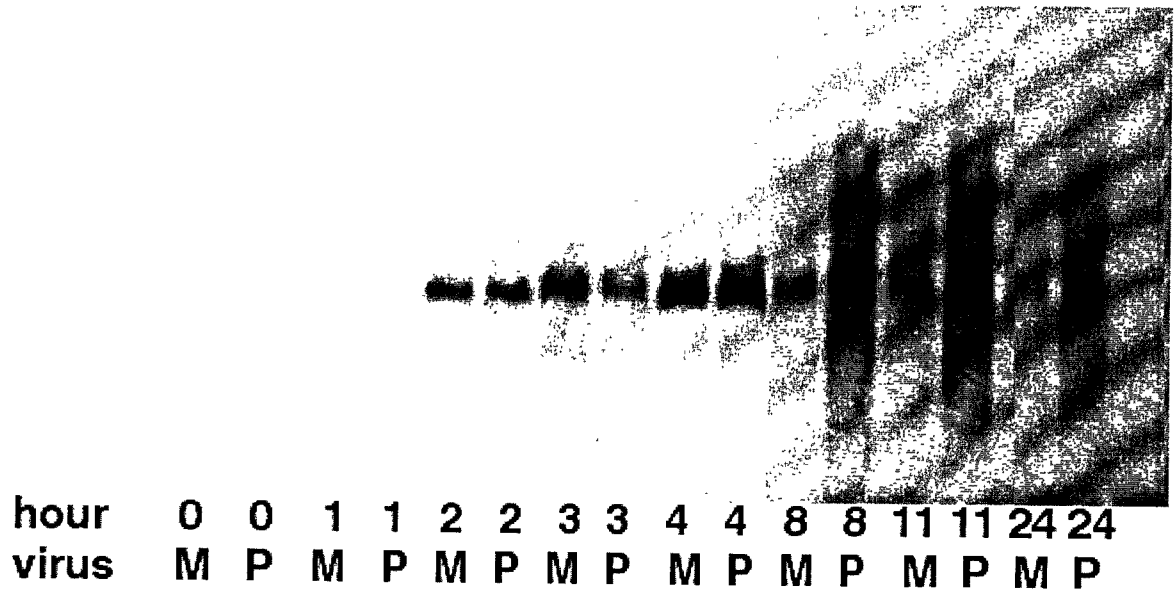

Fig. 2
Northern blot analysis of the early viral mRNA synthesized in CV1 cells infected with recombinant vaccinia viruses P13-E or MVA-E
The early viral mRNA was assayed at 0, 1, 2, 3, 4, 8, 11, and 24 hrs after infection. For the experimental procedure see the text. P13-E (P) and MVA-E (M) viruses.

in the presence of GM-CSF (Lutz *et al.*, 1999), were infected with MVA-E or P13-E viruses at MOI of 0.5–10. The cells (5.10^5 cells per well) were cultivated in the presence of araC in 96-well microplates for 24 hrs in quadruplicate. The cell extracts prepared by three cycles of freezing and thawing were assayed for ß-galactosidase activity. Numbers of infected cells were determined in cytospins after overnight staining with X-gal (Nielsen *et al.*, 1983).

The results of this experiment are shown in Fig. 3. In contrast to other cell cultures tested, the early expression in MVA-E-infected dendritic cells was not higher than that in cells infected with P13-E virus.

To see, whether the difference between P-13 and MVA in early expression depends on the growth rate of viruses in cell lines used, we performed an one step virus growth test. Cell monolayers were infected with MVA or P-13 parental viruses at an MOI of 1. After 1hr of virus adsorption the inoculum was removed, the cells were washed and a fresh medium was added. The infected cells were harvested at 24 hrs p.i. and the virus was released by three cycles of freezing-thawing. The lysates were titrated on CEF cells in case of MVA or on CV1 cells in case of P13, as CV1 cells had the best plating efficiency for P13. Multiplication of the viruses is shown in Table 1. In concordance with earlier results

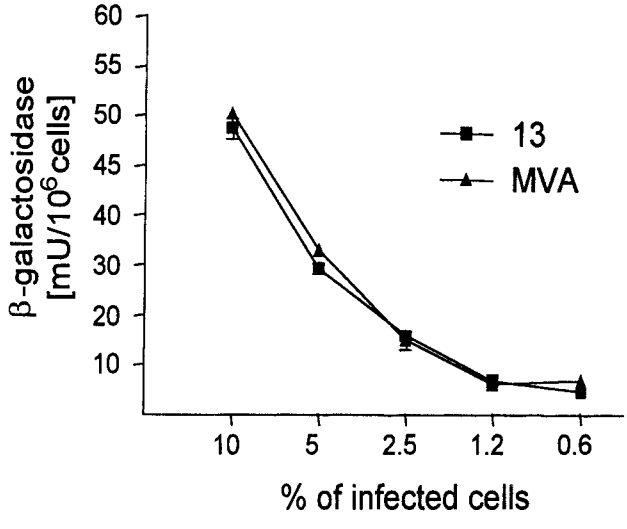

Fig. 3
ß-galactosidase synthesis in dendritic cells infected with recombinant vaccinia viruses P13-E and MVA-E
ß-galactosidase activity (each value is the mean of two measurements ± SD) is plotted against percentage of infected cells. P13-E (squares) and MVA-E (triangles). For the experimental procedure see the text.

Table 1 Multiplication of MVA-E and P13-E viruses in different cells

Virus	Infected cells [virus titer ratio][a]		
	CEF [T_{24}/T_0]	CV1 [T_{24}/T_0]	LEP [T_{24}/T_0]
MVA-E[b]	484	71	59
P13-E[c]	ND	187	78

For the experimental procedures see the text. For the abbreviations see their list on the front page of the article.
[a]Titer at 24 hrs (T_{24}) p.i. divided by titer at 0 hr (T_0).
[b]MVA-E was titrated on primary CEF cultures.
[c]P13-E was titrated on CV1 cell cultures.

(Carroll and Moss, 1997) we found that MVA gave highest yields in CEF cells and that human LEP cells supported very well the growth of P13 (Kutinova *et al.*, 1990). The growth of MVA in CV1 and LEP cells was limited similarly as shown by others (Drexler *et al.*, 1998). Thus the lower early expression in P13 in comparison with MVA did not correlate with growth adaptation of the viruses to particular cell lines.

The presented findings that the early promoter-controlled transcription of reporter gene was higher in MVA than in P13 are in good agreement with our previous observation (Sroller *et al.*, 1998) that MVA produced considerably more 3ß-HSD (another early gene product) than another VV strain. As all so far tested strains (Praha clones 13 and 20, clones obtained from strains Wyeth, WR and LIVP) produced similar levels of 3ß-HSD *in vitro* (Sroller *et al.*, 1998), we used for further comparison only one of them, namely P13. The higher MVA expression under the control of early promoter was confirmed using the lacZ as reporter gene. Our results corroborate with the finding of Ramirez *et al.* (2000), who have also found that the expression of a reporter gene within 6 hrs p.i. was higher in tissues of mice infected with MVA virus than in those infected with the wild type virus. The expression from the early promoter of each virus was in the presence of araC higher than in its absence when the cells were infected at a MOI above 4. We found similar effects not only in CV1 but also in LEP and CEF cells (data not shown). The enhancing effect of inhibitors of DNA replication on early gene expression has been explained earlier by accumulation of early mRNA in consequence of inhibition of late protein synthesis (Baldick and Moss, 1993). The difference between the MVA and P13 early expression was not demonstrated at the level of early transcripts by Northern blot analysis. The enhanced MVA early expression was not a specific feature of certain cell line used in our study. It was observed in CV1 and CEF cells supporting the growth of MVA but also in LEP cells in which the MVA growth is limited. However, such a difference in early expression was not found in mouse bone marrow-derived dendritic cells. It is well known that only the antigen expressed under the control of early promoter can be efficiently presented to T cells in complex with MHCI molecules (Townsend *et al.*, 1988). Therefore, the elevated early expression in MVA-infected cells could be particularly important for induction of cell-mediated immunity by recombinant viruses.

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References

Antoine G, Scheiflinger F, Dorner F, Falkner FG (1998): The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. *Virology* **244**, 365–396.

Baldick CJJr., Moss B (1993): Characterization and temporal regulation of mRNAs encoded by vaccinia virus intermediate-stage genes. *J. Virol.* **67**, 3515–3527.

Carroll MW, Moss B (1997): Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: propagation and generation of recombinant viruses in a nonhuman mammalian cell line. *Virology* **238**, 198–211.

Chomczynski P (1992): Solubilization in formamide protects RNA from degradation. *Nucleic Acids Res.* **20**, 3791–3792.

Davison AJ, Moss B (1990): New vaccinia virus recombination plasmids incorporating a synthetic late promoter for high level expression of foreign proteins. *Nucleic Acids Res.* **18**, 4285–4286.

Drexler I, Heller K, Wahren B, Erfle V, Sutter G (1998): Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells. *J. Gen. Virol.* **79**, 347–352.

Joklik WK (1962): The purification of four strains of poxvirus. *Virology* **18**, 9–18.

Kutinova L, Nemeckova S, Hamsikova E, Press M, Zavadova H, Hirsch I, Nemecek V, Krchnak V, Smrt J, Slonim D (1990): A recombinant vaccinia virus expressing hepatitis B virus middle surface protein. Restricted expression of HBV antigens in human diploid cells. *Arch. Virol.* **112**, 181–193.

Kutinova L, Ludvikova V, Simonova V, Otavova M, Krystofova J, Hainz P, Press M, Kunke D, Vonka V (1995): Search for optimal parent for recombinant vaccinia virus vaccines. Study of three vaccinia virus vaccinal strains and several virus lines derived from them. *Vaccine* **13**, 487–493.

Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, Schuler G (1999): An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* **223**, 77–92.

Mayr A, Danner K (1978): Vaccination against pox diseases under immunosuppressive conditions. *Dev. Biol. Stand.* **41**, 225–234.

Mayr A, Stickl H, Muller HK, Danner K, Singer H (1978): Der pockenimpfstamm MVA: Marker, genetische Struktur, Erfahrungen mit der parenteralen Schutzimpfung und Verhalten im abwehrgeschwachten Organismus. *Zbl. Bakt. Hyg.* **167**, 375–390.

Moss B (1996): Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. *Proc. Natl. Acad. Sci. USA* **93**, 11341–11348.

Nielsen DA, Chou J, MacKrell AJ, Casadaban MJ, Steiner DF (1983): Expression of a preproinsulin-beta-galactosidase gene fusion in mammalian cells. *Proc. Natl. Acad. Sci. USA* **80**, 5198–5202.

Ramirez JC, Gherardi MM, Esteban M (2000): Biology of attenuated modified vaccinia virus Ankara recombinant vector in mice: virus fate and activation of B- and T-cell immune responses in comparison with the Western Reserve strain and advantages as a vaccine. *J. Virol.* **74**, 923–933.

Sambrook J, Maniatis T, Fritsch EF (1989): *Molecular Cloning.* CSH Laboratory Press.

Sroller V, Kutinova L, Nemeckova S, Simonova V, Vonka V (1998): Effect of 3-beta-hydroxysteroid dehydrogenase gene deletion on virulence and immunogenicity of different vaccinia viruses and their recombinants. *Arch. Virol.* **143**, 1311–1320.

Sutter G, Moss B (1992): Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proc. Natl. Acad. Sci. USA* **89**, 10847–10851.

Sutter G, Moss B (1995): Novel vaccinia vector derived from the host range restricted and highly attenuated MVA strain of vaccinia virus. *Dev. Biol. Stand.* **84**, 195–200.