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Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence

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Different passages of the vaccinia virus strain Ankara (CVA wild-type) during attenuation to MVA (modified vaccinia virus Ankara) have been analysed to detect alterations in the genome. Physical maps for the restriction enzymes *Hin*dIII and *Xho*I have been established. Six major deletions relative to the wild-type strain CVA could be localized. They reduce the size of the entire genome from 208 kb (CVA wild-type) to 177 kb for the MVA strain. Four deletions occurred during the first 382 passages and the resulting variant (CVA 382) displays an attenuated phenotype similar to that of the MVA strain. The deletions are located in both terminal fragments, affect two-thirds of the host range gene K1L and eliminate 3·5 kb of a highly conserved region in the *Hin*dIII A fragment. During the next 190 passages leading to MVA two additional deletions appeared. Again, one is located in the left terminal fragment, and the other includes the A-type inclusion body gene. Neither of the deletions appear to participate in further attenuation of the virus. Rescue of the partially deleted host range region with the corresponding wild-type DNA restored the ability of the attenuated strains MVA and CVA 382 to grow in some non-permissive tissue cultures. Nevertheless, the complete host range of the wild-type strain was not recovered. Also, plaque-forming behaviour and reduced virulence were not influenced. From the data presented it may be concluded that the partially deleted host range gene is not solely responsible for attenuation.

Introduction

The use of vaccinia viruses as carriers for foreign genes has led to a renewed interest in vaccinia virus research (Moss, 1985). Owing to side-effects observed during the smallpox eradication campaign there are strong concerns about introducing recombinant vaccinia viruses into human vaccination programmes (Kaplan, 1989). Therefore recent studies have focused on so-called strongly attenuated vaccinia virus strains and on virus attenuation. Deletion or insertion of certain DNA sequences, as well as inactivation of single genes have been used to demonstrate their influence on virulence (Flexner *et al.*, 1987; Kotwal *et al.*, 1989; Rodriguez *et al.*, 1989). Mutants of vaccinia virus with a thymidine kinase-negative (TK^-) or small plaque phenotype exhibited a marked decrease in virulence (Buller *et al.*, 1985; Gong *et al.*, 1989). Also the generation of deletions at the left end of the genome contributes to reduced pathogenicity (Dallo & Esteban, 1987; Kotwal & Moss, 1988; Buller *et al.*, 1988).

However, none of these viruses has been tested extensively in a large number of animals. Altenburger *et al.* (1989) investigated the highly attenuated and well characterized strain MVA (modified vaccinia virus Ankara), which had been attenuated by Mayr *et al.* (1975) by more than 570 serial passages in primary chick embryo fibroblasts (CEFs). The host range of this strain is severely restricted as it replicates only in CEFs. MVA has been proven to be avirulent in a variety of animals even under immunosuppression (for review see Mayr *et al.*, 1978; Stickl *et al.*, 1974). It has been used without complications in primary vaccinations in over 120000 humans (Mayr & Danner, 1979). Three major deletions, relative to vaccinia virus Western Reserve, have been identified so far (Altenburger *et al.*, 1989). One affects two-thirds of the 32K human host range gene; this gene is called the K1L gene following recommended nomenclature (Rosel *et al.*, 1986). The other deletions are located in the left and right terminal fragments.

In this study we establish and compare physical maps (*Hin*dIII, *Xho*I and *Sma*I) of MVA (574 passages), a precursor CVA 382 (382 passages) and the original wild-type virus CVA (two passages on CEFs). Marker rescue experiments were conducted to evaluate whether the

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partially deleted host range gene is responsible for the attenuated phenotype.

Methods

Viruses and cells. Vaccinia viruses used for this study were the reference strain Elstree and the second, 382nd and 574th passage of vaccinia virus Ankara on CEFs (Mayr *et al.*, 1975). In the text they are referred to as CVA wild-type, CVA 382 and MVA respectively. Cell cultures used were: CEFs, chick fibroblast LSCC-H-32 (a gift of O. R. Kaaden, Munich, Germany), bovine embryonic lung (BEL) cells and Madin-Darby bovine kidney (MDBK) cells, the human HeLa, HRT 18, MRC 5 and Hep-2 cells, the monkey cell lines Vero and MA 104, rabbit kidney (RK_{13}), equine dermal (E-derm), mouse DBT cells and Madin-Darby canine kidney (MDCK) cells. The cells were grown as monolayers in Eagle's basal medium (EMEM) supplemented with 5% foetal bovine serum.

Virus propagation and purification. For the preparation of viral stocks CEFs were infected with the appropriate plaque-purified (three times) virus (1 p.f.u. per cell) and incubated for 2 to 3 days at 37 °C. The virus was liberated from cells by freezing and thawing and brief sonication followed by low speed centrifugation to remove cellular debris. Virus stocks were titrated on CEFs, and stored in aliquots at −70 °C. Virus was purified as described by Joklik (1962) with some modifications (Czerny & Mahnel, 1990). The host range of CVA wild-type and the attenuated MVA was compared with the recombinant viruses rec.MVA and rec.CVA 382 by infecting cell monolayers at a multiplicity of 0·05 p.f.u. per cell. Adsorption of virus was allowed to occur for 45 min at 37 °C. After removal of the inoculum, the cell monolayer was washed once with EMEM and incubated with fresh medium (3% FCS in EMEM) at 37 °C. At 0, 48 and 72 h post-infection (p.i.) virus was harvested by freezing and thawing and brief sonication. Virus obtained was titrated on CEFs.

Cloning reagents. Viral DNA was isolated from purified virions and cleaved with restriction enzymes purchased from Boehringer Mannheim and used as specified by the manufacturer. Physical maps for the restriction endonucleases *Hin*dIII, *Xho*I and *Sma*I were established by Southern blot hybridization (Maniatis *et al.*, 1982) using the Digoxygenin labelling and detection system (Boehringer Mannheim). For restriction site mapping, sequences of CVA wild-type, CVA 382 and MVA were cloned by standard procedures into the vector pTZ19R (Pharmacia/LKB) using T4 DNA ligase [Gibco/Bethesda Research Laboratories (BRL)]. Transformation of competent *Escherichia coli* bacteria, strain DH5α (Gibco/BRL), was done according to Hanahan (1983). Plasmid DNA was isolated by the procedure of Birnboim & Doly (1979).

Marker rescue. The 5·2 kb *Eco*RI fragment of CVA wild-type, spanning the K1L host range gene (Gillard *et al.*, 1985), was cloned. Marker rescue experiments were done on the E-derm cell line which is non-permissive for the attenuated strains MVA and CVA 382. Subconfluent cells, infected with 0·05 p.f.u. of either MVA or CVA 382 were transfected with calcium phosphate-precipitated plasmid DNA (pTZ 5.2) according to Graham & van der Eb (1973). After 90 min of adsorption fresh medium was added to the cells and incubation was continued for 3 to 5 days. Recombination events could be detected easily by a developing c.p.e. After plaque purification, DNA of the recombinant viruses rec.MVA and rec.CVA 382 was analysed with *Hin*dIII.

Mouse experiments. For the experiments, approximately 6-week-old female NMRI mice and 2- to 3-day-old baby mice were used. Mice were inoculated either intraperitoneally (i.p.) with 3×10^6 or intracranially (i.c.) with 2×10^4 $TCID_{50}$, baby mice with 1×10^5 and 1×10^4 respectively. Virus strains used were MVA, CVA wild-type and rec.MVA.

Results

Mapping of six major deletions

To demonstrate possible alterations of the genome during continuous propagation we compared the *Hin*dIII restriction profiles of CVA wild-type, CVA 382 and MVA (Fig. 1*a* and *b*). Compared to CVA wild-type DNA a slightly higher mobility of the largest *Hin*dIII A fragments could be observed in CVA 382 and more prominently in MVA. Additionally the fragments B and C migrated faster in CVA 382. Compared to CVA 382 the size of the MVA B fragment remained constant, whereas the C fragment was again reduced in size. Regarding the smaller fragments (Fig. 1*b*), two bands (2·1 and 1·5 kb) of CVA wild-type were missing in CVA 382 but an additional fragment (1·0 kb) appeared. The existence of a recently identified 0·3 kb *Hin*dIII fragment located in the central part of the genome could be demonstrated for CVA wild-type, CVA 382 and MVA in a polyacrylamide gel (data not shown). To determine the exact size of the entire genome we isolated the largest *Hin*dIII and *Xho*I fragments from the gel and cleaved them with *Xho*I, *Hin*dIII or *Eco*RI (data not shown). Using M_r standards and a digitizer-aided computer program (Microgenie, Beckman) the length of the entire genomes adds up to 208 (CVA wild-type), 188 (CVA 382) and 177 (MVA) kb. *Hin*dIII, *Xho*I and *Sma*I genomic maps of CVA wild-type, CVA 382 and MVA were determined by cross-hybridizations (Fig. 2). The location and size of six major deletions is indicated by arrowheads. There are four deletions in the CVA 382 genome relative to CVA wild-type. Deletions I (2·9 kb) and IV (10·2 kb) are located at the left (*Hin*dIII C) and right (*Hin*dIII B) end of the viral genome. This could be shown by cross-hybridizations using CVA wild-type *Xho*I fragments F, H, M and N as well as CVA 382 *Xho*I I, E and F (Fig. 2). Deletion II has already been described (Altenburger *et al.*, 1989) and affects a 55K polypeptide as well as two-thirds of the 32K human host range gene product. Deletion III is located at the right-hand side of the *Hin*dIII A fragment and leads to the loss of 3·5 kb including the only *Sma*I restriction site. Detailed restriction site mapping of this region was carried out using cloned *Eco*RI–*Hin*dIII fragments from the right side of *Hin*dIII A of CVA wild-type and CVA 382 (Fig. 3). Although CVA 382 and MVA display a haemagglutinin-negative phenotype (HA^-), the haemagglutinin gene (Shida, 1986) does not seem to be affected by this deletion.

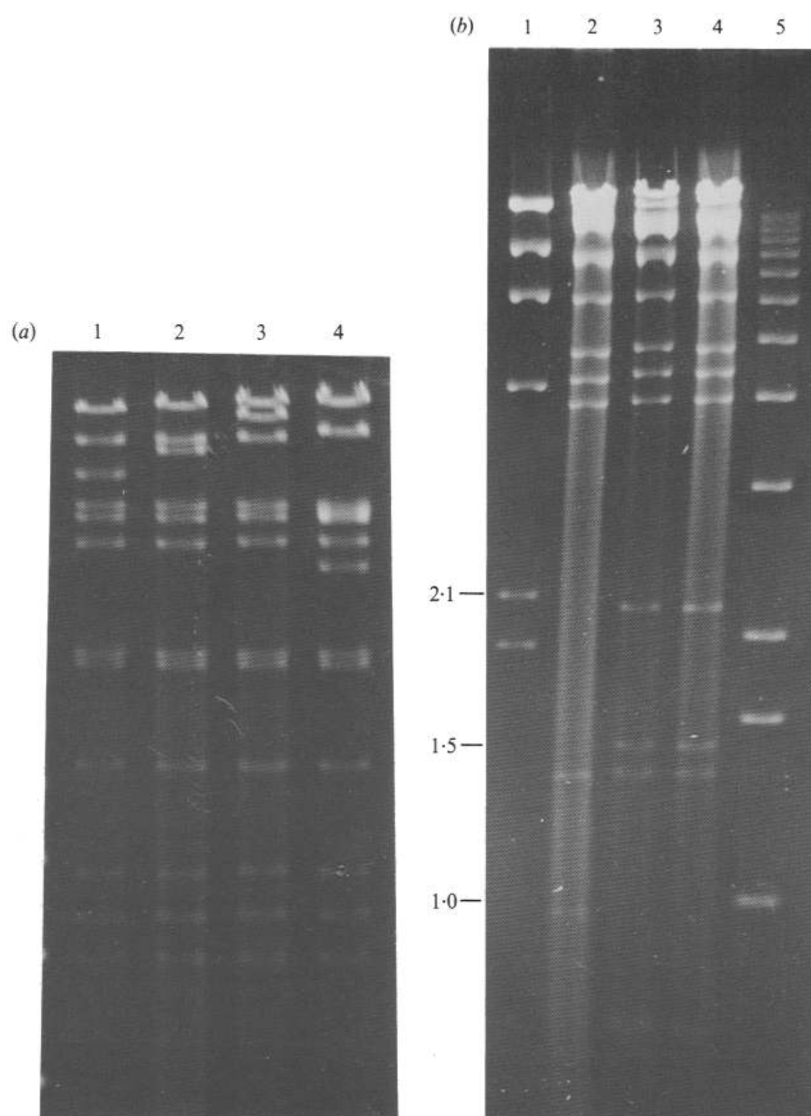
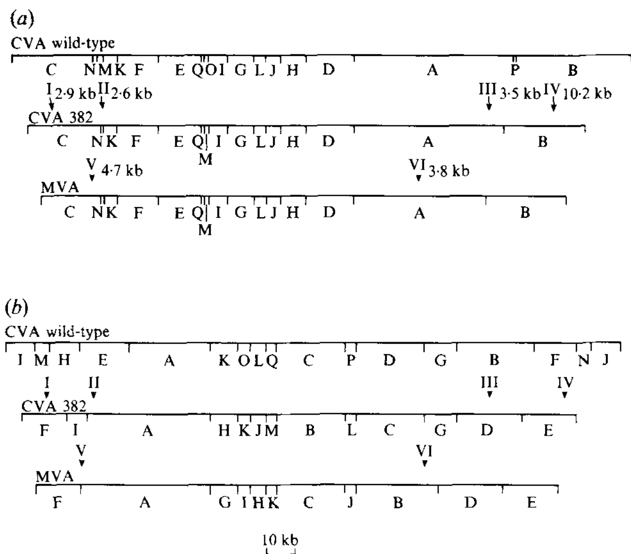


Fig. 1. (*a*) Electrophoresis of *Hin*dIII digests of DNAs from vaccinia virus MVA (lane 1), CVA 382 (lane 2), CVA wild-type (lane 3) and vaccinia virus Elstree (lane 4). The digests were electrophoresed on a 0·5% agarose gel. (*b*) Electrophoresis of *Hin*dIII digests of DNAs from CVA 382 (lane 2), CVA wild-type (lane 3) and vaccinia virus Elstree (lane 4). M_r standards: λ *Hin*dIII DNA (lane 1) and kb size ladder (lane 5). The digests were electrophoresed on a 1·2% agarose gel.

After a further 190 passages leading to MVA, two additional deletions occurred, one being located again in the *Hin*dIII C fragment (deletion V: 4·7 kb), the other in the middle of the *Hin*dIII A fragment (deletion VI: 3·8 kb). *Eco*RI clones of the *Hin*dIII C fragment of MVA and CVA 382 were used to map deletion V. This deletion reduces the size of the 5·8 kb *Eco*RI fragment, located at the very right end side, to 1·1 kb (Fig. 4). Deletion VI eliminates sequences of two adjacent *Eco*RI fragments (3·6 and 2·0 kb) and results in the formation of a new 1·8 kb fragment (Fig. 4), thus deleting nearly the entire A-type inclusion body gene (Funahashi *et al.*, 1988).

Marker rescue of the partially deleted host range gene

Non-permissive E-derm cells were infected with either MVA or CVA 382 and then transfected with plasmid pTZ 5.2, as we had hoped that by directly transfecting a non-permissive cell line only recombinant viruses would be able to replicate, allowing easy isolation. In fact, accumulation of rounded cells could be seen 2 to 4 days after infection. Subsequently, virus was isolated and after plaque purification named rec. MVA and rec. CVA 382, respectively. However cells infected with MVA or CVA 382 alone displayed no c.p.e., even after five



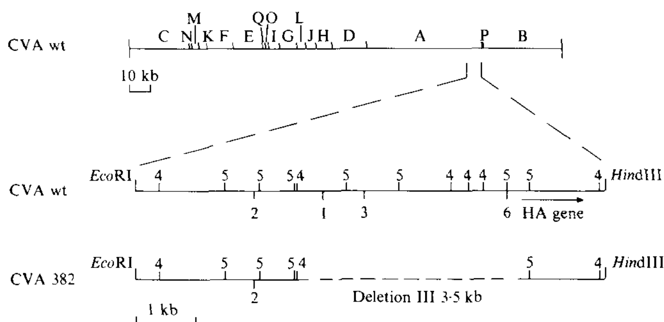


Fig. 2. Physical arrangement of (*a*) *Hin*dIII and (*b*) *Xho*I genome DNA fragments of vaccinia virus CVA wild-type, CVA 382 and MVA. Fragments are labelled alphabetically according to size. Location and size of six major deletions (I to VI) are indicated by arrowheads.

Fig. 3. *Hin*dIII restriction map of CVA wild-type DNA. The cloned 7·8 kb *Eco*RI–*Hin*dIII fragment of CVA wild-type containing the haemagglutinin gene (HA gene) is compared to the corresponding 4·3 kb *Eco*RI–*Hin*dIII clone of CVA 382. Dashed lines indicate the location of deletion III. Restriction sites are abbreviated as follows: 1, *Sma*I; 2, *Bam*HI; 3, *Pst*I; 4, *Cla*I; 5, *Acc*I; 6, *Sal*I.

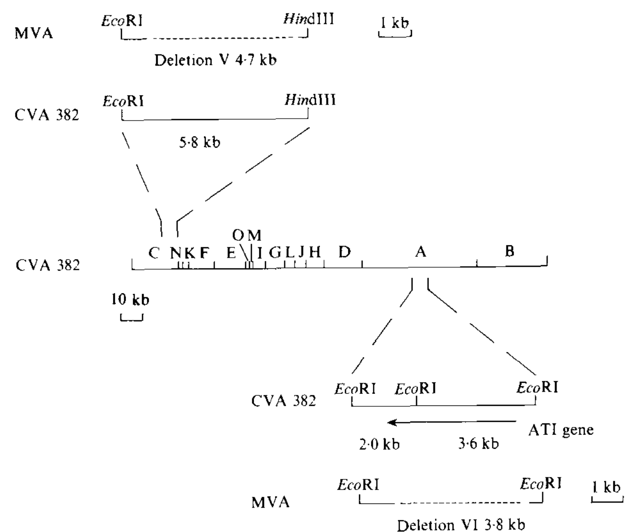


Fig. 4. *Hin*dIII restriction map of CVA 382 DNA. Deletions V and VI in the MVA relative to the CVA 382 genome are indicated by dashed lines.

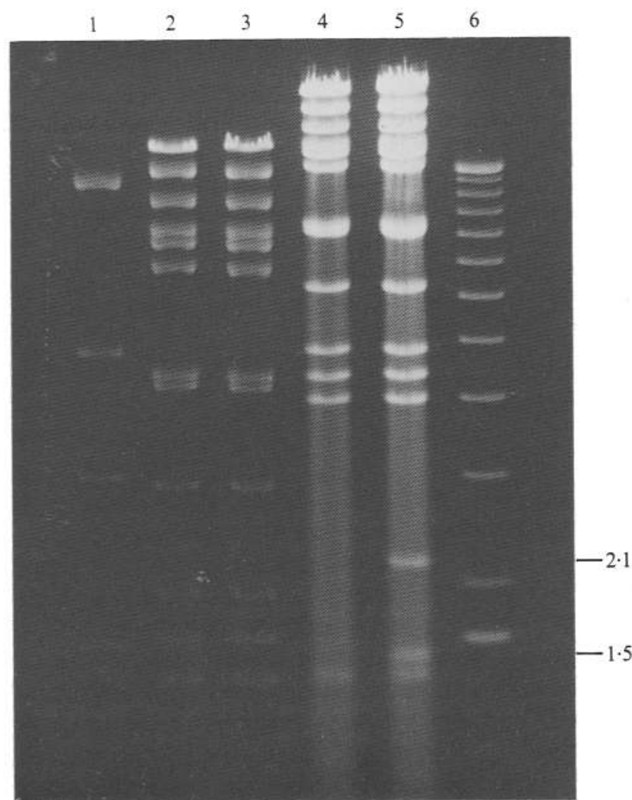


Fig. 5. *Hin*dIII digests of DNA from vaccinia virus MVA (lanes 2 and 4) and recombinant rec. MVA (lanes 3 and 5) in a 0·6% agarose gel. The digests were electrophoresed for 36 h (lanes 1, 2 and 3) and 18 h (lanes 4, 5 and 6). M_r standards: λ *Hin*dIII DNA (lane 1) and kb size ladder (lane 6).

continuous passages. To identify the rearranged fragments, DNA from rec. MVA and rec. CVA 382 was cleaved with *Hin*dIII (Fig. 5, data for CVA 382; data for rec. CVA 382 are not shown). Gel electrophoresis demonstrated that the deletion had been restored since both recombinant viruses possessed the expected *Hin*dIII 2·1 and 1·5 kb fragments characteristic of CVA wild-type.

Phenotypic characterization

The host range was tested on 14 different cell lines (Table 1). CVA wild-type virus was able to multiply in a broad range of different cell lines. Seventy-two hours p.i., titres measured on CEFs had usually increased 100- to 3000-fold compared to titres at the end of the adsorption period. However, CVA wild-type was not able to

Table 1. *Multiplication of vaccinia virus CVA wild-type, MVA and recombinant viruses rec.MVA and rec.CVA 382 in different cell lines*

Cell type	CVA wt	MVA	rec.MVA	rec.CVA 382
Chick				
CEF	1778*	13335	16125	10000
LSCC-H-32	100	316	ND†	ND
Human				
HeLa	1334	0·6	4·2	4
MRC 5	1778	0·4	1·4	4
HRT 18	316	0·8	7·5	8
Hep-2	3162	0·2	42	56
Monkey				
Vero	1000	4	56	56
MA 104	316	237	177	133
Rabbit				
RK_{13}	237	0·6	3162	4217
Equine				
E-derm	422	0·8	1000	1778
Bovine				
BEL	316	4	0·3	0·2
MDBK	2	0·6	ND	ND
Canine				
MDCK	1334	0·2	1	6
Mouse				
DBT	0·6	0·4	ND	ND

* The values represent the ratio of the titres obtained after 72 h p.i. over the titres in the cultures at the end of the adsorption period.
† ND, Not determined.

replicate in mouse DBT or bovine MDBK cells. In contrast, the attenuated strain MVA was restricted to primary and permanent chick fibroblasts and to the monkey cell line MA 104. Three days after infection virus yields were comparable to the CVA wild-type strain or in the case of the CEFs 10 times higher. In parallel the same experiments were done for the recombinant viruses rec.MVA and rec.CVA 382. Reintroduction of the K1L and the adjacent gene, encoding a 55K protein, extended the host range of the recombinant viruses. Virus yield obtained on non-permissive E-derm and RK_{13} cells were even higher than the yield of CVA wild-type. Vero and Hep-2 cells were also permissive for multiplication, albeit to a limited extent. The existence of three kinds of infections with the recombinant viruses, i.e. permissive, semi-permissive or non-permissive, is clearly illustrated in comparative growth curves (Fig. 6).

After infection, pocks produced on the chorioallantoic membrane (CAM) of embryonated eggs by CVA wild-type were large with a deep, central necrosis. On the other hand pocks produced by MVA, CVA 382 as well as by the recombinant viruses rec.MVA and rec.CVA 382 were small, proliferative and without necrosis.

Baby mice infected i.p. or i.c. with CVA wild-type died 2 to 3 days after infection (Table 2). In contrast, all

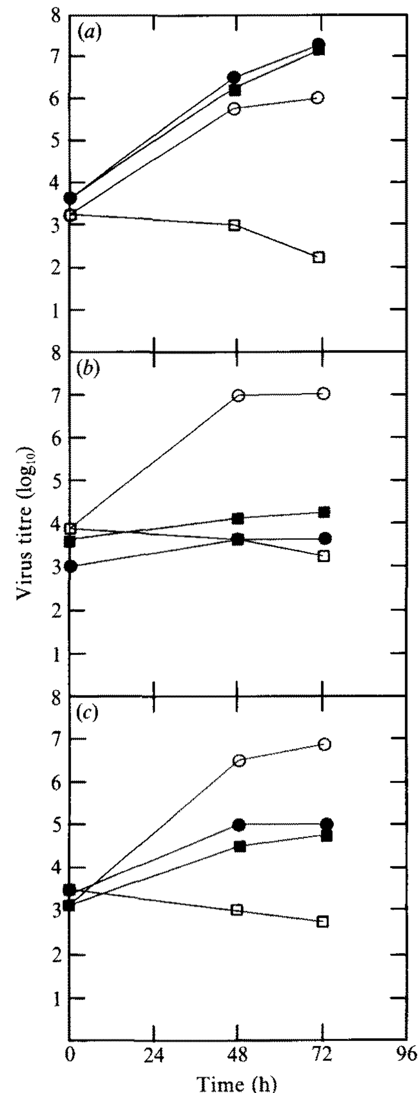


Fig. 6. Multiplication of vaccinia virus CVA wild-type (○) and MVA (□) as well as recombinants, rec.MVA (■) and rec.CVA 382 (●) in three different cell types: (*a*) RK_{13}; (*b*) HeLa; (*c*) Hep-2.

Table 2. *Virulence of vaccinia virus CVA wild-type, MVA and recombinant rec.MVA after i.p. or i.c. inoculation**

	Virus	No. of animals	Application i.c. Ill	Application i.c. Died	Application i.p. Ill	Application i.p. Died
Baby mice	CVA wt	30	30/30	30/30	30/30	30/30
	MVA	15	0/15	0/15	0/15	0/15
	rec.MVA	30	0/30	0/30	0/30	0/30
Mice 6-week	CVA wt	10	10/10	3/10	10/10	0/10
	MVA	10	0/10	0/10	0/10	0/10
	rec.MVA	30	0/30	0/30	0/30	0/30

* NMRI mice were inoculated either i.p. with 3×10^6 or i.c. with 2×10^4 $TCID_{50}$, baby mice with 1×10^5 and 1×10^4 respectively.

animals infected with either MVA or rec. MVA survived without any clinical symptoms. In adult mice all animals infected i.c. with wild-type virus showed marked signs of illness and three out of 10 died. After i.p. infection all animals developed a generalized infection with multiple pocks starting 7 days after infection. Animals infected with MVA or rec. MVA never showed any signs of illness.

Discussion

In this study we compared the genomes of the strongly attenuated strains MVA and CVA 382 with the ancestral CVA wild-type strain. We provide evidence that during attenuation several alterations in the genome have occurred. Owing to limitations of the technology used, deletions smaller than 0·3 kb are difficult to detect and point mutations would be missed. However, the size of six major deletions identified so far adds up to about 30 kb. In other words, nearly 15% of the entire genome is non-essential for virus replication *in vitro*. Mutants of vaccinia virus strains have been described with as much as 21·7 kb of DNA deleted from the left terminus (Drillien *et al.*, 1981; Perkus *et al.*, 1989). The sizes we determined for the genomes of CVA wild-type and vaccinia virus Elstree (208 and 200 kb respectively) have been obtained after subcleavage of isolated large *Hin*dIII and *Xho*I fragments. Our values are higher than data from other authors (Esposito & Knight, 1985; Mackett & Archard, 1979). They determined the size by using comigrating λ *Hin*dIII fragments as an M_r marker only. However, our results are confirmed by sequence data published recently (Goebel *et al.*, 1990) and match those of Bostock (1988) who determined genomic sizes by pulsed-field electrophoresis.

Mapping of the deletions and marker rescue experiments are first steps to defining functions involved in attenuation. During the first 382 passages four major deletions could be identified, decreasing the size of the CVA wild-type genome from 208 to 188 kb. Two deletions (I and IV) are located in the terminal fragments. Neither could be mapped precisely because of transposition of sequences (unpublished data). This will be the subject of further research. The presence of long stretches of non-essential DNA near the two ends of the genome is still an unexplained structural feature of the orthopoxviruses (Gangemi & Sharp, 1976; Panicali *et al.*, 1981; Paez *et al.*, 1985). It is assumed that these sequences encode a variety of proteins which interact with the host (Moyer *et al.*, 1980; Moss *et al.*, 1981; Pickup *et al.*, 1986; Kotwal & Moss, 1988; Smith *et al.*, 1989).

Therefore deletions or mutations can attenuate virus pathogenicity (Buller *et al.*, 1985; Dallo & Esteban, 1987). Additionally both terminal regions can readily undergo complex sequence rearrangements or transpositions during continuous propagation in cell cultures (Moyer *et al.*, 1980; Esposito *et al.*, 1981; Pickup *et al.*, 1984). Deletion II has been described for the MVA strain by Altenburger *et al.* (1989): a 2·6 kb deletion eliminates most of the two adjacent 2·1 and 1·5 kb fragments resulting in a new 1·0 kb fragment. From our findings it is clear that this deletion occurred during the first 382 passages and remained stable throughout the next 190 passages leading to MVA. During attenuation, 3·5 kb from the right side of the *Hin*dIII A fragment have been eliminated. Although no deletions or mutations within this region have been described so far, it is non-essential for replication *in vitro*. According to Goebel *et al.* (1990), in the vaccinia virus strain Copenhagen these sequences contain three proposed open reading frames but their function has not been defined. Although CVA 382 displays an HA^- phenotype in contrast to CVA wild-type, the deletion does not affect the haemagglutinin gene. Hovever, an HA^- phenotype can be caused by a single point mutation (Shida, 1986).

During the next 190 passages two deletions occurred and reduced the size from 188 to 178 kb for the MVA strain. Once again the left end of the genome was affected. Whether this deletion contributes to a further decrease in virulence cannot be evaluated because CVA 382 displays the same attenuated phenotype as MVA. The second deletion affects the gene encoding the major protein of the A-type inclusion body (ATI). Despite the morphological absence of ATIs in vaccinia virus compared to cowpox, monkeypox or ectromelia viruses, an antigenically related protein is induced (Kitamoto *et al.*, 1986). The ATI gene of cowpox virus has been mapped and sequenced recently (Funahashi *et al.*, 1988). In vaccinia virus, approximately 400 bp starting at the initiation codon of the vaccinia virus ATI gene equivalent have been sequenced by Patel *et al.* (1988). Compared to the corresponding sequences of cowpox virus there are only two bases which are different. From several conserved restriction enzyme sites the authors assume that both ATI genes are probably located in the same position. Although the ATI gene is one of the most strongly expressed genes it is not essential for replication in cell culture (Patel *et al.*, 1988). Mature viruses are occluded into the ATI and it has been assumed that such bodies protect the virus during dissemination from animal to animal. Our findings are confirmed by the use of a monoclonal antibody which was raised against the major protein of the ATI and reacts with CVA 382 but not with MVA (C. P. Czerny, personal communication).

To understand more precisely the significance of a restricted host range in virulence, marker rescue experi-

ments of the host range phenotype were undertaken. For our experiments we used an *Eco*RI fragment which upon insertion into the TK gene locus of the Copenhagen host range mutant restored a wild-type phenotype (Gillard *et al.*, 1985). According to Altenburger *et al.* (1989) this fragment overlaps the entire host range deletion of the MVA strain as well as some undeleted sequences flanking both ends. In our experiments we transfected MVA- or CVA 382-infected cells with the corresponding 5·2 kb CVA wild-type fragment. It has been shown that insertion of the K1L gene which leads to an increased host range can be used as a selection system for recombinant viruses expressing foreign genes (Perkus *et al.*, 1989). However, our results indicate that insertion of the wild-type host range gene did not restore the wild-type phenotype; in fact the recombinants rec. MVA and rec. CVA 382 combined properties of the wild-type and the attenuated strain. On the one hand their yield on a few cell lines was comparable to that of the CVA wild-type, and on the other hand c.p.e., plaque morphology on the CAM and complete avirulence for mice were identical to the attenuated strains MVA and CVA 382.

This strongly implies that one or more other genes determining the host range of CVA wild-type are affected. One possible gene is the recently identified 'second' host range gene (ORF C7L) which has been described for the vaccinia virus Copenhagen strain by Perkus *et al.* (1990). Either C7L or the K1L host range gene is necessary and sufficient to allow replication. From hybridization data (G. Sutter, unpublished results) the C7L gene seems to be conserved in the MVA strain but it is not yet clear whether it is functionally active. In cowpox virus a 'third' host range gene has been identified which is responsible for multiplication in Chinese hamster ovary cells and which is independent of the K1L gene (Spehner *et al.*, 1988). From transfection experiments all three host range genes appear to play overlapping roles in determining replication competence on various cell lines.

From the data obtained, marker rescue of the host range gene had no effect on the small plaque size phenotype and on the pock morphology of the attenuated strains. One might speculate whether reduced virulence is caused by a restricted host range and subsequently by the inability to multiply *in vivo* or whether it is related to the altered phenotype in cell cultures and on the CAM or a combination of both.

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