

Tissue Culture Adaptation of Foot-and-Mouth Disease Virus Selects Viruses That Bind to Heparin and Are Attenuated in Cattle

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Isolates of foot-and-mouth disease virus (FMDV) exist as complex mixtures of variants. Two different serotype O1 Campos preparations that we examined contained two variants with distinct plaque morphologies on BHK cells: a small, clear-plaque virus that replicates in BHK and CHO cells, and a large, turbid-plaque virus that only grows in BHK cells. cDNAs encoding the capsids of these two variants were inserted into a genome-length FMDV type A12 infectious cDNA and used to produce chimeric viruses that exhibited the phenotype of the original variants. Analyses of these viruses, and hybrids created by exchanging portions of the capsid gene, identified codon 56 in VP3 (3056) as the critical determinant of both cell tropism and plaque phenotype. Specifically, the CHO growth/clear-plaque phenotype is dependent on the presence of the highly charged Arg residue at 3056, and viruses with this phenotype and genotype were selected during propagation in tissue culture. The genetically engineered Arg 3056 virus was highly attenuated in bovines, but viruses recovered from animals inoculated with high doses of this virus had lost the ability to grow in CHO cells and contained either an uncharged residue at 3056 or a negatively charged Glu substituted for a Lys at a spatially and antigenically related position on VP2 (2134). Comparison of these animal-derived viruses to other natural and engineered viruses demonstrated that positively charged residues are required at both 2134 and 3056 for binding to heparin. Taken together, these results indicate that *in vitro* cultivation of FMDV type O selects viruses that bind to heparin and that viruses with the heparin-binding phenotype are attenuated in the natural host.

Foot-and-mouth disease (FMD), a highly infectious disease of cloven-hoofed animals, remains a major economic concern for livestock health in many developing countries and a continued threat to countries that are disease free. The etiological agent of this disease, the FMD virus (FMDV), belongs to the *Aphthovirus* genus of the *Picornaviridae*. The virion consists of an icosahedral shell composed of 60 copies each of four structural proteins, VP1 to VP4, surrounding a single-stranded, positive-sense RNA (37). Multiple antigenic sites have been found on the capsid of the virus, and several of these consist of conformational epitopes made up of portions of more than one capsid protein (24).

RNA viruses such as FMDV exist as a quasispecies, with individual members of the population expressing different genotypic and phenotypic properties (26). The presence of a mixture of genomes within the virus population allows for the rapid selection of variants upon changes in environment, such as immune pressure *in vivo* or *in vitro*, or the introduction into a new host. Selection of variants with more fit genomes from FMDV populations can occur during persistent infection of animals (13) or during propagation in cell culture (8, 40). We have shown that for FMDV type A12, viruses with specific differences in VP1 that affect cell binding can be selected upon introduction of animal-derived viruses into cell culture (31), and Meyer and coworkers have demonstrated that FMDV

variants present in a mixed population were selected by growth on different cell culture systems or in different animals (28).

A highly immunogenic loop between the G and H beta strands of VP1 (1, 20) contains a conserved Arg-Gly-Asp (RGD) sequence that has been implicated in cell interaction, since RGD-containing synthetic peptides inhibit virus binding to cells (3, 11). Moreover, the RGD sequence of type A12 virus is required for cell binding and infectivity, since viruses with mutations (23) or deletions (27) of this sequence are noninfectious for cells in culture. In addition, type A12 viruses with deletions in this sequence are avirulent in cattle (27) even when administered at high doses (33). The essential role of the RGD sequence in infection by FMDV was also supported by studies showing that antibodies to the RGD-binding integrin, $\alpha_v\beta_3$, can inhibit binding of FMDV type A12 to cells in culture (7). Integrins have also been identified as receptors for two other picornaviruses, ECHO virus (6) and coxsackievirus type A9 (CAV9) (36).

Acquisition of RGD-mediated reception of CAV9 appears to be a recently acquired property of the virus, since the RGD is encoded by a sequence inserted at the C terminus of VP1, which is not present in other coxsackievirus serotypes or subtypes (9). Furthermore, CAV9 can attach to cells in an RGD-independent manner (35), and genetically engineered CAV9 which lacks the RGD is infectious (14), indicating that an alternative cell surface molecule can serve as the receptor for this picornavirus. Thus, it appears that picornaviruses can evolve to utilize more than one method to enter susceptible cells.

FMDV can also enter cells through a non-integrin-mediated

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pathway. Specifically, antibody-complexed virus can enter cells that express the Fc receptor (4, 22). In addition, we have engineered a novel receptor for type A12 virus, which consists of the virus-binding fragment of an FMDV-specific monoclonal antibody (MAb) fused to ICAM-1 (33). The ability to genetically engineer an alternative receptor for FMDV suggests that this virus could acquire a new receptor specificity, although the acquisition of an affinity for a new receptor would be expected to alter viral tropism *in vitro* as well as *in vivo*.

Recently, Jackson et al. (15) investigated the binding of FMDV serotype O1 to heparan sulfate (HS), a ubiquitous cell surface glycosaminoglycan (GAG). They demonstrated specific affinity of the virus for this GAG and postulated that HS binding was required for efficient infection of cells via a two-step interaction of virus binding to HS followed by cell surface integrin-mediated internalization.

Here, we identify capsid residues of FMDV serotype O1 viruses which simultaneously affect plaque phenotype, virus host range in cell culture, and binding to heparin. We demonstrate that heparin-binding viruses are selected by cultivation *in vitro* and are attenuated in bovines. In addition, viruses recovered from bovines inoculated with high doses of a heparin-binding virus contain changes in the capsid that abolish their binding to heparin.

MATERIALS AND METHODS

Cell lines, viruses, and cDNAs. Baby hamster kidney (BHK) cells, strain 21, clone 13, and Chinese hamster ovary (CHO) cells were maintained as previously described (22, 34). For virus adaptation to CHO cells, the cell monolayers were rinsed with a low-pH buffer (140 mM NaCl, 25 mM morpholineethanesulfonic acid [pH 6.0]) after 1 h of virus adsorption, to inactivate virus that had not been internalized. For serial passages, infected 35-mm-diameter BHK cell monolayers were frozen and thawed, and a portion (1×10^6 to 10×10^6 PFU) was used to inoculate a fresh monolayer. Following virus adsorption (with rocking periodically for 60 min at 37°C), Eagle's basal medium (BME) with 0.5% calf serum was added, and the culture was maintained overnight at 37°C and then frozen for subsequent passage. Plaque assays were performed as previously described (34), and cells were stained at 48 h (BHK) or 72 h (CHO). O1 Campos Vallee vaccine seed originated from infected tongue epithelium supplied by the Brazilian Ministry of Agriculture to Vallee, Montes Claros, Minas Gerais, Brazil, where it was propagated three times on BHK cells for use as a vaccine production seed. This virus was passed three times more on BHK cells, and the BHK passage 6 virus is designated O1CVa throughout this work. A second O1 Campos vaccine seed was obtained from Vecol, Bogota, Colombia, as a suspension of infected bovine tongue epithelial tissue. O1 BFS 1860 was obtained from F. Brown, Plum Island Animal Disease Center (PIADC), and the O1 Campos isolate, O1att, was obtained from D. Moore, PIADC. The genome-length infectious cDNA plasmid of FMDV type A12 (pRMC35 [34]) and derivatives of this plasmid (32) have been described elsewhere. pAMP-O1P1/2, a pAMP (Life Technologies, Gaithersburg, Md.) derivative containing the P1-2B region obtained from the fifth BHK passage of the O1 Campos virus obtained from the Brazilian Ministry of Agriculture via Vallee (see above), will be described in detail elsewhere.

RNA isolation cDNA synthesis, PCR amplification, and sequencing. RNA was isolated from animal tissues, infected cell lysates, culture fluids harvested from infected cells, or individual viral plaques by using Trizol (Life Technologies). Viral cDNAs were synthesized with Moloney murine leukemia virus reverse transcriptase (Life Technologies), using random hexamers as primers, and cDNA fragments were amplified by using PCR (38) and specific oligonucleotides. In some cases, amplified fragments were ligated to plasmid DNA vectors by using standard techniques, and resulting plasmid DNAs were sequenced with either Sequenase version 2.0 (Amersham, Arlington Heights, Ill.) or the Fidelity sequencing system (Oncor, Gaithersburg, Md.). In other cases, the PCR-amplified cDNAs were sequenced directly by using a Sequenase PCR sequencing kit (Amersham).

Construction of genome-length infectious cDNAs of type A12, containing the type O1 capsid sequences. cDNA fragments corresponding to the type O1 capsid region were obtained by PCR amplification of viral cDNA or DNA from plasmid pAMP-O1P1/2. These cDNAs were amplified in two pieces, to improve the fidelity of amplification. One piece, corresponding to VP0, VP3, and a portion of VP1, was amplified by using a sense oligonucleotide (CCG CTT AAG GGC GCC GGA CAA TCC AGT CCA GCG ACC) containing an *Afl*III restriction endonuclease site at the 5' end of the VP0 sequence and an antisense oligonucleotide in VP1. The second piece, corresponding to the remainder of VP1, was amplified by using a sense oligonucleotide in VP3 and an antisense oligonucleotide (CGG CAG CTG TTT CAC CGG TGC CAC) containing a *Pvu*II site

added at the 3' end of the VP1 sequence. By using the added sites and a *Bam*HI site within VP1, the type O1 capsid sequences were substituted for the corresponding type A12 sequences in a derivative of pRMC35 (34), which contains the type A12 cDNA followed by a unique *Stu*I restriction site.

In vitro RNA synthesis and transfection. Plasmids containing genome-length cDNAs were linearized at the *Stu*I site following the poly(A) tract and used as templates for RNA synthesis, using a MegaScript T7 kit (Ambion, Austin, Tex.) as instructed by the manufacturer. BHK cells were transfected with these synthetic RNAs by using Lipofectin (Life Technologies) (34) or electroporation (23).

Radioimmunoprecipitation (RIP) and cell binding assays. Viruses were radiolabeled with [³⁵S]methionine and purified by sucrose density gradient centrifugation as previously described (5). Purified virions were immunoprecipitated with MAbs prepared against O1 Campos (2) or O1 Brugge (42) by incubating approximately 5,000 cpm of sucrose density gradient-purified virus with 50 μ l of hybridoma culture fluid and then precipitating the antigen-antibody conjugates with formalin-fixed *Staphylococcus aureus* (Calbiochem, San Diego, Calif.). Cell binding assays were performed with BHK or CHO cells as previously described (5).

Animal experiments. Eighteen- to twenty-month-old Hereford steers or heifers were inoculated by the intradermal lingual (IDL) route with three to five dilutions of virus prepared in BME. Fifty microliters of each dilution tested was inoculated into five sites on the surface of the tongue, and samples from the same dilution series were titrated on BHK cells. After inoculation, animals were examined daily and sedated on selected days for vesicular fluid collection.

Heparin-Sepharose binding studies. Heparin-Sepharose (Pharmacia, Uppsala, Sweden) was equilibrated in binding buffer (BME containing 0.1% bovine serum albumin and 25 mM HEPES [pH 7.3]) and resuspended as a 10% (vol/vol) slurry in binding buffer. Four to eight thousand cpm of sucrose density gradient-purified virus or 100,000 PFU of unlabeled, unpurified virus was diluted in binding buffer and added to the slurry. The virus/heparin-Sepharose suspension was mixed, incubated for 10 min at room temperature, and centrifuged for 1 min at $1,000 \times g$. Bound virus was eluted from the Sepharose matrix by mixing with various buffers, followed by incubation and centrifugation as described above. Virus recovered in the eluted fractions was quantitated by liquid scintillation counting or plaque assay.

RESULTS

Two different O1 Campos stocks contain readily distinguishable variants. Examination of an O1 Campos vaccine seed (BHK passage 6) from Vallee (O1CVa) revealed at least two plaque morphologies on BHK monolayers. One was small and clear (approximately 80% of the population; diameter ≤ 2 mm) and the second was large and turbid (approximately 20% of the population; plaque size = 5 mm). CHO cells were also susceptible to infection by O1CVa, but plaque assays performed on CHO cell monolayers revealed only a single small, clear-plaque phenotype. Virus selected by two serial passages on CHO cells (O1CVa/CHO) produced only small, clear plaques on BHK cells, indicating that passage in CHO cells efficiently selected this population from the original mixture. The O1CVa/CHO virus had a titer on BHK cell monolayers 10 times higher than that on CHO cell monolayers, indicating a 10-fold-higher plaque efficiency on BHK cells.

Viruses capable of growing on CHO cells were also detected in another O1 Campos vaccine seed. This seed, an infected bovine tongue epithelium homogenate obtained from the Vecol, contained a high titer of virus which displayed large (5- to 8-mm), turbid plaques when titered on BHK cells and a low titer of virus that formed small (2- to 4-mm), clear plaques on CHO cells (Table 1). However, virus selected from this homogenate by propagation on CHO cells formed clear plaques of 2 to 4 mm on BHK cell monolayers (Table 1).

Genome-length synthetic RNAs encoding O1 Campos P1 genes in a type A12 background are infectious. Since it appeared likely that the two variants found in the O1CVa seed would differ at many genetic loci, including those unrelated to plaque morphology, we devised a strategy based on reverse genetics to determine the basis of this phenotype. To this end, we substituted the O1 capsid-encoding regions for the capsid sequences in a type A12 infectious cDNA clone (pRMC35 [34]) (see Materials and Methods). Plasmid pCRM8 was con-

TABLE 1. Properties of O1 Campos viruses in cell culture

Virus source	Titer (PFU/ml)		Plaque phenotype on BHK cells	Genotype ^a			
	BHK	CHO		2130	3056	3060	1133
O1CVa ^b	6.8×10^7	7.5×10^6	Mixed	Cys	Arg or His	Ala, Gly, or Asp	Val or Glu
O1CVa/CHO ^c	9.0×10^7	8.5×10^6	Small/clear	Cys	Arg	Ala	Val
O1CVa/BHKp10 ^d	1.8×10^8	4.1×10^6	Small/clear	Cys	Arg	Ala or Gly	Val or Glu
O1C Vecol ^e	1.0×10^6	5.5×10^2	Large/turbid	Arg or Cys	His	Asp	Glu
O1C Vecol/CHO ^f	2.3×10^7	6.5×10^5	Small/clear	Cys	Arg	Gly	Glu

^a Coding capacity determined by reverse transcription-PCR sequencing in the regions where differences were found between pCRM4 and pCRM8 (see text and Fig. 3; regions sequenced included capsid codons 1131 to 1160, 2121 to 2140, and 3051 to 3065).

^b O1 Campos vaccine seed from Vallee, sixth BHK passage.

^c O1CVa passed two times in CHO cells.

^d Tenth BHK passage of passage of O1CVa.

^e O1C Vecol seed.

^f O1C Vecol seed passed two times in CHO cells. The sequenced regions of O1C Vecol/CHO revealed an additional substitution of Tyr for Cys at position 1134.

structed with a cDNA obtained from the pAMP-O1P1/2 plasmid, containing a cDNA of the BHK passage 5 virus (see Materials and Methods), and pCRM4 was made with the cDNA derived from the O1CVa/CHO (BHK passage 6, CHO passage 2) virus (see Materials and Methods).

The properties of synthetic RNAs obtained from these two plasmid DNAs were tested in cell culture. The specific infectivities of these RNAs on BHK cell monolayers, determined by transfection using Lipofectin, were similar to each other and RNA of pRMC35 (34). BHK cells transfected with these RNAs by using electroporation displayed clear cytopathic effect within 16 h. Viruses (vCRM4 and vCRM8) obtained from cells transfected with their respective RNAs were serially passed at least two times to ensure elongation of their poly(C) tracts (34). Following passages, both viruses achieved titers of approximately 10^8 PFU/ml on BHK cells, similar to titers of O1CVa, demonstrating that A12 nonstructural proteins can functionally replicate O1 capsids. vCRM8, which was made with the BHK-grown virus, produced only turbid, large plaques on BHK cells and was not able to grow in CHO cells, whereas vCRM4 produced small, clear plaques on both cell types. Therefore, each chimera represents one of the phenotypes observed in the O1CVa seed, and sequences in the O1 capsid regulate both plaque morphology on BHK cells and ability to grow on CHO cells.

Antigenic characterization of vCRM4 and vCRM8. O1 Campos is recognized by MABs which have been used for vaccine quality control at PanAftosa (2) and antibodies developed on Plum Island to O1 Brugge (42). We used a subset of these MABs, which react well with O1CVa in RIP assays (results not shown), to compare the antigenic structures of vCRM4 and vCRM8. These assays showed that vCRM4 and vCRM8 were distinguished by MAb 34CH4, suggesting that the determinant of plaque phenotype and host range *in vitro* overlaps with the 34CH4 epitope (Fig. 1).

Cell binding properties of vCRM4 and vCRM8. To determine if differences in cell tropism and plaque phenotype resulted from differences in affinity for cells, the cell binding properties of vCRM4 and vCRM8 were investigated. The data in Fig. 2A show that vCRM8 was unable to bind to CHO cells but did bind to BHK cells. As expected, vCRM4 bound to both cell types, and binding to BHK cells was much greater than that observed for vCRM8 (Fig. 2A). Cross-competition studies performed with an excess of unlabeled virus particles (100,000 per cell) showed that both vCRM4 and vCRM8 could prevent vCRM8 from binding to BHK cells, but neither of the unlabeled viruses could significantly lower binding of radiolabeled vCRM4 to these cells (Fig. 2B). These latter data suggest that

vCRM4 can bind to the same receptor as vCRM8 and also to a second receptor, of very high copy number per cell (resulting in the inability to saturate this receptor with unlabeled vCRM4), that is not bound by vCRM8. Binding of vCRM8, but not vCRM4, could be inhibited with unlabeled type A12 virus, suggesting that the receptor utilized by vCRM8 is the integrin $\alpha_v\beta_3$ (results not shown).

Genetic mapping of the residues involved in CHO binding and plaque morphology. Determination of the sequences of the capsid-encoding portions of pCRM4 and pCRM8 revealed four differences in predicted amino acid sequence, two in VP3 and one each in VP1 and VP2 (Fig. 3). To define the residues responsible for growth on CHO cells and plaque phenotype on BHK cells, we engineered additional chimeras by exchanging portions of pCRM4 and pCRM8 and tested the properties of viruses derived from these cDNAs (Fig. 3). Sequences of virus stocks derived from the synthetic transcripts of all of the genomes shown in Fig. 3 were verified in the regions surrounding codons 2130, 3056, 3060, and 1133, demonstrating that all five genetically engineered viruses were identical to their respective recombinant plasmids at these four positions.

Analyses of the growth properties of these five viruses revealed that the amino acids in the β B bulge of VP3 (3056/3060 [1]) conferred both plaque phenotype and ability to grow on CHO cells (Fig. 3). Specifically, viruses with a greater positive

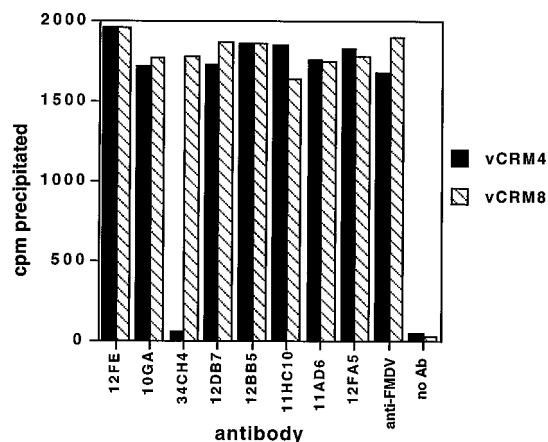


FIG. 1. Reactivities of chimeric viruses vCRM4 and vCRM8, which express type O1 Campos capsids, with different MABs. MABs are labeled as originally described (2, 42); anti-FMDV refers to a convalescent serum from a bovine inoculated with multiple serotypes of FMDV; no Ab indicates no antibody.

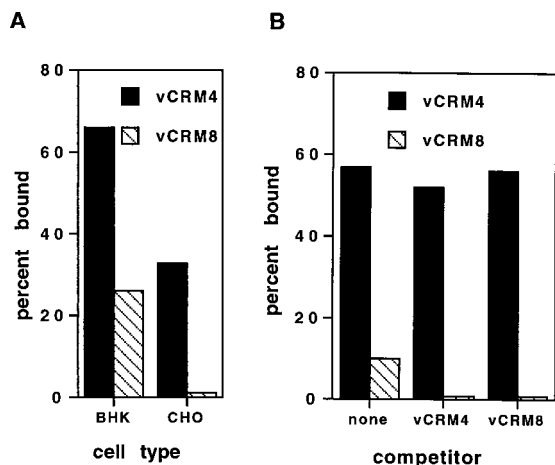


FIG. 2. Binding of different genotypes of O1/A12 chimera to BHK and CHO cells. (A) Binding of radiolabeled vCRM4 and vCRM8 to BHK or CHO cells; (B) binding of vCRM4 or vCRM8 to BHK cells alone or following the addition of the indicated unlabeled competitor virus (added at a concentration of 100,000 particles per cell).

charge in this region (Arg/Ala versus His/Asp) grew on CHO cells and exhibited small, clear plaques on BHK cells (Fig. 3). In addition, the presence or absence of a Cys residue at 2130, which can form an intermolecular disulfide bond with residue 1134 (1), or substitution of Val for Glu at 1133 had no influence on plaque morphology or CHO growth (Fig. 3). RIP analyses performed with vCRM48, vCRM84, and vCRM848 showed that only the virus with His/Asp at 3056 and 3060 (vCRM84) was capable of binding 34CH4. These results indicate that MA b 34CH4 reacts with antigenic site 4 of O1 (17), which is included within antigenic site D defined for type C viruses by Mateu et al. (25).

Adaptation of O1 Campos to cell culture. To determine the fate of the O1CVa population in BHK cells, a serial passage experiment was performed in these cells with this virus stock. After 10 passages of O1CVa, a virus with the small, clear-plaque phenotype on BHK cells became the principal population (>99%), and the large, turbid-plaque virus was undetectable. RNA extracted from this passage 10 virus stock was sequenced by reverse transcription-PCR, to reveal Cys 2130, Arg 3056, Ala or Gly 3060, and Val or Glu 1133 (Table 1). A similar result was obtained by passaging a 1:1 mixture of vCRM4 and vCRM8; vCRM4 completely overgrew vCRM8 after 10 passages on BHK cells, indicating that the competitive advantage of the O1 small, clear-plaque phenotype was due to differences in capsid sequences and independent of other viral genes. Taken together, these data suggest a competitive advantage of the Cys 2130 and Arg 3056 genotype during propagation in BHK cell cultures (Table 1).

Adaptation to CHO cells appears to occur more quickly. Sequence evaluation of O1CVa/CHO revealed that it represented a unique genotype present in O1CVa that was, as expected, identical to vCRM4 at positions 2130, 3056, 3060, and 1133 (Table 1). The O1C Vecol seed propagated on CHO shared the Cys 2130 and Arg 3056 genotype with vCRM4 and O1CVa/CHO but differed at 3060 and 1133, supporting the conclusion (see above [Fig. 3]) that substitutions at these latter two positions are not important determinants of BHK plaque phenotype or ability to grow in CHO cells.

Adaptation of O1 Campos to cattle. O1CVa, vCRM8, and vCRM4 were inoculated into bovines. O1CVa was inoculated

into animal 72 by the IDL route with five inoculations at each of the following dilutions of virus: 8, 80, and 800 PFU, introducing an aggregate dose of approximately 5×10^3 PFU. By 24 h, bovine 72 showed signs of FMD, with fever and lesions at some inoculation sites. By 48 h, lesions had developed at all 800-PFU inoculation sites, three of five 80-PFU sites, and none of five 8-PFU sites, establishing a 50% cattle infectious IDL dose (CIID₅₀) of approximately 80 PFU. By day 3, lesions had developed on all four feet, the nose, gums, and other sites on the tongue. Vesicular fluid was collected from the interdigital cleft (IDC) of the left fore foot of animal 72 on day 2 postinoculation. This IDC fluid contained a high titer of virus that produced turbid plaques on BHK cells and was almost completely free of virus able to form plaques on CHO cells (Table 2), indicating that the viral population was dramatically altered by a single animal passage. Sequence analyses of this animal-selected virus revealed that it contained multiple codons at positions 2130, 3056, 3060, and 1133 (Table 2). This genetic heterogeneity was evaluated by limited sequencing of six plaques obtained from BHK monolayers infected with the IDC fluid. Two genotypes were detected among these plaques (all were large/turbid): three were Arg 2130, His 3056, Asp 3060, and Glu 1133, identical to vCRM8, and three were Cys 2130, Cys 3056, Ala 3060, and Val 1133 (Table 2). This latter group of plaques was similar to vCRM4 except that they contained a codon for an uncharged Cys residue at position 3056, in place of the Arg codon found in vCRM4. Thus, all viruses recovered from bovine 72 encoded amino acids other than Arg at position 3056 (Table 2).

vCRM8 was administered to two bovines, 8 and 43. The tongue of animal 8 was given five inoculations of 5, 50, and 500 PFU (total dose of approximately 3×10^3 PFU), and animal 43 tongue was given five inoculations each of 7, 70, 700, and 7,000 PFU (total dose approximately 1.5×10^4 PFU). Both animals developed FMD with a time course similar to that for animal 72, inoculated with O1CVa (see above), with severe oral and pedal lesions. Evaluation of the lesions formed at 48 h postinoculation of bovines 8 and 43 yielded a CIID₅₀ of between 5 and 50 PFU for vCRM8, similar to results for O1CVa.

vCRM4 was inoculated in two animals, 41 and 44. Animal 41 was inoculated five times each with 2, 20, 200, 2,000 and 20,000 PFU of vCRM4 (total dose approximately 10^5 PFU). During the subsequent 6 days, this animal did not become febrile or express any other signs of FMD. On day 6 postinoculation, the animal was reinoculated with approximately 10^8 PFU of the same virus. The next day, this animal developed FMD, including fever, gum, and pedal lesions. Over the next few days, secondary lesions developed on the tongue but not at any of the inoculation sites, indicating that the inoculated virus had a CIID₅₀ of greater than 10^7 PFU. Virus present in IDC fluid collected on day 7 from an IDC lesion of animal 41 showed a high titer of large-plaque virus on BHK cells, and no virus capable of forming plaques in CHO cells was detected in this sample (Table 2). Sequence analyses of the virus in this IDC fluid revealed that the Arg codon at 3056 found in the inoculated virus had been replaced by a codon for an uncharged Cys residue (Table 2).

Animal 44 was inoculated five times with 560, 5,600, 56,000, and 560,000 PFU of vCRM4 (total dose approximately 3.1×10^6 PFU). This animal developed a fever 2 days postinoculation and a lesion on one foot at 3 days postinoculation. Lesions on the tongue were slow to develop and were fully manifest at only two of five sites of the highest dose at 4 days postinoculation, indicating a very high CIID₅₀ (approximately 5.6×10^5)

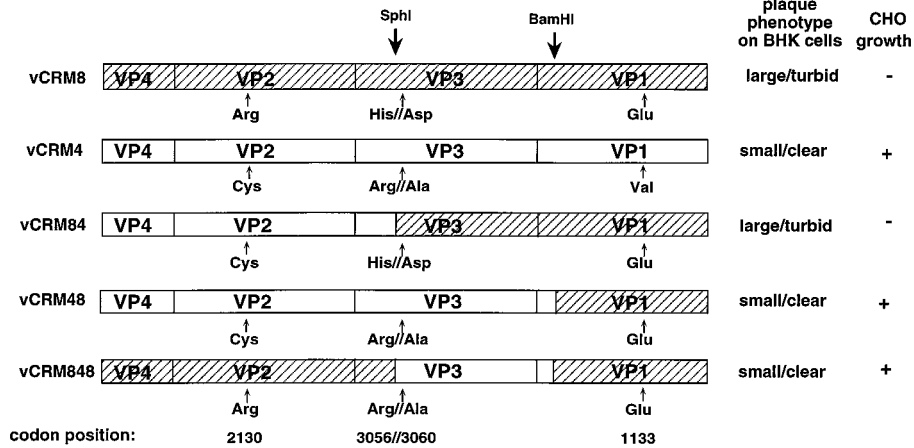


FIG. 3. Schematic diagram of the capsid regions of vCRM4, vCRM8, vCRM48, vCRM84, and vCRM848, showing the differences at the indicated codons, plaque phenotype, and CHO growth ability. Fragments derived from pCRM8 are shown as hatched bars, fragments derived from pCRM4 are shown as white bars, and the restriction endonucleases used to construct the vCRM48, vCRM84, and vCRM848 are shown at the top.

for this virus. As with animal 41, bovine 44 developed lesions on the gums and secondary lesions on the distal extremity of the tongue, but lingual lesions at the inoculation sites did not develop into the severe vesicles caused by vCRM8 or O1CVa. The IDC fluid collected on day 3 postinoculation contained a high titer of virus capable of forming plaques on BHK cells and a very small amount of virus able to form plaques on CHO cells (Table 2). RIP analyses performed with radiolabeled virus prepared from cells inoculated with bovine 44 IDC fluid did not react with MAb 34CH4, suggesting that the bovine 44 virus had an Arg at position 3056, as found for vCRM4, which was confirmed by direct sequencing of the PCR product obtained from the IDC fluid (Table 2). This was surprising since the bovine 44 virus was unable to grow on CHO cells and exhibited the large, turbid-plaque phenotype indicative of an uncharged residue at position 3056 (Table 2). However, further sequence analyses revealed that the virus in the bovine 44 IDC fluid contained a substitution of a negatively charged Glu for a Lys normally found at residue 2134, in the E-F loop of VP2. This positively charged Lys is in close proximity to the Arg 3056 in the structure of O1 BFS (Fig. 4), and both residues are in antigenic site D defined by Mateu et al. (25).

Tissue culture-adapted viruses bind heparin. Recently, Jackson et al. (15) proposed that cell surface HS is required for

efficient infection of FMDV serotype O1. To investigate the affinity of our type O1 viruses for HS, we examined their binding to heparin immobilized on Sepharose. Figure 5 shows that vCRM4 bound tightly to heparin at physiological salt concentrations but was eluted from the resin in high salt. Binding of radioactive vCRM4 to heparin-Sepharose could be reduced by the addition of soluble heparin at the binding step, confirming the specificity of the binding (Fig. 5A). vCRM8, on the other hand, did not display any binding to heparin-Sepharose under these conditions (Fig. 5), and neither vCRM4 nor vCRM8 bound to unconjugated Sepharose (results not shown). Evaluation of binding of several type O1 viruses revealed that all viruses capable of growing in CHO cells bound to heparin-Sepharose, including an O1 BFS which had been passaged on BHK cells more than 10 times (8a). However, all of the viruses that could not grow in CHO cells, including two viruses (bovine 41 and bovine 44) recovered from bovines inoculated with vCRM4, did not bind to heparin-Sepharose under these conditions (Fig. 5B).

DISCUSSION

Based on mapping of MAb-binding sites and the high conservation of the RGD sequence in FMDV VP1, Pfaff et al. (30)

TABLE 2. Properties of viruses recovered from bovines^a

Virus source (animal no.)	Titer (PFU/ml of fluid recovered from IDC vesicles)		Genotype ^b			
	BHK	CHO	2130	3056	3060	1133
72	3.4 × 10 ⁹	4.0 × 10 ²	Arg or Cys	YRC ^c	Ala or Asp	Glu or Val
Plaques 2, 3, and 5 ^d	ND ^e	ND	Arg	His	Asp	Glu
Plaques 4, 6, and 7 ^d	ND	ND	Cys	Cys	Ala	Val
41	2.8 × 10 ⁹	<2.5 × 10 ^{2f}	Cys	Cys	Ala	Val
44 ^g	2.7 × 10 ⁹	2.5 × 10 ³	Cys	Arg	Ala	Val

^a The plaque phenotype on BHK cells was large/turbid in all cases.
^b Coding capacity determined by reverse transcription-PCR sequencing in the regions where differences were found between pCRM4 and pCRM8 (see text, Fig. 3, and footnote a of Table 1); wherever mixtures of bases were detected, the possible codons are all listed.
^c Actual nucleotide sequence detected: pyrimidine (Y), purine (R), cytosine (C), encoding Arg, Cys, His, or Tyr.
^d Properties of six different plaques (2 to 7) picked from bovine 72 virus, grouped by genotype.
^e ND, not determined.
^f No plaques detected in the lowest dilution tested.
^g The sequenced regions of the bovine 44 virus revealed an additional substitution of Glu for a Lys at position 2134 (see text and Fig. 4).

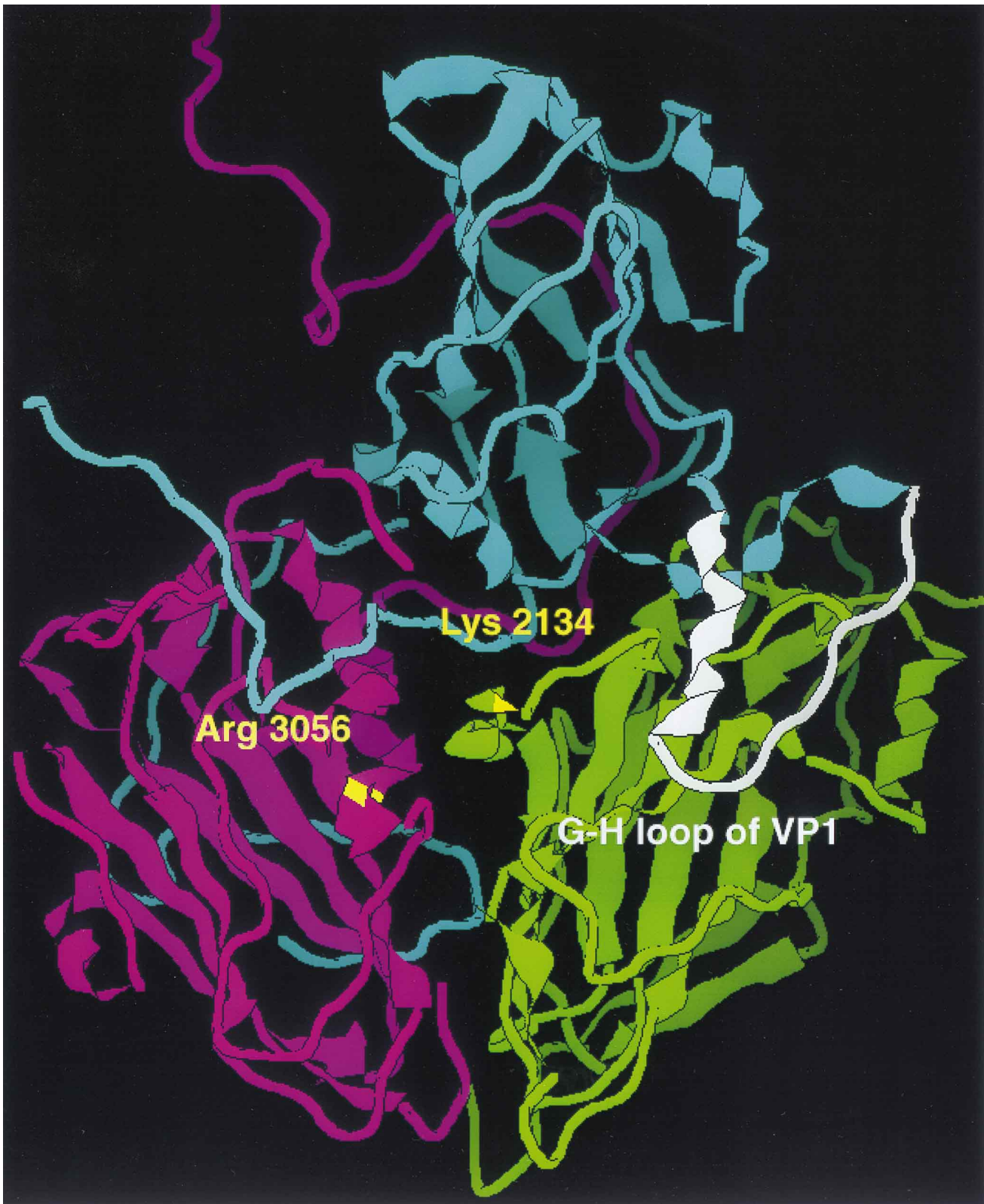


FIG. 4. Positions of the Arg 3056 and Lys 2134 on a ribbon diagram of the biological protomer of FMDV type O1, reduced (20). The distance between these two residues is 9 Å (measured between the guanidinium C of Arg 3056 and the terminal N of Lys 2134). VP1 is represented in cyan, VP2 is represented in green, and VP3 is represented in violet. Residues 3056 and 2134 are shown in yellow, and the G-H loop of VP1 is shown in white. The figure was generated by using MolView 1.3 (39).

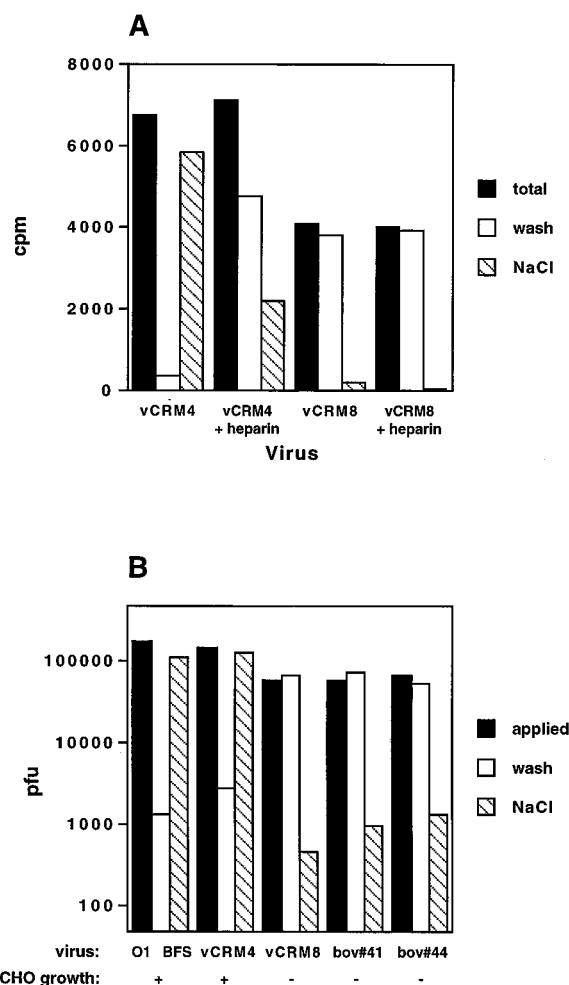


FIG. 5. Binding of different type O1 viruses to heparin-Sepharose. (A) Binding of radiolabeled vCRM4 or vCRM8 to heparin-Sepharose in the presence or absence of heparin (1 mg/ml). Total, sum of cpm recovered in the unbound, binding buffer, binding buffer plus 1 M NaCl, and binding buffer plus 0.5% sodium dodecyl sulfate fractions; wash, sum of cpm recovered in the unbound and binding buffer fractions; NaCl, cpm recovered in the binding buffer plus 1 M NaCl fraction. (B) Binding of unlabeled viruses. Applied, virus added to the resin; wash, virus recovered in the combined unbound and binding buffer fractions; NaCl, virus recovered in the combination of two successive binding buffer plus 1 M NaCl fractions. bov#41 and bov#44, isolates from bovines 41 and 44.

proposed that the RGD sequence of FMDV could function in cell binding. The importance of this sequence in virus interaction with cells has been demonstrated by molecular genetic approaches and biochemical studies showing that synthetic peptides and anti-integrin antibodies can inhibit virus binding to cells (see the introduction). Recently it has been suggested that cell binding via the RGD sequence on viruses of serotypes O1 could be facilitated by initial interaction of the virus with HS (15), a cell surface molecule that has been implicated as a cellular receptor for several other viruses, including several herpesviruses (41, 44) and human immunodeficiency virus (29).

Here we show that clear-plaque variants of FMDV type O1 Campos are readily selected upon passage of virus stocks in cell culture, and we demonstrate that cell culture adaptation is accompanied by acquisition of an Arg at 3056 and a change in antigenic structure. Genetically defined virus populations carrying Arg 3056 displayed the following characteristics: (i) at-

TABLE 3. Summary of published sequence data for positions 3056 and 3060 of other FMDV type O1 isolates

Virus isolate	Passage(s) ^a	Genotype		Reference
		3056	3060	
O1K	BHK, 7 and 64	Arg ^b	Gly ^b	10
O1K	Low	His	Asp	17
O1K (B64)	BHK, 64	Arg	Gly	17
O1K	BHK, 65	Arg	Gly	21
O1K	Low cell	His	Gly	21
O1K	Low animal	His	Gly	21
O1 Lausanne	? ^c	Arg	Gly	30
O1 Lausanne	Low	His	Asp	17
O1 Wettmar	Low	His	Gly	21
O1 BFS 1860	Low	His	Gly	17
O1 BFS 1860	?	Arg	Gly	1

^a See reference.

^b Ambiguity reported at these positions; see reference 10 and text.

^c ?, not indicated in reference.

tenuation in bovines, (ii) increased affinity for BHK cells, (iii) ability to bind to and grow in CHO cell cultures, and (iv) affinity for heparin. Previous work on Plum Island with a tissue culture-passaged variant obtained from a different seed of O1 Campos demonstrated that the Arg codon at position 3056 of this attenuated virus (O1att) was replaced by a His codon following passage through bovines (16). As expected, O1att exhibited tight binding to cells in tissue culture (16) and is able to form plaques on CHO cells (results not shown).

Examination of the literature reveals significant amino acid sequence variation at position 3056 and 3060 of other type O1 FMDVs (summarized in Table 3). In the case of O1 Kaufbeuren (O1K), Forss et al. (10) identify these residues as Arg and Gly but point out ambiguity at these positions due to differences between cDNA molecules derived from low- and high-passage-number virus stocks (Table 3). Kitson et al. (17) reported that their high-passage-number O1K (B64) encodes Arg/Gly at 3056/3060, whereas their low-passage-number O1K encoded a His and Asp at these positions (Table 3). Sequencing of one O1 Lausanne isolate revealed His/Asp at 3056/3060 (17), although Pfaff et al. (30) reported an O1 Lausanne sequence of Arg/Gly (Table 3). In addition, analyses by Marquardt et al. (21) of one high-passage-number and two low-passage-number samples of O1K and one low-passage-number isolate of O1 Wettmar revealed an Arg at 3056 in the high-passage-number stock and a His at this position in all three low-passage-number isolates (Table 3). In the case of the closely related O1 BFS strain 1860, both of these codons have also been reported by different groups for this position (Table 3). Taken together, these data demonstrate that the 3056 position is a major site for genetic variation among type O1 viruses, with the acquisition of an Arg residue readily occurring upon tissue culture propagation.

Interestingly, the O1 viruses used by Jackson et al. (15) for their examination of binding to HS (B64 and O1 BFS 1860) display the ability to form plaques on CHO cells, suggesting that these strains contain Arg residues at 3056. In the case of B64, the sequence of 3056 is reported to be Arg, and for O1 BFS 1860, an Arg has been reported for the 1860 isolate used by this laboratory (Table 3). Our evaluation of viruses selected by growth either in cell culture or in bovines indicates that the overall charge at or near position 3056 plays an important role in determining if the virus can bind to heparin. This is clearly demonstrated by the complete correlation of heparin binding and CHO growth by genetically engineered

viruses that contain an Arg at 3056. The surprising results obtained with viruses recovered from animals inoculated with vCRM4 make the point even more clearly. One of the virulent revertants detected appears to have lost its heparin affinity by a back-mutation at 3056, while the other animal-derived virus maintained the Arg at 3056 but also contained the substitution of a codon for a Glu for a Lys at a neighboring residue in VP2 (residue 2134) (Fig. 4). This latter substitution reduces the net positive charge in the 2134/3056 region of the capsid and thus could account for the loss of binding to negatively charged heparin or HS molecules. Although our analyses strongly suggest a role for residues 2134 and 3056 in binding the virus to GAGs, complete sequence data were not obtained for the entire capsid region of the bovine-derived viruses, and so it remains possible that undetected substitutions also influence the observed alteration in heparin binding that resulted from passage through cattle.

Several interesting pieces of structural and antigenic information exist for position 3056. First, changes at this site are frequently detected in MAb escape variants of both type O (17) and type C (19). Second, this position is included in antigenic site D, which contains portions of all three external capsid proteins (Fig. 5), including the E-F loop of VP2 (2131 to 2134), the B-C loop of VP2 (2070 to 2080), residues 58 to 61 of VP3, and residues 193 to 197 of VP1 (25). Third, Arg 3056 was found complexed to a sulfate ion in crystals of type O1 BFS (12), and comparison of the structure of a MAb escape mutant virus generated from an early-passage O1K isolate (His at 3056) and an O1 BFS virus (Arg at 3056) demonstrated large differences in the structure at this site (18). Finally, X-ray data from crystals of O1 BFS incubated with heparin have demonstrated a direct binding of the heparin sulfate to several residues in this area, including 3056 and 2134 (43).

The quasispecies nature of the FMDV genome allows for the rapid adaptation to a new environment. In this study, type O1 viruses with new properties were rapidly selected when virus stocks were introduced into CHO cells. This adaptation appears to result from the absence of a functional integrin receptor on these cells, which prevents them from growing type A12 virus (22, 23, 33). Adaptation is accompanied by the acquisition of ability to bind heparin, strongly suggesting that the selected viruses utilize cell surface HS to gain entry to these cells. The adaptation to this new receptor appears to alter plaque morphology and host range in vitro, even though the heparin-binding viruses still appear to be able to bind to the integrin (as shown by the ability to prevent non-heparin-binding viruses from binding to cells [Fig. 2B]). Adaptation that accompanies serial passage in BHK cells (a cell line typically used to propagate this virus) occurs more slowly and appears to result from the selection of viruses that can compete more effectively in cell culture as a result of their affinity for heparin. Interestingly, heparin-binding viruses are dramatically attenuated in bovines, indicating that the acquisition of binding to a new receptor can abrogate disease by sequestering the virus to sites that are not favorable for replication.

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