The gene downstream of the gC homologue in feline herpes virus type 1 is involved in the expression of virulence


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Feline herpesvirus type 1 (FHV-1) mutants were constructed, carrying a β-galactosidase marker gene integrated into the region downstream of the gene encoding the homologue of glycoprotein C (gC) of herpes simplex virus type 1. In cell culture, no differences in replication were observed between mutants and the parent FHV-1 strain. However, in experimentally infected cats, mutants caused fewer clinical signs after oronasal administration although they replicated to the same extent as the parental strain. Sequence analysis in the region of the U₅ segment surrounding the insertion site revealed an open reading frame (ORF 2) encoding a putative polypeptide of 21K. RNA analysis indicated a corresponding transcript of 0.8 kb that was detected late after infection of cells in culture. This particular U₅ locus downstream of the gC gene has not been thoroughly investigated in any of the herpesviruses. The putative gene product showed only limited evolutionary conservation since similarity could be found only with the assumed homologue of equine herpesvirus type 1. Further characterization of this newly identified FHV-1 gene involved in virulence may provide insight into the development of disease owing to herpesvirus infection.

Introduction

Feline rhinotracheitis is a ubiquitous disease of cats caused by an alphaherpesvirus. In adult cats, feline herpesvirus-1 (FHV-1) infection causes serious upper respiratory tract and conjunctival disorders, while in kittens the disease can generalize, resulting in high mortality rates. Periodic reactivation of latent infections is a major mechanism for horizontal spread of the virus.

FHV-1 has a genome of approximately 126 kb subdivided into long and short components of 99 and 27 kb, respectively (Grail et al., 1991). The restriction enzyme cleavage patterns of the genomes of different FHV-1 isolates appear to be extremely well-conserved (Herrmann et al., 1984). There have been several reports on the protein composition of FHV-1 (Fargeaud et al., 1984; Maes et al., 1984), although little is known about the molecular organization of the genome. The thymidine kinase (TK) gene of FHV-1 was the first gene identified and mapped in the viral genome (Nunberg et al., 1989). TK is known to be involved in the virulence of herpesviruses (Field & Wildy, 1978) and belongs together with ribonucleotide reductase (RR) (Jacobson et al., 1989) and dUTPase (Pyles et al., 1992) to a class of enzymes that function at the level of DNA synthesis. Another well-characterized group of virulence markers is the glycoproteins. These structural proteins, most of which are anchored in the viral envelope or in the membranes of infected cells, contribute in a complex manner to viral processes such as adsorption, penetration, cell-to-cell spread and cell tropism. Several glycoproteins are non-essential for virus replication but most of them do affect pathogenesis to a certain extent.

FHV-1 is an interesting model in the search for new genes involved in virulence and pathogenicity, since the syndrome caused in susceptible cats is relatively well-defined and limited to the oropharynx and conjunctiva. Mutant strains derived from wild-type FHV-1 by insertion of a marker gene into one or more positions of the viral genome can be tested under standard conditions for reduced virulence in the natural host animal. This report describes two mutants developed independently by gene insertion into a newly defined locus in the U₅ segment of the FHV-1 genome.

Methods

Cells and viruses. Crandell Rees feline kidney (CRFK; Crandell et al., 1973) cells were cultured in a combination of Glasgow's and Eagle's modified MEM supplemented with 5% fetal calf serum and a cocktail of the antibiotics neomycin (50 μg/ml), polymixin (50 μg/ml), pima-
patterns of the inserted FHV-1 DNA and the map positions of clones infected CRFK cells. Individual clones were analysed for restriction p.f.u., except for the titres in oral swabs which were determined by were established based on comparison with previously published data.

All virus titrations were performed on CRFK cells and expressed as p.f.u., except for the titres in oral swabs which were determined by TCID₅₀ either on CRFK cells or feline embryo fibroblasts.

**Cloning of subfragments from the FHV-1 genome.** A lambda phage library in EMBL4 was prepared using DNA purified from FHV-1-infected CRFK cells. Individual clones were analysed for restriction patterns of the inserted FHV-1 DNA and the map positions of clones were established based on comparison with previously published data (Grail et al., 1991). The lambda clone selected for the experiments described in this report was designated pFHV01. Partial sequence analysis of the 5.1 kb BamHI fragment revealed three ORFs that are indicated below the restriction map of pFHV01. The nucleotide sequence of a major part of the 5.1 kb BamHI insert from plasmid pFHV01 was determined after insertion of the marker gene at the unique BamHI site of pFHV01.

**Sequence analysis.** The nucleotide sequence of a major part of the 5.1 kb BamHI insert from plasmid pFHV01 was determined after generating a series of subclones containing unidirectional deletions starting from either end of the fragment (Henikoff, 1984). Dideoxy-nucleotide chain termination reactions were performed on dsDNA templates using T7 polymerase (Pharmacia). Incomplete or ambiguous readings were resolved by specific priming within the insert DNA of pFHV01. Sequence data were assembled using a shot-gun handler and analysed with Gene-Master (Bio-Rad) or PC/Gene (Intelligenetics) software packages. Database searches were done on SWISS-PROT (release 23) using the FASTP algorithm (Lipman & Pearson, 1985). Optimal alignment between protein sequences was done as described previously (Needleman & Wunsch, 1970) with the constant and linear gap penalty scores set at 10.0. Dot matrix analysis based on a proportional match algorithm (Staden, 1982) was used occasionally to detect minor levels of similarity between specific regions of two protein sequences. Parameters for this program were set at a window of 19 amino acids in combination with a minimum score of 220. Screening for characteristic patterns and signatures in the primary structure of herpesvirus proteins was done in PROSITE (Bairoch, 1991).

**RNA analysis.** RNA was prepared from CRFK cells that had been infected for either 8 h or 24 h with FHV-1 at a multiplicity of 3. Total RNA was extracted in high molar guanidine buffers (Chirgwin et al., 1979), subjected to electrophoresis on agarose-formaldehyde gels and transferred to nitrocellulose membranes (Schleicher & Schuell). Blots were hybridized with [x-32P]CTP-labelled complementary RNA probes transcribed from phage promoters of Gemini-derived plasmid constructs (Promega). Procedures for probe labelling and blot hybridization have been described elsewhere (Sambrook et al., 1989). Non-specific hybridization particularly to the ribosomal RNA bands was removed by 15 min incubation at room temperature in 2 x SSC with 1 μg/ml RNase A. Primer extension assays were done in 10 μl reactions containing 10 μg of total RNA and 10 ng of 32P-labelled primer using Moloney murine leukaemia virus reverse transcriptase according to the conditions as recommended by the enzyme manufacturer (Gibco-BRL). The oligonucleotides 5'-CGG TGG GTT AGG CTC-3' (I) and 5'-ATA GCTGTC GTC AAC-3' (II), hybridizing to the proximal region of the coding sequence of ORF 2, were phosphorylated with [γ-
to a specific activity of 110 TBq/mmol. Reaction products were separated on denaturing sequencing gels and the sizes of the transcripts were calculated relative to size markers run simultaneously.

Generation and identification of recombinant virus. To generate viable virus from FHV-1 DNA, the calcium phosphate method (Graham & van der Eb, 1973) was used with the following modifications. In vivo recombinations were performed using CRFK cells in 28 cm² dishes receiving a coprecipitate of 7.5 μg of total infected cell DNA and 0.5 μg plasmid construct, carrying the β-galactosidase gene flanked by FHV-1 DNA. After 5 h incubation, cells were treated with 15% glycerol for 2 min and overlaid afterwards with 0.75% agarose in culture medium. Three days after cotransfection, when FHV-1-specific c.p.e. had developed, cells received a second agarose overlay in growth medium supplemented with 200 μg/ml Blue-gal (Gibco-BRL). Blue plaques were isolated for use in subsequent platings and blue plaque assays. Recombinant virus was plaque-purified several times until the preparation was homogeneous.

Inoculation of FHV-1 strains in cats. Specific pathogen-free cats, 10 to 12 weeks of age were infected with approximately 1 × 10⁶ p.f.u. of FHV-1 virus, either subcutaneously in the neck or oronasally by applying 0.3 ml per nostril and 0.4 ml orally. Animals were observed daily for clinical signs characteristic of FHV-1 infection and scored using the following point system: pyrexia between 39.7 and 40.0 °C (1 point); sneezing infrequent (1 point) or frequent (2 points); heavy respiration (1 point) and mouth breathing (2 points); conjunctivitis, either mild (1) or moderate (2); ocular discharge, either serous (1 point) or mucopurulent (2 points); nasal discharge, either serous (1) or mucopurulent (2); ulcerations (2 points) or bleeding ulcerations (3 points). Oropharyngeal swabs of each cat were taken for a period of 2 weeks starting 1 day before infection. Re-isolated virus from the swabs was titrated in TCID₅₀ on microtitre plates. Plates were read 4 to 5 days post-infection (p.i.) and scored for c.p.e. typical of FHV-1 infection. Titrations of swabs from cats infected with the mutant strain were fixed with 70% methanol and stained in an immunofluorescence assay to confirm the stability of the mutant during replication in the host animal. A monoclonal antibody directed against the enzyme β-galactosidase (Promega) was used in a 1:1000 dilution and detected using anti-mouse IgG–fluorescein isothiocyanate conjugate (Nordic).

Results

Insertional mutagenesis

Potential sites in the genome of FHV-1 that could be mutated without debilitating functions essential for virus replication were identified by random integration of a marker gene at one or more restriction sites in subfragments of the viral genome. The gene encoding β-galactosidase driven by the simian virus 40 early promoter was selected for this purpose, since it would allow sensitive and reliable identification of recombinant virus in cultures of infected cells. The expression cassette carrying the marker gene was flanked by BamHI restriction endonuclease sites and was derived from plasmid pCH110 as described previously (Sondermeijer et al., 1993). Live recombinant herpesvirus was generated by homologous recombination in vivo between genomic viral DNA and the plasmid construct containing the marker gene flanked by FHV-1 sequences.

Although several positions including sites in the U₅ segment were tried using this approach, results presented in this report will be focused on an insertion made into the unique Bg/II site present in a 5.1 kb BamHI restriction fragment of the FHV-1 genome. The position of this fragment is located between 0-10 and 0-14 m.u. in the U₅ of the viral genome (Fig. 1).

The marker gene insertion was made in pFHV01 harbouring the 5.1 kb BamHI fragment subcloned in pGEM3Z and resulted in pFHV05, carrying the β-galactosidase gene orientated towards the left end of the U₅ (Fig. 1). Cotransfection with DNA from plasmid pFHV05 resulted in the establishment of recombinant virus expressing β-galactosidase activity that could be plaque-isolated and stably maintained through serial passages in tissue culture. Several recombinants were selected from the transfection and purified to homogeneity. One of these isolates, designated strain 05-4-1-1, was evaluated for virulence in experimentally infected animals.

The growth characteristics of recombinant strain 05-4-1-1 in cell culture concerning the replication rate and maximum attainable titre in a single-step infection experiment did not differ substantially from the parental FHV-1 strain (Fig. 2). The correct position of the marker gene in this mutant strain was confirmed by Southern blot analysis (data not shown).

Fig. 2. Growth curves of the parent strain G2620 (■) and the insertion mutant 05-4-1-1 (Δ). Monolayers of CRFK cells were infected with either virus at a multiplicity of 3-8. Samples were taken from the culture supernatant at the time points indicated and titrated in duplicate on CRFK cells. The zero time point was taken from the virus suspension made in serum-free medium prior to preadsorbing the inoculum onto the monolayer.
Nucleotide sequence analysis in the region used for integration of the marker gene revealed three major open reading frames (ORFs) of which the first two were directed towards the right end of the UL (Fig. 3). Databank searches with the deduced amino acid sequences showed significant homology scores with several of the herpesvirus gene products, particularly those from equine herpesvirus type 1 (EHV-1).

The first ORF, ORF 1, encoding 236 amino acids and assumed to be in incomplete, shared a low level of identity (18.6%) with the sequence of the C-terminal sequence of gC from herpes simplex virus type 1 (HSV-1) (Frink et al., 1983) and 40.1% identity with glycoprotein 13 from EHV-1 (Allen & Cooley, 1988).

The amino acid sequence of ORF 2, the reading frame containing the BgII site used for insertion, showed a shared a low level of identity (18.6%) with the sequence of the polypeptide encoded by gene 15 from EHV-1. Extensive alignment with the relevant protein sequence data from other alphaherpesviruses, as far as these were available for the specific region of the viral genome, did not reveal additional significant similarities with the 193 amino acids encoded by ORF 2 from FHV-1. A more sensitive method for detection of identity between ORF 2 from FHV-1 and gene 15 from EHV-1 was performed by dot matrix analysis using a proportional match algorithm. Under these conditions, the central regions of both protein sequences showed a striking match in similarity (Fig. 4). A search for the conserved presence of putative sites involved in post-translational modifications (Bairach, 1991), such as the serine residues around nucleotide (nt) 1089 that could serve as a substrate for protein kinase C, further confirmed the suggestion that ORF 2 from FHV-1 and gene 15 from EHV-1 was performed by dot matrix analysis using a proportional match algorithm.

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Structural analysis of the insertion region

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Reduced virulence of FHV-1

Fig. 4. Dot matrix analysis using a proportional match algorithm on the amino acid sequence encoded by ORF 2 from FHV-1 and the sequence encoded by gene 15 from EHV-1 (Telford et al., 1992).

suggested that the protein sequence of FHV-1 ORF 2 is less conserved relative to those of the flanking genes.

A search for transcriptional control elements within the sequence as shown in Fig. 3 revealed polyadenylation signals downstream of ORFs 1 and 2 at positions 942 and 1556, respectively. In the reverse orientation, an AATAAA-sequence, serving as the poly(A) site for the transcript containing the coding sequence of ORF 3, is located at nt 1606. Several promoter motifs were found upstream of ORF 2, from which transcription of the putative gene could be initiated.

Transcription pattern in the region of ORF 2

FHV-1-encoded transcripts were analysed by Northern blotting and primer extension assays. RNA samples were isolated both early and late after infection of CRFK cells with FHV-1 at 8 and 24 h p.i., respectively. Northern blots were hybridized with a 1·4 kb RNA probe complementary to the region between nt 764 and 2121 (Fig. 3) containing the complete ORF 2 and the C-terminal part of ORF 3 that was orientated in the opposite direction. Two transcripts of 2·4 and 0·8 kb were detected late during infection (Fig. 5a). Small amounts of the 2·4 transcript were seen occasionally in RNA isolated early during infection. The apparent size of this transcript indicated that it comprised not only the coding sequence for gC, assumed to be approximately 1·7 kb, but in addition contained ORF 2 and adjacent 3' non-coding sequences up to one of the polyadenylation sites located downstream of the translational stop codon at nt 1542. The size of the second transcript detected in these RNA blots was estimated at 0·8 kb which appeared to agree with the length of ORF 2 predicted from the nucleotide sequence. The relative intensity of the 2·4 and 0·8 kb bands in RNA blots of FHV-1-infected cells suggested that the levels of transcription of ORF 2 and the gene encoding the gC homologue were comparable to each other. Expression of the predicted polypeptide
### Table 1. Clinical observations and virus excretion after inoculating cats with FHV-1 mutant strain 05-4-1-1 and parent strain G2620

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cats</th>
<th>Dose*</th>
<th>Strain</th>
<th>Clinical signs</th>
<th>Score†</th>
<th>Average score‡</th>
<th>Virus excretion§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>5.8 (o.n.)</td>
<td>05-4-1-1</td>
<td>Sneezing</td>
<td>2</td>
<td>0.5</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>5.8 (s.c.)</td>
<td>05-4-1-1</td>
<td>Pyrexia</td>
<td>11</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>6.0 (o.n.)</td>
<td>G2620</td>
<td>Sneezing</td>
<td>12</td>
<td>7</td>
<td>19 ± 0.3</td>
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<td></td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>6.0 (s.c.)</td>
<td>G2620</td>
<td>None</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

* Dose in log₃0 p.f.u. and administration routes either oronasally (o.n.) or subcutaneously (s.c.).
† Numbers represent the summation per group of the daily score for each of the signs over a period of 17 days p.i.
‡ Average of the total score per cat for the complete observation period.
§ Daily mean averaged per animal (± S.D.) in log₁₀ TCID₅₀/ml of swabbing fluid.
|| Body temperature in one animal at day 3 p.i. was 39.7 °C and therefore scored as one point, although the significance of this single observation is questionable.

encoded by ORF 2 is most likely to occur by translation of the 0.8 kb RNA. The results for the 0.8 kb RNA in Northern blots were confirmed in primer extension reactions. In addition, this assay enabled a more accurate positioning of the transcript relative to the coding sequence of ORF 2. Two oligonucleotides, I and II, were designed that hybridized in tandem at 99 and 122 bp respectively, downstream of the ATG initiator of ORF 2. Analysis of the reaction products on denaturing gels revealed several bands of which only those migrating at 297 and 322 bp, primed with oligonucleotides I and II respectively, were specifically present in RNA samples isolated late during infection (Fig. 5b). It was determined that the most likely start for the synthesis of an RNA that could potentially direct the expression of ORF 2 occurred at nt 762 or 764. The presence of several TATA elements in the region 30 to 50 bp upstream of this position and at least one canonical polyadenylation signal downstream of the TGA codon of ORF 2 seems to be in agreement with the synthesis of a putative messenger with an estimated size of 0.8 kb.

Pathogenicity of 05-4-1-1 mutant virus in infected cats

Previous observations of the behaviour of mutant and parental viruses in CRFK cell cultures revealed no differences in growth characteristics. However, marked differences were observed in clinical signs when these two viruses were administered to cats by the oronasal route. As shown in Table 1, cats in group 3 receiving the parental strain developed moderately severe clinical signs including some ulcer development. Conversely, animals in group 1 which had received the mutant strain, developed only mild clinical signs indicating that there was a reduction in virulence. However, despite the marked difference in virulence between the viruses, the re-isolation data showed that both types were excreted from the oropharynx at similar levels (last column in Table 1). Following subcutaneous inoculation, no clinical signs were observed in cats given either strain 05-4-1-1 or G2620 with the exception of one animal in group 2 that had a slightly elevated body temperature on a single day (Table 1). Occasionally, low levels of excreted virus were detected in cats infected subcutaneously. Nevertheless, these results were not consistent enough to allow a more quantitative comparison between the animals in groups 2 and 4.

All viruses re-isolated from cats that had received the mutant strain contained the β-galactosidase marker, indicating that this virus was stable in the natural host (data not shown).

Confirmation of the ORF 2 locus encoding a virulence factor

The above results do not unequivocally prove that the locus in which the marker was inserted is involved in the virulence of FHV-1 for the following reasons. Firstly, it could not be excluded that the insertion in strain 05-4-1-1 had affected the expression of the gC homologue encoded by ORF 1, since the corresponding transcript is coterminal with the transcript of ORF 2 in which the insertion was made. Hence, Northern blot analysis was performed on RNA isolated 24 h p.i. from CRFK cells infected with either FHV-1 strain G2620 or 05-4-1-1, using a riboprobe containing the first 0.6 kb of pFHV01 up to the EcoRV site at position 370 (Fig. 3). Only RNAs containing the sequence of the coding strand of ORF 1 could be detected by this probe. For quantification of the ORF 1 transcripts, the RNA samples of infected cells were loaded in equal amounts by comparing the intensity of the ribosomal RNA bands.
Reduced virulence of FHV-1

Fig. 6. Northern blot analysis of ORF 1 transcripts in CRFK cells infected with strains G2620 and 05-4-1-1. The 32P-labelled riboprobe was chosen such that only the largest of the two coterminal transcripts could be detected in cells infected with the parent virus G2620. Identical amounts of total RNA from G2620- (lane 1) and 05-4-1-1 (lane 2)-infected cells were loaded onto the gel. Sizes of the transcripts are indicated to the right.

Northern blots of strain 05-4-1-1 hybridized with this riboprobe resulted in two RNA species: an abundant band estimated at 6.2 kb and a minor band of 1.7 kb (Fig. 6). The major RNA species represented a transcript initiating upstream of ORF 1 and terminating downstream of ORF 2 which carried the 4.1 kb marker gene insertion. The minor transcript of 1.7 kb most likely transcribes ORF 1 until the polyadenylation site at position 942, a signal not recognized in the parent strain. No significant difference could be observed when comparing the abundance of the gC-encoding transcripts of mutant and parent FHV-1 strains. Therefore, the insertional mutation at the ORF 2 locus in strain 05-4-1-1 did not significantly interfere with the transcription of messengers encoding the gC homologue. Secondly, mutational events occurring during the recombination between the viral genome and transfer plasmid might have contributed to the reduced virulence observed with strain 05-4-1-1. Therefore a new insertion mutant was constructed using a recombination plasmid containing the region of ORF 2 limited to the sequence between positions 763 and 1644 (Fig. 3). The plasmid again contained the β-galactosidase cassette inserted at the BglII restriction site, but in the reversed orientation as was used for 05-4-1-1. In this configuration, expression of the β-galactosidase gene was unlikely to interfere with the transcripts of flanking genes since transcripts initiated from within the cassette would have to be terminated downstream of ORF 2, similarly to the 2.4 and 0.8 kb coterminal RNAs previously mapped in this region of the wild-type viral genome. The structure of the resulting FHV-1 mutant 25B3B11 was confirmed by Southern blot analysis. The growth characteristics on CRFK cells of strain 25B3B11 revealed no significant differences compared to those of strains 05-4-1-1 or G2620 (data not shown).

In a second animal experiment, groups of five cats were oronasally infected with strain 05-4-1-1, 25B3B11 or parent strain G2620. Clinical observations of the cats inoculated with either of the insertion mutants, again revealed a considerable reduction in the virulence of the viruses compared to that of the parent strain G2620 (Table 2). However virus excretion was not significantly impaired indicating that mutants were still replicating efficiently in the upper respiratory tract of infected

Table 2. Clinical observations and virus excretion after oronasal infection with insertion mutant strains 05-4-1-1 and 25B3B11*

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cats</th>
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<th>Dose</th>
<th>Clinical signs</th>
<th>Score</th>
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* See footnotes Table 1 for explanation of column headings.
† Relatively high score originates from one animal which developed a nasal discharge in the second half of the observation period although it did not simultaneously excrete high levels of FHV-1 virus. The possibility exists that these signs were in fact due to complications caused by a secondary bacterial infection.
animals. The results obtained with 25B3B11, together with those on the transcription of the gC homologue in mutant 05-4-1-1, provide strong evidence that the ORF 2 locus actually encodes a virulence factor of FHV-1.

Discussion

The virulence of herpesvirus appears to be determined by a complex interaction of multiple proteins and can involve both structural components such as the envelope glycoproteins as well as enzymes involved in virus-directed nucleotide synthesis. Well-established examples are ICP34.5 from HSV-1 which determines the neurovirulence of the virus in mice (Chou et al., 1990; MacLean et al., 1991) and RR which is required for pathogenicity of HSV-1 in mice (Cameron et al., 1988) and more specifically for ocular virulence of HSV-1 in humans (Brandt et al., 1991). Another example relates to glycoprotein gI from pseudorabies virus that affects spreading of the virus into major parts of the central nervous system (Kimman et al., 1992).

For FHV-1, the scarce amount of relevant data is limited to the TK gene in which a deletion was introduced in order to create an attenuated, safe viral vector that could be used in a multivalent live vaccine for cats (Cole et al., 1990; MacLean et al., 1991) and EHV-1 (Allen & Coogle, 1988) genomes. However no results have yet been reported in detail that indicate the role of this gene in FHV-1 virulence in general.

A 5.1 kb region in the U₁ segment, which was identified and characterized in this report, contains sequences that affect the virulence of FHV-1 when the virus is applied by the natural route of infection. These results are based on animal studies with the two independent mutant strains 05-4-1-1 and 25B3B11 in which a reading frame designated ORF 2 was disrupted by insertion of a β-galactosidase expression cassette. Both mutants showed a remarkably reduced virulence when applied oronasally to cats. The insertion in ORF 2 did not change the growth rate of the virus in tissue culture and did not significantly reduce virus replication in mucosal tissues of the respiratory tract. Therefore, we conclude that the presumptive protein encoded by ORF 2 is truly involved in the expression of virulence, in a way that differs from other virulence factors such as TK and RR that are primarily required for efficient propagation of the virus in specific tissues of the host animal. Given the pronounced tropism of FHV-1 for the epithelium of the upper respiratory tract and the limited spreading into other organs of the animal, it is not surprising that this attenuation in virulence was not observed after systemic infection of the host by subcutaneous inoculation with FHV-1. Even the parent FHV-1 strain used in this study did not induce any signs of disease after parenteral administration, although when applied oronasally, it was able to generate many of the clinical manifestations typical of FHV-1 infection.

Structural analysis in the region of the FHV-1 genome where the insertion was made, revealed a 579 nt ORF, ORF 2, positioned between two oppositely orientated genes that shared significant sequence homology with the proteins encoded by HSV-1 UL44 and UL46 respectively (McGeoch et al., 1988). However, highest scores for identical residues in the flanking regions were obtained when aligning the protein sequence data of FHV-1 and EHV-1.

Initially, we expected that ORF 2 in FHV-1 would be the homologue of UL45 from HSV-1 encoding a 18K protein component of the virion (Visalli & Brandt, 1993), shown to be non-essential for growth in Vero cells (Visalli & Brandt, 1991). This assumption was based on a similar size, position and orientation of ORF 2 within the FHV-1 genome but could not be confirmed by sequence alignment with UL45 from HSV-1 owing to the absence of significant homology at the amino acid level.

The predicted amino acid sequence of ORF 2 contains no typical structures such as a signal peptide or a transmembrane anchor region that are reminiscent for the group of herpesvirus glycoproteins. PROSITE analysis indicated potential sites involved in post-translational modifications of which at least those recognized by protein kinase C might be of biological relevance since equivalent sites are also present at a similar position in the protein encoded by gene 15 of EHV-1. However the significance of these findings clearly needs to be demonstrated in biochemical studies of the protein as it is synthesized and processed in FHV-1-infected cells. In addition, specific sera raised against the polypeptide encoded by ORF 2 will be necessary to determine the kinetics of expression in FHV-1-infected cells. Eventually these experiments may also elucidate the role of ORF 2 in viral pathogenesis. The first data concerning the expression of ORF 2 in FHV-1-infected cells have been presented in this report. The sequence of ORF 2 is transcribed from a promoter located about 200 nt upstream of the start codon of the reading frame resulting in a 0.8 kb RNA which potentially directs the synthesis of a 21K polypeptide. A second, much larger transcript of 2.4 kb contains in addition and upstream relative to the sequence of ORF 2, the coding sequence for a glycoprotein sharing homology with the gC from HSV-1. Both the 24 and 0.8 kb RNA probably use the same 3'-terminal polyadenylation site and are synthesized late during infection of tissue culture cells. A similar nested organization of transcripts has been described in the corresponding regions of HSV-1 (Fink et al., 1983) and EHV-1 (Allen & Coogle, 1988) genomes.

The newly identified virulence factor reported here
Reduced virulence of FHV-1

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could have several consequences for the development of vaccines against FHV-1 and perhaps for vaccines against related herpesviruses. The apparent reduction in virulence provides a new concept for a vaccine virus that follows the natural route of infection in contrast to the existing FHV-1 vaccines, most of which can only be used subcutaneously due to residual pathogenicity when applied oronasally. Both mutants did not affect virus replication in the oropharynx of infected cats which is essential for the induction of a protective immune response. The major advantage of the natural administration route is that vaccinated animals would be better protected against local replication and invasion of wild-type virus and consequently should develop less severe clinical disease upon challenge.

Ultimately the ORF 2 locus would be a preferred insertion site in the context of using FHV-1 as a live viral vector (Cole et al., 1990; Wardley et al., 1992), since integration of foreign genes at this position in the U₃ simultaneously will result in a significant reduction of the virulence. Pathogens that share the same port of entry with FHV-1, for example feline leukaemia virus and calcivirus, are considered good candidate suppliers of relevant genes in the assemblage of a multivalent vaccine virus based on FHV-1. However, detailed studies need to be performed on the biochemical and biological properties of the gene product encoded by this particular locus in the U₃ of the FHV-1 genome. Results presented in this report showed strongly reduced virulence of the mutant strains 05-4-1-1 and 25B3B11 which already suggests an important role of the protein encoded by ORF 2 in the pathogenesis of FHV-1 infection.

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