Feline Syncytium-forming Virus: DNA Provirus Size and Structure

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SUMMARY

An infectious DNA assay has been used to investigate the size and structure of the genome of feline syncytium-forming virus (FSFV). The dose response between DNA extracted from FSFV-infected cells and plaque number on feline embryo cells followed two-hit kinetics and the mol. wt. of the proviral DNA was estimated as approx. $6 \times 10^6$.

INTRODUCTION

Feline syncytium-forming virus (FSFV) has been isolated from both normal and diseased cats and has been classified as a foamy virus (Riggs et al. 1969; Jarrett et al. 1974; Hooks & Gibbs, 1975). The virions of foamy viruses have been shown to contain an RNA genome and to possess a reverse transcriptase activity (Scolnick et al. 1970; Parks et al. 1971; Hruska & Takemoto, 1975). These properties have led to the classification of the foamy viruses as a sub-family (Spumavirinae) of the Retroviridae (Fenner, 1976). FSFV has been included in the foamy viruses on the basis of biological properties and morphology (Riggs et al. 1969; Hooks & Gibbs, 1975) and the demonstration of a reverse transcriptase activity (Todaro et al. 1971; Chiswell & Pringle, 1979). The observation that infectious DNA could be isolated from FSFV-infected feline embryo (FEA) cells (Chiswell & Pringle, 1977) has further substantiated the inclusion of the foamy viruses in the Retroviridae.

The genome structure of the foamy viruses is relatively unknown because of the difficulty of obtaining adequate amounts of purified virus. Hruska & Takemoto (1975), however, have reported that the virion of a hamster foamy virus contained a number of different species of RNA ranging in size from 62S to 4S. They postulated that the 62S RNA species was composed of 18 to 20S sub-units. Here a different approach has been adopted and an infectious DNA assay used to investigate the size and structure of the DNA provirus of FSFV, and hence the organization of the genome of FSFV.

METHODS

Cells and viruses. Secondary feline embryo (FEA) cells and FSFV were provided by Dr O. Jarrett of the Department of Veterinary Pathology, University of Glasgow. The FEA cells were grown in the Glasgow modification of Eagle's minimum essential medium supplemented with 10% foetal calf serum (EFC10).

DNA extraction. DNA was extracted from FEA cells grown at 37 °C and infected 4 to 5 days previously with FSFV at a m.o.i. of 0·1. The method of DNA extraction was a
Fig. 1. Dose–response relationship between FSFV DNA concentrations and plaque number. Various concentrations of FSFV DNA were added to FEA monolayers using the enhanced calcium phosphate precipitation technique and the number of plaques produced was counted after 5 days. The data are plotted on arithmetic (a) and logarithmic (b) axes. The theoretical line representing one-hit kinetics is plotted in (b). ●–●, Plaque number; ---, one-hit kinetics (theoretical).

**RESULTS**

**Concentration dependence of the infectious DNA assay**

DNA was extracted from FEA cells 4 to 5 days p.i. by FSFV at a m.o.i. of 0.1. Cell fusion was extensive at this time. Since the Marmur extraction method was used, the extracted DNA represents the total cellular DNA ('FSFV DNA').

The relationship between different amounts of FSFV DNA added per plate and the number of infectious events (plaques) observed is illustrated in Fig. 1. Fig. 1(a) represents
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Fig. 2. Dose-response relationship between FSFV DNA and plaque number keeping the total amount of DNA added per plate constant. Various concentrations of FSFV DNA were added to two series of FEA monolayers using the enhanced calcium phosphate precipitation technique and the number of plaques produced was counted after 5 days. The total amount of DNA added to one series of plates was kept at 10 μg/plate by the addition of carrier (calf thymus) DNA where necessary. The data are plotted on arithmetic (a) or logarithmic (b) scales. ▲—▲, Without carrier; •—•, with carrier.

the data plotted on arithmetic axes showing that the dose-response relationship was non-linear, i.e. plaque formation depended upon more than one event. The slope of a line derived from the data plotted on logarithmic axes (Fig. 1 b) can be used to give an estimate of the kinetics of the dose-response curve and therefore the number of events needed to initiate the formation of a plaque. The solid line in Fig. 1 (b) represents a linear regression analysis (best fit curve) for the initial portion of the dose-response curve (0 to 2 μg/plate). The slope of this line is 2.19 ± 0.07, indicating that the formation of a plaque required two independent events. The broken line represents a theoretical curve following single-hit kinetics.

The two-hit nature of the dose-response curve may reflect a dependency of transfection efficiency on the total mass of DNA applied to a culture. If this is the case, it should be possible to obtain one-hit kinetics by the addition of carrier DNA to keep the total amount of DNA added per monolayer constant. Fig. 2 presents data of such an experiment plotted on linear (a) and logarithmic (b) axes. The slopes of the two lines present in Fig. 2 were 1.87 (± 0.25) without carrier and 1.77 (± 0.02) with added carrier. The average mol. wt. of the carrier DNA used in these experiments was 7.5 to 11 x 10^6 and this DNA was shown to act as efficient carrier for herpes simplex virus type 1 DNA in transfection experiments (N. Stow, personal communication). Thus, the addition of carrier DNA did not significantly alter the kinetics of the dose-response relationship and only affected the specific infectivity of the DNA, presumably by a dilution effect. Therefore the non-linearity of the dose-response curve does not seem to be a function of the total amount of DNA added to a culture.

Size of DNA provirus

In principle, the infectious DNA assay can be used to estimate the size of the FSFV provirus, either by measuring directly the size of the unintegrated DNA provirus, or by establishing the limiting size of the total cellular DNA necessary for infectivity. Obtaining
Table 1. Infectivity of FSFV after shearing with various size needles

<table>
<thead>
<tr>
<th>Needle size</th>
<th>No. of plaques*</th>
<th>% no. of plaques †</th>
<th>% Infectious DNA ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsheared</td>
<td>9.5</td>
<td>40.4</td>
<td>—</td>
</tr>
<tr>
<td>18 g</td>
<td>23.5</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>21 g</td>
<td>15.25</td>
<td>64.9</td>
<td>81</td>
</tr>
<tr>
<td>23 g</td>
<td>13.5</td>
<td>57.9</td>
<td>76</td>
</tr>
<tr>
<td>25 g</td>
<td>5.2</td>
<td>22.1</td>
<td>47</td>
</tr>
<tr>
<td>26 g</td>
<td>5.5</td>
<td>23.5</td>
<td>48</td>
</tr>
<tr>
<td>27 g</td>
<td>0</td>
<td>1%</td>
<td>10%</td>
</tr>
<tr>
<td>30 g</td>
<td>0</td>
<td>0</td>
<td>10%</td>
</tr>
</tbody>
</table>

* 2 µg of FSFV DNA used per monolayer.
† 100% chosen as 23.5 (see text).
‡ Derived from $P = K(D)^x$ (see text).
§ One plaque on four plates (i.e. 0.25/plate) is the minimum detectable. This represents 1% of 23.5 or 10% of infectious DNA.

Sufficient quantities of unintegrated FSFV provirus proved difficult and we therefore decided to estimate the size of the provirus by investigating the minimum infectious size of DNA from infected cells. Hydrodynamic shearing by passage of DNA through various sizes of syringe needles was used to produce DNA molecules of different sizes and the specific infectivity of each size class was estimated using 2 µg of FSFV DNA per monolayer (Table 1).

The number of plaques obtained with a given concentration of DNA can be expressed as $P = K(D)^x$, where $P$ = the number of plaques, $D$ = concentration of DNA, $x$ = the kinetics of the dose–response relationship, and $K$ = a constant (Graham & Van der Eb, 1973). It has been shown in Fig. 1 and 2 that $x = 2$. Therefore, assuming that DNA sheared through an 18 g needle is all above the genome size [i.e. $D = 100\%$, $P = 100\%$ (23.5 plaques)], $K$ can be shown to equal 1/100. By expressing plaque number as a percentage of 23.5 and substituting for $K$ and $x$ the percentage of DNA that remains infectious after shearing through the different needles can be estimated (Table 1, column 4).

Approximately 1.5 µg of each DNA sample was run on a 0.5% agarose gel, using herpes simplex virus type 1 DNA cleaved by HindIII restriction endonuclease as mol. wt. markers. The DNA was visualized by ethidium bromide staining and photographed under u.v. light. Microdensitometer scans of the negative of the gel are presented in Fig. 3, with the positions of some of the marker bands indicated. As the DNA population decreased in size, the specific infectivity, i.e. the number of plaques per µg of DNA, decreased, with the exception that DNA sheared through the 18 g, 21 g and 23 g needles was more infectious than unsheared DNA. Hydrodynamic shearing of large DNA molecules (>30 × 10⁶ mol. wt.) has been shown to increase the specific infectivity of RSV (Levy et al. 1974) and visna virus (Haase et al. 1976) proviral DNA, probably because of increased efficiency of uptake. Since FSFV DNA late in the growth cycle has been shown to be almost all associated with high mol. wt. DNA, as analysed using DNA extracted by both the Hirt and Marmur extraction methods (Chiswell & Pringle, 1978), we believe that these results reflect a similar property in this system.

A crude estimate of the size of the proviral DNA can be obtained by plotting average mol. wt. (taken as the peaks of the size distributions in Fig. 3) against percentage of DNA remaining infectious from Table 1. The mol. wt. obtained by this method was approx. 6 × 10⁷ (Fig. 4). This method, however, makes no allowance for the size distribution within each DNA sample. To allow for this size distribution, the areas under the peaks of Fig. 3 were calculated and the amount of DNA above specific mol. wt. estimated (Fig. 5). If the assumption is made that the percentage of DNA remaining infectious (Table 1) represents
Fig. 3. Size of sheared DNA. FSFV DNA was sheared through various sizes of syringe needles and samples run on a 0.5% agarose gel as described in the Methods. Herpes simplex virus type I DNA cleaved with HindIII restriction endonuclease was used as a mol. wt. marker. The gel was photographed and the negative scanned with a Joyce-Loebel microdensitometer. The scans are presented with the positions of some of the mol. wt. markers indicated. DNA was either unsheared (a), or sheared through 18 g (b), 21 g (c), 23 g (d), 25 g (e), 26 g (f), 27 g (g) or 30 g (h) syringe needles. Electrophoresis was from right to left.

Fig. 4. Estimation of DNA provirus size (I). The mol. wt. of the peaks given for each scan in Fig. 3 were compared to the percentage DNA remaining infectious (Table 1, column 4) to obtain an estimate for the DNA provirus size. The limit of detection is indicated by the dotted line.

Fig. 5. Estimation of DNA provirus size (II). The areas under the peaks shown in Fig. 3 were determined by weighing. Sections were then progressively removed and the difference in weight used to determine the amount of DNA in each sample above specific mol. wt. V—V, DNA sheared through a 18 g needle; O—O, DNA sheared through a 21 g needle; +++, DNA sheared through a 23 g needle; ——, DNA sheared through a 25 g needle; ———, DNA sheared through a 26 g needle; ———, DNA sheared through a 27 g needle; ———, DNA sheared through a 30 g needle.
the amount of virus DNA remaining unsheared (i.e. above the genome size), then by equating the amount of DNA remaining infectious for each needle size (Table 1) with the size of DNA in that sheared sample (Fig. 5), a value for the size of the FSFV DNA provirus can be obtained. For example, with a 21 g needle, 81% of the DNA remained infectious (Table 1). Fig. 5 shows that the mol. wt. of the 81% of the DNA in that sample was greater than $7 \times 10^6$. Therefore, the mol. wt. of the provirus can be estimated at approx. $7 \times 10^6$. This reasoning can be used to obtain further mol. wt. estimates from Table 1. The estimates with DNA sheared through 23 g, 25 g, 27 g and 30 g needles were $5.1 \times 10^6$, $6.6 \times 10^6$, $5.9 \times 10^6$, $>6.4 \times 10^6$ and $>4.9 \times 10^6$, respectively. The average of these estimates is $6.2 \times 10^6$. Using data accumulated from other similar experiments (unpublished results), an average value for the mol. wt. of the FSFV DNA provirus of $6.1 \times 10^6 \pm 0.8 \times 10^6$ (± standard deviation from 18 determinations) was obtained.

**DISCUSSION**

The dose–response relationship between FSFV DNA and plaque number using the enhanced calcium phosphate technique appeared to follow two-hit kinetics. The dose-response of FSFV DNA using the DEAE dextran pre-treatment could not be accurately determined because of the low efficiency of the technique in this system (unpublished data). The addition of carrier DNA to keep constant the total amount of DNA added per monolayer did not significantly alter the kinetics of the dose-response curve, indicating that the two-hit nature of the curve was probably not due to the process of DNA uptake per se. Also, dose–response curves showing single-hit kinetics have been reported in several other systems, e.g. Rous sarcoma virus (RSV) and reticuloendotheliosis virus (REV) DNAs assayed by diethyl-aminoethyl dextran (DEAE-D) pre-treatment (Cooper & Temin, 1974); with herpes simplex virus type 1 DNA assayed using DEAE-D pre-treatment (Sheldrick et al., 1973), calcium phosphate precipitation (Graham et al., 1973), and enhanced calcium phosphate precipitation (Stow & Wilkie, 1976). Similarly, the dose–response curve obtained using in vitro synthesized murine leukaemia virus DNA and calcium phosphate precipitation followed single-hit kinetics (Rothenberg et al., 1977). Although in all the above cases the kinetics of the dose–response relationship reflected the number of DNA molecules required to initiate a complete cycle of replication, the data presented in this paper do not exclude the possibility that the two-hit nature of the relationship described was the result of some uncontrolled effects of the transfection technique. However, it is also possible that the dose–response relationship could reflect an inherent property of FSFV provirus, for example, the unique genetic information could be distributed over two sub-units of the DNA provirus. This novel type of genome structure has been proposed for visna virus (Haase et al., 1976), but does not appear to be typical of other retroviruses (Billeter et al., 1974; Cooper & Temin, 1974; Quade et al., 1974; Duesberg et al., 1975; Weissmann et al., 1975; Beemon et al., 1976). Obviously further study is required to clarify this issue.

In principle, if DNA of different sizes can be produced, the infectious DNA assay can be used to estimate the size of the FSFV DNA provirus. DNA of different sizes was produced by hydrodynamic shearing through syringe needles of various diam. The amount of DNA remaining infectious when sheared to different sizes was estimated, with a correction for the two-hit nature of the dose–response curve. Initially, the amount of infectious DNA was compared to the average mol. wt. of each DNA preparation to obtain one estimate of the size of the FSFV proviral DNA. However, this method takes no account of the size distribution within each population of DNA. In an attempt to allow for this distribution, the amount of DNA of various sizes was estimated for each population of DNA from microdensitometer scans of the polaroid negatives of ethidium bromide stained agarose gels. This was then
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compared to the amount of DNA remaining infectious to obtain another, perhaps more accurate, estimate of the size of the FSFV proviral DNA.

The validity of this approach depends on the assumptions that (a) ethidium bromide staining and its representation on polaroid film responds in a linear fashion to increasing DNA concentration and (b) DNA sheared below an average size of $26 \times 10^6$ daltons does not significantly increase in specific infectivity. The first assumption has been shown to hold, at least for the concentrations of DNA used in these experiments (Clements et al. 1976).

An increase in the specific infectivity of DNA has been reported when very high mol. wt. DNA was sheared (Levy et al. 1974; Haase et al. 1976; Table 1), but DNA with an average mol. wt. of $25 \times 10^6$ was shown to have the same specific infectivity as DNA with an average mol. wt. of $10 \times 10^6$ (Haase et al. 1976). Also, the validity of this approach in estimating genome size has been established by work on other retroviruses (Cooper & Temin, 1974; Haase et al. 1976). Therefore, although the estimates must be considered of limited accuracy, it appears that the genome of FSFV in its DNA form has a mol. wt. in the region of $6 \times 10^6$. Thus, the RNA genome (assuming it to be single-stranded) would have a mol. wt. of approx. $3 \times 10^6$. The size of the DNA provirus of FSFV (and by implication the RNA form of the genome) is similar to that of other members of the Retroviridae (Haase et al. 1976; Vogt, 1977).

No information is available at present to indicate whether this genome structure is typical of foamy viruses in general, apart from an observation of Hruska & Takemoto (1975) that the 62S virion RNA of hamster foamy virus can be denatured to 18 to 20S RNA, indicating segmentation of the genome.

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REFERENCES


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