Assay of Defective-interfering Semliki Forest Virus by the Inhibition of Synthesis of Virus-specified RNAs

By A. D. T. Barrett,* C. F. Crouch and N. J. Dimmock
Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, U.K.

(Accepted 20 January 1981)

Summary
We describe a simple, rapid and reproducible assay for defective-interfering Semliki Forest virus (DI SFV) which is based on the inhibition of synthesis of virus-specified RNAs in SFV-infected cells. Using the assay, we have been able to show that DI virus is generated by a single passage in baby hamster kidney (BHK) cells in an inoculum which contained no detectable DI virus and we have calculated the u.v. target size of the interfering activity.

Introduction
Defective-interfering (DI) virus particles accumulate during serial passage of many animal viruses at a high multiplicity of infection (Huang & Baltimore, 1970, 1977), including the alphaviruses Semliki Forest virus (SFV) and Sindbis virus (Bruton & Kennedy, 1976; Schlesinger et al., 1972). All DI viruses have a portion of their genome deleted and can only replicate in the presence of standard (infectious) virus and this interferes with the multiplication of the standard virus. Although much is known about the structure of DI RNAs particularly of vesicular stomatitis virus (VSV) (Reichmann & Schnitzlein, 1979) and alphaviruses (Stollar, 1979), understanding of the molecular mechanisms of interference and generation, even for VSV, is incomplete (Faulkner & Lazzarini, 1980; Kang, 1980) and the possible role of DI virus in modulating infection is unknown. Investigation of these aspects has been handicapped by the lack of a suitable quantitative assay for biologically active DI virus. At present, assays measure the reduction in yield of standard virus or the inhibition of infectious centre formation. The former was originally described for DI VSV (Bellett & Cooper, 1959) and adapted recently to assay DI Sindbis virus (Kowal & Stollar, 1980; Fuller & Marcus, 1980) while the latter has been used for DI lymphocytic choriomeningitis (LCM) virus (Welsh et al., 1972), DI Sindbis virus (Johnston et al., 1975) and DI influenza virus (Janda et al., 1979). A focus-forming assay based on the ability of some DI viruses to inhibit cytopathic effects has been used to measure DI LCM virus (Popescu et al., 1976) and DI VSV (Winship & Thacore, 1980). The assay of DI SFV by the inhibition of synthesis of standard virus-induced RNA species identified by polyacrylamide gel electrophoresis (Dimmock & Kennedy, 1978) is too cumbersome for routine use.

This report describes a novel assay for measuring the activity of DI SFV which is based on its ability to interfere with the intracellular synthesis of virus-induced RNAs. The assay is simple, rapid and reproducible and has been used to study properties of DI SFV including its propagation and generation in BHK cells and its effect on the multiplication of standard SFV.

Methods
Virus. We used ts+ SFV (standard virus) (Dimmock & Kennedy, 1978) which was propagated in chick embryo fibroblast (CEF) or baby hamster kidney (BHK21) cell monolayers inoculated at an m.o.i. of 0.01 to 0.1. Monolayers were incubated at 33 °C for 18
h and the tissue culture fluids (TCF) were then clarified by low-speed centrifugation before storage at −70 °C. DI SFV was prepared by serial passage in BHK cells at an m.o.i. of 50 according to Bruton & Kennedy (1976), and this was the direct descendant of the passage 8 virus used by Dimmock & Kennedy (1978). In this report DI SFV stocks of passages 12 and 13 were used except where noted.

**Cells.** Primary CEF cells were grown according to Morser *et al.* (1973). BHK21 and mouse L929 cells were grown as monolayers by standard methods. Virus was plaque assayed in CEF cells (Kennedy & Burke, 1972) at 33 °C, the overlay medium containing 0.02% DEAE-dextran. These conditions allow plaque formation on occasions when cells are refractory.

**Interference assay.** Mouse L929 cells were seeded at 2 × 10^5 cells/ml growth medium (GMEM, containing 10% newborn calf serum; Flow Laboratories) in flat-bottomed glass tubes of approx. 12 mm diam. and 50 mm high (Regina Industries, Stoke-on-Trent, U.K.) and were used after overnight incubation at 37 °C. Assays were in quadruplicate and details are given in Results. Cells were pulsed with 10 μCi/ml ^3H-uridine (sp. act. 29.7 Ci/mmol; The Radiochemical Centre, Amersham), and trichloroacetic acid (TCA)-insoluble radioactivity determined after washing cells twice with ice-cold 5% TCA and once with ice-cold ethanol. Cells were then dissolved in 100 μl Soluene-350 (Packard Instruments), diluted threefold in toluene. To this was added 3 ml acidified scintillation fluid [0.5% (w/v) PPO, 0.1% (w/v) POPOP and 0.1% acetic acid in toluene] and radioactivity was determined using a Packard Tri-carb Model 3220 liquid scintillation spectrometer.

**Ultraviolet (u.v.) irradiation.** This was as described by Dimmock & Kennedy (1978) except that the dosage was 800 μW/cm².

### RESULTS

**Interference assay**

The assay is based on the observation that DI SFV inhibits the synthesis of virus-specified RNAs in cells co-infected with standard SFV. By inhibiting cellular DNA-dependent RNA synthesis with actinomycin D (Act. D) virus-specified RNA synthesis could be measured by the incorporation of TCA-precipitable ^3H-uridine. This gave the 'virus control' value and non-infected cells the 'cell control' value.

Interference was measured by diluting a DI SFV preparation into maintenance medium containing 4 × 10^7 p.f.u./ml standard virus and 1 μg/ml Act. D. Inoculation of 250 μl/tube gave the m.o.i. of 50. Cells were incubated at 37 °C in an atmosphere of 5% CO₂ in air and at 4 h post-infection (p.i.), the inoculum was removed and fresh medium (250 μl) containing 2.5 μCi ^3H-uridine and Act. D was added for 1 h. Monolayers were then processed for TCA-precipitable radioactivity.

In a typical assay (Fig. 1) uninfected cell controls incorporated 5 × 10³ to 20 × 10³ ct/min compared with virus-infected controls (standard SFV alone) in excess of 2 × 10⁵ ct/min. Inhibition of incorporation of ^3H-uridine approached the value of the cell control when sufficient DI virus was assayed; on dilution, it followed a sigmoidal response to the level of the virus control. The response over the intermediate dilution series was linear and 50% inhibition was chosen as the interference titre. This is determined by interpolation and expressed as the reciprocal of the dilution giving 50% reduction of ^3H-uridine incorporation and is equivalent to 1 defective-interfering virus unit (DIU) per 250 μl. The possibility that interference resulted from DI SFV preventing attachment of infectious standard SFV was tested by adding standard SFV first, followed by DI SFV at 1 h later. The same interference titres were obtained in this and the control assay (10^{2.3} and 10^{2.4} DIU/250 μl respectively).

The interference titre can be converted into an estimate of the number of DI particles (DIP)
Assay of DI SFV

Fig. 1. Interference assay for DI SFV. The DI virus sample was diluted in medium containing $4 \times 10^7$ p.f.u. standard SFV and 1 $\mu$g/ml actinomycin D. L929 cells ($2 \times 10^5$ per assay tube) were inoculated with 250 $\mu$l and subsequently radiolabelled with $^3$H uridine from 4 to 5 h p.i. at 37 °C. ○. Radioactivity incorporated into tubes receiving dilutions of DI SFV; the standard virus alone (virus control: A) and uninfected cells (cell control: B) are as shown. A 50% inhibition of $^3$H-uridine incorporation is interpolated here (arrow) to a dilution of $10^{-2.5}$ of DI virus which thus has a titre of $10^{2.5}$ defective-interfering units (DIU) per 250 $\mu$l. Error bars show ± 1 S.E.M.

Fig. 2. Effect of increasing the m.o.i. of standard virus on the sensitivity of the interference assay. Conditions are as described in the text and Fig. 1.

by assuming that interference is all-or-nothing (Sekellick & Marcus, 1980), so that at the 50% endpoint, according to the Poisson distribution, the monolayer of $2 \times 10^5$ cells will have received $1.4 \times 10^5$ DIP. Thus, in Fig. 1 the titre of $10^{2.5}$ DIU/250 $\mu$l represents a minimum of $10^{8.2}$ DIP/ml.

The assay proved to be reproducible with a standard error (S.E.) of $6.3 \pm 2.0 \times 10^7$ from titrations on five separate occasions. In assays of different samples containing various amounts of DI virus, the linear regions of the interference curves were parallel, suggesting that in each case interference was mediated by the same mechanism.

Effect of varying some parameters of the DI SFV assay

Cell type

Different cell types were used in the assay described above to determine the one in which interference was greatest. Table 1 shows that interference titres varied by nearly 100-fold, indicating that the selection of the cell type is crucial to this type of assay. The basis of DI virus interference is obscure and cells at either end of the spectrum appeared to have little in common. For instance, different interference titres were seen in the BHK21 and CHO hamster cells but similar titres were seen in CV-1 and BSC-1 monkey cells. The finding that DI SFV generated in BHK cells interferes in the G8 clone of Aedes albopictus cells is contrary to the findings of Eaton (1975) and Igarashi & Stollar (1976). However, this may merely reflect the properties of the G8 clone compared with uncloned A. albopictus cell cultures.
Table 1. *Assay of DI SFV in different cell types*

<table>
<thead>
<tr>
<th>Cell Origin</th>
<th>Interference titre*†</th>
</tr>
</thead>
<tbody>
<tr>
<td>L929 Mouse</td>
<td>2.9</td>
</tr>
<tr>
<td>MDBK Bovine</td>
<td>2.6</td>
</tr>
<tr>
<td>NRK Rat</td>
<td>2.6</td>
</tr>
<tr>
<td>BHK21 Hamster</td>
<td>2.5</td>
</tr>
<tr>
<td>BSC-1 Monkey</td>
<td>2.4</td>
</tr>
<tr>
<td>CV-1 Monkey</td>
<td>2.35</td>
</tr>
<tr>
<td>L spinner Mouse</td>
<td>2.35</td>
</tr>
<tr>
<td>BGM Monkey</td>
<td>2.35</td>
</tr>
<tr>
<td>HFF Human</td>
<td>2.3</td>
</tr>
<tr>
<td>NIH/3T3 Mouse</td>
<td>1.95</td>
</tr>
<tr>
<td>G8 Mosquito</td>
<td>1.85</td>
</tr>
<tr>
<td>CHO-α3 Hamster</td>
<td>1.25</td>
</tr>
<tr>
<td>HeLa Human</td>
<td>1.15</td>
</tr>
</tbody>
</table>

* Conditions of assay were those described in the text except that G8 cells were incubated at 28 °C.
† Titres (log10 DIU/ml) were normalized by reference to a parallel titration of DI virus of known titre in L929 cells and to a constant number of cells (2 × 10^5) per assay tube.

Table 2. *Failure to measure interferon (IFN) activity by the DI virus interference assay in L929 cells*

<table>
<thead>
<tr>
<th>Interference titre (log_{10} DIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IFN</td>
</tr>
<tr>
<td>Hamster IFN</td>
</tr>
<tr>
<td>DI SFV, pH 2*</td>
</tr>
<tr>
<td>DI SFV</td>
</tr>
</tbody>
</table>

* DI virus was dialysed against pH 2 buffer solution for 5 days and then against PBS (pH 7.4) for 12 h at 4 °C.

Table 3. *Failure to detect interferon (IFN) in DI virus preparations*

<table>
<thead>
<tr>
<th>Hamster IFN assay* (log_{10} IFN titre)</th>
<th>Mouse IFN assay (log_{10} IFN titre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI SFV, pH 2†</td>
<td>≤0.6</td>
</tr>
<tr>
<td>Hamster IFN</td>
<td>≤0.6</td>
</tr>
<tr>
<td>Mouse IFN</td>
<td>≤0.6</td>
</tr>
<tr>
<td>ND‡</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* Hamster and mouse IFNs were assayed in CHO-α3 cells and mouse L929 cells respectively by the method of Atkins et al. (1974).
† DI SFV was dialysed against pH 2 buffer as described in Table 2.
‡ ND, Not done.

**Multiplicity of infection**

Fig. 2 shows that maximum interference occurred at an m.o.i. of 50. Both lowering and increasing the m.o.i. decreased the titre. The explanation is unknown but possibly intracellular contact between replicating DI and standard viruses occurs less frequently at low m.o.i. and at high values standard virus genomes are present in excess. At lower multiplicities (<5) there was insufficient incorporation of ^3H-uridine.

**Time of radiolabelling and temperature of incubation**

Pulsing from 4 to 5 h p.i. was optimal and gave significantly higher levels of interference than at later times. This was also the time of maximum viral RNA synthesis. Likewise, with a labelling time of 4 to 5 h, a temperature of 37 °C was preferred to 33 or 39 °C.

**Presence of interferons**

Mouse and hamster interferons (each a mixture of IFN-α and IFN-β: see Interferon
Assay of DI SFV

Fig. 3. Growth curves of SFV infectivity and interfering activity in BHK cells inoculated with (a) standard virus alone or (b) standard virus at an m.o.i. of about 260 and DI virus at an m.o.i. of 2 DIP. Rolled burler bottles containing $2 \times 10^6$ cells were inoculated, rinsed and incubated at 33 °C with 100 ml medium. Samples of tissue culture fractions were withdrawn at the times indicated and titrated for infectivity (O) and interference (■). The DIU were converted to DIP by multiplying by $10^5$, the number of cells infected at 50% inhibition of $^3$H-uridine incorporation into infected cells (see text). Data shown are the mean (+1 S.E.M. of individual titrations from three replicate burler bottles receiving standard virus plus DI virus and two receiving standard virus alone. Arrows show that no DIP were detected.

Nomenclature, 1980) used in place of DI virus in the interference assay caused no reduction of RNA synthesis in the virus control (Table 2). This was expected since the presence of Act. D throughout the assay would prevent induction of an antiviral state. Conversely, assay for the presence of interferon in DI virus samples by the method which inhibits incorporation of TCA-precipitable $^3$H-uridine into cells infected with a challenge virus (Atkins et al., 1974) was negative (Table 3).
Propagation of DI SFV in BHK cells and its generation de novo

The interference assay permitted detailed measurements of the production of DI SFV and its effect on the multiplication of standard virus at 33 °C (Fig. 3). A high m.o.i. of standard virus alone resulted in an exponential increase of infectivity from 4 to 16 h p.i. The titre remained constant up to 48 h p.i. and then declined abruptly, due probably to cessation of progeny virus production coupled with thermal inactivation. In cells receiving the same m.o.i. of standard virus together with DI virus, exponential increase in infectivity was delayed until 10 h p.i. and reached a plateau at 16 h. Some further increase took place but the titre remained at about 10% of that of virus from cells receiving standard virus alone. The very steep decline in infectivity was not apparent even at 72 h p.i. Cytopathic changes were extensive at 24 h p.i. with standard virus but even at 72 h p.i. a few apparently healthy cells remained. In the presence of added DI virus cytopathic effects progressed slowly and did not extend to the majority of cells until 72 h p.i. Thus, DI virus appeared to give some protection.

The titre of DI virus, measured by the interference assay, rose in parallel with standard virus from the same culture and also reached a plateau at 16 h. However, over the next 46 h the DI titre rose slowly by 10-fold, a useful finding when large amounts of DI virus are required. DI virus was also detected in the culture receiving standard virus alone at 40 h p.i., some 26 h after peak production of infectivity. The maximum value was attained at 54 h p.i., which occurred later than the rapid decrease of infectivity. It is possible that DI virus was produced by the few surviving cells (see above) which persisted even at 72 h p.i. Experiments at 37 °C gave similar results but the yield of DI virus and infectivity were both lower than at 33 °C.

U.v. irradiation of DI SFV

A sample containing DI virus was irradiated and assayed at intervals for remaining interference and infectivity (Fig. 4). DI alphaviruses were more resistant than infectious virus as reported earlier for SFV (Dimmock & Kennedy, 1978) and for Sindbis virus (Kowal & Stollar, 1980) but underwent significant inactivation apparently with single-hit kinetics (about $1.3 \log_{10}/100$ s).

DISCUSSION

The aim of any assay is to be simple, sensitive, rapid and reproducible. The interference assay for DI SFV which we have described involves standard laboratory procedures and takes 5 h. Thus, the titre of an unknown sample can be determined within a working day.
There are advantages over infectious centre assays which require laborious cell counting and plating-out procedures and take some 3 days to achieve a result. Our assay is reproducible with a standard error of $6.3 \pm 2.0 \times 10^7$. Sensitivity is governed by the number of cells/assay tube. Thus, assuming at the 50% dilution endpoint of inhibition of $^3$H-uridine incorporation that 50% of the cells (i.e. $10^5$ cells) receive at least one DI virus particle, the assay can detect a minimum titre of $1.4 \times 10^5$ DIP per 250 μl. We found that the use of subconfluent monolayers increased sensitivity of the assay but with an unacceptable lack of reproducibility (data not shown). At present we are transferring the assay to microtitre trays which, with about $2 \times 10^4$ cells/well, should give an increase in sensitivity in proportion to the reduction in the number of cells. The problem of sensitivity applies equally to the infectious centre and infectious virus yield assays as sufficient cells have to receive DI virus for the extent of the inhibitory effects to become significant. However, our assay compares very favourably with recently published methods which measure the reduction in yield of infectious alphaviruses in the presence of dilutions of DI virus. These assume that a concentration of 1 DIP/cell reduces the yield by 63%. Hence, they can detect DIPs equivalent to 63% of the number of cells in the monolayer, i.e. $1.3 \times 10^6$ DIP (Kowal & Stollar, 1980) and $6.3 \times 10^6$ DIP (Fuller & Marcus, 1980) and are thus at least 10-fold less sensitive than the assay reported here. Only the focus-forming assays (Popescu et al., 1976; Winship & Thacore, 1980) offer higher sensitivity but are suited to few virus systems and are slow to produce a result.

Clearly, our assay works only because DI SFV causes a reduction in the synthesis of virus-specified RNAs in infected cells. This also implies that the view of interference as a competition between DI and standard virus RNAs which results in faster replication of the former, does not apply to DI SFV since it predicts that DI RNA synthesis supplants that of the standard virus.

The assay has allowed the measurement of the u.v. sensitivity of interference. As with other viruses (LCM virus: Welsh et al., 1972; SFV: Dimmock & Kennedy, 1978; influenza virus: Nayak et al., 1978; VSV: Bay & Reichmann, 1979; and Sindbis virus: Kowal & Stollar, 1980) interference was more resistant than infectivity, confirming that the u.v. target size was in agreement with the relative sizes of DI and standard virus genomes. The inactivation rate of interference was 13% of that of infectivity. Hence, assuming that the u.v. target and genome sizes of standard virus are equal, the size of the DI genome required for interference is about $0.6 \times 10^6$. This value is close to the mol. wt. ($0.8 \times 10^6$) of DI RNA of the same virus at passage 8 (Bruton & Kennedy, 1976), suggesting that the entire genome is required for interference.

The interference assay enabled us to examine the kinetics of generation and production of DI virus, about which little is known except for the VSV system (Kang, 1980; Faulkner & Lazzarini, 1980). Interference and infectivity rose in parallel in cultures inoculated with DI and standard viruses together, and infectivity was delayed compared with cells receiving standard virus alone. However, after the initial exponential increase, DI virus rose slowly by 10-fold over the next 2 days. Standard virus gave a conventional one-step growth curve with DI virus being undetectable until 40 h p.i. This has to be compared with the six high m.o.i. passages of SFV, also in BHK cells, which were required before Bruton & Kennedy (1976) had evidence of DI virus production from the decreased yield of infectious virus. It also emphasizes the importance of prolonged incubation in the generation of DI virus noted for Sindbis virus (King et al., 1979). Infected cells apparently continue to synthesize viral products after the exponential increase in infectivity has ended.

This work was supported by the Medical Research Council. We thank P. J. Rees and K. B. Logan for helpful discussions and Caroline Woodford for assistance in the laboratory. Merck, Sharpe and Dohme kindly donated actinomycin D.
REFERENCES


(Received 20 October 1980)