Defective-Interfering Particles of Semliki Forest Virus: Intracellular Events During Interference

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(Accepted 30 January 1976)

SUMMARY

Purified defective-interfering (DI) particles of Semliki Forest virus are unable to carry out any of the steps in virus multiplication except uncoating. Cells co-infected with DI particles and standard virus contain several virus-specified RNA species (DI particle-specific species) absent from cells infected with standard virus alone. Moreover, synthesis of all the virus-specified components distinctive of standard virus-infected cells is reduced. The DI particle-specific RNA species comprise two poly A-containing single-stranded RNAs (D1ss1 and D1ss2), identical to those found in purified DI particles, two double-stranded RNAs (RFs) and a new size class of replicative intermediate (RI).

Hybridization experiments showed that the nucleotide sequences of D1ss1 and D1ss2 (i) are present in the 42S genome of standard virus but absent from the 26S RNA – the RNA from standard virus-infected cells which encodes the structural proteins of the virion (Clegg & Kennedy, 1975a) and (ii) are complementary to the negative strands of the DI particle-specific RFs and RI. Oligonucleotide fingerprinting revealed extensive nucleotide sequence homology between D1ss1 and D1ss2. Analysis of the mRNA complement of standard virus-infected, co-infected and uninfected cells strongly indicated that neither D1ss1 nor D1ss2 can serve as a functional messenger RNA.

From these studies we propose a mechanism for the multiplication of and interference by DI particles of Semliki Forest virus.

INTRODUCTION

We have reported (Bruton & Kennedy, 1976, see previous paper) the accumulation of defective-interfering (DI) particles of Semliki Forest virus (SFV) during high multiplicity passaging in BHK cells. These DI particles differ from standard virus in that whereas each standard virus particle contains a molecule of infectious single-stranded RNA of mol. wt. \( \sim 4.1 \times 10^6 \) (Levin & Friedman, 1971; Martin & Burke, 1974), each DI particle contains several molecules of RNA of mol. wt. 0.81 and 0.75 \( \times 10^6 \). Moreover, unlike the DI particles of many other animal virus systems, SFV DI particles are denser (on CsCl gradients) than standard SFV. Infectious RNA assays indicate that the low mol. wt. RNA species of SFV DI particles are the agents responsible for interference.

Over the last few years many of the steps in the multiplication of standard alphaviruses such as SFV and Sindbis virus have been elucidated. After uncoating the input 42S genome is translated to give an RNA-dependent RNA polymerase(s) (Martin & Sonnabend, 1967;
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Sreevalsan & Yin, 1969; Friedman et al. (1972) which, through an intermediate 42S negative strand (Simmons & Strauss, 1972; Segal & Sreevalsan, 1974; Bruton & Kennedy, 1975) mediates the synthesis not only of progeny 42S RNA but also of 26S RNA. This latter RNA species represents a unique one third of the genome (Simmons & Strauss, 1972a) and is the messenger for the structural proteins of the virus particle (Clegg & Kennedy, 1974a; Cancedda, Swanson & Schlesinger, 1974; Wengler, Beato & Hackemack, 1974; Simmons & Strauss, 1974a; Clegg & Kennedy, 1975a, b). Translation of this polycistronic RNA yields the core protein of the virus nucleocapsid and, through a precursor, the three glycoproteins of the virus envelope (Clegg, 1975; Clegg & Kennedy, 1975b). Newly synthesized core protein rapidly associates with progeny 42S RNA giving intracellular nucleocapsids (Söderlund, 1973). Virus maturation occurs by budding of the nucleocapsids through patches of plasma membrane which have been modified by insertion of the virus glycoprotein (Sefton, Wickus & Burge, 1973; Waite, 1973). Many of these biosynthetic events are carried out either on or in close association with cytoplasmic membranes which undergo extensive rearrangement during the course of virus multiplication (Grimley, Berezesky & Friedman, 1968; Grimley et al. 1972).

Against this background of events in standard virus multiplication, we have examined the virus-specified events which occur in cells infected either with SFV DI particles (Bruton & Kennedy, see accompanying paper) alone or co-infected with DI particles and standard virus. We report that DI particles by themselves are unable to carry out any of the events in multiplication except uncoating. In addition we show that the pattern of virus-specified components in co-infected cells differs markedly from those in cells infected with standard virus alone. Of particular interest, is the appearance of several new species of virus-specified multi-stranded and single-stranded RNAs and a marked reduction in the intracellular level of all the standard virus-specified components. By comparing the properties of the DI particle-specific RNAs with those of standard virus we conclude that SFV DI particles arise by a 'mistake(s)' in the synthesis of virus-specified single-stranded RNA and that this 'mistake' leads not only to interference but also to the selective propagation of DI particles.

METHODS

Materials. Actinomycin D was a generous gift from Merck, Sharp and Dohme Research Laboratories, N.J., U.S.A. Sodium dodecyl sulphate (SDS, especially pure grade), naphthalene-1,5-disulphonic acid (disodium salt; NDS) and formamide (AnalaR) were obtained from British Drug Houses Ltd, Poole, Dorset. Agarose was supplied by L'Industrie Biologique Française S.A., Grennevières, France and oligo(dT)-cellulose by Collaborative Biochemicals, Inc., Waltham, Mass., U.S.A. 32P-orthophosphate (92 to 110 Ci/mg phosphorus), 5-3H-uridine (24 Ci/mmol), 35S-methionine (67 to 310 Ci/mmol) and guanosine-5,32P-triphosphate (7.4 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks. The source of all media, enzymes and other chemicals was as reported by Bruton & Kennedy (1975). Phenol, ethanol and ether were all redistilled and formamide recrystallized at −8 °C before use.

Virus and cells. The standard SFV (passage 0 virus), purified DI particles and passage 8 virus used in the present study were those described by Bruton & Kennedy (see accompanying paper). Monolayer cultures of BHK cells, clone 13 and of primary chick embryo fibroblasts were grown in 14 cm plastic Petri dishes or in 2.5 l smooth-walled roller bottles as described by Morser, Kennedy & Burke (1973).

Infection and labelling. Cultures were infected with 20 to 100 p.f.u. of standard virus or
passage 8 virus per cell. The 'input' multiplicity of purified DI particles, calculated from the ratio of the $^3$H-uridine radioactivity in purified DI particles to identically labelled and purified standard virus of known infectivity, was equivalent to 100 p.f.u./cell. After adsorption in the presence of 1 μg/ml of actinomycin D for 1 h at 37 °C the fluids were replaced with maintenance medium (Morser et al. 1973) containing 1 μg/ml actinomycin D. All labelling was performed in 10 ml of EDA medium (Morser et al. 1973) buffered with 20 mM-HEPES (HEDA). Labelling times are expressed relative to the end of the adsorption period. Virus-specified RNA was labelled from 1 to 6 h post-infection (p.i.) with (i) 500 μCi $^3$H-uridine or 5 to 6 mCi $^{32}$P-orthophosphate per roller bottle or (ii) 50 μCi $^3$H-uridine or 10 μCi $^{14}$C-uridine per Petri dish culture. For cultures labelled with $^{32}$P-orthophosphate, phosphate-free HEDA was used. Virus and host-specified polypeptides were labelled from 3 to 4 h p.i. with 50 μCi of $^{35}$S-methionine/Petri dish culture.

Fate of the input RNA of standard virus and DI particles. $^{32}$P-labelled standard virus and DI particles, grown, labelled with 1 mCi $^{32}$P-orthophosphate/culture and purified by banding on CsCl under identical conditions (Bruton & Kennedy, 1975) were dialysed against maintenance medium (Morser et al. 1973) and then used to infect two Petri dish cultures of BHK cells. After 1 h at 4 °C the fluids were removed and after washing the monolayers six times with maintenance medium the cultures were incubated at 37 °C for 1 h. The cultures were again washed six times with maintenance medium and cytoplasmic extracts prepared as described by Burge & Pfefferkorn (1967). TCA precipitation and ribonuclease digestion were performed as described by Clegg & Kennedy (1974b).

Assay of SFV-specified RNA polymerase activity. Cytoplasmic extracts were prepared from infected or uninfected roller bottle cultures of BHK cells and assayed for RNA-dependent RNA polymerase activity as described by Friedman et al. (1972).

Isolation and purification of virus-specified RNA. At the end of the labelling period nucleic acid was isolated using the NDS/SDS/phenol/chloroform techniques previously described (Clegg & Kennedy, 1974a, b). Virus-specified multi-stranded RNA species, i.e. replicative form (RF) and replicative intermediate (RI), were purified as described before (Bruton & Kennedy, 1975). Virus-specified single-stranded RNA species were purified by centrifuging on linear 6 to 30 % (w/v) sucrose gradients and by oligo(dT)-cellulose chromatography as described by Clegg & Kennedy (1974b) except that the binding buffer for the oligo(dT)-cellulose column was 0·3 M in LiCl. Single-stranded RNA species were further purified by polyacrylamide gel electrophoresis on 150 × 2·8 mm slabs cast to a height of 160 mm (see below). Gels composed of 1·7 % (w/v) acrylamide–0·5 % (w/v) agarose were used for the purification of the 42S and 26S species – gels of 2·6 % (w/v) acrylamide for DIss1 and DIss2. RNA was eluted from strips with 0·15 M-sodium acetate containing 0·5 % SDS (pH 6·0) by gentle stirring at 37 °C overnight. After a further extraction with 10 ml of the same buffer for 4 h at 37 °C, gel pieces were removed by centrifuging at 20000 g for 30 min at 10 °C. Finally oligo(dT)-cellulose chromatography (Clegg & Kennedy, 1974b) was used to concentrate the RNA and to remove soluble acrylamide and the purified RNA species were precipitated with 2·5 vol. ethanol at −20 °C. Before use the integrity of each species was checked by analytical polyacrylamide gel electrophoresis.

Analytical sucrose gradient sedimentation of RNA. The RNA from cells infected with standard virus, passage 8 virus and from uninfected cells was analysed by velocity sedimentation on 4·8 ml 6 to 30 % (w/v) sucrose gradients as described by Atkins, Samuels & Kennedy, (1974).
Analytical polyacrylamide gel electrophoresis

RNA electrophoresis in aqueous conditions. Seventeen cm cylindrical gels of 6 mm diam. or 16 cm vertical slab gels (Studier, 1973) each with four 20 × 17 mm sample slots were used. Gels of either 1.7 % (w/v) acrylamide + 0.13 % bisacrylamide + 0.5 % agarose or 2.6 % (w/v) acrylamide + 0.13 % bisacrylamide were prepared and run as previously described (Clegg & Kennedy, 1974b).

RNA electrophoresis in formamide conditions. Electrophoresis on 3.4 % (w/v) acrylamide + 0.6 % bisacrylamide gels prepared in diethyl barbiturate buffered formamide was performed in 160 mm vertical slab gels using the conditions of Staynov, Pinder & Gratzer (1972) except that 0.02 M-NaCl containing 0.8 mM-EDTA (pH 7.0) was used as electrode buffer. Electrophoresis was for 30 h at 90 V fixed potential.

Polypeptide analysis. 35S-methionine labelled polypeptides were extracted from cells, reduced, alkylated and analysed on 16 cm 10 % (w/v) acrylamide + 0.27 % bisacrylamide gels as described by Laemmli (1970).

After electrophoresis cylindrical gels were sliced into 1 mm segments which were dissolved and counted as previously described (Kennedy & Burke, 1972). Slab gels containing 32P-labelled RNA were covered in polythene sheeting and autoradiographed without drying. Other slab gels were dried under vacuum at 90 °C before autoradiography. Autoradiography was on Kodirex X-ray film or on Kodak RP Royal X-Omat film.

Hybridization reactions. RNA samples were recovered from alcohol by centrifugation, dried, dissolved in 1 mM-EDTA pH 7.4 and their concentration determined from E250 measurements. All hybridization reactions were carried out in sealed sterile 60 × 6 mm internal diam. borosilicate tubes.

Reactions involving 42S and 26S RNAs as competitors. Into each tube was placed 860 ng RFI (this RF which consists of a duplex of a 42S positive and negative strand (Bruton & Kennedy, 1975) was used as a source of negative strands), approx. 45 ng 32P-labelled DI single-stranded RNA (15,470 ct/min of Dlss1 and 11,780 ct/min of Dlss2) and from 0 to 32,000 ng of either 26S or 42S RNA all in a volume of less than 100 µl.

Reactions involving DI RFs and RIs. Into each tube was placed 50 to 78 ng of 32P-labelled DIdsl, DIds2 or low mol. wt. RI (between 21,000 and 48,000 ct/min) and 32,000 ng of 42S, 26S, Dlss1 or Dlss2 RNA all in a volume of less than 100 µl.

After the addition of 0.6 ml 0.4 M-NaCl the tubes were sealed and incubated at 128 °C for 3.5 min then at 80 °C for 16 to 18 h for the competition experiments and for 2 h for other experiments. After cooling, the contents of each tube were quantitatively transferred to 2 ml of 10 mm-tris (pH 7.2) containing 500 mm-NaCl, 10 mm-MgCl2, 100 units ribonuclease T1 and 20 µg pancreatic ribonuclease, digested at 37 °C for 30 min and then TCA precipitated and counted as previously described (Clegg & Kennedy, 1974b). All reactions were performed in triplicate. Preliminary experiments showed that under these incubation conditions: (i) SFV RFs completely melt; (ii) reannealing in the competition experiments is complete by 12 h; (iii) in the absence of added single-stranded RNA 1 to 3 % of the radioactivity in DI RFs and RIs is ribonuclease resistant after 3 h at 80 °C and (iv) in the absence of nuclease digestion 80 to 95% of the input radioactivity is recovered in TCA precipitable counts.

Analysis of RIs. Samples were recovered from alcohol by sedimentation, dried, dissolved in 10 mm-tris containing 0.1 mm-NaCl, 0.5 % SDS and 40 % (w/v) sucrose and layered on to 12 cm 1 % (w/v) agarose gels cast in 6 mm internal diam. perspex tubes. Gels were pre-
pared in 40 mM-sodium acetate containing 10 mM-tris and 1 mM-EDTA (pH 8.0; Hewlett & Baltimore, personal communication) which was also used as reservoir buffer. Electrophoresis was for 10 h at 8 mA/gel at room temperature. After electrophoresis, gels were sliced and counted as previously described (Kennedy & Burke, 1972).

Analysis of polyribosomal RNAs. The mRNAs present on polyribosomes from uninfected, standard virus infected and passage 8 virus-infected BHK cells were analysed as described by Kennedy (1972) with the modification that the mRNAs from unfractionated polyribosomes, i.e. free plus membrane-bound polyribosomes, were examined.

Oligonucleotide fingerprinting. DIss1 and DIss2 were each digested with about 1/10 by weight of ribonuclease T1 and the resultant nucleotides fractionated using electrophoresis on cellulose acetate at pH 3.5 in 7 M-urea in the first dimension and ionophoresis on DEAE-paper at pH 1.9 in the second dimension (Sanger, Brownlee & Barrell, 1965). Between 3 and 4 × 10⁶ ct/min of each RNA was fingerprinted. After autoradiography on Kodirex X-ray film selected oligonucleotides were cut out eluted, digested with pancreatic ribonuclease (100 μg/ml for 30 min at 37 °C) and the products analysed by electrophoresis at 60 V/cm at pH 3.5 on Whatman No. 540 paper. The identity of the pancreatic ribonuclease products was established from markers of known structure.

RESULTS

Events in cells infected with purified DI particles

In order to determine to what extent DI particles of SFV are capable of independent multiplication, we first compared the RNA and polypeptide species synthesized in cells infected with purified DI particles (Bruton & Kennedy, see accompanying paper) with those synthesized in standard virus-infected and in uninfected cells. Whereas standard virus-infected cells contained the two major species of single-stranded RNA reported previously (Simmons & Strauss, 1972a; Martin & Burke, 1974), namely 42S and 26S RNA, no discrete RNA species larger than ~ 4S were detected in either uninfected or DI particle-infected cells (Fig. 1a). This marked difference between standard virus-infected and DI particle-infected cells was also apparent from the polypeptide analysis (Fig. 1b). Indeed the polypeptides synthesized in DI particle-infected cells were indistinguishable from those synthesized in uninfected cells. This observation indicates that DI particles are not only unable to direct the synthesis of significant amounts of the standard virus-specific polypeptides but also that they are unable to shut off host protein synthesis. However, since nothing is known about the polypeptide composition of the SFV RNA-dependent RNA polymerase(s) and since the polymerase(s) is likely to be made in very small amounts compared to the structural proteins and their precursors, it could be argued that the DI particles specify a replicase, albeit a replicase incapable of synthesizing discrete sized RNA. To investigate this possibility we compared the activity of the cytoplasmic RNA-dependent RNA polymerase from standard virus-infected cells with that from DI particle-infected and uninfected cells (Friedman et al. 1972). The replicase activities were 137417, 2717 and 2320 (expressed as ct/min from ³²P-GTP incorporated into TCA precipitable product/mg protein) from standard virus-infected, DI particle-infected and uninfected cells respectively. Thus it appears that the genetic material of the DI particles cannot be translated to give a functional replicase. In the light of these findings it was important to determine if the DI particles can uncoat and if so the fate of the input genetic material. To do this we infected BHK cells
Fig. 1. Analysis of the RNA and polypeptide species labelled in mock-infected BHK cells and in cells infected with purified standard SFV or DI particles. Two pairs of Petri dish cultures of BHK cells were infected with approx. 100 p.f.u./cell of purified SFV and its equivalent of purified DI particles respectively. Two cultures were mock-infected. Actinomycin D (1 μg/ml) was present throughout incubation. (a) One of each pair of cultures was labelled from 1 to 6 h p.i. with 100 μCi of 3H-uridine and total nucleic acids extracted and analysed on 6 to 30 % (w/v) linear sucrose gradients as described in Methods. Sedimentation is from left to right. Radioactivity in: ○—○, standard virus-specified RNA; ●—●, DI particle-specified RNA; △—△, uninfected cell RNA, was determined by TCA precipitation. (b) The remaining cultures were labelled from 3 to 4 h p.i. with 50 μCi 35S-methionine and their polypeptides extracted, reduced, alkylated and electrophoresed on a 10 % polyacrylamide slab gel as described in Methods. The same number of cts/min (15,000) in the polypeptides from: S, standard virus infected; DI, DI particle infected and U, uninfected cells were applied to the gel. Autoradiography was for 5 days. The polypeptides p120 (formerly NVP 97), p68 (formerly NVP 63), E1(E2) and C are all virus-specified (Morser et al. 1973).
Fig. 2. Polyacrylamide gel electrophoresis of the RNA species from cells infected with standard virus or passage 8 virus. Two pairs of Petri dish cultures were infected with approx. 20 p.f.u./cell of standard virus and passage 8 virus respectively. The cultures were labelled from 1 to 6 h p.i. with 100 μCi ³H-uridine and total nucleic acid extracted and electrophoresed in parallel on 17 cm 1.7% (w/v) acrylamide + 0.5% agarose gels as described in Methods. Migration in this and all subsequent electrophoretograms is from left to right. After electrophoresis the gels were cut into 1 mm sections and counted for radioactivity in: O—O, standard virus-specified RNA; ●—● passage 8 virus-specified RNA.

Events in cells co-infected with standard virus and DI particles

Since it would appear, therefore, that SFV DI particles are unable to independently carry out any step in virus multiplication except uncoating, we turned our attention to an examination of the virus-specified intracellular events occurring in cells co-infected with standard virus and DI particles. Because (i) of the damaging effect of caesium chloride on
Fig. 3. Polyacrylamide gel electrophoresis of the RNA species from purified DI particles and cells infected with passage 8 virus. The RNA from cells infected with passage 8 virus (○—○) prepared as described in the legend to Fig. 2 was mixed with 32P-labelled RNA from purified DI particles (●—●) prepared as described by Bruton & Kennedy (1976; see accompanying paper) and analysed by electrophoresis on a 2-6 % (w/v) polyacrylamide gel.

infectivity (Bruton & Kennedy, see accompanying paper) and (ii) because we wished to study the appearance of DI particles during passaging we used the fluid from cells infected with passage 7 virus (referred to as passage 8 virus) as inoculum in the co-infection studies. This fluid, which was the source from which purified DI particles were prepared, also contained 6·2 × 10^6 p.f.u./ml of standard virus (Bruton & Kennedy, see accompanying paper). The RNA species found in BHK cells infected with standard virus or passage 8 virus were analysed in parallel by electrophoresis on 1·7 % (w/v) polyacrylamide + 0·5 % agarose gels. As Fig. 2 shows, standard virus-infected cells contained the multi-stranded RI and RFs and four single-stranded species of RNA observed previously (Levin & Friedman, 1971; Martin & Burke, 1974). By contrast, the major species of RNA isolated from cells infected with passage 8 virus was of lower mol. wt. than any of the standard virus-specific species which, in turn, were present in markedly reduced amounts despite the fact that essentially identical m.o.i. (in terms of p.f.u. of standard virus) were used for the standard virus and passage 8 virus-infected cultures. Thus interference occurs at the level of RNA synthesis.

We now compared the low mol. wt. RNA of passage 8 infected cells with that found in purified DI particles using 2·6 % (w/v) polyacrylamide gel electrophoresis (conditions which resolved two low mol. wt. RNA species from purified DI particles; Bruton & Kennedy, 1976, see previous paper). These conditions (Fig. 3) resolved the low mol. wt. RNA from passage 8 infected cells into two species which had identical electrophoretic mobilities to
those found in purified DI particles and it seems likely that the genetic content of the intracellular species and the DI particle species is the same. Hereafter we shall refer to these RNA species as DIss1 and DIss2 corresponding respectively to the 0.81 and 0.75 × 10⁶ mol. wt. RNAs of DI particles (Bruton & Kennedy, see previous paper). Before attempting to further characterize these RNAs we investigated the possibility that they might be conformational variants of one another. We did this because (i) of the reported presence of only a single low mol. wt. RNA in cells co-infected with Sindbis DI particles and standard virus (Weiss et al. 1974) and (ii) the 26S and 33S RNAs from cells infected with standard Sindbis virus appear to be conformational variants (Simmons & Strauss, 1974b).

Conformational isomerism was investigated using a polyacrylamide gel electrophoresis
in formamide: a technique where the electrophoretic mobility of RNA is independent of its secondary structure (Staynov et al. 1972). Under these conditions partially purified low mol. wt. RNA from passage 8 infected cells was resolved into two bands (Fig. 4) indicating that Dlss1 and Dlss2 are not conformational variants. After purification on a preparative polyacrylamide gel (see Methods) Dlss1 and Dlss2 bound to the extent of 84% and 80% respectively to oligo(dT)-cellulose (Clegg & Kennedy, 1974b), indicating that both species contain a poly A tract.

We now examined the relationship between the nucleotide sequences present in Dlss1 and Dlss2 and those of the 42S and 26S RNAs from cells infected with standard virus. This was done using competition hybridization by taking Dlss1 and Dlss2 in turn and determining to what extent their annealing to 42S negative strands (from purified RFI; Bruton & Kennedy, 1975) could be competed out by either 42S or 26S RNA. As Fig. 5 shows, 42S RNA was able to compete out both Dlss1 and Dlss2, confirming that the nucleotide sequences of the DI particle RNAs are contained in the sequence of the standard virus genome. By contrast, however, neither Dlss1 nor Dlss2 were competed out to any significant extent by 26S RNA. This result indicates that the nucleotide sequences of Dlss1 and Dlss2 are not present in the 26S region of the virus genome.

Cells infected with standard SFV contain a single major species of RF (RFI) which consists of a duplex of a 42S positive and negative strand (Martin & Burke, 1974; Bruton & Kennedy, 1975). In order, therefore, to further characterize the RNA species of cells co-

![Graph](https://example.com/graph.png)
Fig. 6. Polyacrylamide gel electrophoresis of the double-stranded (RF) species isolated from BHK cells infected with various passages of SFV. Six roller bottle cultures of BHK cells were infected with 20 p.f.u./cell of standard virus (passage 0), passage 3, passage 6, passage 7 and passage 8 virus respectively and labelled from 1 to 6 h p.i. with 500 µCi/culture of ³H-uridine. After extraction of total nucleic acids, double-stranded RNA was purified by LiCl precipitation and CF₁₁ cellulose chromatography (Bruton & Kennedy, 1975) and then analysed in parallel on 17 cm 1·7 % (w/v) acrylamide +0·5 % agarose gels.

infected with standard virus and DI particles and in an attempt to gain insight into the genesis of DIss₁ and DIss₂, we analysed the purified RFs present in cells infected with 20 p.f.u./cell of various passages of SFV. The result of this analysis (Fig. 6) shows that passage 0 and passage 3 infected cells contain only RFI but that by passage 6 smaller RFs appear and that cells infected with passage 8 contain very little RFI and in its place two
Fig. 7. Agarose gel electrophoresis of the RI(s) isolated from cells infected with standard virus or passage 8 virus. Two roller bottle cultures of BHK cells, infected with 10 p.f.u./cell of standard virus and passage 8 virus respectively were labelled from 1 to 6 h p.i. with 500 μCi/culture of 3H-uridine and total nucleic acid extracts prepared. The RI(s) were purified from those extracts by Sepharose 2B chromatography (Bruton & Kennedy, 1975) and electrophoresed on 12 cm 1% (w/v) agarose gels as described in Methods. Radioactivity in the RI(s) from: O—O, standard virus; ●—●, passage 8 virus infected cells.

smaller RFs. The nature of the intermediate sized RF – at fraction 41 of the analysis of the RFs from passage 6 infected cells – has not been characterized. Thus cells infected with passage 8 virus contain not only two new species of single-stranded RNA, but also two new species of double-stranded RNA which we shall refer to as Dlds1 and Dlds2 in order of decreasing electrophoretic mobility. The mol. wt. of these RFs (together), estimated from sedimentation analysis (Bruton & Kennedy, 1975), was found to be 1.6 × 10^6 (data not presented).

Since the status of double-stranded RNA in the synthesis of alphavirus single-stranded RNA is unclear, we examined the size of the replicative intermediate(s) isolated from cells infected with standard virus and passage 8 virus. Using agarose gel electrophoresis we observed that the size of the purified RI(s) from standard virus-infected cells was considerably larger than the major RI(s) from passage 8 virus-infected cells (Fig. 7).

To determine the relationship between the single-stranded species of both standard virus and passage 8 virus infected cells and the negative strands of Dlds1, Dlds2 and the low mol. wt. RI(s), these multi-stranded species, labelled with 32P-orthophosphate were purified, mixed with a 5000-fold weight excess of each of the purified single-stranded species in turn and after melting and reannealing the ribonuclease resistance of the RNA was measured. Reannealing conditions were such that in the absence of added single-stranded RNA only 1 to 3% of the input radioactivity became ribonuclease resistant (see Methods). Table 1, which shows the result of this experiment, clearly indicates that the negative strands of Dlds1, Dlds2 and the low mol. wt. RI(s) can form a ribonuclease-resistant hybrid with 42S RNA or with either DIss1 or DIss2 but not with 26S RNA. Two points emerge from this experiment. Firstly,
Table 1. Hybridization properties of the negative strand of
Dlds1, Dlds2 and the low mol. wt. RI(s)

<table>
<thead>
<tr>
<th>Added RNA</th>
<th>Dlds1</th>
<th>Dlds2</th>
<th>RI(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None*</td>
<td>3 ± 1</td>
<td>2 ± 2</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>42S</td>
<td>48 ± 1</td>
<td>50 ± 3</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>26S</td>
<td>7 ± 1</td>
<td>8 ± 2</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>DIss1</td>
<td>50 ± 3</td>
<td>53 ± 4</td>
<td>37 ± 5</td>
</tr>
<tr>
<td>DIss2</td>
<td>47 ± 1</td>
<td>48 ± 3</td>
<td>34 ± 3</td>
</tr>
</tbody>
</table>

* Concentrations of Dlds1 and Dlds2 and conditions of renaturation were such as to minimize the self-
annealing of the positive and negative strands of the DI double-stranded RNAs (see Methods for details).
† Values are expressed as the mean ± standard error of the mean of five determinations.

since the negative strands of Dlds1 and Dlds2 formed a hybrid with both DIss1 and DIss2
and knowing that the mol. wt. of the double-stranded RNAs (together) is almost exactly
twice the average mol. wt. of DIss1 and DIss2, then there must be extensive sequence
homology between these two single-stranded RNAs. Secondly, since the negative strand of
the low mol. wt. RI(s) can also be protected by DIss1 or DIss2 we propose that the templates
for the synthesis of the DI particle RNAs are the component negative strand(s) of the low
mol. wt. RI(s). Dlds1 and Dlds2 may be intermediates in, or products of, this process. This
situation would then differ markedly from that in cells infected with standard virus alone
where a 42S negative strand appears to serve as template for the synthesis of both 42S and
26S positive strands (Simmons & Strauss, 1972b; Martin & Burke, 1974; Segal &
Sreevalsan, 1974; Bruton & Kennedy, 1975).

In the light of these hybridization experiments we directly examined the degree of
sequence homology between DIss1 and DIss2 by ribonuclease T1 oligonucleotide finger-
printing. In addition, selected oligonucleotides from each of these fingerprints (Fig. 8) were
recovered and characterized by analysis of their pancreatic ribonuclease digestion products
(Table 2). Together these experiments clearly show that the nucleotide sequences of DIss1
and DIss2 are very closely related indeed.

From about 2 h p.i. the major mRNA in cells infected with standard SFV is 26S RNA.
This RNA codes for all the structural proteins of the virus particle (Simmons & Strauss,
1974a; Clegg & Kennedy, 1974a, 1975a). Since, as was noted above, cells infected with
passage 8 virus contain large amounts of DIss1 and DIss2, it is pertinent to ask if either of
these species is present as mRNA on polyribosomes. Analysis of the total mRNA
complement of standard virus-infected, passage 8 virus-infected and uninfected cells (Fig. 9)
indicated that neither DIss1 nor DIss2 was present on polyribosomes and it seems, there-
fore, that neither acts as a functional messenger. (Most of the 42S RNA from standard virus-
infected cells is probably derived from nucleocapsids, Kennedy, 1972.) This finding which
is consistent with the observations on the fate of the input RNA of purified DI particles (see
earlier) was also supported by the failure of both DIss1 and DIss2 to function as messengers
in an in vitro protein synthesizing system (J. C. S. Clegg, personal communication).

Finally we compared the polypeptide species synthesized in cells infected with standard
virus with those synthesized in passage 8 virus-infected and in uninfected cells. In these
experiments, as in the RNA experiments (Fig. 2), the same m.o.i. (in terms of infectious
particles) of standard virus and passage 8 virus was used. As can be seen from Fig. 10, none
Fig. 8. Oligonucleotide fingerprinting of DIss1 and DIss2. DIss1 and DIss2 were purified from five roller bottle cultures of BHK cells infected with 20 p.f.u./cell of passage 8 virus and labelled with 6 mCi/culture of 32P-orthophosphate from 1 to 6 h p.i., by sucrose gradient sedimentation and slab gel electrophoresis as described in Methods. Approx. 3 x 10⁶ ct/min (about 20 μg) of each RNA species together with 100 μg carrier yeast tRNA was then digested with 10 μg of ribonuclease T₁ and the resultant nucleotides fractionated by electrophoresis on cellulose acetate in the first dimension (left to right) and by DEAE-paper ionophoresis in the second dimension (top to bottom). ○, denotes the origin. The fingerprint of DIss1 is on the left. Each of the marked oligonucleotides were recovered for pancreatic ribonuclease digestion.
SFV DI particle interference

Table 2. Pancreatic ribonuclease digestion products of the oligonucleotides isolated from the fingerprints of DIssI and DIss2

<table>
<thead>
<tr>
<th>Spot number</th>
<th>Pancreatic ribonuclease product*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>AU, U</td>
</tr>
<tr>
<td>L2</td>
<td>C, U</td>
</tr>
<tr>
<td>L2a</td>
<td>A_2C, AU, U</td>
</tr>
<tr>
<td>L3</td>
<td>A_2C, AU, C, U</td>
</tr>
<tr>
<td>L3b</td>
<td>A_2C, A_2U, A_2C, AU, AC, C, U</td>
</tr>
<tr>
<td>L3c</td>
<td>A_2U, A_2C, AU, AC, C, U</td>
</tr>
<tr>
<td>L4</td>
<td>A_2U, AU, C, U</td>
</tr>
<tr>
<td>L5</td>
<td>A_2N, AU, C, U</td>
</tr>
<tr>
<td>L6</td>
<td>A_2G9R, AU, C, U</td>
</tr>
<tr>
<td>L7</td>
<td>A_2N, A_2N, A_2U, AU, AC, C, U</td>
</tr>
<tr>
<td>L8</td>
<td>A_2C, AU, U</td>
</tr>
<tr>
<td>L9a</td>
<td>A_2C, AU, AC, C, U</td>
</tr>
<tr>
<td>L9b</td>
<td>A_2N, A_2C, AU, AC, C, U</td>
</tr>
<tr>
<td>L9c</td>
<td>A_2N, AC, C, U</td>
</tr>
<tr>
<td>L9e</td>
<td>(AU), C</td>
</tr>
<tr>
<td>L9g</td>
<td>A_2U, C, U</td>
</tr>
<tr>
<td>H1</td>
<td>AU, U</td>
</tr>
<tr>
<td>H2</td>
<td>C, U</td>
</tr>
<tr>
<td>H2a</td>
<td>A_2C, AU, U</td>
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<tr>
<td>H3</td>
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<tr>
<td>H3a</td>
<td>A_2C, A_2U, A_2C, AU, C, U</td>
</tr>
<tr>
<td>H3c</td>
<td>A_2U, A_2C, AU, AC, C, U</td>
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<td>H4</td>
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<tr>
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<td>H8</td>
<td>A_2C, AU, U</td>
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<td>H9a</td>
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<td>H9d</td>
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<tr>
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<td>A_2U, C</td>
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<tr>
<td>H9g</td>
<td>A_2U, C</td>
</tr>
</tbody>
</table>

* Products in parentheses were only tentatively identified. N denotes an unidentified nucleotide.

of the polypeptides distinctive of standard virus-infected cells can be detected in BHK cells infected with passage 8 virus. Indeed the spectrum of polypeptides synthesized in passage 8 virus-infected BHK cells was indistinguishable from that of uninfected cells. Since, however, passage 8 virus-infected cells do release small amounts of both standard virus and DI particles, they must contain small amounts of the virus proteins. Indeed, small amounts of core and of p68 can be seen in passage 8 virus-infected chick cells. This result shows that, as was found for the standard virus-specific RNA species (Fig. 2), all of the standard virus-specific polypeptides are made in markedly reduced amounts in cells co-infected with standard virus and DI particles. Indeed, the failure to synthesize normal amounts of 26S RNA probably accounts not only for the low level of all the structural polypeptides but also for the failure of passage 8 virus to shut off host protein synthesis (Wengler & Wengler, 1975). Moreover, the apparently identical polypeptide profile of passage 8 virus-infected and uninfected BHK cells, and in particular the absence of any polypeptide of mol. wt. 10000 to 80000 (equivalent to the coding potential of DIssI/DIss2) in passage 8 virus-infected cells, again suggests that DIssI and DIss2, which passage 8 virus-infected cells contain in large amounts, does not act as a functional mRNA.
Fig. 9. Polyacrylamide gel electrophoresis of the RNA species present on polyribosomes isolated from uninfected cells and from cells infected with standard virus and passage 8 virus. Total polyribosomes were prepared from three sets of 3 roller bottle cultures of BHK cells as described in Methods. One set was mock-infected, the other two were infected with 20 p.f.u./cell of standard virus and passage 8 virus respectively and all 3 sets were labelled from 1 to 6 h p.i. with 500 μCi ³H-uridine/culture. The mRNA was released from these polyribosomes by puromycin (Kennedy, 1972) and electrophoresed in parallel on three 1.7 % (w/v) acrylamide + 0.5 % agarose gels. Labelled mRNA from: ○—○, standard virus infected, ct/min × 10⁻³; —•—•, passage 8 virus-infected and △—△, uninfected cells, ct/min × 10⁻³. Note the difference in scales on the ordinate axes.

**DISCUSSION**

Since purified DI particles of SFV are unable to independently carry out any step in virus multiplication except uncoating, their propagation requires most, if not all, the intracellular functions performed by standard virus. This stringent dependence on standard virus distinguishes SFV DI particles from those of both poliovirus and vesicular stomatitis virus. For poliovirus DI particles, standard virus provides coat protein (Cole & Baltimore, 1973) and for at least one class of DI vesicular stomatitis virus standard virus provides the machinery for RNA replication (Palma,Perlman & Huang, 1974).

We shall now attempt to answer three questions about SFV DI particles. These are firstly how are SFV DI particles propagated, secondly, how do SFV DI particles interfere with the multiplication of standard virus, and thirdly, what mistake occurs during high multiplicity passing that leads to the appearance and selective accumulation of SFV DI particles?

Since DI particles of SFV differ from standard virions only in that instead of the 42S
SFV DI particle interference

Fig. 10. Polyacrylamide gel electrophoresis of the polypeptides synthesized in mock-infected BHK and chick cells and in cells infected with standard virus and passage 8 virus. Petri dish cultures of chick and BHK cells either mock-infected or infected with 20 p.f.u./cell of standard virus or passage 8 virus were labelled from 3 to 4 h p.i. with 50 μCi/culture of 35S-methionine and their polypeptides extracted, reduced, alkylated and electrophoresed on a 10 % polyacrylamide slab gel as described in Methods. Approx. the same number of ct/min (170 000 to 200 000) in the polypeptides from: 0, standard virus-infected; 8, passage 8 virus-infected and U, uninfected cells were applied to the slab. Autoradiography was for 3 days. See the legend to Fig. 1 for the identity of the marked bands. The arrow indicates a mol. wt. of 80 000 – see text for significance.

RNA genome they contain DIss1 and DIss2 (Bruton & Kennedy, 1976, see previous paper) then their propagation requires synthesis of the structural proteins and of the low mol. wt. RNAs. As Fig. 2 shows, passage 8 virus-infected cells contain large amounts of both DIss1 and DIss2. Since 42S and not 26S RNA is found in standard virus, our finding (Fig. 5) that the nucleotide sequence of DIss1 and DIss2 is not represented in 26S RNA is consistent with
the idea that these RNAs contain the recognition site for nucleocapsid assembly (Bruton & Kennedy, 1976, see previous paper). Moreover, the nucleotide sequence of the low mol. wt. RNA from cells infected with DI particles of Sindbis virus is present in the 26S RNA (Weiss et al. 1974), so our finding further distinguishes SFV DI particles from those of Sindbis virus. Although we were unable to detect the structural proteins in BHK cells directly (Fig. 10), passage 8 virus-infected cells do contain a small amount of 26S RNA (the messenger for the structural proteins) on polyribosomes (Fig. 9) and do release virus particles, albeit to a titre about 3 log_{10} units less than standard virus-infected cells (Bruton & Kennedy, see previous paper). Our failure to detect the structural polypeptides on polyacrylamide gels is probably due therefore to (a) their markedly reduced synthesis compared to standard virus-infected cells and (b) the inability of passage 8 virus to significantly shut off host protein synthesis in BHK cells. Indeed, small amounts of both core and p68 can be detected in chick cells infected with passage 8 virus. This raises the interesting question of the extent to which the expression of interference is dependent on the host cell.

On the question of interference in BHK cells it is clear that SFV DI particles interfere with the synthesis of all the components distinctive of standard virus-infected cells (Fig. 2 and 10). Interference in the synthesis of the structural proteins is, however, probably indirect and merely a consequence of interference in the synthesis of 26S RNA. Interference is not, however, limited to the synthesis of 26S RNA; synthesis of 42S RNA is also severely impaired. A possible hypothesis to explain this interference in RNA synthesis is suggested by our studies on the nature of the negative strand(s) found in passage 8 virus-infected cells. Since neither DIss1 nor DIss2 appear to be functional messengers (Fig. 9), the enzyme(s) responsible for the synthesis of the negative strand(s) of the low mol. wt. RI (DI negative strand(s)) which is complementary to, and therefore probably acts as template for, the synthesis of DIss1 and DIss2 (Fig. 7; Table 1) must be encoded by the 42S genome of the standard virus component of passage 8 virus. If the intracellular amount of this enzyme is limited, and there is evidence that this is so (Friedman & Grimley, 1969; Wengler & Wengler, 1975) then its activity in passage 8 infected cells appears to be towards synthesizing DI negative strand(s) rather than 42S negative strand (Fig. 7). Since the template for this enzyme may be 42S genomic RNA, mol. wt. 4 × 10^6, DIss1, mol. wt. 0.81 × 10^6, or DIss2, mol. wt. 0.75 × 10^6, then in a given period of time and assuming the same rate of transcription of each of these RNAs, more DI negative strand(s) than 42S negative strand will be synthesized. Likewise in an analogous manner but considering 42S negative and DI negative strand(s) as templates, more DIss1 and DIss2 than 42S or 26S RNAs will be synthesized by the enzyme(s) responsible for positive strand synthesis. Thus the low mol. wt. self-complementary negative and positive strands characteristic of cells infected with standard virus and DI particles effectively 'compete' for the available RNA synthesizing enzymes encoded by the standard virus genome. Clearly this hypothesis predicts that DIss1 and DIss2 both contain the nucleotide sequence to which the negative strand synthesizing enzyme binds and that the DI negative strand(s) contains the nucleotide sequence to which the positive strand synthesizing enzyme(s) binds. Moreover, since the former of these two binding sites is located at the 3' end of the 42S positive strand then the nucleotide sequence of both DIss1 and DIss2 must encompass this region of the genome.

To account for the appearance of DI particles we propose that a 'mistake(s)' occurs during virus-specified RNA synthesis resulting in the formation either of DI negative strand(s) or of DIss1 and DIss2. (Although DIss1 and DIss2 are extremely closely related [Fig. 8; Table 2] we need to know the exact relationship between these RNAs before determining the number of different mistakes which occur). As soon as DIss1 and/or DIss2 appear
they can be encapsidated and together with standard virus form the inoculum for the next passage in the series. The kinetics of appearance of DIss1 and DIss2 during passaging (Fig. 6) are entirely consistent with this model. Since our hypothesis accounts for the selective synthesis of the genetic material of the DI particles their accumulation at the expense of standard virus is assured. There will, however, be a limit to the DI particle/standard virus ratio since only cells co-infected with DI particles and standard virus produce progeny DI particles.

Our hypothesis both for the appearance of and interference by DI particles of SFV rests on the mechanism of virus-specified RNA synthesis. This process is poorly understood even in cells infected with standard SFV. In order, therefore, to test our hypothesis we need to know more not only about the enzymes responsible for RNA synthesis and the architecture of the RNA synthesizing complexes in the cell, but also the nucleotide sequence relationship between the 42S RNA genome, the 26S RNA messenger for the structural proteins, and both DIss1 and DIss2.

We thank the Medical Research Council for support.

REFERENCES


(Received 10 November 1975)