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The Genomic Sequence of Defective Interfering Semliki Forest Virus (SFV) Determines Its Ability to Be Replicated in Mouse Brain and to Protect against a Lethal SFV Infection *in Vivo*

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We have recently cloned and sequenced two genomes of defective interfering (DI) Semliki Forest virus (SFV), DI-6 (2146 nt), and DI-19 (1244 nt). These are similar in that both contain two large central deletions (encompassing the 5′ part of the nsP1 gene and the 3′ part of the nsP2 gene and all of the structural genes), and all the sequence of the latter is represented in the genome of SFV DI-6. RNA was transcribed from both and transfected into SFV-infected BHK-21 cells. RT–PCR analysis of tissue culture fluid harvested 18 h after transfection suggested that SFV DI virions had been rescued from the cloned genomes. Unlike the genomes of noncloned DI SFV, these genomes bred true for at least 7 serial passages. Cloned DI-6 and DI-19 viruses interfered to a similar extent with the multiplication of SFV in cultured cells, but only DI-19 protected mice from a lethal intranasal dose of SFV. Further investigation by RT–PCR analysis showed that DI-19 but not DI-6 genomes were replicated in mouse brain after direct intracerebral injection of DI virus together with an excess of infectious helper SFV. Thus the replication and hence antiviral activity of two closely related DI SFV genomes appears to be exquisitely sequence specific and cell specific. These findings mark a significant step on the way to using DI genomes as antivirals and also may explain why so few animal-protecting DI viruses have been identified.

INTRODUCTION

Defective interfering (DI) viruses have been described for most families of viruses (reviewed by Perrault, 1981; Dimmock, 1991; Roux *et al.,* 1991; Holland, 1990). They possess a genome that is a deleted form of the standard (infectious) virus genome and thus require standard virus to provide replicative and encapsidative proteins for propagation. In addition, they have the property of interfering with the multiplication of standard virus. DI viruses are generated on serial passage at both low and high multiplicities of infection of standard virus in tissue culture, but because they are unable to replicate autonomously, their propagation is favoured by high multiplicity infection. Although by definition all DI viruses have the property of being able to interfere with the multiplication of standard virus in cell culture, in only a few systems has protection of animals from infection *in vivo* been solidly demonstrated (SFV: reviewed by Barrett and Dimmock, 1986; vesicular stomatitis virus (VSV): Doyle and Holland, 1973; Holland *et al.,* 1976; Jones and Holland, 1980; Fultz *et al.,* 1982; Cave *et al.,* 1985; influenza A virus: reviewed by Dimmock, 1996).

Recently we described two DI SFV genomes (DI-6: 2146 nts and DI-19: 1244 nts) that were obtained from a cell culture-generated DI preparation that was able to protect mice from a lethal infection with SFV. These genomes were derived from three noncontiguous regions of the SFV genome: the 5′ untranslated region and the nsP1 coding region, part of the nsP2 coding region, and the 3′ untranslated region (Thomson and Dimmock, 1994; see Fig. 1). In addition to having a similar basic structure, all the sequence of DI-19 is present in DI-6. In contrast to clones of DI-301 and DI-309 viruses (Lehtovaara *et al.,* 1981, 1982), our clones did not possess extensive repeats or sequence rearrangements and were thought to be full-length DI genomes. Because previous work showed that DI SFV preparations produced by serial passage of SFV in tissue culture are biologically and physically heterogeneous (Kääriäinen *et al.,* 1981; Barrett *et al.,* 1984a), DI genomes were cloned with a view to regenerating naturally occurring DI viruses that could be defined on the basis of their constituent RNA sequences and hence be used to study sequence–function relationships.

In this report we show that molecularly cloned preparations of SFV DI-6 and DI-19, generated by transcription and transfection of transcripts into SFV-infected cell lines, were stable on serial passage and interfered with the propagation of standard virus in cell lines. However, only DI-19 protected mice from an otherwise lethal intranasal infection of the central nervous system with a highly neurovirulent strain of SFV. Failure of DI-6 to protect mice was probably due to its inability to be replicated in brain, even after direct intracerebral injection with an excess of infectious virus.

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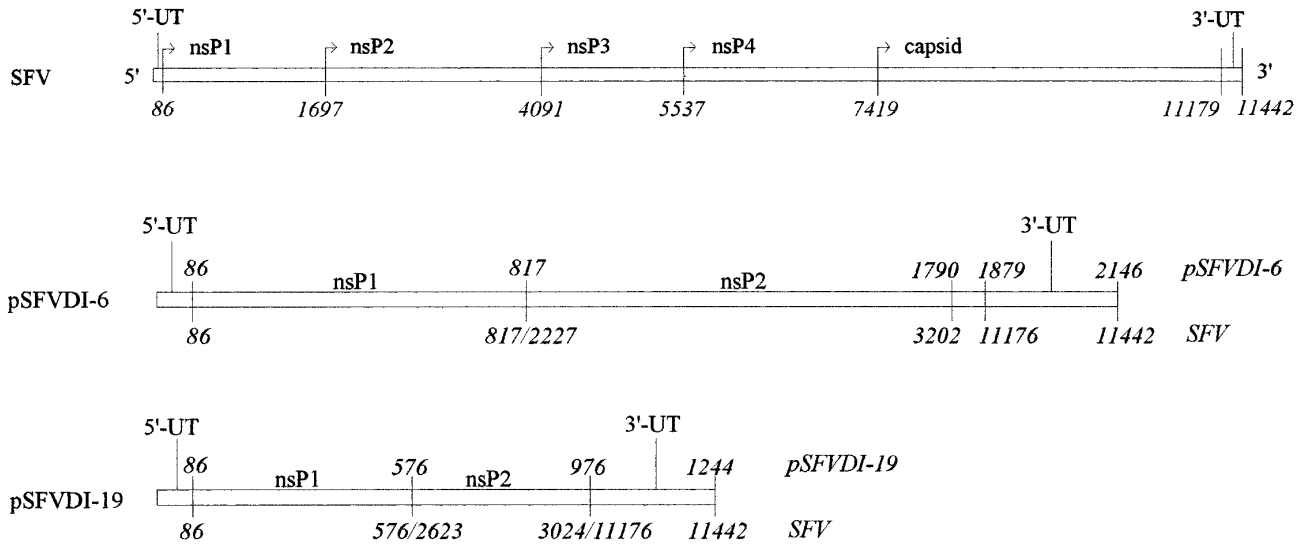

FIG. 1. Comparison of pSFVDI-6 and pSFVDI-19 with the SFV genome (Thomson and Dimmock, 1994). Both clones have parts of the genes encoding nsP1 and nsP2. The top numbers in pSFVDI-6 and pSFVDI-19 refer to the nucleotide position in the DI sequence. The bottom numbers refer to the corresponding nucleotide in the SFV genome. In pSFVDI-6, the region 1791–1814 could not be assigned to SFV, and the region 1828–1879 is an imperfect repeat of 1880–1930.

RESULTS

Rescue and passage of pSFVDI-6 and pSFVDI-19 transcripts as virions

pSFVDI-6 and pSFVDI-19 were linearised at the 3′ termini with *Nco*I and transcribed with T3 RNA polymerase. This yielded RNAs of 2.1 and 1.2 kb, respectively. Transcripts were not polyadenylated and had an additional guanylate residue at the 5′ terminus as a consequence of the promoter used. RNA transcripts were capped with methylated diguanosine triphosphate, although subsequent experiments found this to be unnecessary. Transcripts were transfected into BHK-21 cells which had been infected previously with SFV. After a single passage, RNAs of the same length as the DI-6 and DI-19 transcripts were detected in tissue culture fluid by RT–PCR. This and a later DI-19 passage is shown in Fig. 2. Faint 0.6- and 1.1-kbp products were also observed after RT–PCR amplification of tissue culture fluid containing SFV, probably resulting from nonspecific binding of the 5′ primer to viral RNA. They were not seen if a primer specific for a region just within the 5′ terminus of the SFV genome was used instead (data not shown).

After transfection of DI-19 RNA, a single RNA species of the expected size of 1.2 kb was found to persist in the virus population through at least 7 passages of tissue culture fluid containing putative DI virus and SFV (Fig. 3a). The genome of DI-6 (2.1 kb) was similarly stable (data not shown). These data indicate successful rescue of the original RNA transcripts into DI virus particles and their propagation without the generation of any other DI genomes. RT–PCR products all had the same restriction fragment patterns after digestion with *Acc*I (Fig. 3b). These were also similar to the restriction pattern of the pSFVDI clone except for the absence of the 0.3-kbp band. The latter is derived from the 3′ terminus of the clone and is included in the 3.0-kbp band of mainly vector sequence. Northern blot analysis of RNA extracted from passage 3 of SFV and DI-19 was performed using a RNA$^-$ probe transcribed from pSFVDI-6 and

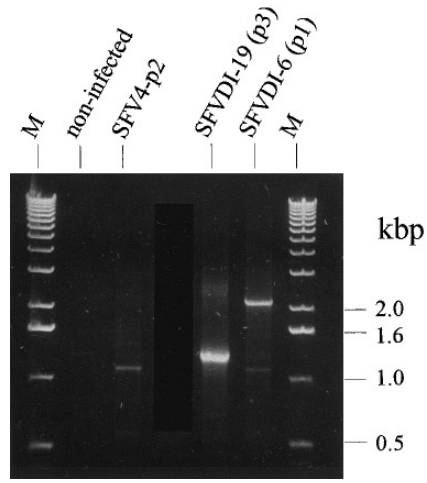

FIG. 2. Transfection of RNA transcribed from pSFVDI-6 and pSFVDI-19 into SFV-infected BHK-21 cells. pSFVDI-6 and pSFVDI-19 were transcribed with T3 RNA polymerase and approximately 20 μg RNA transfected by electroporation into BHK-21 cells previously infected with 10 PFU/cell SFV. Culture medium was harvested after 18 h and was passaged serially in BHK-21 cells. RNA was detected in culture medium by RT–PCR with primers specific for the termini of the SFV genome. The RT–PCR products from DI-19 after 3 passages in BHK-21 cells (p3) and DI-6(p1), of 1.2 and 2.1 kbp, respectively, corresponded to the sizes of the two cloned molecules. The faint 1.1-kbp band obtained from the SFV sample and DI-6 was derived through nonspecific hybridisation of the primers to SFV sequences (see text). The SFV genome is too large to be amplified by the RT–PCR method used. M, marker DNA.

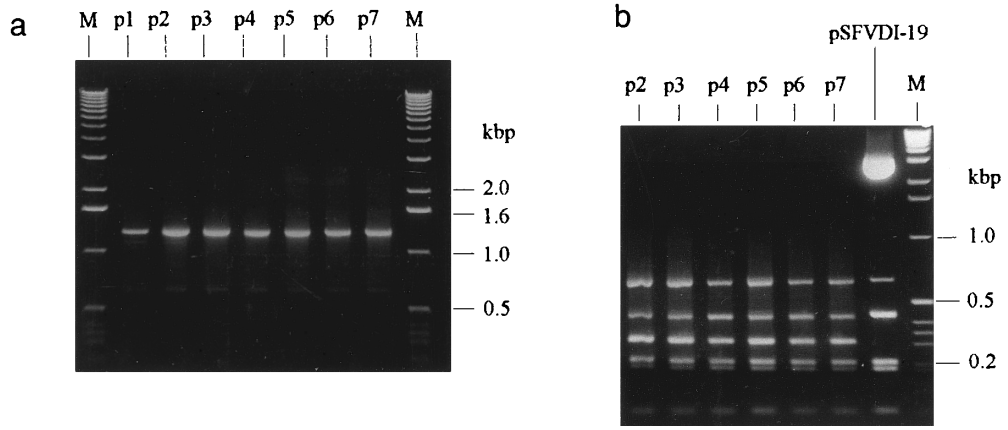

FIG. 3. Detection of defective SFV RNA in passages 1 to 7 of DI-19. (*a*) Culture medium containing putative DI SFV derived from pSFVDI-19, produced as described in Fig. 2, was serially passaged in BHK-21 cells. RNA was extracted from tissue culture fluid and amplified by RT–PCR using primers specific for the termini of the SFV genome. DNA of 1.2 kbp was detected in the RT–PCR products derived from passages (p) 1 to 7 of DI-19. M, marker DNA. (*b*) RT–PCR products derived from passages 2 to 7 of DI-19 were digested with *Acc*I. The band patterns produced after agarose gel electrophoresis were the same in all samples. Digestion of pSFVDI-19 with *Acc*I is shown along side. The additional 3.0-kbp band comprised vector sequences and included 0.3 kbp that derived from the 3′ terminus of the clone.

showed that only virion RNA and an RNA of 1.2 kb were present. A similar analysis of passage 3 of SFV and DI-6 using the same probe showed the expected 2.1-kb band (data not shown).

Rescue and passage of uncloned transcripts from RNA extracted from uncloned DI-p7 yields an evolving population of DI SFV genomes

DI SFV DNA sequences (DI-p7 transcript) were obtained by RT–PCR from RNA extracted from DI-p7 using the primers *5′SFV-T3* and *3′SFV* that are specific for the termini of SFV. DI-p7 virus is the mouse-protecting tissue culture preparation from which pSFVDI-6 and pSFVDI-19 were derived. The RT–PCR products obtained comprised a predominant DNA species of 1.2 kbp and a minor one of 2.1 kbp, which are equivalent in size to pSFVDI-19 and pSFVDI-6, respectively. DI-p7 DNA has a terminal T3 promoter, and direct transcription with T3 polymerase produced RNAs of 1.2 and 2.1 kb. These were transfected into SFV-infected BHK-21 cells. Tissue culture fluid was harvested at various passages after transfection for analysis of putative DI virus particles derived from transfected RNA by RT–PCR. The predominant RT–PCR product from passage 2 of DI-p7-transcript was 1.2 kbp, the same size as that from DI-19 (Fig. 4a). After passage 3 (Fig. 4b) several DNA species, including those of 1.1, 1.2, and 1.4 kbp, could be seen in contrast to the single RT–PCR product of molecularly cloned DI-19.

Cloned DI-6 and DI-19 have interfering activity in tissue culture

DI-6 and DI-19 were UV-irradiated under conditions that just removed all infectivity. Table 1 shows that such uvDI-6 and uvDI-19 have interference activities in both BHK-21 and L929 cells that after 4 passages are comparable with that of the uncloned DI-p7, with maximum titres of approximately 1/100. DI virus preparations were produced under standard culture and incubation conditions, using the same quantity of input DI RNA; thus, any

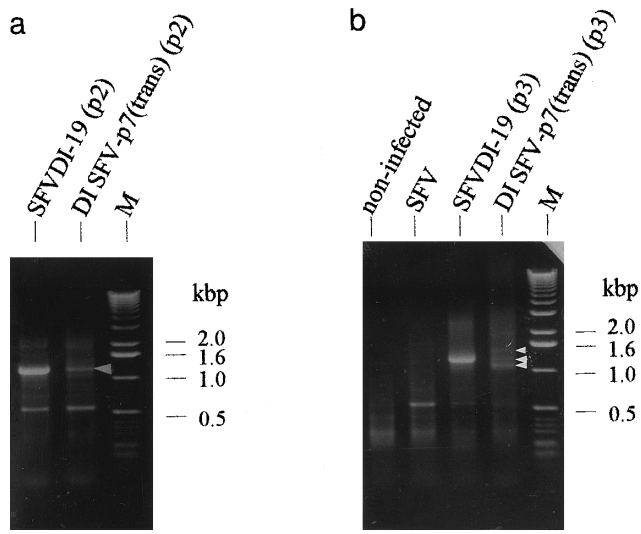

FIG. 4. Transfection of RNA transcribed from DI-p7-derived RT–PCR products into SFV-infected BHK-21 cells. DI SFV DNAs were amplified from DI-p7 RNA using *5′SFVT3* and *3′SFV* primers and RNA transcribed using T3 polymerase. RNA was transfected into SFV-infected BHK-21 cells and culture medium harvested after 18 h and serially passaged in BHK-21 cells. RNA was detected in culture medium by RT–PCR with the primers specific for the termini of the SFV genome. In (*a*), the predominant RT–PCR product from the second passage of the DI-p7-derived preparation (DI-p7 transcript (p2)) is 1.2 kbp (arrowed) and is the same size as that from DI-19(p2), but in lower quantities. In (*b*), arrows indicate 1.1-, 1.2-, and 1.4-kbp products that appear on amplification of RNA extracted from the third passage of the DI-p7-derived preparation (DI-p7 transcript (p3)), in contrast to the single band from DI-19(p3). There is a nonspecific RT–PCR product of 0.6 kbp (see text).

TABLE 1

Interference by DI SFV with the Multiplication of SFV in BHK or L929 Cells

Virus preparation[a]	Number of passages in BHK cells	Interference titre[b]
uvDI-6	3	16
uvDI-6*	4	62
uvDI-19	3	64
uvDI-19*	6	136
uvDI-p7	7	128
uvDI-p7 transcript	2	4
uvDI-SFV4	3	<2
uvDI-SFV4	7	64

[a] DI-p7 is uncloned DI virus; DI-6 and DI-19 are molecularly cloned DI viruses; DI-p7 transcript is DI virus produced by transcription and transfection of the RT–PCR product derived from uncloned DI p7; DI-SFV4 is DI virus obtained from the infectious clone. Each virus preparation was UV-irradiated to remove infectivity, as indicated by the prefix uv. Each well was inoculated with 10 $TCID_{50}$ SFV. No CPE was observed in wells inoculated with uvDI SFV alone.

[b] Titres are defined as the reciprocal of the highest dilution showing no CPE. Assays were carried out in BHK cells, apart from those denoted *, which were in L929 cells. The two cell lines gave similar amounts of interference. Titres are the mean of 4 replicate assays.

differences in their titres reflect a combination of their abilities to be propagated and to mediate interference. Table 1 also shows that the preparation derived by RT–PCR and transcription from the uncloned DI-p7 (DI-p7 transcript) (see above) interfered poorly here and at other passage levels (data not shown) compared with the DI SFV preparations derived from the molecular clones. As a control, SFV4 derived from the infectious clone was passaged in parallel with the cloned preparations of DI SFV. In contrast with uvDI-6 and uvDI-19, interference was not observed until 4–6 passages after transfection and did not reach maximum titre until passage 7. The possibility that the induction of interferons by UV-irradiated DI-6 and DI-19 viruses contributed to interference was not investigated. However, no interferons were found in earlier studies when cells were inoculated with noncloned DI SFV or DI SFV + SFV or when the effects of putative interferons on interferon-sensitive heterologous viruses were examined in mice co-inoculated with noncloned DI SFV or DI SFV + SFV (Dimmock and Kennedy, 1978; Barrett and Dimmock, 1984, 1986).

Molecularly cloned DI-19, but not DI-6, protected mice from a lethal SFV infection and only DI-19 was replicated in the brain

Random-bred CFLP and CD1 and the inbred BALB/c mice were protected from a lethal intranasal infection with SFV by administration of DI-19 (Table 2). Protected mice showed no clinical signs and were indistinguishable from noninfected mice for the whole duration of the experiment. At around 1 month after infection, they had developed no protective immunity to homologous challenge with 100 LD_{50} (data not shown). Thus DI-19 resembles the uncloned DI SFVp4, rather than DI SFVp13a that left mice solidly immune (Barrett and Dimmock, 1984). Nonprotected mice developed the same disease with the same time span as infected controls. Inactivation of the DI genome with BPL or prolonged UV irradiation abrogated protection, showing that protection required an active DI genome and was not mediated by DI virus competing for cell receptors or stimulating host defence responses. However, DI-6 gave no detectable protection in any of the mouse strains, even though its interfering ability in cell culture was not significantly different from that of DI-19 according to the Mann–Whitney *U* test. Possible reasons for this include the inability of DI-6 to exert interfering activity in cells of the central nervous system, the main target tissue of SFV, or failure to be replicated in these cells. To test the latter, approximately equal amounts of DI-6 and DI-19 virus, normalized by reference to their interference titres, were mixed with 10^6 PFU SFV and injected directly into the right cerebral hemisphere of CD1 and CFLP mice. No protection was expected from infection with this very large dose of SFV (10^6 intracerebral LD_{50}), which was chosen to give max-

TABLE 2

Protection of Mice from a Lethal SFV Intranasal Infection by Administration of Molecularly Cloned DI Virus[a]

Inoculum		Mouse survival (%)		
SFV	DI preparation	CFLP	Balb/c	CD1
+	None	0[b]	0	0[b]
+	uvDI-p7[c]	75	75	nd
+	BPL-uvDI-p7	0	0	nd
+	uvDI-19	71	88	67
+	BPL-uvDI-19	11	0	0
+	uvDI-6	0	0	0
+	BPL-uvDI-6	0	0	11
−	uvDI-p7	100	100	100
−	BPL-uvDI-p7	100	100	100
−	uvDI-19	100	100	100
−	BPL-uvDI-19	100	100	100
−	uvDI-6	100	100	100
−	BPL-uvDI-6	100	100	100
−	None	100	100	100

Note. nd, not done.

[a] All DI SFV preparations were UV-irradiated for 80 s to remove virus infectivity but not cell culture-based DI-mediated interference. Mice were inoculated intranasally under light ether anaesthesia 2 h before infection with the DI virus preparation shown and then at 0 h with 10 LD_{50} *ts*$^+$ SFV + DI SFV also intranasally. Infected groups consisted of 7–9 mice and noninfected control groups of 2–3 mice. Noninfected mice inoculated with a variety of DI virus preparations showed no sign of disease. Infected mice died at 5–6 days pi.

[b] 13% (1/8) CFLP and CD1 mice survived inoculation with SFV alone and this value was subtracted from the number surviving inoculation with SFV + uvDI SFV.

[c] DI-p7 is uncloned DI virus.

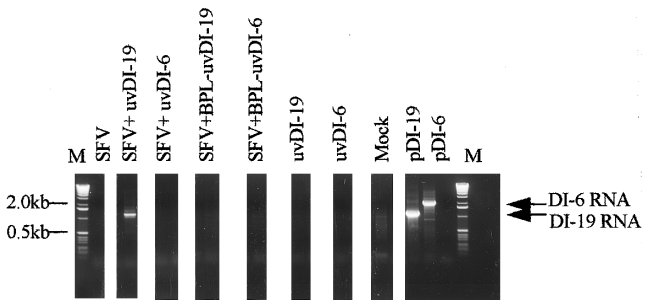

FIG. 5. DI-19 RNA but not DI-6 RNA is replicated in mouse brain. Mixtures of DI virus and SFV (1000 intranasal LD_{50}) or DI virus alone were injected intracerebrally (approximately 50 μl) into CD1 mice. DI-6 (passage 4) and DI-19 (passage 6) viruses had interference titres of 1/62 and 1/136, respectively (not significantly different by the Mann–Whitney *U* test). RNA was extracted from brains at 3 days pi, and, RT–PCR products were generated with *5′SFV* and *3′SFV* primers and analysed by agarose gel electrophoresis. uvDI is a DI virus preparation that has been UV-irradiated to remove virus infectivity but not DI virus activity. BPL-uvDI-19 and BPL-uvDI-6 are DI preparations that were treated with BPL to inactivate the DI genome.

imum replication of DI virus. Using the primers *5′SFV* and *3′SFV,* a RT–PCR product of the size expected for DI-19 was first detected at 2 days pi. Data for the CD1 mice are shown in Fig. 5. The RT–PCR band increased in intensity at 3 days and declined by 4 days pi (data not shown). Mice died on day 5. Brains injected with DI-6 + SFV were analysed in parallel but no evidence of replication was found in either strain of mouse. The RT–PCR analysis was repeated using *5′SFV* and the internal primer *594-ve* that give a product of 1229 bp with DI-6 RNA. This is similar in size to DI-19 (1244 nt) and controls for any affect that size difference between DI-6 and DI-19 RNAs may have on the sensitivity of the assay. However, the DI-6 samples again proved negative (data not shown). Mice injected with DI-6 + SFV also died on day 5, demonstrating that virus replication was unaffected. Clearly DI-6 was at a replicative disadvantage in mouse brain compared to DI-19. This result was surprising as all the sequence of DI-19 is present within that of DI-6 (Thomson and Dimmock, 1994). Possible reasons for the apparent tissue-specific requirement for the replication of the DI-6 genome are discussed below.

DISCUSSION

Following transcription of DI RNAs from pSFVDI-6 and pSFVDI-19 and their transfection into SFV-infected cells, interfering activity could be detected after 2 serial passages. In contrast, interfering activity was detected in standard SFV preparations derived from the infectious clone only after 4 to 6 passages (Table 1; data not shown). The sizes of RT–PCR products, restriction analysis, and Northern blot analysis showed that these preparations contained only the DI RNA expected. It is evident therefore that DI-6 and DI-19 possess sequences required for propagation and interference *in vitro* and therefore represent genuine DI genomes.

The genomes of cloned DI-6 and DI-19 viruses were maintained stably through at least 7 passages of the DI viruses without the appearance of any other detectable DI genomes. Thus they neither appeared to evolve nor allowed the establishment of other DI genomes *de novo.* The stability of cloned defective RNA contrasts with the instability on passage of the genomes derived by RT–PCR from noncloned DI SFV-p7 (Fig. 4b). Such dominance by a single DI genome has also been seen with DI VSV (reviewed by Holland, 1990) and DI influenza virus (reviewed by Nayak *et al.,* 1989) and is of obvious practical importance in maintaining stocks of DI particles containing only a single species of RNA. Restriction analysis of the RT–PCR products of DI-6 and DI-19 derived from serially passaged material suggested that the cloned defective genomes did not alter during their propagation *in vitro.* Analysis of DI-6 and DI-19 RNAs by Northern blotting failed to reveal the presence of any other DI RNA sequences (data not shown).

Although DI-6 and DI-19 stocks gave similar interfering titres in BHK and L cells, only DI-19 protected mice from lethal intranasal infection with a virulent strain of SFV. SFV is highly tropic for the central nervous system and after intranasal inoculation is thought to gain access to the brain via the olfactory nerves (Dimmock and Kennedy, 1978; Kaluza *et al.,* 1987). SFV-infected mice that were protected by administration of DI-19 showed no sign of infection, as reported before with noncloned DI-SFV, and those few mice that were not protected showed exactly the same course of disease as infected mice that received no DI virus (Barrett and Dimmock, 1984). The ability of both DI viruses to be replicated in the brain was tested by direct intracerebral injection together with a very high dose of SFV (10^6 PFU or 10^6 intracerebral LD_{50}). Only DI-19 could be detected by RT–PCR. Poor detection of the larger DI-6 genome did not appear to be the reason, as use of an internal primer that reduced its RT–PCR product to the size of the DI-19 genome also failed to give a positive signal and, in any case, DI-6 was easily detected in cell culture fluids with terminal primers. Thus it is likely that DI-6 could not be replicated in brain tissue. As both DI-6 and DI-19 were cloned from the same stock of DI virus it is clear therefore that this stock was heterogeneous. Direct demonstration of a DI virus that interfered in cell culture but did not protect mice is in agreement with the earlier failure to consistently propagate mouse-protecting DI virus despite the preparations having constant amounts of interfering activity (Barrett *et al.,* 1984a).

The explanation that DI-6 failed to replicate in mouse brain has a number of implications. Amongst these is the inference that the biological activity of DI viruses *in vivo* is cell specific. Some cell lines are also restricted for DI virus in that they fail to generate, propagate, or allow DI

genomes to interfere (Perrault and Holland, 1972; Eaton, 1975; Holland *et al.,* 1976, 1978; McLaren and Holland, 1974; Stark and Kennedy, 1978; Philips *et al.,* 1980; Barrett *et al.,* 1981; Steacie and Eaton, 1984). In turn, this implies that DI genomes that protect *in vivo* are best isolated from the cell or tissue of interest and that attempts to create protecting DI genomes by deletion of the infectious genome are unlikely to be successful. Because there is a clear distinction between the interfering activity of DI genomes *in vitro* and *in vivo,* we suggest that those that protect animals should be classified separately as defective protecting (DP) genomes or virus. The demonstration of a DI genome that fails to protect animals *in vivo* may go some way to explaining the paucity of such systems, despite the abundance of DI viruses *in vitro.*

The reason that DI-6 and DI-19 can be replicated in hamster (BHK) and mouse (L929) cells but not in mouse brain is intriguing, as both have the same general genome structure, and all the sequence of DI-19 (1244 nts) is contained within the genome of DI-6 (2146 nts: Thomson and Dimmock, 1994; Fig. 1). Also DI-6 has some sequence peculiarities, as nts 1791–1814 could not be assigned to the SFV genome, and the region 1828–1879 is an imperfect repeat of 1880–1930. The significance of these sequences is not known. Clearly the genome of DI-6 is viable in an appropriate cell background, and both DI viruses have the same coat proteins as infectious SFV. Thus it seems likely that the failure of DI-6 RNA to be replicated in brain resides in the DI-6 genome sequence or conformation. It is possible that a product of the nsP1 open reading frame or its fusion product with a central fragment of nsP2 is responsible for the dominant negative effect exerted by DI-19, but this seems unlikely, as the same initial nsP1 sequence is present (in an extended form) in DI-6, and it is not easy to reconcile this with a cell-specific effect. An alternative hypothesis for the observed cell specificity is that infectious virus and DI-19 genomes interact successfully with cellular factors in the brain, while DI-6 does not. Such cellular factors are likely to be involved in the replication of viral RNAs and contribute to the structure of the viral polymerase itself (as found in other RNA virus systems: Dmitrieva *et al.,* 1979; Blumenthal and Carmichael, 1979; Baron and Baltimore, 1982; Dasgupta, 1983; Hey *et al.,* 1987; Hayes and Buck, 1990; David *et al.,* 1992; Quadt *et al.,* 1993; Shimizu *et al.,* 1994). The hypothesis can be tested both positively and negatively by making chimeras of DI-6 and DI-19 RNAs and testing for the gain and loss, respectively, of replicative ability in mouse brain.

Almost all viruses make DI genomes. The discovery reported here of a molecularly cloned DI genome that protects against a life-threatening virus infection, together with the demonstration that a (very similar) DI genome is not protective under identical conditions, is a significant step forward to understanding how to harness this vast reservoir of natural antivirals against human and other diseases of animals and plants. This particular branch of intracellular immunization (Baltimore, 1988) may become a reality after all.

MATERIALS AND METHODS

Cells

BHK-21 cells (clone 13-3P) and L929 cells were obtained from the European Collection of Animal Cell Cultures (CAMR, Porton Down, Wilts, UK) and grown as monolayers by standard methods.

Virus

SFV (standard virus, strain ts^+; Tan *et al.,* 1969) was propagated in BHK-21 cell monolayers. Cultures were inoculated at a multiplicity of infection (M.O.I.) of 0.1 PFU per cell and incubated for 18 h at 37°C. Tissue culture fluid was harvested and clarified by low-speed centrifugation and stored at −70°C. Stocks of SFV derived from the full-length infectious clone pSP6-SFV4 were generated by transcription and transfection into BHK-21 cells, as described by Liljeström *et al.* (1991). Virus of the second passage after transfection was used for propagation of DI virus.

DI virus

Clones of DI genomes, pSFVDI-6 and pSFVDI-19, were derived from a tissue culture preparation of DI SFV that was produced by seven high multiplicity passages of SFV in BHK-21 cells (DI-p7). Besides interfering with virus multiplication in cell culture, this preparation was able to protect mice against a lethal dose of SFV (Dimmock and Kennedy (1978); Barrett and Dimmock, 1984). RNA was extracted from DI-p7 and amplified by reverse transcription and polymerase chain reaction (RT–PCR) with *Taq* and primers specific for the termini of SFV (see below). The 5′ primer incorporated a promoter for T3 RNA polymerase *(5′SFV-T3)* and the 3′ primer incorporated the restriction enzyme site *Nco*I *(3′SFV-Nco).* These features were designed to permit runoff transcription of RNA (see below). The nucleotide overhang was removed with the large fragment of DNA polymerase I (Klenow) and the RT–PCR products cloned into the *Sma*I site of pUC13. Two clones, pSFVDI-6 and pSFVDI-19, were sequenced and the regions of the SFV genome from which these two clones were derived are indicated in Fig. 1 (Thomson and Dimmock, 1994). RNA transcribed from cloned DI DNA was rescued as virus particles by transfection into SFV-infected BHK-21 monolayers (see below). This DI virus was subsequently passaged with exogenous SFV added at a M.O.I. of 60. Culture fluids were collected after 24 h at 37°C.

Infectivity titration

Virus was plaque assayed in BHK-21 cell monolayers by incubating at 37°C for 48 h with an agar overlay

containing 0.08% DEAE–dextran. Live cells were stained with neutral red (0.01% (w/v) in PBS) for 2 h at 37°C. Alternatively, infectivity was estimated by an endpoint assay in BHK-21 or L cells in 96-well plastic plates (2×10^4 cells/well; Nunclon; Gibco UK, Ltd.). CPE was determined by light microscopy after 3 days and the $TCID_{50}$ calculated according to the Spearman–Kärber equation (Rhodes and van Rooyen, 1968).

UV irradiation to inactivate infectious virus in DI virus preparations

DI and infectious SFV cannot be physically separated, so preparations were UV irradiated to remove most of the infectivity but minimal amounts of DI activity (Dimmock and Kennedy, 1978). DI viruses were irradiated in 1-ml aliquots in a 5-cm petri dish for 40, 50, or 60 s, titrated in cell culture, and inoculated intranasally into mice (see below). The lowest UV dose that gave no detectable infectivity was then used. One $\log_{10}$ PFU was inactivated in about 8 s with the UV source employed (Gelman Sciences, Northampton, UK). Interference was measured (see below) after UV treatment, to compensate for differences in rates of inactivation that arise from the different sizes of DI genomes.

Inactivation of DI RNA

The RNA component of DI virus was inactivated by either prolonged UV irradiation for 5 min or 0.1% (v/v) β-propiolactone (BPL grade II; Sigma) in a final concentration of 0.05 M Tris–HCl (pH 7.5) for 12 h at 4°C (Barrett *et al.,* 1984b). BPL was removed by dialysis against PBS overnight at 4°C and inactivation verified by testing for interfering activity.

RNA extraction from tissue culture fluid

RNA was extracted from small volumes of tissue culture fluid (typically 75 μl) with an equal volume of 10 mM Tris–HCl, pH 7.9, 350 mM NaCl, 10 mM EDTA, 7 M urea, 1% (w/v) SDS followed by phenol extraction.

RNA extraction from brain tissue

Mouse brains were removed from the animal, frozen in dry ice, and stored at −70°C. These were then homogenised in 4 M guanidinium isothiocyanate. Equal volumes of phenol (heated to 60°), chloroform, and 0.1 M NaAc (pH 5.2) were added to the brain lysate and mixed into a single phase. The tubes were centrifuged at 650 *g* for 15 min and the supernatant was then reextracted. RNA was precipitated with ethanol at −70°C and recovered by centrifugation. The pellet was resuspended in distilled water and incubated in proteinase K in proteinase K buffer at 37°C for 20 min. This was extracted twice with an equal quantity of phenol–chloroform at 60°C and once with chloroform alone at room temperature. RNA was again ethanol-precipitated, recovered by centrifugation, and resuspended in sterile distilled water.

Reverse transcription and polymerase chain reaction

For production of cDNA, RNA was reverse transcribed and amplified by PCR in 100 μl reaction buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.01% gelatine, 4.5 mM $MgCl_2$, 0.5 mM each dNTP, 2 U AMV reverse transcriptase, 1.5 U *Taq* polymerase [Perkin–Elmer Cetus (ILS Ltd.), London, UK], 0.1 μg each primer) and overlaid with 100 μl paraffin. For analysis of DI SFV RNA, the primers 5′-ATG GCG GAT GTG TGA CAT ACA CGA C-3′ *(5′SFV),* and 5′-GGA AAT ATT AAA AAC CAA TTG CAA AAT AAA ATA-3′ *(3′SFV),* which were homologous to the 5′ terminus and complementary to the 3′ terminus of SFV virion RNA, respectively, were used. To abrogate any effect of the difference in size of DI genomes, *5′SFV* was used in conjunction with the internal primer 5′-ACG CGT GCA ACG TCT GGA T-3′ *(594-ve),* which is complementary to nts 2623–2641 of the virion positive strand. These make 594- and 1229-bp products with DI-19 and DI-6 RNAs, respectively. For production of cDNA that could be used as a template for RNA transcription, RNA was amplified with a 5′ primer that incorporated the T3 polymerase promoter (underlined) 5′-GCA ATT AAC CCT CAC TAA AGA TGG CGG ATG TGT GAC ATA CA-3′ *(5′SFV-T3)* and a 3′ primer that incorporated a *Nco*I restriction site (underlined) 5′-TTC CAT GGG AAA TAT TAA AAA CCA ATT CCA ATT GCA-3′ *(3′SFV-Nco).* For analysis of DI SFV RNA from brain tissue, the primers *5′SFV* and *3′SFV* were used. Typical reaction times were 40 min at 42°C for reverse transcription followed by 30 cycles of 60 s at 93°C, of 60 s at 58°C, and of 80 s at 72°C. A final step of 5 min at 72°C was included before cooling to 25°C. RT–PCR products were analysed by electrophoresis on 1% (w/v) agarose gels.

Transcription and analysis of transcription products

Runoff, capped transcripts were made by linearising DI SFV plasmid DNA with *Nco*I and then synthesizing RNA at 37°C for 1 h with 20–50 U T3 RNA polymerase in 50 μl reaction buffer (40 mM Tris HCl pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine-$(HCl)_3$, 25 mM NaCl, 10 mM dithiothreitol, 5 mM ATP + TTP + CTP, 2.5 mM GTP, 1 mM sodium $m^7G(5')pppG$ (Pharmacia Biosystems Ltd., Milton Keynes, UK), 10 U human placental RNase inhibitor, 1 μg template DNA). DNA was degraded prior to transfection by incubation at 37°C for 10 min with 5 U RNase-free DNase (Promega Ltd., Southampton, UK). Transcribed RNA was analysed by electrophoresis on 1% agarose gels after denaturation by glyoxal (see below; McMaster and Carmichael, 1977). Transcription of pSP6-SFV4 was performed as described by Liljeström *et al.* (1991).

Transfection

For rescue of DI SFV transcripts into DI particles, BHK-21 monolayers were first infected with 10 PFU SFV per cell for 45 min at 25°C and electroporated with 20 μl of transcription reaction (approximately 20 μg RNA) using a Gene Pulser apparatus (Bio-Rad) as described by Liljeström *et al.* (1991).

Northern blot analysis

RNA extracted from tissue culture preparations was analysed by Northern blotting using standard methods. Briefly RNA was denatured with 1.08 M glyoxal and 53% dimethyl sulphoxide at 50°C for 60 min and then analysed by agarose gel electrophoresis (after McMaster and Carmichael, 1977). The RNA was then transferred to a hybridisation filter and fixed by exposure to UV light for 5 min. For a probe, pSFVDI-6 was cloned in a pBluescript (KS^+) vector (Stratagene, Cambridge, UK) and negative-strand RNA was produced from this labelled with [α-^{32}P]UTP (Amersham Int., plc, Aylesbury, UK). This detected DI and virion RNAs but not 26S mRNA.

Interference assay

This assay measured the ability of DI SFV to prevent the cytopathic effect (CPE) caused by infectious virus. L929 or BHK cell monolayers in 96-well plates (2×10^4 cells/well; Nunclon) were inoculated with 10 $TCID_{50}$/well SFV for 1 h at 37°C. The medium was then aspirated and replaced with maintenance medium. UV-irradiated DI SFV (see above) was then titrated on the plate in two-fold serial dilutions. Plates were incubated at 37°C for 48–60 h and CPE assessed. Assays were performed in quadruplicate and the interference titre was calculated according to the Spearman–Kärber equation. Differences of ≥fourfold were significant according to the Mann–Whitney *U* or nonparametric test (Campbell, 1967).

Mouse experiments

Young adult 5-week-old male CFLP, CD1, or Balb/c mice (B & K Universal Ltd., Grimston, Aldborough, Hull; Harlan UK., Bicester, Oxon) were used for these experiments. The virulent ts^+ strain of SFV was titrated to determine the PFU:LD_{50} ratio for intranasal inoculation of each strain of mouse (Dimmock and Kennedy, 1978). One intranasal LD_{50} contained approximately 10^3 PFU. Groups of 10 mice were lightly anaesthetised with ether and inoculated with 10 LD_{50} in 20 μl divided between nostrils. Mice developed encephalitis and died in 5–6 days, and the LD_{50} was determined according to Spearman–Kärber (Rhodes and van Rooyen, 1968). Clinical signs of disease have been described by Barrett and Dimmock (1984) and these varied in detail between both individual animals and strains. Briefly these were first apparent at 3 days pi and included malaise, random walking, and hyperactivity. More severe clinical signs developed and by 4 days pi these had extended to circling and proceeded to immobility with or without paralysis. All mice showed raised fur and a hunched, though relaxed, posture. To determine its protective ability, mice were inoculated first with DI virus which had been UV irradiated to remove virus infectivity (uvDI; see above) and then 2 h later with a mixture of uvDI virus + 10 LD_{50} SFV (Dimmock and Kennedy, 1978). DI viruses were used before passage 7, up to which time DI genomes bred true (see Fig. 3), and were normalized by interference titre. To control for protective host responses directed against viral protein and also the possibility of DI virus blocking virus-specific receptors, the experiment was repeated with uvDI virus that had been inactivated with BPL or by prolonged UV irradiation (see above). Attempts to propagate DI virus in the brain were made by intracerebral injection into the right cerebral hemisphere of a mixture of DI virus and 10^6 intracerebral LD_{50} of SFV (approximately 10^6 PFU, determined by titration as described for intranasal inoculation: unpublished data). This very large dose of SFV was used to ensure that DI virus had every chance of being replicated. These mice died at 5 days pi. All animal experimentation followed the guidelines laid down by the UK Coordinating Committee for Cancer Research.

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