The Short Sendai Virus Leader Region Controls Induction of Programmed Cell Death

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The replication of nonsegmented minus-strand RNA genomes, like that of Sendai paramyxovirus (SeV), is controlled by the short leader regions present at each end of the linear genomes and antigenomes; the left and right promoters (Ple and Pr), respectively. Wild-type SeV is highly cytopathic in cell culture, because it induces programmed cell death (PCD). We have found that a recombinant SeV (rSeVGP42), in which the first 42 nt of le sequences at PLe were replaced with the equivalent sequences of Ple, and which produces infectious virus in amounts comparable to wild type, does not kill cells. Further, the increasing replacement of the terminal le sequences at Ple with le sequences led to a decreasing fraction of infected cells being apoptotic. This property (PCD −), moreover, is dominant in cells co-infected with SeVwt and rSeVGP42, and the mutant virus therefore appears to have gained a function which prevents PCD induced by SeVwt. Even though this virus has not been selected for naturally, it excludes SeVwt during co-infections of cultured cells or embryonated chicken eggs. The noncytopathic nature of cells infected or co-infected with rSeVGP42 leads automatically to stable, persistent infections. The mutation in rSeVGP42 is not in the protein coding regions of the viral genome, but in the 55-nt-long leader region which controls antigenome synthesis from genome templates. The SeV leader regions, which are expressed as short RNAs, thus appear to control the induction of PCD.

INTRODUCTION

Apoptosis, or programmed cell death (PCD), is a physiological process in which unwanted cells are eliminated from a multicellular organism (Kerr et al., 1972; Raff et al., 1993). This process consists of a well defined morphological sequence of events in which the contents of the cell are packaged within membranes prior to their budding from the cell as apoptotic bodies. These bodies are subsequently eliminated by macrophages, and as the contents of the apoptotic cells are not liberated into the extracellular space, this process occurs without the induction of an inflammatory response (Arends and Wyllie, 1991). PCD is now accepted as a critical element in the repertoire of potential cellular responses to many different situations, including virus infection. To promote survival of the host organism, infected cells may be motivated to commit "altruistic" suicide. This pathway has been postulated to be an important host defense mechanism, leading to the reduction of the organism’s total virus burden. As obligate intracellular parasites, many viruses have therefore evolved strategies for preventing or delaying PCD (Gillet and Brun, 1996). Some viruses, for example, encode gene products which directly inhibit cell death pathways [adenovirus E1B 19K (Rao et al., 1992; White et al., 1992), baculoviruses p35 and OpiAP (Clem et al., 1991; Bimbaum et al., 1994), cowpox CrmA (Ray et al., 1992; Kamita et al., 1993), and Epstein–Barr virus BHRF1 (Gagliardini et al., 1994)], whereas other viral gene products upregulate the expression of cellular "anti-death" genes [e.g., Epstein–Barr virus LMP (Henderson et al., 1993)]. In addition, a growing number of viruses are now known to actively induce apoptosis late in the infection, which may represent an important step in the spread of the progeny to neighboring cells which can endocytose the apoptotic bodies (Teodoro and Branton, 1997).

This paper reports that Sendai virus (SeV), a respiratory pathogen of mice and a model paramyxovirus, also kills cells in culture by inducing PCD. SeV might therefore be expected to encode a function(s) which interferes with this pathway. We have no evidence that this is so, but we have found a mutant virus (rSeVGP42) which grows normally but does not kill cells in culture. This property (PCD −), moreover, is dominant in cells co-infected with SeVwt and rSeVGP42, and the mutant virus appears to have gained a function which prevents PCD induced by SeVwt. Curiously, the mutation in rSeVGP42 lies not in the protein coding regions of the viral genome, but in the 55-nt-long leader region which controls antigenome synthesis from genome templates. This region is expressed as short RNAs, and the work described here suggests that these RNAs may play a role in controlling the induction of PCD.

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RESULTS

Organization of paramyxovirus genomes

The linear genomes of nonsegmented negative-strand ([-]) RNA viruses (Rhabdoviridae, Paramyxoviridae, and Filoviridae) vary from 11 to 19 kb in length and express from 5 to 10 genes (or transcription units). One feature Mononegaviruses share, and which distinguishes them from segmented [-] RNA viruses, is that the 5’ end of the first mRNA is recessed from the 3’ end of the [-] genome template, and this interval is made up by the short [+] leader (le+) region (Fig. 1). The very 3’ end of the [-] genome is, nevertheless, expressed as a short le-

RNA(s) (ca 55 nt for SeV), which terminates near the start of the first mRNA (reviewed in Banerjee and Barik, 1992; Lamb and Kolakofsky, 1996). The polymerase that has made the le+ RNA may then continue on, to sequentially synthesize the linear array of mRNAs (shown as a single generic mRNA in Fig. 1). An analogous [-] leader (le-) RNA is expressed from the 3’ end of the antigenome (57 nt for SeV), containing the sequences up to the end of the L gene (Leppert et al., 1979; Vidal et al., 1989; Chandrika et al., 1995). As there are no other reinitiation sites downstream of the le- region on the antigenome, the le- RNAs are the only subgenomic length transcripts of the antigenome (Fig. 1).

RNA synthesis from the le+ and le- regions is thought to play a central role in controlling antigenome synthesis from genome templates, and vice-versa (Fig. 1). The le regions can thus be considered as RNA promoters for genome replication. The le- region (or antigenomic) promoter will also be referred to as P0, and that of the le+ region (or genomic promoter) as P+, in reference to their representation in Fig. 1 in the convention used for protein-encoding DNA. For SeV, the genomic promoter (at P+) includes not only the le+ sequence up to the start of the N gene (nt56), but at least part of the adjacent 5’ nontranslated region (Pelet et al., 1996; Tapparel and Roux, 1996). Similarly, the antigenomic promoter (at P0) includes not only the le- sequence up to the end of the L gene, but at least part of the adjacent 3’ nontranslated region (Murphy et al., 1998; Tapparel and Roux, 1998). We use the term P+ (and P0) to refer to the cis-acting sequences at the 3’ end of the genome as well as their complement at the 5’ end of the antigenome (and vice versa). Similarly, le+ refers to the sequences present both at the 3’ end of the genome and the 5’ end of the antigenome. This is because sequences on both strands most likely control the rate-determining steps for genome/antigenome synthesis; i.e., (1) the rate at which the polymerase starts at the very ends of the templates, and (2) the rate at which the nascent leader chains are assembled with N subunits. Failure to assemble the nascent leader chains results in polymerase termination near the le+/N and L/le- junctions, to generate the free le RNAs.

Defective interfering (DI) genomes are deletion mutants generated by a copy-choice mechanism, in which the viral replicase (carrying its nascent chain) either jumps forward on its template, creating an internal deletion (e.g., DI-E307, Fig. 1), or jumps back on the nascent chain itself, creating a terminal complementary repeat upon finishing the chain (e.g., H4, Fig. 1) (Leppert et al., 1977; Perrault, 1981; Lazzarini et al., 1981; Re, 1990; Engelhorn et al., 1993). These latter DIs are called ‘‘copybacks,’’ and all natural examples have replaced their genomic promoters at P+ with the antigenomic promoter and therefore contain the antigenomic promoter at both ends (Fig. 1). SeV copyback DI genomes have a competitive advantage in replication over the nondefective genome (and over DI-E307), and this has led to the notion that the antigenomic promoter is more efficient
than the genomic promoter. For example, the amplification efficiencies of the H4 and E307 DI constructs vary by ca. 20-fold in favor of H4 in the vTF7-3 infected/transfected cell system (Fuerst et al., 1986). Calain and Roux (1995) examined the cis-acting sequences responsible for this difference by progressively swapping the sequences at P L of the H4 and E307 DI genomes with the alternate le region. They found that the relative amplification advantage of the copybacks was determined primarily by whether the DI genomes contained the le sequences at both ends, independent of whether or not they expressed mRNAs.

One of the chimeric DI-E307 derivatives used in this study was designated E307 GP42, as the first 42 nt of the genomic promoter at P L was replaced with the corresponding sequence of P R (DI-E307 GP42 is thus a copyback over 42 nt). DI-E307 GP42 was of particular interest because it replicated to almost the same high levels as DI-H4 (copyback over 110 nt (Calain et al., 1992)), whereas the expression of its single fusion mRNA was reduced only slightly (Calain and Roux, 1995). Using a recombinogenic system for the recovery of infectious virus from DNA (Schnell et al., 1994), P L of DI-E307 GP42 was crossed into the nondefective viral genome, creating the recombinant rSeV GP42 (Garcin et al., 1995). rSeV GP42 appeared to replicate less well in embryonated chicken eggs than wild-type virus (SeV wt), but had the remarkable property of excluding SeV wt in co-infections of eggs, i.e., rSeV GP42 had retained the interfering properties of the defective genome. Interference thus appeared to be due to the presence of le sequences at both P L and P R. The present study was undertaken to understand the replicative advantage of rSeV GP42 in mixed infections and to investigate why this highly robust copyback virus has not been detected in nature.

Replication of rSeV GP42 and SeV wt in cultured cells

To compare the replication of SeV wt and rSeV GP42 in cell culture, BHK cells were infected with 10 PFU/cell of SeV wt; allantoic fluid containing an equivalent amount of rSeV GP42 (Materials and Methods, and see below), or a mixture of the two stocks. Cytoplasmic extracts were prepared at 9, 24, and 48 hpi and separated on CsCl density gradients into fractions containing the viral mRNAs or genomes/antigenomes, and the levels of the various viral RNAs were determined by Northern blotting and primer extension (Materials and Methods). We found that genomes accumulated roughly to equivalent levels in all three infections (Figs. 2 and 3; slightly more genomes were found when rSeV GP42 is present, presumably because little or no CPE occurs at later times, see below). This is consistent with the fact that the antigenomic promoter at P R (which directs genome synthesis) has not been altered between SeV wt and rSeV GP42 (Fig. 1). Figure 2 also shows that the shorter (1794 nt) DI-E307 GP42 (used to generate rSeV GP42) is not detected in the rSeV GP42 infections; hence the properties of this virus stock cannot be ascribed to contaminating DI genomes. Antigenomes and mRNAs (Fig. 3), on the other hand, accumulated in lesser amounts in rSeV GP42 compared to SeV wt-infected cells. This is consistent with the fact that the promoter at P L (which directs antigenome and mRNA synthesis from genome templates) differs in these two viruses. This difference in antigenome levels, however, is in the direction opposite to that expected (see Discussion). In the mixed virus infection, antigenome levels remained low similar to those in the rSeV GP42 infections, whereas N mRNAs accumulated to levels approaching those of the SeV wt infection. The presence of the co-infecting rSeV GP42 genome appears to suppress antigenome synthesis from the normal (noncopyback) genome, but has a smaller inhibitory effect on mRNA accumulation from the SeV wt genome.

The SeV wt used in this study is strain H (SeV H), whereas rSeV GP42 is derived from the very closely related SeV Z. SeV H was chosen because its N protein (N H) is electrophoretically distinguishable from that of strain Z. The N proteins from both viral genomes can thus be monitored simultaneously by SDS-PAGE. When equal amounts of cell extracts prepared at 9, 24, and 48 hpi were examined by immunoblotting, slightly less N H than N Z was found intracellularly in their respective infections.
(Fig. 4, top), consistent with the lower N mRNA levels in the rSeVGp42-infected cells (Fig. 3). Both N proteins were also present in roughly similar amounts during the mixed virus infection (lanes mix). Culture supernatants were harvested at 24 and 48 hpi and their virus particles were examined. Again, roughly equal amounts of N\textsuperscript{H} and N\textsuperscript{Z} were found extracellularly in virus particles in their respective infections, as well as in the mixed virus infection (Fig 4, medium). Genome-specific RT/PCR amplification showed that both viral genomes were also present throughout the mixed infection (Fig 5). We conclude that SeV\textsuperscript{wt} and rSeVGp42 infections of BHK cells accumulate roughly similar amounts of viral proteins intracellularly and liberate similar amounts of virus into the medium. Further, cells co-infected with both viral genomes also secreted both viral N proteins into the medium. Only the intracellular levels of antigenomes and mRNAs appear to differ significantly in these virus infections.

rSeVGp42 produces noncytopathic persistent infections

We previously reported that we were unable to accurately titrate rSeVGp42, as this virus produced pin-hole plaques at best, whose titer was at least 2 logs diminished relative to the amount of viral proteins and genomes present (Garcin et al., 1995). We therefore normalized the rSeVGp42 and SeV\textsuperscript{wt} infections of cell cultures to the amounts of virus particles (pelletable N protein and viral genomes) in the allantoic fluid stocks. We found that cells infected with SeV\textsuperscript{wt} showed clear cytopathic effects (CPE) by 24 hpi, which then increased in severity, and the vast majority of the cells had died by 72 hpi (Fig. 6). Cells infected with rSeVGp42, in contrast, exhibited only mild CPE by 48 hpi, which did not increase on further incubation. Rather, the CPE was transient and had disappeared by 72 hpi, and the cultures could then be passaged with almost the same plating efficiency as...
mock-infected cultures (cf. Fig. 6). Immunoblot analysis of cytoplasmic extracts of these cultures showed that they were persistently infected (data not shown). Remarkably, cells co-infected with equal amounts of SeVwt and rSeVGP42 behaved similarly to those infected with rSeVGP42 alone; i.e., CPE was mild and transient, and the cells could be passaged and were persistently infected (Fig. 6). The time course of the ability of rSeVGP42 to prevent CPE of cells infected with SeVwt was examined. When the copyback virus was added to cells infected with SeVwt either 75 or 150 min earlier, these infections behaved similarly to those infected with SeVwt alone, i.e., CPE was extensive by 48 hpi and none of the cells survived (Fig. 6). rSeVGP42 must therefore be present at the earliest times of infection of cells by SeVwt, to protect against the CPE of the wild-type virus.

When both viruses are added to cells simultaneously and these cultures (with minimal CPE) are passaged at 48 or 72 hpi, the persistently infected cells grow a little more slowly (Fig. 6). This difference in growth rate is lost by the second passage, and the persistently infected cells now divide at least as quickly as uninfected cells. Moreover, they can be passaged at much higher dilutions than control BHK cells. The degree to which a confluent culture can be divided for passage (its plating efficiency) varies among different cell lines. The BHK cells we used were routinely passaged at a dilution of 1:8 and grew to confluence within 3 days. Plating at higher dilutions (1:12) resulted in slower growth, and at a dilution of 1:20, these cells did not divide and the culture could not be continued. Cultured cells are thought to require a minimal level of stimulation from other cells to stay alive, which condition the medium with growth factors. In the absence of a given threshold of growth stimulation, the (default) PCD pathway is activated (Raff, 1992). The baculovirus p35 anti-apoptotic gene is known to increase cell proliferation by inhibiting PCD, similar to the action of oncogenic phorbol esters (reviewed in M-
whereas infection with rSeVwt was highly cytopathic, the CPE of cells infected with rSeV<sup>GP42</sup> was inapparent to mild, the vast majority of these cells survived (as determined by staining with vital dyes), and the cells could be passaged at high plating efficiency. Three conclusions can be drawn from these experiments: (i) infection of several cultured cells with rSeV<sup>GP42</sup> is relatively non-cytopathic and leads to long-term persistent infections at high frequency; (ii) rSeV<sup>GP42</sup> somehow prevents or attenuates the cytopathic effects of SeV<sup>wt</sup> in mixed infections, also leading to persistent infections; and (iii) rSeV<sup>GP42</sup> has a replicative advantage over SeV<sup>wt</sup> in cell culture (as well as in eggs) and even in the absence of the severe CPE of SeV<sup>wt</sup>, which would be expected to limit viral replication and budding. The nature of this advantage is discussed below.

FIG. 6. Cytopathicity of SeV<sup>wt</sup> and rSeV<sup>GP42</sup>. Parallel confluent cultures of BHK cells in 9-cm dishes were either mock-infected (ctl) or infected with 10 PFU/cell (or equivalent amounts) of SeV<sup>wt</sup> (wt), rSeV<sup>GP42</sup> (GP42), or both viruses (wt + GP42). rSeV<sup>GP42</sup> was added to the mixed infections either together with SeV<sup>wt</sup> (0'), 75 min after SeV<sup>wt</sup> (75'), or 150 min after SeV<sup>wt</sup> (150'). Two dishes were trypsinized at the times indicated, their cells stained with trypan blue, and the average number of viable cells is shown. The vertical arrow at 48 hpi indicates that either the entire culture, or 2 × 10<sup>6</sup> cells at this time, were replated and followed until 96 hpi.

In contrast to uninfected cells, BHK cells persistently infected with rSeV<sup>GP42</sup> could be passaged at a dilution of 1:40 without significant effect on their plating efficiency. This result is consistent with the notion that the threshold of growth stimulation below which the default PCD pathway is activated has been significantly lowered in these persistently infected cells (see below). The relative presence of the co-infecting genomes was monitored by RT-PCR. Whereas both viral genomes were present at roughly equal levels at early times, there appeared to be more rSeV<sup>GP42</sup> than SeV<sup>wt</sup> at 48 hpi (Fig. 5B, lanes 5 and 6). Further, rSeV<sup>GP42</sup> clearly predominated over SeV<sup>wt</sup> by passage 1 (P1; lanes 7 and 8) and was the only genome detectable by passage 3 (P3; lanes 9 and 10). Both the rSeV<sup>GP42</sup> and mixed virus-infected cultures remained persistently infected to at least passage 9 (determined by RT-PCR and immunoblotting (not shown)). One persistently infected BHK culture has now been kept continuously for 8 months and contained the same level of viral macromolecules throughout this time as those found 48 hpi (not shown). The intracellular accumulation of viral macromolecules in itself is therefore insufficient to induce CPE. Moreover, basically the same results were obtained with LLC-MK2 (monkey kidney), HeLa, A549, and 293 (human) cell lines; i.e., whereas infection with rSeV<sup>wt</sup> was highly cytopathic, none of these cells survived subsequent passage, and
The above characteristics of PCD are, however, difficult to quantify. We therefore examined other properties that are more indicative of the fraction of the cells undergoing PCD. One method for detecting apoptosis, and which can distinguish it from necrosis, is based on the changes which occur in the plasma membrane during this process of physiological death, so that these cells can be eliminated by phagocytosis before they can elicit an inflammatory response. These changes maintain the integrity of the plasma membrane and dyes like trypan blue and propidium iodide are excluded. However, there is a looser packing of the phospholipids in the membrane and the appearance of phosphatidylserine in the outer leaflet, among other changes. This latter property has been developed by Vermes et al. (1995) into a flow cytometric assay using annexin V, a Ca\(^{2+}\)-dependent phospholipid binding protein. Studies with annexin V showed that phosphatidylserine exposure begins early after the onset of apoptosis in all cell types, under the action of a wide variety of initiators of apoptosis. Although this assay has normally been used to measure apoptosis in cells in suspension, it has recently been adapted for adherent cells (van Engeland et al., 1996). Parallel cultures of HeLa cells were infected with 8 PFU/cell of SeV\(^{wt}\), rSeVGP42, or both viruses and examined by flow cytometry after reaction with annexin V and propidium iodide staining of DNA as an indicator of necrosis. As a negative control, mock-infected cells were examined, and as a positive control, cells were treated with UV light. As shown in Fig. 8A, 15.7% of the SeV\(^{wt}\)-infected cells were judged to be apoptotic by this test vs 0.6% of the mock-infected control. In contrast, only 2.8% of the rSeVGP42-infected cells and 3.5% of the mixed virus-infected cells were found to be apoptotic.

Another early and specific marker of apoptosis is the caspase-induced conversion of the U1 snRNP 70-kDa protein, essential for splicing of pre-mRNAs, to its 40-kDa cleavage product (Casciola-Rosen et al., 1994). The relative levels of the U1 70- and 40-kDa proteins in extracts of the various virus-infected and control cell cultures...
were therefore examined by immunoblotting (Materials and Methods). As shown in Fig. 8B, the vast majority of the U1 gene product was the 40-kDa form in the UV-treated cells, whereas only 3% was cleaved in the untreated cells. SeVwt infection led to the cleavage of the majority of these proteins (80±85%), whereas infection with rSeVG42, or both viruses, led to the cleavage of a much smaller fraction of the protein (16±23%). Infection with VSV, another mononegavirus known to induce PCD (Koyama, 1995), also efficiently led to the accumulation of the 40-kDa product (lane 9, Fig. 8B). Unexpectedly, the auto-immune patient sera used here were found to also react with the SeV N protein, and this serves as an internal control for the general level of virus replication (i.e., accumulation of viral genomes) in the various infections. We conclude that SeVwt kills cells in culture, as in human blood monocytes, by inducing PCD, and that rSeVG42 is noncytopathic because it does not induce PCD. Moreover, in co-infections of rSeVG42 and SeVwt, rSeVG42 appears to prevent SeVwt from inducing PCD.

Mapping the leader region required to induce PCD

Replacement of the terminal 42 nt of the le1-sequence normally present at P1 with the equivalent le2-sequences (normally present only at P8) leads to a virus which cannot induce PCD. We therefore investigated the extent of this replacement necessary to prevent this induction. rSeVG viruses in which the terminal 24, 30, 33, and 48 nt of the le1-sequence at P1

FIG. 8. The extent of apoptosis in SeVwt, rSeVG42, and mixed virus-infected cells. FACS analysis of HeLa cells infected with the various viruses as indicated, after staining with annexin V and propidium iodide. The upper right quadrant indicates that fraction of the population of cells undergoing necrosis, and the lower right quadrant indicates that fraction of the population of cells undergoing apoptosis. UV1 and UV2 are cultures irradiated with 25 and 50 J/m² of 254-nm light (Materials and Methods). The relative levels of the U1 70- and 40-kDa proteins in HeLa cells infected with the various viruses as indicated, as well as VSV, were determined by immunoblotting with an autoimmune patient sera directed against the U1 70-kDa protein (serum B175). The fraction of the total represented by the 40-kDa protein (% cleaved) was determined by densitometry. Lanes 3±5 show the results of infection with 2 PFU/cell of each virus, lanes 6±8 show the results of infection with 10 PFU/cell, and lane 9 shows the infection with 10 PFU/cell of VSV-Indiana. Only 1/5 as much material was loaded in lane 9.
were replaced with the equivalent $le^{-}$ sequences were also prepared (similarly to rSeV$^{GP_{42}}$ (Garcin et al., 1995)), and their ability to kill cells, as well as to induce PCD, were studied. As shown in Fig. 9A, rSeV$^{GP_{24}}$ and rSeV$^{GP_{30}}$ were highly cytopathic like SeV$^{wt}$, whereas rSeV$^{GP_{33}}$, rSeV$^{GP_{42}}$, and rSeV$^{GP_{48}}$ were in-
creasingly less cytopathic. Similarly, the increasing replacement of the terminal le+ sequences at P+ with le− sequences led to a decreasing fraction of infected cells appearing apoptotic by flow cytometry (Fig. 9B). A decreasing fraction of their U1 70-kDa protein was also cleaved (Fig. 9C), even though the viral proteins had accumulated to wt levels in all the rSeV-infected cells (Fig. 9C). These results suggest that the second half of the le sequence at P+ are important in modulating PCD.

**FIG. 9.** The effects on apoptosis of replacing increasing amount of le+ sequences at P+ with the equivalent le− sequences. (A) Parallel cultures of HeLa cells were infected with 10 PFU/cell of the various rSeV indicated. At 48 hpi, the cultures were trypsinized and passaged with a 1 to 4 dilution. After 48 h of further culturing, the cells were stained with Giemsa. (B) Parallel cultures of HeLa cells were infected with 10 PFU/cell of the various rSeV indicated in duplicate, or treated with 9 and 50 J/m² of 254-nm light (UV1 and UV2). At 48 hpi, the cells were examined by FACS analysis as for Fig. 8, and the average of the fraction of the cells in the lower right quadrant is shown (the bar indicates the range). M refers to mock-infected cells. (C) The same samples as in B were analyzed by immunoblotting for the percentage cleavage of the U1 70-kDa protein to its 40-kDa product as for Fig. 8B, except that autoimmune patient serum K20, which also reacts with the lower host band indicated, and anti-L antibody, which also reacts with the upper host band indicated, was used. A separate membrane (bottom) was stained with a mixture of anti-L, anti-P, and anti-N sera (Materials and Methods).

**DISCUSSION**

rSeVGP42 grows slightly less well than SeVwt in eggs (Garcin et al., 1995) or in cultured cells (Figs. 3 and 4). Nevertheless, when these same cells are co-infected with both viruses, SeVwt is progressively excluded by the presence of the copyback nondefective genome. This exclusion appears to be due to the intracellular interference of SeVwt genome amplification by that of rSeVGP42. This phenomenon is not entirely unexpected, as rSeVGP42 has in part duplicated the le−
sequences at P\(^{L}\), like copyback DI genomes. In the case of rSeV\(^{GP42}\) and SeV\(^{wt}\), the intracellular interference is unlikely to be due to a stronger (antigenomic) promoter now being present at P\(^{L}\), as rSeV\(^{GP42}\)-infected cells actually accumulate less antigenomes relative to genomes (Fig. 3). The interference with SeV\(^{wt}\) genome amplification may act via the promoter-specific inhibitory effects of the viral C proteins, which are mostly nonstructural proteins expressed from the P gene. C protein expression strongly inhibits the amplification of DI-E307 (in transfected cells) under conditions where there is little or no effect on that of copybacks DI-H4 and DI-E307\(^{GP42}\). Further, whereas C expression prevents the recovery of SeV\(^{wt}\) from DNA in this transfected cell system, it does not prevent the recovery of the copy-back nondefective rSeV\(^{GP42}\) from DNA (Cadd et al., 1996). The copyback arrangement of promoter ends (Fig. 1) is apparently resistant to the C protein inhibition of RNA synthesis, because the C-sensitive sequences of P\(^{L}\) have been replaced by the equivalent but relatively insensitive sequences of P\(^{R}\).

We also note that the replicative advantage of rSeV\(^{GP42}\) over SeV\(^{wt}\) is one that is limited to co-infections, where both genomes normally express C proteins, but rSeV\(^{GP42}\) is less sensitive to their inhibitory effects. This advantage is presumably limited in the natural environment where direct competition of two similar viruses in animals is rare. A virus with a larger burst might be more successful on the whole, and this may account for why copyback nondefective viruses have not been found in nature.

We have found that SeV kills cells in culture by inducing PCD, similar to its effect on human peripheral blood monocytes (Tropea et al., 1995). The major finding of this study, however, is that rSeV\(^{GP42}\) is unable to induce PCD and that rSeV\(^{GP42}\) is also able to prevent SeV\(^{wt}\) from inducing this cellular response in co-infected cells. Further, the increasing replacement of the terminal le\(^{−}\) sequences at P\(^{L}\) with le\(^{−}\) sequences led to a decreasing fraction of infected cells appearing apoptotic by flow cytometry. A decreasing fraction of their U1 70-kDa protein was also cleaved (Fig. 9), even though the viral proteins had accumulated to wt levels in all the rSeV-infected cells. The dominant nature of rSeV\(^{GP42}\) in preventing PCD induced from infection with SeV\(^{wt}\) (and possibly also from insufficient growth stimulation at low plating densities) would normally classify this virus as a gain-of-function mutant, i.e., rSeV\(^{GP42}\) appears to have acquired the capacity to prevent PCD. While this is true in one respect, it is also misleading, as it surmises an evolutionary advantage of the mutated virus, for which there is no evidence. The acquired ability to prevent PCD is also unusual, given that the protein coding regions of the viral genome are unchanged. We found that the different sequences at P\(^{L}\) between rSeV\(^{GP42}\) and SeV\(^{wt}\) result in relatively modest differences in the amounts of viral mRNAs during the course of the infection, and even smaller differences in the amounts of viral proteins and genomes, especially in the coinfections. It is therefore unlikely that the acquired capacity to inhibit PCD is due to the relative amounts of these macromolecules, although this cannot at present be excluded. It appears more likely that the gain of function results from the le sequences themselves, which are expressed as short le RNAs from P\(^{L}\) and P\(^{R}\). It has been known for some time that the presence of copyback DI-H4 genomes in SeV stocks also prevents the CPE of SeV\(^{wt}\) and leads to persistent infections. This is so even though the amounts of viral proteins and genomes intracellularly are similarly unchanged or in fact often augmented (Roux and Holland, 1979; Roux and Waldvogel, 1981). It thus seems likely that DI-H4 and rSeV\(^{GP42}\) prevent PCD induced by SeV\(^{wt}\) similarly.

The common characteristic of the DI-H4/SeV\(^{wt}\) and rSeV\(^{GP42}/SeV\(^{wt}\) mixed infections is the copyback arrangement of promoters on the co-infecting genome, such that le\(^{−}\) RNAs (or RNAs with mostly le\(^{−}\)sequences) are now expressed from P\(^{L}\) as well as P\(^{R}\) (Fig. 1). This greatly decreases the ratio of le\(^{+}\)/le\(^{−}\)RNAs. The relative absence of le\(^{+}\) RNAs in these mixed infections could conceivably account for why the copyback genome can prevent PCD. For the closely related rhabdovirus VSV whose infection of HeLa cells also induces PCD (Koyama, 1995), the analogous le\(^{−}\) RNA is known to interact with the cellular La protein and to enter the nucleus (Kurilla and Keene, 1983). Here, le\(^{+}\) RNA is thought to participate in the shut-off of host RNA and DNA synthesis following infection (Weck et al., 1979). Infection with VSV copyback DI particles (which also produce only le RNAs), on the other hand, does not shut off the host cell (Weck and Wagner, 1979). Furthermore, purified le\(^{−}\) RNA (but not le\(^{+}\) RNA) can partially inhibit RNA synthesis when added to mammalian cell extracts (McGowan et al., 1982; see Keene, 1985, for review). The earliest known effect of the VSV le\(^{−}\) RNA on cellular metabolism is the inhibition of U1 snRNP maturation, which occurs 15–30 min postinfection (Crone and Keene, 1989). We have found that VSV infection also induces the cleavage of the U1 70-kDa protein to its 40-kDa product, an early marker for PCD (Fig. 8). Remarkably, this inhibition by VSV occurs even when there are as few as 100 le\(^{−}\) RNAs/cell, consistent with the notion that recognition of the VSV le\(^{−}\) RNA is the primary step leading to the shut-off of host macromolecular synthesis, and to PCD. The induction of PCD by SeV\(^{wt}\) also appears to be a very early event in the infection (Fig. 6). It will be of interest to determine whether the paramyxovirus le RNAs also induce the cleavage of the U1 70-kDa protein when introduced into cells by means other than SeV infection and to identify the host proteins with which they interact.
MATERIALS AND METHODS

Virus levels

Virus levels were determined by:

1. Pelleting the virus in 1 ml allantoic fluid through 500 μl of 25% glycerol in TNE, at 12,000 rpm for 30 min in an Eppendorf centrifuge. The pellet was dissolved in sample buffer, separated by 10% SDS–PAGE, and the gel stained with Coomassie brillant blue.

2. By infecting BHK cells with 1/10 dilution of the different stocks. At various times postinfection, cells were harvested, lysed with 20 mM Tris±Cl, pH 7.4, 150 mM NaCl, 2 mM EDTA (TNE), plus 0.5% NP-40; and the cytoplasmic extracts were centrifuged on 20±40% (w/w) CsCl density gradients to purify the nucleocapsids, which band at 1.31 g/ml, and the mRNAs which pellet. The viral RNAs were examined as described below.

3. Plaque titration on LLC-MK2 cells covered with 0.3% agarose overlay containing 1.2 μg/ml acetyl-trypsin, for 3±4 days at 33°C.

Primer extension and RT-PCR

RNA from viral nucleocapsids, or mRNA pellets, purified on CsCl density gradients (from 1/4 of a 9-cm-diameter petri dish) was used for primer extension and RT-PCR with the following primers: NP126, 5’-32P-GGCCCATCGTGAACTTT-15,270-3’, (genomic polarity); le21, 5’-21GTATGGAATATATA-15,285-3’, (antisense polarity); tr-15, 5’-ATTGGATC-ACCAGACAAGAGTTTAAGAGATATGTATC-3’, the sequence in italics is a T7 promoter and that in boldface type is nucleotides 15,384±15,356 (antisense orientation); L515,270, 5’-15,270GAAGCTCCGCGGTACC-13’, (antisense orientation).

Reverse transcription reactions were carried out with the M-MLV enzyme, as described by Gibco/BRL, using 200,000 cpm of 5’-32P-oligonucleotides purified by PAGE. The products of the reaction were separated on 8% sequencing gels.

Nonradioactive reverse transcription reactions were carried out as described above with 10 pmol of cold primers. One-tenth of the reaction was then used directly for DNA amplification with 1 U Taq DNA polymerase in 50-μl reactions. After 20 cycles (denaturation 30 s at 90°C; annealing 30 s at 50°C; elongation 20 s at 72°C), the products were separated directly on a 10% polyacrylamide gel and visualized with ethidium bromide.

Virus infection and induction of apoptosis in cell culture

HeLa cells were trypsinized and passaged one to two in MEM containing 5% heat-inactivated fetal calf serum the day before. Control apoptosis was induced by irradiation with 9 to 50 J/m² of 254-nm light, followed by incubation at 33°C in MEM containing 5% FCS. HeLa cells were also infected with the indicated PFU/ml of the various virus stocks for 1 h in 2 ml of medium without FCS, followed by incubation at 33°C in medium containing 5% FCS. A mock-infected control was treated identically.

Gel electrophoresis and immunoblotting

HeLa cells (in a 9-cm petri dish) were scraped into their medium, centrifuged at 500g for 5 min, washed once in cold PBS, and then lysed in 150-μl buffer containing 0.5% Nonidet P40, 20 mM Tris±Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and a cocktail of protease inhibitors (pepsatin A, leupeptin, antipain, chymostatin, and PMSF). Fifty microliters (plus 20 μl of protein loading buffer) was used for protein analysis, the rest for RNA extraction. Five microliters of each sample was electrophoresed on 10% SDS–polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. Membranes were reacted with monoclonal antibodies to various viral proteins, anti-L, anti-P (1–180), and anti-N (N/877) (Curran et al., 1991), and with auto-immune patient sera directed against the U1 70-kDa protein and its cleavage product U1 40 kDa (sera B-175 and K-20, from Dr. W. J. van Venrooij, Amsterdam (van Venrooij et al., 1990). Antibody reaction was detected by enhanced chemiluminescence (ECL; Amersham).

Measurement of phosphatidylserine exposure by annexin-V fluorescence

At 48 h postinfection, medium from 9-cm petri dishes containing adherent HeLa cells (6 × 10⁶ cells) was removed, a quick rinse was performed with 1 ml of 0.05% trypsin, and 1 ml of 0.05% trypsin was added for 2±3 min. Once the cells had lifted off, 10 ml of complete medium containing 10% fetal bovine serum was added, and the cell suspension was transferred to a 13-ml centrifuge tube and centrifuged at 500g for 5 min. The supernatant was decanted and cells gently resuspended in 10 ml of MEM containing 10% FCS and centrifuged again as above. Cells were washed twice with cold PBS and then resuspended in 500 μl of annexin V binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl₂); 100 μl of this suspension was used for annexin V staining by adding 2 μl annexin-V-fluos reagent (Boehringer) and 2 μl of a 50 μg/ml propidium iodide solution for 10±15 min prior to flow cytometry analysis. For excitation, 488 nm light was used; a 515-nm bandpass filter was used for fluorescein detection and a filter > 560 nm for PI detection.

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REFERENCES


