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Abstract

Many paramyxoviruses express small basic C proteins, from an alternate, overlapping open reading frame of the P gene mRNA, which were previously found to inhibit mRNA synthesis. During recent experiments in which infectious Sendai virus (SeV) was recovered from cDNA via the initial expression of the viral N, P, and L genes from plasmids, the abrogation of C protein expression from the plasmid P gene was found to be necessary for virus recovery. We have investigated the effect of C coexpression on the amplification of an internally deleted defective interfering (DI) genome directly in the transfected cell, for which, in contrast to virus recovery experiments, genome amplification is independent of mRNA synthesis carried out by the SeV polymerase. We find that C protein coexpression also strongly inhibits the amplification of this DI genome but has little or no effect on that of a copy-back DI genome (DI-H4). We have also characterized the C protein from a mutant SeV and found that (i) it had lost most of its inhibitory activity on internally deleted DI genome amplification and (ii) its coexpression no longer prevented the [...]
The Sendai Paramyxovirus Accessory C Proteins Inhibit Viral Genome Amplification in a Promoter-Specific Fashion

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Many paramyxoviruses express small basic C proteins, from an alternate, overlapping open reading frame of the P gene mRNA, which were previously found to inhibit mRNA synthesis. During recent experiments in which infectious Sendai virus (SeV) was recovered from cDNA via the initial expression of the viral N, P, and L genes from plasmids, the abrogation of C protein expression from the plasmid P gene was found to be necessary for virus recovery. We have investigated the effect of C coexpression on the amplification of an internally deleted defective interfering (DI) genome directly in the transfected cell, for which, in contrast to virus recovery experiments, genome amplification is independent of mRNA synthesis carried out by the SeV polymerase. We find that C protein coexpression also strongly inhibits the amplification of this DI genome but has little or no effect on that of a copy-back DI genome (DI-H4). We have also characterized the C protein from a mutant SeV and found that (i) it had lost most of its inhibitory activity on internally deleted DI genome amplification and (ii) its coexpression no longer prevented the recovery of SeV from DNA. However, consistent with the insensitivity of copy-back DI genomes to C protein inhibition, C coexpression did not prevent the recovery of copy-back nondefective viruses from DNA. The inhibitory effects of C coexpression thus appear to be promoter specific.

Sendai virus (SeV) is a member of the Paramyxovirus genus of the Paramyxovirinae subfamily. This subfamily also includes the Morbillivirinae (e.g., measles virus) and the Rubulavirus (e.g., mumps virus, simian virus 5, and Newcastle disease virus) genera. The more distantly related pneumoviruses, such as respiratory syncytial virus (RSV), are now classified in a separate subfamily (Pneumovirinae) of the Paramyxoviridae (29a). The Paramyxovirinae have been recently reclassified on the basis of their P gene organizations, including the presence of a C open reading frame (ORF). The C proteins were first found in SeV-infected cells and, as they were apparently absent in virions, were termed nonstructural proteins (reviewed in reference 26). Subsequent work with specific antisera showed that the C proteins could be detected in virions, in which they appear to be associated with nucleocapsids. They are, however, greatly underrepresented in virions relative to their amounts intracellularly (26, 31, 39). In SeV-infected cells, the C proteins are relatively abundant and are the major products of the P/C gene on a molar basis (11). Their expression can also be very complex. For SeV, there are four independently initiated proteins which are expressed (Fig. 1), via a non-AUG start site (C) and both scanning-dependent (C) and -independent (Y1/Y2) ribosomal initiation (reviewed in reference 11). However, morbilliviruses such as measles virus appear to express only a single C protein and in significantly lesser amounts than P (1, 2). Rubulaviruses such as simian virus 5 and mumps virus, in further contrast, do not contain a C ORF. For RSV, there is no overlapping ORF in the P gene, but most pneumoviruses contain four accessory genes, 1B, 1C, 1A/SH, and 22K/M2, in which a similar function might reside (9). For the even more distantly related rhabdovirus vesicular stomatitis virus, however, a C-like ORF overlapping the N-terminal end of the P ORF is again present, and two C proteins are expressed from this frame (35).

The C proteins are relatively small (175 to 215 residues), basic proteins expressed from all the P genes of the SeV group and the morbilliviruses (19). (As “paramyxovirus” now applies to a family, a subfamily, and a genus [29a], the term “SeV group” is used for this genus.) There appears to be little specificity in their localization intracellularly; they are evenly distributed in the cytoplasm by immunofluorescence and are present in all cytoplasmic fractions by cell fractionation (31). However, in other studies, C was also found to specifically colocalize with viral nucleocapsids (39). Consistent with this latter association, the C proteins were recently found to play a role in viral RNA synthesis. Elimination of C expression from the SeV P/C gene (by placing a stop codon just downstream of the Y2 AUG [10]) was found to strongly increase mRNA synthesis from nondefective genomes in vitro. The further expression of C from a separate plasmid was found to eliminate this increase (12). Because the abrogation of C coexpression had no effect on the amplification of a copy-back defective interfering (DI) genome in transfected cells, the inhibitory effect of C on viral RNA synthesis appeared to be specific for mRNA synthesis.

This paper reports that C protein coexpression also inhibits amplification of nondefective genomes and internally deleted DI genomes (but not copy-back DI genomes) in transfected cells, and hence the inhibitory effect of C is more general than appreciated previously. We have also characterized a mutant C protein from a SeV variant, which maps to a single amino acid substitution [Phe-170 substituted by Ser (F170S)] in C, as being defective in its ability to inhibit viral RNA synthesis. Taken
together, our results suggest that C normally functions during virus infection to limit both genome amplification and mRNA synthesis.

MATERIALS AND METHODS

DI genomes and P/C genes. Plasmids PS-DIH/Rbz (H4) and PS-DIH/E307 (E307) expressing T7 antigenome transcripts were described previously (3, 14). The nucleotide exchanges to generate the E307 derivatives (GP24-53 and GP1-53 [[see Fig. 3]]) in which genomic promoter sequences were replaced by the upstream AUG for PA. The HA tag, initiated from the C’ start codon, is shown in black. The regions of the gene derived from the H strain (residues 1 to 74 of C) and the M strains (residues 75 to 204) in the P/C and P/CMV constructs are indicated. The presence of a Phe (F) or Ser (S) at position 170 of C is also indicated.

RESULTS

C protein coexpression inhibits DI-E307 genome amplification. We have recently described a transfected cell system for the recovery of infectious SeV from a full-length cDNA copy of the viral genome (18). Virus recovery was carried out by expressing the viral N (formerly called NP), P, and L proteins from plasmids, to initiate encapsidation of the T7 RNA polymerase-generated plus-sense antigenome RNA and its replication to minus-sense genomes. All of the viral proteins are then generated from the recombinant RNA genome. The system is particularly robust and has failed to generate recombinant SeV (rSeV) only four times in >100 attempts. In each of these failed attempts, a wild-type copy of the P/C gene was used. On the other hand, parallel transfections with P/C genes in which C protein expression was severely limited, either (i) by the introduction of a stop codon just downstream of the Y2 initiation codon (P/C<sup>stop</sup>, Fig. 1) or (ii) by the fusion of the N-terminal end of the P ORF to a 47-nt-long sequence coding for the influenza virus hemagglutinin (HA) tag (which itself is initiated from the C’ start codon which has been changed from ACG to AUG) (P/C<sup>HA</sup> [[Fig. 1]]), consistently led to rSeV recoveries. It therefore appeared that normal levels of C protein expression (relative to P) prevented infectious virus recovery.

To more closely examine this effect of the C protein(s), we replaced the FL-3 plasmid containing the full-length (15,384-nt) genome with one containing the internal-deletion DI-E307 genome (1,794 nt [[14]]), whose shorter T7 antigenome transcript is assembled into nucleocapsids and amplified more readily than that of FL-3 (4). These two antigenome constructs are presumably identical except for the 13,590-nt deletion which fuses the beginning of the N gene to the end of the L gene. However, in contrast to virus recovery from cDNA experiments, the amplification of E307 is independent of mRNA synthesis carried out by the SeV polymerase. E307 amplifications were carried out in parallel cell cultures using various P/C gene constructs, and the levels of the DI genomes (and antigenomes) generated within the transfected cell were determined (by primer extension) after their isolation as nucleocapsids on CsCl density gradients (Fig. 2). The levels of the various P and C proteins in cytoplasmic extracts of the transfected cells were monitored by immunoblotting. The various P proteins were found to have accumulated to levels similar to each other and that found in an acute natural virus infection (Fig. 2B, lane SeV) or in a persistently infected culture (Fig. 2B, lane SeV-PI). The four C proteins (C’, [21 amino acids [[aa]]], C [204 aa], Y1 [181 aa], and Y2 [175 aa]) can all be detected in the lower region of the immunoblot. C (initiated from the third

![FIG. 1. Schematic representation of the SeV P/C gene. (A) The three ORFs which are expressed as protein are shown as rectangles, drawn roughly to scale. The +1 ORF is shaded. The positions of relevant restriction sites, the cotranscriptional editing site, and the loss-of-function mutation in C are indicated. (B) Structures of the N-terminal ends of the various P/C gene constructs. The positions of the five ribosomal start sites are indicated at the top. The C ORF of P/C<sup>HA</sup> is shown as an unshaded dotted box, as it is not expressed as protein because the upstream AUG for PA. The HA tag, initiated from the C’ start codon, is shown in black. The regions of the gene derived from the H strain (residues 1 to 74 of C) and the M strains (residues 75 to 204) in the P/C and P/C<sup>HA</sup> constructs are indicated. The presence of a Phe (F) or Ser (S) at position 170 of C is also indicated.](image-url)
FIG. 2. Effect of C protein coexpression on DI-E307 amplification. vTF7-infected HeLa cells were transfected with pE307 (which is transcribed by T7 RNA polymerase) to generate a plus-sense ([+]) antigenome with three extra G residues at the 5' end (AG + 3), pGEM-N, pGEM-L, and the various pGEM-P/C genes as indicated (see Fig. 1). At 24 h postinfection, cytoplasmic extracts were prepared and their nucleocapsids were isolated on CsCl density gradients. The amounts of the assembled minus-sense ([−]) genomes (AG) (and T7-generated N mRNAs) were estimated by primer extension (Materials and Methods) on an equal sample (one-eighth of a dish) of the transfected cells (A). The relative intensities of the minus-sense genome bands are listed below the gel in panel A. The amounts of P and C proteins in the cytoplasmic extracts were estimated by immunoblotting in which the top and bottom halves of the gel were reacted separately with the anti-P and anti-C antisera, respectively (B). SeV-PI, persistently SeV infected culture.

start codon) is the major form, followed by C' and the Y proteins. The C proteins from the transfected P/Cwt and P/C<sup>Mlu</sup> genes [P/C<sup>Mlu</sup> Fig. 1] contains two base changes in the P ORF at codon 445, well downstream of the C ORF, and is used as another example of a wild-type gene) had accumulated to about twice the level of that found in the natural virus (SeV) genome. The relative intensities of the minus-sense genome bands are those found in the natural virus (SeV) infection. The OH-M1 strain of SeV (SeVM) is one of the plaque-purified clones of Ohita-M, obtained from an epidemic of laboratory mice, and is unusually lethal for these animals (38). OH-MVC11 (SeV<sup>MVC</sup>) is a mutant derived from OH-M1 (after five serial passages in LLC-MK2 cells) which formed clear plaques rather than the turbid plaques of the parent strain (SeV<sup>M</sup>). SeV<sup>MVC</sup> is highly adapted to LLC-MK2 cells; it produces 20-fold more virus than SeV<sup>M</sup> in cell culture, and this phenotype was associated with significantly higher levels of viral mRNAs and proteins intracellularly (23). Sequencing of the SeV<sup>M</sup> and SeV<sup>MVC</sup> genomes revealed a U-to-C change in codon 170 of the C ORF, where Phe was changed to Ser (this base change is silent in the P ORF) (22a). The carboxy-terminal half of the C ORF region of our P/C<sup>wt</sup> strain (H) construct [the Sall-EcoRI fragment [Fig. 1]] was therefore replaced with that from the parental M and mutant MVC viruses, and these hybrid constructs were assayed alongside the other P/C genes. As shown in Fig. 2, expression of C proteins containing the F170S mutation led to E307 genome levels fourfold higher than those of proteins containing Phe at this position, even though all C protein levels were very similar to each other for both M strain genes and to those found in the natural virus (SeV) infection. We also note that the hybrid H/M strain C' protein and P protein migrate slightly more slowly than those of the H strain. When the P/C<sup>wt</sup>, P/C<sup>Mlu</sup>, and P/C<sup>MVC</sup> genes are considered, there is a good correlation between their C protein levels in the cell extracts and the extent of the inhibition of genome amplification. Only the C protein of the P/C<sup>MVC</sup> gene accumulated to normal levels, but it does not appear to inhibit E307 genome amplification. We also introduced the F170S mutation into P/C<sup>wt</sup> and found that C expression from this construct had lost enzyme because it contains three extra G residues at its 5' end which are eliminated during its replication by the SeV enzyme. The levels of the SeV-generated antigenomes parallel those of the genomes in these transfections, but they are difficult to quantify because they are considerably less abundant than the assembled T7-generated antigenomes and migrate too close to them. There is also a strong 5' end just below the position of the minus-sense genome band, which corresponds to the end of the T7 transcript generated from pGEM-N and is absent from natural virus infections (Fig. 2, lanes SeV). The assembly of the pGEM-N mRNA by its translation product appears to be a common feature of these recovery systems (it also occurs in LaCrosse bunyavirus and Tacaribe arenavirus systems [data not shown]) and is due to the illegitimate assembly of RNAs in the transfected cell cytoplasm (illegitimate in the sense that the assembly of these RNAs is uncoupled from their synthesis by the SeV enzyme). The relatively constant levels of these latter plus-sense RNAs, however, show that T7 transcript expression from pE307 and pGEM-N is unaffected when different P/C genes are expressed and that they serve as internal controls. These results also indicate that only a minority of the assembled T7 antigenomes appear to act as templates for the SeV replicase, possibly because of their excess over the SeV replicase. In this and other experiments (see Fig. 4), we determined that the N and L proteins had also been expressed invariably in the presence of all of these P/C gene constructs. Further, the same relative levels of E307 minus-sense genomes (in relation to the various P/C constructs) were found in three separate experiments, indicating that our primer extension reactions are likely to be semiquantitative. The available evidence thus suggests that it is the level of C protein(s) expression itself that is detrimental to genome amplification.

A mutant C protein. Support for the notion that C expression inhibits genome replication has also been obtained from a mutant virus whose defect appears to map to the C proteins. The Oh-M1 strain of SeV (SeV<sup>M</sup>) is one of the plaque-purified clones of Ohita-M, obtained from an epidemic of laboratory mice, and is unusually lethal for these animals (38). Oh-MVC11 (SeV<sup>MVC</sup>) is a mutant derived from Oh-M1 (after five serial passages in LLC-MK2 cells) which formed clear plaques rather than the turbid plaques of the parent strain (SeV<sup>M</sup>). SeV<sup>MVC</sup> is highly adapted to LLC-MK2 cells; it produces 20-fold more virus than SeV<sup>M</sup> in cell culture, and this phenotype was associated with significantly higher levels of viral mRNAs and proteins intracellularly (23). Sequencing of the SeV<sup>M</sup> and SeV<sup>MVC</sup> genomes revealed a U-to-C change in codon 170 of the C ORF, where Phe was changed to Ser (this base change is silent in the P ORF) (22a). The carboxy-terminal half of the C ORF region of our P/C<sup>wt</sup> strain (H) construct [the Sall-EcoRI fragment [Fig. 1]] was therefore replaced with that from the parental M and mutant MVC viruses, and these hybrid constructs were assayed alongside the other P/C genes. As shown in Fig. 2, expression of C proteins containing the F170S mutation led to E307 genome levels fourfold higher than those of proteins containing Phe at this position, even though all C protein levels were very similar to each other for both M strain genes and to those found in the natural virus (SeV) infection. We also note that the hybrid H/M strain C’ protein and P protein migrate slightly more slowly than those of the H strain. When the P/C<sup>wt</sup>, P/C<sup>Mlu</sup>, and P/C<sup>MVC</sup> genes are considered, there is a good correlation between their C protein levels in the cell extracts and the extent of the inhibition of genome amplification. Only the C protein of the P/C<sup>MVC</sup> gene accumulated to normal levels, but it does not appear to inhibit E307 genome amplification. We also introduced the F170S mutation into P/C<sup>wt</sup> and found that C expression from this construct had lost
most of its ability to inhibit mRNA synthesis from nondefective templates in vitro and was indistinguishable from the hybrid P/C<sup>CIV</sup>C<sup>NIV</sup> gene in this respect (data not shown). Taken together, these results suggest that (i) the C protein normally inhibits genome expression, (ii) the overexpression phenotype of SeV<sup>NIV</sup> is due to a functional defect in the C protein, and (iii) Phe-170 is critical for this effect.

The amplification of different DI genomes is unequally inhibited by C protein. One feature shared by nonsegmented minus-strand RNA viruses is that the 5′ end of the first mRNA is recessed from the very 3′ end of the minus-sense genome template (Fig. 3), and this interval is made up by the short plus-sense leader (le<sup>+</sup>) region. The 3′ end of the minus-sense genome is nevertheless expressed as a short le<sup>−</sup> RNA (ca. 55 nt for SeV) which terminates near the start of the N mRNA (reviewed by Banerjee and Barik [1a] and Lamb and Kolakofsky [25]). The polymerase which has made the le<sup>−</sup> RNA then continues on, to sequentially synthesize the linear array of mRNAs. An analogous minus-sense leader (le<sup>−</sup>) RNA is expressed from the 3′ end of the antigenome (the le<sup>−</sup> region, 57 nt for SeV [Fig. 3]), containing the sequences up to the end of the (last) L gene (4a, 28, 37). RNA synthesis from the le<sup>+</sup> and le<sup>−</sup> regions is thought to control genome replication, as these sequences at the 3′ ends of genomes and antigenomes are thought to serve as replicase promoters, and their complements at the 5′ ends of the nascent chains are thought to serve as nucleocapsid assembly promoters.

DI-H4 is a copy-back rather than an internally deleted DI genome and thus contains the le<sup>−</sup> region at the 3′ genome end as well as at its normal position at the 3′ antigenome end (Fig. 3) (28). We previously reported that the amplification of DI-H4 was essentially unaffected when C protein expression was restricted (P/C<sup>CIV</sup>) in a slightly different system (DI-H4<sub>UV</sub> [10]) in which the DI genome is delivered to the cell via infection with a natural SeV stock, freed of nondefective helper virus by selective UV inactivation. We therefore compared H4 and E307 amplification in parallel transfections in our present system (in which the DI genomes are introduced as plasmid DNAs), in the presence (P/C<sup>CIV</sup>) and absence (P/C<sup>NIV</sup>) of C expression. As shown in the RNA blots in Fig. 3, E307 genome levels were increased ca. 20-fold in the absence of C protein expression, whereas those of H4 were unaffected by similar conditions. The amplification of different DI genomes can clearly be inhibited to very different extents by the coexpression of C.

It is also clear, however, that H4 can accumulate in these transfections to ca. 10-fold higher levels than E307, even in the absence of C. The lack of an inhibitory effect of C on H4 amplification may then simply be due to the robust nature of this DI. The higher amplification levels of H4 have been ascribed to stronger promoter sequences of the le<sup>−</sup> region, which are now present at the 3′ ends of both genomes and antigenomes. The progressive exchange of le<sup>−</sup> sequences at the genome 3′ end of either H4 or E307 led to the progressive increase or decrease in their relative amplification levels, and in all cases the le<sup>−</sup> sequences were found to be stronger than or equal to the le<sup>+</sup> sequences by this test (4). Two of these E307 constructs were examined for their sensitivity to C coexpression. E307<sub>GIP1-53</sub> which contains le<sup>−</sup> positions 1 to 53 (le<sup>−</sup> [1-53]) at its 3′ genome end, was found to accumulate to low levels like E307 (in the absence of C), but its amplification is nevertheless insensitive to C protein coexpression (E307<sub>GIP1-53</sub> [Fig. 3]). The lack of an inhibitory effect of C on genome amplification is thus not solely associated with the robust nature of the DI and appears in this case to be determined by which le sequences are present at the 3′ genome end. Moreover, sensitivity to C inhibition is restored when nt 1 to 23 of this hybrid DI are changed back to the le<sup>−</sup> sequences (E307<sub>GIP24-53</sub>) [Fig. 3]), suggesting that nt 1 to 23 are critical in determining sensitivity to C protein inhibition. However, in other experiments using DI-H4 in which le<sup>−</sup> [24-53] at the 3′ genome end was replaced with le<sup>−</sup> [24-53], this substitution both lowered amplification levels of the H4 derivative and restored sensitivity to C coexpression (not shown). These latter results suggest, in contrast, that nt 24 to 53 are critical in this respect for DI-H4. It appears that the le sequences which are critical for conferring C sensitivity, as well as whether this sensitivity correlates with weaker promoters, are different for the internal deletion and copy-back DI genomes. An explanation for this complexity will probably require a better understanding of these replication promoters, which for SeV also appear to extend beyond the le regions (35a). Nevertheless, as the sensitivity to C inhibition can be gained or lost by simply exchanging 23 nt at the 3′ genome end of E307, this effect is likely to be (at least partly) determined during the initiation of genome replication. We also note that when C coexpression is ablated, the replicative advantage in having the le<sup>−</sup> sequences at the 3′ genome end of the E307 derivatives is largely lost, as
E307, E307GP24-53 and E307GP1-53 all accumulate to similar levels (well below that of H4). Promoter strength for SeV is thus affected by C coexpression.

**C protein inhibition of DI-H4 amplification in vitro.** We previously reported that C coexpression strongly inhibited mRNA synthesis (from nondefective genomes) but had little or no effect on DI-H4 amplification in vitro (12). Genome amplification, however, occurred at the limit of detection in these experiments. The efficiency of our in vitro amplification reactions has increased considerably since (>50-fold), and we now find that C coexpression can significantly inhibit H4 amplification in vitro (ca. 20-fold [Fig. 4B]), similar to its effect on mRNA synthesis (data not shown). As before, we have controlled the levels of N, P, and L proteins in our transfected cell extracts, and these appear to be relatively invariant (Fig. 4A). Thus, under our present in vitro conditions, C coexpression can also limit DI-H4 amplification. Moreover, when the carboxy-terminal half of the C ORF of our P/C44 construct was replaced with that from the mutant MVC strain, the mutant C proteins had lost most of their ability to inhibit the reaction, even though the C\textsuperscript{MVC} proteins had accumulated to levels comparable to those of the C\textsuperscript{44} proteins in the extracts. In contrast, the C\textsuperscript{CM} proteins were found to be highly inhibitory, similar to C\textsuperscript{44} (Fig. 4B). Thus, all of the various types of RNA synthesis that we have investigated, only copy-back DI amplification in vivo is relatively insensitive to C protein inhibition.

**rSeV recovery from cDNA.** To determine whether the function of C that is lost in the C\textsuperscript{MVC} protein was that which was responsible for preventing the rescue of infectious virus from FL-3 DNA, virus recoveries were carried out in parallel with the P/C\textsuperscript{strep}, P/C\textsuperscript{M}, and P/C\textsuperscript{MVC} genes (18). The transfected cells were injected into embryonated chicken eggs as before, and these stocks were further passaged in eggs to amplify the recovered viruses. The presence of virus in the allantoic fluids was determined by infecting HeLa cells with these stocks. Cytoplasmic extracts were prepared and centrifuged on CsCl density gradients to isolate viral nucleocapsids. Not only are these gradients useful for purifying the nucleocapsids, but they are diagnostic of SeV infections, as relatively small amounts (e.g., containing 1 \(\mu\)g of genome RNA) are visible to the naked eye with indirect light (they scatter blue light) at their characteristic buoyant density. When the P\textsubscript{2} (passage 2) egg stocks from the recoveries specified above were used to infect HeLa cells, a visible nucleocapsid band (whose identity was confirmed by immunoblotting [not shown]) was present when the P/C\textsuperscript{strep} and P/C\textsuperscript{MVC} genes had been used. However, the nucleocapsid band was absent when the P/C\textsuperscript{M} gene had been used in the initial recovery. To determine whether a very small amount of virus had been generated, the P/C\textsuperscript{M} P\textsubscript{2} stock was passaged again, undiluted, and those of the P/C\textsuperscript{strep} and P/C\textsuperscript{MVC} series were passaged after a 1/1,000 dilution. When these P\textsubscript{2} stocks were examined, however, the results were unchanged; i.e., only the P/C\textsuperscript{M} sample did not yield a visible nucleocapsid band. RNA was nevertheless extracted from this region of all three CsCl gradients and used for reverse transcription-PCR (RT-PCR) amplification, the most sensitive of our tests for the presence of viral genomes. As shown in Fig. 5A, the viral genomes recovered in the P\textsubscript{2} stocks of the P/C\textsuperscript{strep} and P/C\textsuperscript{MVC} series were recombinant genomes, containing the marker Bg\textsubscript{II} site in the le\textsuperscript{+} region (lanes 5 and 11) and had lost the marker NsiI site in the 5\textsuperscript{\textprime} untranslated region of the N gene (lanes 6 and 12), as found previously (18). Even RT-PCR, however, was unable to show the presence of viral genomes from the P/C\textsuperscript{M} series (lanes 7 to 9). The normal expression of C proteins appears to completely suppress the recovery of infectious virus. The function of C that is lost in the C\textsuperscript{MVC} protein also appears to be that which is responsible for preventing virus recovery from cDNA.

When E307GP1-42 is included as a supplementary plasmid in virus recovery from FL-3, a 42-nt copy-back nondefective virus (rSeV-GP42) is generated along with the normal rSeV (18). rSeV-GP42 also eliminates the normal virus on subsequent passage, presumably because the substitution of the normal 3\textsuperscript{\textprime} genome end le\textsuperscript{[1-42]} with le\textsuperscript{[1-42]} confers a selective advantage to the copy-back virus in natural infections. We were interested in whether recovery of rSeV-GP42 from pFL-3 (by recombination with E307\textsuperscript{GP1-42}) would also be insensitive to C coexpression, since recovery of infectious viruses (in contrast to E307 amplification) requires mRNA synthesis as well as genome amplification from the T7-generated template, and both are normally sensitive to C coexpression. E307GP1-42 was therefore included as a supplementary plasmid in half of the virus recoveries containing either P/C\textsuperscript{strep} or P/C\textsuperscript{strep}, and the presence of recovered viruses was examined as for Fig. 5A except that RT-PCRs specific for both normal rSeV and rSeV-GP42 were carried out. We found that C\textsuperscript{44} coexpression no longer prevented virus recovery when E307GP1-42 was present and moreover that only rSeV-GP42 could be detected in the recovered stocks, even at the earliest passages (Fig. 5B, lane 3). Parallel transfections with P/C\textsuperscript{strep}, in contrast, produced stocks in which both rSeV and rSeV-GP42 were present (lane 2). The presence of le\textsuperscript{[1-42]} at the 3\textsuperscript{\textprime} end of the nondefective genome thus appears to relieve the inhibitory effects of C coexpression on both mRNA synthesis and genome amplification. This relief is presumably one of the reasons for the selective advantage of rSeV-GP42 in competition with normal SeV.
DISCUSSION

The C\textsuperscript{MVC} protein has lost its ability to inhibit DI-E307 genome amplification in vivo and that of DI-H4 in vitro, as well as mRNA synthesis from nondefective genomes in vitro (not shown), suggesting that C inhibits these reactions by a similar mechanism. Moreover, the ability of C to inhibit mRNA synthesis or H4 amplification in vitro depended on its coexpression with P and L; simply adding C back to an extract in which P and L had been expressed did not reproduce the inhibitory effect (reference 12 and data not shown). Together, these results suggest that the inhibitory effects of C coexpression act indirectly on the SeV polymerase, by somehow conditioning the activity of the enzyme made during the transfection. However, it is puzzling that the amplification of the copy-back DI-H4 genome is more sensitive to C inhibition in vitro than it is in vivo. Since this inhibition appears to occur during initiation of replication, and amplification estimations in vivo measure multiple rounds of synthesis whereas those carried out in vitro are more limited, one might have expected the opposite result. We have no clear explanation for why the inhibitory effects of C coexpression appear to be exaggerated in vitro. Although highly speculative, it is possible that amplification of DI-H4 (and that of some of the hybrids) in vivo is insensitive to C inhibition as a result of a mechanism that does not operate in vitro, e.g., that a kinase (or phosphatase, etc.) acts on a protein such as P differentially in vivo and in vitro. This mechanism must somehow be regulated by the promoter sequences (Fig. 3). The manner in which C exerts its inhibitory effects thus remains to be determined. We expect that the F170S mutation will help these investigations.

The mutant (F170S) C protein was found to have lost most of its ability to inhibit SeV RNA synthesis, and its normal coexpression from the P gene no longer prevented the recovery of rSeV from DNA. This P gene was derived from a virus originally isolated from a laboratory epidemic of mice (SeV\textsuperscript{N}), which was then adapted to grow on LLC-MK2 cells in culture (SeV\textsuperscript{MVC}). There is no direct evidence that the F170S mutation was important in this adaptation to cell culture, as this will require the isolation of virus revertants. However, these results are consistent with the notion that C inhibition also operates during natural virus infection. These infections presumably benefit from the relative absence of C during primary transcription, since there is relatively little C protein in virions. Later in infection, when the C proteins have accumulated to their steady-state levels, they might act to restrain genome amplification and expression and possibly to attenuate the cytopathic effects of the infection. The proposed role for the SeV C protein during the infectious cycle has interesting parallels with that of the long form of the hepatitis delta virus (HDV) \(\delta\) antigen. This HDV protein is also required late in the infectious cycle (for packaging the minus-sense strand HDV RNA genome), but \(\delta\) is presumably counterproductive during the early stages of the infection, as it inhibits genome replication. For HDV, this early inhibitory effect is thought to be circumvented by the expression of only the short form of \(\delta\) during the early phase of the infection (reviewed in reference 27). The SeV C proteins will also accumulate during virus recovery from cDNA once they begin to be expressed from the recombinant RNA genome. It is presumably the presence of high levels of C during the initial stages of the virus recovery from cDNA that is most detrimental to the generation of infectious virus, but this remains to be demonstrated.

All Paramyxovirinae contain at least six genes (or mRNA transcription units), for nucleocapsid (N), phosphoprotein (P), matrix (M), two surface transmembrane glycoproteins (F and HN), and the large (L) polymerase protein (invariably in this order) (25), representing the minimal essential genes of this virus subfamily. Many of the viruses also express accessory proteins (accessory in the sense that these proteins are not expressed by all the viruses of this subfamily), either from overlapping ORFs within the P gene mRNA or as separate transcription units. Besides C, which is expressed by all but the rubulaviruses, the V proteins are expressed by all but human parainfluenza virus types 1 and 3 (hPIV1 and hPIV3) (both members of the SeV group), the D proteins (equivalent to but larger than W [Fig. 1]) are expressed only by hPIV3 and bovine parainfluenza virus type 3 (bPIV3), and the SH protein is expressed only by some of the rubulaviruses (mumps virus and simian virus 5) (reviewed in reference 34). Remarkably, the expression of these accessory genes can be quite different even among the most closely related viruses; e.g., SeV (also called murine parainfluenza virus type 1) and hPIV1, and bPIV3 and hPIV3, are two pairs of very related viruses, yet SeV and bPIV3 express V proteins (30, 37) whereas hPIV1 and hPIV3 do not (15, 29, 30).

Pneumoviruses like RSV contain four transcription units in addition to the six ubiquitous or minimal essential genes, only
of which (1A/SH) appears to have a counterpart among the Paramyxovirinae, and another (22K/M2) contains two ORFs (ORF1 and ORF2) which briefly overlap in the middle (9). The recovery of infectious RSV from cDNA has also recently reported (7), and here another of the accessory proteins has been found to play an important role in the recovery, but in a very different fashion. The presence of the 22-kDa protein (ORF1) of RSV has been found to be necessary for virus recovery, apparently acting to increase the processivity of the viral polymerase during mRNA synthesis (8). For SeV, only the viral N, P, and L proteins are required for virus recovery from cDNA (18). It is striking that RSV, which grows in cells has been found to lay an important role in the recovery, recently reported (7), and here another of the accessory proteins may thus be required for other viral functions, such as the ability to grow productively in animals, in analogy with the analogues of Sendai virus from cloned cDNA: generation of a novel copy-back nondefective paramyxovirus from cDNA: generation of a novel copy-back nondefective paramyxovirinae; the viruses and their replication, p. 1313–1352. In B. N. Fields et al. (ed.), Virology, 3rd ed. Raven Press, New York.


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