JOURNAL OF VIROLOGY, Aug. 1996, p. 5067–5074 Vol. 70, No. 8
0022-538X/96/$04.00+0
Copyright © 1996, American Society for Microbiology

The Sendai Paramyxovirus Accessory C Proteins Inhibit Viral Genome Amplification in a Promoter-Specific Fashion

TAMARRA CADD,[1] DOMINIQUE GARCIN,[1] CAROLINE TAPPAREL,[1] MASAE ITOH,[2] MORIO HOMMA,[3] LAURENT ROUX,[1] JOSEPH CURRAN,[1] AND DANIEL KOLAKOFSKY[1]*

Department of Genetics and Microbiology, University of Geneva School of Medicine, CH-1211 Geneva, Switzerland,[1] and Department of Microbiology, Kobe University School of Medicine, Chuo-ku, 650 Kobe,[2] and Department of Microbiology, Kobe Womens University, Higashisuma, Suma-ku, 654 Kobe,[3] Japan

Received 12 December 1995/Accepted 12 April 1996

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 *Morbillivirus* (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 stomatis 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 reexpression 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

* Corresponding author. Mailing address: Department of Genetics and Microbiology, University of Geneva School of Medicine, CMU, 9 Ave de Champel, CH-1211 Geneva, Switzerland. Fax: (41 22) 702 5702. Electronic mail address: kolakofs@cmu.unige.ch.

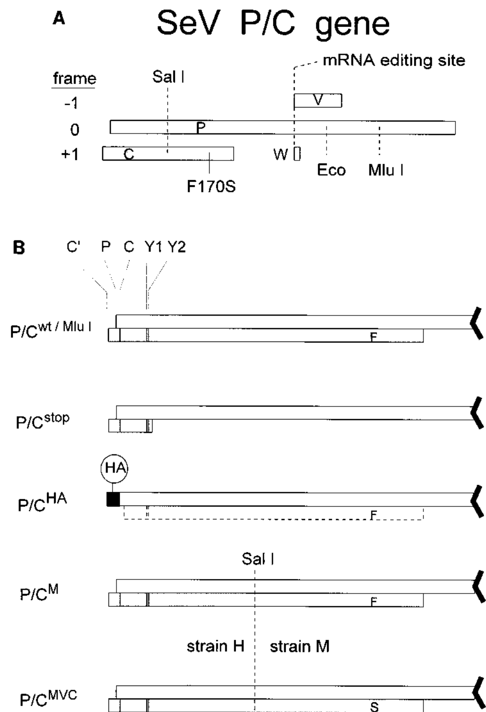

FIG. 1. Schematic representation of the SeV P/C gene. (A) The three ORFs which are expressed as protein are shown as rectangles, drawn roughly to scale. The +1 C ORF is shaded. The positions of relevant restriction sites, the cotranscriptional editing site, and the loss-of-function mutation in C are 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^{HA} is shown as an unshaded dotted box, as it is not expressed as protein because the upstream AUG for P^{HA}. 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^{M} and P/C^{MVC} constructs are indicated. The presence of a Phe (F) or Ser (S) at position 170 of C is also indicated.

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 pSV-DIH4/Rbz (H4) and pSP65-E_{307} (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 antigenomic promoter sequences derived from DI-H4 were done by fusion PCR (21). First-round PCR products were obtained by using amplimers composed of 18 3′-end nucleotides complementary to the donor strand and 18 5′-end nucleotides complementary to the recipient strand, along with amplimers common to both DI-H4 and E307 RNA, including a *Sal*I site to the left of the T7 RNA polymerase promoter and a *Stu*I site in the L gene. These first PCR products were then combined and amplified with the amplimers common to both RNAs (*Sal*I and *Stu*I). The recombined final products flanked by *Sal*I and *Stu*I sites were finally subcloned back into pSV-E307 into the original *Sal*I-*Stu*I sites. All modifications were verified by sequencing.

The construction of the P/C^{stop} and P/C^{HA} genes from the wild-type P/C (P/C^{wt}) gene (19) was previously described (10, 13). The P/C^{M} and P/C^{MVC} genes were constructed by replacing the *Sal*I-to-*Eco*RI fragment (nucleotides [nt] 334 to 1130) of P/C^{wt} with those of the M and MVC genes (22a) as shown in Fig. 1.

Primer extension analysis. E307 genome and antigenome levels were estimated by extending primers L15,270 (5′ CTTCGAGGCGCCATGG), whose 5′ end is 114 nt from the end of the genome, and NP126 (5′ CGGCCATCGT GAACTTTGGC), whose 5′ end is 126 nt from the end of the SeV polymerase-generated antigenome and 129 nt from the end of the T7 polymerase-generated antigenome, on CsCl gradient-banded nucleocapsid RNA as previously described (17).

In vitro replication. In vitro replication assays were conducted essentially as described previously (22). In short, 5-cm-diameter dishes of A549 cells were preinfected with a vaccinia virus expressing T7 RNA polymerase (vTF7-3) and transfected with the viral protein expression vectors pGEM-L (0.5 μg) and pGEM-N (2.5 μg) and various pGEM-P genes (2.5 μg). Cytoplasmic extracts were prepared in a modified reaction mixture containing 0.1 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 8.5), 50 mM NH_4Cl, 7 mM KCl, 4.5 mM magnesium acetate, 20 μg of actinomycin D per ml, 1 mM each creatine phosphate, dithiothreitol, spermidine, ATP, UTP, and CTP, 0.04 U of creatine phosphokinase per ml, and 0.14 U of RNasin per ml. One-tenth of the cytoplasmic extracts was used for Western blot (immunoblot) analysis with monoclonal anti-N, -L, and -P antibodies and polyclonal rabbit anti-C antiserum. The remaining cytoplasmic extract was incubated with [α-^{32}P]GTP (250 mCi/ml; Amersham) and approximately 1 μg of purified DI-H4 template for 3 h at 30°C. The DI-H4 template was purified from egg allantoic fluid by pelleting the virus and banding the nucleocapsids twice on CsCl gradients. The replication products were purified on 20 to 40% CsCl step gradients (40,000 rpm for 16 h at 12°C in a Beckman SW60 rotor). The nucleocapsid band at the 20 to 40% interface was collected and pelleted at 50,000 rpm for 1 h, resuspended, phenol extracted, and analyzed on a 1.5% agarose-formaldehyde gel. The products were quantitated with a PhosphorImager.

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^{stop}; 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^{HA} [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′ [215 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

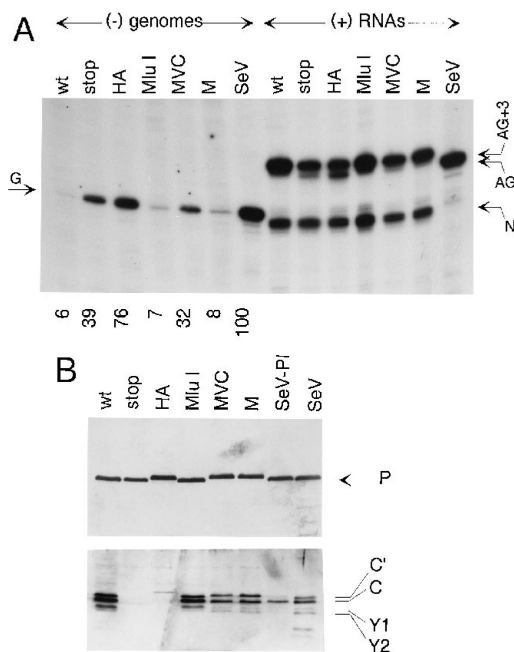

FIG. 2. Effect of C protein coexpression on DI-E307 amplification. vTF7-infected HeLa cells were transfected with pE307 (which is transcribed by T7 RNA polymerase to generate a plus-sense [(+)] antigenome with three extra G residues at the 5′ end (AG+3), pGEM-N, pGEM-L, and the various pGEM-P/C genes 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 (G) and plus-sense antigenomes (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/C^{wt} and P/C^{Mlu} genes [P/C^{Mlu} [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) acute infection, and Y1, rather than Y2, was now the predominant Y protein. None of the C proteins could be detected in extracts of the P/C^{stop}- and P/C^{HA}-transfected cells, indicating that there was little or no suppression of the UAG codon which follows the Y2 AUG and that conversion of the 5′-proximal ACG start codon to AUG had effectively prevented most of the ribosomes from reaching the downstream AUGs.

Analysis of the E307 genome levels in the transfected cells (by detection of their 5′ ends) is straightforward, as these minus-sense RNAs are generated only by the SeV polymerase. These were near the limit of detection when P/C constructs normally expressing the C proteins (P/C^{wt} or P/C^{Mlu}) were used. Genome levels, however, clearly increased when C expression was restricted (Fig. 2A; 6-fold for P/C^{stop} and 12-fold for P/C^{HA}). In this latter case, the DI genome levels approached those of nondefective genomes in a natural SeV infection carried out in parallel (Fig. 2, lanes SeV). Analysis of the levels of plus-sense RNAs found assembled with N protein is more complex, because the plus-sense RNAs are generated by both the SeV and T7 RNA polymerases. The T7-generated antigenome can be distinguished from that made by the SeV 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 invariantly 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^{M}) 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^{MVC}) 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^{M}). SeV^{MVC} is highly adapted to LLC-MK2 cells; it produces 20-fold more virus than SeV^{M} in cell culture, and this phenotype was associated with significantly higher levels of viral mRNAs and proteins intracellularly (23). Sequencing of the SeV^{M} and SeV^{MVC} 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^{wt} (strain H) construct [the *Sal*I-*Eco*RI 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^{wt}, P/C^{Mlu}, and P/C^{M} 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^{MVC} gene accumulates to normal levels, but it does not appear to inhibit E307 genome amplification. We also introduced the F170S mutation into P/C^{wt} and found that C expression from this construct had lost

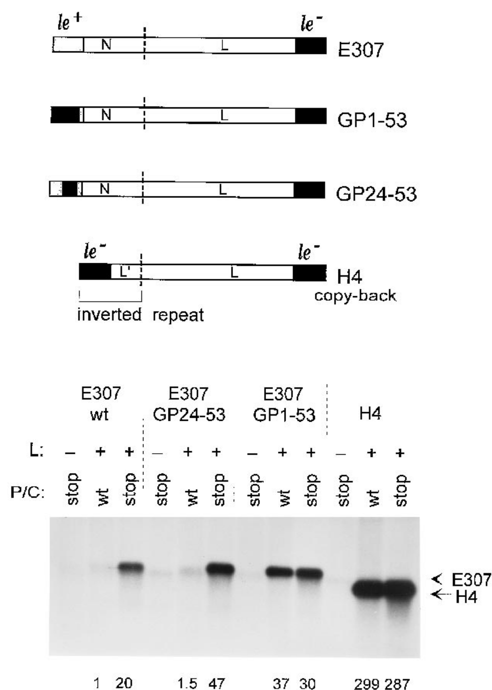

FIG. 3. Effect of C protein coexpression on the amplification of various DI genomes. The various DI genomes examined are schematized at the top. Their orientation is that of double-stranded DNA with respect to gene expression; i.e., the 3′ end of the minus-sense genome and the 5′ end of the plus-sense antigenome are on the left. The 55-nt-long leader regions (le^+ and le^-) are shown as grey and black boxes, respectively. The le^+ region refers to the sequences that are present at both the 3′ end of the genome and the 5′ end of the antigenome. The position where the replicase crossed over during the generation of the internally deleted E307 (1,794-nt) and the copy-back H4 (1,410-nt) genomes are indicated with dashed lines. GP1-53 and GP24-53 are derivatives of E307 in which the indicated positions of the le^+ region were replaced with those le^- region (Materials and Methods). Transfections of the plasmids carrying the various DI genomes (all generating plus-sense antigenomes without extra 5′ G residues) were carried out (as for Fig. 2) with either the P/C^{wt} or P/C^{stop} gene (as indicated above the bottom panel); the amounts of minus-sense genomes present in nucleocapsids from an equal sample of the cells (one-fourth of a dish) were estimated by Northern (RNA) blotting (4) and are listed below the bottom panel. Plasmid pGEM-L was withheld from some of the transfections (indicated above) as a negative control.

most of its ability to inhibit mRNA synthesis from nondefective templates in vitro and was indistinguishable from the hybrid P/C^{MVC} 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^{MVC} 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^+) region. The 3′ end of the minus-sense genome is nevertheless expressed as a short le^+ 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^+ RNA then continues on, to sequentially synthesize the linear array of mRNAs. An analogous minus-sense leader (le^-) RNA is expressed from the 3′ end of the antigenome (the le^- 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^+ and le^- 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^- 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^{stop}) in a slightly different system ($DI\text{-}H4^{uv}$ [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^{wt}) and absence (P/C^{stop}) 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^- region, which are now present at the 3′ ends of both genomes and antigenomes. The progressive exchange of $le^{+/-}$ 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^- sequences were found to be stronger than or equal to the le^+ sequences by this test (4). Two of these E307 constructs were examined for their sensitivity to C coexpression. $E307^{GP1\text{-}53}$, which contains le^- positions 1 to 53 (le^-[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 ($E307^{GP1\text{-}53}$ [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^+ sequences ($E307^{GP24\text{-}53}$ [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^-[24-53] at the 3′ genome end was replaced with le^+[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^- sequences at the 3′ genome end of the E307 derivatives is largely lost, as

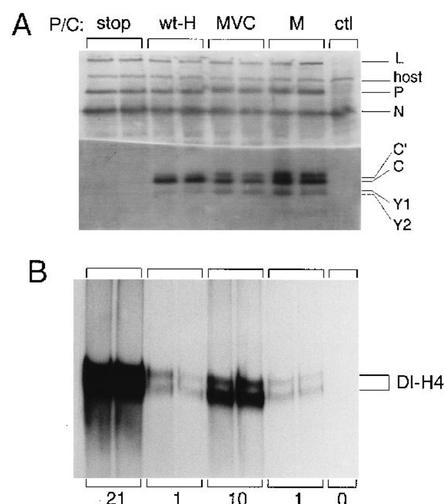

FIG. 4. Effect of C protein coexpression on the amplification of DI-H4 genomes in vitro. (A) Various pGEM-P/C genes (as indicated at the top) were coexpressed along with pGEM-N and pGEM-L in vTF7-infected A549 cells. A cytoplasmic extract of these transfected cells was combined with CsCl-banded DI-H4 core nucleocapsids, and RNA synthesis was carried out in the presence of [α-^{32}P]GTP (Materials and Methods). The reaction products were reisolated as nucleocapsids on CsCl density gradients, and their RNAs were separated on HCHO-agarose gels (B). DI-H4 resolves into two bands when these gels are electrophoresed quickly, but their nature has not been determined. The (average) relative intensities of the DI bands are listed at the bottom. The levels of the L, P, N, and C proteins in the various cell extracts were determined by immunoblotting with a mixture of specific antibodies; the top and bottom halves of the gel were reacted separately (A). The double lanes show duplicate transfections and in vitro reactions. A single transfection (lane ctl), in which the pGEM-L plasmid was withheld, is included as a negative control.

E307, E307$^{GP24-53}$, and E307^{GP1-53} 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 have 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/C^{H} 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^{MVC} proteins had accumulated to levels comparable to those of the C^{H} proteins in the extracts. In contrast, the C^{M} proteins were found to be highly inhibitory, similar to C^{H} (Fig. 4B). Thus, of 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^{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^{stop}, P/C^{M}, and P/C^{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 μg of genome RNA) are visible to the naked eye with indirect light (they scatter blue light) at their characteristic buoyant density. When the P_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^{stop} and P/C^{MVC} genes had been used. However, the nucleocapsid band was absent when the P/C^{M} gene had been used in the initial recovery. To determine whether a very small amount of virus had been generated, the P/C^{M} P_2 stock was passaged again, undiluted, and those of the P/C^{stop} and P/C^{MVC} series were passaged after a 1/1,000 dilution. When these P_3 stocks were examined, however, the results were unchanged; i.e., only the P/C^{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_3 stocks of the P/C^{stop} and P/C^{MVC} series were recombinant genomes, containing the marker *Bgl*II site in the le^{+} region (lanes 5 and 11) and had lost the marker *Nsi*I site in the 5′ 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^{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^{MVC} protein also appears to be that which is responsible for preventing virus recovery from cDNA.

When E307^{GP1-42} 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′ genome end le^{+}[1-42] with le^{-}[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^{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. E307^{GP1-42} was therefore included as a supplementary plasmid in half of the virus recoveries containing either P/C^{wt} or P/C^{stop}, 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^{H} coexpression no longer prevented virus recovery when E307^{GP1-42} 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^{stop}, in contrast, produced stocks in which both rSeV and rSeV-GP42 were present (lane 2). The presence of le^{-}[1-42] at the 3′ 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.

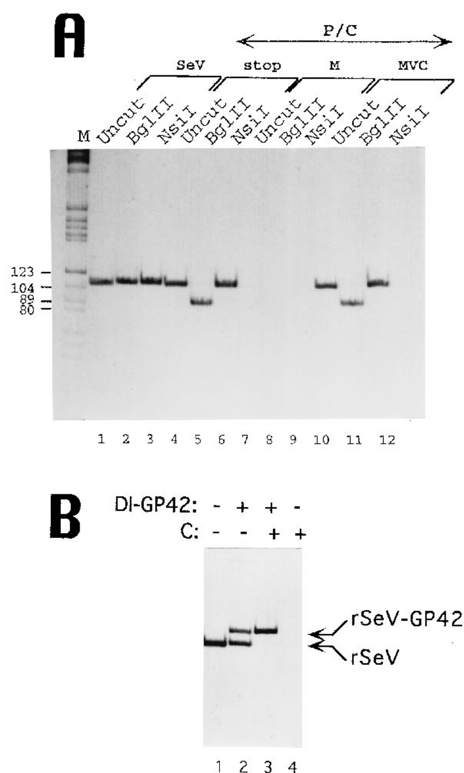

FIG. 5. SeV recoveries from cDNA with various P/C genes. (A) SeV recoveries were carried out with the three P/C genes listed at the top as previously described (18), and the progeny were amplified in chicken eggs. The P_3 stocks (see text), as well as natural SeV, were used to infect BHK cells, and an equal fraction of the resulting nucleocapsid RNAs (from one-eighth of a dish) were amplified by RT-PCR (18). Each reaction mixture was divided into three parts, which were either digested with *Bgl*II or *Nsi*I or not digested as indicated above the lanes. The DNAs were then separated by electrophoresis on an 8% polyacrylamide nondenaturing gel and detected by ethidium bromide staining. Lane M shows DNA length markers; positions are indicated in nucleotides. (B) SeV recoveries were also carried out in the presence (P/C^{wt}) or absence (P/C^{stop}) of C coexpression as indicated, with and without the addition of DI-$E307^{GP42}$ as a supplemental plasmid, also as indicated. The P_2 stocks were used to infect HeLa cells, and equal fractions of the resulting nucleocapsid RNAs (from one-eighth of a dish) were amplified by RT-PCR with primers specific for both the le^+ (rSeV) and le^- (rSeV-GP42) regions.

DISCUSSION

The C^{MVC} protein has lost its ability to inhibit DI-E307 genome amplication 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^M), which was then adapted to grow on LLC-MK2 cells in culture (SeV^{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) δ antigen. This HDV protein is also required late in the infectious cycle (for packaging the minus-sense strand HDV RNA genome), but δ 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 δ 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

one 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 been 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 so poorly in cell culture, codes for an accessory protein to help gene expression, whereas SeV, which replicates relatively well, codes for an accessory protein to restrain this expression.

The possible advantages to the *Paramyxovirinae* in coding for proteins which attenuate the attendant cytopathic effects of the infection are obvious (16, 33), as for other viruses which code for proteins which prevent apoptosis (5, 6, 20, 32). Given the very different levels of C expression among the different *Paramyxovirinae*, they may have different requirements for the down-regulation of viral genome amplification and expression at the later stages of infection. A measles virus unable to express its C protein has recently been found to be viable and essentially without phenotype in cell culture (31a), as has the analogous VSV (24a). The inhibitory role of the SeV C proteins does not, of course, preclude other functions. It may not be coincidental that the only C protein mutation described so far (C^{MVC}), although a loss-of-function mutation, is present in a gene which continues to express normal levels of C protein. The adaptation of SeV^{M} to cell culture, moreover, gave rise to progeny (SeV^{MVC}) which produce 20-fold-higher titers in these cells than the parent SeV^{M}, as expected. Unexpectedly, whereas SeV^{M} is particularly virulent for mice, SeV^{MVC} was found to be virtually apathogenic, growing to a titer 5 log units lower than that of SeV^{M} in mouse lungs (22a). A protease activation variant of SeV^{MVC} has, in fact, been proposed as a more relevant live vaccine for laboratory animals (38). The C proteins may thus be required for other viral functions, such as the ability to grow productively in animals, in analogy with the Nef protein of simian immunodeficiency virus (24), another accessory (retrovirus) protein (reviewed in reference 36). We are presently attempting to construct an rSeV containing the P/C^{stop} gene to determine whether such a virus is viable. We are also attempting to construct an rSeV containing the F170S mutation to determine whether this mutation alone is responsible for the unusual phenotype of SeV^{MVC}.

ACKNOWLEDGMENTS

We thank Jean-Baptiste Marq and Genevieve Mottet for excellent technical assistance. We are also grateful to Philippe Calain, who first pointed out the promoter component of the inhibitory effect of C.

This work was supported by grants from the Swiss National Science Fund and The Human Frontiers Science Program.

REFERENCES

1. **Alkhatib, G., B. Massie, and D. J. Briedis.** 1988. Expression of bicistronic measles virus P/C mRNA by using hybrid adenoviruses: levels of C protein synthesized in vivo are unaffected by the presence or absence of the upstream P initiator codon. J. Virol. **62:**4059–4069.

1a. **Banerjee, A. K., and S. Barik.** 1992. Gene expression of vesicular stomatitis virus genome RNA. Virology **188:**417–428.

2. **Bellini, W. J., G. Englund, S. Rozenblatt, H. Arnheiter, and C. D. Richardson.** 1985. Measles virus P gene codes for two proteins. J. Virol. **53:**908–919.

3. **Calain, P., and L. Roux.** 1993. The rule of six, a basic feature for efficient replication of Sendai virus DI RNA. J. Virol. **67:**4822–4830.

4. **Calain, P., and L. Roux.** 1995. Functional characterisation of the genomic and antigenomic promoters of Sendai virus. Virology **212:**163–173.

4a. **Chandrika, R., S. M. Horikami, S. Smallwood, and S. A. Moyer.** 1995. Mutations in conserved domain I of the Sendai virus L polymerase protein uncouple transcription and replication. Virology **213:**352–363.

5. **Chou, J., and B. Roizman.** 1992. The gamma 1(34.5) gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programed cell death in neuronal cells. Proc. Natl. Acad. Sci. USA **89:**3266–3270.

6. **Clem, R. J., M. Fechheimer, and L. K. Miller.** 1992. Prevention of apoptosis by a baculovirus gene during infection of insect cells. Science **254:**1388–1390.

7. **Collins, P. L., M. G. Hill, E. Camargo, H. Grosfeld, R. M. Chanock, and B. R. Murphy.** 1995. Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the M2(ORF1) transcription elongation factor in gene expression and provides a new capability for vaccine development. Proc. Natl. Acad. Sci. USA **92:**11563–11567.

8. **Collins, P. L., M. G. Hill, J. Cristina, and H. Grosfeld.** 1996. Transcription elongation factor of respiratory syncytial virus, a nonsegmented negative strand RNA virus. Proc. Natl. Acad. Sci. USA **93:**81–85.

9. **Collins, P. L., K. McIntosh, and R. M. Chanock.** 1996. Pneumonvirinae; the viruses and their replication, p. 1313–1352. *In* B. N. Fields et al. (ed.), Virology, 3rd ed. Raven Press, New York.

10. **Curran, J., R. Boeck, and D. Kolakofsky.** 1991. The Sendai virus P gene expresses both an essential protein and an inhibitor of RNA synthesis by shuffling modules via mRNA editing. EMBO J. **10:**3079–3085.

11. **Curran, J., and D. Kolakofsky.** 1990. Sendai virus P gene produces multiple proteins from overlapping open reading frame. Enzyme **44:**244–249.

12. **Curran, J., J.-B. Marq, and D. Kolakofsky.** 1992. The Sendai virus nonstructural C proteins specifically inhibit viral mRNA synthesis. Virology **189:**647–656.

13. **Curran, J., T. Pelet, and D. Kolakofsky.** 1994. An acidic activation-like domain of the Sendai virus P protein is required for RNA synthesis and encapsidation. Virology **202:**875–884.

14. **Engelhorn, M., R. Stricker, and L. Roux.** 1993. Molecular cloning and characterization of a Sendai virus internal deletion defective RNA. J. Gen. Virol. **74:**137–141.

15. **Galinski, M. S., R. M. Troy, and A. K. Banerjee.** 1992. RNA editing in the phosphoprotein gene of the human parainfluenza virus type 3. Virology **186:**543–550.

16. **Garcin, D., M. de Melo, L. Roux, D. Kolakofsky, and J. Curran.** 1994. Presence of a truncated form of the Sendai virus P protein in a long-term persistent infection; implications for the maintenance of the persistent state. Virology **201:**19–25.

17. **Garcin, D., and D. Kolakofsky.** 1990. A novel mechanism for the initiation of Tacaribe arenavirus genome replication. J. Virol. **64:**6196–6203.

18. **Garcin, D., T. Pelet, P. Calain, L. Roux, J. Curran, and D. Kolakofsky.** 1995. A highly recombinogenic system for the recovery of infectious Sendai paramyxovirus from cDNA: generation of a novel copy-back nondefective interfering virus. EMBO J. **14:**6087–6094.

19. **Giorgi, C., B. M. Blumberg, and D. Kolakofsky.** 1983. Sendai virus contains overlapping genes expressed from a single mRNA. Cell **35:**829–836.

20. **Gregory, C. D., C. Dive, S. Henderson, C. A. Smith, G. T. Williams, J. Gordon, and A. B. Rickinson.** 1991. Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. Nature (London) **349:**612–614.

21. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.

22. **Horikami, S. M., J. Curran, D. Kolakofsky, and S. A. Moyer.** 1992. Complexes of Sendai virus NP-P and P-L proteins are required for defective interfering particle genome replication in vitro. J. Virol. **66:**4901–4908.

22a. **Itoh, M.** Unpublished data.

23. **Itoh, M., and M. Homma.** 1993. A mechanism of adaptation of Sendai virus to LLC-MK2 cells; single point mutation of C protein elevated the level of viral mRNA, p. 74. *In* Abstracts of the IXth International Congress of Virology.

24. **Kestler, H. W., D. J. Ringler, K. Mori, D. A. Panacali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1991. Importance of the nef gene for maintaining high virus loads and for development of AIDS. Cell **65:**651–662.

24a. **Kretzschmar, E., R. Peluso, M. J. Schnell, M. A. Whitt, and J. K. Rose.** 1996. Normal replication of vesicular stomatitis virus without C proteins. Virology **216:**309–316.

25. **Lamb, R. A., and D. Kolakofsky.** 1996. *Paramyxoviridae*: the viruses and their replication, p. 1177–1204. *In* B. N. Fields et al. (ed.), Virology, 3rd ed. Raven Press, New York.

26. **Lamb, R. A., and R. G. Paterson.** 1991. The nonstructural proteins of paramyxoviruses, p. 181–210. *In* D. W. Kingsbury (ed.), The paramyxoviruses. Plenum Press, New York.

27. **Lazinski, D. W., and J. M. Taylor.** 1995. Regulation of HDV ribozymes. RNA **1:**225–233.

28. **Leppert, M., L. Kort, and D. Kolakofsky.** 1977. Further characterization of Sendai virus DI-RNAs: a model for their generation. Cell **12:**539–552.

29. **Matsuoka, Y., J. Curran, T. Pelet, D. Kolakofsky, R. Ray, and R. W. Compans.** 1991. The P gene of human parainfluenza type 1 virus encodes P and C proteins, but not a cysteine-rich V protein. J. Virol. **65:**3406–3410.

29a. **Murphy, F. A., C. M. Fauquet, and D. H. L. Bishop.** 1994. Sixth report of the International Committee on Taxonomy of Viruses. Arch. Virol. Suppl. **10:** 268–274.

30. **Pelet, T., J. Curran, and D. Kolakofsky.** 1991. The P gene of bovine parainfluenza virus 3 expresses all three reading frames from a single mRNA editing site. EMBO J. **10:**443–448.

31. **Portner, A., K. C. Gupta, J. M. Seyer, E. H. Beachey, and D. W. Kingsbury.** 1986–1987. Localization and characterization of Sendai virus nonstructural C and C′ proteins by antibodies against synthetic peptides. Virus Res. **6:**109–121.

31a. **Radecke, F., and M. A. Billeter.** 1996. The nonstructural C protein is not essential for multiplication of Edmonston B strain measles virus in cultured cells. Virology **217:**418–421.

32. **Rao, L., M. Debbas, P. Sabbatini, D. Hockenbery, S. Korsmeyer, and E. White.** 1992. The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. Proc. Natl. Acad. Sci. USA **89:**7742–7746.

33. **Re, G. G.** 1990. Deletion mutants of paramyxoviruses, p. 275–295. *In* D. W. Kingsbury (ed.), The paramyxoviruses. Plenum Press, New York.

34. **Rochat, S., H. Komada, and D. Kolakofsky.** 1992. Loss of V protein expression in human parainfluenza virus type 1 is not a recent event. Virus Res. **24:**137–144.

35. **Spiropoulou, C. F., and S. T. Nichol.** 1993. A small highly basic protein is encoded in overlapping frame within the P gene of vesicular stomatitis virus. J. Virol. **67:**3103–3110.

35a. **Tapparel, C., and L. Roux.** 1996. The Sendai virus replication promoter: importance of potential RNA end complementarity or RNA primary sequence. Submitted for publication.

36. **Trono, D.** 1995. HIV accessory proteins: leading roles for the supporting cast. Cell **82:**189–192.

37. **Vidal, S., J. Curran, and D. Kolakofsky.** 1990. Editing of the Sendai virus P/C mRNA by G insertion occurs during mRNA synthesis via a virus-encoded activity. J. Virol. **64:**239–246.

38. **Wang, X.-L., M. Itoh, H. Hotta, and M. Homma.** 1994. A protease activation mutant, MVCES1, as a safe and potent live vaccine derived from currently prevailing Sendai virus. Virology **68:**3369–3373.

39. **Yamada, H., S. Hayata, T. Omata-Yamada, H. Taira, K. Mizumoto, and K. Iwasaki.** 1990. Association of the Sendai virus C protein with nucleocapsids. Arch. Virol. **113:**245–253.