JOURNAL OF VIROLOGY, May 1993, p. 2772–2778
0022-538X/93/052772-07$02.00/0

Vol. 67, No. 5

Rescue of Synthetic Analogs of Genomic RNA and Replicative-Intermediate RNA of Human Parainfluenza Virus Type 3

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Received 13 November 1992/Accepted 3 February 1993

The genome of human parainfluenza virus type 3 (PIV3) is a single negative-sense RNA strand (vRNA) that is 15,463 nucleotides in length. A cDNA was constructed to encode an 898-nucleotide, internally deleted version of PIV3 vRNA, PIV3-CAT vRNA, in which the viral genes were replaced with the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. The CAT gene was flanked in turn by sequences representing (i) nontranslated sequences of the first and last genes in the PIV3 genome, (ii) PIV3 gene-start and gene-end sequences, which are presumed to be transcription signals, and (iii) 3′ extracistronic (leader) and 5′ extracistronic (trailer) terminal regions of PIV3 vRNA. A second cDNA was constructed to encode the exact complement of PIV3-CAT vRNA; this positive-sense RNA, PIV3-CAT vcRNA, would correspond to the predicted replicative intermediate of PIV3-CAT vRNA. When synthesized in vitro by runoff transcription with T7 RNA polymerase and transfected separately into PIV3-infected cells, both PIV3-CAT vRNA and vcRNA were rescued with similar efficiencies; that is, they were expressed to yield CAT and were packaged into particles that could be used to infect fresh cells. Rescue of PIV3-CAT vRNA was strictly dependent on complementation by PIV3; PIV3 could not be replaced by respiratory syncytial virus or, unexpectedly, by a bovine strain of PIV3. Passage was blocked by prior incubation with neutralizing monoclonal antibodies specific to the PIV3 attachment protein. Also, during nine serial passages, the expression of CAT by PIV3-CAT vRNA increased more than 3,000-fold. These results indicated that the 3′-terminal 111 nucleotides and the 5′-terminal 115 nucleotides of PIV3 vRNA, which are present in PIV3-CAT vRNA, contained all of the *cis*-acting RNA sequences required for replication, gene expression, and transmission.

The ability to introduce desired mutations into the genomes of viruses is critical for defined genetic studies and holds great promise for the production and characterization of engineered vaccine strains. For some viruses, such as vaccinia virus, adenovirus, or coronaviruses, this can be readily achieved by taking advantage of homologous recombination to replace genome segments with surrogate copies that have been cloned in bacteria and manipulated in vitro. For many of the positive-strand RNA viruses, the complete viral genome can be synthesized from engineered cDNA and transfected into cells to initiate productive infection.

However, until recently, methods for direct manipulation of the genomic RNA (vRNA) of negative-strand viruses were not available. This is because (i) homologous recombination is not detectable for these viruses and (ii) naked vRNA is not infectious on its own. Instead, infection by negative-strand RNA viruses is initiated by a ribonucleoprotein complex, the nucleocapsid, which consists of vRNA encapsidated by nucleocapsid protein (NP) and associated with the viral polymerase. A transcriptionally active nucleocapsid is thought to be the minimum unit of infectivity.

Recently, a new genetic approach was developed for a segmented negative-strand virus, influenza A virus (18). When an RNA gene segment, synthesized in vitro from cDNA, was mixed with influenza virus nucleocapsid proteins and transfected into influenza virus-infected cells, the synthetic RNA was replicated, expressed, and incorporated into infectious virus. This was accomplished both with synthetic genome segments representing heterologous strains of influenza virus (22) and with a chimeric segment containing the bacterial chloramphenicol acetyltransferase (CAT) reporter gene (18) flanked by influenza virus terminal sequences. Improved and modified versions of this methodology have been used to analyze influenza virus signals for transcription, replication, and encapsidation of vRNA (9, 10, 17, 27, 28), to study packaging of genome segments (11), and to produce novel viruses with altered biological properties (3, 16). These studies were likely facilitated by the ready ability of influenza virus genome segments to undergo reassortment. Production of a complete cDNA-encoded influenza virus has not yet been described.

The application of this approach to nonsegmented negative-strand viruses poses additional difficulties because (i) the nonsegmented nature of the genome precludes reassortment and, ultimately, requires manipulation of the genome in toto and (ii) the association of NP with vRNA in the nucleocapsid appears to be more intimate and extensive than is the case for influenza virus (8, 15). In vitro encapsidation has been described for RNA isolated from defective interfering (DI) particles of vesicular stomatitis virus (VSV) (19), but this approach has not been widely used and might not apply to complete vRNA. In a different approach to the same problem, an efficient system has been described in which VSV DI RNA was transcribed from cDNA intracellularly and encapsidated by VSV proteins expressed in *trans* from plasmid vectors (24). In the presence of a complete set of VSV proteins, the synthetic DI RNA was replicated and packaged into authentic DI particles. However, systems

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based on DI RNAs have the disadvantages that (i) DI RNA is nontranscribing and (ii) replication of DI RNA differs from that of standard vRNA in ways that are incompletely understood.

The success with synthetic influenza virus RNA containing the CAT gene has stimulated similar approaches with the nonsegmented paramyxoviruses Sendai virus (SeV) (23) and respiratory syncytial virus (RSV) (5). cDNAs were constructed to encode vRNA in which all of the viral genes were replaced by a negative-sense copy of the CAT gene. When transfected into cells infected with the corresponding standard virus, these short vRNA analogs were replicated, transcribed, and packaged into particles that could be passaged to fresh cells. Importantly, these experiments identified short terminal RNA segments that contain the *cis*-acting signals involved in replication and transcription. Also, it was shown that these signals could be attached to foreign sequences without loss of function. This methodology, as it currently stands, has the drawback that it involves only a small population of vRNA analogs against a large background of standard vRNA molecules but has the advantage that the synthetic analog appears to participate in all aspects of the viral replicative cycle.

These model systems offer new experimental strategies for direct characterization of *cis*-acting RNA sequences and *trans*-acting proteins involved in transcription, replication, and packaging of negative-strand virus RNAs. Also, vRNA analogs bearing convenient marker genes may be critical for the development of methods for producing helper-independent viruses from complete cDNA copies of RNA. In view of this potential, we have developed a similar experimental system for the paramyxovirus human parainfluenza virus 3 (PIV3), an important agent of pediatric respiratory tract disease.

MATERIALS AND METHODS

Viruses and cell culture. PIV3 (Wash57/47885; ATCC strain C-243), bovine PIV3 (BPIV3; strain SF/4), and RSV (strain A2) were plaque purified three times and propagated in monolayer cultures of Hep-2 cells. 293 cells grown in six-well culture dishes were used for transfections.

Construction of PIV3-CAT(−) cDNA. PIV3-CAT(−) cDNA (Fig. 1A and Fig. 2A and B) was constructed from RSV-CAT cDNA (5) by using three pairs of synthetic oligonucleotides as primers in three consecutive polymerase chain reactions (PCR). The primers were as follows: for the first PCR, 5′-AAAAGGGAACTCTATAATTTCAAAAATC TAGAATGGAGAAAAAAATCA-3′ (positive sense) and 5′-AGGTTAGGATATATCTTTATTGTATTTGTATTCTG CAGTTACGCC-3′ (negative sense); for the second PCR, 5′-GAAATATAAATTTAAATTAAAATTAACTTAGGAT TAAAGACATTGACTAGAAGGTCAAGAAAAGGGAAC TCTATA-3′ (positive sense) and 5′-GTTTGGTATATATAT ATTACATGTTTTTCTTACTTTTTGTCTATCTTTAGG CTTAATGATAAAGGTTAGGATATATC-3′ (negative sense); and for the third PCR, 5′-TTCGAGCTCGGTAC CGACGCAGTTAACCAAACAAGAGAAGAAACTTGTT TGGAAATATAAATTTAAATTAAA-3′ (positive sense) and 5′-TGATTACGCCAAGCTTAATACGACTCACTATA ACCAAACAAGAGAAGAACTCTGTTTGGTATATAT ATA-3′ (negative sense) (7, 12, 14). The final PCR product was cloned into the *Kpn*I and *Hin*dIII window of pUC19. Sequences were confirmed by dideoxynucleotide sequencing. A second plasmid was constructed by blunt-end ligation of the filled-in *Asp* 718-*Hin*dIII fragment of PIV3-CAT cDNA into the filled-in *Bss*HII site of pBluescript II KS+ (Stratagene). Since these two plasmids are identical with respect to the encoded vRNA analog, they were used interchangeably and are referred to as pPIV3-CAT(−).

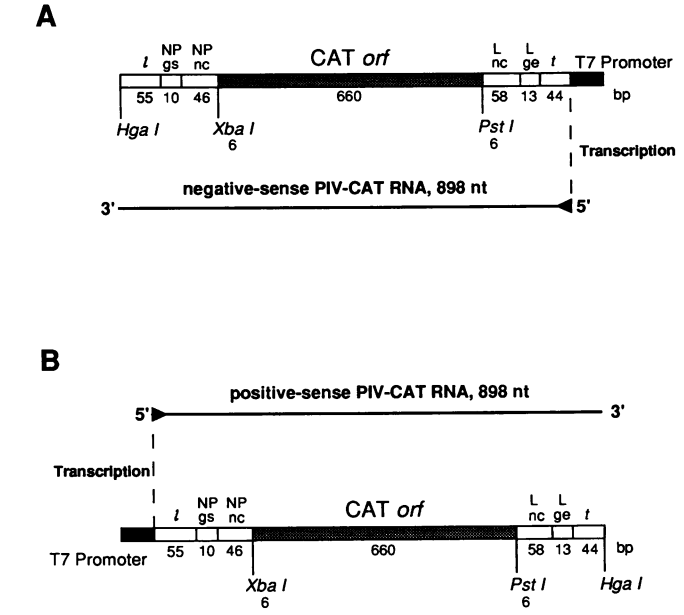

FIG. 1. PIV3-CAT(−) and PIV3-CAT(+) cDNAs and their encoded PIV3-CAT RNA analogs. Diagrams of PIV3-CAT(−) (A) and PIV3-CAT(+) (B) cDNAs and their predicted in vitro RNA transcripts are presented. Black boxes represent the bacteriophage T7 RNA polymerase promoter, white boxes represent PIV3 sequences, and shaded boxes represent the CAT ORF. The CAT ORF is shown in the 5′ to 3′ orientation from left to right. The positions of PIV3 leader (*l*) and trailer (*t*) sequences as well as the gene-start (gs), gene-end (ge), and non-protein-coding (nc) sequences of the PIV3 NP and polymerase (L) genes are also depicted. The length of each of the different DNA sequences is shown below the corresponding box. Plasmid DNA was digested with *Hga*I and transcribed in vitro with T7 RNA polymerase to yield either a negative-sense analog of the PIV3 genome (A) or a positive-sense analog of the PIV3 replicative intermediate (B), both of which are predicted to be 898 nt in length. The drawings are not to scale.

Construction of PIV3-CAT(+) cDNA. pPIV3-CAT(+) cDNA was constructed by using 5′-GAGCTCGGTACCTAA TACGACTCACTATAACCAAACAAGAGAAGAAACT TGTTTGGAAATATAAATT-3′ and 5′-TACGCCAAGCTT GACGCATATTACCAAACAAGAGAAGAACTCTGTT TGGTATATATATATTAC-3′ as primers for PCR mutagenesis and amplification of the PIV3-CAT sequences in PIV3-CAT(−) cDNA. The PCR product was directly cloned into a modified form of pBluescript II KS+ that lacked the T7 promoter, pKS-SHORT (a gift of Lili Kuo), to yield pPIV3-CAT(+) (Fig. 1B and Fig. 2C and D).

In vitro transcription and transfection. *Hga*I-digested plasmid DNA (2 μg/100-μl reaction) was transcribed in vitro with T7 RNA polymerase according to protocols provided by the supplier (Promega). 293 cells were infected with 5 to 10 PFU of PIV3 per cell in a volume of 1 ml per well and incubated for 1 h at 37°C. The cells were washed twice with Opti-MEM I (GIBCO/BRL) and incubated for 3 h at 37°C with a mixture containing 1 ml of Opti-MEM I (GIBCO/BRL), 12 μl of TransfectACE (GIBCO/BRL), and approximately 5 to 10 μg of synthetic RNA (10 to 20 μl of a transcription reaction).

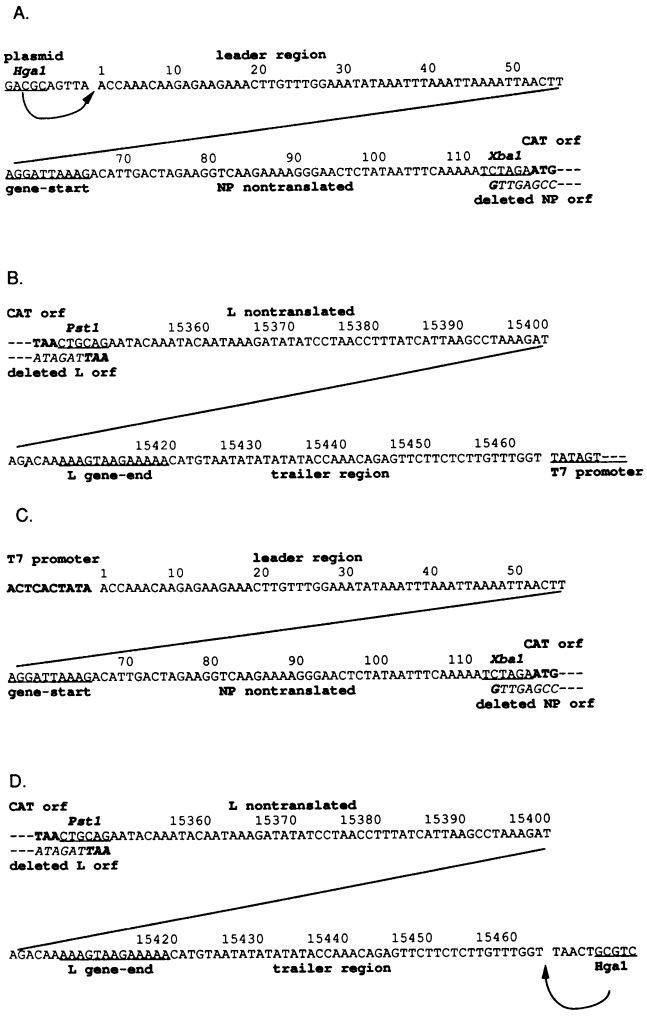

FIG. 2. PIV3-specific and flanking sequences in the PIV3-CAT cDNAs. All sequences are of the positive-sense strand, 5′ to 3′, and represent the following regions: the leader-containing (A) and trailer-containing (B) ends of PIV3-CAT(−) cDNA and the leader-containing (C) and trailer-containing (D) ends of PIV3-CAT(+) cDNA. In panels A and C, the partial sequence labeled "deleted NP orf" marks the point where the PIV3-CAT sequences begin to diverge from the parental vRNA sequence; the translational initiation codon of the CAT ORF and the third nucleotide of the initiation codon of the NP ORF are in bold. In panels B and D, the partial sequence labeled "deleted L orf" also indicates the point of divergence between PIV3-CAT sequences and that of parental vRNA; the translational termination sites of the CAT ORF and the L ORF are in bold type. Restriction sites and the gene-start, gene-end, and T7 promoter sequences are underlined. The site of *Hga*I cleavage is indicated with an arrow. PIV3-specific sequence is numbered according to its position in the complete 15,463-nt vRNA; where shown as a single strand, the PIV3 sequences were identical in the parental and analog RNAs.

The transfection mixture was replaced with 1.5 ml of Eagle modified minimal essential medium supplemented with 10% fetal bovine serum and 2 mM glutamine (culture medium). At 20 to 24 h after infection, cells were harvested for CAT assays. In experiments involving subsequent passage of culture fluids, cells were washed twice with 1.5 ml of culture medium following transfection to remove input RNA and transfection reagent.

Serial passage of virus. The medium (1.5 ml) from transfected cell cultures was harvested 24 h after infection and clarified, and 1 ml was transferred to fresh 293 cells. After a 1-h incubation, cells were washed twice with 1.5 ml of culture medium. In some experiments, cells were subsequently superinfected with 5 to 10 PFU of PIV3 per cell. Cells were harvested for CAT assays 24 h after the initial infection. PIV3 in culture supernatants was quantitated by plaque assay in Hep-2 cells.

Virus neutralization. Supernatants of transfected cells were collected 24 h after infection and clarified; 1 ml was incubated for 1 h at room temperature with 50 μl of the indicated dilution of a mixture of three neutralizing monoclonal antibodies specific for either PIV3 or RSV (see Fig. 4). The PIV3 antibodies, 170/7, 403/7, and 128/9, were specific for the PIV3 HN glycoprotein (4) and the RSV antibodies, 1129, 1243, and 1269, were specific for the RSV F glycoprotein (2). Each sample was then transferred undiluted to a well of fresh cells. Following a 1-h adsorption, the cells were washed, superinfected with PIV3, incubated for 22 h, and harvested.

CAT assays. Cells were dislodged from the plate by pipetting and washed, and a 100-μl lysate was prepared from each well by three cycles of freezing and thawing. Aliquots of 50 μl were assayed for CAT activity, using D-*threo*-[dichloroacetyl 1-^{14}C]chloramphenicol (50 to 60 mCi/mmol; Amersham) as the substrate according to standard procedures (1, 13).

RESULTS

Expression of CAT by PIV3-CAT vRNA, an analog of PIV3 vRNA. cDNA was constructed to encode a version of PIV3 vRNA in which all of the viral genes were replaced by the CAT reporter gene; the resulting PIV3-CAT(−) cDNA and sequences are shown in Fig. 1A and 2A and B. PIV3-CAT(−) cDNA was designed to be transcribed by T7 RNA polymerase and contained a T7 promoter immediately adjacent to the PIV3 trailer region and an *Hga*I site next to the PIV3 leader region. Transcription of *Hga*I-digested DNA would yield negative-sense RNA, PIV3-CAT vRNA, with the correct 5′ and 3′ ends of PIV3 vRNA and the following structural features (listed in 3′ to 5′ order): (i) the leader region of PIV3 vRNA (55 nucleotides [nt]); (ii) the gene-start (10 nt) and 5′ nontranslated (46 nt) sequences of the PIV3 NP gene; (iii) an *Xba*I restriction site (6 nt); (iv) the CAT coding sequence (660 nt); (v) a *Pst*I site (6 nt); (vi) the 3′ nontranslated (58 nt) and gene-end (13 nt) sequences of the PIV3 L gene; and (vii) the trailer region of PIV3 vRNA (44 nt). The CAT open reading frame (ORF) would be under the control of PIV3 gene-start and gene-end sequences, which are presumed to be PIV3 transcription signals. This chimeric expression cassette, in turn, would be flanked by the PIV3 3′ extracistronic leader region and the 5′ extracistronic trailer region. Therefore, the 898-nt PIV3-CAT vRNA transcript, which contained 226 nt of PIV3-specific sequence, was 5.8% of the length of the authentic PIV3 genomic RNA (15,463 nt).

To test whether PIV3-CAT vRNA could direct expression of the CAT reporter gene, 293 cells were infected with PIV3 and then transfected with the vRNA analog. Following a 24-h incubation, cells were harvested and assayed for CAT activity. CAT activity was observed only in cells that had been both infected with PIV3 and transfected with PIV3-CAT vRNA (Fig. 3A); CAT could not be detected if the infection was omitted or if PIV3 was replaced with RSV (Fig. 3A) or BPIV3 (not shown).

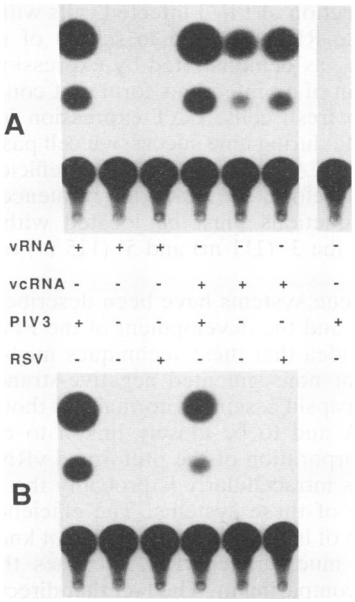

FIG. 3. Transfection and passage of negative- and positive-sense versions of PIV3-CAT RNA. Uninfected 293 cells or cells infected with PIV3 or RSV were transfected with either PIV3-CAT vRNA or PIV3-CAT vcRNA; 24 h after infection, cells were collected and cell lysates were prepared (A). Culture supernatants were used to infect fresh 293 cells, and after a 24-h incubation, the second set of cells was harvested and cell lysates were prepared (B). Cell lysates were then assayed for CAT activity. Each lane in panel A (transfection) is aligned with its counterpart in panel B (passage), and the conditions are indicated between the panels.

CAT activity was totally dependent upon transfection with PIV3-CAT vRNA. If PIV3-CAT vRNA was omitted from the transfection mixes (Fig. 3A) or if transcription reactions were treated with RNase A (50 μg/ml) prior to transfection (not shown), CAT gene expression was abolished. Cells transfected with either *Hga*I-digested or supercoiled PIV3-CAT cDNA without prior transcription were negative for CAT activity (not shown), and digestion of the DNA template with RNase-free DNase (250 U/ml) prior to transfection did not diminish the level of CAT gene expression (not shown). Furthermore, incubation of cells with concentrations of actinomycin D ranging from 0.2 to 5 μg/ml beginning 1 h prior to infection had no significant effect on the level of CAT gene expression (not shown). Together, these results strongly support the interpretation that CAT expression was mediated by the input vRNA analog and by PIV3 RNA-dependent RNA polymerase, which is insensitive to actinomycin D.

A positive-sense version of PIV3-CAT RNA also directs expression of CAT. A second cDNA, PIV3-CAT(+), was constructed to encode an analog of the predicted replicative intermediate (PIV3 vcRNA) of the PIV3 genome, as shown in Fig. 1B and 2C and D. In PIV3-CAT(+) cDNA, the T7 promoter was placed adjacent to the leader region and the *Hga*I site was placed next to the trailer region. Transcription of *Hga*I-digested DNA, therefore, would yield the exact complement of PIV3-CAT vRNA, PIV3-CAT vcRNA, containing the correct 5′ and 3′ termini.

To determine whether PIV3-CAT vcRNA could also direct CAT gene expression, it was transfected into PIV3-infected 293 cells in parallel with its negative-sense counterpart. Both PIV3-CAT vRNA and PIV3-CAT vcRNA directed expression of the CAT gene, and the levels of CAT expression were approximately equal (Fig. 3A). In the case of PIV3-CAT vcRNA, however, CAT activity was also detected in uninfected cells, or in RSV-infected cells, at levels representing 10 to 20% of the level in PIV3-infected cells (Fig. 3A), as measured by liquid scintillation counting of spots cut from thin-layer plates. This background level of helper virus-independent CAT activity probably was due to direct translation of the input RNA.

PIV3-CAT RNA can be passaged to fresh cells. To investigate whether PIV3-CAT RNA was incorporated into a form that could be transmitted to fresh cells, culture medium from transfected, PIV3-infected 293 cells was clarified and used to infect fresh cells. Twenty-four hours later, cells were harvested and assayed for CAT activity. PIV3-infected cells transfected with either PIV3-CAT vRNA or PIV3-CAT vcRNA produced particles capable of transmitting the CAT gene (Fig. 3B). However, transmission by particles produced with PIV3-CAT vcRNA was only about 15% as efficient as with PIV3-CAT vRNA. The reasons for this difference are unclear at this time. The production of transmissible particles was strictly dependent on PIV3 infection, since supernatants from uninfected or RSV-infected cells that had been transfected with PIV3-CAT vRNA or PIV3-CAT vcRNA were unable to transmit CAT activity to secondary cultures. Thus, the low level of CAT expression detected following transfection with PIV3-CAT vcRNA in the absence of helper virus was not associated with the production of infectious particles.

To investigate the nature of PIV3-CAT transmission, supernatants from transfected, PIV3-infected cells were incubated with serial dilutions of a mixture of three neutralizing monoclonal antibodies representing three different antigenic sites on the PIV3 HN protein. The treated supernatants were then used to infect 293 cells. To ensure that any inhibition of CAT transmission was not due simply to neutralization of progeny helper PIV3 present in the culture supernatants, these secondary cultures were washed and superinfected with fresh PIV3 immediately after incubation with culture fluid from transfected cells. CAT activity in the secondary cultures was inhibited by prior incubation of the culture supernatants with neutralizing antibodies; the amount of CAT expression increased with increasing dilution of the neutralizing monoclonal antibodies (Fig. 4). PIV3 replication in all secondary cultures was roughly equal, as indicated by the similar amounts of virus produced by each culture (not shown). The specificity of the PIV3 antibodies was confirmed by the lack of inhibition of RSV-CAT transmission in parallel experiments (not shown). In a reciprocal experiment, RSV-specific monoclonal antibodies neutralized RSV-CAT transmission (not shown) without inhibiting passage of PIV3-CAT (Fig. 4). These results support the idea that PIV3 particles containing PIV3-CAT RNA were released from transfected cells.

PIV3-CAT RNA was amplified during serial passage. Serial passage of culture supernatants was also performed. In one passage series, the cells were superinfected with fresh PIV3 after each passage, while in a parallel series, superinfection was omitted. In both series, CAT expression was transmitted via culture fluid for the duration of the experiment (nine passages). During passage, the level of CAT expression increased dramatically over that in the original transfected culture (Table 1). CAT gene expression increased steadily throughout the experiment if cells were superinfected with PIV3 after each passage, reaching a level of activity more

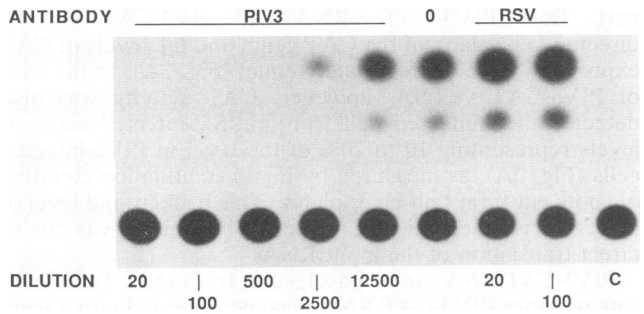

FIG. 4. Inhibition of passage of PIV3-CAT vRNA by PIV3-specific neutralizing monoclonal antibodies. 293 cells were infected with PIV3 and transfected with PIV3-CAT vRNA; 24 h after infection, culture supernatants were collected, clarified, and then incubated with serial dilutions of a mixture of three neutralizing monoclonal antibodies specific for either the PIV3 HN glycoprotein or the RSV F glycoprotein or (lane 0) were mock treated in parallel. Supernatants were then used to infect fresh cultures of 293 cells, after which cells were superinfected with PIV3; 24 h later, the cells were assayed for CAT gene expression. The specificities and dilutions of antibodies used are indicated above and below the lanes, respectively. C, nontransfected control cells infected with PIV3.

than 3,000 times higher than in the original transfected culture (Table 1). If cultures were not superinfected with fresh PIV3 during passage, CAT activity rose more quickly, peaked after the fifth passage at a level more than 3,000-fold greater than in the original transfected culture, and then diminished (Table 1). The infectious titer of PIV3, however, did not reflect the large changes seen for CAT activity and remained relatively constant or diminished marginally during both passage series.

DISCUSSION

cDNA was constructed to encode an internally deleted version of PIV3 vRNA bearing the foreign reporter gene CAT. Transfection of PIV3-infected cells with the synthetic negative-sense RNA resulted in rescue of the PIV3-CAT vRNA analog, as demonstrated by expression of CAT and the production of an infectious form that could transmit the CAT gene to fresh cells. CAT expression increased more than 3,000-fold during nine successive cell passages, indicating that PIV3-CAT vRNA replicated efficiently and was amplified. Therefore, the *cis*-acting sequences required for these viral functions must be located within the 226 nt derived from the 3′ (111 nt) and 5′ (115 nt) termini of PIV3 vRNA.

TABLE 1. Amplification of PIV3-CAT vRNA upon serial passage in 293 cells

Passage no.	Passage without superinfection[a]		Passage with superinfection[b]	
	CAT activity[c]	Titer[d]	CAT activity	Titer
0[e]	1	7.1	1	7.0
1	4	7.0	1	7.3
2	50	7.0	4	7.5
3	350	7.0	20	7.5
4	1,500	7.0	120	7.5
5	3,300	6.8	560	6.9
6	2,300	7.0	1,400	7.0
7	1,700	6.6	1,100	6.8
8	1,300	6.8	3,000	7.0
9	770	6.8	3,400	6.9

[a] 293 cells were infected with approximately 5 to 10 PFU of PIV3 per cell in a final volume of 1 ml and then transfected with PIV3-CAT vRNA; 24 h after infection, the culture fluids were collected and clarified, and two-thirds of each supernatant (1 ml) was used to infect fresh cultures. Passage was repeated at 24-h intervals for 9 days.

[b] Infections and passages were performed in the same way except that following each passage, the cells were superinfected with PIV3 for 1 h immediately after incubation with culture supernatants.

[c] Relative to the original transfected culture.

[d] Log_{10} PFU released from the culture assayed for CAT activity.

[e] The original transfected culture.

Similar rescue systems have been described for SeV (23) and RSV (5), and the development of the PIV3-CAT model supports the idea that these techniques might have general application for nonsegmented negative-strand viruses. Because nucleocapsid assembly normally is thought to involve nascent RNA and to be closely linked to elongation, the apparent incorporation of the preformed vRNA analog into nucleocapsids intracellularly is probably the most unanticipated feature of these systems. The efficiency with which encapsidation of input vRNA occurs is not known, since it is unclear how much intact vRNA reaches the appropriate intracellular compartment. The fact that direct translation of PIV3-CAT vcRNA contributed a significant proportion of total CAT synthesis (Fig. 3) suggests that only a small fraction of the RNA that enters the cytoplasm is encapsidated and amplified.

In the SeV and RSV systems described previously, rescue of negative-sense genome analogs containing the CAT gene was demonstrated. Here, we showed that positive-sense PIV3-CAT vcRNA, which corresponds to the replicative intermediate, can also be rescued. However, in contrast to what was observed for the negative-sense analog, PIV3-CAT vRNA, CAT activity was also detected in the absence of helper virus in cells transfected with PIV3-CAT vcRNA. This is probably due to direct translation of the input mRNA-sense vcRNA. The first ATG from the 5′ end of the vcRNA analog is that of the CAT gene (Fig. 2C), which would be consistent with it being able to function as a translational start site. Furthermore, we found that PIV3-CAT vcRNA can direct the synthesis of CAT in an in vitro translation system, although it was translated less efficiently than a subgenomic version containing a shorter 5′ nontranslated region (unpublished data). Given that PIV3-CAT vcRNA can function as an mRNA, albeit inefficiently, we cannot rule out the possibility that the CAT expression detected during rescue was due solely to translation of input and newly synthesized PIV3-CAT vcRNA rather than to newly synthesized subgenomic PIV3-CAT mRNA. We do note that in the case of RSV-CAT, evidence for the synthesis of a subgenomic polyadenylated CAT mRNA has now been obtained by reverse transcription and PCR (unpublished data). Therefore, there is a precedent for the idea that this type of analog does indeed execute transcription.

The production of particles that could transmit CAT to fresh cells was detected only when cells were infected with PIV3, consistent with the idea that both negative- and positive-sense PIV3-CAT RNA is rescued and enters the PIV3 replicative pathway via the same helper virus-dependent mechanism. Unexpectedly, CAT expression following passage of virus from cells transfected with PIV3-CAT vcRNA appeared to be lower than with PIV3-CAT vRNA. We are investigating whether there are differences in the efficiency of rescue of positive- versus negative-sense RNA analogs.

RSV was unable to complement PIV3-CAT vRNA, which

is a reciprocal confirmation of our previous observation that PIV3 could not complement RSV-CAT vRNA (5). BPIV3 also was inactive in rescuing PIV3-CAT vRNA. This was somewhat surprising, given (i) the extensive nucleotide and amino acid sequence identity between the two viruses (for example, there are only seven nucleotide differences in the 55-nt leader regions of the two viruses) and (ii) the observation that BPIV3 could support replication of an SeV DI genome (6). However, in other work (unpublished data), rescue was ablated by insertion of a dinucleotide at any of several different sites throughout the leader region. This finding suggested that the leader region is highly sensitive to nucleotide changes, and thus the seven nucleotide differences between the human and bovine strains of PIV3 might be significant. In addition, it is possible that BPIV3 might be competent to direct replication but not transcription of PIV3-CAT RNA; replication in the absence of transcription would not be detected by monitoring CAT activity. Clearly, there is a high degree of specificity imposed upon the formation of RNP complexes that are competent for rescue of paramyxovirus genomic RNA analogs.

Expression of CAT by PIV3-CAT vRNA increased more than 3,000-fold during nine serial passages. When fresh helper PIV3 was added at each passage, CAT activity increased steadily. In the parallel series without added PIV3, CAT activity increased more rapidly to the same high level and then diminished. One interpretation is that the continued addition of helper had two effects: the helper might slow the increase in PIV3-CAT by competition, but it also would stabilize replication of PIV3-CAT by providing a steady supply of *trans*-acting proteins. Without added helper virus, PIV3-CAT might function with the characteristics of a DI RNA, competing and interfering with replication of the helper virus and eventually leading to a decline in replication of the helper and, as a consequence, itself. We did not observe any dramatic changes in PIV3 titer that would support this interpretation, but this may not be surprising since PIV3 DI particles frequently do not exhibit high levels of interference (20, 21). Given that PIV3 does not yield a large number of infectious virions per cell, the production of such particles might be an insensitive indicator of intracellular interference. Alternatively, PIV3-CAT vRNA might not compete effectively against helper virus vRNA once amplified to the point where replication factors became limiting. PIV3-CAT vRNA has properties which have been suggested to diminish the ability of DI RNAs to interfere with standard vRNA: (i) the 3′ terminus of vRNA rather than vcRNA, (ii) (presumed) transcriptional activity, and (iii) small size (25, 26). The nature of the effects, if any, of synthetic vRNA on PIV3 replication remains to be characterized further. It will be important to examine intracellular viral macromolecular synthesis and the rate of virus release in mixed infections to detect possible effects on virus replication that might not be reflected in the virus yield. It may also be possible to increase the efficiency of replication of the vRNA analog by, for example, eliminating its ability to transcribe. This would test the theory that one of the replicative advantages of certain DI RNAs is their transcriptional silence.

We were interested in establishing the PIV3-CAT system because it will facilitate development of conditions for rescue of complete synthetic PIV3 RNAs. In addition, there are differences in the molecular genetics of RSV and PIV3 that can be explored by comparing analogous in vitro vRNA transcripts in the same host cell line. These differences have been described in detail elsewhere and include (i) differences in encoded proteins (specifically, four RSV proteins, the NS1, NS2, SH, and M2 proteins, are not produced by PIV3, and conversely, two or three PIV3 proteins, the C and D [and the predicted V] proteins, are not produced by RSV) and (ii) conserved intergenic sequences in the PIV3 genome versus variable intergenic sequences in the RSV genome. We are now mapping *cis*-acting sequences in RSV-CAT and PIV3-CAT constructs. The ability to rescue vRNA analogs of the two different paramyxoviruses indicates that in both cases, the *cis*-acting signals for encapsidation, replication, transcription, and packaging are contained in short terminal RNA segments that can be linked to foreign sequences without loss of function. However, studies in progress, involving mutagenesis of the leader sequences of both viruses (unpublished data), suggest that the RSV signals are relatively circumscribed and are unaffected by nearby mutations, whereas those of PIV3 are more extensive and render most of the terminal sequences sensitive to inactivation. Further characterization of PIV3, RSV, and other nonsegmented negative-strand viruses using these experimental systems will provide insight into both the similarities and differences in the mechanisms utilized by these diverse viruses to accomplish a common task: the expression and replication of a negative-sense vRNA.

ACKNOWLEDGMENTS

We thank Lili Kuo and Haim Grosfeld for helpful discussions of the data, Susan L. Hall and Mark Connors for the monoclonal antibodies, and Ena Camargo and Myron Hill for excellent technical assistance. We thank Brian Murphy and Robert Chanock for support and for reviewing the manuscript.

K.D. is the recipient of a career development award from the Ontario Ministry of Health and acknowledges the support of the Medical Research Council of Canada (grant MA-8688).

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