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An Infectious Clone of Human Parainfluenza Virus Type 3

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A full-length clone of the human parainfluenza virus type 3 (HPIV-3) genome (called pHPIV-3) was constructed, and recombinant, infectious HPIV-3 was generated by transfecting pHPIV-3 and support plasmids encoding the HPIV-3 NP, P, and L proteins into HeLa cells infected with a vaccinia virus recombinant which expresses T7 RNA polymerase. T7 RNA polymerase promoters on the transfected plasmids direct the synthesis of transcripts encoding the NP, P, and L proteins and a full-length, positive-sense copy of the HPIV-3 genome. Generation of virus was dependent on transfection of pHPIV-3 and the HPIV-3 P- and L-encoding plasmids. However, a plasmid encoding the NP protein was not required since NP was expressed from pHPIV-3. Recovered virus was neutralized by anti-HPIV-3 antisera and shown to contain specific base substitutions characteristic of pHPIV-3. Recombination was shown to occur during recovery, as viruses with two distinct genotypes and phenotypes were isolated. The ability to produce infectious HPIV-3 engineered to contain specific alterations within the HPIV-3 genes and *cis*-acting elements expedites the study of all aspects of the virus replication cycle. Additionally, analysis of mutations may lead to the identification of attenuating genotypes, a key step in the development of a live virus vaccine.

Parainfluenza viruses are enveloped, single-stranded, negative-sense RNA viruses within the family *Paramyxoviridae* (3). Recognized in 1956 as a cause of respiratory infection in humans, human parainfluenza viruses (HPIV) are believed to account for 4 to 22% of the respiratory illnesses in children, second only to respiratory syncytial virus in this regard (10, 36). These viruses are important causes of lower respiratory tract diseases such as pneumonia and bronchiolitis and are the most common causes of croup in young children (10, 18, 26, 34). Of the four HPIV serotypes, HPIV type 3 (HPIV-3), a member of the paramyxovirus genus of the *Paramyxoviridae*, appears to be the most virulent, frequently causing bronchiolitis and pneumonia during the first month of life (34). Effective vaccines or antiviral therapies against any of the HPIV serotypes are not presently available.

Replication of the HPIV-3 RNA genome is similar to that of other members of the *Paramyxoviridae* and the closely related *Rhabdoviridae* (1, 22). Upon infection of a cell, transcription is the major RNA synthesis event, resulting in the production of the viral mRNAs from the negative-sense genome. Later in infection, a transition to RNA replication occurs, resulting in synthesis of antigenomic, positive-sense RNA, which serves as the template for synthesis of additional negative-sense genomic RNA. Transcription and replication of the genomic RNA is dependent on formation of a ribonucleoprotein complex (RNP) consisting of the 15,462-nucleotide genomic RNA encapsidated by the nucleocapsid protein (NP), the closely associated phosphoprotein (P), and the large (L) polymerase protein. Several cellular factors are also involved in the HPIV-3 replicative cycle (7, 8).

The requirement of an intact RNP for RNA synthesis has hindered analysis of paramyxovirus and rhabdovirus transcription and replication in a cell-free system. Efforts to encapsidate viral RNA in vitro have failed, and unlike the case for positive-sense RNA viruses, naked paramyxovirus RNA is not infectious. This problem led to the development of systems allowing the intracellular expression of required viral proteins, either from helper virus or from plasmids, and genomic analogs. In these systems, the genome analog could be encapsidated, transcribed, replicated, and, in some cases, packaged into virus-like particles (2, 5, 6, 9, 19, 27, 33, 37). The plasmid-based system utilizes T7 RNA polymerase expressed from a recombinant vaccinia virus to drive the synthesis of viral proteins from support plasmids. Recently, this system has been used to show that coexpression of antigenomic full-length RNA and the required viral proteins NP, P, and L (also M2 in the case of respiratory syncytial virus [4]) can lead to production of infectious virus. The use of antigenomic rather than genomic RNA to initiate the infectious cycle appeared to be the key step in the development of infectious clones for the *Paramyxoviridae* and *Rhabdoviridae* (32). The inability to recover virus with genomic-sense RNA may be due to annealing of the genomic RNA with the positive-sense NP, P, and L transcripts also present in the cells. An additional reason may be that there appear to be cryptic T7 RNA polymerase transcription termination signals in the genomic RNA (29, 35). Currently, infectious cDNA clones have been derived for the rhabdoviruses rabies virus and vesicular stomatitis virus and the paramyxoviruses Sendai virus, measles virus, and respiratory syncytial virus (4, 17, 23, 29, 32, 35). Here, we show that coexpression of a full-length, antigenomic-sense transcript of HPIV-3 and the HPIV-3 NP, P, and L proteins leads to production of infectious HPIV-3.

MATERIALS AND METHODS

Plasmid constructions. The infectious clone pHPIV-3 was constructed by first creating a positive-sense minireplicon [pPIV3-MG(+)], into which reverse transcription-PCR (RT-PCR) fragments derived from HPIV-3 genomic RNA were inserted. The vector pOCUS-2 (Novagen) was chosen as the starting plasmid because of its small size (1,930 bp), which may increase the stability of a full-length clone.

The positive-sense minireplicon was constructed by generating PCR products encoding the leader and trailer regions flanked by a T7 promoter and hepatitis delta virus antigenomic ribozyme, respectively. The primers used for synthesis of the T7 promoter/leader region were 5′-TAGTCGGCCCTAATACGACTCACTATAGGACCAAACAAGAGAAGAAACT-3′ and 5′-GAAATTATAGAGCTCCCTTTTCT-3′. The first primer encodes an *Eag*I site and the T7 promoter (underlined), and the second primer introduced an A-to-G base change at virus

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base 94 (boldface), within the 5′ untranslated region (UTR) of the NP mRNA, which creates a *Sac*I site. The template for this reaction was pHPIV3-CAT, a previously described HPIV-3 minireplicon (6). The resulting PCR product was cloned into the *Eag*I and *Sac*I sites of pOCUS-2. The primers used for synthesis of the trailer/ribozyme region were 5′-TAA**G**GCCTAAAGATAGACAAAAA GTAAGAAAAACATGTAATATATATATACCAAACAGAGTTCTTCTCTT GTTTGGTGGGTCGGCATGGCATCTC-3′ and 5′-CTGGGTACCTCCCTT AGCCATCCGAGT-3′. The first primer contains sequence from the 3′ UTR of the L mRNA, through the trailer, and primes synthesis of the ribozyme (underlined). Also, an A-to-G change at virus base 15389 (boldface), which creates a *Stu*I site within the 3′ UTR of the L mRNA, is encoded by this primer. The second primer encodes the 3′ end of the ribozyme (underlined) and a *Bgl*II site. The template for this PCR was pSA1, a previously described plasmid containing the ribozyme sequence (28). The PCR product derived from this reaction was cloned into the *Stu*I and *Bgl*II sites of pOCUS-2. The leader and trailer regions were combined into a single clone by transferring the *Eag*I/*Pst*I fragment of the T7/leader clone into the *Pac*I/*Pst*I sites of the trailer/ribozyme clone.

To prevent possible interference by transcription from cryptic vaccinia virus promoters, vaccinia virus polymerase transcription stop signals (TTTTTNT) were inserted upstream and downstream of the replicon near *Pvu*II and *Ssp*I sites within pOCUS-2. A T7 transcription termination signal was removed from pET-17b by digestion with *Blp*I and *Bsp*EI and inserted into the *Ssp*I site (blunted with T4 DNA polymerase) of pOCUS-2. A luciferase reporter gene was then inserted into the *Sac*I and *Stu*I sites to create pPIV3-MG(+).

Five RT-PCR products were generated from HPIV-3 strain 47885 virion RNA. These PCR products, encompassing the remainder of the HPIV-3 genome, were identified by restriction enzyme analysis, cloned in either pUC19 or pOCUS-2, and then inserted into pPIV3-MG(+). A PCR product containing virus bases 83 to 27281 was inserted into the *Sma*I site of pUC19. The 83/2721 clone was then digested with *Sac*I and *Xmn*I, removing virus bases 94 to 553, which were inserted into the *Sac*I and *Sph*I (blunt with T4 DNA polymerase) sites of pPIV3-MG(+). The 83/2721 clone was then digested with *Pst*I to remove a fragment containing virus bases 540 to 2274, which was then inserted into the *Pst*I site of the pPIV3-MG(+) clone containing the 94/554 fragment. A second PCR product encompassing virus bases 13395 to 15397 was cloned into the *Sma*I site of pUC19. This 13395/15397 clone was then digested with *Stu*I and *Pac*I, and the resulting fragment containing virus bases 13632 to 15381 was inserted into the *Stu*I and *Pac*I sites of the pPIV3-MG(+) clone containing the virus sequence to base 2274. The resulting clone contained virus bases 1 to 2274 and 13632 to 15462 in the pPIV3-MG(+) context.

A third PCR product containing virus bases 7403 to 11513 was digested with *Bsp*MI (blunted with T4 DNA polymerase) and *Xho*I to produce a fragment containing bases 7437 to 11444, which was inserted into the *Xho*I and *Ssp*I sites of pOCUS-2. A fourth PCR fragment containing virus bases 10904 to 13773 was digested with *Pvu*II and *Bam*HI (virus bases 10918 to 13733) and inserted into the *Eco*RI (blunted with T4 DNA polymerase) and *Bam*HI sites of pUC19. The two viral segments were combined by digesting the 7437/11444 clone with *Sac*I (blunted with T4 DNA polymerase) and *Eco*NI and inserting it into the 10918/13733 clone digested with *Eco*RI (blunted with T4 DNA polymerase) and *Eco*NI. The resulting clone contained virus bases 7437 to 13733 in a pUC19 background. The remainder of the virus sequence was derived from a fifth PCR product encompassing virus bases 83 to 7457 which had been digested with *Xmn*I and *Xho*I (virus bases 553 to 7437) and cloned into the *Stu*I and *Xho*I sites of pOCUS-2. The 7437/13733 clone was then digested with *Bam*HI, blunted with T4 DNA polymerase, and digested with *Xho*I to release a fragment that was inserted into the *Eag*I (blunted with T4 DNA polymerase)- and *Xho*I-digested 553/7437 clone. The resulting clone contained virus bases 553 to 13733. This clone was then digested with *Psh*AI and *Pac*I, and the resulting fragment containing virus bases 2143 to 13632 was inserted into the same sites of the pPIV3-MG(+) clone containing virus bases 1 to 2274 and 13632 to 15462. This generated pHPIV-3, the infectious clone.

The pPIV3-NP and pPIV3-L clones (15, 16) were in a pGEM-4 background. To insert the P gene into pGEM-4, P sequences were transferred from a P-*lac* fusion clone (38) by digestion with *Xba*I (blunted with T4 DNA polymerase) *Bam*HI and inserted into the *Kpn*I (blunted with T4 DNA polymerase) and *Bam*HI sites of pGEM4. The pPIV3-L clone was also modified. In the natural L mRNA sequence, there is a noninitiating AUG 11 nucleotides from the 5′ end of the transcript. This was removed from pPIV3-L by mutational PCR, changing virus bases 8636 and 8637 from AT to TA.

Transfection and recovery of recombinant HPIV-3. Confluent monolayers of HeLa cells in six-well plates were infected with recombinant vaccinia virus vTF7-3 (12, 14), which expresses T7 RNA polymerase, at a multiplicity of infection of 2. After 1 h at 37°C, pPIV3-NP, pPIV3-P, pPIV3-L, and pHPIV-3 were transfected by using Lipofectin (Bethesda Research Laboratories) according to the manufacturer's instructions. After 3 h, the transfection medium was removed and replaced with 1.5 ml of Dulbecco's modified Eagle's medium–5% fetal bovine serum. After 40 to 48 h, the plates were frozen, thawed, and scraped. The clarified medium supernatant (250 μl) was then used to infect fresh HeLa cell monolayers in six-well plates. Dulbecco's modified Eagle's medium (1.5 ml) containing 25 μg of 1-β-D-arabinofuranosylcytosine (AraC) per ml to inhibit vaccinia virus replication was added after a 1-h attachment. After 40 h, the plates were frozen, thawed, and scraped. The clarified medium supernatant was then titered for HPIV-3. During the titering, isolated plaques were picked as agar plugs. The agar plugs were placed in 500 μl of Opti-MEM at 4°C for 4 h; 250 μl was then used to infect fresh HeLa cell monolayers for amplification of the plaque isolates for 40 h.

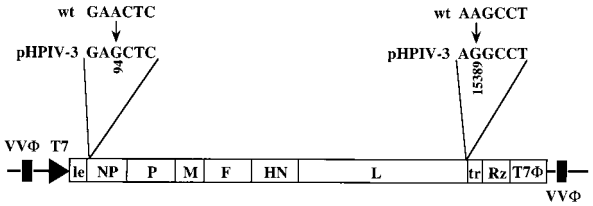

FIG. 1. Structure of the full-length infectious clone, pHPIV-3. VVΦ, vaccinia virus polymerase stop signal (TTTTTNT); T7, T7 RNA polymerase promoter; le, HPIV-3 leader sequence; NP, P, M, F, HN, and L, HPIV-3 protein-coding regions; tr, HPIV-3 trailer sequence; Rz, the hepatitis delta virus antigenomic ribozyme; T7Φ, T7 RNA polymerase terminator signal. Regions containing substitution mutations are expanded and shown above, with the specific changes indicated. The A-to-G change at virus base 94 creates a *Sac*I site, and the A-to-G change at virus base 15389 creates a *Stu*I site. wt, wild type.

Characterization of recombinant HPIV-3. The plaque-purified and amplified virus isolates and appropriate controls were analyzed in neutralization assays. Rabbit polyclonal antiserum (5 μl) to whole HPIV-3 (30) or preimmune serum (5 μl) was incubated with virus on ice for 30 min prior to a standard plaque assay. To allow maximal plaque development, the plates were incubated at 37°C for 66 h prior to staining with crystal violet.

For RT-PCR analysis, viral RNA was isolated from approximately 2×10^7 PFU. Reverse transcription was carried out with Superscript II reverse transcriptase (Boehringer Mannheim Biochemicals) at 44°C for 1 h, using oligonucleotides which primed at virus base 23 or 15100. The PCR was carried out with Expand Long polymerase (Boehringer Mannheim Biochemicals), using second primers which result in amplification of virus bases 23 to 303 or 15100 to 15440.

Proteins produced from the pPIV3-NP and pHPIV-3 plasmids were analyzed by Western blotting. Cell extracts were prepared 48 h posttransfection from HeLa cells infected with vTF7-3 and transfected with the appropriate plasmid. Extracts (equivalent to 6×10^4 cells) were run on sodium dodecyl sulfate–10% polyacrylamide gels and transferred to nitrocellulose membranes. The primary antibody was a rabbit polyclonal anti-RNP antiserum diluted 1:1,000. The secondary antibody was a 1:1,000 dilution of a goat anti-rabbit antibody conjugated to horseradish peroxidase. Visualization was by chemiluminescence (ECL kit; Amersham).

RESULTS

Construction of a full-length cDNA clone of HPIV-3. The construction of a full-length infectious clone of HPIV-3 was achieved by a two-step process. The initial step was the generation of a positive-sense replicon containing the following: a T7 promoter which directed the synthesis of two nonviral G residues, followed by the positive-sense leader region, part of the NP 5′ UTR (to virus base 97), the luciferase gene, part of the L 3′ UTR (starting at virus base 15387), and trailer sequences. The hepatitis delta virus antigenomic ribozyme followed and effected precise cleavage after the 3′-terminal HPIV-3-specific base. A T7 RNA polymerase terminator followed the ribozyme sequence. Additionally, vaccinia virus polymerase termination signals were inserted immediately upstream and downstream of the aforementioned sequences (Fig. 1). During the construction, single base changes were created in the regions encoding the NP 5′ UTR and the L 3′ UTR. The A-to-G change at virus base 94 and the A-to-G change at base 15389 create *Sac*I and *Stu*I sites, respectively, which serve as genetic tags to identify virus as being of recombinant origin. This construction [pPIV3-MG(+)] conforms to previously discovered rules governing HPIV-3 replication, namely, that extra bases at the 5′ terminus of an HPIV-3 genome analog were tolerated while those at the 3′ terminus were not (6). Indeed, this construct has been shown to express high levels of luciferase activity when transfected in combination with plasmids

TABLE 1. Recovery of HPIV-3 from pHPIV-3[a]

Plasmids transfected				Virus recovery[b]
HPIV-3	NP	P	L	
+	+	+	+	14/15[c]
−	+	+	+	0/2
+	−	+	+	3/3[d]
+	+	−	+	0/3
+	+	+	−	0/2

[a] HeLa cell monolayers were infected with vTF7-3 and transfected with the indicated plasmids. After 40 h, cells were lysed and supernatants were added to fresh HeLa cell monolayers in the presence of AraC to inhibit vTF7-3 replication. These monolayers were then lysed, and the supernatants were assayed for HPIV-3.

[b] Number of experiments for which HPIV-3 was recovered divided by number of attempts.

[c] The single experiment that did not yield HPIV-3 used 0.1 μg of the P plasmid.

[d] The omission of the NP plasmid resulted in three- to fivefold-lower titers of HPIV-3.

encoding the HPIV-3 NP, P, and L proteins into vTF7-3-infected HeLa cells (data not shown).

To generate the full-length HPIV-3 clone, five RT-PCR products were generated from HPIV-3 virion RNA and cloned. These fragments were subsequently inserted into pPIV3-MG(+), replacing the luciferase coding sequences, to create pHPIV-3, the full-length clone.

Transfection and recovery of HPIV-3. pHPIV-3 was transfected along with plasmids encoding the HPIV-3 NP, P, and L proteins into HeLa cells infected with vTF7-3, a vaccinia virus recombinant that expresses the T7 RNA polymerase (14). After 40 h, the transfected cells were frozen and thawed to release any cell-associated HPIV-3, and the clarified medium supernatant was used to infect fresh HeLa cell monolayers for amplification. vTF7-3 replication was inhibited by inclusion of AraC in the medium during the amplification. After 40 h, the monolayers were frozen and thawed and the clarified medium supernatants were titered for HPIV-3 in the presence of AraC. Optimal transfection conditions were 1 μg of pHPIV-3, 2 μg of pPIV3-NP, 4 μg of pPIV3-P, and 0.1 μg of pPIV3-L. Under these conditions, approximately 1,000 PFU per 6×10^5 cells was obtained during the initial transfection and 10^6 PFU per 6×10^5 cells was obtained after the amplification.

Support plasmid requirements. When individual plasmids were omitted from the transfection step, it was observed that the pHPIV-3, pPIV3-P, and pPIV3-L plasmids were required for recovery of virus but, surprisingly, pPIV3-NP was not (Table 1). Perhaps this should not have been a surprise since the positive-sense minireplicon pPIV3-MG(+), the precursor to pHPIV-3, displayed significant levels of luciferase expression, even in the absence of any support plasmids (data not shown). This expression, like that from the NP, P, and L plasmids, is probably the result of the T7 transcripts being capped in the cytoplasm by the vaccinia virus capping enzyme (13). Translation of luciferase occurs since the luciferase AUG is the initial AUG of the transcript. Because the NP coding sequence of pHPIV-3 is in the same position as luciferase in pPIV3-MG(+), we investigated whether NP expression from pHPIV-3 could be detected.

pHPIV-3 and pPIV3-NP were transfected into vTF7-3-infected HeLa cells, and cell lysates were prepared after 48 h. The lysates were then analyzed by Western blotting using an anti-HPIV-3 RNP antiserum (Fig. 2). This antiserum, which recognizes primarily NP and reacts poorly with P (5a), recognized NP from the pPIV3-NP- and pHPIV-3-transfected cell extracts and from purified HPIV-3 RNP. No proteins were recognized in a mock-transfected HeLa extract. Thus, it appears that NP is expressed from pHPIV-3, presumably being translated from the T7-directed, antigenomic RNA transcript.

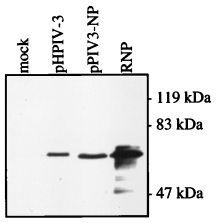

FIG. 2. Expression of NP from pHPIV-3. HeLa cell extracts were prepared from cells infected with vTF7-3 and transfected with no plasmid (mock), pHPIV-3, or pPIV3-NP. The extracts were examined by Western blotting using an antiserum raised against HPIV-3 RNP. Reaction with HPIV-3 RNP is also shown.

Characterization of recovered virus. To purify HPIV-3 from vTF7-3, plaques of which are only slightly smaller than those of HPIV-3, isolated plaques suspected to be HPIV-3 were picked and amplified. The amplified virus was then tested for neutralization by anti-HPIV-3 antiserum (Fig. 3). The plaque-purified virus was completely inhibited by the anti-HPIV-3 antiserum, while vTF7-3 was not. The virus was also tested for growth in the presence of AraC. The HPIV-3 isolates were not inhibited by AraC, whereas the vTF7-3 virus was completely inhibited. Interestingly, of the eight recombinant HPIV-3 isolates, four had plaque sizes identical to that of the parental HPIV-3 stock whereas four were slightly larger. In Fig. 3, note that the plaque size of isolate 3 is slightly larger than that of isolate 5 and wild-type HPIV-3.

RNA was then extracted from wild-type and plaque-isolated viruses and used for RT-PCR analysis using primers flanking the substitution mutations (Fig. 4). Results are shown for four of the eight isolates. PCR products of the expected sizes were generated in a reverse transcription-dependent manner, indicating that the PCR products were derived from RNA rather than contaminating plasmid DNA. Digestion with *Sac*I (lanes 1 to 5) showed that the mutation at base 94 was not present in the wild-type virus but was present in the plaque-isolated viruses, indicating they are of recombinant origin. Similarly, PCR product derived from the region encompassing virus base 15389 of wild-type HPIV-3 was not cleaved by *Stu*I (lane 6). However, only four of the eight plaque-isolated viruses, and two of the four shown in Fig. 4, contained the mutation which creates the *Stu*I site (lanes 7 to 10). Direct sequencing of the PCR products confirmed these results (data not shown).

DISCUSSION

We have constructed a full-length plasmid clone of the HPIV-3 genome, pHPIV-3. Upon transfection of pHPIV-3 and plasmids encoding the viral NP, P, and L proteins into vTF7-3-infected HeLa cells, recombinant HPIV-3 bearing genetic markers was efficiently recovered. Several interesting features of this system were noted during this study. First, we showed that the viral NP protein could be expressed from the infectious clone and that this expression obviated the need for an NP support plasmid. Similar expression of NP from a paramyxovirus or rhabdovirus infectious clone has not been previously noted. At this stage, we speculate that the NP protein is synthesized directly from the primary antigenomic transcript after it is capped by the vaccinia virus capping enzyme (13). Unlike other paramyxovirus infectious clones, the

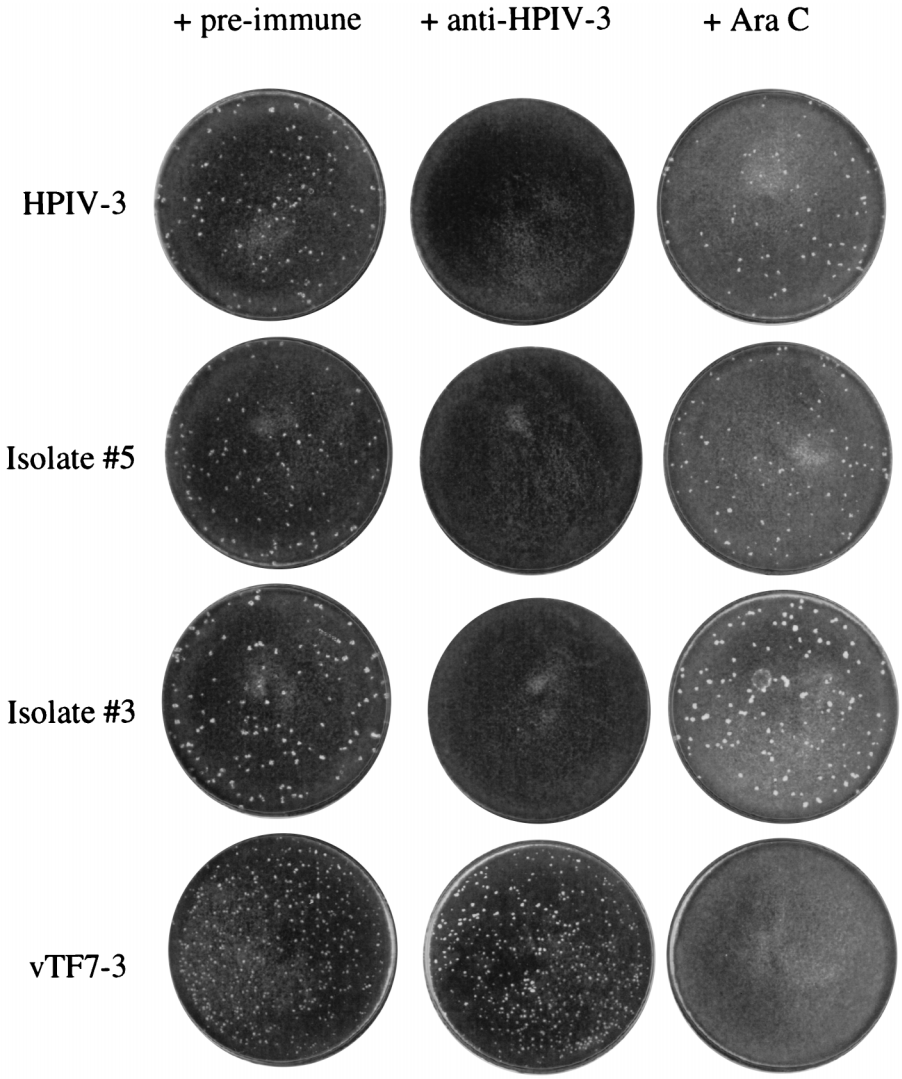

FIG. 3. Identification of recovered virus. Plaques were isolated from virus stocks generated by amplification of the original transfection supernatant. Plaque-purified virus was then amplified in HeLa cells and analyzed by plaque assay. Virus (isolates 3 and 5) was preincubated (30 min on ice) with 5 μl of rabbit preimmune serum or 5 μl of rabbit anti-HPIV-3 antiserum (29) or assayed in the presence of AraC (25 μg/ml). HPIV-3 denotes the parental HPIV-3 strain 47885, while vTF7-3 is the recombinant vaccinia virus which expresses the T7 RNA polymerase.

pHPIV-3 antigenomic transcript contains no AUG codons before the NP initiating codon. The leader sequences of the measles, respiratory syncytial, and Sendai virus infectious clones all contain AUG codons which would interfere with translation initiation at the NP AUG on the antigenomic transcript, making it unlikely that NP could be expressed from these clones.

Second, we showed that two recombinant viruses with distinct genotypes and phenotypes were produced, probably due to recombination between plasmids pHPIV-3 and pPIV3-L, although the possibility that the reversion arises during RNA replication cannot be excluded. pPIV3-L contains the entire L 3′ UTR and part of the trailer region, extending to base 15437, an overlap of 48 bp beyond the *Stu*I site. This is ample room for recombination between plasmids to occur readily in vaccinia virus-infected cells (11, 24, 25). In fact, recombination has been observed in a paramyxovirus infectious clone system (17). The Sendai virus infectious clone was shown to undergo recombination with a support plasmid when a deleterious mutation was present in the full-length construction. Additionally, recombination could even be detected in the absence of any apparent selection.

From our results, there appears to be selection in the HPIV-3 system. All of the large-plaque virus isolates had reverted to a wild-type sequence at base 15389 while retaining the change at base 94. Since A94G is the only known alteration in these viruses from the parental virus, it appears that the mutation at position 94 was beneficial. The isolates which retained both mutations had a plaque size identical to that of the parental (wild-type) virus, but when the 15389 mutations was lost, plaque size increased, indicating that the mutation at base 15389 was detrimental in the context of the A94G mutation. There was one other known change between pHPIV-3 and the support plasmids. A noninitiating AUG exists in the natural L protein message. Since this AUG is only 11 nucleotides from the 5′ end of the L mRNA and in a poor translation initiation context (21), it may not cause much interference with L mRNA translation. However, in the support plasmid pPIV3-L, this

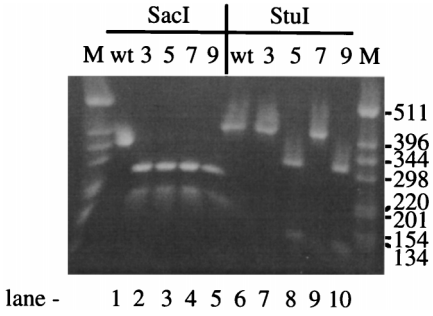

FIG. 4. Identification of genetic tags in recovered virus. Virion RNAs were extracted from plaque-purified virus isolates 3, 5, 7, and 9 and wild-type (wt) HPIV-3 strain 47885 and used as templates for reverse transcription and PCR. PCR products encompassing virus bases 1 to 324 (lanes 1 to 5) and 15080 to 15462 (lanes 5 to 10) were generated from the indicated isolates, digested with *Sac*I and *Stu*I, respectively, and analyzed on a 1.4% agarose gel. Positions of DNA size markers (M) are shown in base pairs. The sizes of the 1 to 324 and 15080 to 15462 PCR products are increased by 21 and 22 bp, respectively, over the length of the virus-specific regions due to the inclusion of restriction enzyme sites in the amplification primers.

noninitiating AUG is much further from the 5′ end of the transcript, where it is more likely to be recognized by ribosomes. This AUG was removed from pPIV3-L by changing bases 8636 and 8637 from AT to TA, destroying an *Sph*I site and creating an *Nhe*I site. To investigate whether this change was present in the recombinant virus and could be responsible for the large-plaque phenotype, RT-PCR analysis was done. PCR products encompassing this site and derived from both the wild-type and the plaque-isolated viruses retained a wild-type sequence, indicating that recombination had not occurred over this region and that these changes could not account for any variance in plaque size.

The observation that the A94G and A15389G mutations, within the 5′ UTR of the NP message and 3′ UTR of the L message, respectively, could alter plaque size demands closer examination. The location of the A94G mutation near the leader and the leader/NP junction suggests a possible role in the regulation of transcription and/or replication. As part of the NP or L mRNA, these mutations could alter the translational efficiency or stability of the mRNA. Currently we are investigating whether these mutations, when engineered into the infectious clone singly, confer increased or decreased plaque sizes, and we are attempting to define the mechanism by which virus containing the A94G mutation replicates with greater efficiency.

The finding that recombination may occur between transfected plasmids is not novel, but it does indicate that care must be taken when one is introducing mutations into the paramyxovirus or rhabdovirus infectious clone systems. Mutations introduced within the NP, P, or L sequence should be carried by both the support plasmids and the infectious clone. Otherwise, the resultant virus may not carry the desired mutation. The only possible exception to this is the HPIV-3 system, in which the HPIV-3 infectious clone expresses NP, negating the need for the NP support plasmid. Still, it may be best to include the HPIV-3 NP support plasmid, since significantly greater yields of HPIV-3 were obtained when the support NP plasmid was included in the transfection.

An infectious clone for HPIV-3 will enhance understanding of the molecular biology of HPIV-3 and may lead to the development of an effective vaccine for this important pathogen. The ability to generate specific mutations within HPIV-3 makes all aspects of HPIV-3 replication amenable to study. Any mutation, including those studied previously in other contexts (20), can now be examined in the most relevant and important context, that of the virus life cycle. The ability to introduce specific mutations also permits the possibility of revertant analysis, which could refine our understanding of protein-protein or protein-RNA interactions.

Analysis of numerous mutations with the infectious clone will probably identify mutations which attenuate the virus. These mutations could be used to develop new vaccine strains of HPIV-3. Alternatively, mutations present in a current candidate vaccine strain of HPIV-3 could be inserted into pHPIV-3. One such HPIV-3 strain was passaged serially at low temperatures and found to contain mutation(s) within the L gene that attenuate growth at 37°C (30, 31). The precise mutation or mutations resulting in the attenuated phenotype are not known, but by inserting specific alterations into pHPIV-3, it should be possible to distinguish between silent mutations and the attenuating mutation(s). Through identifying multiple deleterious mutations, it should be possible to engineer several mutations affecting various steps in the virus life cycle into a single HPIV-3 strain. Such a virus may be highly attenuated and not readily able to revert.

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