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Temperature-Sensitive Phenotype of the Human Parainfluenza Virus Type 3 Candidate Vaccine Strain (cp45) Correlates with a Defect in the L Gene

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We have previously demonstrated that the temperature sensitivity of a human parainfluenza virus type 3 (HPIV-3) candidate vaccine strain (cp45), which is currently under evaluation in humans, is associated with poor transcriptional activity of the virus at the nonpermissive temperature (R. Ray, K. Meyer, F. Newman, and R. B. Belshe, J. Virol. 69:1959–1963, 1995). In this study, the temperature sensitivity of cp45 virus was further investigated by the complementation of a specific gene function. CV-1 cells were transfected with cloned genes from wild-type HPIV-3 encoding the large protein (L), phosphoprotein (P), and nucleocapsid protein (NP), alone or together, for the expression of biologically active proteins. Only cells expressing the L gene were able to rescue cp45 replication when incubated at the nonpermissive temperature (39.5°C), whereas cells transiently expressing NP or P were incapable of rescuing the virus. The virus titers obtained following complementation of the L protein were 190 to 2,300 PFU/ml of culture medium, compared with the undetectable growth of the cp45 temperature-sensitive mutant at the nonpermissive temperature. Rescued progeny virus still maintained the temperature-sensitive phenotype. Results from this study suggest that the temperature sensitivity of the cp45 candidate vaccine strain is associated primarily with L-protein function and that the defect can be complemented by transient expression of the wild-type protein. This study underscores the importance of the L protein in RNA polymerase activity and its critical role in virus replication.

Human parainfluenza virus type 3 (HPIV-3) is second only to respiratory syncytial virus as a cause of severe lower respiratory tract disease in neonates and young infants. The HPIV-3 genome is a single-stranded negative-sense RNA and encodes at least six structural proteins [3′-NP-P(+C)-M-F-HN-L-5′] (48, 50). The most abundant structural protein, the nucleocapsid protein (NP), encapsidates the genomic RNA and is believed to maintain the structural integrity and template function of the genome (19). Two additional proteins, a large protein (L) and a phosphoprotein (P), are associated with the NP-RNA template (nucleocapsid core), which together constitute the nucleocapsid complex. The L protein is believed to function as the RNA-dependent RNA polymerase, and P may function as an auxiliary regulatory protein essential for the function of L (44). During primary transcription, the L-P complex interacts with the nucleocapsid core to transcribe the genomic RNA into individual mRNAs (12). In addition, during replication in an infected cell, NP may form a soluble complex with P. This complex is thought to interact with transcribing nucleocapsid complexes to switch from primary transcription to replication of the viral RNA (25, 56).

A cold-adapted HPIV-3 isolate is currently under evaluation in humans as a candidate vaccine (3, 7, 10). The parent virus (JS strain of HPIV-3) was passaged at reduced temperatures 45 times, resulting in a temperature-sensitive (*ts*) virus (cp45) that was highly attenuated for growth in the respiratory tract of hamsters. The reduction of virus replication was directly related to the cold passage level of the virus. Studies using seronegative chimpanzees showed that vaccinated animals were protective to challenge infection (21). Evaluation in children has revealed the virus to be highly attenuated (1a).

Understanding the molecular mechanisms involved in attenuation of cp45 may provide a basis for the continued development or design of an efficacious parainfluenza virus vaccine. We have previously demonstrated that the *ts* property of cp45 is associated with poor transcriptional activity of the virus at a nonpermissive temperature (42). Virus mRNA synthesis is markedly reduced at 39.5°C, and as a result, polypeptide synthesis and virus growth are significantly affected. Our results show that although the transcriptional activity of cp45 is reduced, viral glycoproteins were transported to the cell surface and limited viral morphogenesis occurred at the nonpermissive temperature. The biological properties and antigenic sites of the envelope glycoproteins, defined by reactivity to a panel of monoclonal antibodies (8, 9, 40, 41), remained unaffected as a result of cold adaptation.

In this study, we have further defined the molecular mechanism involved in the temperature sensitivity of cp45 by the complementation of a specific gene function. We observed that the virus could be rescued at the nonpermissive temperature in cells transiently expressing the wild-type L protein, which supports the concept that the reduced transcriptional activity of cp45 is associated with mutations in the L gene.

Expression of HPIV-3 NP, P, and L proteins from cloned DNAs. The molecular cloning and sequence analysis of the HPIV-3 NP, P, and L genes have been previously described (16–18). All genes were removed from their recombinant vectors by restriction endonuclease digestion and ligated into the appropriate sites of pcDL-SR beta 8.2 vector DNA (15a). This

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vector is derived from pcDL-SR alpha-296 (51) and contains a polyvalent restriction site with flanking T7 and SP6 promoter sequences downstream from the SR alpha promoter. pcDL-SR beta 8.2 is a multifunctional vector, and gene expression can be driven by using a simian virus 40 (SV40) early promoter or alternatively by using a vaccinia virus expressing T7 RNA polymerase.

Plasmid pSP18-NP, which contains the NP gene, was first digested with *Sph*I, and the resulting cohesive end was repaired with T7 DNA polymerase. The gene was then released from the vector with *Bam*HI and ligated into pcDL-SR beta 8.2 which had been digested with *Eco*RI, subsequently repaired with Klenow fragment, and then further treated with *Bam*HI. Plasmid pSP19-P, which contains the P gene, was digested with *Bam*HI and *Pvu*II to release the P gene. The gene was subsequently ligated into pcDL-SR beta 8.2 which had been digested with *Xba*I, subsequently repaired with Klenow fragment, and then further treated with *Bam*HI. Plasmid pGEM3-L, containing the L gene, was first digested with *Hin*dIII, and the resulting cohesive end was repaired with Klenow fragment. The L gene was then released from the vector with *Sac*I and cloned into pcDL-SR beta 8.2 which had been digested with *Eco*RI, subsequently repaired with Klenow fragment, and then further treated with *Sac*I. All ligation reactions consisted of vector and gene fragments with compatible ends which would force ligation of the inserts in the desired orientation relative to the SR alpha and T7 promoters. Recombinant clones were randomly picked and further analyzed by restriction endonuclease digestion to confirm the orientation and efficacy of the subcloning. The gene termini were confirmed by dideoxy sequence analysis (U.S. Biochemical, Cleveland, Ohio) to ensure that the initiating methionine and termination codons were maintained.

To examine the biological properties of the various nucleocapsid-associated proteins, we initially tested for the protein expression of L (L-11), P (P-1), and NP (NP-1) in a transient expression system (15), using a recombinant vaccinia virus containing the bacteriophage T7 RNA polymerase gene (vTF7-3). HeLa-T4 cells, which are relatively resistant to the cytopathic effect of vaccinia virus, were infected with vTF7-3 and transfected with plasmids containing the HPIV-3 L, P, or NP gene, using Lipofectamine (Bethesda Research Laboratories, Gaithersburg, Md.) in a procedure similar to one described previously (26). Expression of the viral proteins was detected after 20 h in [^{35}S]methionine-[^{35}S]cysteine-labeled transfected cell lysates by immunoprecipitation with a hyperimmune rabbit antiserum to HPIV-3 or a monoclonal antibody to NP (Fig. 1). Immunoprecipitates were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and autoradiography. To obtain better resolution of the large-molecular-size L protein, immunoprecipitates were also separated in a lower-percentage polyacrylamide gel (Fig. 1A). Immunoprecipitated L, P, and NP polypeptides from the corresponding DNA-transfected cells were indistinguishable in size from the authentic viral proteins (38). The amount of the L protein appeared to be lower than that of the P or NP protein immunoprecipitated by the antiserum from transfected cell lysates.

Complementation of cp45 replication by HPIV-3 gene-transfected cells. Although the vaccinia virus system provided excellent levels of expression, because of the concern regarding the use of a viral vector which causes extensive cytopathic effect in the cell monolayer and the difficulties inherent to measuring HPIV-3 replication in vaccinia virus coinfection experiments, we used an expression system which did not require the use of vaccinia virus to drive transcription of our plasmid vectors. The vector pcDL-SR beta 8.2 contains a novel promoter, designated SR alpha, consisting of the SV40 early promoter and the R segment and part of the U5 sequence (R-U5′) of the long terminal repeat of human T-cell leukemia virus type 1 (51). The vector also contained the SV40 origin of replication and gives significant levels of protein expression in COS-1 or COS-7 cells, in which the plasmids are amplified by the endogenous levels of T antigen. The utility of this expression system was extended to CV-1 cells by coexpression in *trans* of the large T antigen from another plasmid vector, pRSV-T (35), kindly provided by James Pipas, University of Pittsburgh.

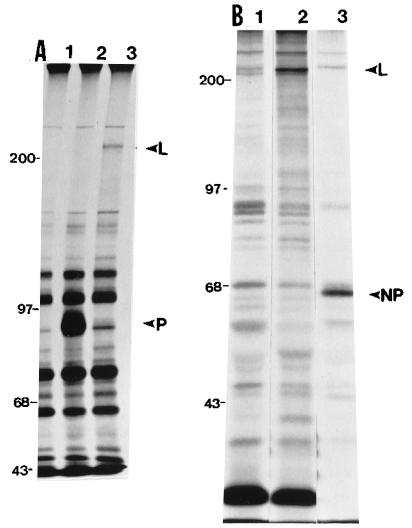

FIG. 1. Immunoprecipitation of HPIV-3 proteins expressed by using the vaccinia virus-T7 system. HeLa-T4 cells were transfected with plasmids containing the HPIV-3 L, P, or NP gene. Approximately 20 h posttransfection, the cells were radiolabeled with [^{35}S]methionine-[^{35}S]cysteine at 37°C for 1 h. The HPIV-3 proteins from cell lysates were immunoprecipitated with a rabbit antiserum to HPIV-3 or a monoclonal antibody to NP and analyzed by SDS-PAGE. Lanes: 1 (A and B), vector DNA-transfected cell lysates immunoprecipitated with a rabbit antiserum to HPIV-3; 2 (A), P-gene-transfected cell lysates immunoprecipitated with a rabbit antiserum to HPIV-3; 3 (A) and 2 (B), L-gene-transfected cell lysates immunoprecipitated with a rabbit antiserum to HPIV-3; 3 (B), NP-gene-transfected cell lysates immunoprecipitated with a specific monoclonal antibody. Labeled proteins were electrophoresed on SDS–7.5% (A) and SDS–10% (B) polyacrylamide gels in the presence of the reducing agent 2-mercaptoethanol. Positions of molecular weight markers are shown on the left in kilodaltons.

CV-1 cells were cotransfected with plasmid vector pRSV-T (encoding the SV40 large T antigen driven by a Rous sarcoma virus long terminal repeat) and recombinant plasmid DNA (L, P, or NP). The transfected cells were tested initially for intracellular expression of the viral proteins by immunofluorescence, using a procedure similar to one described previously (39). Briefly, cells were fixed with acetone-methanol (1:1) for 10 min at −20°C. A hyperimmune rabbit antiserum to HPIV-3 (thoroughly preadsorbed with mock-transfected cells) was used as the primary antibody, and a fluorescein isothiocyanate-tagged mouse anti-rabbit immunoglobulin G was used as the second antibody. Cells were examined at a magnification of ×600 on a Nikon microscope equipped for epifluorescence, and digital photographs were captured by using a computer imaging system (Oncor Image Systems, Inc.). L and P appeared to form small inclusions, whereas NP seemed to give a more homogeneous, punctate staining pattern (Fig. 2). Negative control cells did not show any detectable immunofluorescence. These HPIV-3 genes were tested to determine whether CV-1 cells which were transiently expressing the proteins could rescue cp45 at the nonpermissive temperature. cp45 was derived from the JS strain, an isolate originally cultured from a

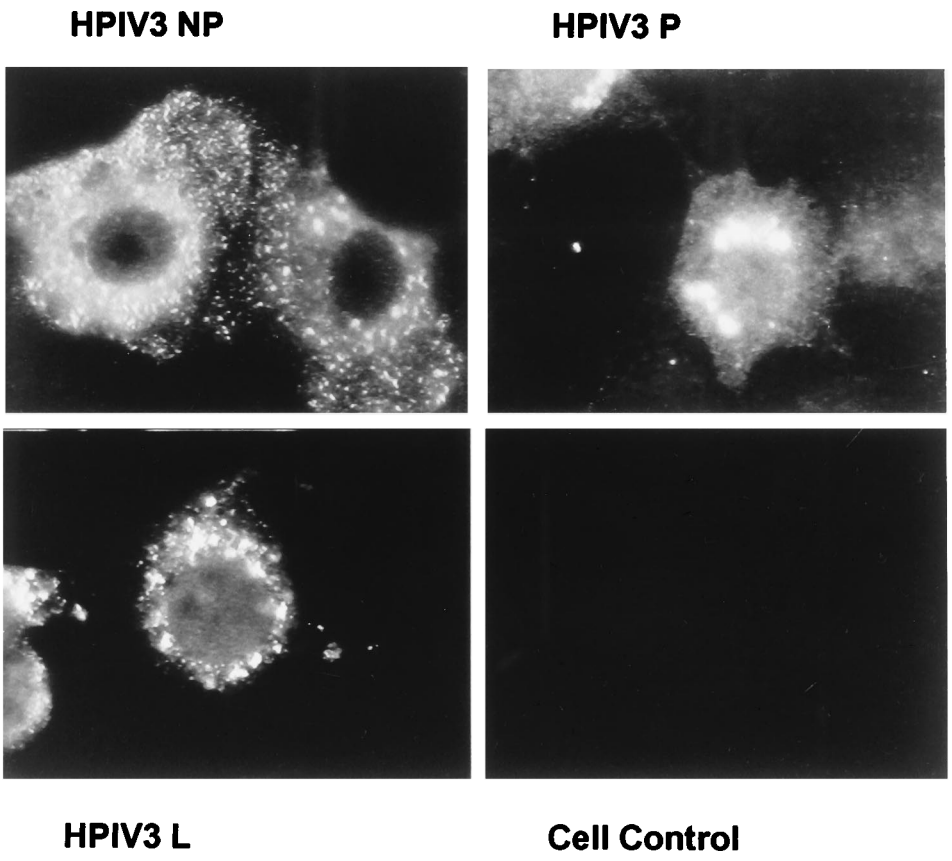

FIG. 2. Indirect immunofluorescence staining of cells transiently expressing HPIV-3 NP, P, and L proteins. Cells expressing HPIV-3 NP, P, L, and control cells transfected with vector DNAs were fixed and reacted with a primary rabbit anti-HPIV-3 antibody and a secondary mouse anti-rabbit immunoglobulin G-fluorescein isothiocyanate conjugate for immunofluorescence.

child with febrile respiratory disease. A cold-adapted mutant was selected after serial passage of the virus 45 times at 20°C and isolated by plaque purification (2). The cp45 virus was subsequently grown at 32°C in continuous cell lines (2). Previously we demonstrated (42) that the *ts* mutation of cp45 is associated with poor transcriptional activity of the virus at a nonpermissive temperature. The entire genomic RNA of cp45 has been sequenced and is known to contain three amino acid changes in the L protein but does not exhibit any amino acid changes in either the NP or P protein (49). Evaluation of the ability of recombinantly expressed wild-type HPIV-3 L protein to complement the *ts* mutation of the cp45 isolate was performed to further examine whether the mutations in the L protein were responsible for the altered phenotype.

CV-1 cells were cotransfected with the plasmid vector pRSV-T and one or more recombinant plasmids containing the NP, P, or L gene and incubated at 37°C. Twenty hours posttransfection, the expressing cells were infected with cp45 or wild-type virus at a multiplicity of infection of 1, and infected cells were incubated for an additional 28 h at 39.5°C. Following incubation, cell culture medium was harvested and HPIV-3 titers were determined by plaque assay in L-132 cells at permissive (32°C) and nonpermissive (39.5°C) temperatures, using a methodology similar to one described previously (2). Infected cell monolayers were stained with hematoxylin and eosin-Y or, alternatively, overlaid with 0.9% agar and 0.005% neutral red (50) for visualization of viral plaques. The results of a typical complementation experiment are shown in Table 1; similar results were achieved in assays performed in triplicate. Virus yield at 39.5°C showed significant levels of cp45 replication at the nonpermissive temperature. These results indicate that the wild-type L protein was biologically functional and could complement the *ts* mutation of the cp45 L protein, whereas expression of P or NP in the absence of L was nonfunctional. The virus titer represents a complementation efficiency of approximately 950 and 11,500 PFU of virus per 3×10^6 cells. Cells transfected with L, P, and NP show a 10-fold increase in cp45 virus yield compared with cells transfected with L alone or with

TABLE 1. Complementation assay for recovery of cp45 virus at the nonpermissive temperature (39.5°C)

Gene(s) used in complementation[a]	Virus recovery titer (PFU/ml of culture medium) at 32°C[b]
None	<1.0
L, P, and NP	2.3×10^3
L and P	1.9×10^2
L	3.3×10^2
P	<1.0
NP	<1.0

[a] CV-1 cells were cotransfected with plasmid DNA (pRSV-T) encoding the SV40 large T antigen and the recombinant plasmid DNAs (L, P, and/or NP). Cells were infected with cp45 20 h posttransfection and incubated at 39.5°C for 28 h.

[b] Virus replication was measured from culture medium by plaque assay on L-132 cells.

L and P. This may be due to interactions among these proteins for the formation of the nucleocapsid complex for efficient virus replication. However, their specific interactions during virus replication remain to be determined. Results from this study further support the role of the L protein as an RNA-dependent RNA polymerase activity essential for transcription and the life cycle of HPIV-3. Other cell lines, not transfected with the L gene, failed to produce detectable virus titers. In addition, L-132 or primary rhesus monkey kidney cells, when coinfected with HPIV-1 and cp45, did not rescue growth of cp45 at the nonpermissive temperature. This result indicated heterotypic exclusion of L-protein complementation for cp45. cp45 virus produced from L-gene-transfected CV-1 cells at the nonpermissive temperature should remain *ts* for growth despite their ability to replicate in L-expressing cells. At least 10 plaque-purified virus stocks of the progeny rescued virus were examined, and all virus stocks were found to have maintained their *ts* property.

The capacity to express individually cloned genes has allowed investigations into the protein functions of a number of mutant viruses (27, 29–32, 43, 55). In this study, we have shown that the recombinant L protein of wild-type HPIV-3 expressed in CV-1 cells is able to transcribe the viral genome, resulting in replication of cp45 at the restrictive temperature.

Comparison of the nucleotide and predicted amino acid sequences of cp45 and the parent wild-type JS strain revealed seven amino acid substitutions in genes encoding four different polypeptides, M, F, HN, and L (49). Results from our previous studies with cp45 did not show changes in the biological properties of the virus glycoproteins when cells were grown at permissive and nonpermissive temperatures as observed with *ts* mutants of other paramyxoviruses (36–38, 52, 53) or influenza virus (46). However, the *ts* phenotype appears to correlate with changes in the transcriptional activity of cp45, perhaps due to alterations in the L protein. Three unique changes in the L protein were reported: Tyr to His, Leu to Phe, and Thr to Ile, with substitutions at residues 942, 992, and 1558, respectively (49). While the results reported here support the idea that the L-gene mutations are responsible for the *ts* phenotype, viewed from another context, these mutations may function to enhance the transcriptional activity of L at reduced temperatures and may confer the cold adaption phenotype to cp45. Although the idea is speculative, the His and Phe substitutions, at residues 942 and 992, may represent the critical residues involved in the determination of the *ts* phenotype. It has been reported that histidine-phenylalanine interactions may be involved in stabilizing the C-peptide helix of RNase A (47) and in a model alanine-based alpha helix (1). Further, these interactions are pH dependent. Since intracellular pH changes are affected by temperature (20), the ability of the L protein to function at a reduced temperature may be moderated through conformational changes in L and dependent on a histidine-phenylalanine interaction. The 3′ noncoding leader sequence, which is important in gene regulation and in virus propagation, also showed several nucleotide changes. Characterization of a cold-adapted influenza A virus vaccine strain suggested conformational changes in the RNA structure facilitating advantageous growth at 25°C (24). Mutations in the noncoding regulatory regions, which occurred as a result of the attenuation process, have been suggested to affect transcription and/or replication of polioviruses (4, 54). Recently, mutations within the 3′ noncoding terminal sequences of Sendai virus have been shown to be sensitive to mutagenesis (23). Similarly, nucleotide changes in the 3′ leader region of cp45 may affect the temperature sensitivity or attenuation property of the virus and require further investigation.

The genetic stability of cp45 and the multiple defects suggest that cp45 will be a suitable vaccine. To date, clinical trials suggest this possibility. The *ts* defect clearly is not the only lesion involved with attenuation (22). Revertant non-*ts* viruses from an earlier clone (cp18) were still attenuated when evaluated in primates (21). Studies in infants are in progress, and cp45 is highly attenuated and immunogenic (1b). A reverse-genetics approach recently developed for influenza A virus has allowed for direct genetic manipulation of specific viral genes (14). This general approach has been adapted and used successfully to establish similar transfection systems employing parainfluenza viruses (5, 6, 11, 13, 33, 34). More recent success with the generation of infectious rabies virus and vesicular stomatitis virus (28, 45) by reverse genetics hold promise for similar approaches with HPIV-3. The *ts* cp45 mutant may find use as a helper virus for the rescue process in reverse-genetics engineering of HPIV-3. This will allow us to answer a number of important fundamental questions involving the further characterization of the specific mutations in the viral genome of cp45 and for the recovery of clonal virus stocks from a defined sequence, if necessary, for the further development of this live attenuated human parainfluenza virus vaccine.

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