Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

VIROLOGY 186, 543–550 (1992)

RNA Editing in the Phosphoprotein Gene of the Human Parainfluenza Virus Type 3

MARK S. GALINSKI, ROBERTA M. TROY, AND AMIYA K. BANERJEE

Department of Molecular Biology, Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195

Received May 14, 1991; accepted October 28, 1991

RNA editing of the human parainfluenza virus type 3 (HPIV3) phosphoprotein (P) gene was found to occur for the accession of an alternate discontinuous cistron. Editing occurred within a purine-rich sequence (AAUUAAAAA-AGGGGG) found at the mRNA nucleotides 791–805. This sequence resembles an HPIV3 consensus transcription termination sequence and is located at the 5′-end of the putative D protein coding sequences. Editing at an alternate site (AAUUGGAAAGGAAAGG), mRNA nucleotides 1121–1136, for accession of a conserved V cistron, which is present in a number of paramyxovirus P genes, was not found to occur in HPIV3. In contrast with many other paramyxoviruses, editing was indiscriminate with the insertion of 1–12 additional G residues not present in the gene template. RNA editing was found to occur in both *in vivo* (HPIV3 infected cells) and *in vitro* (purified nucleocapsid complexes) synthesized mRNAs. Further, the *in vitro* prepared mRNA was edited regardless of whether the nucleocapsid complexes were transcribed in the presence or absence of uninfected human lung carcinoma (HLC) cell lysates. These results support the notion that RNA editing appears to be exclusively a function of viral proteins. © 1992 Academic Press, Inc.

INTRODUCTION

The phosphoprotein genes of members of the *Paramyxovirus* and *Morbillivirus* genera of the *Paramyxoviridae* family are unique among the nonsegmented negative-strand RNA viruses in their capacity to encode multiple proteins (Kingsbury 1990, 1991). Interestingly, the protein coding information is contained within a variety of continuous, discontinuous, and overlapping cistrons. Accession of this information is dependent upon transcriptional editing of the primary mRNA transcripts (discontinuous cistrons) as well as translation initiation at alternate codons (overlapping and continuous cistrons).

Molecular cloning and sequence analysis of the simian virus type 5 (SV5) phosphoprotein (P) gene/mRNA revealed that insertion of nontemplated nucleotides during transcription of the P mRNA was necessary for the fusion of two discontinuous (and overlapping) cistrons in order to produce a template capable of encoding a complete P protein (Thomas *et al.*, 1988). The addition of nontemplated nucleotides in the P mRNA occurred within a guanosine track just downstream from a purine-rich sequence which closely resembles an SV5 transcription terminator. Subsequently, similar observations for RNA editing were made for measles virus (Cattaneo *et al.*, 1989); mumps virus (Elliott *et al.*, 1990; Paterson and Lamb, 1990); human parainfluenza viruses types 2 (Ohgimoto *et al.*, 1990; Southern *et al.*, 1990), 4A, and 4B (HPIV4A and HPIV4B; Kondo *et al.*, 1990); bovine parainfluenza virus type 3 (BPIV3; Pelet *et al.*, 1991); and Sendai virus (Vidal *et al.*, 1990). While SV5, HPIV2, HPIV4A, HPIV4B, and mumps viruses require the insertion of nontemplated nucleotides into their respective P mRNAs for the synthesis of complete P proteins, measles virus (Bellini *et al.*, 1985), HPIV1 (Matsuoka *et al.*, 1991), HPIV3 (Galinski *et al.*, 1986b; Luk *et al.*, 1986; Spriggs and Collins, 1986b), BPIV3 (Sakai *et al.*, 1987; Pelet *et al.*, 1991) and Sendai virus (Giorgi *et al.*, 1983) do not. This latter group of viruses encode their P proteins in single continuous cistrons; however, in addition to the continuous P cistrons, these genes contain overlapping (continuous) C protein cistrons which are accessed via independent translation initiation.

A notable feature of the 5′-proximal (mRNA) cistron in SV5 (designated as protein V in SV5), mumps, HPIV4A, and HPIV4B is the presence of a conserved amino acid sequence (approximately 55 amino acids) at the C-termini of the encoded proteins. A prominent feature among the residues in this domain is the presence of six highly conserved cysteine residues. The similarity of these sequences to motifs found in nonrepetitive zinc fingers has been noted (Thomas *et al.*, 1988). The existence of similar amino acid sequences in "cryptic" cistrons, present in the P genes of other paramyxoviruses, suggests some functional or biological significance for the maintenance of these sequences within this group of viruses. These observations, together with the known association of the P protein with the nucleocapsid complex (Chinchar and Portner, 1981; Deshpande and Portner, 1985; Hamaguchi *et al.*,

0042-6822/92 $3.00

1983; Ryan and Kingsbury, 1988; Ryan *et al.,* 1990; Ryan and Portner, 1990), would suggest a transcriptional regulatory function for this domain.

The HPIV3 P gene/mRNA nucleotide sequence has been detailed in a number of independent reports (Galinski *et al.,* 1986b; Luk *et al.,* 1986; Spriggs and Collins, 1986b). The molecular organization of the P gene allows for the encoding of continuous cistrons for the P, C, and putative D proteins. And, as described above, a cryptic V cistron, which shares 33% amino acid identity with the C-terminus of the SV5 V protein, is also present. A sequence, similar to the SV5 RNA-editing site, is adjacent to the cryptic V cistron and an additional sequence, similar to an HPIV3 transcription terminator, is present upstream of the D cistron. In both instances, purine-rich tracts are followed by a string of guanosine residues. Interestingly, the number of G nucleotides reported from the various laboratories in the mRNA sequences proximal to the D cistron differ, with 5, 8, or 11 Gs being reported.

In this report we characterize the transcriptional editing that occurs in HPIV3. The results presented demonstrate that RNA editing occurs proximal to the D cistron and not at the V cistron, and that the number of nucleotides inserted at the D editing site is indiscriminate. In addition to mapping the location of transcriptional editing, we provide evidence that this process is solely a function of viral proteins and does not appear to require any cellular factor(s).

MATERIALS AND METHODS

Cell lines and virus

The preparation and maintenance of the CV-1 cell line and viral stock of HPIV3 (HA-1; NIH 47885; catalog number V323-000-020) was as previously described (Galinski *et al.,* 1986a). Radiolabeling of uninfected and HPIV3-infected CV-1 cells was as previously described (Wechsler *et al.,* 1985) using [^{35}S]methionine.

Preparation of RNA

The various RNA species, including total cytoplasmic RNA and nucleocapsid-associated RNA, were prepared from uninfected and HPIV3-infected CV-1 cells as previously described (Galinski *et al.,* 1986a). The recombinant clone, 33-9, containing a full-length copy of the P mRNA in the transcription vector pSP19, was used to synthesize RNA with a "fixed" sequence as previously described (Galinski *et al.,* 1986b).

Nucleocapsid-associated genomic RNA (vRNA) was obtained from HPIV3-infected CV-1 cells by banding nucleocapsid cores in cesium chloride as previously described (Udem and Cook, 1984; Galinski *et al.,* 1986a). Free cytoplasmic RNA, which pelleted through the cesium chloride, consisted of total cytoplasmic RNA depleted of any viral genomic/antigenomic RNA.

In vitro synthesized mRNA, a generous gift from Bishnu De (Cleveland Clinic Foundation), was prepared as previously described (De *et al.,* 1990, 1991) using purified nucleocapsid complexes in the presence or absence of cell lysates. Transcribed mRNA was removed from the genomic vRNA following oligo dT-cellulose chromatography.

cDNA amplification and recombinant library construction

cDNA was prepared using various RNA templates and synthetic oligodeoxyribonucleotides complementary to the genomic viral RNA (vRNA) nucleotides 701–720 (5′-TGCTGCGACACCAGATGATG; P + 701) or complementary to the P mRNA nucleotides 1221–1240 (5′-TTGTCTTGATATAAATCTAG; P-1240), as previously described (Galinski *et al.,* 1986a). Briefly, the RNA templates were mixed with 200 ng of each primer in water, incubated at 95° for 1 min, and allowed to cool on ice. The reactions were adjusted with 10× stocks to a final concentration of 50 m*M* Tris–HCl, pH 8.0, 50 m*M* KCl, 10 m*M* dithiothreitol, 5 m*M* magnesium chloride, 30 U/30 μl RNAsin, 20 U/30 μl AMV reverse transcriptase, and incubated at 37° for 60 min.

Four (30 μl) cDNA reactions were performed using the following RNA templates (and 200 ng primer): (1) total cytoplasmic RNA from uninfected CV-1 cells (P-1240), (2) total cytoplasmic RNA (depleted of nucleocapsid vRNA) from HPIV3 infected CV-1 cells (P-1240), (3) nucleocapsid vRNA (P + 701), and (4) SP6 *in vitro* transcribed P specific mRNA (P-1240) as previously described (Galinski *et al.,* 1986a). Following first strand cDNA synthesis, the entire reaction was transferred to a 500 μl eppendorf tube and adjusted with 10× stock solution to make a final concentration of 10 m*M* Tris–HCl, pH 8.0, 50 m*M* potassium chloride, 1.5 m*M* magnesium chloride, 0.250 m*M* each deoxyribonucleotide triphosphate, 200 ng/100 μl P + 701/P-1240 primer, and 2U/100 μl Taq DNA polymerase. The reaction was overlaid with mineral oil and amplified by polymerase chain reaction (PCR) in a Perkin–Elmer Cetus DNA Thermal Cycler using the following parameters: initial denaturation, 94°, 7 min; cycle denaturation, 94°, 3 min; cycle annealing, 37°, 2 min; cycle extension, 72°, 3 min; final extension, and 72°, 3 min. The PCR reaction was run for 35 cycles and then held at 4°. Following the amplification of the cDNA, the ends of the amplified DNA were repaired by the addition of 1 U of Klenow fragment to the PCR reaction and further incubated at 37° for 30 min. The amplified cDNA was puri-

fied following electrophoresis in low melting 1% agarose gels as previously described (Galinski *et al.*, 1983).

The PCR amplified DNA was cloned into the *Sma*I site of the vector pGEM-3. Following ligation with T4 DNA ligase, the samples were treated with *Sma*I to reduce the background intramolecular religations of the vector. Plasmids were transformed into the bacterial host JM83 and selected with ampicillin as previously described (Galinski *et al.*, 1986a). Recombinant clones were identified using P gene-specific probes which were prepared by end-labeling of restriction endonuclease fragments. The fragments were separated on 1% low melting agarose gels and then used to probe recombinant colonies by *in situ* hybridization as previously described (Galinski *et al.*, 1986a). Mini plasmid preparations of positive colonies were prepared and sequenced by dideoxy sequence analysis (U.S. Biochemical Sequenase Kit, following the manufacturer's protocol) using the same oligonucleotides utilized above for cDNA amplification.

Preparation of transcription vectors for specific P mRNAs

Since the PCR-generated clones were not full-length copies of the P gene/mRNA, reassembly of transcription vectors containing defined sequences was performed. Selected PCR generated clones, with either six or seven Gs were used to reconstruct full-length P gene sequences. A *Fok*I to *Cla*I restriction endonuclease fragment spanning the editing/chatter site was reassembled into the plasmid 33-9 by religation of a *Bam*HI to *Fok*I fragment from the clone 33-9 (N-terminus P) and a *Cla*I to *Bam*HI fragment from the clone 33-9 (C-terminus of P and pSP19 vector). Recombinant clones were identified by *in situ* hybridization and sequenced across the fused fragments domains as previously described (Galinski *et al.*, 1986b).

Transcription, translation, and protein gel electrophoresis

The recombinant pSP19 plasmids 33-9 (containing a full-length P protein gene) and pPG6 and pPG7 (containing six and seven Gs, respectively, at the D editing site) were used to produce RNA transcripts, following restriction endonuclease digestion with *Pvu*II, as described by Konarska *et al.* (1984). The RNA transcripts, recovered following DNAse I treatment, phenol extraction, and successive ethanol precipitations, were then used to charge a translation system.

Translation of *in vivo* and *in vitro* produced RNA transcripts was performed in a rabbit reticulocyte lysate as directed by the manufacturer (Promega Biotec) using

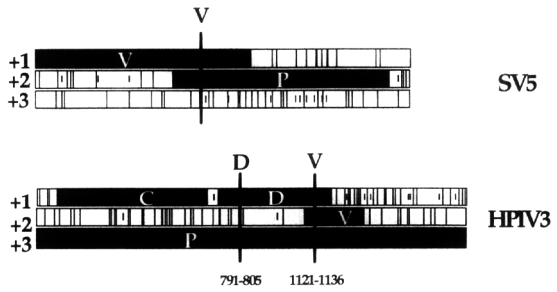

FIG. 1. The stop codon usage map and cistron placement of SV5 and HPIV3 P genes. Termination and methionine codons are indicated by complete and partial vertical lines, respectively. Cistrons encoding the V and P proteins of SV5 are filled. The RNA editing site at the carboxyl-end of the V cistron is denoted with a vertical line spanning the three alternate reading frames. Similarly, the cistron placement of the P, C, D, and V proteins of HPIV3 are indicated. The putative editing sites for accession of the V (nucleotides 1121–1136) and D (nucleotides 791–805) cistrons are shown with vertical lines spanning the three alternate reading frames.

[^{35}S]methionine as the radiolabel. The translation products, total or immune precipitated, were resolved by polyacrylamide gel electrophoresis on 10% polyacrylamide gels (Maizel, 1971), followed by fluorography (Bonner and Laskey, 1974).

Computer analysis

Analysis of nucleotide and protein sequences was performed using algorithms built by Queen and Korn (1984; MicroGenie) and Devereux *et al.* (1984; Genetics Computer Group).

RESULTS

The utilization of discontinuous cistrons for encoding a paramyxoviral P protein was first described for SV5 (Thomas *et al.*, 1988). Figure 1 illustrates the cistron usage map of the SV5 P gene as compared with that for HPIV3. HPIV3 follows the more general pattern of a single continuous P cistron with an alternate overlapping C cistron similar to that first described for Sendai virus (Giorgi *et al.*, 1983; Shioda *et al.*, 1983). Cloning and sequence analysis of the HPIV3 mRNA/genes by a number of labs (Galinski *et al.*, 1986b; Luk *et al.*, 1986; Spriggs and Collins, 1986b; Galinski, unpublished observations) revealed a heterogeneity in the number of G nucleotides between position 801 and 805, which is proximal to the 5′-end of the D cistron (Fig. 1, labeled D). Interestingly, the sequence just upstream of this G tract, AATTAAAAAA, resembles the consensus transcription termination sequence, AATAACAAAAA, found in the gene-end boundaries of HPIV3 (Spriggs and Collins, 1986a). Together with the observation that transcriptional editing occurs in a

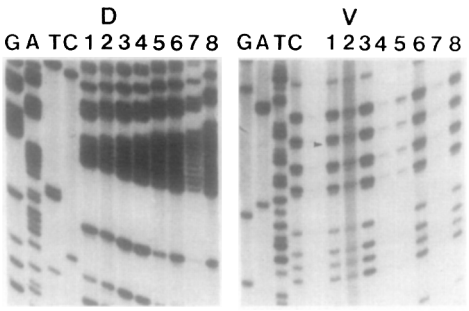

FIG. 2. Composite nucleotide sequence analysis of molecularly cloned PCR amplified cDNA. The PCR amplified cDNA using total mRNA as the template was molecularly cloned into the SmaI site of pGEM-3. Recombinant clones were identified and utilized for sequence analysis as described under Materials And Methods. The same oligodeoxyribonucleotides used for the amplification of the cDNA were used to generate the dideoxy sequences shown in this figure. The sequences spanning the putative editing sites for accession of the D and V cistrons (separate panels) are shown as composites. The complete sequence is indicated as GATC, while only the ddG (D) or ddC (V) reactions from several different clones are shown. The insertion of numerous G residues into the mRNA template is readily apparent in (D), while the sequences in (V) are invariant in all the clones. The putative position for RNA editing in (V) is indicated with an arrowhead. All of the cDNA clones derived from nucleocapsid RNA contained invariant sequences at both the (D) and (V) editing sequences.

number of paramyxoviruses at similar sequences suggests that editing is occurring at this site for accession of the D cistron. Since the cryptic V cistron is also present in the HPIV3 P gene, the question of whether transcriptional chattering occurs in the accession of the D and/or V cistrons needed to be resolved.

Initially, restriction fragment length polymorphisms present in PCR amplified cDNA was examined. The results of restriction site analysis indicated that the PCR amplified cDNA was homogeneous (data not shown). Thus it was necessary to analyze individual cloned molecules to determine whether any sequence heterogeneity was present. The amplified cDNA was cloned into the *SmaI* site of pGEM-3 and recombinant clones were identified by *in situ* hybridization using radiolabeled P gene-specific sequences as a probe. Individual clones were then sequenced across the putative editing sites.

Approximately 50 clones, derived from either vRNA or mRNA templates, were examined. All the clones from the vRNA template had invariant sequences spanning both putative editing sites (the sequence was identical to that determined by dideoxy sequence analysis of the vRNA). In contrast, clones derived from the mRNA template had variable numbers of G residues at the D editing site and an invariant sequence at the V editing site. The results for the mRNA derived clones are illustrated in Fig. 2. This figure is a composite of information (dideoxy G or dideoxy C sequencing reactions only) from several representative mRNA clones aligned with the precise sequence spanning the two editing sites derived from a genomic RNA clone. Panel D shows the sequence across the D editing site while panel V shows the sequence across the V editing site. This figure serves to illustrate the indiscriminate insertion of additional G residues into the mRNA template at the D editing site beyond the 5 Gs which are present in the genomic template and, further, the absence of any additional G insertions at the V editing site. The number of Gs inserted into the mRNA template varied from 5–14 residues. These results demonstrate that RNA editing occurs exclusively at the D editing site.

Inspection of Fig. 1 shows that downstream of the D editing site there are a number of stop codons in the same reading frame (+2) as the V cistron. Thus any editing which occurs with the insertion of 1, 4, 7, or 10, additional G residues would result in the generation of a truncated P cistron following fusion to the termination codons.

The role of host cell factors in optimal HPIV3 transcription has recently been described (De *et al.*, 1990, 1991). Extension of these observations to address the question of whether host cell factors participate in RNA editing, or if this process is dependent solely upon viral components was pursued. Nucleocapsid complexes were isolated from purified HPIV3 virions and used in an *in vitro* transcription system. Transcription was performed either in the presence or absence of uninfected cell lysates. The mRNA was separated from the genomic RNA by oligo dT-cellulose chromatography. The *in vitro* synthesized mRNA was then used as a template for cDNA synthesis, PCR amplification, and molecular cloning and sequence analysis as described above for the *in vivo* synthesized RNAs. Individual clones were identified and then sequenced across the two putative editing sites. As was observed with the *in vivo* synthesized mRNA, sequence heterogeneity was observed only at the D editing site, and not at the V editing site. Fig. 3A summarizes the results of the *in vivo* and *in vitro* produced RNAs. Since the putative editing site for the accession of the V cistron was invariant in all clones, only the results for the D editing site are shown in this figure.

The effects on cistron usage following the insertion of nontemplated nucleotides in the mRNA are shown in Fig. 4. In this figure, the three potential reading frames (+1, +2, +3) in the faithfully transcribed P gene and following the addition of one or two nontemplated nucleotides in the edited transcripts are indicated in (A). In all instances following editing, the V cistron is never fused to any other cistron and thus would not be expected to be functional. In addition, since the D editing

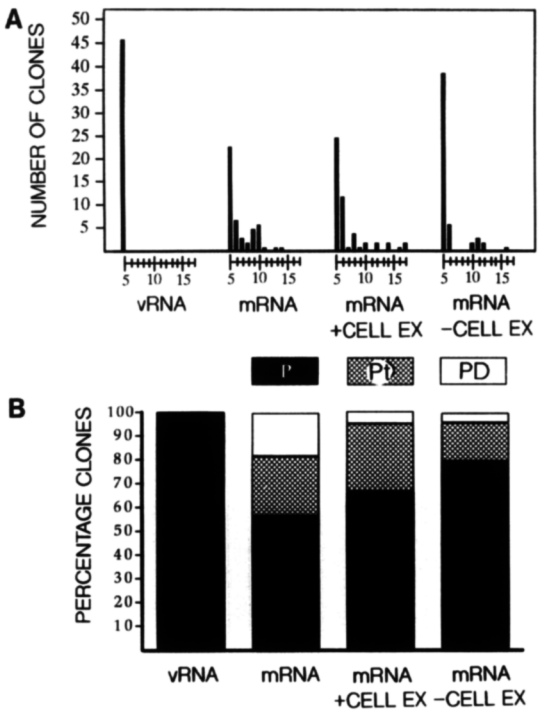

FIG. 3. Summary analysis of RNA editing, produced *in vitro* and *in vivo*, at the D cistron. (A) The number of recombinant clones containing specific numbers of G residues at the D editing site. Clones were obtained from cDNA prepared from various templates including nucleocapsid RNA (vRNA), HPIV3-infected CV-1 cellular mRNA (mRNA), and *in vitro* synthesized mRNA made in the presence (+Cell Ex) or absence (−Cell Ex) of uninfected cell lysates as described under Materials and Methods. Approximately 50 clones were examined from each group. (B) Summarizes the percentage of clones (shown in A) encoding an intact P protein (P), truncated P protein (Pt), or fused P and D protein (PD) following RNA editing.

site is downstream of the C cistron termination codon, this cistron is never affected by the addition of nontemplated nucleotides.

The addition of a single nucleotide at the D editing site results in the introduction of a stop codon and the subsequent truncation of the P cistron. This truncated protein consists of 242 N-terminal residues of P and six additional residues (KRERLV) followed by the stop codon. Further, the C-terminal 361 amino acids of the P cistron are fused to nine of the N-terminal residues of the D cistron including an initiating methionine (MTKELKKGG). Displacement of the remaining portion of the D cistron does not provide any methionine codon for internal translation initiation.

The most noticeable feature following the addition of two nontemplated nucleotides at the D editing site is the fusion of the main portion of the D cistron (residues 10–140) onto the N-terminal 241 residues of the P cistron. Displacement of the C-terminal 361 residues of the P cistron does not provide for an initiating methionine within 1300 nucleotides of the 5′-end of the mRNA, and thus, this portion of the P cistron would not be expected to be functional.

Since the insertion of three (or multiples of three) nucleotides into the mRNA will add a new codon, multi-

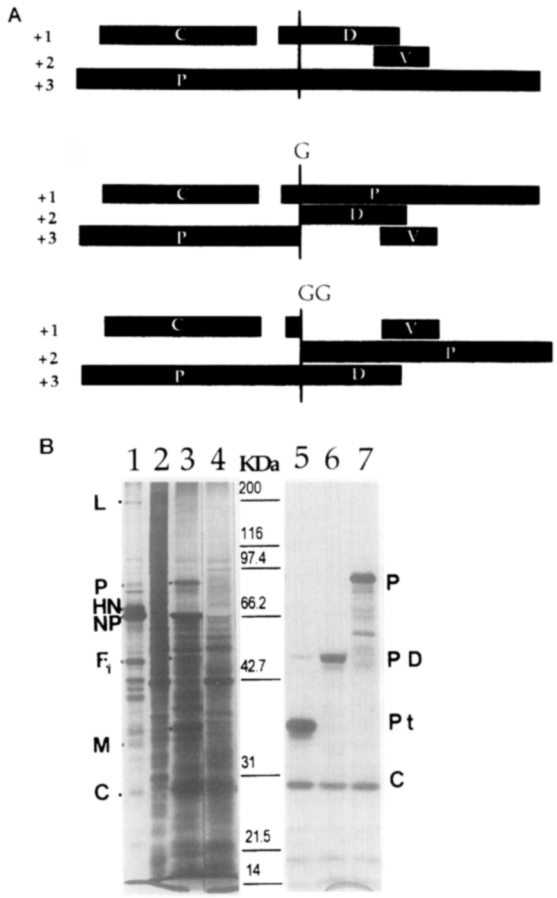

FIG. 4. Accession of alternate cistrons following RNA editing of the HPIV3 P mRNA. (A) The cistron usage map in all three reading frames of the faithfully transcribed HPIV3 P gene mRNA and the mRNAs produced following the insertion of one or two additional G residues at the D editing site (denoted with a vertical line). The primary mRNA transcript (faithful copies of the P gene) encodes intact P and C proteins. The principal products following the introduction of a single G are truncated P and C proteins, while the introduction of two G residues leads to fusion of the D cistron and the amino terminus of the P cistron. Recombinant transcription vectors containing genes with fixed number of G residues at the D editing site were assembled as described under Materials And Methods. Each of these clones was transcribed and used to charge a rabbit reticulocyte lysate system for *in vitro* translation of the mRNAs encoding a full-length P (lane 7), PD (lane 6), and truncated P (Pt; lane 5). Control HPIV3-infected (lane 3) and uninfected (lane 4) CV-1 cell mRNAs were prepared and similarly translated. The position of the various P gene products from the *in vivo* synthesized mRNAs are indicated. HPIV3-infected and uninfected CV-1 cells, [^{35}S]methionine labeled as described under Materials And Methods, were analyzed in lanes 1 and 2, respectively. The proteins in (B) were resolved on 10% discontinuous SDS–polyacrylamide gels. The position of stained molecular weight markers (M) are shown. Lanes 1–4 were resolved on a different gel from lanes 5–7.

ple "codon" insertions in any reading frame will result in the production of nearly identical proteins except for the addition of a glycine residue. Thus, the insertion of G residues can be viewed as the accession or fusion of three cistrons, the intact P proteins, truncated P (P_t) proteins, and a fused P/D protein. Fig. 3B summarizes these details by charting the percentage of recombinant clones encoding P, P_t, and P/D.

In order to investigate the expression of the various cistrons, P, C, P_t, and P/D, we reassembled recombinant genes containing a fixed number of Gs at the D editing site to duplicate the various mRNA species illustrated in Fig. 4A. Three clones, containing either six, seven, or eight G residues in the editing site, were individually transcribed with SP6 RNA polymerase following restriction endonuclease digestion with *Pvu*II at a site downstream of the P genes. The RNAs were then recovered and used to charge a rabbit reticulocyte lysate system for the production of various proteins. Proteins were resolved by SDS–PAGE using 10% polyacrylamide resolving gels. The results of this analysis are shown in Fig. 4B, which compares the proteins produced by each of the fixed mRNA sequences (Lanes 5, 6, and 7) to the proteins synthesized from HPIV3-infected and uninfected CV-1 cell mRNA (lanes 3 and 4, respectively). In addition to the *in vitro* synthesized proteins, *in vivo* synthesized proteins from HPIV3-infected and uninfected cells are shown (lanes 1 and 2, respectively). The results show that the encoded proteins translated from the recombinant P mRNAs are also present in the translation products of the infected cell mRNAs and that proteins with identical electrophoretic mobilities are present in HPIV3-infected cell lysates.

DISCUSSION

Fusion of discontinuous cistrons through RNA editing for the expression of an intact P protein appears to be an obligatory process for SV5, mumps, HPIV2, HPIV4A, and HPIV4B (PII group; indicating two discontinuous cistrons). In contrast, measles, Sendai, BPIV3, HPIV1, HPIV3, NDV, and CDV encode their respective P proteins in a single cistron (PI group). For many of these latter viruses, RNA editing and fusion of discontinuous cistrons have been shown to be a feature for the accession of alternate V or D cistrons. The distinction in the molecular organization and expression of the P genes between these two groups is congruent with the amino acid similarities shared among the P proteins from each group of viruses. Thus, the P proteins of the PII group appear to be more closely related to one another than they are to the PI group. Conversely, the PI group appears to be more closely related to one another then they are to the PII group.

The distinction between these two groups is also apparent when analysis is extended to the nucleotide sequence found at the RNA editing site. Alignment of the viral RNA sequences accommodates two related consensus sequences:

$$\begin{matrix} G \\ U \\ A \end{matrix}\!\!\begin{matrix} UAA \\ CGU \end{matrix}\!\!UUUUUUCCC\ \textbf{PI} \qquad \begin{matrix} G \\ UU \\ A \end{matrix}\!\!\begin{matrix} A \\ G \end{matrix}\!\!AUUCUCCC\ \textbf{PII}$$

In the PI sequences the stretch of U residues appears to vary with either five for Sendai, NDV, CDV, and MV, or six for BPIV3 and HPIV3 (indicated with an arrow). These sequences, which are pyrimidine-rich, resemble transcription termination sequences found at the gene-end boundaries of these viruses (Galinski, 1991). This resemblance suggests that the insertion of nontemplated nucleotides in the P mRNA may occur in a fashion similar to that for polyadenylation of the mRNAs. However, unlike polyadenylation, a more limited number of residues is added to the 3′-end and transcription termination does not occur.

In paramyxoviruses, the gene-end boundaries are composed of three domains, transcription termination, intergenic, and transcription initiation sequences. If the addition of nontemplated nucleotides is occurring through a mechanism analogous to aberrant transcription termination, then a role for the intergenic and transcription initiation sequences in proper transcription termination is suggested since these sequences are not present in the editing site. In further support of this notion is the observation that the viral RNA template, which has an invariant sequence, would need to suppress transcriptional editing as the polymerase complex crossed the RNA editing region to make viral complementary RNA. The utilization of a common control mechanism for these processes would be a parsimonious means for the virus to control transcription (both polyadenylation and polyguanylation) and replication (replicative and progeny RNAs).

The results of the *in vitro* synthesized mRNA suggest that RNA editing is a function solely of virion-associated proteins. That is, the addition of host cell lysate did not appreciably alter the pattern of clones containing additional nontemplated nucleotides. While these data are not conclusive in that host cell factors packaged in the virions (i.e., actin, tubulin, or other as yet uncharacterized proteins) might participate in this process, it is certainly suggestive that viral proteins contain all the requisite functions. Until an *in vitro* transcription system using sufficiently defined products

(i.e., reconstituted nucleocapsid complexes from recombinant proteins and RNA) is available, the exclusive role of viral proteins in this process will remain circumstantial. Finally, these results are also in agreement with the observations that RNA editing is a function of (primary) transcription as discussed above.

The results presented here indicate that the cryptic V cistron in HPIV3 is not expressed through transcriptional editing. However the D cistron is accessed in a similar fashion as the V cistron is in other paramyxoviruses. There are no significant amino acid sequence similarities between the D and V cistrons. In contradistinction, the D cistron contains a single cysteine and is extremely rich in glutamine (17%), properties which are divergent with those observed for the V cistron. Clearly, accession of the D cistron was not the result of selection for a protein with similar properties as V.

Frameshifting by eucaryotic ribosomes for the accession of discontinuous cistrons has been shown to occur in both retroviruses (Craigen and Caskey, 1987) and coronaviruses (Brierly *et al.,* 1989). The formation of a pseudoknot downstream of a "slippery" RNA sequence (UUUAAAC) appears to be essential for effective shifting of the reading frame and translational fusion of an alternate cistron. Comparison of the RNA sequence downstream of the D editing site did not disclose any sequence homologies with the frameshift sequences. This result supports the notion that accession of the V cistron does not occur via ribosomal frameshifting.

Recently the HPIV1 P gene/mRNA nucleotide sequence has been determined. In contrast with other paramyxoviruses, HPIV1 does not contain an intact V cistron (Matsuoka *et al.,* 1991). Thus, although remnants of the amino acid sequence of V can be found within the P gene, there are several stop codons spanning this region. In addition, there is no RNA editing sequence proximal to the 5′-end of the cistron. These results, together with the results reported here for HPIV3, indicate that V is nonessential for replication in these two viruses.

Interestingly, there is another cistron, just downstream of the C cistron in HPIV1, remarkably similar in position to that observed for the D cistrons in HPIV3 and BPIV3. However, the encoded amino acids show no significant homology with the D proteins, and there is no RNA editing site present for the accession of this cistron.

The BPIV3 virus was recently shown to access both the D and V cistrons by utilizing an RNA editing site proximal to the BPIV3 D cistron (Pelet *et al.,* 1991). In this virus, the number of G residues inserted into the mRNA template was found to be indiscriminate as was reported here for HPIV3. However, there are no stop codons in any of the three potential reading frames between the RNA editing site and either the D or V cistrons. This results in the fusion of either the D cistron or V cistron to the amino terminus of the P cistron. Thus, BPIV3 has selected an alternate editing site for the accession of two different discontinuous cistrons.

It would appear that there is a spectrum of "types" of P genes in the paramyxoviruses including: (1) those which obligately require RNA editing for the expression of an intact P protein and normally access the V cistron; (2) those which edit their RNA, presumably for access of the V cistron; (3) BPIV3 which accesses both the V and D cistrons; (4) HPIV3 which accesses only the D cistron; and finally (5) HPIV1, which does not edit its P mRNA and expresses the intact P (and C) protein only.

A search of the Swiss, GenPept, and National Biomedical Research Foundation data banks for proteins with similar amino acids to the P/D proteins did not reveal any significant homologies with nonparamyxoviral proteins. In addition, a search for similarity to 18 proteins with defined structural motifs did not reveal any resemblance with these domains. The P proteins of Paramyxoviridae are extremely divergent at the amino acid level (Galinski and Wechsler, 1991). In fact, the P proteins are the least conserved of all of the viral proteins. If one seeks to define functionally important domains within these proteins by sequence alignment then this wide divergence rapidly obscures any salient features. Thus, it is not unexpected that sequence similarity searches of the protein data banks did not reveal any significant similarities. These results again underscore the uniqueness of the P proteins.

ACKNOWLEDGMENTS

The authors thank Bishnu De for providing the HPIV3 *in vitro* transcribed mRNA for analysis, and Beverly R. Heminway for technical support. This work was supported in part by Public Health Service Grant AI-26256.

REFERENCES

BELLINI, W. J., ENGLUND, G., ROZENBLATT, S., ARNHEITER, H., and RICHARDSON, C. D. (1985). Measles virus P gene codes for two proteins. *J. Virol.* **53,** 908–919.

BONNER, W. M., and LASKEY, R. A. (1974). A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46,** 83–88.

BRIERLEY, I., DIGARD, P., and INGLIS, S. C. (1989). Characterization of an efficient coronavirus ribosomal frameshifting signal: Requirement for an RNA pseudoknot. *Cell* **57,** 537–547.

CATTANEO, R., KAELIN, K., BACZKO, K., and BILLETER, M. A. (1989). Measles virus editing provides an additional cysteine-rich protein. *Cell* **56,** 759–764.

CHINCHAR, V. G., and PORTNER, A. (1981). Functions of Sendai virus nucleocapsid polypeptides: Enzymatic activities in nucleocapsids

following cleavage of polypeptide P by *Staphylococcus aureus* protease V8. *Virology* **109,** 59–71.

CRAIGEN, W. J., and CASKEY, C. T. (1987). Translational frameshifting: Where will it stop? *Cell* **50,** 1–2.

DE, B. P., GALINSKI, M. S., and BANERJEE, A. K. (1990). Characterization of an *in vitro* system for the synthesis of mRNA from human parainfluenza virus type 3. *J. Virol.* **64,** 1135–1142.

DE, B. P., LESOON, A., and BANERJEE, A. K. (1991). Human parainfluenza virus type 3 transcription *in vitro:* role of cellular actin in mRNA synthesis. *J. Virol.* **65,** 3268–3275.

DESHPANDE, K. L., and PORTNER, A. (1985). Monoclonal antibodies to the P protein of Sendai virus define its structure and role in transcription. *Virology* **140,** 125–134.

DEVEREUX, J., HAEBERLI, P., and SMITHIES, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12,** 387–395.

ELLIOTT, G. D., YEO, R. P., AFZAL, M. A., SIMPSON, E. J. B., CURRAN, J. A., and RIMA, B. K. (1990). Strain variable editing during transcription of the P gene of mumps virus may lead to the generation of non-structural proteins NS1 (V) and NS2. *J. Gen. Virol.* **71,** 1555–1560.

GALINSKI, M. S. (1991). Paramyxoviridae: Transcription and replication. *In* "Advances in Virus Research" (K. Maramorosch, F. A. Murphy, and A. J. Shatkin, Eds.), Vol. 39, pp. 129–162. Academic Press, San Diego.

GALINSKI, M. S., MINK, M. A., LAMBERT, D. M., WECHSLER, S. W., and PONS, M. W. (1986a). Molecular cloning and sequence analysis of the human parainfluenza 3 virus RNA encoding the nucleocapsid protein. *Virology* **149,** 139–151.

GALINSKI, M. S., MINK, M. A., LAMBERT, D. M., WECHSLER, S. L., and PONS, M. W. (1986b). Molecular cloning and sequence analysis of the human parainfluenza 3 virus mRNA encoding the P and C proteins. *Virology* **154,** 46–60.

GALINSKI, M. S., and WECHSLER, S. L. (1991). The molecular biology of the *Paramyxovirus* genus. *In* "The Paramyxoviruses." (D. W. Kingsbury, Ed.), pp. 41–82. Plenum Press, New York.

GALINSKI, M. S., STANIK, V. H., ROHRMANN, G. F., and BEAUDREAU, G. S. (1983). Comparison of sequence diversity in several cytoplasmic polyhedrosis viruses. *Virology* **130,** 372–380.

GIORGI, C., BLUMBERG, B. M., and KOLAKOFSKY, D. (1983). Sendai virus contains overlapping genes expressed from a single mRNA. *Cell* **35,** 829–836.

HAMAGUCHI, M., YOSHIDA, T., NISHIKAWA, K., NARUSE, H., and NAGAI, Y. (1983). Transcriptive complex of Newcastle disease virus. I. Both L and P proteins are required to constitute an active complex. *Virology* **128,** 105–117.

KINGSBURY, D. W. (1990). *Paramyxoviridae* and their replication. *In* "Virology" (B. N. Fields, D. M. Knipe, *et al.,* Eds.), 2nd ed., pp. 945–962. Raven Press, New York.

KINGSBURY, D. W. (1991). "The Paramyxoviruses." Plenum Press, New York.

KONARSKA, M. M., PADGETT, R. A., and SHARP, P. A. (1984). Recognition of cap structure in splicing *in vitro* of mRNA. *Cell* **38,** 731–736.

KONDO, K., BANDO, H., TSURUDOME, M., KAWANO, M., NISHIO, M., and ITO, Y. (1990). Sequence analysis of the phosphoprotein (P) genes of human parainfluenza type 4A and 4B viruses and RNA editing at transcript of the P genes: The number of G residues added is imprecise. *Virology* **178,** 321–326.

LUK, D., SANCHEZ, A., and BANERJEE, A. K. (1986). Messenger RNA encoding the phosphoprotein (P) of human parainfluenza virus 3 is bicistronic. *Virology* **153,** 318–325.

MAIZEL, J. V., JR. (1971). Polyacrylamide gel electrophoresis of viral proteins. *In* "Methods in Virology" (K. Maramorosch and H. Koprowski, Eds.), pp. 179–246. Academic Press, New York.

MATSUOKA, Y., CURRAN, J., PELET, T., KOLAKOFSKY, D., RAY, R., and COMPANS, R. W. (1991). The P gene of human parainfluenza virus type 1 encodes P and C proteins but not a cysteine-rich V protein. *J. Virol.* **65,** 3406–3410.

OHGIMOTO, S., BANDO, H., KAWANO, M., OKAMOTO, K., KONDO, K., TSURUDOME, M., NISHIO, M., and ITO, Y. (1990). Sequence analysis of P gene of human parainfluenza type 2 virus: P and cysteine-rich proteins are translated by two mRNAs that differ by two nontemplated G residues. *Virology* **177,** 116–123.

PATERSON, R. G., and LAMB, R. A. (1990). RNA editing by G-nucleotide insertion in mumps virus P-gene mRNA transcripts. *J. Virol.* **64,** 4137–4145.

PELET, T., CURRAN, J., and KOLAKOFSKY, D. (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.

QUEEN, C., and KORN, L. J. (1984). A comprehensive sequence analysis program for the IBM personal computer. *Nucleic Acids Res.* **12,** 581–599.

RYAN, K. W., and KINGSBURY, D. W. (1988). Carboxyl-terminal region of Sendai virus P protein is required for binding to viral nucleocapsids. *Virology* **167,** 106–112.

RYAN, K. W., MURTI, K. G., and PORTNER, A. (1990). Localization of P protein binding sites on the Sendai virus nucleocapsid. *J. Gen. Virol.* **71,** 997–1000.

RYAN, K. W., and PORTNER, A. (1990). Separate domains of Sendai virus P protein are required for binding to viral nucleocapsids. *Virology* **174,** 515–521.

SAKAI, Y., SUZU, S., SHIODA, T., and SHIBUTA, H. (1987). Nucleotide sequence of the bovine parainfluenza 3 virus genome: its 3′ end and the genes of NP, P, C and M proteins. *Nucleic Acids Res.* **15,** 2927–2945.

SHIODA, T., HIDAKA, Y., KANDA, T., SHIBUTA, H., NOMOTO, A., and IWASAKI, K. (1983). Sequence of 3,687 nucleotides from the 3′ end of Sendai virus genome RNA and the predicted amino acid sequences of viral NP, P, and C proteins. *Nucleic Acids Res.* **11,** 7317–7330.

SOUTHERN, J. A., PRECIOUS, B., and RANDALL, R. E. (1990). Two nontemplated nucleotide additions are required to generate the P mRNA of parainfluenza virus type 2 since the RNA genome encodes protein V. *Virology* **177,** 388–390.

SPRIGGS, M. K., and COLLINS, P. L. (1986a). Human parainfluenza virus type 3: messenger RNAs, polypeptide coding assignments, intergenic sequences, and genetic map. *J. Virol.* **59,** 646–654.

SPRIGGS, M. K., and COLLINS, P. L. (1986b). Sequence analysis of the P and C protein genes of human parainfluenza virus type 3: Patterns of amino acid sequence homology among paramyxovirus proteins. *J. Gen. Virol.* **67,** 2705–2719.

THOMAS, S. M., LAMB, R. A., and PATERSON, R. G. (1988). Two mRNAs that differ by two nontemplated nucleotides encode the amino coterminal proteins P and V of the paramyxovirus SV5. *Cell* **54,** 891–902.

UDEM, S. A., and COOK, K. A. (1984). Isolation and characterization of measles virus intracellular nucleocapsid RNA. *J. Virol.* **49,** 57–65.

VIDAL, S., CURRAN, J., and KOLAKOFSKY, D. (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.

WECHSLER, S. L., LAMBERT, D. M., GALINSKI, M. S., HEINEKE, B. E., and PONS, M. W. (1985). Human parainfluenza virus 3: Purification and characterization of subviral components, viral proteins and viral RNA. *Virus Res.* **3,** 339–351.