The EMBO Journal vol.10 no.2 pp.443-448, 1991

The P gene of bovine parainfluenza virus 3 expresses all three reading frames from a single mRNA editing site

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Communicated by D.Kolakofsky

The P gene of bovine parainfluenza virus 3 (bPIV3) contains two downstream overlapping ORFs, called V and D. By comparison with the mRNA editing sites of other paramyxoviruses, two editing sites were predicted for bPIV3; site a to express the D protein, and site b to express the V protein. Examination of the bPIV3 mRNAs, however, indicates that site b is non-functional whereas site a operates frequently. Insertions at site a give rise to both V and D protein mRNAs, because a very broad distribution of Gs is added when insertions occur. This broad distribution is very different from the editing sites of Sendai virus or SV5, where predominantly one form of edited mRNA containing either a one or two G insertion respectively is created, to access the single overlapping ORF of these viruses. A model is proposed to explain how paramyxoviruses control the range of G insertions on that fraction of the mRNAs where insertions occur. The bPIV3 P gene is unique as far as we know, in that a sizeable portion of the gene expresses all 3 reading frames as protein. bPIV3 apparently does this from a single editing site by removing the constraints which control the number of slippage rounds which take place.

Key words: bPIV3/mRNA editing/overlapping genes

Introduction

The P genes of paramyxoviruses are unusual in that they express multiple proteins from overlapping ORFs. Based on their pattern of P gene expression, these viruses can be divided into 2 groups. The first, which includes SV5 (Thomas *et al.*, 1988), parainfluenza viruses (PIV) 2 (Ohgimoto *et al.*, 1990; Southern *et al.*, 1990) and 4 (Kondo *et al.*, 1990), and mumps virus (Elliott *et al.*, 1990; Paterson and Lamb, 1990), do not contain a single ORF spanning the entire gene. Rather, the gene is spanned by two ORFs of about equal length which overlap in the centre, and separate mRNAs are produced to express the alternative downstream ORFs. One mRNA, an exact copy of the gene, expresses the N-terminal ORF alone, and this protein is called V. Another, which contains a 2G insertion within a short run of Gs, fuses the two ORFs near the start of the overlap, and expresses a longer protein, called P. The C-terminal portion of V which is not shared with P contains a Cys-rich domain, and this domain is the most highly conserved of all the P gene ORFs among the paramyxoviruses (Thomas *et al.*, 1988).

The second group, which includes measles virus (MV) (Cattaneo *et al.*, 1989), Sendai virus (SEN, Vidal *et al.*, 1990a), PIV1 (Matsuoka,Y., Curran,J., Pelet,T., Kolakofsky,D., Ray,R. and Compans,R., submitted for publication), and PIV3, contains a single ORF which spans the entire gene, and the unedited mRNA is translated into the P protein (Figure 1). Except for PIV1, the Cys-rich V ORF is conserved in these viruses at the same relative location (the middle of the gene), but in the −1 frame relative to P. For MV and SEN, the V ORF is expressed from an edited mRNA which contains a single G insertion within a short run of Gs. The resulting V protein then contains the same N-terminal sequences as P, fused to the V ORF sequences at the insertion site (Figure 1). This second group of viruses also contain a C ORF overlapping the N-terminus of P and upstream of the insertion site (Figure 1), which is expressed from both inserted and uninserted mRNAs by independent ribosomal initiation (Curran and Kolakofsky, 1989). For SEN and PIV1, multiple C proteins are also made, again by ribosomal choice, which include non-AUG start codons (Curran and Kolakofsky, 1988; Matsuoka,Y., Curran,J., Pelet,T., Kolakofsky,D., Ray,R. and Compans,R., submitted for publication).

The G insertions take place in the P gene mRNAs of most paramyxoviruses, and for SEN evidence has been presented that the insertions occur co-transcriptionally, by controlled polymerase stuttering (Vidal *et al.*, 1990a and b). A remarkable feature of this editing mechanism is that for each virus group, the requisite number of Gs is added at high frequency to provide access to the alternative downstream ORF, i.e. two Gs are added in the SV5/mumps group to go from V to P and mRNAs with only one G inserted are very rare, whereas +1G is the predominant event for the SEN/MV group to go from P to V. The stuttering model, in which the polymerase pauses before completing the G run and the 3′ end of the nascent mRNA slips upstream on the template (see Figure 5), can explain this feature (Vidal *et al.*, 1990b). For the SEN/MV group, the editing site is 3′UUUUCCC (written as the template strand). Here, the misalignment intermediate created by a 1 base slippage is more stable than that of a 2 base slippage, hence 1G is added at high frequency. For the SV5/mumps group whose editing site is 3′UUCUCCC, a 2 base slippage is preferred as it avoids the creation of an unstable A:C pair, hence two Gs are predominantly inserted when slippage has occurred.

PIV3 is slightly more complicated than SEN and MV. The P gene sequences of both the human (h) (Spriggs and Collins, 1986; Galinski *et al.*, 1986; Luk *et al.*, 1986) and bovine (b) (Sakai *et al.*, 1987) strains are known, and these contain one more ORF (called D). The D ORF follows shortly after that of C, in the same reading frame (Figure 1). On the basis of sequence comparisons, Cattaneo *et al.* (1989) predicted two editing sites for hPIV3 (c.f. Figure 1), both of which would favour single base slippages according to the above rule. Insertions at the first site cannot produce a functional

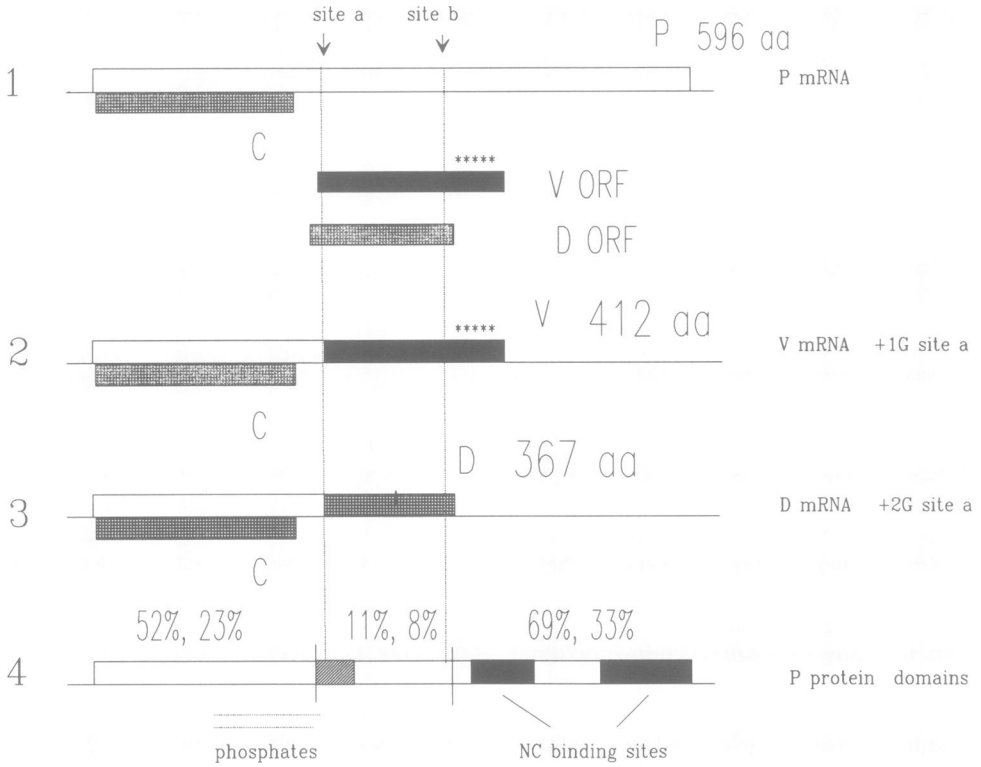

Fig. 1. Schematic representation of the bPIV3 gene and its mRNAs. Line 1 shows the mRNA which is an exact copy of the gene, coding for the P and C proteins. The two predicted editing sites are shown above. Below are shown the V and D overlapping ORFs which would be fused to the P ORF upon insertion of 1 or 2 Gs, respectively (lines 2 and 3), if only site a were to operate. The asterisks above the V ORF indicate the position of the highly conserved Cys-rich domain. Line 4 shows the domains of the P protein. The percentages above the three regions (divided by vertical lines) refer to amino acid identity of the SEN/PIV1 and SEN/bPIV3 alignments, respectively. The shaded block in the central hypervariable region, which contains site a, is absent in SEN and PIV1. The sites at which the SEN protein is phosphorylated (Vidal *et al.*, 1988), and by which the protein binds to nucleocapsids (Ryan and Portner, 1990), are indicated below.

V mRNA for hPIV3, as there are several stop codons between this site and the Cys-rich domain. However, a two G insertion here would give rise to an mRNA which has fused the N-terminus of P to 131 amino acids of the D ORF. A second insertion site, ~300 nt downstream and at the same relative location as the site which operates in MV and SEN, lies downstream of the stop codons and can produce a functional V mRNA. hPIV3 would then appear to use two insertion sites; the first to express the D ORF exclusively, and the second for V.

The P gene of bPIV3 is organized identically to that of hPIV3 and contains the same two putative insertion sites. However, there are no stop codons between the first site and the Cys-rich domain of the V ORF. This creates the very unusual situation where a block of 320 nt can potentially be expressed in all three reading frames. As it is unlikely that such a situation would exist by chance, this suggests that the first editing site will be used to express both the V and D proteins (Figure 1). Moreover, if the second site also operates in bPIV3, there could potentially be three different forms of V as well as multiple forms of P. To learn more about this mRNA editing mechanism, and in particular to determine whether a single editing site can in fact express all three reading frames, we have examined the mRNAs of bPIV3.

Results

Two editing sites have been predicted for the P gene of hPIV3, and by extension for bPIV3. The first site (5′AAAAAGGGG, site a) is at nt 794–803, and the second (5′AAAGGAAAGAGAGG, site b) at nt 1105–1118. These editing sites are flanked by unique *Eco*RV (nt 757) and *Pst*I (nt 1191) sites. It is useful in characterizing mRNA clones to translate plasmid derived transcripts *in vitro*. The P gene mRNAs were therefore copied into DNA using an oligonucleotide downstream of the *Pst*I site, and the *Eco*RV–*Pst*I fragments were cloned into a plasmid containing the entire P gene from which the *Eco*RV–*Pst*I fragment had been removed (Materials and methods). As no positive colonies could be isolated in the absence of the reverse transcription step (not shown), all our positive colonies resulted from cDNA inserts.

The regions encompassing both editing sites of 7 plasmids were sequenced. We found that none had insertions at site b, whereas 5 of the 7 had insertions of 1, 2, 4, 4, and 7 Gs, respectively, at site a. This distribution of the number of Gs inserted was very different from that found for SEN and MV, and so site a of 13 more plasmids were sequenced. As shown in Figure 2A, the distribution of Gs inserted at site a is indeed very broad, and there is even one example of a 12G insertion. The RNA transcripts from the 20 plasmids were translated *in vitro*. Those with 0, 3 or 12 Gs inserted made a protein which co-migrated with the SEN P protein (lane 2, Figure 3), those with 1, 4, or 7 Gs inserted made a smaller protein whose migration is consistent with the P–V fusion protein or V protein (lane 3), and those with two Gs inserted made a even smaller protein whose migration is consistent with the P–D or D protein (lane 4). All these mRNAs also made a C protein (not shown), which has run

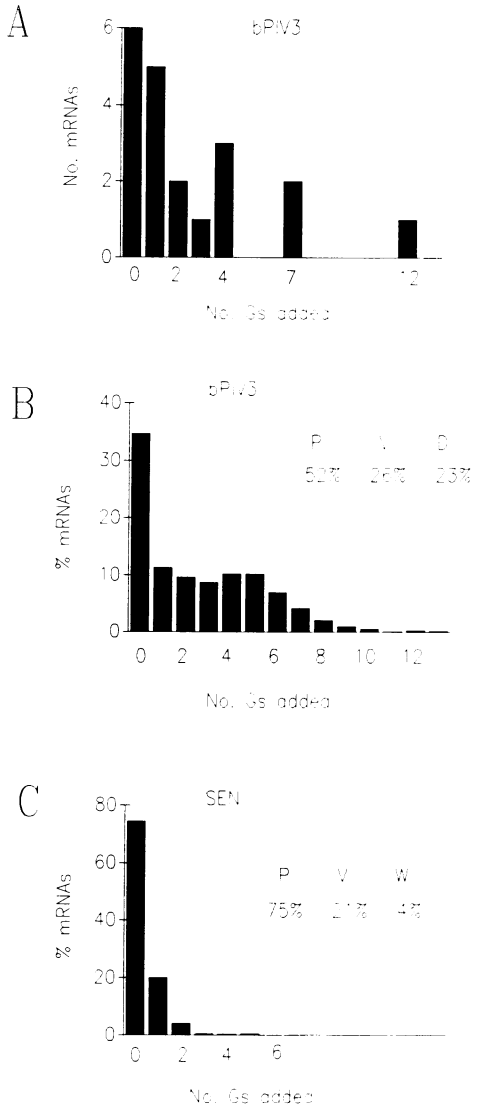

Fig. 2. Frequency and distribution of G insertions. Panel **A**. Histogram of G insertions at site a of 20 mRNAs, determined by sequencing and confirmed by *in vitro* translation. Panels **B** and **C**. Histograms of the bPIV3 and SEN mRNAs determined by primer extension (Figure 4B and C), given as % of the total population. The results have been normalized for the small amount of +1 band in the control RNAs. The fraction of the mRNAs which would code for the 3 proteins are indicated within the graph.

into the dye front in Figure 3. Since these results are as expected based on the insertions at site a alone, if there were any insertions at site b, they must be multiples of three.

The broad distribution of Gs inserted at site a was unexpected, in that the same sequence of SEN and MV produces only single G insertions at high frequency (Cattaneo *et al.*, 1989; Vidal *et al.*, 1990a) . The apparent absence of insertions at site b was also unexpected, as a virtually identical site should operate in hPIV3 to produce a V protein. It was therefore necessary to examine a larger number of mRNAs to document these findings with more confidence. We have previously examined groups of 200 SEN mRNAs by oligonucleotide typing (Vidal *et al.*, 1990a). However, the presence of two sites, and more importantly the broad distribution of insertions at the first site, makes this approach unwieldy here. An alternate approach was suggested by the work of Driscoll *et al.* (1989) on the editing of the apolipoprotein B mRNA. Complementary oligonucleotides

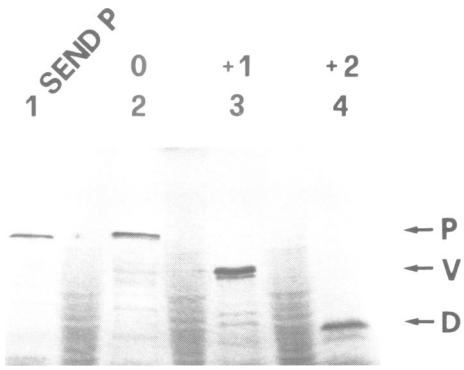

Fig. 3. Protein products of the various mRNAs. Plasmid derived *in vitro* transcripts from the uninserted SEN P gene (lane 1), or bPIV3 clones containing either 0, 1, or 2Gs inserted at site a (lanes 2, 3, and 4, respectively), were translated in both wheat germ and rabbit reticulocyte systems in the presence of [^{35}S]methionine. The translation products were separated on a 10% SDS–polyacylamide gel. The arrows on the right refer to the major translation products in the reticulocyte lysate. The alternate lanes show the unsuccessful wheat germ translations.

whose 3′ ends were located opposite the first C or U residue downstream of each site on the mRNA were made. These were then extended on total mRNA with reverse transcriptase in the presence of only dCTP and dTTP to cover the A and G residues of each site, and ddATP was included so that synthesis would terminate at the first U base on the mRNA upstream of the insertion site (Figure 4A). The length of the extended primer would then depend on the number of insertions that had occurred, and the distribution of lengths would reflect the distribution of the number of bases inserted within the entire population of mRNAs. To control the specificity of this method, the same primers were extended on *in vitro* transcripts from plasmids containing the genomic copy of the gene, which should represent a homogeneous population of uninserted mRNAs. In addition, the *in vitro* transcript was combined with the same amount of uninfected cell mRNA present in the infected cell samples, so that the reactions would take place under equivalent conditions.

As shown in Figure 4B, lane 4, primer extension at site a of the control mRNA yielded predominantly a single band at the expected position (marked as the 0 band), but there were also minor bands which were one base longer and shorter. These minor bands are unlikely to be due to T7 polymerase errors during transcription, as the same bands are formed on both T3 and SP6 polymerase transcripts for SEN (Figure 4C, lanes 3 and 4), and we found no examples of insertion among 395 SEN mRNAs transcribed by the vaccinia virus polymerase (Vidal *et al.*, 1990a). It is more likely that these minor bands are due to other nucleotides which contaminate the dNTP and particularly the ddNTP solutions, since the use of ddGTP rather than ddATP exacerbates this problem (not shown). In any event, the extra bands are relatively minor when ddATP is used, and do not prevent interpretation of the results.

Extension of the primer across site a of intracellular mRNA yielded a major band at position 0, i.e. uninserted mRNA, but also a ladder of bands which extended from +1 to +10 (Figure 4B, lane 6). The ladder extended to +13 on longer exposure (not shown). As expected, there were no extension products when uninfected mRNA was used as template (lane 5). Moreover, it is clear that insertions of +1

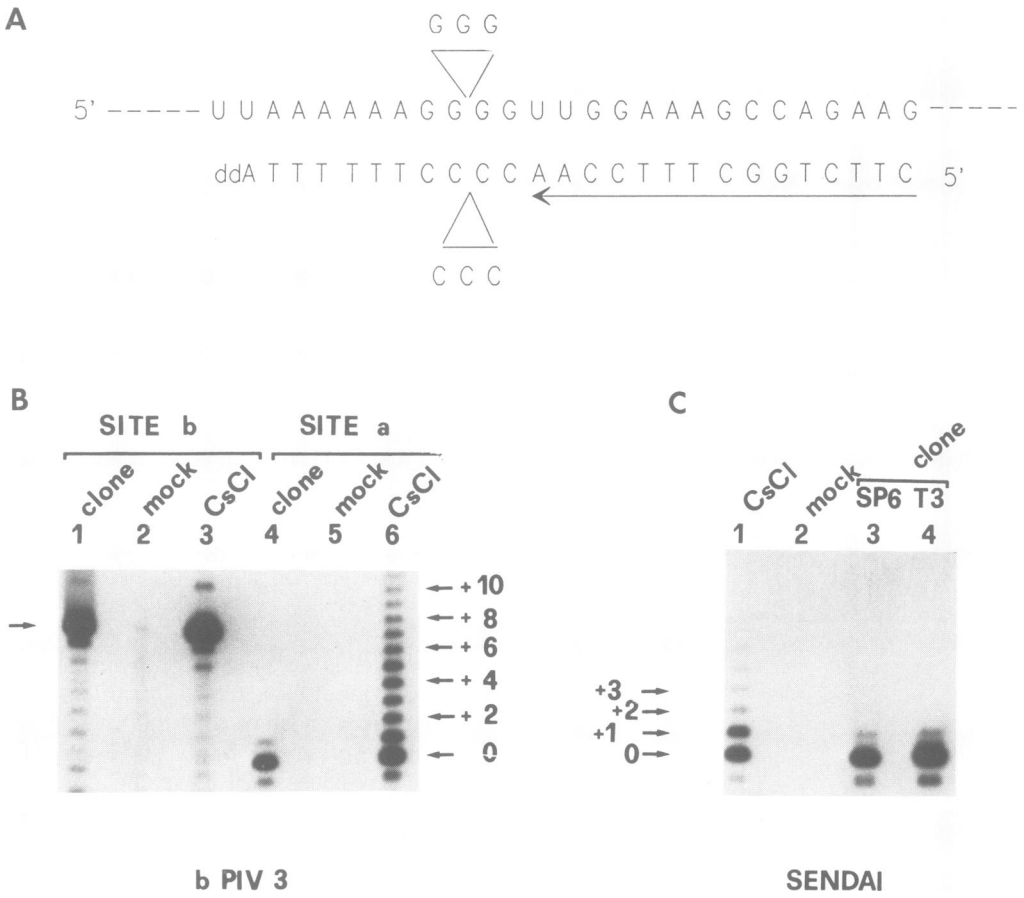

Fig. 4. Primer extension analysis of insertions within the mRNA population. Panel **A**. The method is indicated for site a of bPIV3. The mRNA is the top line, and the G insertion site is indicated with a triangle. The primer, underlined with an arrow, is extended with dCTP, dTTP, and ddATP which terminates extension at the first U residue of the mRNA upstream of the insertion site. Panel **B**. Examination of sites a and b of bPIV3. Primers specific for either site a or b, as indicated above, were extended on either *in vitro* transcripts of genomic clones made with T7 polymerase (clone, lanes 1 and 4), CsCl pellet RNA from uninfected cells (mock lanes 2 and 5), or CsCl pellet RNA from bPIV3 infected cells (CsCl, lanes 3 and 6). The arrow on the left refers to the 0 band with site b; those on the right refer to the various bands with site a, as indicated. Panel **C**. Examination of the SEN editing site. The same experiment as in panel **B**, except that CsCl refers to SEN infected cell RNA, and SP6 and T3 refer to two separate plasmids containing the uninserted P gene from which transcripts were made *in vitro* with SP6 and T3 polymerase.

are not by far the predominant event when insertions occur, as the bands at +4 and +5 are almost as intense as that at +1. The relative intensities of the bands were determined by densitometry, and their distribution is shown in Figure 2B. When the same experiment was repeated with site b, on the other hand, we found no evidence that any insertions had occurred. On the overexposure shown in Figure 4B, lane 3, there is a minor band at position +3, but this band is present with the control transcript as well (lane 1). Thus, with this method which measures the grand average of insertion events, the conclusions have not changed. Site b does not appear to operate in bPIV3, and the distribution of Gs inserted at site a is very different from that at the SEN and MV editing sites.

To confirm this second point, we examined SEN and MV intracellular mRNAs with this method. The control transcripts again yielded minor bands which were one base longer and shorter than the major band at position 0 (Figure 4C, lanes 3 and 4), presumably for the reasons discussed above. Extension across the SEN mRNA yielded a prominant +1 band, which was about one-fourth as intense as the 0 band, as well as a very faint ladder of bands from +2 to +5 (Figure 4C, lane 1, and Figure 2C). However, only 25% of the SEN mRNA contained insertions by this test, and of these, 80% contained a single base insertion. For bPIV3, in contrast, 65% of the mRNAs contained insertions, and of these only 18% contained a 1 base insertion. For MV, there was again a prominant band at +1, but no subsequent ladder could be detected at all (not shown). The difference in the distribution of insertions between bPIV3 and the other viruses remains striking, even when the same method is applied to all three viruses.

Discussion

A primer extension method to quantify the fraction of mRNAs with insertions, and the distribution of the number of bases inserted, has been described. There are several advantages of this method over the cloning methods previously used. The method is considerably faster and simpler, it examines the entire mRNA population at the same time, and it avoids the otherwise obligatory cloning step which may introduce a bias. For example, we have previously noted that when >1G is inserted with SEN mRNAs, there is a bias for the +4G insertion (Vidal *et al.*, 1990a and b). However, inspection of Figure 4C does not show this bias, which may be due therefore, to the cloning, and PCR amplification before cloning will further bias the

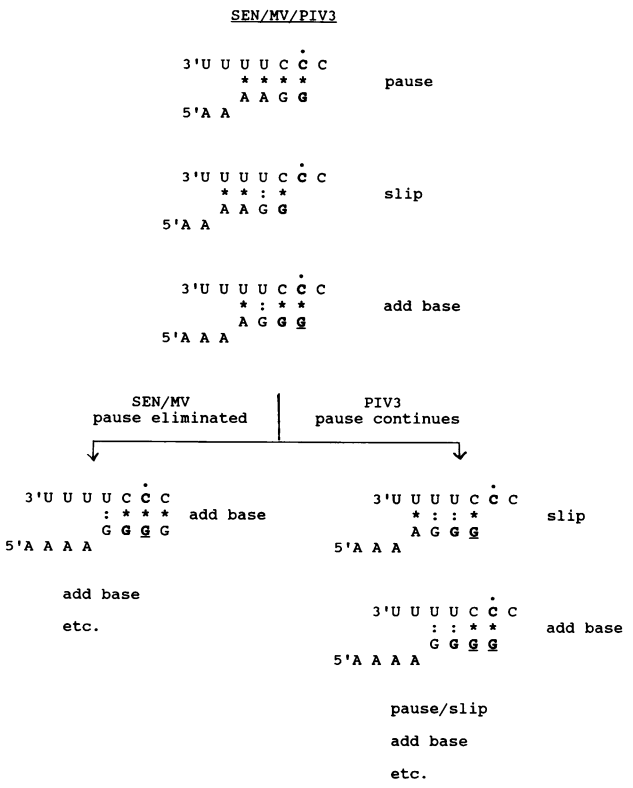

Fig. 5. A model to explain the different frequencies of multiple G insertions for SEN/MV and PIV3. The polymerase pauses after adding the second G to the mRNA (in bold, bottom line), and the mRNA slips one base upstream, creating a single G:U pair (double dots). The length of the pause determines the total fraction of mRNAs with insertions, and presumably the pause is stronger for bPIV3 than for SEN. Addition of the pseudotemplated G brings the polymerase back to the pause site (the template C with dot above), creating a single base insertion (underlined). For SEN/MV, the pause is presumably eliminated at this point, the next truly templated G is added, and the polymerase continues in a strictly processive mode. For PIV3, the pause is not eliminated. Because the G:U pair makes it easier to break the bonds between the mRNA and the template for slippage to recur, multiple rounds of slippage and insertion are favoured, and a large majority of the mRNAs with insertions contain multiple insertions. After ~6 rounds, the probability that the pause is eliminated eventually increases, or the process ceases as oligo-G formation becomes self-limiting.

results (S.Vidal, unpublished). We note that the exact method used here does not distinguish between A and G insertions, but it can be adapted to look only at G insertions within the G run. One disadvantage of this method is that it is highly dependent on the purity of the dNTPs and ddNTPs.

Among the paramyxoviruses, PIV3 is most closely related to SEN and PIV1, and with the recent sequence of the PIV1 P gene (Matsuoka,Y., Curran,J., Pelet,T., Kolakofsky,D., Ray,R. and Compans,R., submitted), it is now possible to align the P proteins of these three viruses with some confidence. The alignment (a summary of which is shown in Figure 1D) shows that the protein is composed of N- and C-terminal conserved blocks, joined in the middle by a hypervariable region of ~100 amino acids, which presumably acts as a bridge or a tether. The SEN editing site and site b of PIV3 are found at the C-terminal end of the hypervariable region, whereas site a of PIV3 is found within an additional 36 amino acids at the N-terminal end of this region which is absent in SEN and PIV1. Of the two editing sites predicted for bPIV3, we have been unable to find any evidence that site b operates at all. There would then be only one editing site in this gene, and its complexity would not extend to multiple forms of the P and V proteins which are significantly different. Site a, in contrast, operates frequently, and mostly adds multiple Gs. The extra glycines introduced here into the P proteins probably have little effect on protein function, as they fall within the hypervariable region. We had assumed that site b would function, since a virtually identical site must operate in the closely related hPIV3 to provide a V protein mRNA. However, recent examination of the P gene mRNAs of hPIV3 has also found that site b is apparently non-functional (M.Galinski, personal communication).

The bPIV3 P gene is unique to our knowledge, in that a sizeable region of the gene (320 nt) expresses all three reading frames as protein. This can take place from a single editing site, because a broad range of numbers of Gs is inserted when insertions occur. From these studies, P protein mRNAs would make up half, and V and D protein mRNAs would almost equally make up the other half of the mRNAs. For SEN, where there is only one overlapping ORF downstream of the editing site (insertion of two Gs places a stop codon in frame almost immediately, creating a truncated P protein referred to as W), the V protein mRNA again makes up about a quarter of the total, whereas the +2 (plus +5, etc) form of the mRNA accounts for <5%. In other viruses such as SV5 and mumps, two Gs are inserted at high frequency from a single two base slippage event, again to express the one overlapping ORF. The mRNA editing mechanism of paramyxoviruses is thus remarkably adaptable to the genetic organization of each of these P genes. We also note that even though a particular P gene mRNA is rare, it will nevertheless be expressed. The mumps virus P gene derived NS2 or I protein has long been known in infected cells. Recently, Paterson and Lamb (1990) have shown that it is translated from a rare mRNA with a 4G insertion.

Although SEN, MV, and bPIV3 all insert Gs within the common sequence 5′AAAAGGG, bPIV3 does this somewhat differently, and there is a plausible mechanism for how this could occur in the stuttering model. In this model, the polymerase would pause after incorporating the second G at the editing site (see Figure 5). In some cases, the pause would be too short for the base pairing between the 3′ end of the nascent mRNA and the template to be broken. Here the polymerase would continue on, without creating insertions. When the pause is long enough, the base pairing is transiently interrupted, and there is some pressure to move the 3′ end of the mRNA upstream. For bPIV3 the pause would presumably be longer than for SEN, as 65% of the bPIV3 mRNAs are inserted versus 25% for SEN. However, once the initial round of slippage/insertion(s) has occurred, the polymerase is back to the same position as before, except that at least one G:U pair has been formed in the misalignment. A second round is then favoured if nothing else has changed, since it should be easier to break the new base pairs for slippage to recur, and there is experimental evidence for this with SEN (Vidal *et al.*, 1990b). Nevertheless, both *in vivo* and *in vitro*, 80% or more of SEN mRNAs which contain insertions have inserted only a single G. For 1G insertions to predominate, something else must have changed when the polymerase returns to the pause site after the initial events, to prevent a second round. For example, protein modification could have occurred during the first round,

which eliminates the pause. It is apparently this something else which has failed to occur in large part during bPIV3 mRNA editing, because here insertions of >1G are now more than 5 times as frequent as those with only 1G. Somewhat similar situations can be created with SEN *in vitro*, by altering reaction conditions which are thought to either extend the pause, or decrease the time required for slippage to occur (Vidal *et al.*, 1990b).

Paramyxovirus mRNA editing, like ribosomal frameshifting of retroviruses (Jacks and Varmus, 1985) or coronaviruses (Brierley *et al.*, 1987), appears to be composed of two parts; a pause, and a slippery sequence which allows for alternate base pairing (Brierley *et al.*, 1989). The slippery sequence for paramyxoviruses (3'UUYUCCC) is fairly clear. However, the counterpart to the downstream template structure which causes ribosomal pausing is difficult to even guess at. DNA dependent RNA polymerases have long been known to pause in the middle of genes (Chamberlin, 1976; von Hippel *et al.*, 1984; Platt, 1986), and although there is some evidence that this is due to an intrinsic property of the template (Reines *et al.*, 1987), its nature is equally unclear. Further progress in this form of mRNA editing will require some understanding of the nature of the pause.

Materials and methods

Cells and viruses

Stocks of bPIV3 (from Hiroshi Shibuta, Tokyo) were prepared in CV1 cells and used to infect BHK cells. SEN (Harris strain), and MV (human 3 strain) stocks were prepared in embryonated chicken eggs and CV1 cells respectively, and used to infect BHK cells and CV1 cells, respectively.

Cloning method

The *Bsp*MII–*Pvu*II fragment (nt 1727–3685 from the 3' end of the genome) containing the entire P gene of bPIV3 from the clone of Sakai *et al.* (1987), was subcloned into the *Ava*I–*Eco*RV sites of pBluescript KS in which the *Xho*I site had been inactivated by fill-in to provide a unique *Ava*I site. The *Eco*RV–*Pst*I fragment (nt 757–1191 from the start of the P gene (nt 1705 on the genome)) containing the two predicted editing sites was then removed to create the cloning vector. cDNA was prepared from infected cell CsCl pellet RNA using the oligo-nt 3'GGACATGGTTCTGTTCTCTC (nt 1203–1222) as previously described (Vidal *et al.*, 1990a), cut with *Eco*RV and *Pst*I, and cloned into the above vector. Positive colonies were detected by hybridization, and plasmid derived mRNAs were translated *in vitro* (Vidal *et al.*, 1990a).

Primer extension method

5' end-labelled primers (0.5 pmol) were co-precipitated with 10 μg of CsCl pellet RNA from either bPIV3, SEN, or MV infected cells, and extended with 200 U for MLV reverse transcriptase (RNase H^-, BRL) in the presence of 0.5 mM dCTP and dTTP, and 0.32 mM ddATP, for 45 min at 42°C in a total volume of 30 μl. The control reactions included the same amount of uninfected cell RNA, and 20 ng of genomic plasmid derived transcript combined with 10 μg of uninfected cell RNA. After phenol extraction and ethanol precipitation, samples were separated on a 12% sequencing gel. In all cases, the appropriate sequence ladder was run alongside for length determination. The primers used were: 3'AACCTTTCGGTCTTC (nt 803–818, site a, bPIV3), 3'GTGTCTTCTCTCGTG (nt 1119–1133, site b, bPIV3), 3'CCCGTATCCTCTC (nt 1051–1063, SEN, Giorgi *et al.*, 1983), and 3'GTGTCTCGCGTCTAA (nt 755–769, MV, Bellini *et al.*, 1985). Except for the SEN primer, whose 3' end covers the three templated Gs of the editing site, the others have 3' ends which begin just downstream of the editing site.

Acknowledgements

We are indebted to Hiroshi Shibuta, Tokyo, for providing us with bPIV3 and the clone containing the P gene, and to Martin Billeter and Roberto Cattaneo, Zurich, for their clone pePM(N) containing the MV P gene. We also thank the reviewers for their helpful suggestions. This work was supported by a grant from the Swiss National Science Fund.

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Received on September 28, 1990; revised on November 15, 1990