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A stuttering model for paramyxovirus P mRNA editing

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Paramyxovirus P genes are transcribed into two mRNAs which differ from each other by either one (measles and Sendai virus) or two (SV5 and mumps virus) G insertions, and which code for either the P or V proteins. The G insertions always occur within a short run of Gs, and a stuttering mechanism for the insertions has been suggested in which the viral polymerase reiteratively copies a template C residue during mRNA synthesis. Support for this mechanism was obtained by varying the reaction conditions during Sendai virus mRNA synthesis *in vitro*. A stuttering model is proposed which accounts for how the ratio of inserted to uninserted mRNAs is controlled, and why some paramyxoviruses insert one G and others two Gs when insertions occur.

Key words: paramyxovirus/mRNA editing/stuttering

Introduction

A form of mRNA editing has recently been found for the P gene mRNAs of simian virus 5 (SV5, Thomas *et al.*, 1988), measles virus (Cattaneo *et al.*, 1989), Sendai virus (Vidal *et al.*, 1990) and mumps virus (R.Paterson and R.Lamb, personal communication), and similar P mRNA modifications have been predicted for other paramyxoviruses. For the four above mentioned viruses, a unique P gene gives rise to two mRNAs. One mRNA is an exact copy of the genome, whereas the other contains either one (measles and Sendai virus) or two (SV5 and mumps virus) Gs inserted within a run of 3–6 Gs. The insertions take place in the middle of the gene, and the frameshifts thus created allow ribosomal access to a second reading frame downstream. Two proteins with common N-terminal sequences, and alternate C-terminal sequences of different length, are translated from the two mRNAs. The longer protein in each case is the highly phosphorylated P protein, a component of the viral polymerase. The shorter protein, which is referred to as V, contains a conserved Cys-rich region near the C terminus which is possibly a metal binding domain, but is otherwise poorly described to date. For SV5 and mumps virus, the uninserted mRNA codes for the V protein and the inserted mRNA codes for P, whereas the reverse situation applies to Sendai and measles virus.

The addition of 'non-templated' bases to these mRNAs is not unique to the G insertions. Like most mRNAs paramyxovirus mRNAs are polyadenylated and this is also true when they are made *in vitro* with the polymerase and template present in purified virions (Kingsbury, 1974). When the 3′ ends of the mRNAs are mapped on the genome, they are always found to end at a run of 5–7 Us on the template (Giorgi *et al.*, 1983; Gupta and Kinsbury, 1984). For the closely related rhabdovirus vesicular stomatitis virus (VSV) mutants exist which form abnormally long poly(A) tails, and in *in vitro* reconstitution studies this phenotype is associated with the L protein, the viral polymerase (Hunt *et al.*, 1984). Furthermore, Iverson and Rose (1981) have shown that the VSV polymerase pauses at the gene junctions during mRNA synthesis. Taken together, these results suggest that the poly(A) tails are added by the viral polymerase during synthesis, by reiteratively copying the U run at the end of each cistron. This process is referred to as stuttering. With this precedent, it was suggested that the G insertions would also result from polymerase stuttering (Thomas *et al.*, 1988), even though the number of Gs inserted was small and controlled in a very precise manner. The 5′ poly(A) tails or leader sequences on late vaccinia virus mRNAs are also thought to be formed by a stuttering mechanism (Schwer and Stunnenberg, 1988).

Sendai virus P mRNAs made *in vitro* with purified virions also contain the same G insertions found *in vivo*. This suggests that the insertions are due to virus structural protein(s). Moreover, when the P mRNA is expressed from a vaccinia virus (vv) recombinant in cells co-infected with Sendai virus, in conditions in which each P mRNA can be distinguished, the natural mRNA is modified as usual whereas that made from vv-DNA is not modified at all (Vidal *et al.*, 1990). The inability of the insertion activity to act in *trans* supports the idea that the insertions occur during mRNA synthesis from the Sendai viral genome, rather than on preformed mRNA. However, it is difficult to rule out the possibility that this inability was due to the extra vv sequences present at both ends of the recombinant mRNA.

This paper directly tests the notion of a stuttering mechanism for the paramyxovirus G insertions. An *in vitro* system was used to test certain predictions of stuttering, by modifying the reaction conditions. We have found strong support for this mechanism, and propose a model which accounts both for how the frequency of inserted mRNAs is controlled, and why certain paramyxoviruses insert one G, and others two Gs, when insertions occur.

Results

The stuttering mechanism implies that the viral polymerase reiteratively copies one of the three C residues on the Sendai genome template (nucleotides 1051–1053). This presumably occurs when the 3′ end of the nascent mRNA at the insertion site, which is base paired to the template over a few nucleotides, slips backwards or upstream on the template together with the polymerase before the next base is incorporated (Figure 1B). The frequency with which the P mRNAs are inserted appears to be tightly controlled. For

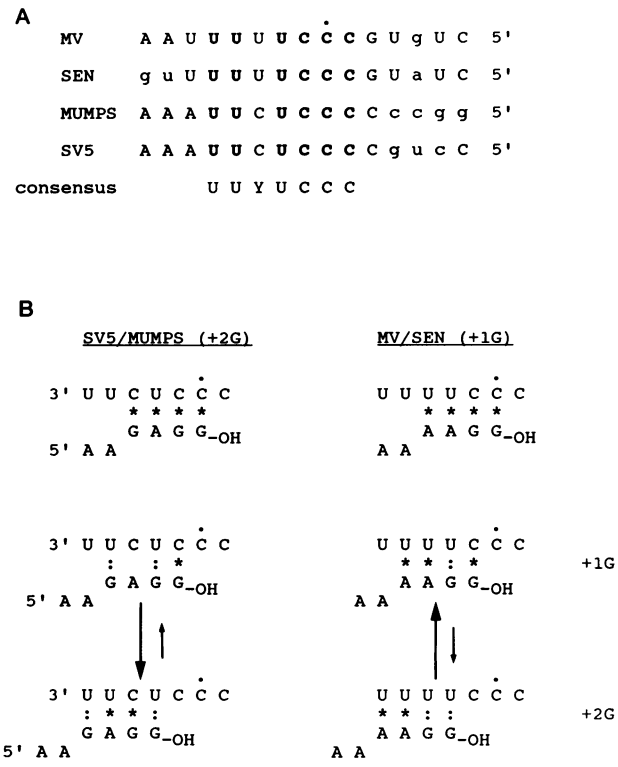

Fig. 1. Stuttering model for paramyxovirus P mRNA insertions. **Panel A.** The four paramyxovirus minus-strand genome sequences where G insertions are known to occur, written 3′ to 5′, are shown. Exact homologies are highlighted in bold script, and other homologies in capital letters. A consensus sequence is given underneath. **Panel B.** The proposed events at the insertion site for each virus group are shown. The top line in each case is the genome, and only the consensus sequence is shown, with a dot above marking the proposed pause site. The bottom line is the nascent mRNA, with its 3′-OH end indicated. Only 4 bp between the mRNA and the template are shown, with normal pairs indicated with asterisks, and U:G pairs with two dots. The top duplexes show the nascent chains at the pause site before slippage, the middle and bottom duplexes after a 1 and 2 bp slippage respectively. Incorporation after slippage which brings the polymerase back to the pause site leads to the G insertions, and incorporation beyond the pause site fixes the events.

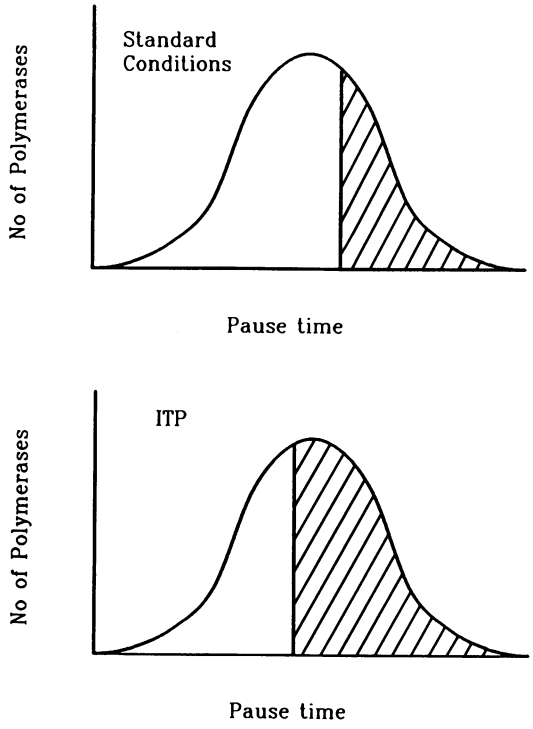

Fig. 2. Polymerase pausing and the frequency of G insertions. The distribution of the length of the pause at the insertion site (before slippage/insertion) for individual polymerases is shown as a bell-shaped curve. The vertical line within the curve indicates the minimum time necessary for slippage to occur, and the shaded area to the right indicates the fraction of polymerases which will insert one or more Gs. The difference between the fraction of inserted mRNAs *in vivo* (31 ± 2%) and under standard conditions *in vitro* (20 ± 2%) could be due to a difference in either of these two parameters. The effect of replacing guanosine with inosine (below) is shown as lowering the minimum time required for slippage.

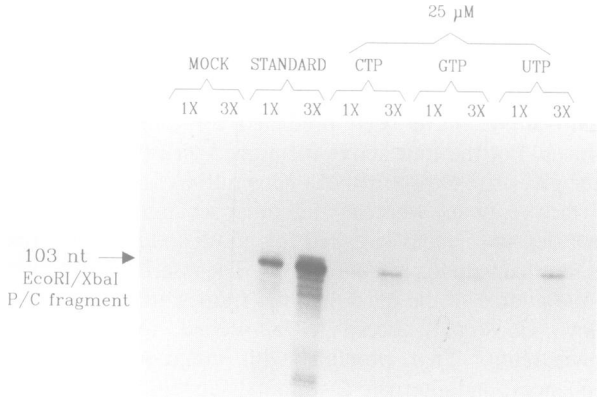

Fig. 3. Estimation of polymerase products by RNase mapping. The mRNA products of standard polymerase reactions (1 mM all four NTPs), of those containing either 25 μM CTP, GTP or UTP, or of mock reactions, were isolated by pelleting through CsCl density gradients. Two different amounts of each sample, presenting 5 μl and 15 μl (1 × and 3 ×) of each reaction (250 μl), were used to protect a 139 nucleotide riboprobe containing nucleotides 1028–1130 of the genomic P/C sequence as minus-strand RNA from RNase digestion. The remaining RNA was examined on an 8% sequencing gel and estimated by densitometry.

Sendai virus *in vivo*, 31 ± 2% of the mRNAs contain a one G insertion (five determinations, Vidal *et al.*, 1990). In a stuttering mechanism, it seems reasonable that the polymerase must pause at the insertion site, otherwise there would not be time for the slippage to occur. RNA polymerases are known to pause during transcription on both prokaryotic and eukaryotic DNA not only at termination sites, but well within the mRNA (von Hippel, 1984; Platt, 1986; Reines *et al.*, 1987), and pause times of 10 s to several minutes have been estimated (Chamberlin, 1976; Krakow *et al.*, 1976). The reasons for the polymerase pausing are unclear, but pausing occurs *in vitro* even in the presence of high NTP concentrations (von Hippel *et al.*, 1984), and for RNA polymerase II the pausing is independent of the structure of the nascent mRNA (Reines *et al.*, 1987). These findings suggest that pausing is an intrinsic property of the template at these sites.

As a working hypothesis, we suggest that the length of the pause is described by a bell-shaped curve (Figure 2). A plausible mechanism to explain how the frequency of inserted mRNAs is controlled can then be based on this distribution. Assuming for example an average pause time of 10 s, if the minimum time necessary for slippage were slightly longer (e.g. 13 s), then insertions would occur at a fixed frequency of less than half. The fraction of mRNAs

Table I. mRNA synthesis *in vitro*

Reaction conditions	Total colonies[a]	Colonies examined	No. Gs inserted at nucleotides 1051–1053			Total frequency insertions
			0	1	>1	
Exp. 1						
Mock	34	34	22 (65%)	9 (26%)	3 (9%)	35%
Standard	2688	224	174 (78%)	44 (18.5%)	6 (2.7%)	21.2%
25 μM CTP	408	170	135 (79%)	32 (18.8%)	3 (1.8%)	20.6%
25 μM UTP	362	151	112 (74%)	34 (22.5%)	5 (3.3%)	25.8%
			average frequency	19.9 ± 1.7%	2.6 ± 0.5%	22.5 ± 2.2%
25 μM GTP	192	160	128 (80%)	19 (11.8%)	13 (8.1%)	19.9%
Exp. 2						
Mock	10	0				
Standard	2688	176	130 (73.8%)	43 (24.4%)	3 (1.7%)	26.1%
40% Br-UTP	952	167	133 (79.6%)	32 (19.1%)	3 (1.8%)	20.9%
40% ITP	317	176	74 (42%)	67 (38%)	35 (19.8%)	57.8%

[a]Neutral oligo$^+$ colonies

with G insertions is then determined by the fraction of the polymerases which pause longer than the time required for slippage, and changes in either of these two parameters would alter the frequency of G insertions. Conditions which increase the pause, for example, by so limiting the NTP required to continue elongation that further incorporation becomes rate limiting (Ruteshouser and Richardson, 1989), might then increase the frequency of G insertions.

We therefore examined the effect of limiting NTP concentrations during Sendai virus mRNA synthesis on the frequency of G insertions. Virion polymerase reactions were carried out under standard conditions (1 mM of all four NTPs), or when CTP, UTP or GTP were individually lowered to 25 μM. The concentration of ATP was not lowered, since all viral RNAs start with ATP and its relatively high K_m for synthesis (300 μM) reflects its requirement for initiation rather than internal incorporation. Under these conditions total mRNA synthesis was decreased significantly, yet the RNAs made *de novo* were still several fold more abundant than those already present in purified virions, presumably as cellular contaminants. This experiment also examines the effect of varying relative NTP concentrations 40-fold.

The effects on mRNA synthesis was first examined by RNase mapping, using a riboprobe containing nucleotides 1028–1130 of the P gene. A mock polymerase reaction was also examined, to determine the levels of pre-existing mRNAs. As shown in Figure 3, the products of a standard reaction are relatively abundant by this test, whereas those of the mock reaction are not detectable at this exposure. When CTP or UTP were present at 25 μM, RNA synthesis was decreased by 6- to 9-fold, and when 25 μM GTP was used, the products were decreased by 15-fold. The stronger effect of lowering GTP may reflect the fact that this nucleotide is also used to cap the mRNAs, and that capping and mRNA synthesis are coupled.

To determine the effect on the frequency of G insertions, we cloned the P mRNA of the reaction products and examined the resulting colonies by hybridization with three oligonucleotides (oligos) as before (Vidal *et al.*, 1990). One oligo is complementary to a region just downstream of the insertion site where no changes have been found (neutral oligo), and identifies those colonies which carry the P gene fragment. The other two are complementary to the insertion site, but can distinguish whether there are three Gs (genomic) or four Gs (+1 G) at this position. The vast majority of the neutral oligo$^+$ colonies are also either genomic oligo$^+$ and +1 G oligo$^-$, or vice versa, and need not be confirmed further. A minority, however, are neither genomic nor +1 G oligo$^+$, and these were confirmed by plasmid sequencing. Most of these colonies have previously been shown to result from a smaller fraction of the mRNAs which have multiple G insertions (Vidal *et al.*, 1990).

As shown in Table I, a few colonies are obtained when the mock reaction products are cloned, and their distribution according to whether 0, 1 or >1 G has been inserted is similar to that found on larger samples of intracellular mRNAs. The standard reaction yielded 80 times more colonies and their distribution is again similar to that reported previously (except that there are less in the >1 category, possibly because fresh virus was used here). The reaction containing 25 μM GTP gave the least number of colonies as expected, but even here there were still 5.7 times as many colonies as in the mock reaction. The vast majority of the clones are therefore derived from mRNA made *de novo*.

We found that neither the frequency of single G insertions nor that of multiple G insertions were basically different from the standard reaction when either CTP or UTP were decreased to 25 μM (Table I). When treated as a group, 19.9 ± 1.7% contained +1 G, and 2.6 ± 0.5% contained >1 G. Only when GTP was lowered to 25 μM were significant differences apparent, but in an unexpected fashion. The frequency of single G insertions was decreased by half, whereas those with multiple G insertions were increased 3-fold. The net result, however, was that the total fraction of inserted mRNAs varied little, if at all.

In terms of the stuttering model we did not expect any differences at 25 μM UTP, as the first U after the G run is three nucleotides downstream on the mRNA (5′GGGCAU 3′). Causing the polymerase to wait longer before it can incorporate this base should have no effect because it is too far from the insertion site. By the same criteria, the lack of any effect at 25 μM CTP (5′GGGCAU 3′) would argue that if the stuttering

Table II. mRNAs with A modifications

Reaction conditions	Total no. clones examined	Clones with modifications +1G	>1G	+/−A	
Standard	224	44	6	1	ucaacAAAA GGGcauagg (−2 A)
25 μM CTP	170	32	3	1	ucaacAAAAAAAGGGcauagg (+1 A)
25 μM UTP	151	34	5	1	ucaacAAAAAAAGGGcauagg (+1 A)
25 μM GTP	160	19	13	0	
	705	129	27	3	

mechanism applies, stuttering would not take place at the third C on the template, but presumably on the first or second C (3′CCCGUA 5′). If this were so, at low GTP there might be more time for slippage before the 2nd or 3rd G was incorporated (5′GGGCAU 3′), and so the frequency of insertions should increase. This is in essence what has occurred, even though the total fraction of inserted mRNAs is unchanged. Under standard conditions, one G is added 18.5% of the time, and an average of six Gs are added at 2.7%, such that overall an average of 1.6 Gs are added when insertions occur. The same calculation when 25 μM GTP is used shows that an average of 3.6 Gs are added when insertions occur. In terms of the stuttering model, it would appear that 25 μM GTP cannot significantly lengthen that initial pause at the insertion site, and so the total fraction of inserted mRNA is unchanged. However, 25 μM GTP appears to extend the pause after the 1st insertion has occurred, hence a greater fraction of the inserted mRNAs contain multiple insertions.

The results of this experiment also support the stuttering model in other ways. The fact that the frequency of insertions is similar in all respects at low CTP or UTP, even though 6- to 9-fold less mRNA is made, is inconsistent with the insertions occurring on mature mRNAs. The ratio of insertion activity (viral proteins) to mRNA would be 6- to 9-fold higher here. Thus, *in vitro*, the insertions also appear to occur during mRNA synthesis, and unlike the *in vivo* studies (Vidal *et al.*, 1990), there is no ambiguity here of whether the insertion activity cannot act on preformed mRNAs because of the extra vv sequences at each end of the transcript. A second support for the insertions taking place during synthesis at the predicted site was more unexpected. As listed in Table II, we examined a total of 705 colonies from the four reactions, of which 129 contained +1 G, and 27 contained >1 G. There were, however, 30 colonies in the neutral oligo$^+$, genomic and +1 G oligo$^-$ group. The three extra colonies were found to have three Gs (the genomic sequence) at the insertion site, but fell into this category because they did not contain six As before the G run. Two were found to contain seven As, and one had only four As (Table II). Besides the G insertions at 1051−1053, these A modifications were the only other changes within the 103 nucleotides routinely sequenced (1028−1130). The polymerase can apparently also stutter once on the U run of the template, albeit at a much lower frequency than at the three Cs just downstream. Moreover, the polymerase also appears to be able to skip two bases while transcribing the U run, whereas we have never seen a G deletion in the more than 50 colonies sequenced to date.

Stuttering implies that during the polymerase pause at the insertion site, the base pairing between the 3′ end of the nascent mRNA and its template will be broken transiently,

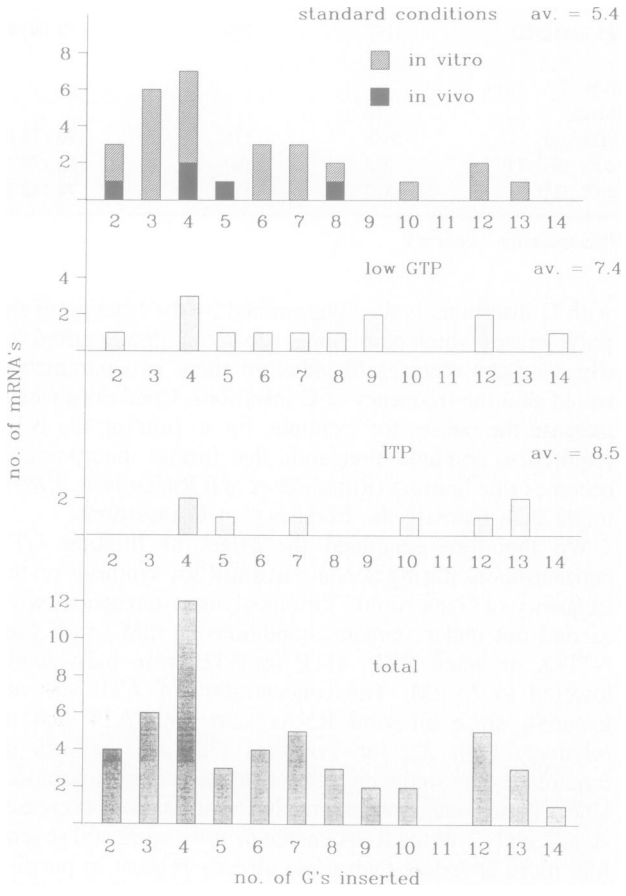

Fig. 4. Distribution of mutliple G inserted mRNAs. The number of Gs inserted in the neutral oligo$^+$ but genomic and +1 G oligo$^-$ colonies were determined by plasmid sequencing. Their distribution is shown according to the reaction conditions used. The graph for standard conditions includes the low UTP and low CTP reactions, as well as five *in vivo* mRNAs indicated by the black portion of the bars.

for slippage to occur. The precise number of these base pairs and their composition would then be important. We therefore examined the effect of incorporating base analogues into the mRNA chain which alter the strength of these interactions. Inosine (I) incorporation in place of guanosine will decrease the stability of the base pairing (and there must be at least one G:C pair available here for substitution), whereas 5-bromo-uridine incorporation will have the opposite effect. Parallel transciption reactions were carried out under standard conditions, and in which 40% of the UTP was replaced with Br-UTP, or GTP with ITP. Nearest neighbour analysis of [^{32}P]CTP-labelled samples confirmed that a roughly similar proportion of the natural bases were in fact replaced with their analogues (not shown).

When the frequency of G insertions in the mRNA was examined (Table I), Br-U substitution for U was found to have little or no effect (a 1 G insertion frequency of 19.1% versus 24.4% for the parallel control, and 19.9% average in experiment I, and a >1 G insertion frequency of 1.8% versus 1.7% and 2.6%). In contrast, inosine substitution for G increased the frequency of one G insertions 2-fold (38%), and the multiple G insertions were 10-fold more frequent (19.8%). When eight of these plasmids were sequenced, G insertions were found only within the G run at nucleotides 1051–1053, and so the presence of ITP did not lead to insertions at other sites within the mRNA. The incorporation of the analogues would have two effects; they would alter the stability of the intramolecular pairing upstream of the 3′ end of the nascent chain, as well as that to the template at the 3′ end. The absence of an effect when U is substituted with Br-U argues that the folding of the nascent chain has little effect on the insertion frequency. The effect of substituting inosine for G is then more likely to be due to the pairing with the template. In terms of the model in Figures 1 and 2, this can be viewed as a decrease in the minimum time necessary for the initial slippage to occur, as well as subsequent slippages, because it would be easier to break the I:C pairs within the polymerase domain.

Discussion

When the above results are considered together, it is difficult to conceive of a mechanism other than stuttering that is plausible. All attempts to show that the insertions can occur on preformed mRNAs were negative, and manipulations of the *in vitro* system can all be interpreted in a coherent way for the predicted insertion site in terms of a stuttering model. The model can account for how the fraction of one G inserted mRNAs is tightly controlled both *in vivo* (31 ± 2%, five determinations), and *in vitro* under standard conditions (20 ± 2%, four determinations). Can it also offer any clues as to why two Gs are inserted at high frequency in SV5 and mumps virus when insertions occur, rather than the one G inserted in Sendai and measles virus?

An alignment of the demonstrated insertion regions of the four paramyxovirus templates is shown in Figure 1A. They all contain a minimum of three Cs on which the polymerase could pause and slip. There is little homology in the alignment downstream of the three Cs, but the 1st, 3rd and 4th bases upstream are all Us. A consensus sequence 3′UUYUCCC 5′ is evident, in which the Y is a U in those viruses where one G is inserted, and a C where two Gs are inserted, and it is this difference which may be involved. Figure 1B shows a schematic representation of the insertion mechanism for each virus group. We do not know which template C is the pause site, nor the number of base pairs between the nascent chain and the template. However, we would expect the latter to be limited to the minimum in this region, to facilitate the transient melting required for slippage. For demonstration, 4 bp starting at the middle C are chosen. There is presumably some pressure following the pause to displace the nascent chain upstream. For SV5/mumps, displacement upstream by 1 bp is unstable as it includes an A:C pair, whereas displacement by 2 bp creates a more stable intermediate. For the measles/Sendai group, on the other hand, an A:U rather than an A:C pair occurs at this position upon displacement by a single base pair, and this intermediate is more stable than that obtained on a shift of 2 bp (G:U base pairs would presumably be tolerated in this system). Thus, the relative stabilities of the 1 and 2 bp misalignment intermediates for each virus could determine whether one or two Gs are inserted at high frequency when insertions occur. This scheme would also hold for all the other paramyxovirues (CDV, bovine and human PIV3, and NDV; all one G insertions) where insertions have been predicted (Cattaneo *et al.*, 1989; Paterson *et al.*, 1989). It may soon be possible to test these predictions for measles virus, as Ballart *et al.* (1990) have developed a system in which infectious virus is produced from measles DNA. A mechanism with many similar features has previously been proposed to account for the repeated TTGGGG sequences in *Tetrahymena* telomers (Greider and Blackburn, 1989). These are added by a telomerase which contains an RNA template for this sequence within a larger chain. What is similar here is that the enzyme is proposed to pause on the template and then slip 6 nucleotides upstream and this distance is determined by the base pairing to the template.

The G insertion mechanism for Sendai virus is relatively precise; >80% of the inserted mRNAs contain a single extra G, either *in vivo* or *in vitro*. The vast majority of the remainder have added two or more Gs, and are of interest both because this may be a way to introduce further diversity into these proteins and because of what they can tell us of the insertion mechanism. When twelve >1 G inserted mRNAs were previously examined (Vidal *et al.*, 1990), those with +2 Gs did not predominate as expected, but mRNAs with +2 to +8 Gs inclusive were found. A larger number of these mRNAs made *in vitro* has now been examined, and their distribution is not dissimilar (Figure 4). What is striking here is that 12 Gs are as likely to be incorporated as two Gs when multiple insertions occur (with a curious bias for +4 Gs). This distribution with averages of 5.4 Gs (standard conditions), 7.4 Gs (25uM GTP) and 8.5 Gs (40% ITP) added, suggests that the multiple insertions are not the result of simple repetition on the initial G insertion, where one would expect a distribution in which +2 Gs > +3 Gs > +4 Gs etc. Once the initial slippage/insertion has occurred, however, one G:U bond has been formed, and upon two insertions two G:U bonds are formed, and these would be expected to increase the likelihood of further rounds of slippage, leading to the broad distribution observed. The finding that low GTP concentrations or ITP substitution for GTP have stronger effects on subsequent slippages rather than the initial slippage, is consistent with the notion that the initial and subsequent insertions are not the same. For the one G insertion in Sendai and measles virus (or the two G insertion in SV5 and mumps virus) to predominate, however, a second round of slippage must be avoided. This could occur by the initial insertion(s) somehow relieving the polymerase pause >80% of the time, in contrast to what would happen during polyadenylation. The multiple G insertions then presumably arise because sometimes the initial insertion fails to relieve the pause. In this respect, the G insertion mechanism would approach the polyadenylation process, and the fact that an apparent maximum of 14 Gs are added under these conditions may in part reflect the self-limiting nature of poly(G) addition. For polyadenylation, the pause is also presumably much stronger, and can only be relieved by chain termination and

not continued elongation. In contrast, we have not seen any evidence for chain termination at the insertion site under any conditions, using RNase mapping.

The G insertions do not represent mRNA editing in a strict sense, as the mRNA has not been altered after its synthesis. However, it is a form of editing in that it results in a specific change of the mRNA relative to its template which is important for translation. Compared to the two forms of editing which take place post-transcriptionally, the stuttering mechanism appears to have little in common with that which simply causes C to U transitions in the apolipoprotein B mRNA (Powell *et al.*, 1987; Chen *et al.*, 1987) and certain plant mitochondrial mRNAs (Gualberto *et al.*, 1989; Covello and Gray, 1989), but which has not as yet been determined. The mechanism which causes U insertions (and deletions) in trypanosome mitochondrial mRNAs (reviewed in Benne, 1989; Simpson and Shaw, 1989), however, has recently become clearer (Bakalara *et al.*, 1989; Blum *et al.*, 1990). In this system from 1 to 8 Us are inserted at given sites in the primary transcript by a cleavage, insertion, and religation mechanism, and no slippage is required. The information for these insertions is contained within a separate guide RNA, in which G:U pairs are allowed in either direction, and either one or two guide RNAs are required for each edited region. For paramyxoviruses where the insertions occur co-transcriptionally, there is no separate guide RNA but this function would be assumed by the template itself after slippage, and here too G:U pairs would play a prominent role. There is thus only limited similarity between these two insertion mechanisms. However, for the extreme cases of massive mRNA editing or pan-editing, it remains possible that a slippage mechanism will be used in addition, thereby limiting the number of guide RNAs required for the many different sites.

Materials and methods

In vitro mRNA synthesis

All polymerase reactions contained 250 μg of purified virions in a total volume of 250 μl and 1 mM of all four NTPs except as described in the text, and were carried out as previously described (Vidal and Kolakofsky, 1989). Reactions in which CTP, GTP or UTP were reduced to 10 μM were also carried out, but under these conditions insufficient mRNA was made relative to that which contaminates the virions. When ITP or Br-UTP were used, the analogues were present at 400 μM and GTP or UTP were reduced to 600 μM. Further replacement of the natural triphosphates with their analogues led to severely reduced mRNA synthesis. Mock reactions contained no added NTPs and 10 mM EDTA (a 2-fold excess). After 3 h at 30°C, 0.5 M NaCl and 1% Nonidet P-40 (NP-40) were added, the reactions heated for 2 min at 37°C, and their products isolated by pelleting through 20–40% CsCl density gradients. The viral genomes are retained at the middle of these gradients. The amounts of P mRNAs made were estimated by RNase mapping as previously described (Vidal and Kolakofsky, 1989).

Determination of the frequency of G insertions at nucleotide 1051–1053

The method is described in detail in Vidal *et al.* (1990). Briefly, nucleotides 1028–1130 (*Xba*I–*Eco*RI) of the P mRNA in the CsCl pellets were specifically cloned in a one tube reaction using a primer downstream of nucleotide 1130. The cDNA was then cut with *Xba*I and *Eco*RI (both unique in the P gene) and cloned into the same site of pGEM or pBluescript. Under these conditions, more than a third of the colonies contained the specific insert. The resulting colonies were then screened by filter hybridization with an oligo just downstream of the insertion site (1068–1072, neutral oligo) to identify colonies with the insert, and two 19 mers which can distinguish three or four Gs at the insertion site. Ambiguous colonies (5%) were confirmed by a second round of screening or by plasmid sequencing.

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Two new viruses, PIV2 (Southern *et al.*, 1990, Virology, in press) and PIV4 (Kondo *et al.*, 1990, Virology, in press), as well as CDV (Barret *et al.*, 1990, in *The Paramyxoviruses*, Plenum Press, NY) have since been shown to edit their P gene mRNA by adding 2, 2, and 1 G respectively. In all cases the sequence at the editing site adds further support to the model.