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Scanning independent ribosomal initiation of the Sendai virus Y proteins *in vitro* and *in vivo*

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The Sendai virus P/C mRNA contains five ribosomal initiation sites between positions 81 and 201 from the 5′ end. One of these sites initiates in the P open reading frame (ORF) (ATG/104), whereas four initiate in the C ORF (ACG/81 and ATGs/114, 183, 201), to give a nested set of C proteins (C′, C, Y1, Y2). Introduction of new ATGs or physically breaking the mRNA upstream of these natural sites was used *in vitro* to prevent ribosomal scanning downstream. The results suggest that a minority of the ribsomes which initiate C (ATG/114) and all of those which initiate Y1 and Y2 (ATGs/183 and 201) do so by a scanning independent mechanism. When the leaky ACG/81 site is changed to a non-leaky ATG site in *in vivo* experiments, ribosomal initiation at Y is again not diminished, whereas that at C as well as at P becomes undetectable. Ribosomal initiation at Y appears to be scanning independent *in vitro* and *in vivo*. That at C is partly independent *in vitro*, but completely dependent *in vivo*. These results are discussed in terms of a model of internal initiation at Y.

Key words: internal initiation/ribosomes/Sendai virus

Introduction

Sendai virus, a paramyxovirus, contains a non-segmented minus strand RNA genome of 15.3 kb, from which six mRNAs are transcribed (see Kolakofsky and Roux, 1987, for a recent review). Five of the mRNAs are monocistronic and code for only a single primary translation product. The P/C mRNA, however, is polycistronic and contains two overlapping ORFs which start near the 5′ end of the chain (Giorgi *et al.*, 1983). The longer P ORF begins with the 5′ most proximal ATG at position 104 (ATG/104) and continues for 568 amino acids, yielding the P protein (Figure 1). The last 95 amino acids of this ORF are also expressed as a separate protein (X), which is independently initiated by ribosomes >1500 nt from the 5′ end (Curran and Kolakofsky, 1988b).

The shorter C ORF is also responsible for a nested set of proteins, called C′, C, Y1 and Y2, which are carboxy co-terminal, and which we refer to collectively as the C proteins. Site directed mutagenesis has shown that C′ is initiated on a non-ATG codon, ACG/81, whereas C, Y1 and Y2 start on ATGs at positions 104, 183, and 201 respectively. When each of these codons is changed to non-initiators in a SP6 vector carrying the gene, only the expression of its cognate protein is ablated in *in vitro* translations (Curran and Kolakofsky, 1988a; Gupta and Patwardhan, 1988; Patwardhan and Gupta, 1988). Including P (ATG/104), five proteins (C′, P, C, Y1 and Y2) are therefore initiated near the 5′ end and one (X) near the 3′ end of a single mRNA. Only the P protein has so far been detected in mature virions; the others are considered to be non-structural proteins.

Eukaryotic ribosomal initiation sites are generally the 5′ most proximal ATGs, whose surrounding nt or context conforms to a weak but recognizable consensus, of which the most important elements in the model pre-pro-insulin mRNA, are a purine at position −3, followed by a G at +4 (Kozak, 1983, 1986). The initiation codons are thought to be chosen by a scanning mechanism, in which the 43S ribosomal pre-initiation complex, or initiation factors of the eIF-4 series, or both, traverse the mRNA from its capped 5′ end, and initiate translation when they reach the first such site. To account for unusual situations such as viral mRNAs with overlapping genes, Kozak (1986) proposed a modified or 'leaky' scanning model. Ribosomes could scan through the first ATG some of the time if it were in an unfavourable or poor context for initiation, and initiate on the next ATG, if it was in a good context. These terms refer to how well the key positions (−3 and +4) conform to the consensus. An expanded version of this model could then explain the first three sites of the P/C mRNA; C′ starts on a non-ATG codon, P on an ATG in a poor context (a Py at position −3), and C on an ATG in a good context (an A at −3). Moreover, as the C/ATG is not in the context considered to be the most favourable (it contains a C rather than a G at +4), it is not impossible that Y1 and Y2 are also initiated by leaky scanning. Initiation of the Y proteins, on the other hand, could be due to a scanning independent mechanism, which takes place during X protein synthesis, at least *in vitro* (Curran and Kolakofsky, 1988b).

Whether a particular initiation codon is in a favourable context or not, however, cannot always be predicted from the immediate context. Examples of overlapping genes are known in which the simple context of the initiation codons does not conform to the leaky scanning model (Bos *et al.*,

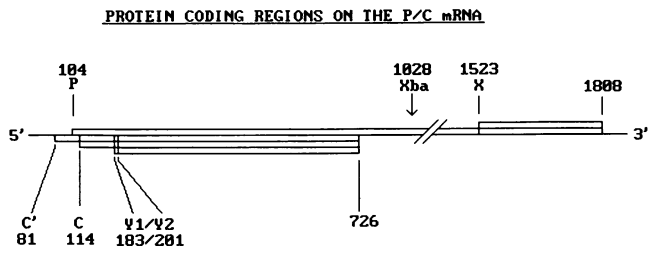

Fig. 1. Schematic representation of the P/C mRNA. The mRNA is shown as a horizontal line. Open boxes above the line refer to proteins coded by the P ORF; those below the line to those coded by the C ORF. The initiation sites are indicated by letters. Numbers refer to the position of the first base of the initiation or termination codons, or the unique *XbaI* site, relative to the 5′ end of the natural mRNA.

1981; Shaw *et al.*, 1983), and in some viral mRNAs the context can be changed in either direction without affecting relative translational efficiency (Munemitsu and Samuel, 1988; Williams and Lamb, 1989). In these cases, other sequences or structures of the mRNA must play a dominant role. This paper reports a study of how the Sendai virus C′, P, C, and Y proteins are initiated, both *in vitro* and *in vivo*, in which the context of the natural initiation codons has not been altered.

Results

When baby hamster kidney (BHK) cells infected with five different strains of virus are labelled with [^{35}S]Met and directly analysed by SDS−PAGE, the P, C′, and Y proteins are found in roughly equimolar amounts, whereas C is 4 to 5 times as abundant (Curran and Kolakofsky, 1988a). To determine whether the relative ratios of the C proteins vary during the time course of the infection, infected cells were pulse labelled at various times, and the C proteins were recovered with a specific antiserum and separated by SDS−PAGE. This antiserum was raised against purified C protein made in *Escherichia coli*. As shown in Figure 2, the ratios of the various C proteins made during the infection do not vary. When quantitated by densitometry and normalized for Met content, we find 6.3 (±0.2) times as much C as C′, and 1.6 (±0.4) and 1.2 (±0.2) as much Y1 and Y2, during the last four times of pulse labelling. Synthesis rates parallel mRNA abundance (not shown) for the first 12 h, and then decrease slowly. The relative ratios of the C proteins are also similar to those seen before, and both Y1 and Y2 are clearly visible, with Y1 slightly more abundant. We sometimes see only a single band of Y *in vivo*. In these cases, it appears that only one of the Y ATGs is being used, but it is also possible that some gels cannot resolve the Y doublet.

One approach to determining whether a scanning mechanism can account for the synthesis of all of these proteins is to introduce a new ATG upstream of the natural initiation codons (Liu *et al.*, 1984). Our assumption is that at least ACG/81 and ATG/104 are designed to be leaky initiation sites. Introduction of a non-leaky site which is now the 5′ most proximal ATG should therefore prevent scanning downstream. Using SP65-P/C, we extended the P ORF by changing CAG/29 to ATG, both in a context considered favourable for initiation (GCT**ATG**G) and one highly unfavourable (TCT**ATG**T, positions −3 and +4 are underlined). Although ribosomes which initiate at ATG/29 will make a P protein of 593 rather than 568 amino acids (P*), we found that P and P* could barely be distinguished by electrophoretic mobility. These bands are just visible in Figure 3, presumably due to incomplete restriction of the plasmid DNAs. To augment their difference in mobility, the plasmids were linearized with *Xba*I which cuts at position 1028, so that mRNAs which code for truncated P proteins (P−Xba and P*−Xba) would be made without affecting the C proteins, which terminate at 726. When parallel translation reactions primed with equal amounts (50 ng) of these mRNAs were examined (Figure 3), the introduction of ATG/29 in a good context (CAG/29-ATG$^+$) was found to eliminate all C′ and P expression, whereas that in a poor context (CAG/29-ATG$^-$) eliminated all of P and decreased C′ expression to 17% of wild type levels, as determined by

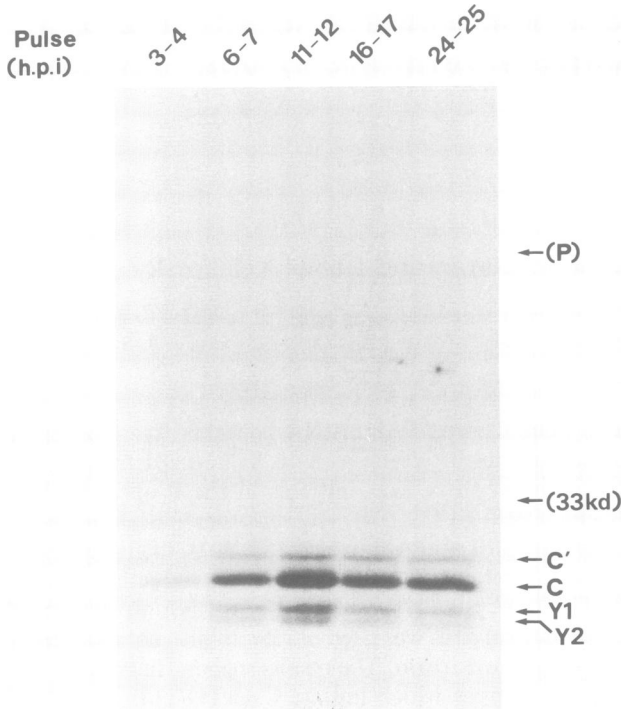

Fig. 2. Kinetics of synthesis of the C proteins during the infectious cycle. Confluent monolayers of the BHK cells were infected with 20 p.f.u./cell of the H strain of Sendai virus, and labelled for 1 h with 45 μCi of [^{35}S]Met at the times indicated above. The cells were directly solubilized after the pulse in 0.5 ml of PBS containing 2% SDS and 1% β-mercaptoethanol, and the DNA was sheared by forced pipetting. Equal samples (75 μl) were diluted 20-fold immunoprecipitated with guinea pig anti C serum, and separated on a 12.5% SDS−polyacrylamide gel. A fluorograph of the gel is shown. The positions where the P and 33 kd proteins would migrate are also indicated.

densitometry. The difference in mobility between P*−Xba and P−Xba is again very limited, but when these samples are run on urea gels for longer times, the separation is sufficient to determine that no P−Xba is made from either mutant mRNA (not shown). C expression, however, is not completely eliminated by either ATG/29, although less C is made when ATG/29 is in a good context (29%) than when in a poor one (54%), again relative to wild type levels. Y2, the predominant Y protein in *in vitro* translation, on the other hand, is decreased by <20% by ATG/29 in either context. Figure 3 also shows the effect of changing ACG/81 to ATG, which naturally lies in an excellent context (GCC**ATG**G). C′ expression is now significantly increased (7-fold) as expected and P expression is again eliminated. C is further decreased relative to ATG/29, and is now only 16% of wild type levels. Y expression, in contrast, is again relatively unaffected (75% of wild type levels). These results indicate that ribosomes which initiate C′ and P at ACG/81 and ATG/104 *in vitro* do so entirely by scanning, those which initiate C at ATG/114 do so mostly by scanning, but those which initiate Y1 and Y2 at ATG/183 and ATG/201 appear to do so almost entirely by a scanning independent mechanism. We also note that the artificial ATGs created at position 29 initiate P* synthesis equally regardless of their context, both initiating 1.8 times as frequently as the natural P ATG/104.

An alternative and more direct approach to determine

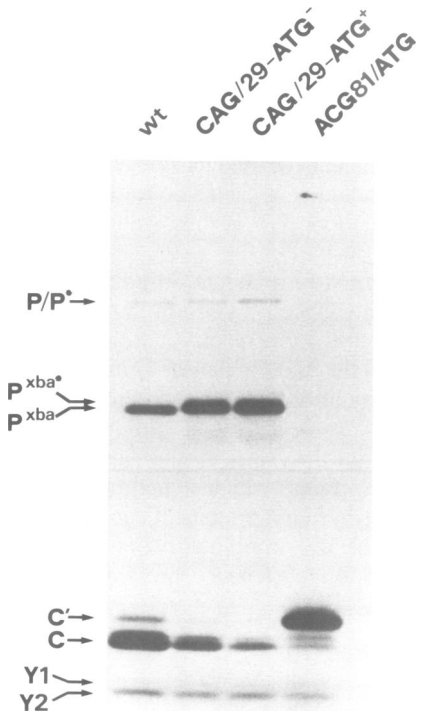

Fig. 3. The effect of additional upstream ATGs on the expression of the C proteins *in vitro*. Equal amounts of mRNAs (20 ng) from SP65 plasmids containing either the wild type P/C gene, or the site directed mutants described in the text, which had been linearized with *XbaI*, were translated in wheatgerm extracts in the presence of [^{35}S]Met. The reaction products were analysed directly on a 12.5% SDS gel. Unprimed translation reactions contained no detectable bands (not shown).

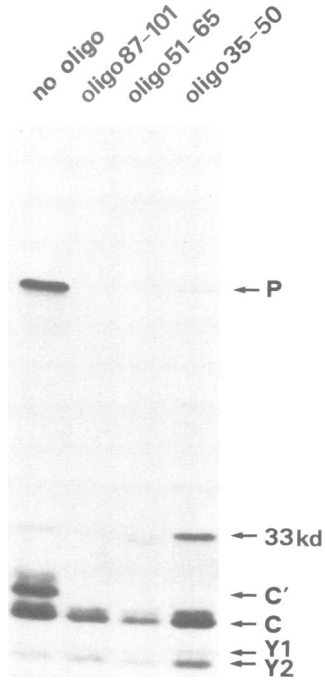

Fig. 4. Effect of chain scission in the 5′ UTR region on expression from downstream initiation sites. 20 pmol of oligonucleotides complementary to positions 87−101, 51−65, 35−50 or no oligos, were combined with 20 ng of complete wild type P/C mRNA and added to wheatgerm extracts. Amino acids containing [^{35}S]Met were then added. After translation, the products were analysed directly as for **Figure 3**.

whether scanning is taking place for all these initiation events *in vitro* is to introduce a physical break in the mRNA between the capped 5′ end and the initiation sites. This can be done simply by adding complementary oligonucleotides to the wheatgerm translation extracts along with the mRNA, as these extracts contain a potent RNase H activity (Minshull and Hunt, 1986; Vidal *et al.*, 1988). We have found that not all oligos cut the mRNA completely in this system when radiolabelled transcripts are examined directly, and so three oligos, complementary to positions 35−50, 51−65 and 87−101, were used (Figure 4). Oligos 51−65 and 87−101 appeared to cut the mRNA completely (this was confirmed for oligo 87−101 in a separate experiment, in which the mRNA was also shown to be cut specifically) and had similar effects. Both C′ and P expression were undetectable. C expression was decreased to 70% (oligo 87−101) and 40% (oligo 51−65), but again Y1 and Y2 expression were comparable to control levels (140% for oligo 87−101 and 80% for oligo 51−65). Oligo 35−50 appeared to cut most but not all of the mRNA, as indicated by a very small amount (4%) of residual P synthesis. However, C′ synthesis was undetectable, whereas C and Y1 + Y2 expression were in fact increased relative to the uncut control, by a factor of 1.8 and 4.2 respectively. Non-complementary oligos added at the same concentration (1 μM) had no effect (not shown). These results complement and support those obtained by introducing upstream ATGs. *In vitro*, some of the ribosomes which initiate C, and most of those which initiate Y1 and Y2, are unlikely to be scanning from the 5′ end, as it is difficult to see how they could cross the gap in the mRNA. By the same criteria, all the ribosomes which initiate C′ and P, on the other hand, are likely to be scanning. An additional band which migrates at ~33 kd is also made in this *in vitro* translation, and is discussed below.

In vitro translation of the uncut P/C mRNA in Figure 4 yields considerably more C′ than that in Figure 3 (Figure 3 is more comparable to the *in vivo* situation). This variance is presumably due to the different extracts used in these two experiments, and this has been noted previously (Curran *et al.*, 1986). The enhancement of C′ synthesis in Figure 4 is probably due to the higher Mg^{2+} concentration of this extract which specifically stimulates ACG initiations (Peabody, 1987; Gupta and Kingsbury, 1985), as C′ synthesis is specifically reduced when this extract is back titrated with EDTA (not shown).

In vivo studies

Our wheatgerm translation system appears to reflect faithfully translation in infected cells, in terms of which initiation sites are used, and sometimes their relative frequencies as well (Figure 3). However, we would like to know whether the mechanism by which ribosomes choose these sites are the same *in vivo* as *in vitro*. Many of the experiments carried out *in vitro* cannot easily be tranposed to *in vivo* studies, e.g. oligo induced cleavage of the mRNA, or use of truncated P genes which might yield unstable proteins *in vivo*. However, some of our studies can be done *in vivo* using vaccinia virus (VV) recombinants. In particular, the ACG/81 to ATG mutation should be informative. At the same time

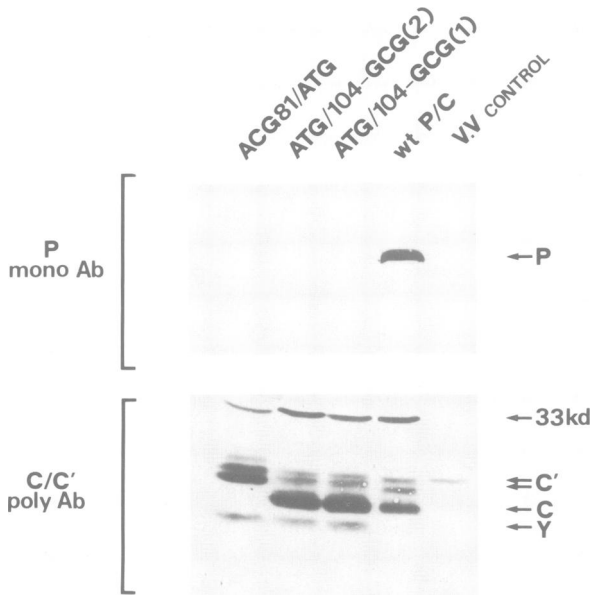

Fig. 5. Effect of site directed mutants on P/C gene expression *in vivo*. Parallel cultures of CV1 cells were infected with 10 p.f.u./cell of either non-recombinant VV (control), or recombinants containing either the wild type P/C gene or site directed mutants as indicated above. Total cell extracts were prepared as for **Figure 2** and 20 μl samples were separated by SDS−PAGE and electroblotted onto a nitrocellulose membrane. The membrane was cut in half. The top was reacted with anti-P monoclonal antibody 1.180 (Oervell and Grandien, 1982; Vidal *et al.*, 1988) and the bottom with rabbit anti-C/C′ serum. The two lanes for the ATG/104−GCG mutant represent two independent isolates of this VV recombinant.

we also examined mutant ATG/104-GCG, which eliminates the P initiation site. These mutants as well as the wild type gene were transferred from SP65 to VV under the control of the 7.5K promoter.

Parallel cultures of CV1 cells were infected with equal p.f.u./cell of the recombinants and wild type virus, extracts were prepared at 18 h when expression is maximal, separated by SDS−PAGE, and examined by immunoblotting (Figure 5). The antisera used here was made against infected BHK cell proteins which migrate at the position of C/C′, and it also detects a single band in the wild type VV infected control, just above C′. However, a band at this position is also present in some Sendai virus infections, especially when defective-interfering (DI) genomes are present, and here yet another band just above is also present (not shown). These extra bands are occasionally present *in vitro* (Figure 4), where they appear to result from initiation at ACG/81.

The wild type gene via VV infection expresses C′, P and C as in natural infections, but only a single band of Y, at the position of Y1, is found. Y also appears less abundant than with Sendai virus infection, in part because this antisera under-estimates Y relative to C′ and C (not shown). When the P protein ATG/104 is changed to GCG, C′ expression is unchanged, whereas P is totally eliminated, as expected. C and Y expression, however, are clearly increased relative to wild type levels, consistent with both being the result of leaky scanning. The change of the weak ACG/81 initiation site to a strong ATG site increased expression of the C′ protein(s) and was as effective as the ATG/104-GCG mutation in eliminating P expression. Moreover, C levels here are not simply reduced as *in vitro*, they are undetectable. Y1 synthesis, on the other hand, is again increased relative to wild type levels and is now as prominent as C′ in the wild type infection. These results indicate that all the ribosomes which initiate at C, as well as P, do so by a scanning mechanism *in vivo*. However, as the absence of both P and C expression in the ACG81/ATG mutant indicates that scanning beyond this initiation site is effectively eliminated, initiation of Y cannot be by the same mechanism. Ribosomal initiation of Y is thus scanning independent *in vivo* as well.

The band which migrates at 33 kd in Figure 4 is also detected by the C specific antisera in this experiment and is thus yet another form of the C proteins. However, we have never seen this band in Sendai virus infected cells, even those which contain DI genomes and multiple forms of C′ and it is not always detectable *in vitro* (Figure 3). We have no explanation for this band, except that it is a modification of one of the C proteins which sometimes takes place in the absence of the other viral proteins. Considering its predicted size, it might be due to ubiquitination of a C protein, as recently found for the tobacco mosaic virus coat protein (Dunigan *et al.*, 1988).

Discussion

The P/C mRNA is unusual even for a viral mRNA, in using six initiation sites, five of which are near the 5′ end with one of these in an alternate reading frame. This situation was exploited to examine how ribosome choose these sites near the 5′ end, as each site is an internal control for the others when changes are made. In this report we examined mutant mRNAs with new initiation sites upstream and wild type mRNA cleaved in the 5′ untranslated region (UTR) *in vitro*, and mutant mRNA in which the ACG of the first site was changed to ATG *in vivo* as well. All our results suggest that initiation at the first two sites (C′ and P) occurs via a scanning mechanism, but the mechanism of Y initiation at the fourth and fifth sites is mostly scanning independent. A minority of initiations of C at the third site are also scanning independent *in vitro*, but *in vivo* our results suggest that this fraction becomes insignificant.

Except for picornavirus translation, which naturally takes place on uncapped mRNA, this paper reports the first study of scanning independent initiation on a capped mRNA which has also been carried out *in vivo*. Scanning independent initiation has previously been suggested to occur for the vesicular stomatitis virus 7 kd protein from the P or NS gene (Herman, 1986), which appears to be similar to the X protein of the Sendai virus P/C gene, as well as the adenovirus-2 polymerase mRNA (Hassin *et al.*, 1986) and segment A of infectious pancreatic necrosis virus (Nagy *et al.*, 1987). In all these cases, the suggestion is based on the inability of DNA complementary to regions upstream of the internal initiation site to arrest this translation *in vitro*, when translation from the upstream region was simultaneously arrested. This paper also provides strong evidence of some scanning independent initiation of the C protein *in vitro*, however, *in vivo* our data suggest that virtually all the ribosomes which initiate C are likely to be scanning. Further *in vivo* evidence will therefore be required to determine whether these other instances, including that of the Sendai virus X protein, occur only *in vitro*.

The best studied example of internal initiation is that of picornaviruses, where sequences from the viral 5′ UTR have

been shown to direct initiation of downstream genes *in vivo*, even in artificial bicistronic mRNAs (Pelletier and Sonenberg, 1988; Jang *et al.*, 1988). Abramson *et al.* (1988) and Pelletier and Sonenberg (1988) have suggested a model for this internal initiation. Initiation factors eIF-4A and B first bind to the internal region, which then directs ribosome binding, thus eliminating the normal requirement of ribosome loading at the 5′ end. This model could of course explain scanning independent initiation of the Sendai virus Y proteins, and it might also explain why we find some evidence of such initiation of C *in vitro*, but not *in vivo*. The picornavirus sequences responsible for internal initiation are located in the 5′ UTR and are not contiguous with the initiation site (Pelletier *et al.*, 1988; Bienkowska-Szewczyk and Ehrenfeld, 1988). If ribosomes bind first to this sequence in the 5′ UTR, they must then migrate to the appropriate initiation site, in this case, downstream. We assume that such a sequence must also exist to allow initiation of Y, somewhere downstream of ATG/114 for C. Ribosomes bound to this site might then be able to migrate backwards to some extent *in vitro* and initiate C (but not P or C′), but this would occur less frequently *in vivo*. We also note that whereas initiation at Y1 predominates *in vivo*, Y2 is preferentially initiated *in vitro*. This latter difference is also consistent with different ribosome migration *in vivo* and *in vitro*.

The use of alternate initiation codons within the same reading frame to produce an N-terminally nested pair of proteins is not uncommon (reviewed recently by Kozak, 1988), and even a nested set of more than two proteins is not unique to Sendai virus. Herpes virus thymidine kinase is initiated on three ATGs near the 5′ end of presumably a single mRNA (Haarr *et al.*, 1985). The three coat proteins of adeno-associated virus are also a nested set, in which the two smallest are initiated from ACG and ATG codons of the same mRNA similar to the C′ and C proteins of Sendai virus and the largest is made from an alternate mRNA with an extra upstream exon (Becerra *et al.*, 1985). In none of these latter cases are the reasons for the nested set clear, but presumably the additional N-terminal sequences are required for specific interactions. The Sendai virus requirement for this nested set, plus the need to express the overlapping P ORF is apparently beyond the capability of leaky scanning, hence the use of a scanning independent form of initiation for the Y proteins. We presume that this internal initiation is similar to that used by picornaviruses. Attempts will now be made to identify the sequences which allow scanning independent initiation at the Y ATGs using the VV ACG/81-ATG construct and deletion analysis. Once identified, we can ask whether this sequence will direct internal initiation on artificial bicistronic mRNAs, as shown for picornaviruses.

Finally, Thomas *et al.* (1988) have recently shown that the P gene of the paramyxovirus SV5 is interrupted in the middle by a stop codon, but that the remainder of the gene can be translated in a second ORF, which partially overlaps the first. The mRNA, which is an exact copy of the gene (coding for a protein called V), terminates at the internal stop codon. However, a second mRNA with a precise 2-base insertion is also made, which frame-shifts upstream of the stop codon and codes for the larger P protein. The sequence at the C-terminus of V which is absent in P, between the insertion and the stop codon, is unusually rich in Cys and is highly conserved in all paramyxoviruses examined, including Sendai virus. Here it exists on a third ORF. These authors have proposed that a P/C mRNA with a similar insertion will also be found in Sendai virus, where it will code for a fusion protein containing the N-terminus of P and the Cys-rich domain. The proposed frame-shift in Sendai virus would be downstream of the C ORF, and therefore should not affect expression of the C proteins. Attempts to examine this proposal are currently underway. Moreover, as the altered P mRNAs are thought to arise during viral transcription and our experiments were done with a clone derived from the viral genome, all the proteins described for the P/C gene of Sendai virus in this report are apparently coded for by a single unaltered mRNA.

Materials and methods

Construction of SP6 subclones

The SP6 subclone expressing the wild type P/C mRNA has been previously described (Curran and Kolakofsky, 1988a). Site directed mutagenesis was performed essentially as described in Curran and Kolakofsky (1988b). Transcription of these constructs and subsequent translation *in vitro* in wheatgerm extracts has also been previously reported (Curran and Kolakofsky, 1988a,b).

Immunoprecipitation and immunoblotting

Immunoprecipitations were performed as described in Curran *et al.* (1986). The antiserum used here, a gift of Drs Yamada, Iwasaki and Shibuta, Tokyo, was raised in guinea pigs against purified C made in *E.coli* and it reacts with the native proteins. Western blotting of cellular extracts was performed as outlined in Curran and Kolakofsky (1988b). The antiserum was raised in rabbits against C/C′ from infected cells by the method of Diana *et al.* (1987), except that no purification was carried out prior to electrophoresis. Briefly, one dish of infected BHK cells was pulse labelled with [^{35}S]Met, separated by SDS–PAGE, and electroblotted onto a nitrocellulose membrane. The position of the C and C′ proteins was determined by autoradiography, and this region was excised. The membrane was pulverized by sonication in 1 ml PBS, and injected s.c. into two rabbits without added adjuvant. The rabbits were re-injected similarly 2 weeks later. Antiserum was prepared 5 weeks later; titres were maximal at 3 weeks. This serum is very efficient for immunoblotting, but not for precipitation.

Construction of recombinant vaccinia viruses

Infectious vaccinia recombinants were prepared essentially as described in Mackett *et al.* (1984). We used the insertion vector pGS62 (kindly provided by G.L.Smith, Cambridge University, England) which contains the vaccinia virus 7.5K early promoter. This plasmid is a modified form of pGS20 described in Mackett *et al.* (1984) in which a single *Eco*RI site now exists downstream of the 7.5K promoter.

P/C constructs were transferred from SP65 to pGS62 by excising the complete P/C coding region with a *Hin*dIII–*Eco*RI (partial) digestion, filling in the fragment using Klenow enzyme in the presence of dNTPs, and cloning into the *Sma*I site of pGS62. For transfection, confluent CV1 monolayers (5 cm diameter) were infected with 0.1 p.f.u./cell of a temperature sensitive mutant of vaccinia virus (ts7). Cells were incubated in 2 ml of MEM containing 5% FBS for 2 h at 33°C (the permissive temperature). Transfection mixes (1 ml), containing 500 ng of wild type vaccinia virus DNA (prepared as described in Esposito *et al.*, 1981) and 250 ng of the pGS62 subclone in 0.5% Hepes pH 7.05, 0.8% NaCl, 0.038% KCl, 0.0134% $Na_2HPO_4.2H_2O$, 0.1% glucose and 285 mM $CaCl_2$were prepared. Medium was removed from the infected CV1 cells and replaced with 250 μl of the transfection mixes. Petri dishes were then left at room temperature for 1 h, then 2 ml of MEM containing 2% FBS was added to each dish and incubation continued for a further 2 h at 39.5°C (the non-permissive temperature). Medium was removed and the monolayers treated with 1 ml of 10% glycerol in MEM containing 2% FBS for exactly 1 min. Cells were then washed twice with PBS before adding 2 ml of MEM containing 5% FBS and incubating at 39.5°C for 2 days. Virus was collected by freeze–thawing and gentle sonication. Recombinants were selected by plaqueing twice in TK^- cells in the presence of 50 μg/ml bromodeoxyuridine. Plaques were screened for recombinant gene expression by Western blotting of infected cell extracts.

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Note added in proof

S.Vidal has determined that an altered P/C mRNA with a single G insertion does in fact exist *in vivo* as predicted, and codes for the Sendai virus equivalent of the SV5 V protein, as well as the C proteins.