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An efficient ribosomal frame-shifting signal in the polymerase-encoding region of the coronavirus IBV

I.Brierley, M.E.G.Boursnell[1], M.M.Binns[1], B.Bilimoria, V.C.Blok, T.D.K.Brown and S.C.Inglis

Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP and [1]Houghton Poultry Research Station, Houghton, Huntingdon, Cambridgeshire, PE17 2DA, UK

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The polymerase-encoding region of the genomic RNA of the coronavirus infectious bronchitis virus (IBV) contains two very large, briefly overlapping open reading frames (ORF), F1 and F2, and it has been suggested on the basis of sequence analysis that expression of the downstream ORF, F2, might be mediated through ribosomal frame-shifting. To examine this possibility a cDNA fragment containing the F1/F2 overlap region was cloned within a marker gene and placed under the control of the bacteriophage SP6 promoter in a recombinant plasmid. Messenger RNA transcribed from this plasmid, when translated in cell-free systems, specified the synthesis of polypeptides whose size was entirely consistent with the products predicted by an efficient ribosomal frame-shifting event within the overlap region. The nature of the products was confirmed by their reactivity with antisera raised against defined portions of the flanking marker gene. This is the first non-retroviral example of ribosomal frame-shifting in higher eukaryotes.

Key words: coronavirus IBV/mRNA translation *in vitro*/ribosomal frame-shifting/RNA-dependent RNA polymerase/translational regulation

Introduction

Ribosomal frame-shifting during translation is a well documented although relatively unusual phenomenon in prokaryotes and is involved in the suppression of frame-shift mutations and in the control of wild type gene expression in a variety of systems (see Roth, 1981; Craigen and Caskey, 1987 for reviews). The phenomenon has also been observed in yeast, where frame-shift mutations of the mitochondrial *oxi-1* gene appear to be suppressed at a low level (Fox and Weiss-Brummer, 1980). In higher eukaryotes all examples so far have been found in the vertebrate retroviruses, or in retroviral-related systems. The best studied to date are those which occur in Rous sarcoma virus (RSV) (Jacks and Varmus, 1985) and mouse mammary tumor virus (MMTV) (Moore *et al.*, 1987; Jacks *et al.*, 1987). In both viruses frame-shifting appears to be a mechanism for the regulation of expression of the viral RNA-dependent DNA polymerase. One termination codon in RSV, and two in MMTV are suppressed by (−1) ribosomal frame-shifts into alternative, overlapping open reading frames (ORF) allowing the synthesis of gag/pol polyproteins, from which the viral polymerases are produced by proteolytic processing. Frame-shifting events seem likely candidates for the production of gag/pol polyproteins in many other retroviruses (see Jacks and Varmus, 1985; Jacks *et al.*, 1987; Craigen and Caskey, 1987). Frame-shifting has also been demonstrated in the retrotransposons Ty*1* and Ty*912* of yeast (Clare and Farabaugh, 1985; Mellor *et al.*, 1985), and is suspected to occur in the copia-like 17.6 element of *Drosophila melanogaster* (Saigo *et al.*, 1984) and in ground squirrel hepatitis virus (GSHV) (Enders *et al.*, 1985). Ty*1*, Ty*912*, 17.6 and GSHV share important structural features with retroviral proviruses, including overlapping reading frames in the same relative positions as the retroviral *gag* and *pol* coding sequences.

In this paper we describe the first non-retroviral, higher eukaryotic example of ribosomal frame-shifting, in the coronavirus avian infectious bronchitis virus (IBV). IBV, a chicken pathogen, is the type species of the family Coronaviridae and like all coronaviruses has a large, single-stranded, continuous RNA genome of positive polarity (Schochetman *et al.*, 1977; Siddell *et al.*, 1983; Stern and Kennedy, 1980a). Recently the complete sequence of the IBV genome has been elucidated (Boursnell *et al.*, 1987). The virion RNA is 27 600 nucleotides in length, is polyadenylated and is infectious (Schochetman *et al.*, 1977), indicating that it must act as a mRNA in infected cells. Since full expression of the IBV genome in infected cells involves the production of a 'nested' set of subgenomic 3′ co-terminal mRNA from a negative-stranded RNA copy of genomic RNA (Stern and Kennedy, 1980a,b; Liebowitz *et al.*, 1981; Siddell, 1983), the initial translation product of the genomic RNA must include components of an RNA-dependent RNA polymerase, although as yet these have not been identified. Sequence analysis of the genomic RNA (Boursnell *et al.*, 1987) has indicated that it contains two very large ORF which are not present in any of the subgenomic mRNA, and which therefore must be expressed from genomic RNA. These ORF, termed F1 and F2, have the capacity to encode 400 kd and 350 kd polypeptides respectively. The ORF of F1 and F2 overlap by 42 nucleotides and are in different reading frames; F2 is in the −1 frame with respect to F1 (see Figure 1). Several considerations have led to the suggestion (Boursnell *et al.*, 1987) that F2 expression *in vivo* may occur by ribosomal frame-shifting, with the ribosome slipping into the −1 frame prior to encountering the F1 opal termination codon and continuing to translate into the F2 ORF, producing an F1−F2 fusion protein. Firstly the 70 or so bases preceding the first AUG codon of F2 were found to have strong codon bias, similar to the bias found in other IBV genes, suggesting that they have coding function. Secondly no subgenomic mRNA with F2 as its 5′-proximal ORF has been detected in infected cells. Furthermore a conserved sequence which signals initiation of each of the other IBV subgenomic mRNA (Brown *et al.*, 1984) by leader-primed transcription (Lai *et al.*, 1983) is not present upstream of F2. Thirdly nucleotide sequence comparison of the F1/F2 overlap region with the *gag/pol* overlap region of RSV revealed a short but significant homology (8 out of 9 bases conserved, see Figure 5) in the region where frame-shifting is suspected to occur in RSV (Jacks and Varmus, 1985).

As neither polymerase activity nor polymerase polypeptides have been detected in IBV-infected cells, it has not been possible to determine whether an F1−F2 fusion protein exists *in vivo*.

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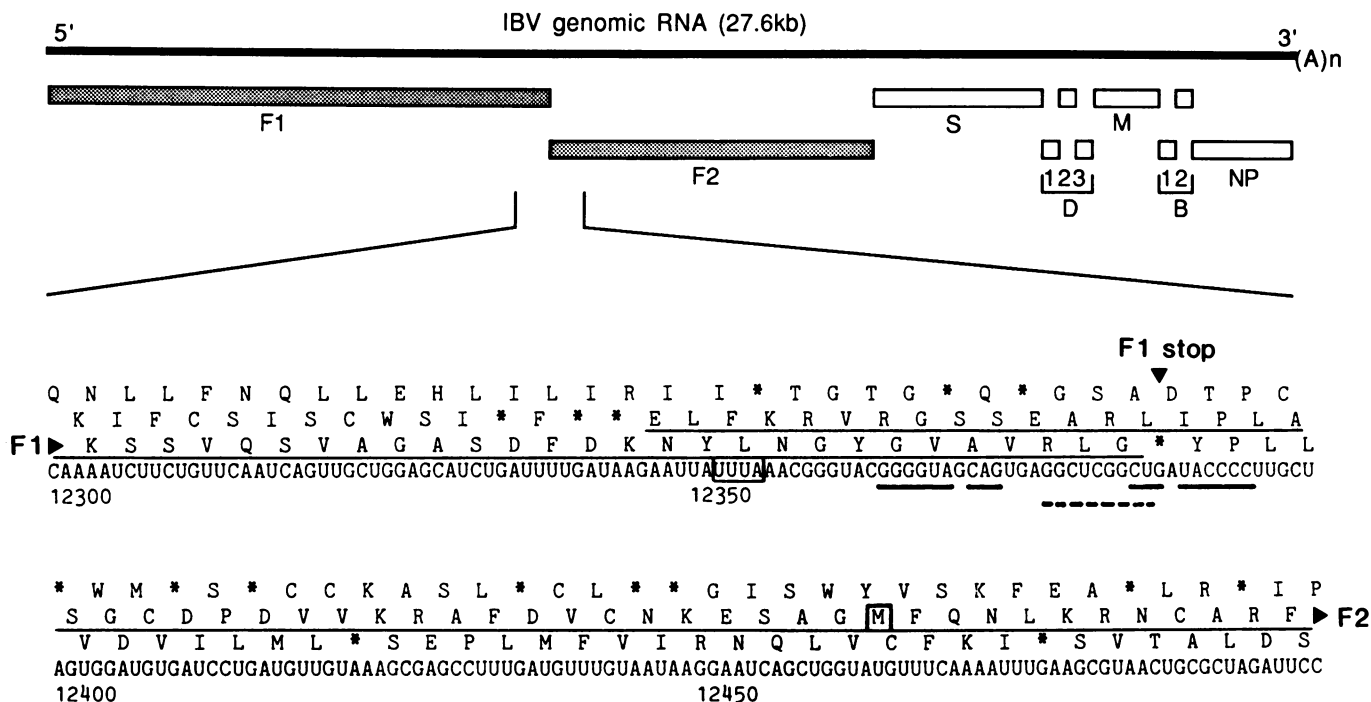

Fig. 1. Nucleotide sequence of the F1/F2 overlap region. The three reading frames have single letter amino acid codes, and stop codons are marked as asterisks. The amino acid is shown above the second base of the appropriate codon. ORF in F1 and F2 are underlined, the RSV homology region is indicated by a dashed line, and the methionine at the start of F2 is boxed. The UUUA consensus is boxed, and complementary bases which may participate in stem-loop formation are heavily underlined.

To test whether a frame-shift event occurs in the F1/F2 overlap region, we have used an approach similar to that used to demonstrate frame-shifting in RSV (Jacks and Varmus, 1985) and MMTV (Moore *et al.*, 1987; Jacks *et al.*, 1987). A cDNA corresponding to the F1/F2 overlap region was cloned into plasmid SP64 (Melton *et al.*, 1984) such that the region could be transcribed *in vitro* with bacteriophage SP6 RNA polymerase. The synthetic mRNA were then translated in cell-free reticulocyte lysate or wheat germ extracts, and their translation products analysed. In these plasmids, the F1/F2 DNA was flanked, in the appropriate reading frames, by sequences comprising the bulk of the influenza virus cap-binding protein, PB2 (Young *et al.*, 1983), since antisera raised against defined regions of the PB2 protein were available. The results obtained support strongly the idea that F2 is indeed produced as a fusion protein with F1 as a result of a highly efficient frame-shifting event. When the F1−F2 DNA was replaced in the transcription vector by a short oligonucleotide containing the sequence homologous to RSV, no frame-shifting was detectable, indicating that a larger sequence context is necessary to induce frame-shifting.

Results

Demonstration of translational frame-shifting in vitro

Plasmid pFS1 (Figure 2), which contains the putative 'frame-shifting region' from the IBV genome flanked by defined regions of the influenza virus PB2 gene was linearized with either *Bgl*I, *Nco*I or *Sma*I, transcribed with SP6 RNA polymerase, and the resulting mRNA were translated in cell-free systems. The logic of the experiment was that RNA generated by transcribing the linearized templates were expected to specify in each case a 21 kd protein corresponding to ribosomes which terminate at the F1 stop codon (the F1, or 'stopped' product). However if occasional ribosomal frame-shifting were to occur in the F1/F2 overlap region, translation would then be expected to proceed into the F2 ORF (and the rest of the PB2 gene) to produce additional 27 kd (*Bgl*I-digested template), 54 kd (*Nco*I) and 95 kd (*Sma*I) products, corresponding to PB2/F1/F2/PB2 (F1/F2) fusion proteins (Figure 3). To confirm their identity, the translation products were immunoprecipitated with antisera (abs 1−5) raised against defined regions of the influenza PB2 protein.

Figure 4A shows the translation products synthesized in the reticulocyte lysate in response to mRNA derived from *Bgl*I-, *Nco*I- or *Sma*I-digested pFS1 and their reactivity with the various PB2 antisera. In each case the major product was a 21 kd polypeptide, but in addition larger products were observed whose size depended on the template used for transcription. The sizes of these larger products were exactly as predicted if frame-shifting were occurring at the F1/F2 junction. The same mRNA were also translated in wheat germ extracts (Figure 4B) and gave essentially the same result, except that the ratio of putative frame-shifted to stopped product was considerably reduced. Immunoprecipitation experiments on the reticulocyte lysate translation products using the PB2 antisera confirmed the nature of the putative frame-shift products. Antiserum ab 1 reacted not only with the 21 kd polypeptide, but also with each of the larger products, indicating that these polypeptides have common N-termini. This is consistent with the larger products being F1/F2 fusion proteins. The reactivity of these proteins with the other PB2 antisera correlated well with the expected size of the fusion. The 27 kd polypeptide (from the *Bgl*I transcript) reacted only with ab 1, the 50 kd polypeptide (from the *Nco*I transcript) with abs 1−3, and the 95 kd polypeptide (from the *Sma*I transcript) with all the PB2 antisera. None of the products could be immunoprecipitated with a control antiserum raised against the unrelated influenza PA protein, and only antiserum ab 1 could precipitate the 21 kd product, as expected from the DNA sequence of pFS1. Immunoprecipitation of the wheat germ translation products with

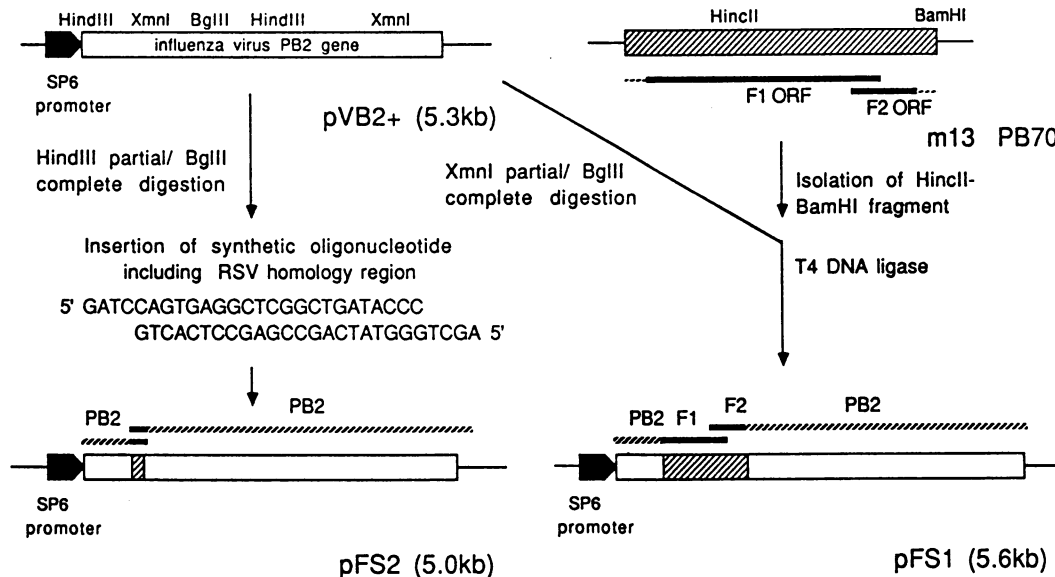

Fig. 2. Construction of plasmids pFS1 and pFS2. To generate pFS1, pVB2+ was linearized with *BglII*, partially digested with *XmnI* and a 5090 bp vector fragment isolated. This was ligated to a 497 bp *HincII*–*BamH*1 fragment from PB70, a fragment containing the region of IBV sequence from bp 12 012–12 509. For construction of pFS2, pVB2+ was linearized with *BglII*, partially digested with *HindIII* and a 5023 bp vector fragment isolated. This was ligated with a complementary pair of oligonucleotides synthesized to include 22 nucleotides corresponding to those from positions 12 368–12 389 of the IBV genome (see Figure 1) and *BamH*1 and *HindIII* overhanging ends.

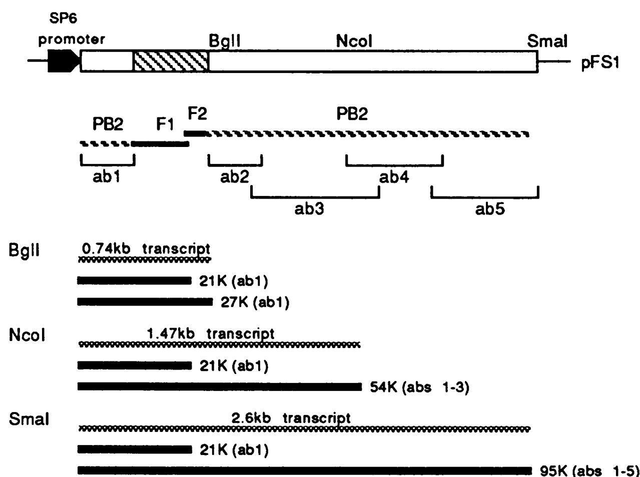

Fig. 3. Diagram showing the predicted sizes of protein products and the reactivity of these products with PB2 antisera which would be expected if a detectable ribosomal frame-shifting event were to occur at the F1/F2 junction during translation of mRNA derived from *BglI*-, *NcoI*- or *SmaI*-digested pFS1 template.

abs 1 and 5, though less efficient, again gave the results expected if frame-shifting had occurred.

We compared the efficiency of frame-shifting in the two systems by measuring the radioactive content of excised gel slices. The bands corresponding to the F1 and F1/F2 fusion proteins from the pFS1/*BglI* RNA translations were excised from dried gels and the [^{35}S]methionine content determined by scintillation counting in a toluene-based scintillant. After correcting for the differential methionine content of the products, the efficiency of frame-shifting was estimated to be 25–30% in the reticulocyte lysate but rather less than 10% in the wheat germ extract.

The conclusion that F2 is expressed by translational frame-shifting rests on the assumption that the mRNA synthesized by the SP6 RNA polymerase is homogeneous and does not contain a subpopulation in which F1 and F2 are in the same frame. In order to rule out this possibility the nucleotide sequence of RNA transcribed from pFS1/*NcoI* template was analysed directly, by primer extension across the 'frame-shifting region'; since the efficiency of frame-shifting in the reticulocyte lysate appeared to be approximately 30%, any mRNA subpopulations with −1 or +2 transcriptional frame-shifts should have been readily detectable. However no heterogeneity was observed. Furthermore this explanation would not account for the markedly different ratio of 'stopped' to 'elongated' products observed in wheat germ extracts.

In addition to the stopped and frame-shifted products seen in these experiments, several minor protein species were apparent. Bands corresponding to proteins less than 21 kd in size were likely to be prematurely terminated translation products, since they were immunoprecipitated with only antiserum ab 1 and hence contained the N-terminal sequences of PB2. However in the reticulocyte lysate translation of pFS1/*SmaI*-derived mRNA, a spectrum of products up to 95 kd (the full length frame-shift product) in size were seen. The immunoprecipitation pattern of these proteins suggests that they may have arisen as a result of internal initiation of translation at many sites along the template rather than by premature termination of translation, since they were all immunoprecipitated by antiserum ab 5, the C-terminal antiserum. Random mRNA degradation in the reticulocyte lysate could not explain the immunoprecipitation pattern observed.

The RSV homology region alone does not direct frame-shifting

The work of other groups (Jacks and Varmus, 1985; Moore *et al.*, 1987; Jacks *et al.*, 1987) has strongly suggested that in RSV and MMTV, signals for frame-shifting are present in the *gag/pol* overlap regions of these viruses. The short region of nucleotide sequence homology shared by IBV and RSV was therefore a potential signal for frame-shifting, since it was similarly located in both cases (Figure 5). To test this experimentally a pair of complementary oligonucleotides, which contained the region of homology (IBV sequence information from positions 12 368 to 12 389, including the TGA stop codon), were cloned within the PB2 gene (see Figure 2) such that frame-shifting could again be

Fig. 4. Panel A. Reticulocyte lysate translation products synthesized in response to RNA transcribed from *BglI*-, *NcoI*- or *SmaI*-digested pFS1. RNA was prepared and translated as described in Materials and methods. Products were analysed directly (total) or immunoprecipitated with antiserum as indicated above each track. Marker tracks contained ^{14}C-labelled mol. wt markers. Polypeptides were labelled with $[^{35}S]$methionine, separated on 15% SDS–polyacrylamide gels and detected by autoradiography. **Panel B.** Wheat germ translation products synthesized in response to *in vitro* transcripts produced as for panel A. Translations were performed as described in Materials and methods. Samples were analysed directly or immunoprecipitated with antiserum as indicated above each track.

```
IBV
                                    F1 →
 G  A  S  D  F  D  K  N  Y  L  N  G  Y  G  V  A  V  R  L  G  *
GGAGCAUCUGAUUUUGAUAAGAAUUAUUUAAACGGGUACGGGGUAGCAGUGAGGCUCGGCUGAUACCCCUUGCUAGUG
: :::    : :   :  :  :        ::  :  :    :   :   :  ::::: :::          ::   :
GUAGCUAUGGUUAGAGGGAGUAUCCUAGGAAGAGAUUGUCUGCAGGGCCUAGGGCUCCGCUUGACAAAUUUAUAGGGA
 V  A  M  V  R  G  S  I  L  G  R  D  C  L  Q  G  L  G  L  R  L  T  N  L  *
                   gag →
RSV
```

Fig. 5. Comparison of nucleotide sequences from the IBV F1/F2 and RSV *gag/pol* junction. The region of IBV sequence included in plasmid pFS2 is overlined.

detected using specific antibodies (Figure 6A). Plasmid pFS2 was digested with *PvuII* or *SmaI* and the template transcribed with SP6 polymerase. The resulting mRNA were translated in the reticulocyte lysate and products immunoprecipitated as before. The results are shown in Figure 6B. For pFS2-derived mRNA, the product corresponding to ribosomes terminating at the in-frame opal stop codon of the oligonucleotide was expected to be 18 kd and any frame-shifted products to be either 33 kd (*PvuII*-digested template) or 75 kd (*SmaI*-digested template). Although both mRNA produced the expected 18 kd product, there was no evidence of frame-shifting; antiserum ab 1 failed to immunoprecipitate any clearly defined 33 kd or 75 kd products. As with pFS1, the *SmaI*-digested template generated a range of protein species; the immunoprecipitation pattern again suggests that internal initiation can occur with this template. No specific products were immunoprecipitated with the control antiserum, as expected. In these experiments, the 18 kd 'stopped' product appeared consistently as a double band. The significance of this is not clear, but it is interesting that a similar phenomenon was noted by Moore *et al.* (1987); several of the *in vitro* translation products terminating at MMTV frame-shift regions were found to migrate as triplets.

Discussion

Our results strongly suggest that expression of the IBV F2 ORF occurs *in vivo* through a ribosomal frame-shifting event during translation of the IBV genomic mRNA. Firstly *in vitro* studies have indicated that the F1/F2 overlap region contains an efficient frame-shifting signal. Secondly similar signals are present in the genome of certain retroviruses, and have been shown to operate *in vivo*. Finally the codon usage in the F1/F2 region suggests that production of an F1/F2 fusion protein does occur *in vivo*. We would therefore predict that translation of IBV genomic RNA gives rise to a major primary product of 400 kd corresponding to the F1 ORF and a less abundant product of 750 kd corresponding to an F1/F2 fusion protein. In the retroviral systems analysed so far, the efficiency of frame-shifting in the reticulocyte lysate has correlated well with the *in vivo* abundance of frame-shift products. In RSV, the 5% *in vitro* efficiency determined by Jacks and Varmus (1985) reflects the ratio of gag to gag/pro

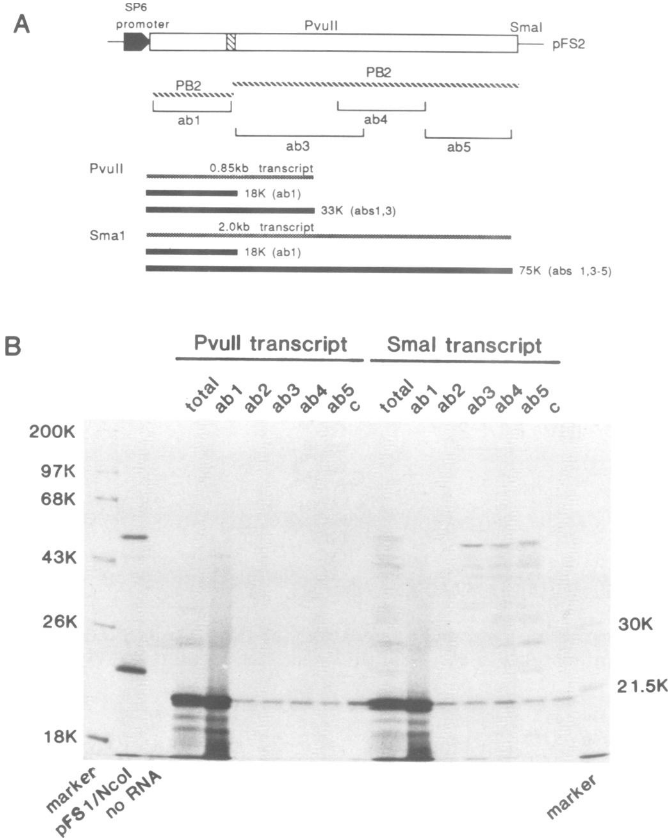

Fig. 6. Panel A. Diagram showing the predicted sizes of protein products and the reactivity of these products with PB2 antisera which would be expected if a detectable ribosomal frame-shifting event were to occur at the F1/F2 junction during translation of mRNA derived from *Pvu*II- or *Sma*I-digested pFS2 template. **Panel B.** Reticulocyte lysate translation products synthesized in response to RNA transcribed from *Pvu*II- or *Sma*I-digested pFS2. RNA was prepared and translated as described in Materials and methods. Products were analysed directly (total) or immunoprecipitated with antiserum as indicated above each track. Marker tracks contained ^{14}C-labelled mol. wt markers. Polypeptides were labelled with [^{35}S]methionine, separated on 15% SDS–polyacrylamide gels and detected by autoradiography. Plasmid pFS2 does not contain the coding sequences for the region of the influenza virus PB2 protein recognized by ab 2.

proteins (20:1) seen in infected cells (Oppermann *et al.*, 1977). In MMTV, frame-shifting at the *gag/pro* and *pro/pol* junctions occurs *in vitro* with efficiencies of 25–30% and 10–15% respectively (Moore *et al.*, 1987; Jacks *et al.*, 1987), and the three MMTV gag-related precursor proteins in virus-infected cells occur in the ratio 30 gag:10 gag/pro:1 gag/pro/pol (Anderson *et al.*, 1979; Dickson and Atterwill, 1979). If this relationship holds for IBV, we would expect from our studies of translation in the reticulocyte lysate that the ratio of F1:F1/F2 products seen in infected cells would be approximately 3:1. The lower efficiency noted in wheat germ extracts suggests that frame-shifting signals are not recognized as well in the plant system, a possible consequence of differences in the translational environment or ribosome structure.

As yet F1- and F2-encoded polypeptides have not been detected in IBV-infected cells. However taking into account the very large size of these predicted primary products and the information available concerning expression of long ORF in other RNA viruses, it seems highly likely that they will be subject to proteolytic cleavage either during or immediately after synthesis. We are currently investigating the precise coding function of the F1- and F2-encoded polypeptides using specific antibodies raised against defined regions of the predicted proteins.

The presence of a frame-shifting signal at the end of F1 provides a simple translational control mechanism for maintaining a defined ratio of gene expression from the upstream and downstream ORF. The significance of such a potential control mechanism is of course not clear at the moment, but since the initial translation products of genomic RNA must include components of the RNA-dependent RNA polymerase, it is possible that the production of different forms of the enzyme might be regulated in this way. In the related coronavirus, mouse hepatitis virus (MHV), two separate polymerase activities have been detected in infected cells (Brayton *et al.*, 1982). Indeed a similar kind of translational control seems to operate during expression of the RNA-dependent RNA polymerase encoding region from another class of positive-strand RNA virus, the togaviridae, although not in this case through ribosomal frame-shifting. In both Sindbis virus and Middelberg virus the long ORF present at the 5′ end of the genomic RNA is interrupted by an in-frame translational stop codon (Strauss *et al.*, 1983; Lopez *et al.*, 1985) which can be suppressed occasionally during translation leading to the production of a longer protein. In retroviruses also, expression of upstream and downstream regions of the 5′-proximal ORF from genomic RNA appears to be regulated through translational suppression of a termination codon; either by ribosomal frame-shifting (see Jacks *et al.*, 1987), or by direct suppression of an in-frame stop codon [e.g. murine leukaemia virus (Yoshinaka *et al.*, 1985a) and feline leukaemia virus (Yoshinaka *et al.*, 1985b)]. Finally expression of the 5′ proximal coding regions from the genomic RNA of certain positive-stranded plant viruses is also apparently regulated through translational suppression of a termination codon, leading to expression of downstream sequences (Pelham, 1978; Morch and Benicourt, 1980; Hamilton *et al.*, 1987). It therefore appears that such translational control mechanisms are common among the positive-stranded RNA viruses.

The first experimental confirmation of a frame-shifting event in higher eukaryotes was provided by Jacks and Varmus (1985), who suggested that the sequence UUUA (present at the *gag/pol* junction) might be an important signal for this event since short, homopolymeric nucleotide runs had been implicated in frame-shifting in other systems, including the yeast mitochondrial *oxi-1* gene (Fox and Weiss-Brummer, 1980), certain *Salmonella* genes (Kohno and Roth, 1978; Atkins *et al.*, 1983) and the bacteriophage T7 gene 10 (Dunn and Studier, 1983). A mechanism was suggested in which slippage of a P-site leucine tRNA reading a UUA codon just 5′ of the *gag* amber stop codon (see Figure 5), followed by mispairing to the UUU codon in the −1 frame generates the frame-shift. Interestingly IBV has the UUUA sequence in the overlap region and requires a −1 frame-shift to generate the F1/F2 fusion protein. Although this sequence is located considerably more 5′ of the F1 stop codon than the same sequence in the RSV *gag* gene, it is feasible that this may be a crucial signal for frame-shifting and that the RSV homology region is merely coincidental. Our observation that the RSV homology region alone was unable to direct frame-shifting would be consistent with this hypothesis, although of course we cannot rule out the possibility that this region constitutes part of the signal.

More recent work (Jacks *et al.*, 1987; Moore *et al.*, 1987) has advanced our understanding of frame-shifting during retro-

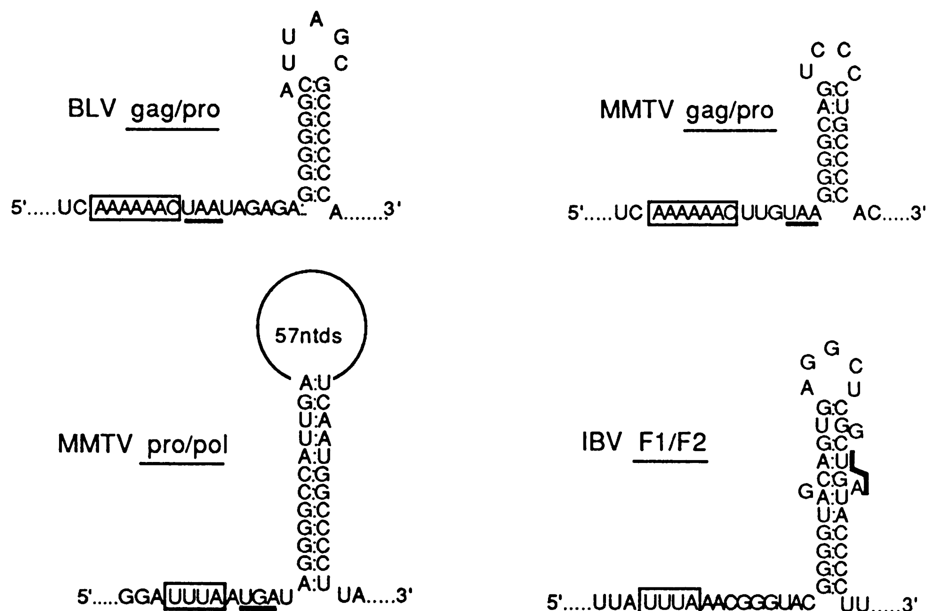

Fig. 7. Predicted RNA secondary structures at ribosomal frame-shift sites. The potential signals for frame-shifting are boxed, and the termination codons for the upstream ORF are indicated by a heavy line. The retrovirus structures shown are taken from Rice *et al.* (1985) (BLV) and Jacks *et al.* (1987) (MMTV). The IBV structure was obtained by using the 'fold' program of Jacobson *et al.* (1984).

virus gene expression. In MMTV two stop codons are suppressed by this mechanism, allowing the viral RNA-dependent DNA polymerase and endonuclease to be synthesized. These authors have suggested that AAAAAAC and UUUA are the probable frame-shifting sites. The former sequence is present at the *gag/pro* junction in MMTV RNA and is also the candidate sequence for frame-shifting at the bovine leukaemia virus (BLV) *gag/pro* junction, both from analysis of the nucleotide sequence (Rice *et al.*, 1985) and from protein mapping of the gag precursor protein (Yoshinaka *et al.*, 1986). The UUUA sequence, common to RSV, is present at the *pro/pol* junctions of both MMTV and BLV. Nucleotide sequence analysis of retroviral *gag/pol* junctions by Jacks and co-workers (1987) has also implicated these sequences in frame-shifting. These authors have cloned the regions containing these sequences into SP6 promoter-based plasmids in order to test their ability to induce frame-shifting during *in vitro* translation. Surprisingly no frame-shifting was observed. The current explanation for this observation is that additional sequences bordering the MMTV overlaps (and other retroviruses known or thought to utilize frame-shifting during replication) may be needed in addition to the specific oligoribonucleotide consensus sequences.

Downstream of many retroviral *gag/pol* junction regions are sequences potentially capable of forming stem-loop structures in the mRNA. Such structures may make important contributions to frame-shifting in MMTV, RSV and BLV (Moore *et al.*, 1987; Jacks *et al.*, 1987; Rice *et al.*, 1985) by acting to stall ribosomes, thereby increasing the chance of a tRNA slippage event. We have analysed potential RNA secondary structure in the F1/F2 region of IBV using the method of Jacobson *et al.* (1984). Figure 7 shows the predicted structure, a moderately stable ($\Delta G^\circ = -17.2$ kcal/mol) stem-loop. The structures predicted for the BLV *gag/pro* junction (Rice *et al.*, 1985) and the MMTV *gag/pro* and *pro/pol* junction regions (Jacks *et al.*, 1987) are shown in comparison. As can be seen the IBV stem-loop is remarkably similar to those predicted for the retroviral overlaps, with the UUUA or AAAAAAC consensus sequences located just 5' of the loop. The predicted stability of the IBV stem-loop is similar to that of the BLV stem-loop ($\Delta G^\circ = -22$ kcal/mol; Rice *et al.*, 1985). A major difference is that the stop codon for the upstream ORF in IBV is buried in the stem-loop, whereas in the other structures it occurs just prior to the loop. The position of the stop codon may therefore be unimportant in the frame-shifting process. Further experiments involving deletion analysis and site-directed mutagenesis will be required to establish the precise sequence requirements of the frame-shifting signal.

Materials and methods

Vector construction

Figure 2 shows the construction of the plasmids used in this study. Plasmid pFS1 was derived from a recombinant M13 phage, PB70, which contained a cDNA insert complementary to the IBV F1/F2 junction region in the *Sma*I site of m13mp8 (Boursnell *et al.*, 1987), and a cDNA copy of the influenza virus A/PR8/34 PB2 gene (Young *et al.*, 1983) inserted into the *Bam*HI site of pSP64 (pVB2+). Plasmid pFS1 thus comprises pSP64/PB2 with the sequence from positions 225–486 bp of PB2 replaced by 497 bp of IBV F1/F2 sequence, such that both the F1 and F2 ORF were in frame with the flanking PB2 sequences, with F1 in frame 1 and F2 in frame 3. The influenza–IBV–influenza junctions were confirmed by nucleotide sequencing after sub-cloning the relevant fragments into phage m13 (Sanger *et al.*, 1977). Plasmid pFS2 was constructed by inserting the sequence 5'-CAGTGAGGCTCGGCTGATACCC-3' into *Bgl*II-linearized, *Hin*dIII-partially digested pVB2+, using a pair of complementary synthetic oligonucleotides (see Figure 2). The resulting plasmid had PB2 sequence information from position 486–798 bases replaced by the oligonucleotide. As with pFS1, pFS2 was designed such that ribosomes could translate the length of the PB2 gene if a frame-shift were to occur from frame 1 to frame 3 of PB2. The junctions were confirmed by nucleotide sequencing as before.

All plasmids were transformed into *Escherichia coli* TG1 by standard procedures (Hanahan, 1983).

Antisera against defined regions of the influenza virus PB2 protein

Antisera against five defined regions of the PB2 protein were prepared by insertion of five overlapping regions of the PB2 gene (covering the entire gene sequence) at the C-terminus of a β-galactosidase gene using the pEX series of bacterial expression plasmids (Stanley and Luzio, 1984). The resulting fusion proteins were purified by elution from polyacryalmide gels and used to immunize rabbits. The sequence information to which each of the five antisera (abs 1–5) were raised was 0–483 bp (ab 1), 418–836 bp (ab 2), 708–1488 bp (ab 3), 1227–1785 bp (ab 4) and 1777–2341 bp (ab 5). An antiserum was also raised against a region of the influenza PR8 PA protein in a similar way and was used as a control serum (c).

Preparation of DNA template and transcription of mRNA

Plasmids for transcription were prepared by alkaline–SDS extraction and purified by banding on caesium chloride–ethidium bromide density gradients (Maniatis *et al.*, 1982). Transcriptions were performed essentially as described by Melton *et al.* (1984) and included a synthetic cap structure, 7mGpppG (New England Bio-Labs) to generate capped mRNA. Following transcription, mRNA were extracted once with phenol:chloroform:isoamyl alcohol (49:49:2), once with chloroform and ethanol-precipitated in the presence of 2 M ammonium acetate. Remaining unincorporated nucleotides were removed by chromatography on Sephadex G-50 in water. The RNA was recovered by ethanol precipitation, dissolved in water and checked for integrity by electrophoresis on 1.5% agarose–formaldehyde gels (Maniatis, 1982).

Translation of mRNA in cell-free extracts

Wheat germ cell-free extracts were prepared and used for cell-free translation as described previously (Inglis *et al.*, 1977). Messenger RNA dependent, micrococcal nuclease treated rabbit reticulocyte lysate, prepared according to Pelham and Jackson (1976) was obtained from Dr T.Hunt (Biochemistry Department, University of Cambridge, Cambridge, UK). The lysate was supplemented with KCl to 0.1 M, $MgCl_2$ to 0.5 mM and creatine phosphate to 10 mM. A mixture of 19 unlabelled amino acids (no methionine) was added at the concentrations used by Hunt and Jackson (1974). The translation reaction mixture (usually 70 μl) contained 85% (v/v) supplemented reticulocyte lysate, 5% (v/v) $[^{35}S]$methionine (usually 1.85 MBq, >30 TBq $mmol^{-1}$) and 10% (v/v) of the mRNA solution under test. Both wheat germ and reticulocyte lysate translations were carried out for 1 h at 30°C. The mRNA concentration in the translations was between 10 and 50 $\mu g\ ml^{-1}$. Translation products were analysed on 15% SDS–polyacrylamide gels according to standard procedures (Hames, 1981). Following electrophoresis, the gels were fixed and stained, destained, dried and labelled proteins detected by autoradiography.

Immunoprecipitation

Translation products were immunoprecipitated using antisera raised against influenza PB2 and PA proteins. Five to ten microlitres of lysate (50–100 000 c.p.m. μl^{-1}, as determined by TCA precipitation) was diluted to 100 μl with lysis buffer [10 mM Tris–HCl pH 8.0, 0.14 M NaCl, 0.05% (v/v) NP40], 10 μl of the appropriate rabbit antiserum added and mixed at room temperature for 30 min. Immune complexes were precipitated from this mixture by adding protein A–Sepharose CL4B (10 mg, preswollen in 25 mM Tris, pH 8.0) and mixing at 4°C for 30 min. The Sepharose beads were washed three times with 0.5 ml of lysis buffer, and bound proteins were eluted in 25 μl SDS-gel sample buffer at 100°C. The beads were briefly centrifuged (30 s, 12 000 *g*) and 20 μl of the supernatant loaded onto 15% SDS–polyacrylamide gels as before.

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