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Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'

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The 2A region of the aphthovirus foot-and-mouth disease virus (FMDV) polyprotein is only 18 aa long. A 'primary' intramolecular polyprotein processing event mediated by 2A occurs at its own C terminus. FMDV 2A activity was studied in artificial polyproteins in which sequences encoding reporter proteins flanked the 2A sequence such that a single, long, open reading frame was created. The self-processing properties of these artificial polyproteins were investigated and the co-translational 'cleavage' products quantified. The processing products from our artificial polyprotein systems showed a molar excess of 'cleavage' product N-terminal of 2A over the product C-terminal of 2A. A series of experiments was performed to characterize our *in vitro* translation systems. These experiments eliminated the translational or transcriptional properties of the *in vitro* systems as an explanation for this imbalance. In addition, the processing products derived from a control construct encoding the P1P2 region of the human rhinovirus polyprotein, known to be proteolytically processed, were quantified and found to be equimolar. Translation of a construct encoding green fluorescent protein (GFP), FMDV 2A and β-glucuronidase, also in a single open reading frame, in the presence of puromycin, showed this antibiotic to be preferentially incorporated into the [GFP2A] translation product. We conclude that the discrete translation products from our artificial polyproteins are not produced by proteolysis. We propose that the FMDV 2A sequence, rather than representing a proteolytic element, modifies the activity of the ribosome to promote hydrolysis of the peptidyl(2A)-tRNA^{Gly} ester linkage, thereby releasing the polypeptide from the translational complex, in a manner that allows the synthesis of a discrete downstream translation product to proceed. This process produces a ribosomal 'skip' from one codon to the next without the formation of a peptide bond.

Introduction

A common, if not ubiquitous, strategy of positive-strand RNA viruses is to encode some, or all, of their proteins in the form of polyproteins. This strategy is not confined to viruses, as both eu- and prokaryotes also (rarely) use polyproteins in protein biogenesis. Cellular polyproteins may comprise different proteins, e.g. proopiomelanocortin (POMC; Douglass *et al.*, 1984) and adrenocorticotropic hormone (ACTH; Scott *et al.*, 1973; Nakanishi *et al.*, 1980), or may contain multiple copies of the same protein, e.g. yeast prepro-α-mating factor (Kurjan & Herskowitz, 1982). Whilst cellular polyproteins are processed by cellular enzymes post-translationally, the presence of proteinases within positive-strand RNA virus polyproteins can bring about both co-translational (in *cis*) and post-translational (in *trans*) proteolytic processing (reviewed by Dougherty & Semler, 1993; Ryan & Flint, 1997; Ryan *et al.*, 1998).

In the case of the picornaviruses, all of the proteins are

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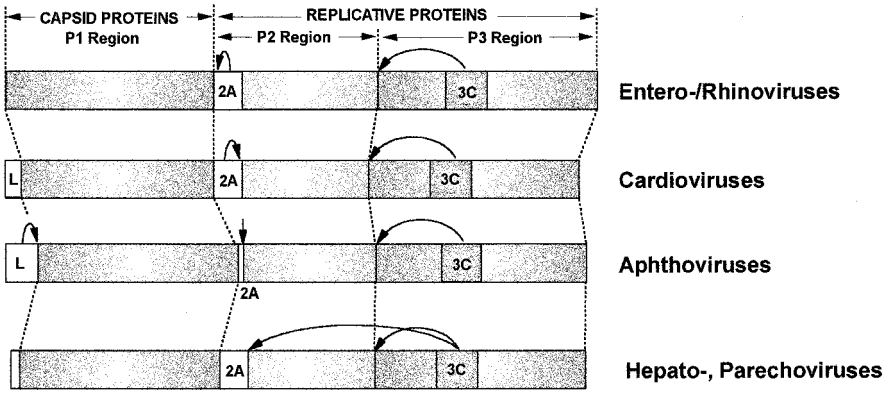

Fig. 1. Picornavirus 'primary' polyprotein cleavages. The genomes of picornaviruses are shown with the single, long, ORF in boxed areas. Primary, intramolecular cleavages (in *cis*) are shown (curved arrows).

encoded in a single, long, open reading frame (ORF). Picornavirus polyproteins undergo a 'primary' co-translational cleavage *in statu nascendi* between domains containing the capsid proteins and domains containing the replicative proteins (Fig. 1). Precursors spanning these primary cleavage sites are not detected during native polyprotein processing. Only in hepatitis A and parechoviruses 1 and 2 does this type of primary cleavage not occur (Jia *et al.*, 1993; Schultheiss *et al.*, 1994, 1995). In the entero- and rhinoviruses the 1D/2A primary cleavage is mediated by a well-characterized virus-encoded proteinase ($2A^{pro}$), of some 17 kDa, acting in an intramolecular fashion (in *cis*) to cleave the nascent polyprotein at its own N terminus (Toyoda *et al.*, 1986; Sommergruber *et al.*, 1989). The analogous primary cleavage in the aphtho- and cardioviruses occurs at the C terminus of the 2A region between the capsid protein precursor ([P1-2A] – aphthoviruses; [L-P1-2A] – cardioviruses) and 2BC/P3 (Fig. 1). Inspection of the cardiovirus 2A protein sequence (ca. 15 kDa) reveals no similarity to $2A^{pro}$ of the entero- and rhinoviruses and none of the characteristic proteinase sequence motifs. The 2A region of the aphthovirus foot-and-mouth disease virus (FMDV) is only 18 aa long – but is highly similar to the C-terminal region of cardiovirus 2A.

Our previous work on the function of the 2A region has demonstrated a number of important features.

(i) The FMDV 2A sequence (together with the N-terminal proline of protein 2B) retained 'cleavage' activity in recombinant FMDV polyproteins when either the upstream or downstream contexts were replaced, but the upstream context influenced the activity (Ryan *et al.*, 1991).

(ii) A single ORF encoding an artificial polyprotein comprising FMDV 2A (plus the N-terminal proline of protein 2B; 19 aa in total) flanked by the reporter proteins chloramphenicol acetyltransferase (CAT) and *β*-glucuronidase (GUS) produced three major translation products – uncleaved [CAT2AGUS], GUS and [CAT2A]. The FMDV 2A sequence was able to mediate a co-translational 'cleavage' in this artificial polyprotein directly analogous to its function in FMDV polyprotein processing such that ~90% of the translation product was in the 'cleaved' forms (Ryan & Drew, 1994).

(iii) 2A-mediated 'cleavage' occurred only co-translationally – upon prolonged incubation the 'uncleaved' translation products did not subsequently cleave (Ryan & Drew, 1994).

(iv) The C-terminal region (19 aa) of the cardiovirus encephalomyocarditis virus (EMCV) 2A (plus the N-terminal proline of protein 2B) was as active as the FMDV 2A sequence (Donnelly *et al.*, 1997).

(v) The artificial [CAT2AGUS] polyprotein did not 'cleave' when expressed in prokaryotic systems (Donnelly *et al.*, 1997).

Our initial working hypothesis was that this short 2A region could mediate a single-turnover proteolysis of the polyprotein, in *cis*, at the 2A/2B site (invariantly a glycine/proline pair). The 2A sequence, together with the N-terminal residue of 2B, would represent an autonomous, self-aligning, nucleophile:electrophile couple that brought about the cleavage of the Gly–Pro peptide bond (not a proteinase:substrate couple *sensu stricto*). In this scenario the sequences in a proteinase which are concerned with imparting substrate specificity could be dispensed with. Similarly, the sequences required to provide the molecular environment whereby an active site nucleophile could be regenerated could also be dispensed with. Thus one might envisage how such a short sequence could bring about this specific (*cis*) proteolytic event.

The co-translational 'cleavage' of the [CAT2AGUS] polyprotein into the [CAT2A] and GUS products was monitored by phosphorimaging. In both rabbit reticulocyte lysate and wheat germ extract *in vitro* translation systems an imbalance in the accumulated translation products was observed. Careful analysis of the translation profiles of the [CAT2AGUS] self-processing artificial polyprotein system showed considerable internal initiation within the CAT sequence in coupled transcription/translation *in vitro* systems (Donnelly *et al.*, 1997), with higher levels of accumulation of [CAT2A] than of

GUS. The substantial amount of the N-terminally truncated forms of [CAT2A] (produced by internal initiation) migrated on gels much more rapidly than [CAT2A] and was taken into account in our quantification of the translation products. When this was done the imbalance became more marked. A hypothesis in which 2A functions as a proteolytic element, however, would predict a 1:1 stoichiometry of the cleavage products.

In this paper we describe detailed analyses of the translation profiles from three types of polyprotein. The first is artificial self-processing polyproteins comprising two reporter proteins flanking FMDV 2A; the second an FMDV polyprotein in which 2A is in its native context; the third a polyprotein containing a defined *cis*-acting proteinase derived from human rhinovirus (HRV). We show striking differences in the polyprotein processing properties of these systems. Whilst the proteolytically processed HRV polyprotein showed equimolar quantities of the cleavage products, the artificial polyproteins showed a molar excess of the translation product N-terminal of the 2A sequence to that C-terminal of 2A. Experiments are described which were designed to eliminate the translational or transcriptional properties of the coupled *in vitro* systems, rather than the properties of the polyproteins themselves, as an explanation for this imbalance in the 'cleavage' of the artificial polyproteins. A model of FMDV 2A 'cleavage' is presented in which the 2A oligopeptide sequence is proposed to promote the hydrolysis of the peptidyl-tRNA ester linkage at a specific site – the C terminus of 2A.

Methods

■ **Plasmid constructs.** All plasmids were constructed using standard methods and confirmed by nucleotide sequencing. All restriction enzymes and coupled transcription/translation systems (TnT) were purchased from Promega whilst oligonucleotides were obtained from commercial suppliers (Oswel DNA Service).

pGFPGUS. Plasmid pCATGUS (Ryan & Drew, 1994) was restricted with *Bam*HI and *Xba*I and the large DNA restriction fragment purified by agarose gel electrophoresis. Plasmid pGFP-N2 (Clontech) was similarly restricted and the smaller restriction fragment (GFP gene) was gel purified. Purified restriction fragments were ligated to form pGFPGUS.

pGFP2AGUS. Plasmid pCAT2AGUS (Ryan & Drew, 1994) was restricted with *Bam*HI and *Xba*I and the large DNA restriction fragment purified by agarose gel electrophoresis. Plasmid pGFP-N2 (Clontech) was similarly restricted and the smaller restriction fragment (GFP gene) was gel purified. Purified restriction fragments were ligated to form pGFP2AGUS.

pGUS2AGFP. A PCR product containing the sequence encoding GUS was amplified from pGFP2AGUS using the oligonucleotide primers GUSfor (5′ AGAGAGGATCCGCCGCCACCATGTTACGTCTTGTA 3′) and GUS23 (5′ ATATAGGGCCCAAATCTAGATTCTTTGCGTCCCTG 3′). The PCR product was restricted with *Bam*HI and *Xba*I, gel purified, and ligated into the similarly restricted pCAT2AGUS to give the intermediate plasmid pGUS2AGUS. A PCR product containing the sequence encoding GFP was then amplified by PCR from pGFP2AGUS using the oligonucleotide primers ApaGFPfor (5′ AGAGAGGGGCCCGGTAAAGGAGAAGAA 3′) and GFPrev (5′ GCGCGCCTGCAGTCATCTAGATCCGGACTTGTATAG 3′). The PCR product was restricted with *Apa*I and *Pst*I, gel purified, and ligated into the similarly restricted pGUS2AGUS to form plasmid pGUS2AGFP.

pAM2. A single nucleotide insertion frame-shift mutation (underlined) was introduced into the GUS sequence immediately following the codon corresponding to the initiating AUG. Sequences encoding GUS within plasmid pGFP2AGUS were amplified by PCR using oligonucleotide primers 186 (5′ TCCAACCCTGGGCCCATGGTTACGTCCT 3′) and SP6 (5′ TATTTAGGTGACACTATAG 3′). The PCR product was restricted with *Apa*I and *Nsi*I, gel purified, and ligated into pGFP2AGUS, similarly restricted, to form the intermediate plasmid pAM1. A two-nucleotide insertion, together with a further point mutation to remove a stop codon (mutations underlined), were introduced into the 2A region. Sequences encoding GFP and 2A were amplified by PCR using primers T7 (5′ TAATACGACTCACTATAGGG 3′) and187(5′CCGCAAGCTTAAGAAGGTCAAAATTAAACAGCTGGCATGCTCCTCTAGATATCCGGACTT3′). The PCR product was restricted with *Bam*HI and *Hin*dIII, gel purified, and ligated into plasmid pAM1, similarly restricted, to form plasmid pAM2.

pHRVP1P2. A PCR product containing the sequence encoding HRV-14 P1P2 was amplified by PCR from HRV-14 cDNA using the oligonucleotide primers OB12 (5′ GGGGGTACCGCCGCCACCATGGGCGCTCAGGTT 3′) and P2-rev (5′ TTTTTTGCGGCCGCCTATTGAAACAGTGTTTCTAG 3′). The PCR product was ligated into pGEMT (Promega) to give plasmid pHRVP1P2.

pFMDVP1P2. A PCR product containing the sequence encoding FMDV P1P2 was amplified by PCR from pMR15 (Ryan *et al.*, 1989) using the oligonucleotide primers FMDVP1for (5′ GAGAGAGGTACCGCCGCCACCATGGGGGCTGGACAATCC 3′) and FMDVP2rev (5′ CCCCCCTCTAGACTACTGCTTGAAGATCGG 3′). The PCR product was ligated into pGEM-T to give plasmid pFMDVP1P2.

■ **Coupled transcription/translation *in vitro*.** Coupled transcription/translation (TnT) reactions were performed as per the manufacturer's instructions (Promega). Briefly, rabbit reticulocyte lysates (20 µl) or wheat germ extracts (20 µl), each containing [^{35}S]methionine (50 µCi; Amersham), were programmed with unrestricted plasmid DNA (0·5 µg) and incubated at 30 °C for 45 min.

■ **Immunoprecipitation.** CAT and GUS translation products were characterized by immunoprecipitation with anti-CAT and anti-GUS antibodies as described previously (Ryan & Drew, 1994). Puromycin-(Sigma) labelled proteins were immunoprecipitated using the same protocol with anti-puromycin antibody (kind gift of J. Brown, Institute of Cell and Molecular Biology, Edinburgh, UK), used at a 1:5 dilution.

■ **Transcription *in vitro*.** Plasmid pGFP2AGUS DNA was restricted with *Not*I and the linearized product purified by agarose gel electrophoresis. Restricted DNA was used to programme a transcription reaction as per the manufacturer's instructions (RiboMAX; Promega). RNA transcripts were purified by Sephadex G-50 column chromatography and the integrity of transcript RNA was checked by agarose gel electrophoresis prior to translation experiments.

■ **Translation *in vitro*.** Translation reactions (20 µl), containing [^{35}S]methionine (50 µCi), were performed as per the manufacturer's instructions (Ambion). Briefly, translation mixtures were programmed with 0·5 µg transcript RNA and incubated at 30 °C for 45 min.

■ **Protein degradation.** Translation reactions (50 µl) were performed for 45 min after which synthesis was arrested by addition of RNase (1 µg) and cycloheximide (50 µg). The mixture was then incubated

further; samples (5 μl) were removed at the times indicated and SDS–PAGE sample buffer (5 μl) added. Samples were stored on ice until the conclusion of the incubations and then analysed by 10% SDS–PAGE and phosphorimaging.

■ **Distribution of radiolabel.** Translation reactions were analysed by SDS–PAGE (10%) and the distribution of radiolabel was determined either by autoradiography or by phosphorimaging using a Fujix BAS 1000. Incorporation of radioactivity into specific products was quantified directly by the latter method.

■ **Calculation of molar ratios of 'cleavage' products.** Using phosphorimaging the photo-stimulated luminescence (PSL) was determined for each translation product. The local 'background' was subtracted (PSL − BG) and this value divided by the number of methionine residues for a given translation product. The calculation of molar ratios of the 'cleavage' products was repeated three times (by integration of profile peaks or encircling the band either by 'freehand' or by using a rectangle) to estimate the error in this method of determination, which was estimated to be ∼ 2%. The methionine contents of the proteins used in our studies are; CAT = 9; GUS = 12; GFP = 6; HRVP1 = 19; HRV P2 = 16; FMDV [P12A] = 15 and FMDV [2BC] = 14.

Results

Characterization of pCAT2AGUS translation products

As we have previously reported, translation of the plasmid construct pCATGUS encoding the reporter proteins CAT and GUS, in a single ORF, produces a band corresponding to the expected [CATGUS] product. Other high molecular mass translation products correspond to N-terminally truncated forms of the protein (see below). Inclusion of the FMDV 2A sequence into this polyprotein ([CAT2AGUS]) resulted in a dramatically different translation profile: the major products were identified as [CAT2A] and GUS, with a small amount (∼ 5%) of the uncleaved form [CAT2AGUS] (Ryan & Drew, 1994; Donnelly *et al.*, 1997).

Translation products derived from pCAT2AGUS (Fig. 2), analysed by 10% SDS–PAGE, showed three protein bands migrating more slowly than the GUS 'cleavage' product (Fig. 3 A). These were identified by the analysis of a series of N-terminally truncated constructs as (i) the faint uppermost band being the full-length, uncleaved, [CAT2AGUS], (ii) a strong (doublet) band corresponding to the two uncleaved products produced by internal initiation within the CAT sequence at Met^{67} and Met^{77} and (iii) a further uncleaved product produced by initiation at Met^{163} (data not shown). The presence of the predicted [ΔCAT2A] 'cleavage' products produced by initiation at Met^{67} and Met^{77} (mol. mass 20·3 and 19·3 kDa, respectively) was confirmed by immunoprecipitation with anti-CAT antibodies (Fig. 3 A). The [ΔCAT2A] cleavage product derived from initiation at Met^{163} (mol. mass 9 kDa) was not resolved in this gel system.

Phosphorimaging analysis of the [CAT2AGUS], GUS and [CAT2A] translation products was performed and when a correction was applied to account for the relative methionine contents of CAT and GUS (but *not* for the internal initiation within the CAT sequence) a 2- to 3-fold molar excess of [CAT2A] was observed relative to GUS. When a further correction was applied to account for the internal initiation within CAT then our calculations showed a greater excess of translation product N-terminal of 2A ([CAT2A]) over that C-terminal (GUS). This imbalance was always observed, although the level of imbalance varied between different batches of rabbit reticulocyte lysate (or wheat germ extract) and between rabbit reticulocyte lysate and wheat germ extract *in vitro* translation systems.

The analysis of the distribution of radioactivity between the various products is complicated by the considerable internal initiation within the CAT sequence. The multiplicity of products together with the inability to resolve all of the products by 10% SDS–PAGE led us to construct another artificial polyprotein system ([GFP2AGUS]).

Characterization of pGFP2AGUS and pGUS2AGFP translation products

Translation profiles derived from pGFP2AGUS (Fig. 2) showed very low levels of internal initiation and produced a much more easily quantifiable protein band pattern (Fig. 3 B). Three major translation products were observed: 'uncleaved' [GFP2AGUS] plus the 'cleavage' products GUS and [GFP2A]. Phophorimaging analyses again showed an excess of translation product N-terminal of 2A ([GFP2A]) to that C-terminal. We found that this excess varied between different batches of rabbit reticulocyte lysates (from 5:1 to 2:1) and between different batches of wheat germ extract (from 10:1 to 3:1). The most noticeable difference occurred between the two different *in vitro* translation systems, rather than batches of the same translation system.

Experiments were performed to eliminate the intrinsic properties of the *in vitro* systems as an explanation for this imbalance. We also wished to show that by reversing the order of the reporter proteins in the artificial polyprotein the same N- to C-terminal imbalance was observed. Phosphorimaging analyses of translation products derived from pGUS2AGFP (Fig. 2) showed that the translation product N-terminal of 2A ([GUS2A]) was present in excess over the product C-terminal of 2A (GFP) by some 2- to 7-fold, even though the order of the proteins in the polyprotein was reversed, thereby eliminating the possibility that translation was being interrupted by an effect of the CAT or GUS sequences themselves (Fig. 2, Fig. 3 B).

Protein degradation studies

One very simple explanation for the observed difference in accumulated protein levels is that of different protein degradation rates. This was addressed by translating constructs for 45 min, arresting further synthesis by addition of RNase plus cycloheximide, and then incubating the (arrested) translation mixture for progressively longer periods at 30 °C. Although

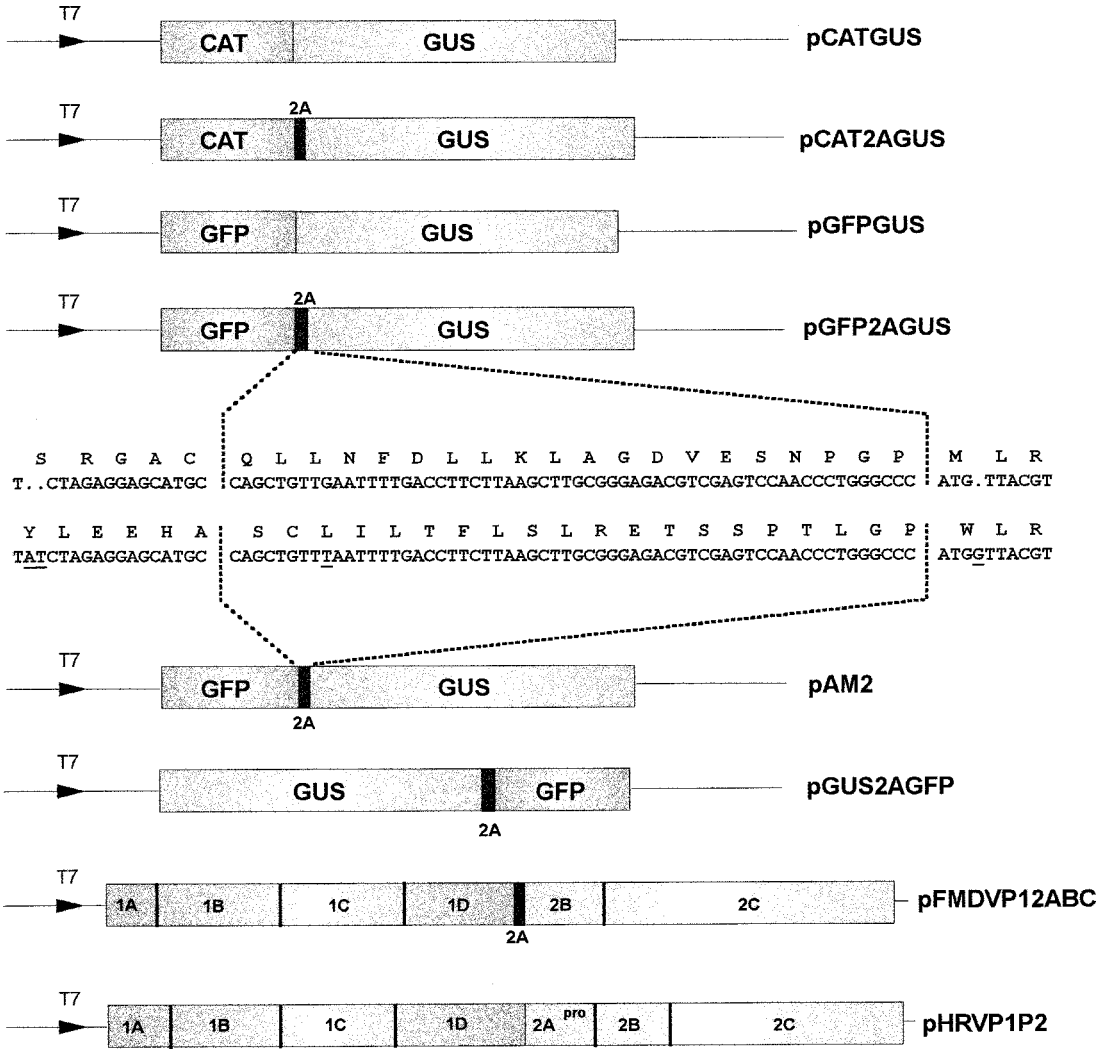

Fig. 2. Plasmid constructs. Regions of plasmids encoding polyproteins (boxed areas) are shown. The sequence of the 2A region of pGFP2AGUS is shown for comparison with that of pAM2. Frame-shift and point mutations are underlined.

the levels of CAT2A, GFP and GUS present in the translations systems decreased to some extent (over much longer incubation periods compared to the 45 min translation), neither the absolute rates nor the relative rates of degradation could account for the imbalance in the accumulated products that we had observed (Fig. 4) – particularly dramatic in wheat germ extracts. The experiment comparing the translation profiles from pGFP2AGUS and pGUS2AGFP also, perhaps, argues against protein degradation as an explanation for the imbalance.

Evaluation of premature termination of transcription or translation

An alternative explanation for our data is that the T7 RNA polymerase, when transcribing from the plasmid templates in the coupled transcription/translation systems, arrests prematurely either at random (producing a nested set of 5′ co-terminal RNA transcripts) or at a specific position at the end of the 2A region. Translation of these shorter, prematurely terminated, RNA transcripts would bias the accumulation of our 'cleavage' products towards those constituting the N-terminal region of the polyprotein.

pGFP2AGUS was transcribed separately *in vitro* to produce a single, discrete, RNA transcript. This defined mRNA was used to programme an uncoupled *in vitro* translation system. Translation profiles from pGFP2AGUS transcribed *in vitro* (and subsequently used to programme an *in vitro* translation system) were compared with those obtained by using the plasmid DNA to programme a coupled transcription/translation system. Phosphorimaging analysis showed that both methods produced a molar excess of [GFP2A]:GUS of 4:1 and 5:1, respectively (data not shown).

Premature termination of translation throughout the length of the RNA transcript template could also account for the

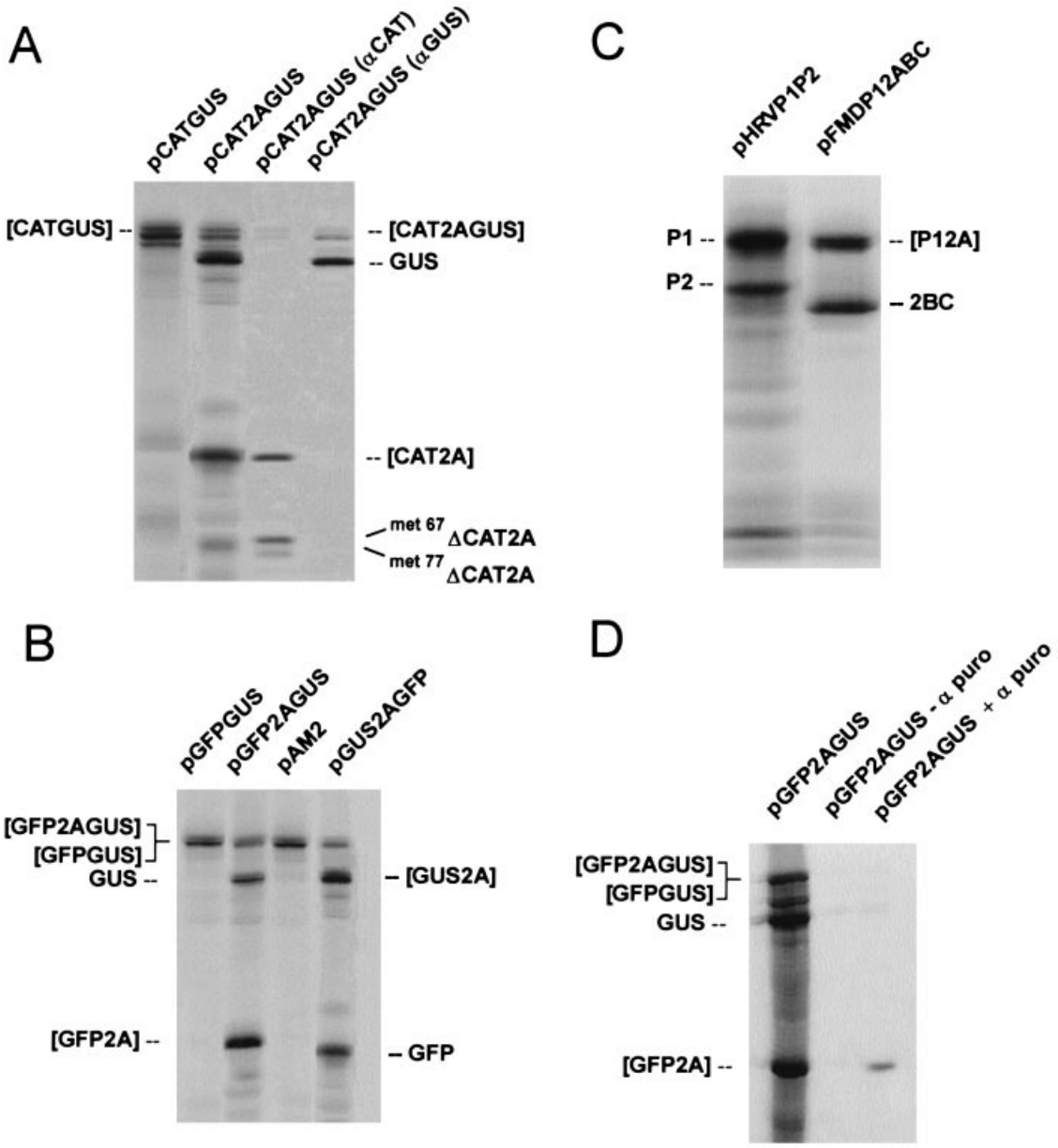

Fig. 3. Translation *in vitro*. Translation products from pCATGUS and pCAT2AGUS are shown together with immunoprecipitated products (A). The translation products from pAM2 are compared with those of pGFPGUS, pGFP2AGUS and pGUS2AGFP (B). (C) Translation products derived from the control constructs pHRVP1P2 and pFMDP12ABC. (D) pGFP2AGUS was translated in the presence of puromycin (50 μg/ml) and the translation products were then immunoprecipitated using null serum (−αpuro) or anti-puromycin antibodies (+αpuro).

observed imbalance in the accumulation of products. Similarly, this effect could produce an excess of N-:C-terminal translation products. One way to address this question was to analyse the translation profile of a system that *should* (i) produce a unitary stoichiometry of proteolytic cleavage products and (ii) be of a size comparable to the artificial polyprotein systems we have been analysing. The 2A protein of HRV is known to be a *cis*-acting proteinase and should, therefore, produce a 1:1 stoichiometry in its cleavage products – in the case of the polyprotein encoded by construct pHRVP1P2 (Fig. 2) the capsid protein precursor, P1, and replicative proteins precursor, P2. The size of the [P1P2] ORF in pHRVP1P2 is 1429 codons compared to the [GFP2AGUS] ORF at 887 codons – over 50% longer. Phosphorimaging analysis of the cleavage products derived from coupled *in vitro* transcription/translation of pHRVP1P2 showed the two expected translation products, P1 (mol. mass 95 kDa) and P2 (mol. mass 64 kDa). The molar ratio of HRVP1:HRVP2 was 1:1, showing that in

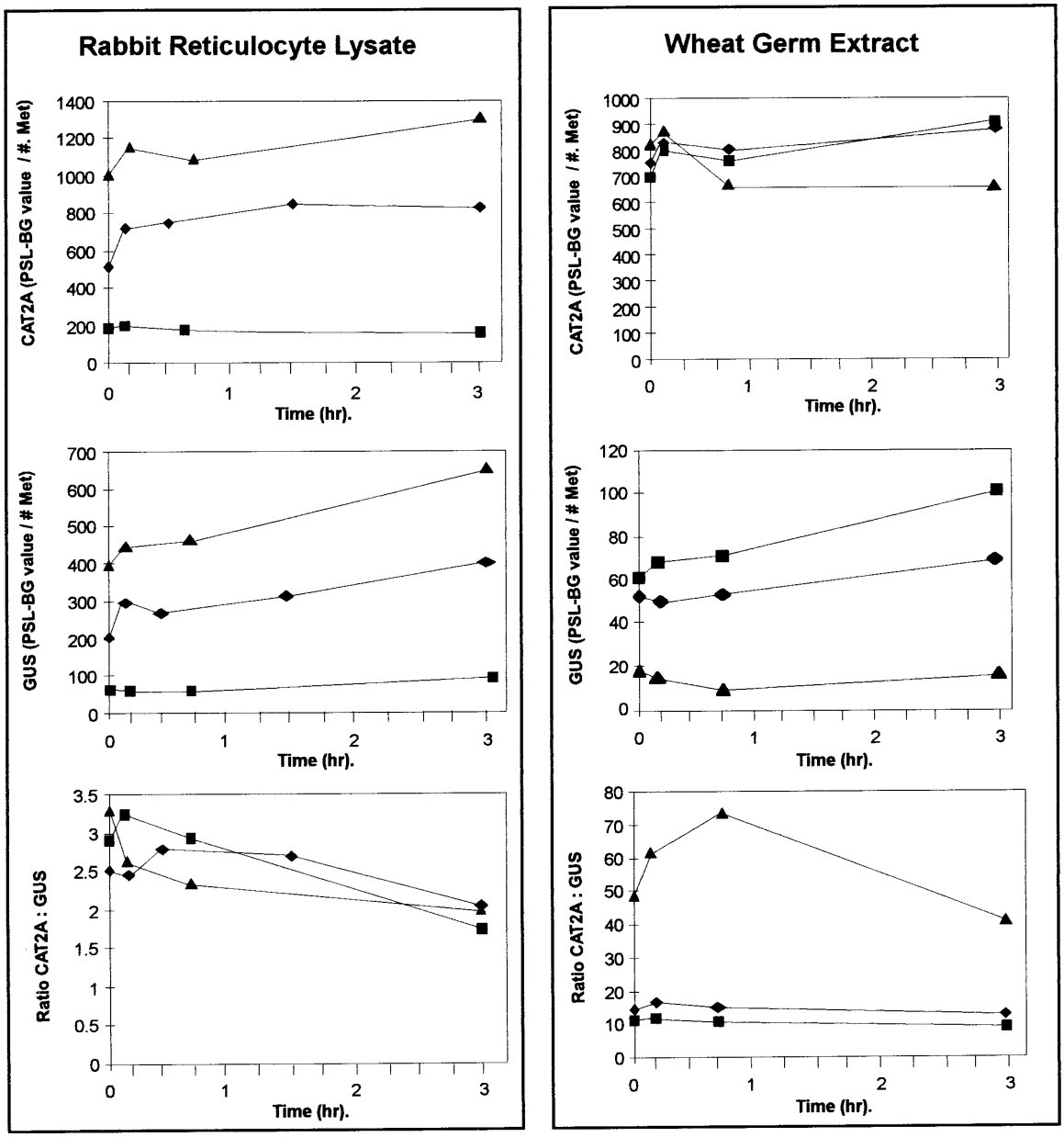

Fig. 4. Rates of protein degradation in rabbit translation systems *in vitro*. Data from three independent experiments are shown (▲, expt 1; ■, expt 2; ◆, expt 3). for each translation system. Phosphorimaging data (photo-stimulated luminescence; PSL) were corrected for methionine contents, the ratio PSL [CAT2A]:PSL [GUS] being shown for each experiment.

the coupled translation systems we were using random premature termination was not producing the 'imbalance' effect observed in the artificial polyprotein systems (Fig. 3C). Interestingly, phosphorimaging analysis of the translation products derived from pFMDP12ABC showed the expected two major products, [P12A] (mol. mass 82 kDa) and [2BC] (mol. mass 52 kDa), to be present in the molar ratio 1:1 (Fig. 3C). This result, again, argues against premature termination effects accounting for the imbalance of translation products derived from our artificial self-processing polyprotein cDNA constructs.

Is 2A-mediated 'cleavage' an RNA or oligopeptidic effect?

A construct (pAM2) was made in which two frame-shifts were introduced into pGFP2AGUS such that the reading frame of GFP and GUS was maintained whilst the 2A region was in an alternative (+2) reading frame (Fig. 2). A further single-base mutation was, of necessity, introduced into the 2A region to remove a stop codon such that the single, long, ORF was maintained. This mutation is in a region of 2A shown not to be required for 2A activity (Ryan & Drew, 1994). Although the

RNA sequence corresponding to the 2A region is present in transcripts derived from this construct the recombinant polyprotein does not contain the 2A peptide sequence. Translation of pAM2 showed a single product of the same size as [GFPGUS]: no 'cleavage' activity was observed (Fig. 3B).

Incorporation of puromycin into nascent translation products

Having eliminated proteolysis as a mechanism for generation of the 'cleavage' products we have proposed a translational model of 2A-mediated 'cleavage' (see below) which we wished to test. Puromycin is added to the C terminus of nascent proteins via an amide linkage but is incorporated independent of the nature of the codon present in the ribosomal A site. Inclusion of puromycin throughout translation should result in its incorporation throughout the synthesis of the polyprotein – leading to the (random) truncation of translation products. Puromycin incorporation could, however, occur preferentially at any significant ribosomal 'pause' sites. When pGFP2AGUS was translated in the presence of puromycin (50 μg/ml) and the translation products immunoprecipitated with anti-puromycin antibodies, a single, discrete product was observed with a gel migration indistinguishable from that of [GFP2A] (Fig. 3D).

Discussion

Our initial working hypothesis that FMDV 2A mediates a co-translational proteolytic cleavage, in *cis*, at its own C terminus gives a simple predicted outcome: the proteolytic cleavage products will be generated, but not necessarily accumulate, in equimolar amounts. In the two artificial polyproteins described here (and all others we have examined) the translation product N-terminal of 2A routinely accumulates in 2- to 5-fold molar excesses over that C-terminal of 2A.

FMDV 2A-mediated 'cleavage' is not mediated by the RNA sequence

Inspection of the aligned nucleotide sequences available for aphtho- and cardiovirus 2A regions shows little similarity other than bases absolutely required to encode the conserved amino acids. Algorithms which predict RNA secondary structures were used to examine all available sequences. No RNA structure was found to be conserved amongst aphtho- and cardioviruses (data not shown). Plasmid pAM2 encodes a single ORF but with the RNA sequence encoding 2A frame-shifted into the +2 reading frame with respect to GFP and GUS. Analysis of translation products showed no 'cleavage'.

Product imbalance is not due to protein degradation

Studies in which artificial polyprotein synthesis was arrested and the mixture subsequently incubated showed that the rates of [CAT2A] and GUS degradation in the *in vitro* translation systems were low and directly comparable, one with another. Identical experiments using pGFP2AGUS produced the same results (data not shown). These experiments were performed for periods much longer than the 'synthetic phase' of the translation reactions, with very little protein degradation, and we concluded that protein degradation rates cannot account for the imbalance in the accumulation of the products.

Product imbalance is not due to premature termination of transcription or translation

An alternative explanation for the artificial polyprotein 'cleavage' product imbalance is specific termination of (T7 RNA polymerase-driven) transcription at the 2A site. Individual (T7) transcription reactions were performed, and the T7 RNA transcripts were characterized and used to programme translation reactions. These experiments produced the same translation profiles as the coupled TnT systems. We conclude that explanations such as RNA degradation or premature termination of transcription in the coupled transcription/translation system did not account for the imbalance of FMDV 2A-mediated 'cleavage'. A good test of whether premature, random, termination of transcription or translation in these systems was producing the observed imbalance of 'cleavage' products was translation of pHRVP1P2. In this polyprotein construct the HRV 2A region encodes a *cis*-acting proteinase cleaving co-translationally at its own N terminus (1D/2A site). In this case we predict a unitary stoichiometry of the proteolytic products (P1 and P2) – and this is what we observed.

FMDV 2A-mediated 'cleavage' is not due to proteolysis

The experiments we have done to characterize the *in vitro* translation systems have shown that the imbalance in the accumulation of translation products from the artificial polyproteins is due to unequal levels of *synthesis*, which cannot be accounted for by the reasons described above. Phosphorimaging analysis of the translation products derived from pFMDP1P2 showed that no translation product spanning 2A could be detected and that the 'cleavage' products [P12A] and [2BC] were present in equimolar quantities. Taken together with the translation profile derived from pHRVP1P2, this showed that translation factors, aminoacyl-tRNAs, metabolites etc. were present in the *in vitro* translation systems (during the synthetic phase of our translation reactions) at a level sufficient to synthesize an ORF considerably longer than our artificial polyproteins without levels of premature termination of transcription/translation sufficient to produce a spurious 'imbalance' result.

Our data are not consistent with 2A-mediated proteolysis of the nascent polyprotein, nor proteolysis by a cellular enzyme. The imbalance in these systems must be due to an effect on the translational machinery, rather than events subsequent to synthesis.

A translational model of FMDV 2A activity

The multiple translation products described above are generated from a single ORF. Our data are not consistent with these being produced by proteolysis of a precursor molecule. The model we have developed for the mechanism of 2A activity on the translational apparatus must account for the three outcomes we observed in the translation of the artificial polyproteins. Firstly, peptide bond formation proceeds throughout the length of the polyprotein (i.e. 'uncleaved' [GFP2AGUS]). Secondly, the translational complex either 'stalling' or dissociating at the C terminus of 2A – in a stop codon-independent manner. Either effect would account for our observation that the [GFP2A] product is synthesized at higher levels than GUS. Thirdly, translation of the upstream product ([CAT2A] or [GFP2A]) followed by translation of the discrete downstream product (GUS) without the synthesis of a peptidic linkage between the two.

The scheme we have proposed is summarized in Fig. 5. The Pro–Gly peptide bond at the C terminus of 2A is synthesized (Fig. 5, steps i–ii). Translocation of the peptidyl(2A)-tRNA^{Gly} from the A to P site, mediated by elongation factor 2 (eEF2), would allow ingress of prolyl-tRNA (Fig. 5, step iii). The nucleophilic attack by the prolyl-tRNA amide nitrogen upon the peptidyl(2A)-tRNA^{Gly} carbonyl carbon is inhibited by 2A. Hydrolysis of the peptidyl(2A)-tRNA^{Gly} ester linkage occurs, releasing the nascent peptide from the ribosome (Fig. 5, steps iv and v). Prolyl-tRNA in the A site is then translocated to the P site, as if a peptide bond had been synthesized (Fig. 5, step vi), and translation of the downstream product can continue.

(*a*) 2A:ribosome exit tunnel interaction. The ribosomal exit channel or tube is made primarily of RNA and has been described as 'non-stick' (Nissen *et al.*, 2000); these authors describe the large entropic penalty that would be paid by a nascent peptide binding to the tunnel wall. The exit tunnel does not, however, appear to be completely 'neutral' for certain nascent peptides. Indeed, such interactions are well documented: nascent peptides have been shown to induce both ribosomal pausing and to inhibit peptidyltransferase activity (Gu *et al.*, 1994; Rogers & Lovett, 1994; Harrod & Lovett, 1995). Virus sequences are known to manipulate events within the ribosome itself: programmed ribosomal frame-shifting (e.g. retro-, coronaviruses) and programmed ribosomal 'hopping' (bacteriophage T4 *gene 60*; reviewed by Farabaugh, 1996; Gesteland & Atkins, 1996). This 'hopping' is also referred-to as 'translational bypassing' or 'subversion of contiguity' (Gesteland & Atkins, 1996). In T4 *gene 60* a ribosomal 'hop' occurs from codon 47 to a matched 'landing' codon 50 nt downstream (Weiss *et al.*, 1990). Of specific relevance to our model is the finding that a requirement for the *gene 60* translational bypass is a 16 aa *cis*-acting sequence in the nascent chain – probably acting in the exit channel of the ribosome (Weiss *et al.*, 1990). This type of translational control has also been implicated in the expression of cellular genes (Matsushita *et al.*, 1991; Benhar *et al.*, 1992; Benhar & Engelberg-Kulka, 1993; Manch-Citron & London, 1994).

Our analyses of site-directed mutagenic and N-terminally extended forms of FMDV 2A (accompanying paper: Donnelly *et al.*, 2001) and other 2A-like sequences (Donnelly *et al.*, 2001) show that amino acid changes some distance from the C terminus of 2A may affect events within the peptidyl-transferase centre. In the case of FMDV 2A we have mapped these influential sequences entirely within a 23–32 aa tract constituting FMDV 2A and the C-terminal region of FMDV protein 1D. It has been proposed that the most probable conformation of a nascent peptide is helical (Lim & Spirin, 1986) and the dimensions of the ribosomal exit tunnel are consistent with this notion (Nissen *et al.*, 2000). The prokaryotic ribosome exit tunnel is some 100 Å in length and straight, although it has a bend some 20–35 Å from the peptidyltransferase centre. Modelling a range of different 2A sequences as α-helixes shows that all of these structures could be easily accommodated entirely within the ribosome exit tunnel.

(*b*) Inhibition of peptidyltransferase activity. Since mutation of the N-terminal proline residue (secondary amino acid) of 2B to primary amino acids resulted in the synthesis of 'uncleaved' polyprotein alone (Hahn & Palmenberg, 1996; Donnelly *et al.*, 2001) any mechanism for the inhibition of peptidyltransferase activity would need to account for this effect only being observed when prolyl-tRNA is the nucleophile. Mutation of the proline residue to a primary amino acid permits the aminoacyl-tRNA to out-compete the hydrolysis of the peptidyl(2A)-tRNA^{Gly} ester linkage. The absolute requirement for a proline residue in this position for 'cleavage' could be explained by the chemical character of proline as a nucleophile. The secondary amino group is sterically hindered relative to the primary amino groups of other amino acids and is conformationally restrained due to its location in a five-membered ring. Indeed, the lower nucleophilicity of proline compared to that of primary amino acids in peptide synthesis and translation is well documented (Nathans & Niedle, 1963; Rychlik *et al.*, 1970; Lenman *et al.*, 1997).

The mechanism of peptidyltransferase activity proposed by Nissen *et al.* (2000) invokes many ribosome components, and the involvement of the 3′ end of tRNAs in this reaction is clear (Samaha *et al.*, 1995; Green *et al.*, 1998). The nature of the interaction of 2A with the exit tunnel clearly plays a role in this inhibition since peptide bond formation between the peptidyl(2A)-tRNA^{Gly} and prolyl-tRNA occurs when the 19 aa version of 2A is used in the artificial polyprotein systems (synthesis of [GFP2AGUS] at ~ 10%), but not when 2A is present in its native context or when 2A bears an N-terminal extension of between 5–14 aa (Donnelly *et al.*, 2001).

(*c*) Hydrolysis of the peptidyl(2A)-tRNA^{Gly} ester linkage. One question addressed by Nissen *et al.* (2000) was 'how is the catalysed hydrolysis of the peptidyl tRNA in the P site

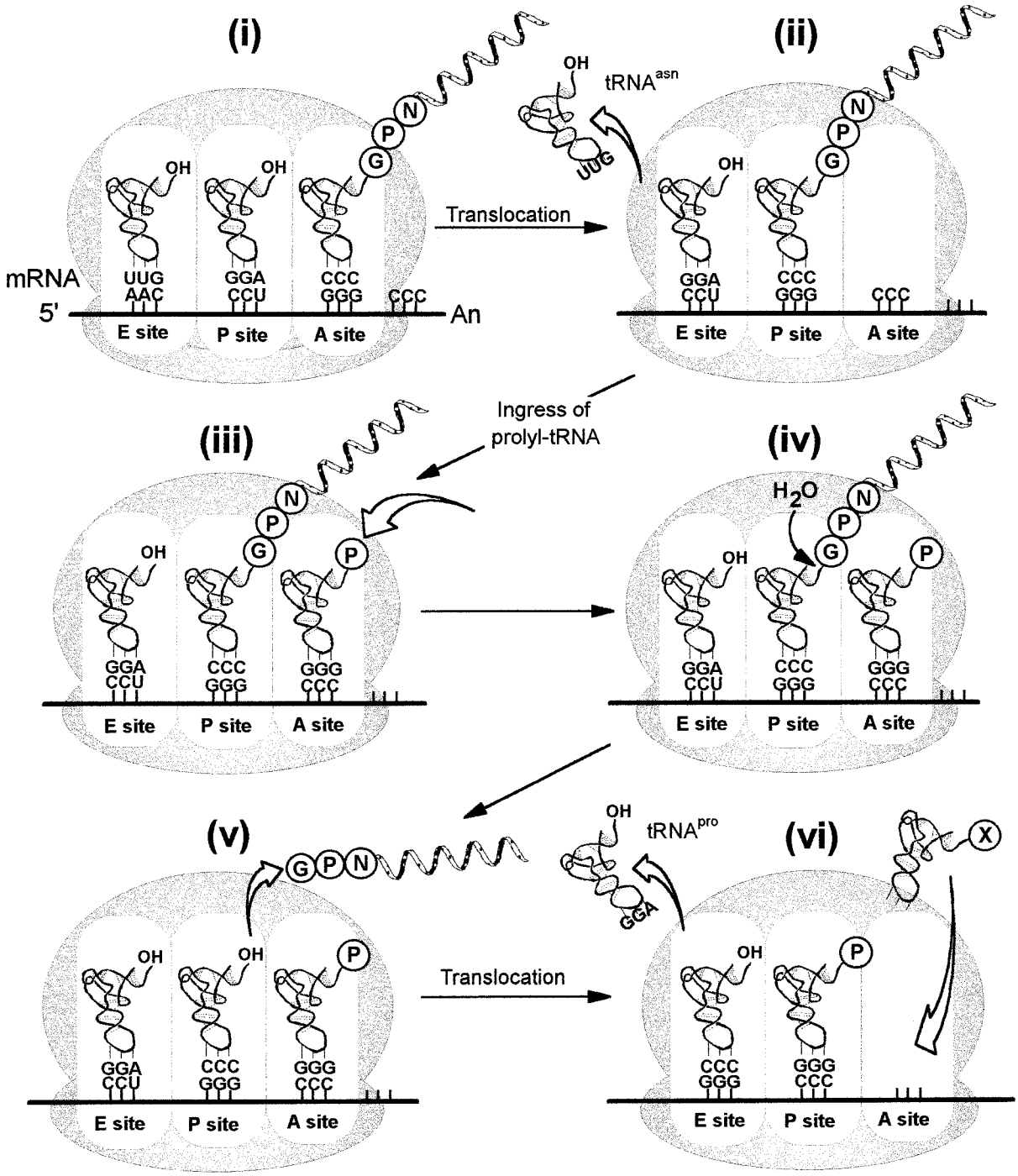

Fig. 5. Scheme of 2A activity. The stage following the addition of the ultimate residue of 2A is shown (step i). Peptidyl(2A)-tRNA is translocated from the A to P site (step ii), allowing the ingress of prolyl-tRNA (step iii). Prolyl-tRNA is unable to attack the peptidyl(2A)-tRNA^{Gly} ester linkage, and hydrolysis of the glycyl-tRNA ester bond releases the nascent peptide (steps iv and v). Deacylated tRNA is now present in the P site (mimicking peptide bond formation) and would allow the translocation of prolyl-tRNA (rather than the normal peptidyl-tRNA) from the A to P sites (step vi). Synthesis of the peptide C-terminal of 2A would proceed as normal, although recommencing (rather than 'initiating' *sensu stricto*) with proline.

prevented prior to the delivery of the next appropriate amino acid-tRNA to the A site?'. They discuss the possibility that a catalytic rRNA base (A2486) and/or the peptidyl-tRNA substrate are not properly oriented for hydrolysis to occur or that the binding site for the amino group is blocked by a reoriented base whilst the A site is unoccupied. Indeed, we have proposed that the C-terminal – NPG – residues of 2A could serve to re-orient the peptidyl(2A)-tRNA^{Gly} substrate to

disfavour peptide bond formation and promote hydrolysis (Ryan *et al.*, 1999), although in our case this would occur when the A site is occupied by prolyl-tRNA. Promotion of the hydrolytic event could be due to a 2A-mediated enhancement of the intrinsic rate of hydrolysis within ribosomes or one could envisage 2A being an 'active' hydrolytic element in its own right: positioning the peptidyl(2A)-tRNA^{Gly} ester linkage to lie beneath the α-helix such that the dipole moment could be harnessed to generate an activated water molecule.

(*d*) Ribosomal A to P site translocation rates. The complexes shown in Fig. 5 (steps v and vi) would not be encountered during the normal course of translation. Were the stability of either, or both, of these complexes to be low then ribosomal subunits might dissociate at this point – generating a molar excess of the upstream translation product. Alternatively, if the 2A peptidyl-tRNA resided for too long in the A site, hydrolysis in this situation would lead to deacylated tRNAs occupying both the P and A sites – termination of translation. Interestingly, translation studies on cardiovirus RNA using Krebs-2 cell-free extracts (containing low levels of eEF-2 activity) showed a translational 'barrier' in the central region of the genome (Svitkin & Agol, 1983). The translation products formed indicate that this barrier prevented translation of products downstream of cardiovirus 2A. The addition of eEF-2 greatly enhanced the synthesis of proteins C-terminal of cardiovirus 2A.

(*e*) Puromycin incorporation. Puromycin competes with all aminoacyl-tRNAs for incorporation onto the C terminus of nascent polypeptide chains. Translation of pGFP2AGUS in the presence of puromycin gave, however, not a uniform pattern of puromycin incorporation, but a single major species. This band represented a significant population of translation complexes which were 'paused' at a specific site and translational state competent to incorporate puromycin. We argue that this corresponds to a kinetically slow step and it is at this stage that a water molecule (or in this case puromycin) can hydrolyse the peptidyl-tRNA ester linkage – step (iv), Fig. 5. These data also provide an additional line of evidence arguing against a proteolytic model – proteolytic cleavage (at the 2A/2B site; glycyl–prolyl pair) of translation products which had been extended past 2A would separate the puromycin 'tag' (present only at C termini) from [GFP2A] which would not, therefore, be immunoprecipitated using the anti-puromycin antibodies – which would not produce a specific [GFP2A] band.

2A activity in native and artificial polyproteins

Differences in 2A activity were apparent when 2A was present in its native, or an artificial, polyprotein context. 2A-mediated 'cleavage' appeared to be complete when the 2A region was present in its normal FMDV polyprotein context (Ryan *et al.*, 1989, 1991), whereas 2A activity in artificial polyproteins showed an imbalance in 'cleavage' products and a low level (~5%) of full-length translation products. Translation of pFMDP12ABC showed the 'cleavage' products [P12A] and [2BC] were present in equimolar quantities. We have shown that FMDV sequences upstream of 2A do, however, play a role in maximizing the 'cleavage' activity, reducing the amount of 'uncleaved' product (Ryan *et al.*, 1991; Donnelly *et al.*, 1997, 2001).

Product imbalance – the translational model

The imbalances in translation products we observed in the artificial polyprotein systems could be accounted for by two, quite possibly functionally independent, aspects of the system. Firstly, the efficiency of the 'pseudo-termination' event and secondly, the efficiency of the 're-initiation' event.

(*a*) The efficiency of 'pseudo-termination'. Here, we have observed two outcomes: either the full-length translation product is synthesized (formation of the peptide bond between 2A and GUS) or, we propose, it is not formed and a stop codon-independent (pseudo-) termination of translation occurs. Two explanations are considered here as to how the efficiency of this event could affect the imbalance in translation products.

(i) The 19 aa 2A tract we have analysed is suboptimal and incorrectly re-aligns the peptidyl-tRNA^{Gly} ester linkage such that in a proportion of cases *neither* hydrolysis of the ester linkage *nor* attack by prolyl-tRNA can occur. This would lead to a 'stall' or arrest of translation at the C terminus of 2A and, following the disruption of this stalled complex by the sample preparation procedure for SDS–PAGE analysis, result in a net N-:C-terminal product imbalance. In its native polyprotein context [and N-terminally extended forms of 2A; see accompanying paper (Donnelly *et al.*, 2001)], however, the proposed re-alignment of the 2A peptidyl-tRNA^{Gly} ester linkage is 'correct' in that it adopts a conformation in which hydrolysis of the bond can occur, but nucleophilic attack by prolyl-tRNA cannot.

(ii) In the same proportion of cases the rate of hydrolysis is increased such that this occurs at a stage (Fig. 5, steps i–ii) prior to the ingress of prolyl-tRNA into the A site. This would result in either deacylated tRNAs in both A and P sites (hydrolysis occurring at step i, Fig. 5) – the situation produced by stop codon-mediated termination of translation – or deacylated tRNA in the P site with the A site unoccupied (hydrolysis occurring at step ii, Fig. 5). Our puromycin incorporation data showed, however, that the C terminus of 2A represents the major translational pause site throughout the ORF.

2A in its native polyprotein context (pFMDVP1P2), however, produces complete 'cleavage'. We have determined that by N-terminally extending the inserted 2A sequence in the [GFP2AGUS] reporter polyprotein system the accumulation of uncleaved material is either reduced (N-terminal extension of 5 aa of 1D) or eliminated (N-terminal extension of 14 aa of 1D) (Donnelly *et al.*, 2001). We would argue, therefore, that the efficiency of the pseudo-termination event is determined

primarily by the nature of the sequences upstream (within ~ 30 aa) of the conserved -DxExNPGP- motif.

(*b*) **The efficiency of 'pseudo-reinitiation'.** Given that the 'cleavage' occurred as shown in step (v) of Fig. 5, what other factors could come into play to affect the efficiency of the 'pseudo-reinitiation' event? The complex shown in step (vi) would not be encountered during the normal course of protein synthesis and the stability of this complex could well determine whether translation terminates at this point or continues to synthesize the (discrete, 'cleaved') downstream product. The *in vitro* translation of constructs encoding 2A in its native polyprotein context (pFMDVP1P2) and N-terminally extended forms of 2A (extension by $\geqslant$ 14 aa 1D; Donnelly *et al.*, 2001) resultes in equimolar ratios of up- and downstream translation products. This argues that the complex shown in step (vi) would lead to continued translation in a highly efficient manner.

The product imbalances we have observed for pSAT1 (suboptimal 20 aa 2A tract) were dramatically different between rabbit reticulocyte lysates and wheat germ extracts, although no such differences between these translation systems were observed for 2A-mediated 'cleavage' when 2A was in its native polyprotein complex. Taking our data together we account for this observation by the ability of the different lengths (and sequences; Donnelly *et al.*, 2001) of 2A to interact with the exit pores of the rabbit or wheat ribosomes to produce the 'correct' realignment of the peptidyl-tRNA^{Gly} ester linkage in the peptidyltransferase centre to promote hydrolysis rather than a complete translational arrest.

In summary, we think that by studying FMDV 2A within artificial polyprotein systems the stoichiometric imbalance in the 'cleavage' products and the low-level synthesis of full-length translation products are a product of its suboptimal functioning, compared to its activity in a native polyprotein context. We believe that the study of 2A activity in these artificial polyprotein systems has, however, provided us with real insights as to its mode of action.

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