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The properties of chimeric picornavirus IRESes show that discrimination between internal translation initiation sites is influenced by the identity of the IRES and not just the context of the AUG codon

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ABSTRACT

The internal ribosome entry segment (IRES) of picornaviruses consists of ~450 nt of 5′-untranslated region, terminating at the 3′ end with an ~25 nt element consisting of an absolutely conserved UUUC motif followed by a more variable pyrimidine-rich tract and G-poor spacer, and finally an AUG triplet, which is considered to be the actual ribosome entry site. Events following entry at this site differ among picornaviruses: in encephalomyocarditis virus (EMCV) virtually all ribosomes initiate translation at this site (AUG-11); in foot-and-mouth-disease virus (FMDV), one-third of the ribosomes initiate at this AUG (the Lab site), and the rest at the next AUG 84 nt downstream (Lb site); and in poliovirus (PV), the AUG at the 3′ end of the IRES (at nt 586 in PV type 1) is considered to be a silent entry site, with all ribosomes initiating translation at the next AUG downstream (nt 743). To investigate what determines this different behavior, chimeras were constructed with a crossover at the conserved UUUC motif: the body of the IRES, the sequences upstream of this UUUC motif, was derived from one species, and the downstream sequences from another. When the body of the FMDV or PV IRESes was replaced by that of EMCV, there was a marked increase in the absolute and relative frequency of initiation at the upstream AUG, the Lab site of FMDV and ^{586}AUG of PV, respectively. In contrast, when the body of the EMCV IRES was replaced by that of PV, initiation occurred with no preference at three AUGs: the normal site (AUG-11), AUG-10 situated 8 nt upstream, and AUG-12, which is 12 nt downstream. Thus although the context of the AUG at the 3′ end of the IRES may influence initiation frequency at this site, as was shown by improving the context of ^{586}AUG of PV, the behavior of the ribosome is also highly dependent on the nature of the upstream IRES. Delivery of the ribosome to this AUG in an initiation-competent manner is particularly efficient and accurate with the EMCV IRES.

Keywords: aphthovirus; cardiovirus; encephalomyocarditis virus (EMCV); enterovirus; foot-and-mouth-disease virus (FMDV); poliovirus (PV); rhinovirus; scanning ribosome model

INTRODUCTION

Picornavirus RNAs are translated by a mechanism of internal ribosome entry dependent on an ~450 nt *cis*-acting RNA element located within the 5′-untranslated region (UTR), originally designated as the "internal ribosome entry site" (IRES). However, it is generally agreed that the actual ribosome entry site is not the total length of this large segment, but, if operationally defined as the most 5′-proximal point at which initiation can occur, the entry or landing site is an AUG codon located 25 nt downstream of the start of a pyrimidine-rich tract at the 3′ end of the IRES (for recent reviews, see Ehrenfeld & Semler, 1995; Hellen & Wimmer, 1995; Jackson & Kaminski, 1995). As has been described previously, the 5′-proximal part of the oligopyrimidine tract is essential for IRES function in several different picornavirus species (Iizuka et al., 1989; Jang & Wimmer, 1990; Kühn et al., 1990; Meerovitch et al., 1991; Nicholson et al., 1991; Pestova et al., 1991; Pilipenko et al., 1992), but, on the other hand, the sequences between the oligopyrimidine tract and the AUG codon are not highly conserved between closely related species or even between different strains of the same species, although the length

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of this segment is conserved (Sangar et al., 1987; Pritchard et al., 1992). It has been suggested that this ~25-nt stretch serves as an unstructured spacer element (Kaminski et al., 1994).

On the basis of their IRES sequences, picornaviruses can be divided into three different groups: (1) hepatitis A virus, (2) the cardiovirus [e.g., encephalomyocarditis virus (EMCV)] and aphthovirus family [e.g., foot-and-mouth-disease virus (FMDV)], and (3) the enterovirus [e.g., poliovirus (PV)] and rhinovirus family. Several differences have been observed in the in vitro translation characteristics between IRESes from these different groups (Borman et al., 1995). While IRES-driven translation of cardio- and aphthovirus RNAs is accurate and extremely efficient in the reticulocyte lysate, translation driven by the poliovirus or rhinovirus IRES is inaccurate and inefficient unless the system is supplemented with HeLa cell or Krebs II ascites cell extracts (Brown & Ehrenfeld, 1979; Dorner et al., 1984; Svitkin et al., 1988; Borman et al., 1993, 1995). Another difference is that cleavage of eIF4G by picornavirus encoded proteases strongly stimulates translation driven by the polio- and the rhinovirus IRESes (Borman et al., 1995; Ziegler et al., 1995a, 1995b), whereas it has little or no effect on protein synthesis driven by the cardio- and aphthovirus IRESes (Borman et al., 1995; Ohlmann et al., 1995; Ziegler et al., 1995a, 1995b).

However, the greatest difference concerns the events that follow internal ribosome entry at the AUG triplet at the 3′ end of the IRES. In the case of cardiovirus IRESes, the overwhelming majority of the ribosomes initiate translation at this AUG codon, with just a few utilizing the next AUG further downstream (Kaminski et al., 1994). However, in the case of enterovirus/rhinovirus IRESes, very little if any initiation occurs at the entry site AUG at the 3′ end of the IRES, and virtually all initiation is at the next AUG codon downstream that is thought to be accessed by scanning from the original entry site (reviewed in Ehrenfeld & Semler, 1995; Jackson & Kaminski, 1995). FMDV represents an intermediate between these two extremes, with translation initiation occurring not only at the AUG at the 3′ end of the IRES but also, at higher frequency, at the next AUG further downstream (Belsham, 1992). Hitherto the explanation advanced for this different behavior relied largely on arguments based on the context of the AUG at the 3′ end of the IRES (Belsham, 1992); a favorable context, defined by the criteria proposed by Kozak (1986), is thought to favor exclusive use of the AUG at the 3′ end of the IRES (as in EMCV), and a poor context is believed to lead to low, or even no, initiation at this site, but instead initiation at the next AUG downstream. However, context considerations alone do not easily account for the differences between FMDV and PV IRESes.

To examine this in a more critical way, we have generated a series of chimeric IRESes, in which the major part (the body) of the IRES is from one picornavirus species, the crossover is made at a conserved UUUC motif at the start of the oligopyrimidine tract, and the sequences downstream of this, which therefore include the actual ribosome landing site, are from a different species. We find that the behavior of the ribosome following internal entry is influenced not just by the downstream sequences that include the actual entry site, but also, unexpectedly, by the origin of the upstream IRES body.

RESULTS

Experimental strategy

The strategy used to design chimeric constructs between the three different classes of picornavirus IRES is depicted in Figure 1. Each of the six chimeric constructs contains the main part, or body, of the IRES element (up to the start of the oligopyrimidine tract) of one class of picornavirus (either PV, EMCV, or FMDV) fused to the downstream segment of another class of virus (Fig. 1A), with the fusion made at the conserved UUUC motif at the start of the oligopyrimidine tract.

The decision to make the fusion or crossover at the UUUC(C) motif at the start of the oligopyrimidine tract was based on several considerations. Of paramount importance was the fact that this (or strictly the UUUC tetranucleotide motif) is the only motif that is absolutely conserved in sequence and relative location amongst all aphtho-, entero-, cardio-, and rhinovirus IRESes, and indeed is the only part of the pyrimidine-rich tract that is absolutely invariant. In contrast, the sequences further downstream of this motif are subject to high genetic drift, not just between different virus species, but even between different isolates of the same strain (Pöyry et al., 1992). Moreover, it has been shown that although point mutations in the UUUC motif are severely debilitating, mutations in the region just downstream of this in the coxsackie B1 and poliovirus IRES, or replacement of this sequence in the poliovirus IRES as a result of deletions or insertions, have at most a minimal influence on infectivity and on IRES activity (Iizuka et al., 1989, 1991; Meerovitch et al., 1991; Nicholson et al., 1991; Pilipenko et al., 1992). A further consideration was that the whole of the region between the conserved UUUC motif and the downstream AUG triplet will be in direct contact with any ribosome that enters at and binds to the AUG triplet. Thus it seemed appropriate that in the chimeric constructs, these sequences immediately upstream of the AUG should be derived from the same virus species as the sequences immediately downstream of it, so that the whole segment in intimate contact with the ribosome would represent the entry site of a single species, and would not, in itself, be chimeric.

(A)

IRES of PV or FMDV or EMCV

cross-over

5' UUUCC / downstream sequences of EMCV, FMDV or PV.

polypyrimidine rich tract

(B)

Series	Construct	IRES	Junction sequence
PV series	PV-EMCV	5'---PV IRES---	**GUGUUUCCUUUU**CCUUUGAAAAACACG<u>AUG</u>(826, #10)AUAAU**<u>AUG</u>**(834, #11)GCCACAACC<u>AUG</u>(846, #12)*GAUCCC*... (*Bam*HI)
	PV-FMDV	5'---PV IRES---	**GUGUUUCCUUUU**CCUUUUAUAACCACUGAACAC**<u>AUG</u>**(442, Lab)AAU......**<u>AUG</u>**(526, Lb)GAAuucccg*GAUCCC*...
FMDV series	FMDV-EMCV	5'---FMDV IRES---	**ACGUUU**CCUUUGAAAAACACG<u>AUG</u>(826, #10)AUAAU**<u>AUG</u>**(834, #11)GCCACAACC<u>AUG</u>(846, #12)*GAUCCC*... (*Bam*HI)
	FMDV-PV	5'---FMDV IRES---	**ACGUUU**CCUUUUAUUUUAUUGUGGCUGCUU<u>AUG</u>(586)GUG......**<u>AUG</u>**(743)gagcucgguacc<u>aug</u>*GAUCCC*...
EMCV series	EMCV-PV	5'---EMCV IRES---	**UGGUUUU**CCUUUUAUUUUAUUGUGGCUGCUU<u>AUG</u>(586)GUG.....**<u>AUG</u>**(743)gagcucgguacc<u>aug</u>*GAUCCC*...
	EMCV-FMDV	5'---EMCV IRES---	**UGGUUUU**CCUUUUAUAACCACUGAACAC**<u>AUG</u>**(442, Lab)AAU......**<u>AUG</u>**(526, Lb)GAAuucccg*GAUCCC*...

FIGURE 1. Design of chimeric constructs and resulting crossover sequences from the oligopyrimidine tract to the downstream AUG codon(s). **A**: The IRES element (up to and including the highly conserved UUUCC motif at the start of the oligopyrimidine tract) of one of the picornavirus species (PV, FMDV, or EMCV) was amplified by PCR and fused to the downstream sequences of another picornavirus species. **B**: Sequences of the junctions of the six chimeric constructs. The AUG triplets contained in the downstream sequences of each chimeric construct are underlined, and the authentic viral initiation codon is also in bold (there are two such sites in FMDV). Residues in bold at the 5′ end of the sequences shown represent the 3′ end of the upstream segment (or body) of the IRES as amplified by PCR. The start of the NS reading frame (...GAUCCC...) is italicized; sequences between the authentic initiation codon and the NS ORF are either authentic viral coding sequences (if shown in upper case), or synthetic linkers (lower case). The nucleotide numbering corresponds to the consensus genomic sequence in the case of poliovirus type 1; for EMCV the numbering system is that of Duke et al. (1992); for FMDV the numbering is simply that of the parent IRES-containing construct. The *Bam*H1 site in downstream EMCV sequences that was in-filled to detect initiation at AUG-10 is indicated.

In the case of EMCV the sequence at the start of the oligopyrimidine tract is actually UUUUCC, rather than UUUCC. Since the additional (first) U residue appears to be base paired with an upstream A residue (Pilipenko et al., 1989), it was retained in all constructs in which the body of the IRES was of EMCV origin.

These crossovers lead to six hybrid constructs: PV-EMCV and PV-FMDV, both containing the body of the PV IRES fused to downstream sequences of EMCV or FMDV; FMDV-EMCV and FMDV-PV, containing the body of the FMDV IRES and downstream sequences of EMCV or PV; and EMCV-PV and EMCV-FMDV, containing the EMCV IRES and downstream sequences of PV or FMDV (Fig. 1B). All these constructs were transcribed in vitro and the resulting RNA was translated in the rabbit reticulocyte lysate system. To allow a direct comparison, they all encode the NS protein from influenza virus as the IRES-dependent reporter cistron, which was in-frame with the authentic viral initiation codon (the downstream Lb initiation site in the case of FMDV). Very small differences in the apparent size of the NS product translated from different constructs are explained by small differences in the length of the linker sequences inserted between the authentic picornaviral initiation codon and the NS reading frame (Fig. 1B).

Translation characteristics of constructs with the PV IRES upstream element

The first series of chimeric constructs studied were those with the body of the poliovirus type 1 (Mahoney) IRES fused to the downstream sequences of EMCV strain R or FMDV strain O1K. Figure 2A shows the translation products resulting from the PV-EMCV and the PV-FMDV clones. As a control, the dicistronic RNA (XLPV) containing virtually the whole poliovirus 5′-UTR (nt 18–745) as the intercistronic spacer and encoding cyclin B2 (first cistron) and NS (second cistron) was trans-

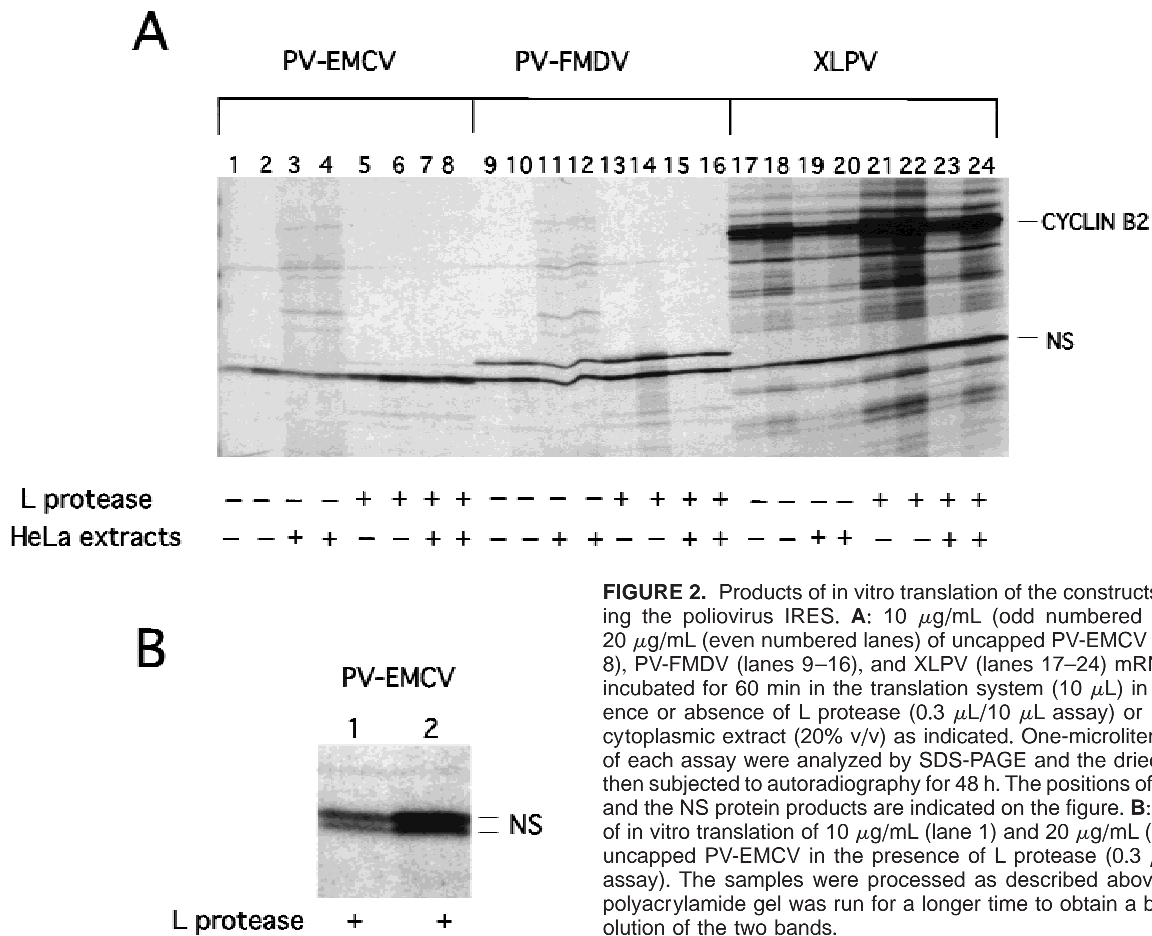


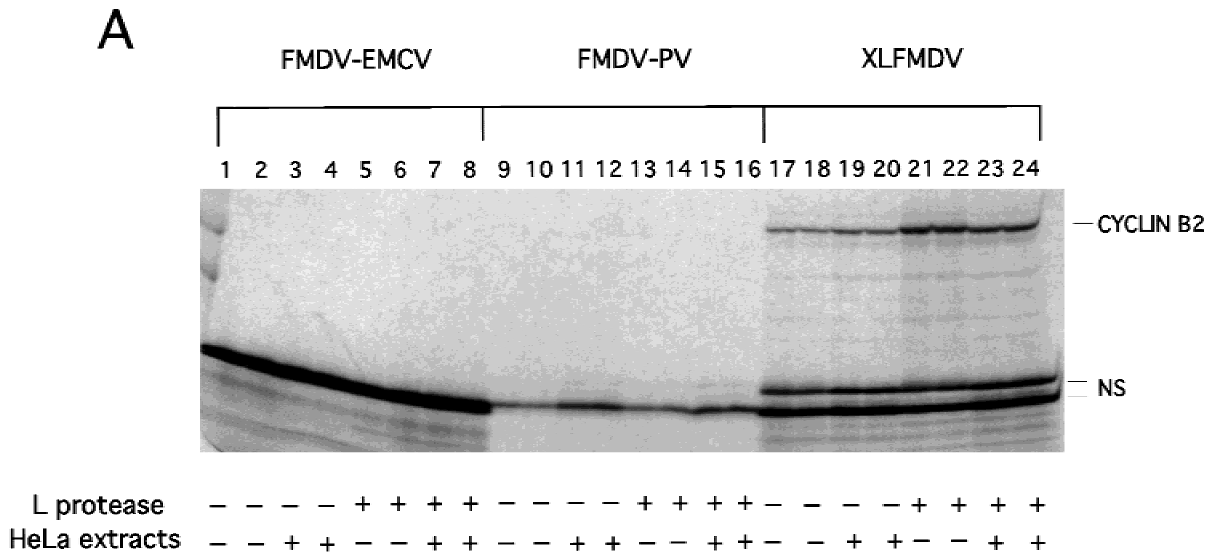
FIGURE 2. Products of in vitro translation of the constructs containing the poliovirus IRES. **A**: 10 μg/mL (odd numbered lanes) or 20 μg/mL (even numbered lanes) of uncapped PV-EMCV (lanes 1–8), PV-FMDV (lanes 9–16), and XLPV (lanes 17–24) mRNAs were incubated for 60 min in the translation system (10 μL) in the presence or absence of L protease (0.3 μL/10 μL assay) or HeLa cell cytoplasmic extract (20% v/v) as indicated. One-microliter samples of each assay were analyzed by SDS-PAGE and the dried gel was then subjected to autoradiography for 48 h. The positions of cyclin B2 and the NS protein products are indicated on the figure. **B**: Products of in vitro translation of 10 μg/mL (lane 1) and 20 μg/mL (lane 2) of uncapped PV-EMCV in the presence of L protease (0.3 μL/10 μL assay). The samples were processed as described above but the polyacrylamide gel was run for a longer time to obtain a better resolution of the two bands.

lated in parallel. The translation of the PV-EMCV construct showed that two forms of the NS protein were made, suggesting that both AUG-11 (nt 834) and AUG-12 (nt 846) of the EMCV ribosome landing site are utilized to initiate protein synthesis (see Fig. 2A, lanes 1–8). Figure 2B shows the samples of an identical experiment that were run longer on a gel to obtain a better resolution of the two bands. Clearly there is only a marginal preference for initiation at AUG-11 rather than AUG-12. This dual initiation site utilization is rather unexpected considering the good context of AUG-11, which should not be bypassed by scanning ribosomes. Indeed, with a wild-type EMCV IRES, there is really quite strong preference for initiation at AUG-11 rather than AUG-12 (see Fig. 4C).

The PV-FMDV clone was also translated in the reticulocyte lysate under the same conditions, and the results are shown in Figure 2A (lanes 9–16). As suggested by the presence of two forms of the NS protein, initiation occurred at both the Lab initiation codon and the Lb initiation site. The second AUG codon was preferentially selected as the initiation site, as is the case with wild-type FMDV IRES (Fig. 3, lanes 17–24), in agreement with the results of Sangar et al. (1987).

As described previously (Borman et al., 1995; Ohlmann et al., 1995; Ziegler et al., 1995b), supplementation of the lysate with in vitro-expressed FMDV L protease quite strongly stimulated translation of both cistrons of the uncapped dicistronic mRNA transcribed from the poliovirus construct XLPV (Fig. 2A, compare lanes 17–20 with lanes 21–24). The same treatment resulted in the stimulation of translation of both the PV-EMCV (Fig. 2A, compare lanes 1–4 with 5–8) and the PV-FMDV mRNAs (Fig. 2A, compare lanes 9–12 and 13–16). These results with chimeric constructs suggest that the stimulation of translation effected by the cleavage of eIF4G is mediated via the body of the poliovirus IRES upstream of the oligopyrimidine tract, rather than via sequences downstream of it.

The addition of 20% (v/v) cytoplasmic HeLa cell extract stimulated the translation of the IRES-dependent cistron of XLPV (Fig. 2A, compare lanes 17, 18 with 19, 20), and this effect was additive with cleavage of eIF4G (Fig. 2A, compare lanes 21, 22 with 23, 24). However, we were surprised to find that addition of the same HeLa extract did not have any marked effect on the translation of either PV-EMCV (Fig. 2A, lanes 3, 4, 7, and 8) or PV-FMDV (Fig. 2A, lanes 11, 12, 15, and 16). It is true that as judged by the reduced yield of cyclin translated from the upstream cistron of XLPV RNA (Fig. 2A, compare lanes 17, 18 with 19, 20), the HeLa cell extract had some nonspecific inhibitory effect on



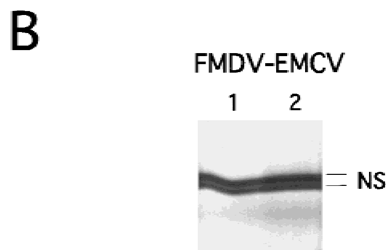


FIGURE 3. Products of in vitro translation of the constructs containing the FMDV IRES. 10 μg/mL (odd numbered lanes) or 20 μg/mL (even numbered lanes) of uncapped FMDV-EMCV (lanes 1–8), FMDV-PV (lanes 9–16), and XLFMDV (lanes 17–24) mRNAs were incubated for 60 min in the translation assay system (10 μL) in the presence or absence of L protease (0.3 μL/10 μL assay) or HeLa cell cytoplasmic extract (20% v/v) as indicated. One-microliter samples of each assay were analyzed by SDS-PAGE and the dried gel was then subjected to autoradiography for 12 h. The positions of the cyclin B2 and NS protein products are indicated. **B**: Products of in vitro translation of 10 μg/mL (lane 1) and 20 μg/mL (lane 2) of uncapped FMDV-EMCV. The samples were processed as described above but the polyacrylamide gel was run for a longer time to obtain a better resolution of the two bands.

translation that might have masked any specific stimulation of IRES-dependent translation, but the fact is that throughout this work stimulation was consistently seen only with the dicistronic XLPV mRNA. It may be noted that supplementation of the assay with HeLa cell extracts resulted in the appearance of some faint bands representing larger translation products (visible in Fig. 2A, lanes 3, 4, 11, and 12). Disappearance of these bands after treatment with L protease (Fig. 2A, lanes 7, 8 and 15, 16), which inhibits the translation of capped mRNAs because it results in cleavage of initiation factor eIF4G (Devaney et al., 1988), suggests that they were the result of translation of some residual endogenous mRNAs present in the HeLa cell extract.

Translation characteristics of constructs with the FMDV IRES upstream element

The second series of constructs had the downstream sequences of PV and EMCV fused to the body of the FMDV IRES (Fig. 1B). Figure 3A shows the pattern of translation products of these constructs compared to the dicistronic XLFMDV mRNA. As was observed for the PV-EMCV clone, the FMDV-EMCV construct also utilized both AUG-11 and AUG-12 to initiate the synthesis of the NS protein, with little discrimination between the two sites (Fig. 3A, lanes 1–8). Figure 3B (lanes 1 and 2) shows the samples of an identical experiment that were run longer on a gel to obtain a better resolution of the two bands. Once again, this result contrasts with wild-type EMCV where AUG-11 is the main initiation site (Fig. 4C).

Figure 3A (lanes 9–16) shows the products obtained on translation of the FMDV-PV mRNA. It should be noted that ^{586}AUG was put in-frame with ^{743}AUG in the downstream poliovirus segment to detect any initiation events that occurred at ^{586}AUG (see Materials and Methods). As seen in the figure, only the NS product resulting from initiation at ^{743}AUG was visible after a 12-h exposure of the autoradiograph. However, a longer exposure (3 days) revealed the presence of two very faint bands migrating more slowly than the main NS product (data not shown); the origin of these products will be discussed later. It also appears that the overall translation efficiency of the FMDV-PV clone is significantly decreased compared to the other constructs containing the FMDV IRES body (Fig. 3A, compare lanes 9–16 with 1–8 and 17–24).

Addition of L protease did not show any noticeable effects on IRES-dependent translation of any of these constructs, although the customary stimulation (Ohlmann et al., 1995) of translation of the uncapped up-

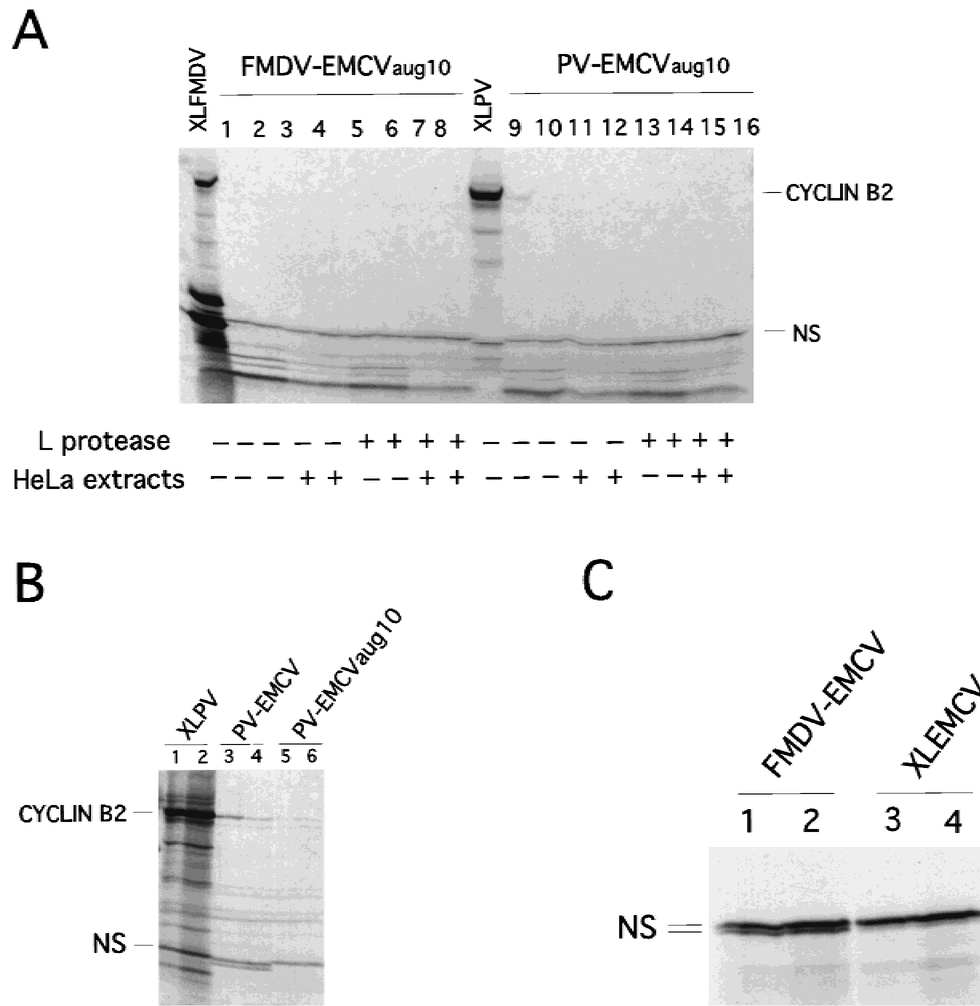


FIGURE 4. In vitro translation products resulting from initiation at AUG-10 of the constructs containing the EMCV downstream element. Uncapped FMDV-EMCVaug10 (lanes 1–8) and PV-EMCVaug10 (lanes 9–16) mRNAs were incubated at 10 μg/mL (odd numbered lanes) or 20 μg/mL (even numbered lanes) in the translation assay for 60 min in the presence or absence of L protease (0.3 μL/10 μL assay) or HeLa cell cytoplasmic extract (20% v/v) as indicated. The uncapped dicistronic XLFMDV and XLPV mRNAs were also translated under the same conditions at 10 μg/mL. One-microliter samples of each assay were analyzed by SDS-PAGE, and the dried gel was then subjected to autoradiography for 12 h. The positions of cyclin B2 and the NS protein products are indicated. Note that the NS protein arising from initiation at AUG-10 is slightly larger, and therefore runs more slowly on SDS-PAGE, than that initiated at AUG-11 (Kaminski et al., 1990). **B**: Uncapped XLPV (lanes 1, 2), PV-EMCV (lanes 3, 4), and PV-EMCVaug10 (lanes 5, 6) mRNAs were incubated at 10 μg/mL (odd numbered lanes) or 20 μg/mL (even numbered lanes) in the translation assay system for 60 min. The samples were processed as described above. **C**: Products of in vitro translation of 10 μg/mL (odd numbered lanes) and 20 μg/mL (even numbered lanes) of uncapped FMDV-EMCV mRNA (lanes 1, 2) and XLEMCV (lanes 3, 4). The assays and the sample processing were carried out as described above, but the polyacrylamide gel was run for a longer time to obtain a better resolution of the two bands.

stream cyclin cistron of XLFMDV mRNA was observed (Fig. 3A, lanes 21–24). Supplementation of this particular assay with 20% HeLa cell extracts resulted in a slight enhancement of translation of FMDV-PV (Fig. 3A, lanes 11, 12 and 15, 16), though this was not observed consistently.

Discrimination between AUG codons at the EMCV ribosome entry site

The utilization of EMCV AUG-12 as a functional initiation codon in the context of both of the chimeric constructs PV-EMCV and FMDV-EMCV raises the question of whether other AUGs present in the EMCV downstream segment also served as initiation sites. The sequence downstream of the oligopyrimidine tract of EMCV strain R has three closely spaced AUG codons: AUG-10 (nt 826), AUG-11 (nt 834), which is the authentic initiation site, and AUG-12 (see Fig. 1B). However, potential initiation at AUG-10 could not be monitored in Figures 2 and 3 because this AUG is not in frame with both AUG-11 and AUG-12. Therefore, we made a 4-nt insertion to put AUG-10 in frame with the downstream coding sequence of NS, which puts both

AUG-11 and AUG-12 out of frame (constructs PV-EMCVaug10 and FMDV-EMCVaug10 described in Materials and Methods). Figure 4A (lanes 1–8) shows the synthesis of the NS protein resulting from initiation at AUG-10 with the FMDV-EMCV construct compared with the translation products of the dicistronic XLFMDV mRNA. If these results are compared with those shown in Figure 3A, it is quite clear that AUG-10 is used by only a minute proportion of the initiating ribosomes. This is quite similar to what happens in the case of the wild-type EMCV IRES when less that 1% of the total initiation events occur at AUG-10 (Kaminski et al., 1990, 1994). However, when the same experiment was carried out with PV-EMCV, the yield of NS translated from AUG-10 of PV-EMCVaug10 mRNA (Fig. 4A, lanes 9–16) seemed of a similar order of magnitude as that translated from the IRES-dependent cistron of the dicistronic mRNA XLPV, and if these results are compared with those of Figure 2, the implication is that AUG-10 of PV-EMCV must be used at quite high frequency. This is confirmed in Figure 4B, which shows a direct comparison of the translation of PV-EMCV mRNA (initiation at AUG-11 and AUG-12), with PV-EMCVaug10 mRNA (initiation at AUG-10). It appears that all three AUG codons are used at similar (and low) efficiency, with just a marginal preference for AUG-11 over the other two, consistent with the slight preference for AUG-11 over AUG-12 seen in Figure 2B.

Figure 4C shows a direct comparison of the utilization of AUG-11 and AUG-12 as initiation sites in FMDV-EMCV mRNA, and XLEMCV mRNA. It is clear that with the wild-type EMCV IRES, there is quite strong preference for initiation at AUG-11 rather than AUG-12. Although the degree of preference is dependent on the particular batch of lysate, there always is a distinct preference and it is independent of whether mono- or dicistronic mRNAs are studied (data not shown). In contrast, in the same batch of lysate, there was almost no discrimination between the two AUGs in FMDV-EMCV mRNA (Figs. 4C and 3B), or PV-EMCV mRNA (Fig. 2B).

Translation characteristics of constructs with the EMCV IRES upstream element

Finally, the third series of chimeric constructs had the body of the IRES of EMCV strain R fused to the downstream sequences of PV or FMDV. Figure 5 shows the translation products of these mRNAs compared to the products of translation of the dicistronic XLEMCV mRNA, and reveals a number of surprises. As observed for the PV-FMDV and the XLFMDV clones, the synthesis of NS from the EMCV-FMDV mRNA was initiated at both the Lab and Lb initiation sites (Fig. 5, lanes 9–16). However, in this case it appears that the first AUG is preferentially chosen by the initiating ribosomes, in contrast to the preference for the downstream Lb initiation site of PV-FMDV or XLFMDV mRNAs (Figs. 2A and 3A, respectively). For all the constructs containing the EMCV IRES, addition of L protease did not show any effects, and supplementation of the lysate with HeLa cell extracts was, if anything, slightly inhibitory.

The analysis of the products of translation of EMCV-PV mRNA showed a very unexpected feature: the presence of a doublet of labeled products migrating more slowly than the main NS protein product (Fig. 5, lanes 1–8). A similar doublet, albeit in lower relative yield, was seen on prolonged exposure of the analysis of the FMDV-PV mRNA translation products shown in Figure 3. The upper band is very likely to arise from

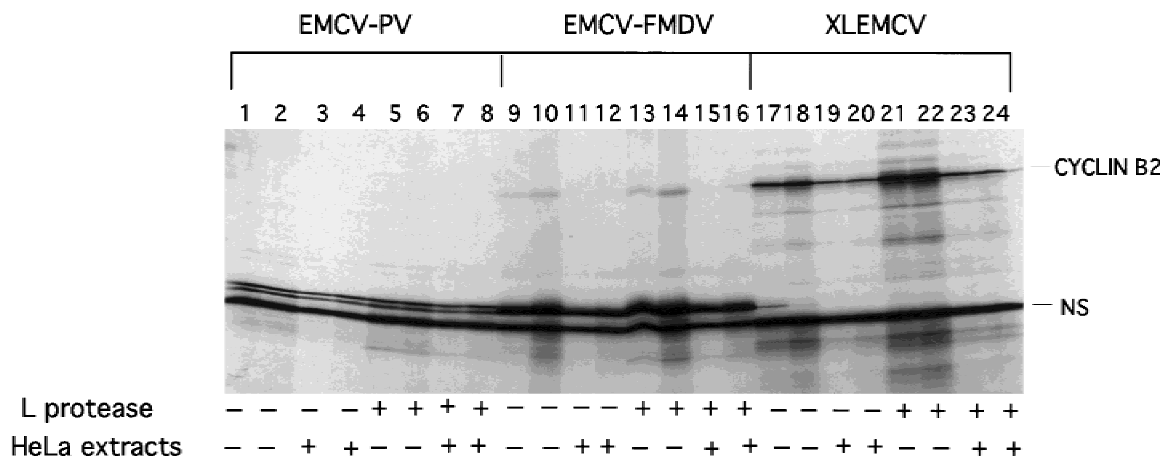


FIGURE 5. Products of in vitro translation of the constructs containing the EMCV IRES. 10 μg/mL (odd numbered lanes) or 20 μg/mL (even numbered lanes) of uncapped EMCV-PV (lanes 1–8), EMCV-FMDV (lanes 9–16), and XLEMCV (lanes 17–24) mRNAs were incubated for 60 min in the translation assay system (10 μL) in the presence or absence of L protease (0.3 μL/10 μL assay) or HeLa cell cytoplasmic extract (20% v/v) as indicated. One-microliter samples of each assay were analyzed by SDS-PAGE, and the dried gel was then subjected to autoradiography for 12 h. The positions of cyclin B2 and NS protein products are indicated.

initiation at ^{586}AUG that had been deliberately put in-frame with ^{743}AUG (see Materials and Methods). However, the nature and origin of the product of intermediate size remains unclear, as there are no AUG codons between ^{586}AUG and ^{743}AUG in the PV sequence.

Initiation at ^{586}AUG in the poliovirus IRES

The synthesis of these two larger products prompted us to investigate whether or not this was a feature peculiar to the chimeric constructs, or if it could also be evidenced in the context of the wild-type poliovirus IRES. Therefore ^{586}AUG and ^{743}AUG of poliovirus type 1 were put in frame, and the resulting monocistronic clone (PVif) was then transcribed and translated under the same conditions as above. Figure 6 shows the translation products obtained with the PVif and the dicistronic XLPV mRNAs. (Note that ^{586}AUG is in-frame with ^{743}AUG in PVif, but not in XLPV.) As with EMCV-PV mRNA, in the case of PVif, we observed a doublet migrating more slowly than the main NS product. However, because of the lower overall translation efficiency of the poliovirus IRES, the intensity of these bands, and of the major NS product translated from ^{743}AUG of PVif, is much less than that observed with the EMCV-PV construct. Nevertheless, this result indicates that the segment of poliovirus mRNA downstream the oligopyrimidine tract contains at least two potential initiation sites (in addition to the authentic one at nt 743) which can be utilized to start protein synthesis.

Although it has been previously claimed that ^{586}AUG is not utilized as an initiation codon (Dorner et al., 1982), our results tend to suggest the contrary. To verify whether or not ^{586}AUG was serving as an initiation site in PVif and EMCV-PV mRNAs, we decided to change its surrounding context into **ACC**AUG**G** in both of these construct backgrounds. As shown in Figure 7A (lanes 9–16), this mutation resulted in an increase in the yield of the larger product translated from PVifaug586 mRNA, confirming that it was indeed the result of initiation at ^{586}AUG. Perhaps as an indirect result of the improved context (Kozak, 1986), the yield of the intermediate-sized product was much reduced, to the point of being almost undetectable. However, and in agreement with previously published results, the improvement of the context of the ^{586}AUG did not prevent ribosomes initiating at ^{743}AUG (Pestova et al., 1994). Addition of L protease to the translation mixture resulted in an overall increase of the translation efficiency that was due to a higher initiation frequency at both ^{586}AUG and ^{743}AUG (Fig. 7A, compare lanes 9–12 with lanes 13–16).

To investigate this effect further, we have also improved the surrounding context of ^{586}AUG in the EMCV-PV chimeric construct. Figure 7B shows that this mutation had the remarkable effect of promoting a massive increase in the yield of product initiated at ^{586}AUG

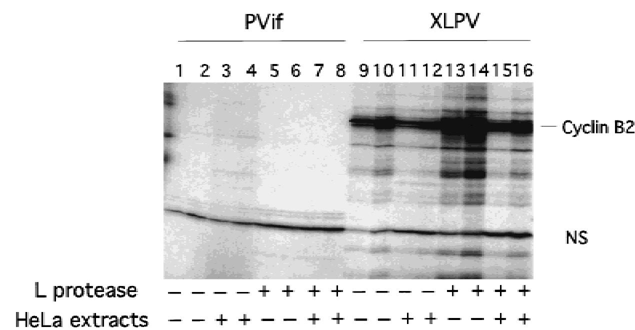


FIGURE 6. Poliovirus type I 5′-UTR has two upstream sites that are utilized for translation initiation. 10 μg/mL (odd numbered lanes) or 20 μg/mL (even numbered lanes) of uncapped PVif (lanes 1–8) and XLPV (lanes 9–16) mRNAs were incubated for 60 min in the translation assay system (10 μL) in the presence or absence of L protease (0.3 μL/10 μL assay) or HeLa cell cytoplasmic extract (20% v/v) as indicated. One-microliter samples of each assay were analyzed by SDS-PAGE, and the dried gel was then subjected to autoradiography for 48 h. The positions of cyclin B2 and NS protein products are indicated.

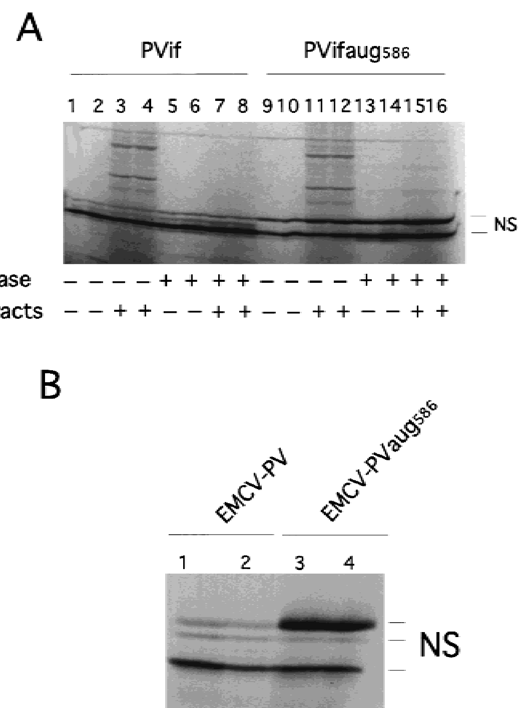


FIGURE 7. The poliovirus type I IRES can promote initiation of protein synthesis at ^{586}AUG in vitro. **A**: 10 μg/mL (odd numbered lanes) or 20 μg/mL (even numbered lanes) of uncapped PVif (lanes 1–8) and PVifaug$_{586}$ (lanes 9–16) mRNAs were incubated for 60 min in the translation assay system (10 μL) in the presence or absence of L protease (0.3 μL/10 μL assay) or HeLa cell cytoplasmic extract (20% v/v) as indicated. One-microliter samples of each assay were analyzed by SDS-PAGE. The dried gel was then subjected to autoradiography for 48 h. The positions of cyclin B2 and NS protein products are indicated. **B**: 10 μg/mL (odd numbered lanes) or 20 μg/mL (even numbered lanes) of uncapped EMCV-PV (lanes 1, 2) and EMCV-PVaug586 (lanes 3, 4) mRNAs were incubated for 60 min in the translation assay system (10 μL). The samples were processed as described above and the gel subjected to autoradiography for 12 h.

(compare lanes 1, 2 with 3, 4), and a large increase in overall translation product yield. In this particular case (in contrast to PVifaug586), the product of intermediate size was still detectable, though slightly reduced in absolute yield, and considerably reduced in relative yield. Moreover, the increase in yield of product initiated at ^{586}AUG did not result in a corresponding decrease in the yield of NS initiated at ^{743}AUG (Fig. 7B).

Identification of ^{643}GUG as a functional initiation codon

The next step was to investigate the provenance of the intermediate band seen upon translation of both PVif and EMCV-PV (Figs. 5–7). As there are no AUG codons between ^{586}AUG and ^{743}AUG in poliovirus type 1, and indeed they have never been found in any isolates of poliovirus (Pöyry et al., 1992), we looked for non-AUG codons that could serve as potential initiation sites. Among the several GUG and CUG codons contained in this 154-nt segment, two of them, ^{637}GUG and ^{643}GUG, seemed to be good candidates (see Fig. 8A), given the apparent size of the intermediate translation product. In PVif and EMCV-PV they are both in the same reading frame as ^{586}AUG, ^{743}AUG, and the NS coding region. Therefore, using site-directed mutagenesis, we have changed these GUG codons into GUA. Three mutants of the monocistronic PVif clone were generated in which (1) ^{637}GUG was changed into GUA, (2) ^{643}GUG was changed into GUA, and (3) both ^{637}GUG and ^{643}GUG were changed to GUA. The result of the translation of these constructs is shown in Figure 8B. Mutation of ^{637}GUG into GUA had no effect whatsoever; we obtained a similar pattern of translation products as with the control PVif mRNA (Fig. 8B, compare lanes 1–4 with lanes 5–8). Mutation of the second GUG (^{643}GUG) into GUA resulted in a decrease in the intensity of the intermediate-sized band (Fig. 8B, lanes 9–12) suggesting that this GUG was used as an initiator codon. Finally, mutation of both GUGs into GUA resulted in the almost, but not quite complete abrogation of the intermediate-sized product. Taking together these results suggest that the second GUG (^{643}GUG) is used as a functional initiation codon in poliovirus type 1 in vitro, albeit at low efficiency. However, the finding that mutation of this GUG did not completely abrogate initiation at this site suggests that either (1) the mutated GUA could also serve to initiate protein synthesis but at even lower efficiency, or (2) in the absence of ^{643}GUG, some of the ribosomes can initiate at the upstream GUG (^{637}GUG). However, the latter explanation does not account for the fact that even when both GUGs were mutated, the intermediate-sized product was still detected, albeit in extremely low

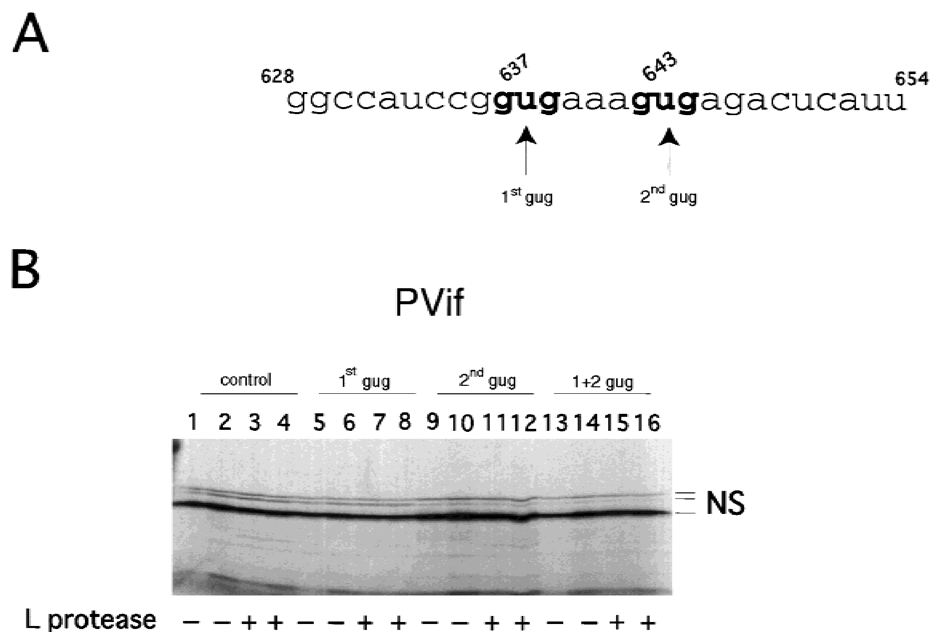


FIGURE 8. Poliovirus type 1 RNA has an upstream reading frame initiated at ^{643}GUG. **A**: The segment of the poliovirus type 1 (Mahoney) sequence containing two GUG codons that could be potentially used to initiate protein synthesis. **B**: Effect on in vitro translation of mutating either one or both of these GUG codons. 10 μg/mL (odd numbered lanes) or 20 μg/mL (even numbered lanes) of uncapped PVif (lanes 1–4), uncapped PVif in which the first GUG (at nt 637) has been mutated to GUA (lanes 5–8), uncapped PVif in which the second GUG (at nt 643) has been mutated to GUA (lanes 9–12), and uncapped PVif in which both GUGs have been mutated to GUA (lanes 13–16) were incubated for 60 min in the translation assay system (10 μL) in the presence (lanes 3, 4, 7, 8, 11, 12, 15, and 16) or the absence (lanes 1, 2, 5, 6, 9, 10, 13, and 14) of L protease (0.3 μL/10 μL assay). One-microliter samples of each assay were analyzed by SDS-PAGE and the dried gel was then subjected to autoradiography for 6 days. The positions of the NS-related products are indicated in the figure.

yield (Fig. 8B, lanes 13–16). Therefore the possibility of a very low frequency of initiation at a GUA codon needs to be considered.

DISCUSSION

All picornavirus IRESes end with a 3′-terminal AUG triplet located ~25 nt downstream of the start of the oligopyrimidine tract, and this AUG is considered to be the actual ribosome entry site (reviewed by Ehrenfeld & Semler, 1995; Hellen & Wimmer, 1995; Jackson & Kaminski, 1995). Where the various picornavirus IRESes differ is in what happens following internal ribosome entry at this site: whether it will be used as the primary translation initiation site, or whether the majority of ribosomes will initiate translation elsewhere. They also differ in other respects: (1) their efficiency in various systems, (2) whether their activity is increased as a result of cleavage of eIF4G, and (3) whether their activity in rabbit reticulocyte lysates is stimulated by supplementation with HeLa cell cytoplasmic extracts. Although the primary aim of this work was to use chimeric IRESes to determine whether the behavior of the ribosome on internal entry is dictated by the nature of the upstream IRES body or by the sequences at the actual entry site, we were also interested in which of these two parts influenced the other properties in which picornavirus IRESes differ.

With regard to efficiency, both in unsupplemented rabbit reticulocyte lysate and also in transfection assays of cultured cells, the hierarchy is generally agreed to be: rhinovirus ≪ enterovirus (e.g. poliovirus) ≪ cardiovirus < aphthovirus IRESes (Borman et al., 1995, 1997b; Roberts et al., 1998). The substitution of heterologous downstream sequences in the chimeric IRESes described here did not, in general, change the efficiency of internal initiation, which must therefore be largely governed by the nature of the upstream sequences, the body of the IRES. What is perhaps most significant is that, apart from one exception, none of the chimeric IRESes were unexpectedly inefficient. This argues that the two parts of the IRES are functionally independent: the upstream IRES segment and the downstream entry site region are not matched to each other in a functionally dedicated manner. The exception was FMDV-PV mRNA, which was distinctly less efficient than FMDV-EMCV or XLFMDV mRNAs (Fig. 3A).

Stimulation of IRES activity by supplementation of in vitro assays with FMDV L protease is seen only with rhino- and enterovirus IRESes (with a very small effect on cardiovirus IRESes), and there is good evidence that stimulation is due to cleavage of eIF4G (Borman et al., 1995, 1997a). Our results unambiguously show that the stimulation of poliovirus IRES activity by eIF4G cleavage is mediated via the body of the IRES, and not via the sequences at the putative ribosome entry site, as neither FMDV-PV mRNA (Fig. 3A) nor EMCV-PV (Fig. 5) showed a significant response to addition of L protease. We did not obtain sufficiently consistent results in assays supplemented with HeLa cell extracts to be able to draw any conclusions as to whether the stimulation of poliovirus IRES activity by such extracts is mediated via the body of the IRES or via the sequences at the actual ribosome entry site.

The main aim, however, of this work was to investigate whether the properties of chimeric IRESes would throw light on what determines the behavior of the ribosome following entry at the AUG triplet at the 3′ end of the IRES. Hitherto, the context of the AUG codon has been invoked as the primary explanation (Belsham, 1992), and, in addition, other data suggest that the distance between the pyrimidine tract and the AUG may play a secondary role (Kaminski et al., 1994). The majority of internal initiation events on the EMCV IRES occur at this AUG codon (AUG-11), which does indeed have a good context (AAU**AUG**GCC) by the recognized criteria (Kozak, 1986). There is no significant use of the upstream AUG-10, probably because it is too close to the oligopyrimidine tract, and only a low frequency of initiation at AUG-12 (Fig. 4C; Kaminski et al., 1990, 1994), which is located 12 nt downstream of AUG-11 and is presumably disfavored (notwithstanding its good context) because the majority of ribosomes are captured by AUG-11. Limited use of a (second) downstream site (equivalent to AUG-12) is also seen in another cardiovirus, Theiler's murine encephalomyelitis virus (Kong & Roos, 1991).

In the case of FMDV, about one-third of the internal initiation events occur at the Lab initiation site at the 3′ end of the IRES, and the rest at the next AUG codon downstream, the Lb site (Sangar et al., 1987; Belsham, 1992), which correlates with a poor context at the Lab initiation site (CAC**AUG**AAU in the O1K strain). However, the equivalent AUG in rhino- and enterovirus IRESes (^{586}AUG in PV type 1) has a context that appears no worse (CUU**AUG**GUG), yet this AUG is considered to be either completely silent, or almost so, and virtually all initiation is at the next AUG codon downstream. The upstream contexts of the FMDV and PV sites seem equally poor, and the G at the +4 position in the PV case should make this more favored than the FMDV Lab site (Kozak, 1986). It is only by invoking some somewhat contradictory data on the influence of the +5 position that the FMDV site is in any way better than that of PV: it has been claimed that a U in the +5 position (as in PV) has a negative influence (Kozak, 1997), whereas others have suggested that an A in this position (as in FMDV) is favorable (Boeck & Kolakofsky, 1994; Grünert & Jackson, 1994). Thus although context considerations can explain the almost exclusive use of AUG-11 in the EMCV IRES, context alone does not readily explain the large difference between the utilization of the FMDV Lab and PV ^{586}AUG sites, particularly as the shorter spacing between the oligo-

pyrimidine tract and the AUG in FMDV (Table 1) should mitigate against the use of the Lab site.

On the other hand context clearly does have some relevance, as shown by the highly increased initiation frequency at ^{586}AUG when its context was improved in PVifaug586 and EMCV-PVaug586 (Fig. 7). However, it is interesting to note that improving the context of the FMDV Lab initiation site (and at the same time increasing its distance from the oligopyrimidine tract by four residues) had only a very small influence on the frequency of initiation at this site, and the ribosomes still preferentially used the next AUG further downstream (Piccone et al., 1995).

The properties of the chimeric IRESes, as reported here, provoke the entirely novel suggestion that quite apart from any context effects, the identity of the upstream IRES body has an important influence on events at the putative ribosome entry site AUG. In particular the EMCV IRES seems to be especially efficient in delivering initiation-competent ribosomes accurately to the AUG located at the appropriate distance downstream of the oligopyrimidine tract. In EMCV-PVaug586, the majority of initiation events were at ^{586}AUG of the poliovirus entry site, whereas in PVifaug586 only about half the initiation events occurred at this site. Moreover, although in FMDV itself (XLFMDV) or PV-FMDV about one-third of initiation events occur at the Lab site at the 3′ end of the IRES and the other two-thirds at the downstream Lb site (Figs. 2A and 3A), in EMCV-FMDV this ratio was reversed with two-thirds of initiation events occurring at the Lab site (Fig. 5), notwithstanding the fact that the spacing between the oligopyrimidine tract and the Lab AUG is exactly the same in EMCV-FMDV as in FMDV itself (Table 1).

In contrast to the almost exclusive use of AUG-11 in EMCV itself, in an FMDV-EMCV chimeric IRES, in which the spacing between the oligopyrimidine tract and the AUG is exactly the same as in EMCV itself (Table 1), AUG-11 and AUG-12 were used with approximately equal frequency, although admittedly initiation at AUG-10 was still negligible (Figs. 4A and 4C). However, in the case of PV-EMCV there was almost equal utilization of all three AUG codons, and although in this case the distance or spacing is greater than in EMCV itself (Table 1), it should be noted that such a high relative frequency of initiation at AUG-10 was not seen when the spacing in the actual EMCV IRES was increased (Kaminski et al., 1994). These comparisons further support the notion that the body of the EMCV IRES delivers ribosomes exceptionally precisely and efficiently to the correct initiation codon, but the body of the FMDV IRES is significantly less precise, while that of PV is arguably even more imprecise.

These results are also pertinent to the question of how ribosomes access the authentic initiation site of PV and FMDV. Hitherto the favored straightforward model has been that all ribosomes enter at ^{586}AUG of PV, yet none of them initiate at this site, and all of them are transferred by scanning to ^{743}AUG; and that all ribosomes which initiate translation at the FMDV Lb AUG accessed this site by first binding at the upstream Lab AUG. This model has arisen largely by default, in an attempt to unify the mechanisms of internal initiation on rhino-, entero-, and aphthovirus IRESes with that of the cardiovirus IRES, which is much better understood. The problem with interpreting the data on the other viral IRESes is that we have no direct means of knowing the frequency and site of ribosome entry, only the frequency and site of actual translation initiation. Moreover, this and other models proposed so far have not allowed for the possibility that there may be more than one route by which ribosomes access the authentic initiation site of PV at nt 743. If, indeed, internal ribosome entry directed by the PV and FMDV IRESes is somewhat imprecise, some ribosomes may enter at non-AUG codons downstream of ^{586}AUG or the Lab AUG respectively.

Another possibility not previously considered is that despite the lack of any AUG triplets in any enterovirus genome in the segment equivalent to nt 589–742 of poliovirus type 1 (Pöyry et al., 1992), there might be some low frequency initiation at non-AUG triplets in this region; yet our results showing some initiation at ^{643}GUG suggest that this can happen. Initiation at this site was not seen in similar experiments conducted by Pestova et al. (1994), perhaps because of differences in the sensitivity of detection of such events: as ^{643}GUG was in-frame with the reporter cistron in our studies, a product initiated at this site would have 10 radiolabeled methionine residues, but in the experiments of Pestova et al. (1994) it would have had only a single labeled cysteine residue and perhaps the initiating methionine. (The detection of a low frequency of initiation at ^{643}GUG raises the further question, not investigated here, of whether there is also initiation at non-AUG triplets in the other two reading frames within the window nt 589–742.)

The most compelling evidence (indeed the only compelling evidence) for ribosome entry uniquely at ^{586}AUG

TABLE 1. Spacing between the oligopyrimidine tract and the AUG codon in the chimeric constructs compared with the parent viral genomes.

Upstream sequences	Downstream sequences		
	PV	FMDV	EMCV
PV	**22**	25	27
FMDV	22	**19**	21
EMCV	22	19	**21**

Spacing is defined as the number of residues *between* the end of the conserved (U)UUCC motif at the 5′ end of the oligopyrimidine tract and the AUG codon. Spacing in the parent viral genomes is shown in bold.

of poliovirus type 1 is the results of Pilipenko et al. (1992), showing that an AUG situated ~25 nt downstream of the start of the oligopyrimidine tract is indispensable for infectivity, and for translation initiation at ^{743}AUG. On the other hand, point mutation of the equivalent AUG (at nt 588) in poliovirus type 2 conferred a small plaque phenotype rather than abrogating infectivity completely (Pelletier et al., 1988), and in the background of a heterologous reporter construct such mutations reduced IRES-dependent translation by 60–70% rather than causing complete loss of activity (Meerovitch et al., 1991; Nicholson et al., 1991). Moreover, in the case of FMDV, mutation of the upstream Lab initiation codon caused only a small reduction in infectivity, whereas mutation of the downstream Lb AUG was lethal (Cao et al., 1995). These results argue that an AUG located 25 nt downstream of the start of the oligopyrimidine tract may not be invariably or absolutely necessary for internal ribosome entry. This in turn raises the possibility that even in the wild-type PV and FMDV genomes some internal ribosome entry could occur at non-AUG codons downstream of this AUG (which had previously been considered to be the unique site for ribosome entry) as a result of imprecision in the delivery of the ribosome by the IRES.

Context changes at ^{586}AUG were introduced in an attempt to circumvent the uncertainties inherent in the fact that we have no direct means of knowing the frequency and site of actual ribosome entry, but only the frequency and site of initiation. In terms of the straightforward model, improving the context of ^{586}AUG would be expected to increase the frequency of initiation at this site, coupled with a compensatory decrease in the utilization of ^{743}AUG, but in fact such mutations always result in an increase in the overall (combined) initiation frequency at the two sites. This was first observed by Pestova et al. (1994), who studied the effect of the context change on translation in HeLa and Krebs 2 cell extracts. They observed a 50% decrease in use of ^{743}AUG coupled with a massive increase in initiation at ^{586}AUG (which appeared to be silent in the absence of the context change); the outcome was that initiation occurred at ^{586}AUG about 3.5-fold more frequently than at the downstream site, implying that the context change had doubled overall initiation at the two sites combined. In our hands, the same context change made in PVifaug586 likewise almost doubled the overall initiation frequency, which was again mainly due to a large increase in utilization of ^{586}AUG coupled with a small (~25%) decrease in initiation ^{743}AUG (Fig. 7A). In the background of EMCV-PV, improving the context of ^{586}AUG resulted in an even smaller reduction in initiation at ^{743}AUG, yet almost quadrupled the combined utilization of the two sites, because there was a massive increase in initiation at ^{586}AUG (Fig. 7B).

This large increase in initiation at the two sites combined rather frustrates the aim of using these context change experiments as a test of the straightforward model that all ribosome entry is normally at ^{586}AUG. Three different explanations for these results can be advanced, each with different underlying assumptions. One is to postulate that all ribosomes do indeed enter at ^{586}AUG, that the frequency of entry at this site is not altered as a result of the change in context, but that for those ribosomes that are subsequently transferred to ^{743}AUG, the transfer process is not 100% efficient. Within these terms, the data of Pestova et al. (1994) showing a 50% decrease in initiation frequency at ^{743}AUG coupled with a doubling in overall initiation at the two sites combined would imply a transfer efficiency of about 30%. However, our own data would imply an even lower transfer efficiency, because the context change in PVifaug586 or EMCV-PVaug586 caused a smaller decrease in initiation at ^{743}AUG (Fig. 7).

On the other hand, if we assume that any transfer to ^{743}AUG is 100% efficient yet still adhere to the assumption that ^{586}AUG is the unique site of ribosome entry, then the only way to explain why the context change causes an overall increase in initiation at the two sites combined is to invoke that the mutation has increased the frequency of actual ribosome entry at ^{586}AUG (i.e., the efficiency of the IRES has increased), quite apart from bringing about the expected increase in the proportion of entering ribosomes that initiate at this site. What is curious is that the outcome of these dual effects of the mutation (an increase in the frequency of ribosome entry coupled with a decrease in the proportion of these ribosomes that are transferred to ^{743}AUG) should so closely counterbalance each other, resulting in rather little change in the frequency of initiation at ^{743}AUG (Fig. 7). This “coincidence” does not necessarily invalidate the interpretation given above, but it does provoke consideration of yet other alternative explanations.

The data are actually compatible with a third model, which posits that in the wild-type IRES, a large proportion of the ribosomes that initiate at ^{743}AUG do not actually enter at ^{586}AUG, but rather at some point between nt 589 and 742; and that the behavior of this cohort of ribosomes is entirely uninfluenced by the context change at ^{586}AUG, which only has the consequence of committing most of the ribosomes that enter at that site to initiate there. Within the terms of this hypothesis, the data of Pestova et al. (1994) would imply that in the wild-type background, 50% of the ribosomes initiating at ^{743}AUG did not enter at ^{586}AUG, whereas our own results would put this figure in the 75–90% range. The idea that ribosomes that initiate at ^{743}AUG need not necessarily have accessed this site via entry at ^{586}AUG followed by a scanning process was first proposed by Hellen et al. (1994) on the grounds that point mutations generating AUG triplets in the window nt 589–742, or insertion mutations generating stem-loop structures in this region, caused a smaller decrease

in initiation at [743]AUG than would be expected. Given the results presented here showing that the body of the PV IRES is not particularly precise in delivering ribosomes to a specific site, and that context changes at [586]AUG have such a small influence on initiation at [743]AUG, and taking into consideration the published evidence that [586]AUG of PV or the Lab AUG of FMDV are not invariably or absolutely essential for initiation at the next AUG codon downstream (Meerovitch et al., 1991; Nicholson et al., 1991; Cao et al., 1995), this proposition of Hellen et al. (1994) perhaps deserves closer consideration than has been the case hitherto.

MATERIALS AND METHODS

Plasmid constructs

For all the constructs described in this study, the experimental approach consisted of making hybrid cDNA clones by using a PCR-based method. The starting materials of these clones were the dicistronic constructs pXLPV, pXLEMCV, and pXLFMDV(Lab), which have the cDNA for *Xenopus laevis* cyclin B2 followed by an IRES and then the influenza virus NS coding region and 3′ UTR, and contain, respectively, the poliovirus type 1 IRES, the EMCV strain R IRES, and the FMDV O1K IRES as the intercistronic spacers. All were cloned in pGEM2 (Promega) as vector, oriented such that linearization with *Eco*RI and transcription by T7 RNA polymerase would generate sense transcripts. The sequences at the junction between the viral IRES segment and the start of the NS ORF are shown in Figure 1B.

In the first stage, the sequences starting with the conserved CC dinucleotide motif near the 5′ end of the oligopyrimidine tract and extending up to the 3′ end of the NS reporter cDNA segment in each dicistronic construct were amplified by PCR and subcloned into pGEM2. The following oligodeoxynucleotides were used as forward primers:

pXLPV: 5′ GGG GCT GCA GTT TTA GG|C CTT TTA TTT TAT 3′
pXLEMCV: 5′ GGG GCT GCA GTT TTA GG|C CTT TGA AAA ACA CGA TGA 3′
pXLFMDV: 5′ GGG GCT GCA GTT TTA GG|C CTT TTA TAA CCA CTG AAC 3′.

For reverse primer, in all three cases an oligonucleotide complementary to the SP6 promoter was used. Three restriction sites were inserted by this PCR amplification, a *Pst*I and a *Stu*I site (the cut site is denoted by a vertical line in the sequences given above) at the very 5′ end and an *Eco*RI site at the 3′ end. This PCR fragment was subcloned between the *Pst*I and *Eco*RI sites of the pGEM2 vector creating, respectively, pPV-NS, pEMCV-NS, and pFMDV-NS. For the pPV-NS clone, [586]AUG was made in frame with the authentic initiation codon ([743]AUG) by cutting the cDNA at the *Mfe*I site (nt 730), removing the 4-nt residue overhangs using mung bean nuclease, and then religating.

For the construction of pPV-EMCV and pPV-FMDV, the cDNA fragment of the poliovirus IRES from nt 18 up to and including the start of the oligopyrimidine tract was amplified by PCR using the following oligonucleotides:

sense (forward) primer: 5′ GGG GCT GCA GCC CCT TGT ACC CAC CCC AGA 3′
antisense primer: 5′ CCC CTT TAA AAG GAA ACA CGG ACA CCC AAA 3′.

Two new restriction sites were introduced by this PCR, a *Pst*I site at the 5′ end and a *Dra*I site at the 3′ end. The amplified fragment was digested with *Pst*I and *Dra*I, whereas both pEMCV-NS and pFMDV-NS were digested with *Pst*I and *Stu*I. After gel purification, the PCR fragment was ligated between the *Pst*I and *Stu*I sites of both pEMCV-NS and pFMDV-NS vectors creating respectively pPV-EMCV and pPV-FMDV.

The construction of pEMCV-PV and pEMCV-FMDV was based on a similar strategy. The fragment of the EMCV 5′-UTR from nt 284 in the numbering system of Duke et al. (1992) up to and including the four consecutive T(U) residues at the start of the oligopyrimidine tract was amplified by PCR using the following oligonucleotides:

sense (forward) primer: 5′ GGG GCT GCA GCG TTA CTG GCC GAA GCC GC 3′
antisense primer: 5′ CCC CTT TAA AAC CAC GTC CCC GTG G 3′.

The amplified fragment was then digested with *Pst*I and *Dra*I, gel purified, and ligated into *Pst*I/*Stu*I cut pPV-NS and pFMDV-NS, creating pEMCV-PV and EMCV-FMDV.

For the construction of the pFMDV-EMCV and pFMDV-PV the strategy was similar except that a *Pme*I site was introduced instead of the *Dra*I site (since *Dra*I cuts within the FMDV IRES sequence). The FMDV IRES sequence up to and including the three T(U) residues at the start of the oligopyrimidine tract was amplified using the following oligonucleotides:

sense (forward) primer: 5′ GGG GCT GCA GCC GTG CAA CTT GAA ACT CCG CC 3′
antisense primer: 5′ CCC CGT TTA AAC GTG CCG GCC TCC GGT C 3′.

The PCR fragment was digested with *Pst*I and *Pme*I, and ligated into *Pst*I/*Stu*I cut pEMCV-NS and pPV-NS creating pFMDV-EMCV and pFMDV-PV.

For the construction of pFMDV-EMCVaug10 and pPV-EMCVaug10, pEMCV-NS plasmid was digested with *Bam*HI, the overhangs in-filled using Klenow fragment, and the plasmid religated to generate pEMCV-NSaug10. This was then cut with *Pst*I and *Stu*I, and then ligated with the cut and gel-purified PCR fragments of the FMDV and PV upstream IRES segments as described above, creating respectively pFMDV-EMCVaug10 and pPV-EMCVaug10. This four-residue in-fill puts AUG-10 in frame with the NS ORF (and takes AUG-11 and AUG-12 out of frame).

For the construction of pPVif, the PCR-amplified fragment of the upstream poliovirus IRES, from nt 18 up to and including the UUUCC motif, was amplified by PCR using the same sense primer as previously described above, and the following antisense primer:

5′ CCC CTT TAA ACA CGG ACA CCC AAA GTA GTC GG 3′.

The PCR fragment was then cut with *Pst*I and *Dra*I, gel purified, and ligated into *Pst*I/*Stu*I cut pPV-NS, which already has ^{586}AUG in-frame with ^{743}AUG.

The sequence of all constructs was verified by dideoxynucleotide sequencing, and all plasmids were propagated in *Escherichia coli* TG1 under ampicillin selection (Sambrook et al., 1989).

Site directed mutagenesis

The surrounding context of the ^{586}AUG in both pPVif and pEMCV-PV was changed from CTTATG into ACCATG by using the QuickChange Site directed mutagenesis kit (Stratagene). The oligonucleotides used were:

sense (forward) primer: 5′ ATT GTG GCT GAC CAT GGT GAC AAT CAC AGA 3′
antisense primer: 5′ TCT GTG ATT GTC ACC ATG GTC AGC CAC AAT 3′.

Using the same kit, both ^{637}GUG and ^{643}GUG in pPVif were changed into ^{637}GUA and ^{643}GUA using the following primers:

Mutation of the first GUG:
sense (forward) primer: 5′ GGC CAT CCG GTA AAA GTG AGA CTC ATT ATC 3′
antisense primer: 5′ GAT AAT GAG TCT CAC TTT TAC CGG ATG GCC 3′
Mutation of the second GUG:
sense (forward) primer: 5′ GGC CAT CCG GTG AAA GTA AGA CTC ATT ATC 3′
antisense primer: 5′ GAT AAT GAG TCT TAC TTT CAC CGG ATG GCC 3′
Mutation of both GUGs:
sense (forward) primer: 5′ GGC CAT CCG GTA AAA GTA AGA CTC ATT ATC 3′
antisense primer: 5′ GAT AAT GAG TCT TAC TTT TAC CGG ATC GCC 3′.

In vitro transcription and translation

All the plasmids utilized in this work were linearized with *Eco*RI, with the exception of pFMDV-EMCV, pFMDV-PV, and pXLFMDV which were linearized with *Pvu*I. Transcription reactions were carried out with bacteriophage T7 RNA polymerase under conditions to produce uncapped transcripts as described previously (Kaminski et al., 1990), with a low concentration of [^{35}S]UTPαS added to the reaction to allow the concentration of the RNA product to be estimated (Dasso & Jackson, 1989). Translation reactions were carried out in 10 μL volumes that contained 10 mM creatine phosphate, 0.3 mM $MgCl_2$, 0.1 mM each amino acid (with the exception of methionine), 3 μCi [^{35}S]methionine, 4 mM 2-aminopurine, and 80 mM KCl, with 50% (by volume) nuclease-treated rabbit reticulocyte lysate; the translation reactions were supplemented with either HeLa cell S100 cytoplasmic extract (20% v/v) or with an equivalent volume of H100 buffer (20 mM HEPES-KOH, 100 mM KCl, 2 mM DTT, pH 7.5). Translation assays were incubated for 60 min at 30 °C, and the products separated on 20% SDS-PAGE. The gel was then dried and subjected to autoradiography for the period of time stated in the individual figure legends using Hyperfilm βMax (Amersham International).

Where indicated, 0.3 μL of in vitro-expressed L protease or a control incubation were added to the translation reactions. The L protease was expressed in reticulocyte lysate at 50 mM added KCl from 20 μg/mL of uncapped pMM1 mRNA, which codes for the FMDV Lab protease plus VP4 and part of VP2 (Medina et al., 1993; Ohlmann et al., 1995). After 50 min incubation, the mixture was treated with 2 mM $CaCl_2$ and 150 U/mL micrococcal nuclease for 10 min at room temperature. This digestion was terminated by the addition of 4 mM EGTA. As a control, a dummy translation reaction was taken through the whole procedure except that pMM1 mRNA was omitted.

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