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Comparison of picornaviral IRES-driven internal initiation of translation in cultured cells of different origins

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ABSTRACT

We recently compared the efficiency of six picornaviral internal ribosome entry segments (IRESes) and the hepatitis C virus (HCV) IRES for their ability to drive internal initiation of translation *in vitro*. Here we present the results of a similar comparison performed in six different cultured cell lines infected with a recombinant vaccinia virus expressing the T7 polymerase and transfected with dicistronic plasmids. The IRESes could be divided into three groups: (i) the cardiovirus and aphthovirus IRESes (and the HCV element) direct internal initiation efficiently in all cell lines tested; (ii) the enterovirus and rhinovirus IRESes are at least equally efficient in several cell lines, but are extremely inefficient in certain cell types; and (iii) the hepatitis A virus IRES is incapable of directing efficient internal initiation in any of the cell lines used (including human hepatocytes). These are the same three groups found when IRESes were classified according to their activities *in vitro*, or according to sequence homologies. In a mouse neuronal cell line, the poliovirus and other type I IRESes were not functional in an artificial bicistronic context. However, infectious poliovirions were produced efficiently after transfection of these cells with a genomic length RNA. Furthermore, activity of the type I IRESes was dramatically increased upon co-expression of the poliovirus 2A proteinase, demonstrating that while IRES efficiency may vary considerably from one cell type to another, at least in some cases viral proteins are capable of overcoming cell-specific translational defects.

INTRODUCTION

For capped eukaryotic mRNAs, translation initiation requires scanning by the ribosome from the 5′ end of the message to the initiator AUG codon (1). In contrast, all picornavirus RNAs examined to date have been found to possess an internal ribosome entry segment (IRES) (2–5). These elements, which comprise ~450–500 nt of highly structured RNA situated in the long viral 5′-untranslated region (UTR), permit the initiation of picornavirus RNA translation in a manner which is both cap and 5′-end independent (6,7). Recently, IRESes have also been demonstrated to exist in other viral messages (8,9) and in some cellular mRNAs (10–12). It has previously been shown that the picornaviral IRESes can be classified into three distinct groups on the basis of primary sequence and secondary structure conservation (for a review see 6), and also on the basis of their requirements for efficient internal initiation of translation *in vitro* (13).

Type I IRESes (those of the enteroviruses and rhinoviruses) are inefficient in driving translation initiation in reticulocyte lysates in the absence of specific cellular proteins, and are sensitive to even slight modifications of KCl and $MgCl_2$ concentrations (13). Furthermore, their efficiency is dramatically enhanced *in vitro* in the presence of either of two picornaviral proteinases, the 2A proteinase of enteroviruses and rhinoviruses or the Lb proteinase of aphthoviruses (13–16). Conversely, type II IRESes (those of the cardioviruses and aphthoviruses) initiate translation efficiently in reticulocyte lysates in the absence of other cell proteins, and are relatively insensitive to fluctuations in salt concentration. Additionally, they are not dramatically affected by the presence of the 2A or Lb proteinases (13,15,17). In these respects, the IRES of hepatitis C virus behaved as a type II IRES (13). Finally, the type III IRES (that of hepatitis A virus) is relatively inefficient in reticulocyte lysates, but to date its activity has not been found to be markedly stimulated by supplementation with additional cell proteins (18). This IRES tolerates a wide range of salt concentrations, and is inhibited by the presence of the viral 2A or Lb proteinases (13,19). The various picornavirus IRESes can be similarly separated on the basis of their organization and location within the viral genome; the essential sequences required for type II and type III IRES activity extend up to, include, or even continue beyond the authentic start site for translation (20–23), whereas the 3′ end of type I IRESes lies between 30 and 150 nt upstream of the translation initiation site (5,24–26).

One of the major determinants for picornaviral species and tissue tropism is the presence or absence of the viral receptor on the cell surface (27). However, several results suggest that the IRES may also represent a determinant of viral tropism. *In vitro* studies have identified different cell factors that bind to the different IRESes and that may be required for translation initiation (28–30). In addition, the analysis of poliovirus IRES mutants showed that translation defects could be cell type-specific,

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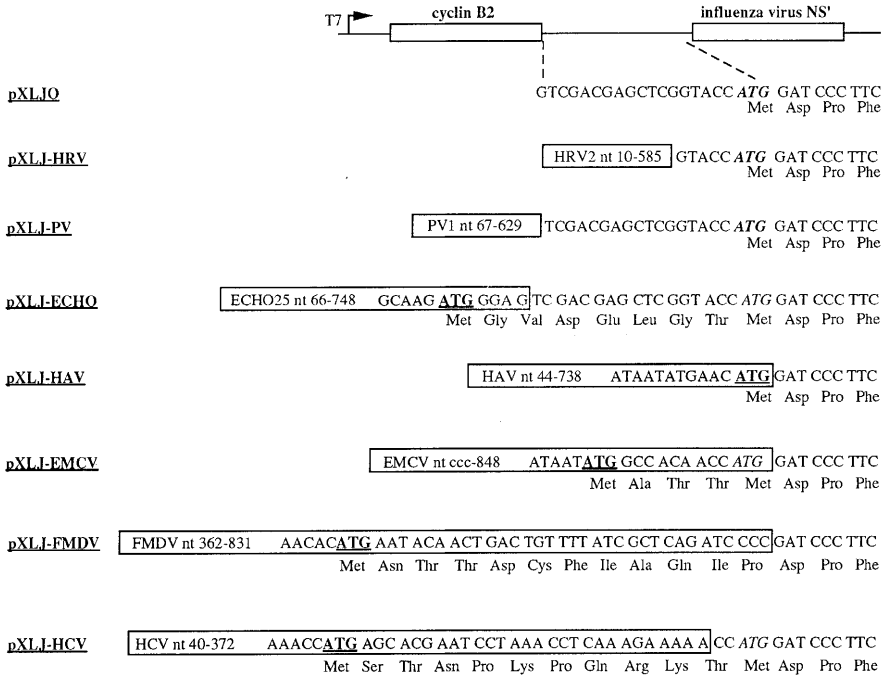

Figure 1. Schematic representation of the dicistronic plasmids used for this work. The *X.laevis* cyclin B2 and influenza virus NS protein coding regions are shown as boxes. The intercistronic spacer elements are shown either as a nucleotide sequence (for the parental pXLJO plasmid) or as a box containing the length (numbers refer to the first and last nucleotides) of the respective viral inserts followed by the sequence of the junction between the IRES sequence and the NS coding region. The initiator codon used for NS protein synthesis is shown in bold in all cases and the ATG codon at the start of the unmodified NS gene is italicised in all clones where it is retained. Where NS synthesis is initiated from the authentic picornaviral initiation codon, it is depicted in bold and underlined. Aligned under the nucleotide sequences are the N-terminal amino acid sequences of the respective NS proteins. These sequences are aligned with respect to the Asp-Pro-Phe motif (amino acids 2, 3 and 4 of the original NS protein) which are retained in all constructions.

the decreased translation capacity of mutant templates being evidenced in cells or extracts of neuronal origin, rather than in HeLa cells or their extracts (31–32). Cell-specific determinants were recently demonstrated to exist in the poliovirus 5′-UTR using viruses with chimeric genomes. Indeed, when the poliovirus IRES was replaced by that of human rhinovirus, neuropathogenicity in a mouse model was abrogated (33).

In the present study we have examined the translation efficiencies of six different picornaviral IRESes in a variety of human and non-human cell lines. Our results indicate that IRES activity differs dramatically according to the cell line used, and that the different IRESes can once again be classified into three distinct groups on the basis of cell-specific efficiencies.

MATERIALS AND METHODS

Plasmids

Escherichia coli strain TG1 was used for the propagation of plasmids. All of the dicistronic plasmids used for the present study are based on the parental construct pXLJO (5) which contains the *Xenopus laevis* cyclin B2 gene followed by a slightly truncated influenza virus NS gene, under the control of the T7 Ø10 promoter. The insertion into this vector of the sequences corresponding to the complete IRESes of poliovirus type 1 Mahoney strain (PV), ECHO virus type 25 JV4 strain (ECHO), human rhinovirus type 2 (HRV), hepatitis A virus p16 HM175 (HAV), encephalomyocarditis virus (EMCV), foot-and-mouth disease virus (FMDV) and hepatitis C virus (HCV) has been described previously (13). The amino acid sequences of the N-terminus of the NS protein as synthesised from the seven different IRES constructions and the control pXLJO are summarised in Figure 1.

pAΔ802 (20) encodes the PV 2A proteinase preceded by a short 5′-UTR, under the control of the T7 Ø10 promoter. Plasmids pM16 (34) and pT7-PV1-52 (35) harbour infectious cDNAs of the Mengo virus and PV genomes, respectively, under the control of the T7 Ø10 promoter.

Cell culture conditions

Neuro-2A (partially differentiated mouse neuroblastoma) and SKNBE cells (minimally differentiated human neuroblastoma) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% by volume of foetal calf serum. HeLa (human cervix epitheloid carcinoma), HepG2 (human hepatocyte), FRhK4 (monkey foetal kidney) and L929 cells (mouse C34/An connective tissue) were maintained in DMEM supplemented with 5% foetal calf serum. BHK21 cells (baby hamster kidney) were maintained in Glasgow MEM, supplemented with 20 mM HEPES, 2 mM glutamine, 10% tryptose phosphate broth and 5% foetal calf serum. HeLa S3 cells were maintained in MEM supplemented with 10% foetal calf serum. After transfection, all cell lines were maintained in DMEM supplemented with 5% foetal calf serum, except after transfection with full-length Mengo virus and PV transcripts, when DMEM was supplemented with 2% foetal calf serum.

Similarly, after infection with Mengo virus and PV, cell lines were maintained in DMEM supplemented with 2% foetal calf serum.

Production of stocks of vaccinia virus

HeLa S3 cells (5×10^7) were infected with 10^8 p.f.u. of recombinant vaccinia virus vTF7-3 which expresses the bacteriophage T7 RNA polymerase (36). After 3 days of incubation, the infected cells were detached from the flask by shaking, and centrifuged for 5 min at 1800 *g*. The supernatant was discarded, and cells were resuspended in 2 ml MEM supplemented with 5% foetal calf serum. Virus was released by three successive freeze–thaw cycles, and the virus stock (~2×10^9 p.f.u./ml) was stored at –70°C.

Analysis of IRES activities in cultured cells

Cells were seeded 24 h before each assay in 35 mm diameter plates (6×10^5 cells). For all assays, cells were infected with vTF7-3 recombinant vaccinia virus at a multiplicity of infection of >10, in DMEM without serum. After 90 min at 37°C, cells were washed with DMEM without serum and were transfected with DNA of the pXLJ series of plasmids. These plasmids encode a dicistronic mRNA in which the first cistron (*X.laevis* cyclin B2 gene) is under the control of its own 5′ UTR and the second cistron (influenza virus NS gene) is under the control of a viral IRES (Fig. 1). For assays using Neuro-2A, HeLa, FRhK4, BHK21 and HepG2 cells, transfections were performed using the DOTAP reagent (Boehringer Mannheim) with either 3 or 4 µg plasmid DNA and 30 µg DOTAP in DMEM with 5% foetal calf serum (final volume of 2 ml per plate). For assays using SKNBE cells, no detectable signal was obtained using DOTAP. Therefore, transfections were performed with the maxifectin kit (gift of Dr Andrej Sourovoi, MBCP, Rottenburg) using 2 µg DNA mixed with 2 µl Enhancer and 7.5 µl Unifectin, in DMEM with 5% foetal calf serum (final volume of 2 ml per plate). For L929 cells, no detectable signal was obtained using either method. In all assays, the transfection reagent was removed by multiple washing with PBS at 18 h post-transfection, and cells were starved of methionine for 90 min with methionine-free DMEM. Labelling of newly synthesised proteins was performed by incubating cells in 1 ml methionine-free DMEM containing 15 µCi [^{35}S]methionine (Amersham, 1000 µCi/mmol) for 2 h. Following labelling, cells were washed twice in ice-cold PBS, lysed with 100 µl lysis buffer (10 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM EDTA and 0.5% NP-40) and centrifuged at 12 000 r.p.m. for 5 min to pellet the nuclei. Proteins in the cytoplasmic extract supernatant were analysed by 20% SDS–PAGE, and the dried gels were exposed to β-max film (Amersham).

Quantification of IRES efficiencies

Quantification of translation efficiencies was performed densitometrically with a Sharp JX-330 densitometer using the NIH Image program and Macintosh software. Three different exposures of each gel were scanned to ensure that exposures were within the linear response range of the films. For each assay, the values obtained for IRES-driven translation were measured as the amount of NS product relative to that of cyclin B2, to correct for variations in transfection efficiencies. For each cell-line tested, IRES efficiency was expressed as a percentage of the most efficient IRES in that particular cell type.

RNA transfection and quantification of viruses

Purified pM16 and pT7-PV1-52 plasmid DNAs were linearized with *Bam*HI and *Eco*RI, respectively, and transcribed *in vitro* using T7 RNA polymerase (New England Biolabs) as described (37). Confluent HeLa, L929 and Neuro-2A cell monolayers (corresponding to 10^6 cells) were transfected with 1 µg RNA transcripts, or 10-fold serial dilutions thereof, as described (37). After incubation for 24 or 40 h at 37°C, the cell sheets were scraped into the medium, and intracellular virus liberated by three cycles of freeze–thawing. Virus stocks were then titered on HeLa cell monolayers. Alternatively, to determine the transfection efficiency, transfected cells were incubated under semi-solid medium for 2 days, and plaques quantified after colouring with crystal violet.

RESULTS

Artificial dicistronic mRNAs provide an excellent tool for the analysis of IRES function both *in vitro* and *in vivo*. In the absence of an IRES between the two cistrons, translation of the downstream cistron is extremely inefficient, since it relies on initiation by ribosomes which recommence scanning after completion of upstream cistron translation. However, when an IRES is used to separate the two cistrons, downstream cistron translation is dramatically increased (reviewed in 6). For the study described here, we have used a previously well-characterised dicistronic vector system into which the IRESes of six picornaviruses (PV, ECHOvirus, HRV, HAV, EMCV, FMDV) and a pestivirus (HCV) have been inserted into the intercistronic spacer to drive influenza virus NS protein synthesis (13; Fig. 1). The sequences required for internal initiation of translation extend at least up to the authentic viral initiation codon in the case of the EMCV, FMDV and HAV IRESes, and beyond it in the case of HCV (23). Thus, internal initiation of translation results in the synthesis of NS proteins with N-terminal extensions of 4, 11, 0 and 11 amino acids, respectively (Fig. 1). Similarly, although the 3′ boundary of the ECHO IRES lies upstream of the authentic initiation codon (26), for ease of construction the 5′-UTR fragment used extends beyond the authentic viral AUG codon. Thus ECHO IRES activity results in the synthesis of an NS protein which has an N-terminal extension of eight amino acids (Fig. 1).

Comparison of IRES efficiencies in transfected HeLa cells

The dicistronic cDNAs used are incapable of being replicated in cultured cells. Thus, for the analysis of translation efficiencies *in vivo*, the plasmids were introduced into cells which had first been infected with vTF7-3, a vaccinia virus which expresses the bacteriophage T7 RNA polymerase. RNAs were thus transcribed from the dicistronic plasmids directly in the cell cytoplasm, and subsequently translated. This results in the synthesis of quantities of cyclin B2 and NS proteins which are directly detectable in [^{35}S]methionine labelled cytoplasmic extracts upon migration through polyacrylamide gels (Fig. 2). Transfection efficiency can be standardised on the basis of the level of upstream cistron (cyclin B2) translation. Initial experiments demonstrated that in all cases, IRES-driven translation efficiency in HeLa cells increased linearly over plasmid DNA concentrations in the range of 1–6 µg per 6×10^5 cells (data not shown). Thus for all subsequent experiments, between 2 and 4 µg (depending on the

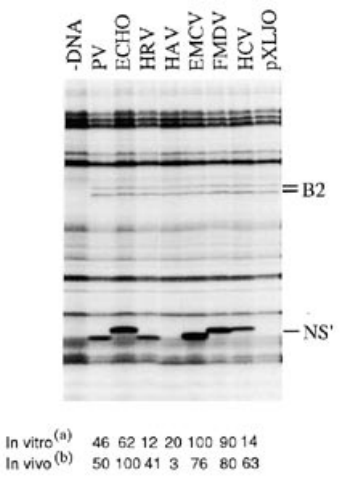

Figure 2. Comparison of the efficiency of translation driven by the different IRESes in HeLa cells. Recombinant vaccinia virus-infected HeLa cells were transfected with 3 µg of the parental dicistronic construct (pXLJO), the equivalent plasmids containing the seven different IRESes (PV through HCV), or were mock transfected (–DNA) as described in Materials and Methods. Labeling of total cell protein synthesis and preparation of cell cytoplasmic extracts for electrophoresis was as described (Materials and Methods). The autoradiograph of the dried 20% SDS–polyacrylamide gel is shown; the positions of the cyclin B2 and influenza virus NS proteins are marked. The results of densitometric analysis of the autoradiograph are shown below the appropriate lanes [*in vivo* (b)]; translation efficiency for each IRES is calculated as a percentage of the most efficient IRES in the assay (ECHO), after adjustment of values of NS yield to take into account fluctuations in upstream cistron translation. (a) The values previously obtained for the different IRESes in an *in vitro* experiment which was designed to mimic physiological conditions for translation (13).

cell line and the transfection procedure used) of each plasmid was introduced into the different cell lines.

Since we previously compared the same seven IRESes *in vitro* in reticulocyte lysates supplemented with cytoplasmic extracts from HeLa cells, the initial comparison of IRES efficiency *in vivo* was performed in HeLa cells (Fig. 2). The ECHO IRES was the most efficient in driving downstream cistron translation, followed by the EMCV, FMDV and HCV IRESes, which were 60–80% as efficient as the ECHO IRES. The PV and HRV IRESes were also relatively efficient in directing internal translation initiation (50 and 41% of the ECHO IRES). In contrast, virtually no HAV IRES-driven NS synthesis could be detected. These results were similar to those obtained previously *in vitro* in conditions chosen to mimic a HeLa cell environment (13; Fig. 2), with the exceptions of the ECHO, HRV and HCV IRESes, which were substantially more efficient *in vivo* than *in vitro*, relative to the other IRESes.

IRES efficiencies in a variety of different primate and non-primate cell lines

In order to investigate the possibility that viral tissue and/or species tropism is to some extent mediated by the nature of the IRES, we extended the comparison to include five different cell lines. SKNBE and Neuro-2A cells (neuronal cells of human and mouse origin respectively) were included in the analysis since PV and ECHOvirus are neurotropic. The HepG2 human hepatocyte cell line and the FRhK4 monkey kidney cell line were chosen as further examples of primate cells, particularly because these cells are permissive for multiplication of HAV (38). Finally, baby hamster kidney cells were used as an example of non-primate, non-neuronal cells. The results of densitometric analyses of the efficiencies of the different IRESes in driving translation in these cell lines are summarised in Figure 3.

The IRESes can once again be classified into the type I, II and III groups defined on the basis of sequence comparisons or *in vitro* translation characteristics. The type III (HAV) IRES exhibited barely detectable activity in any of the cell lines tested, including the human hepatocyte HepG2 cells. Conversely, type II IRESes (the EMCV, FMDV and HCV elements), were relatively efficient in all cell lines examined. However, some cell type-specific variations were observed. This was most dramatic for the EMCV element, which was considerably less efficient than the FMDV or HCV IRESes in BHK21 and SKNBE cells, while being at least as efficient as them in FRhK4, HepG2 and HeLa cells. Finally, the type I IRESes (PV, ECHO and HRV) drove translation efficiently in HeLa, HepG2 and FRhK4 cells, whereas they were extremely inefficient in the non-primate BHK21 and Neuro-2A cell lines and in the human neuronal cell line, SKNBE. Of the three type I IRESes, the ECHOvirus element was the most efficient in all but the human neuronal cell line and the HRV IRES was reproducibly the least so. In some cases the ECHO IRES was even more efficient than the type II IRESes (see for example HeLa and FRhK4 cells in Fig. 3).

Table 1. Virus production in mouse cells during a single growth cycle

	Cell line		
	L929	Neuro-2A	HeLa
Transfection efficiency[a]	1.0×10^3	2.0×10^{3} [b]	2.0×10^{4} [b]
			2.5×10^{4} [c]
Production of infectious poliovirus[d]	220[e]	345[e]	600[f]
Production of infectious Mengo virus[d]	300[f]	440[f]	400[f]

[a]Number of cells productively transfected per µg RNA: [b]calculated after transfection with serial dilutions of pM16 RNA; or [c]pT7-PV1-52 RNA (p.f.u. obtained upon incubation under semi-solid medium). [d]p.f.u. per cell: [e]calculated after transfection with 1 µg RNA (after 24 or 40 h, virus was harvested and titered on HeLa cells. The titer given takes into account transfection efficiency); or after [f]infection with 10 p.f.u. per cell of virus (every 2 h virus was harvested and titered on HeLa cells to obtain a single step growth curve).

Thus, it seems that the cell lines tested can be divided into two broad groups. The first includes those cells which permit efficient translation initiation from all type I and II IRESes (HeLa, FRhK4 and HepG2 cells), whereas the second dramatically restricts translation initiated from type I but not type II IRESes (SKNBE, BHK21 and Neuro-2A cells). In the light of the neurotrophic nature of PV and ECHOvirus, it was surprising that the IRESes from these viruses directed translation so inefficiently in a human neuronal cell line.

Type I IRES activity can be dramatically increased in restrictive cells by co-expression of the poliovirus 2A proteinase

The analysis of picornavirus IRES efficiency using artificial dicistronic vectors is not comparable to the situation seen upon infection of cells with the different viruses, since the relevant viral

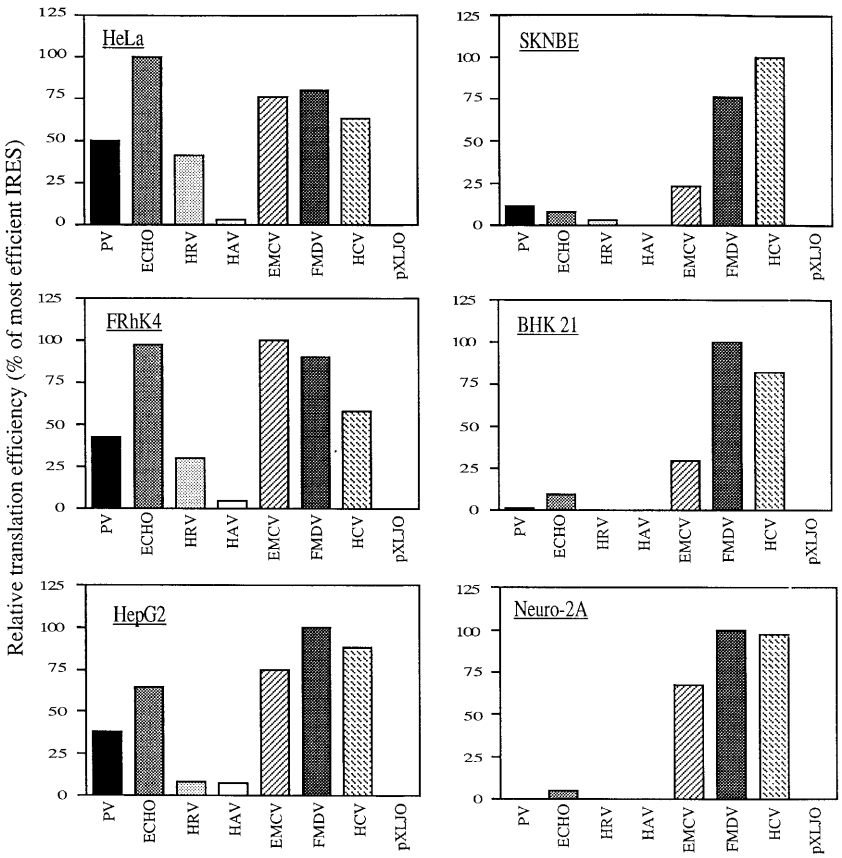

Figure 3. Comparison of the efficiency of the different IRESes to drive translation in five different cell lines. Recombinant vaccinia virus-infected FRhK4, HepG2, Neuro-2A and BHK 21 cells were transfected with 4 µg of the different dicistronic DNAs as described in Materials and Methods. For the assay using SKNBE cells, the analysis was performed using 2 µg of the different plasmids. Labelling of cell proteins and determination of translation efficiencies was performed as described in Materials and Methods and in the legend to Figure 2. The relative translation efficiencies as calculated in the five different cell lines are shown, as is a graphical representation of the results obtained with HeLa cells (Fig. 2). It should be noted that for each cell line, the most efficient IRES is assigned the value of '100% efficient', all other IRESes tested in that cell line are then compared to this one. However, given that the actual translation efficiencies varied substantially from one cell line to another, the 100% values do not represent comparable efficiencies of IRES-driven translation.

structural or non-structural proteins are not expressed. It is possible that translational restrictions imposed upon certain IRESes in the different cell lines could be circumvented if viral proteins were also expressed in the cells. To examine this possibility, we assessed the multiplication of PV in Neuro-2A cells in a single cycle (Table 1). While mouse cells do not possess the PV receptor and thus cannot be infected with PV, they can be transfected with genomic length PV RNA transcripts, and the production of infectious virus can be measured by titration on permissive cells, such as HeLa cells. Thus, L929 cells, which are mouse cells and so lack the PV receptor but which can otherwise support PV multiplication (39), were used as a positive control. Similarly, Mengo virus which multiplies well in mouse cells was used as a positive control. The production of PV in Neuro-2A cells was at least as good as that observed in L929 cells (Table 1), and any difference between the production of infectious poliovirions and that of Mengo virus could be attributed to cell suffering during transfection (compare the titer of PV in infected HeLa cells to that in transfected L929 cells). Thus, even though PV IRES activity is undetectable in Neuro-2A cells using the dicistronic assay, these cells are capable of supporting efficient PV replication, suggesting that viral protein(s) can overcome some block to translation. We and others have recently demonstrated that the entero- and rhinoviral 2A proteinases are capable of stimulating translation driven by type I IRESes (13–16,40). Thus, we examined the possibility that the discrepancy between the results of the dicistronic translation assays and those of transfections with infectious PV RNA might be due to the absence of active 2A proteinase in the former assay. To this end, IRES activity was reassessed in Neuro-2A cells cotransfected with the dicistronic plasmids and with a plasmid which encodes the PV 2A proteinase. Similarly to the dicistronic plasmids, RNA corresponding to the PV 2A gene is transcribed directly in the cytoplasm of cells which are infected with vTF7-3 vaccinia virus. As can be seen from Figure 4, co-expression of the 2A proteinase dramatically stimulated type I IRES-driven translation in Neuro-2A cells, such that the type I IRESes were then almost as efficient as their type II counterparts. In the case of the ECHO IRES, the only type I IRES that gave a detectable signal in Neuro-2A cells in the absence of the 2A proteinase, the stimulation of translation efficiency observed was ~20-fold. Translation mediated by the type II and type III IRESes was largely unaffected by the presence of 2A, although EMCV IRES activity increased by ~1.5-fold. Such a marginal stimulation of EMCV IRES-driven translation

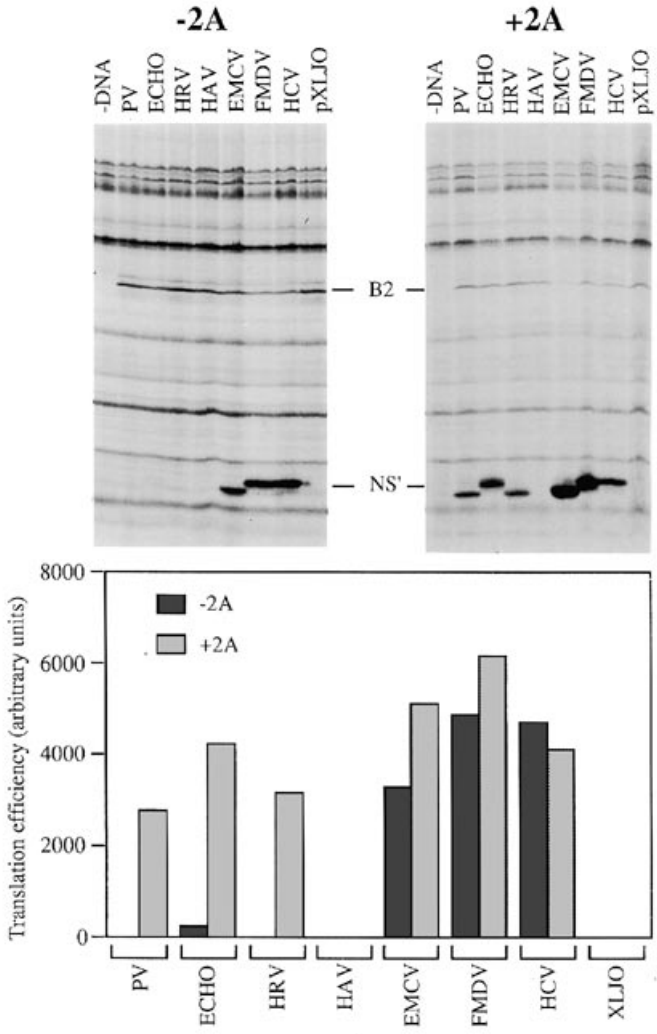

Figure 4. The PV 2A proteinase rescues type I IRES-driven translation in non-permissive Neuro-2A cells. Recombinant vaccinia virus-infected Neuro-2A cells were transfected with 3 µg of the different dicistronic plasmids (–2A) or with 3 µg of these plasmids mixed with 1 µg per well of the pAΔ802 2A expression plasmid (+2A). Cell labelling and analysis of extracts was performed as described in the legend to Figure 2. The positions of the cyclin B2 and NS products are marked. Below are shown the results of the densitometric analysis of the autoradiograph. In this case, translation efficiencies are given in arbitrary units so as to allow an assessment of the effects of 2A on the efficiency of translation as driven by the different IRESes.

with either the Lb or 2A proteinases has also been demonstrated *in vitro* (13,15).

DISCUSSION

The aims of this study were to compare the capacities of different picornaviral IRESes to mediate internal translation initiation in a variety of cultured cells. First, we wished to assess the relative utilities of these elements in the design of IRES-based vectors destined for foreign gene expression in specific cell types. Secondly, this analysis would allow the possible role of these IRESes in viral tissue and species tropism to be examined.

IRES-mediated translation was assayed after transfection of T7 promoter-dependent dicistronic DNAs into cells which had been pre-infected with a recombinant vaccinia virus expressing the T7 polymerase. Thus, dicistronic RNAs were transcribed directly in the cell. When the different IRESes were compared in HeLa cells, similar results were obtained to those found in an earlier *in vitro* study using physiological concentrations of added salt and supplementation of reticulocyte lysate reactions with saturating concentrations of HeLa cell cytoplasmic extracts. Essentially, the type I and type II IRESes were found to be rather similar in their global efficiency in mediating internal initiation of translation (Fig. 2; 13). This confirms that cell-free translation reactions can be a relevant means of examining IRES function. Interestingly, in pilot experiments using HeLa cells, entero- and rhinovirus IRES-driven translation, but not upstream cistron translation or translation driven by cardio- and aphthovirus IRESes, was found to be saturated when >6 µg DNA was used for transfections, and the efficiency of translation was much reduced at even higher concentrations of DNA (data not shown). This is similar to the situation reported for translation driven by entero- and rhinovirus IRESes *in vitro* (5,41). In that case it was concluded that the IRES-containing RNAs were in excess with respect to essential non-canonical translation factors. Thus, it seems that the limiting nature of these specific translation factors is not an artifact of *in vitro* translation systems, but can be reproduced in cells transfected with high concentrations of DNA. For all assays of

IRES efficiency reported here, care was taken to use non-saturating DNA concentrations, ensuring that the activities of the entero- and rhinoviral IRESes on the one hand, and the cardio- and aphthoviral IRESes on the other, could be compared.

When the *in vivo* comparison was extended to incorporate five other cell lines, the picornaviral IRESes could be classified into three distinct groups. These same three groups were previously found on the basis of sequence homologies (6,42,43), and on the characteristics of IRES-driven translation *in vitro* (13). HAV (type III) IRES activity was virtually undetectable in all cell lines tested; even in human hepatocytes (HepG2 cells) this IRES was <10% as active as the most efficient one (that of FMDV). The general inefficiency of the HAV IRES observed here might be one of the reasons for the poor replication of HAV seen in cell culture (44). In this respect, it should be noted that cell culture-adapted variants of HAV have mutations in the IRES which enhance viral replication in certain cell types (38). Thus, our results show that the type III IRES is an extremely poor candidate for expression vector development. Conversely, the type II IRESes (EMCV, FMDV and HCV) seem at first view excellent candidates for such applications, since they functioned efficiently in all cell lines tested. In this respect, the HCV IRES was particularly noteworthy; this element drove internal translation initiation efficiently in all cells lines tested, including those of non-primate origin. Thus, the HCV IRES, as standardised to that of the other type II IRESes, appears to be dramatically more efficient *in vivo* than *in vitro*, where it was consistently one of the weakest elements tested (13). However, the genetic organisation of the type II IRESes makes their use fastidious, as foreign genes have to be fused extremely precisely at the 3′ end of the IRES. In contrast, the type I IRESes allow easier and more flexible foreign gene insertion. Indeed, these elements, particularly that of ECHO virus, are good candidates for expression vectors. Depending on the cell line, the ECHO IRES can direct translation initiation even more efficiently than the type II elements. Nevertheless, care should be taken to ensure that the desired target cell or tissue for foreign gene expression is not restrictive for the chosen type I IRES, since the type I IRESes as a group exhibited the greatest cell type-specific variations in activity, being extremely efficient in several cell lines, and almost inactive in others.

The use of a variety of cell lines, including neuronal cells of mouse and human origin, human hepatocytes and kidney cells from monkeys and hamsters, allowed us to address the question of whether viral tissue or species tropism is mediated by the IRES. The best activity for the HAV IRES was obtained in human hepatocytes (HepG2 cells). This correlates well with both the species and tissue tropism of this virus, which multiplies in the livers of primates (45). However, even in HepG2 cells HAV IRES activity was relatively poor. Thus, it is difficult to ascertain whether the marginally stronger translation obtained compared to other cell lines is a true reflection of tissue tropism. Given the wide tropism of EMCV, and to a lesser extent of FMDV, it was perhaps to be expected that these IRESes function in most cell lines. It is more surprising that the HCV IRES also functioned efficiently in most cell lines, since this virus shows the same restricted tropism as HAV. It should be noted that the EMCV IRES showed significantly reduced activity when compared with the FMDV and HCV elements both in SKNBE and BHK21 cells, suggesting that a certain degree of host-cell translational repression/enhancement may exist amongst the different type II IRESes. The dramatic variations of type I IRES activities in the different cell lines tested suggests that entero- and rhinovirus translational efficiency could be a major contributing factor to viral tissue or species tropism. However, we found no obvious correlation between IRES activity and known virus tropism. For example, although PV is specific for humans and is neurotropic, the PV IRES barely functioned in SKNBE cells.

It seems rather that viral proteins are required for type I IRESes to function efficiently in certain types of cells. The translation-restrictive Neuro-2A cell line is permissive for production of infectious PV after transfection, i.e. translation of the full-length genome must be occurring in these cells. Furthermore, PV production in a one step growth curve after infection of Neuro-2A cells engineered to express the PV receptor was as efficient as virus production in HeLa cells (V.R.Racaniello, personal communication). Indeed, we have shown here that co-expression of the PV 2A proteinase was sufficient to alleviate translational repression of type I IRES activity in Neuro-2A cells. Since the PV 2A proteinase activated translation not only from its own IRES, but also from those of heterologous viruses, some general phenomenon is implicated. The entero- and rhinoviral 2A proteinases have previously been shown to modulate the levels of IRES-driven translation by cleavage of the cellular translation initiation factor eIF4G, via at least two mechanisms. First, translation of capped host cell mRNAs is inhibited upon cleavage of eIF4G, reducing competition for the translation machinery during viral infection (14,46,47). Secondly, the cleavage products of eIF4G specifically stimulate type I IRES-driven translation, at least *in vitro* (48). It is interesting to speculate that these cleavage products can also stimulate type I IRES activity *in vivo*. Nevertheless, it cannot be excluded at this time that the increased type I IRES activity in restrictive cells in the presence of the 2A proteinase is due to decreased competition with cellular mRNAs upon inhibition of host cell translation. Alternatively, it could be that the 2A proteinase acts by the removal of some specific unidentified inhibitor present in restrictive but not permissive cells. However, given the dramatic improvement in type I IRES activity in the presence of the 2A proteinase, it seems unlikely that marked differences in viral tissue/species tropism between these viruses can be assigned to IRES activity *per se*. This contrasts with the conclusions that could be drawn from studies comparing wild-type and attenuated PV. Lowered neurovirulence due to a single mutation in the IRES correlated with reduced translation efficiency in neuronal cells (31). This was interpreted as indicative of mimicry of the host restriction encountered by attenuated PV in the central nervous system of the infected animal. However, the phenotypic effects of such mutations can be suppressed by second-site mutations in the 2A proteinase gene (49), again suggesting interplay between the IRES and viral proteins, rather than between the IRES and cell type-specific factors.

In conclusion, our work should facilitate the choice of the IRES to be used in the construction of vectors for foreign gene expression in different target cells. Additionally, our results highlight the need to examine further the possible roles of the various viral proteins in translation and the mechanism(s) of modulation of IRES activity by such proteins.

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