Absence of Internal Ribosome Entry Site-Mediated Tissue Specificity in the Translation of a Bicistronic Transgene

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The 5’ noncoding regions of the genomes of picornaviruses form a complex structure that directs cap-independent initiation of translation. This structure has been termed the internal ribosome entry site (IRES). The efficiency of translation initiation was shown, in vitro, to be influenced by the binding of cellular factors to the IRES. Hence, we hypothesized that the IRES might control picornavirus tropism. In order to test this possibility, we made a bicistronic construct in which translation of the luciferase gene is controlled by the IRES of Theiler’s murine encephalomyelitis virus. In vitro, we observed that the IRES functions in various cell types and in macrophages, irrespective of their activation state. In vivo, we observed that the IRES is functional in different tissues of transgenic mice. Thus, it seems that the IRES is not an essential determinant of Theiler’s virus tropism. On the other hand, the age of the mouse could be critical for IRES function. Indeed, the IRES was found to be more efficient in young mice. Picornavirus IRESs are becoming popular tools in transgenesis technology, since they allow the expression of two genes from the same transcription unit. Our results show that the Theiler’s virus IRES is functional in cells of different origins and that it is thus a broad-spectrum tool. The possible age dependency of the IRES function, however, could be a drawback for gene expression in adult mice.

Translation of the majority of cellular RNAs is according to the scanning model proposed by Kozak (24). The first steps of this process involve interaction of the eIF-4F initiation factor with the m7G cap at the 5’ extremity of the mRNA, unwinding of surrounding secondary structures and subsequent formation of the 48S preinitiation complex, and scanning to an initiation codon. Picornaviruses are small, positive-stranded RNA viruses that encode a unique polyprotein that yields 11 or 12 mature proteins after proteolytic cleavage (38). The genomic RNAs of these viruses are uncapped, and their 5’ noncoding (NC) regions are usually long and structured and contain several AUG triplets. These features are incompatible with a scanning model for translation. Instead, the genomes of picornaviruses as well as certain other viral and a few cellular RNAs are translated by direct attachment of ribosome subunits to an internal ribosome entry site (IRES) inside the 5’ NC region of the RNA (18–20, 31). Picornavirus IRESs vary in length and have been predicted, by computer folding and biochemical probing, to be organized in a complex series of stems and loops. The picornavirus family can be divided into two major groups based on the nucleotide sequences and proposed secondary structures of their IRESs. The first group includes enteroviruses and rhinoviruses (33, 42), and the second group includes cardioviruses and aphthoviruses (11, 23, 32). The hepatitis A virus IRES is predicted to form a third type of structure with some similarity to that of cardio- and aphthoviruses (8). In vitro translation assays also revealed functional differences of the IRESs of these groups. First, the 3’ border of the IRES element of entero- and rhinoviruses is up to 150 bases upstream from the initiation codon, whereas the 3’ terminus of the cardio- and aphthovirus IRES element is adjacent to the initiation codon. Second, the genomes of the first group are translated inefficiently and inaccurately in rabbit reticulocyte lysates unless HeLa cell extracts are added to the lysate (27). This is not the case for members of the cardio- and aphthovirus group.

Cellular proteins do bind to IRES sequences, and some of them were shown to be essential for IRES activity in vitro (3–5, 16, 21, 27). In view of the requirement of cellular factors for IRES activity, we hypothesized that the IRES might function in a tissue-specific fashion, thereby participating in the tropism of the virus. Several studies support this hypothesis. For instance, the main determinant involved in neurovirulence attenuation of the Sabin vaccine strain of poliovirus was mapped to the IRES (1). Translation driven from the IRESs of attenuated strains in vitro was shown to be specifically inhibited in cell lines of neuronal origin (15). In agreement with this finding, a recent report demonstrated that poliovirus neuropathogenicity in a mouse model was eliminated when the IRES of this virus was replaced by the IRES of a rhinovirus (14). Finally, translation from the IRES of hepatitis A virus in vitro was found to be stimulated 12-fold when fresh liver extracts were added to the assay mixture (13).

In this work, we analyzed mice that are transgenic for a bicistronic construct, where the second cistron is under the control of the Theiler’s murine encephalomyelitis virus (Theiler’s virus) IRES. This virus is a member of the Cardiovirus genus and has a pronounced tropism for the central nervous system (CNS) of the mouse (25, 44, 45). Thus, comparison of the IRES efficiencies in nervous system and other tissues might indicate whether this element participates in the determination of Theiler’s virus tropism. Another aspect of our research was to investigate whether an IRES could function in a bicistronic context in mice. The idea of using an IRES for combining the expression of several proteins in vivo is not without precedent. Two groups have reported that a bicistronic construct with the
TABLE 1. Primers and PCR conditions used for studies of transgenic and infected mice

<table>
<thead>
<tr>
<th>Mouse group and study</th>
<th>Primer paira</th>
<th>Gene fragment</th>
<th>Fragment length (bp)</th>
<th>No. of cycles</th>
<th>Annealing temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td>TM4-TM132</td>
<td>IRES</td>
<td>1,088</td>
<td>30–40</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>TM178-TM179</td>
<td>GAPDH</td>
<td>194</td>
<td>30–40</td>
<td>55</td>
</tr>
<tr>
<td>Transgenic Screening</td>
<td>TM27-TM28</td>
<td>CAT</td>
<td>660</td>
<td>30–40</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>TM4-TM195</td>
<td>IRES</td>
<td>372</td>
<td>30–40</td>
<td>55</td>
</tr>
<tr>
<td>Transcription levels</td>
<td>TM4-TM195</td>
<td>IRES</td>
<td>372</td>
<td>30–40</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>TM178-TM179</td>
<td>GAPDH</td>
<td>194</td>
<td>30–40</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>TM87-TM88</td>
<td>β-Actin</td>
<td>1,059</td>
<td>20–30</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>TM92-TM93</td>
<td>β-Actin</td>
<td>460</td>
<td>20–30</td>
<td>58</td>
</tr>
</tbody>
</table>

*For each pair of primers, the first is sense and the second is antisense. The sequences of the primers are as follows: TM4, TTTCCCTCATCGGAGAAGGACTTTG; TM87, TCCGGATCCACTGGAGAAGAAAAATTTCAGTG; TM178, ATCGGATCCAGGGTACAGGAG; TM27, TGGCCTGTTTGCATCGGAT; TM92, AGCCCTGTCGACAACAAC; TM195, GTGCACTAGTACGAAAAGCA; TM178, CATGGAGAAGGCTGCG; TM179, CAAAGTGGCATCGGATAC; and TM195, CGCGCTGTTTGTGGTGGC.

IRES of encephalomyocarditis virus (EMCV) works stably in mouse embryos (12, 22).

In this study, we showed that the Thieier's virus IRES functions in a bicistronic context in newborn and adult mice. Activity of the IRES was detected in all tissues examined. However, expression of the cistron under IRES control decreased with the age of the mouse, which could indicate an age dependency of the IRES function.

MATERIALS AND METHODS

Analysis of infected mice. Groups of nine 3-week old female FVB/N mice were anesthetized and then inoculated with 10⁵ PFU of the DA1 virus (26). Intracerebral inoculations were performed by injecting 40 μl of virus suspension into the right hemisphere. For intraperitoneal and oral (intragastric) inoculations, 250 μl of virus suspension was used. Groups of three mice infected by the different routes were sacrificed at 1, 3, or 6 weeks postinfection (p.i.). The heart, lungs, liver, spleen, esophagus (from tongue up to the stomach), mesenteries (including pancreas), kidneys, muscle, brain, spinal cord, and intestine were rapidly collected and homogenized in solution D (4 M guanidine thiocyanate, 25 mM sodium citrate [pH 7], 0.5% N-lauroylsarcosine, 0.1 M β-mercaptoethanol), and RNA was extracted as described by Chomczynski and Sacchi (10). The quality of RNA preparations was tested by gel electrophoresis. The presence of the virus in different tissues was monitored by reverse transcription-PCR (RT-PCR) under the conditions summarized in Table 1 and subsequent Southern blotting. As a control for cDNA synthesis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured by comparative RT-PCR.

Protein quantification. Tissues from transgenic mice were homogenized in luciferase lysis buffer (homogenized samples were kept on ice during the dissection of the other tissues) or ground to a powder in liquid nitrogen and then lysed in the same buffer. After a brief centrifugation for clarification, 20 μl of the sample was tested for Luc activity with a luminometer (Lumac Biocounter M 2000). Quantification of the protein concentration in the different samples was done by the Bradford method, and Luc activity was then calculated for 500 μg of total protein. For CAT measurement, a CAT enzyme-linked immunosorbent assay (CAT ELISA; Boehringer Mannheim) was used. In order to improve the sensitivity of the test, 150 μg of protein was tested, samples were incubated for 2 h in the antibody-coated wells, and the enhancer supplied in the kit was added at the reveting step.

Northern blotting. Polyadenylated RNA was isolated from various tissues of transgenic mice (QuickPrep Micro; Pharmacia Biotech). Samples were resuspended in one-third volume of H₂O and two-thirds volume of sample buffer (12.5% SDS; 0.5 M MOPS [morpholinopropanesulfonic acid] buffer [pH 7.4]; 0.4 M MOPS free acid [pH 7.0]; 0.1 M sodium acetate, and 20 mM EDTA). 8.3% formaldehyde, 62.2% deionized formamide, 825 μg of bromophenol blue per ml of 7 μl of ethidium bromide per ml). Samples were then denatured at 55°C for 10 min and run on a 1.2% agarose–37% formaldehyde gel for 6 h at 100 V before being transferred to a positively charged membrane (Porablot NY plus; Macherey-Nagel) with 10× SSC (SSC 20× = 3 M NaCl plus 0.3 M sodium citrate). The membrane was dried and then UV irradiated for 3 min. Prehybridization took place in 5% sodium dodecyl sulfate–0.5 M phosphate buffer (pH 7.4) for 5 to 6 h at 55°C. The membrane was hybridized overnight at 55°C in the same buffer with a 1,605-bp Luc probe labeled with 32P by random priming (Ready To Go; Pharmacia). The membrane was washed four times for 20 min each at 55°C in 1% sodium dodecyl sulfate–0.5 M phosphate buffer (pH 7.4) and exposed. The membrane was hybridized a second time with a 32P-labeled probe corresponding to nucleotides 81 to 1388 of β-actin.
A. Schematic representation (not to scale) of the pCJ9 and pCJ12 vectors. For construction of the pCJ9 vector, the BamHI-SalI fragment carrying the CAT-IRES-LUCIFERASE construct was inserted between the corresponding sites of the pHMG plasmid (28). The cat gene was amplified by PCR with primers that introduced BamHI and EcoRV sites at its 5' and 3' extremities, respectively. The lac gene was also amplified by PCR. An HpaI restriction site was introduced four codons downstream from its 5' extremity, and a SalI site was introduced at its 3' extremity. For the IRES-L fragment, the IRES from plasmid pTMDA (26) was digested at its 5' extremity by Smal at the restriction site at position 12. An HpaI restriction site was inserted by site-directed mutagenesis in the L protein, four codons downstream from the initiator AUG. The IRES-L fragment was fused at its 5' extremity to the cat gene by the EcoRV-Smal junction and at its 3' extremity to the lac gene by the HpaI/Hpal junction. For the pCJ12 vector, the IRES was deleted from the beginning to the ApaI restriction site at position 930 in pTMDA. The cat gene was thus fused to the deleted IRES by the EcoRV/ApaI junction, after filling in of the latter site with the Klenow enzyme. Restriction enzyme sites: EV, EcoRV; Sm, Smal; Ba, BamHI; Hp, HpaI; Sa, SalI; As, AspI. The minor splice donor sites are represented by the small triangles. SV40, simian virus 40.

B. Relative luciferase activity.

FIG. 1. (A) Schematic representation (not to scale) of the pCJ9 and pCJ12 vectors. For construction of the pCJ9 vector, the BamHI-SalI fragment carrying the CAT-IRES-LUCIFERASE construct was inserted between the corresponding sites of the pHMG plasmid (28). The cat gene was amplified by PCR with primers that introduced BamHI and EcoRV sites at its 5' and 3' extremities, respectively. The lac gene was also amplified by PCR. An HpaI restriction site was introduced four codons downstream from its 5' extremity, and a SalI site was introduced at its 3' extremity. For the IRES-L fragment, the IRES from plasmid pTMDA (26) was digested at its 5' extremity by Smal at the restriction site at position 12. An HpaI restriction site was inserted by site-directed mutagenesis in the L protein, four codons downstream from the initiator AUG. The IRES-L fragment was fused at its 5' extremity to the cat gene by the EcoRV-Smal junction and at its 3' extremity to the lac gene by the HpaI/Hpal junction. For the pCJ12 vector, the IRES was deleted from the beginning to the ApaI restriction site at position 930 in pTMDA. The cat gene was thus fused to the deleted IRES by the EcoRV/ApaI junction, after filling in of the latter site with the Klenow enzyme. Restriction enzyme sites: EV, EcoRV; Sm, Smal; Ba, BamHI; Hp, HpaI; Sa, SalI; As, AspI. The minor splice donor sites are represented by the small triangles. SV40, simian virus 40.

RESULTS

Neurotropism of Theiler's virus in FVB/N mice. The mice used in this study for transgenic production were of the FVB/N strain (43). These mice were chosen because they are inbred and their H-2^k haplotype renders them highly susceptible to Theiler's virus (9, 36). We first verified that the tropism of the virus in these mice was restricted mainly to the CNS as reported for other mouse strains (25, 44, 45). Twenty-seven female, 3-week-old mice were inoculated by either the oral, intraperitoneal, or intracerebral route with 10^6 PFU of the DA1 strain of Theiler's virus. At 1, 3, and 6 weeks p.i., groups of three mice inoculated by the different routes were sacrificed and 11 different tissues were collected from each mouse. The presence of the virus in each of these tissues was assessed by RT-PCR for nine mice (one mouse at each time point, inoculated by each route). For the remaining 18 mice (2 mice at each time point, inoculated by each route), virus was tested in tissues having shown a positive signal in previous studies: the heart, esophagus, brain, and spinal cord. The PCR products were identified by Southern blotting with a probe specific for DA1 cdNA. As shown in Fig. 2 and Table 2, Theiler's virus is almost exclusively neurotropic. Indeed, all mice inoculated intracerebrally showed a strong signal in the CNS. After intraperitoneal inoculation, one mouse showed a signal in the spinal cord and brain. After oral inoculation, one mouse showed a low signal in the spinal cord. Apart from the heart, where a low signal was also detected in a few mice, all of the other tissues tested were consistently negative for viral RNA, as they were in one uninfected FVB/N mouse (data not shown).

Construction of a bicistronic vector and analysis of IRES activity in vitro. The bicistronic vector pCJ9 (Fig. 1) was constructed as a tool for investigating the activity of the Theiler's virus IRES in vivo. The 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) promoter was chosen since it has been shown to be active in a large variety of tissues in transgenic mice, including the CNS (28). The 5' NC region of Theiler's virus was inserted between the cat and luc reporter genes. In vitro studies have shown that the first 500 nucleotides of the Theiler's virus 5' NC region are not necessary for efficient IRES activity in vitro (2). However, since neighboring regions might influence the conformation and/or function of the IRES (17), we chose to include the entire 5' NC region in the construct. Furthermore, the first five codons of the L viral protein, directly following the IRES, were fused to the Luc gene to maintain the structure of the IRES around the initiation codon. The activity of the IRES in the pCJ9 construct can be evaluated from the ratio of the Luc and CAT activities or from the ratio of the Luc activity to the amount of bicistronic mRna. A second bicistronic construct (pCJ12) (Fig. 1), in which the IRES is deleted, was also constructed in order to ensure that the expression of Luc from pCJ9 is due to IRES activity and not to splicing or ribosome readthrough. As expected, transfection of the pCJ9 and pCJ12 vectors in BHK-21 cells revealed 34 times less Luc expression from pCJ12 than from pCJ9 after normalization of CAT levels (Fig. 1).

Theiler's virus infection was reported to be restricted in certain cell lines (37). In order to determine if the IRES participates in this restriction, we transfected cell lines of different origins and species with the pCJ9 construct. IRES-mediated translation appeared to be proficient in all cell types tested (data not shown).

We also examined the possibility that the activation state of a cell could influence the activity of the IRES. The P388-D1 macrophage cell line, stably cotransfected with the pCJ9 and pZeosV plasmids, was activated with lipopolysaccharide (10
mu g of Escherichia coli lipopolysaccharide per ml). These cells were clearly activated as demonstrated by measurements of tumor necrosis factor alpha. In spite of this activation, no significant change in the activity of the IRES could be detected (data not shown).

Production of P_HMGCR-CAT-IRES-LUCIFERASE-(A)_n-transgenic mice. Three female founder mice, transgenic for the P_HMGCR-CAT-IRES-LUC-(A)_n construct, were obtained and named Cathy, Lucy, and Therese. Mice derived from the three lineages expressed the transgene, although the level was low.

TABLE 2. Summary of results of DA1 infection of FVB/N mice

<table>
<thead>
<tr>
<th>Inoculation route</th>
<th>Tissue</th>
<th>Presence of virus at wk p.i.:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mouse 1</td>
</tr>
<tr>
<td>Oral</td>
<td>Heart</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Esophagus</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>–</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>Heart</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Esophagus</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>–</td>
</tr>
<tr>
<td>Intracerebral</td>
<td>Heart</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Esophagus</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>+</td>
</tr>
</tbody>
</table>

a The presence of the virus in four different tissues of 27 mice (9 mice for each age group and inoculation route) was detected by RT-PCR followed by Southern blotting.
b –, no signal; +, very weak signal; ++, low signal; ++++, average signal; ++++, strong signal.
The founder mice Therese and Lucy had better expression profiles than the Cathy founder mouse and were thus retained for further study. Extraction of DNA from the tails of F1 Therese mice and digestion with the restriction enzyme PvuII revealed bands of the expected sizes by Southern blot analysis (Fig. 3). We also performed a Northern blot analysis of mRNAs from several tissues of Therese mice. A major transcript of 3.4 kb was detected in the testis (Fig. 4). This confirmed that the bicistronic CAT-IRES-Luc transcript was of the expected size and that no truncated Luc transcripts were present. Due to low transcription levels, no bands were detected in the other tissues. Accordingly, expression of the CAT protein was not detected except in the testes of males. Expression of the Luc protein, on the other hand, could be detected in most tissues, since luminometry tests are about 100 times more sensitive than CAT measurements. The activity of the IRES was therefore estimated from the ratio of Luc activity to the amount of bicistronic mRNA. Although we systematically tested the IRES activity in the testis, these results were not taken into account because expression of the transgene in this tissue was completely out of the range of that for other tissues.

Activity of the IRES in different tissues. We compared the Luc activity to the expression of the bicistronic mRNA transcript in different tissues of the transgenic mice. For each mouse, between 8 and 14 tissues were collected and assayed for CAT (data not shown) and Luc expression. The amount of bicistronic mRNA in these samples was estimated by comparative RT-PCR. GAPDH and/or β-actin was amplified from the various cDNA samples to check the efficiency of RT. Samples for which GAPDH or β-actin was poorly amplified were discarded from the analysis. Two Therese mice and 3 Lucy mice that were 1 day, 1 week, 3 weeks, and more than 3 months old were tested (a total of 206 tissues). Figure 5 shows representative data for 1-day-, 1-week-, 3-week-, and >3-month-old Therese mice.

For 1-day- and 1-week-old Therese mice, Luc expression was generally high in the brain and spinal cord, average in the heart, liver, stomach, and kidneys, and very low in the lungs, intestines, and spleen. Expression of the bicistronic mRNA transcript was consistently detected in the CNS. For a few mice, other tissues expressing reasonable amounts of Luc were also positive for the bicistronic mRNA transcript. For 3-week-old and adult Therese mice, GAPDH was reproducibly amplified from five or six tissues (heart, liver, kidneys, brain, intestines, and spinal cord). Transcription and translation of the bicistronic construct were observed only in the CNS and kidneys.

For Lucy mice we also observed a fairly good correlation...
FIG. 5. Luc activities and levels of bicistronic mRNA in various tissues of transgenic mice. Luc activity was measured by a luminometric assay, and mRNA levels were evaluated by comparative RT-PCR amplifications. Results are shown for Therese mice that were 1 day (A), 1 week (B), 3 weeks (C), and more than 3 months (D) old. The data presented here should be considered per mouse and not compared between different mice, as explained in Materials and Methods. H, heart; Lu, lungs; Lv, liver; Sp, spleen; St, stomach; K, kidneys; Li, large intestine; B, brain; S, spinal cord; I, small intestine.
between Luc activity and mRNA expression, but the results were more variable than those for the Therese lineage. Nevertheless, the IRES activity did not appear to stand out for one particular tissue.

Thus, for mice that were 1 day or 1 week old, our results suggest that there is no tissue specificity of the IRES. For adult mice, we cannot draw a conclusion, since the CNS and kidneys were the only tissues where transcription of the bicistronic construct could be measured.

In order to confirm these results and to get a better quantification of the IRES activity, we tested a second batch of 1-week-old Lucy mouse (A) and an adult Lucy mouse (B) were compared. The comparative RT-PCR amplifications were hybridized by Southern blotting and quantified with a PhosphorImager. The quantity of bicistronic mRNA was normalized to the amount of β-actin mRNA. Scales are in arbitrary units. H, heart; Lu, lungs; Lv, liver; K, kidneys; B, brain.

**FIG. 6.** Luc activity and bicistronic mRNA expression in Lucy mice. Luc activity and bicistronic mRNA expression in different tissues of a 1-week-old Lucy mouse (A) and an adult Lucy mouse (B) were compared. The comparative RT-PCR amplifications were hybridized by Southern blotting and quantified with a PhosphorImager. The quantity of bicistronic mRNA was normalized to the amount of β-actin mRNA. Scales are in arbitrary units. H, heart; Lu, lungs; Lv, liver; K, kidneys; B, brain.

We studied the tropism of Theiler’s virus in the mouse strain used to produce the transgenic mice. This allowed us to relate the results of the activity of the IRES in the transgenic mice to the replication of the virus in different tissues. As expected, Theiler’s virus was highly neurotropic. Indeed, there was much more viral RNA in the CNS than in any other tissue, even after oral or intraperitoneal inoculation. Surprisingly, though, no virus was found in the digestive tract, even though this virus was reported to naturally infect the intestine of its host. This could be due to the presence of RT-PCR inhibitors in that specific tissue, which would lower the detection efficiency. On the other hand, the number of infected cells might be very low and might have gone undetected in the context of the whole intestine. It is possible that the DAI virus strain has a decreased ability to infect the intestine because it was adapted to grow in cell culture. It is clear, though, that this strain is not completely degenerated, since the virus reached the CNS in one of nine mice after either intragastric or intraperitoneal inoculation.

For several reasons, analysis of the IRES activity in vivo proved to be arduous. First and mainly, the transcription level of the bicistronic construct was low. Hence, RNA quantification had to be done by comparative RT-PCR instead of dot blot hybridization. By this method, quantification is difficult, especially in view of the variation due to the difference in the quality of RNA samples from various tissues. However, in spite of this variation, consistent results were obtained from many samples and from mice derived from two transgenic lineages. Second, one cannot exclude the possibility that the turnover of average bicistronic mRNA levels, suggesting poor IRES function in this particular tissue.

**IRES activity in relation to the age of the mouse.** We reproducibly observed that expression of the Luc protein strongly decreased with the age of the mouse. In order to test if this could be due to a decrease in IRES activity, we compared Luc activity to the amount of bicistronic mRNA between tissues from mice of different ages processed in parallel. The tissues selected for this study were the brain and kidneys for the Therese lineage and the heart and brain for the Lucy lineage. We chose tissues that showed sufficient expression of the transgene and gave RNA of consistent and good quality. Total RNAs and proteins were extracted from homogenized tissues of groups of three mice that were 1 day, 1 week, 3 weeks, or more than 3 months old. Luc activity was calculated for 500 μg of total protein, and RNA was subjected to comparative RT-PCR after adjustment of RNA amounts on agarose gels. Figure 7 shows that for mice of the Lucy lineage, expression of the Luc protein diminishes sharply between mice that are 1 day and more than 3 months old. The quantity of the bicistronic mRNA transcript, on the other hand, is stable or increases with the age of the mouse. For mice of the Therese lineage, the quantity of bicistronic mRNA is more variable from mouse to mouse. Nevertheless, calculation of IRES activity from the ratio of Luc activity to the amount of bicistronic mRNA confirms the results obtained with the Lucy lineage in that the important drop in Luc expression could be due to a change in the activity of the IRES. We should, however, point out that the ratio of the total amount of protein to the total amount of RNA was found to increase 1.5- to 4-fold for an adult mouse compared to a 1-day-old mouse (data not shown). Nevertheless, even if this correction factor is taken into account, the difference in IRES activity between 1-day-old and adult mice is still a minimum of sixfold.

**DISCUSSION**

We studied the tropism of Theiler’s virus in the mouse strain used to produce the transgenic mice. This allowed us to relate the results of the activity of the IRES in the transgenic mice to the replication of the virus in different tissues. As expected, Theiler’s virus was highly neurotropic. Indeed, there was much more viral RNA in the CNS than in any other tissue, even after oral or intraperitoneal inoculation. Surprisingly, though, no virus was found in the digestive tract, even though this virus was reported to naturally infect the intestine of its host. This could be due to the presence of RT-PCR inhibitors in that specific tissue, which would lower the detection efficiency. On the other hand, the number of infected cells might be very low and might have gone undetected in the context of the whole intestine. It is possible that the DAI virus strain has a decreased ability to infect the intestine because it was adapted to grow in cell culture. It is clear, though, that this strain is not completely degenerated, since the virus reached the CNS in one of nine mice after either intragastric or intraperitoneal inoculation.

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the Luc protein and RNA transcripts could vary from tissue to tissue or in tissues of different ages. The conclusions of this study thus await confirmation from future studies performed with other reporter genes. To our knowledge, this is the first study to investigate IRES-driven tissue specificity in newborn and adult transgenic mice.

We hypothesized that the IRES might participate in determining Theiler’s virus tropism. Apparently, this is not the case; even if one takes into account the fact that our results are only semiquantitative, there certainly is not sufficient variation in IRES activity between the CNS and other tissues to explain the near-exclusive neurotropism of the virus. These results are in agreement with those of Kim et al. (22), who demonstrated that β-galactosidase under the control of the EMCV IRES is expressed throughout the bodies of transgenic mouse embryos. Accordingly, all of the cellular factors that have been found to control IRES function in vitro turned out to be ubiquitous factors. For poliovirus, the picture seems to be different. Introduction of mutations in the IRES region of this virus was accompanied by a clear decrease in neurovirulence for mice but not for monkeys, even though a wild-type IRES is active in both species (41). Could there be a host specificity but not a tissue specificity of the IRES? It is not unheard of that host factors involved in IRES translation could vary between species (41). Could there be a host specificity but not a tissue specificity of the IRES? It is not unheard of that host factors involved in IRES translation could vary between species (41). Could there be a host specificity but not a tissue specificity of the IRES? It is not unheard of that host factors involved in IRES translation could vary between species (41). Could there be a host specificity but not a tissue specificity of the IRES? It is not unheard of that host factors involved in IRES translation could vary between species (41). Could there be a host specificity but not a tissue specificity of the IRES? It is not unheard of that host factors involved in IRES translation could vary between species (41). Could there be a host specificity but not a tissue specificity of the IRES? 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Interestingly, expression of the polypyrImidine tract binding protein, which is thought to participate in IRES function, was reported to be higher in fetal than in adult mouse tissues (30). Mice that are 1 day or 1 week old die rapidly after infection by Theiler's virus (35). At 3 weeks old, animals survive the infection and the virus persists. Older animals generally manage to clear this virus. It is evident that host factors such as the immune response or the permeability of the blood-brain barrier are the main elements that determine the different outcomes of infection in young and adult mice. It is possible, though, that the virulence of Theiler's virus is equally modulated by certain viral factors, like IRES activity.

In recent years, expression vectors containing picornavirus IRESs have become popular. The advantages of IRES sequences in gene targeting and generation of polycistronic transcripts are indisputable (29). However, several questions concerning the function of an IRES in vivo and even in vitro remain to be answered. In vitro, we observed that the IRES functions in cell lines of different origins and species but also in cells in different activation states (39). In vivo, we tested whether the function of the IRES could be tissue specific or age dependent. As discussed above, IRES activity seemed to be ubiquitous in mouse tissues. This is, of course, essential if IRES would have been interesting for targeting gene expression in vivo. Further studies will be required to confirm these preliminary results and to investigate other important issues such as IRES function at the cellular level or in humans in regard to gene therapy.

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