Alphavirus replicon approach to promoterless analysis of IRES elements

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Abstract

Here we describe a system for promoterless analysis of putative internal ribosome entry site (IRES) elements using an alphavirus (family Togaviridae) replicon vector. The system uses the alphavirus subgenomic promoter to produce transcripts that, when modified to contain a spacer region upstream of an IRES element, allow analysis of cap-independent translation of genes of interest (GOI). If the IRES element is removed, translation of the subgenomic transcript can be reduced >95% compared to the same transcript containing a functional IRES element. Alphavirus replicons, used in this manner, offer an alternative to standard dicistronic DNA vectors or in vitro translation systems currently used to analyze putative IRES elements. In addition, protein expression levels varied depending on the spacer element located upstream of each IRES. The ability to modulate the level of expression from alphavirus vectors should extend the utility of these vectors in vaccine development.

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Introduction

The study of IRES elements has traditionally been accomplished with systems that produce dicistronic RNAs. These systems are usually plasmids that contain an RNA polymerase II (pol II) promoter upstream of two reporter genes that are separated by an IRES element (Macejak and Sarnow, 1991). The pol II promoter is recognized by cellular RNA polymerase II that then transcribes a capped mRNA. The 5′ reporter gene is translated in a cap-dependent manner while the IRES element upstream of the 3′ reporter gene initiates translation in a cap-independent manner. A key to assigning IRES activity to any sequence placed between the two reporter genes is dependent upon the absence of a cryptic promoter or splice acceptor site within the 5′ reporter gene or putative IRES element. The presence of either of these can lead to the production of a second transcript that contains the 3′ reporter gene in a configuration for cap-dependent translation (Van Eden et al., 2004; Holcik et al., 2005). Northern blot analysis is typically used to demonstrate that only one transcript is produced from dicistronic plasmids.

Due to concerns that northern blot analysis is not sensitive enough to detect the existence of low levels of transcript produced by a cryptic promoter or splice acceptor site (Kozak, 2001b, 2003), promoterless dicistronic systems, RNAi-based techniques and RT-PCR analysis have been employed to confirm the activity of IRES elements (Han and Zhang, 2002). Dicistronic plasmids modified to lack pol II DNA dependent RNA promoters have also been used to determine whether any promoter activity can be found associated with a putative IRES element. In addition, in vitro transcribed RNA from the promoterless dicistronic vector can be introduced into cultured cells or used in in vitro translation reactions to monitor putative IRES activity, thus obviating the nuclear aspects of transcription (Han and Zhang, 2002).

Venezuelan equine encephalitis virus (VEE) is a member of the Togaviridae family within the Alphavirus genus. Alphaviruses have a single-stranded positive-sense RNA genome of approximately 11.4 kilobases (kb) in length. The genome is capped at the 5′ end and polyadenylated at the 3′ end. The viral nonstructural proteins (nsP1–nsP2–nsP3–nsP4) are encoded in the 5′ two thirds of the genome, and the structural proteins (capsid–E3–E2–6K–E1) are encoded in the 3′ one third of the genome. The nonstructural proteins are translated in cells
directly from the capped viral genomic RNA. The structural proteins are translated from a subgenomic RNA that is transcribed from a 26S promoter that is present on the full-length negative-stranded RNA replication intermediate (reviewed in Strauss and Strauss, 1994). Replication takes place exclusively in the cytoplasm of cells. A number of alphavirus expression systems have been developed by deleting the structural protein coding region from the genome, thus generating a self-replicating RNA or replicon vector (Bredenbeek et al., 1993; Liljestrom and Garoff, 1991; Pushko et al., 1997; Xiong et al., 1989; Yamaguchi and Shirako, 2002). Heterologous genes may be cloned downstream of the 26S promoter in place of the structural protein genes. When the replicon RNA is introduced into cells, the nonstructural proteins are translated, which then replicate the viral RNA and express the GOI cloned downstream of the 26S promoter to high levels. The robust expression of GOIs is due to the high level production of the subgenomic mRNA transcripts from the 26S promoter. The cytoplasmic localization of alphavirus replication and the ability of the replicon to produce subgenomic RNAs to high levels led us to ask whether such a system could be used to study the functional characteristics of IRES elements. Here we describe an alphavirus replicon system developed to analyze IRES activity that is not confounded by the possibility of either cryptic DNA promoters or RNA splicing events and that offers increased sensitivity over traditional dicistronic DNA vectors.

Results

Expression of CAT from dicistronic IRES replicon vectors

A number of approaches can be used to demonstrate that an IRES element is responsible for programming cap-independent translation of a gene. Three approaches are graphically depicted in Fig. 1. One approach is to reverse the sequence of the element, a second is to make a deletion in a critical stem loop region and a third approach is to simply delete the putative IRES element to inactivate the IRES in the context of a dicistronic RNA (Fig. 1). After reversing, inactivating or deleting the IRES in a dicistronic RNA, the absence or reduction of expression of the second reporter gene, relative to the expression measured from an active IRES, indicates that the IRES is controlling cap-independent translation.

Dicistronic replicon vectors were constructed that produce a subgenomic RNA coding for a 5′ reporter gene, luciferase (LUC), separated from a 3′ reporter gene, chloramphenicol acetyl transferase (CAT), by either a functional EV71 IRES or an inactivated EV71 (ΔEV71) IRES element (Fig. 2A). Dicistronic replicon RNAs were transcribed, electroporated into Vero cells and both LUC and CAT expression were analyzed. The average LUC and CAT activity determined from three separate experiments is summarized in Fig. 2B. The results indicate that the replicon vector coding for the dicistronic RNA with a functional IRES expressed both LUC protein (cap-dependent) and CAT protein (cap-independent). These results also demonstrate that the dicistronic replicon vector with the inactivated IRES expressed CAT at only background levels (Fig. 2B). Northern analysis, of total cellular RNA extracted from the electroporated cells, using a CAT-specific probe revealed that only the expected subgenomic RNA species was detectable (Fig. 3), indicating that the CAT protein detected was not being translated from unexpected RNA transcripts produced by either dicistronic replicon vector. These data indicate that an IRES element can control cap-independent translation of a reporter gene in the context of a dicistronic subgenomic RNA produced by an alphavirus replicon vector.

Fig. 1. Schematic representation of dicistronic RNAs and modifications to the IRES element used to demonstrate IRES control of translation. LUC, luciferase gene; IRES, internal ribosome entry site; CAT, chloramphenicol acetyl transferase.

Fig. 2. Luciferase and CAT expression analysis of dicistronic replicon vectors. (A) Schematic representation of dicistronic replicon vectors. LUC, luciferase gene; EV71, human enterovirus 71 IRES element; CAT, chloramphenicol acetyl transferase gene; Black arrow, 26S subgenomic promoter; solid black circle, 5′ cap structure; p(A), 3′ poly A sequence. (B) Results of luciferase and CAT expression analysis represent the average activity detected from three separate experiments. Error bars represent 1 standard deviation. RLU, relative light units. CAT expression values were normalized based on luciferase activity detected from each replicon. Neg. control; un-transfected Vero cell lysates.
Expression of CAT from IRES replicon vectors lacking a 5′ reporter gene

To determine whether insertion of sequences other than the LUC gene upstream of the IRES would allow for specific cap-independent translation to occur on a capped monocistronic mRNA, additional replicons were constructed. Because the optimal length and nucleotide sequence of the spacer region were unknown, random DNA fragments were cloned between the IRES sequence and the subgenomic promoter in the replicon vectors. Fig. 4A shows a schematic of how the spacer-IRES-CAT replicons were generated. To extend the preceding dicistronic replicon analysis, pairs of replicon constructs containing the same spacer sequence and either an EV71 (type I) or EMCV (type II) IRES sequence were generated. In addition, null replicons were constructed that contained only the spacer region and the CAT gene (Fig. 4A). If CAT expression is cap-independent in the spacer-IRES replicons, there should be little, if any CAT expression in the null replicons which lack an IRES element.

To evaluate CAT expression levels, virus-like replicon particles (VRP) were generated with each spacer-IRES-CAT or null (spacer-CAT) replicon and used to infect Vero cells. The average CAT activity determined from three separate experiments is summarized in Fig. 4B. Protein expression was quantified by CAT-specific ELISA, and subgenomic RNA production was followed by Northern blot analysis using a CAT gene-specific probe. Densitometric analysis of Northern blots was also conducted using a probe specific for the 12S ribosomal RNA to normalize the amount of RNA loaded in each lane. The subgenomic RNA transcripts detected for each null (spacer-CAT) replicon were set to a value of 1.0 and compared to the subgenomic transcript amount from the respective matching spacer-IRES-CAT replicons. An example of Northern blot analysis is shown in the lower portion of Fig. 4B. In most instances, the relative amount of subgenomic transcript for each null replicon was more than for the matched spacer-IRES-CAT replicons (Fig. 4B). Even though the null replicons produced robust amounts of subgenomic RNA transcripts, very little CAT protein was detected by ELISA, demonstrating that the spacer region inhibited cap-dependent translation of the CAT reporter gene. In contrast, even though fewer subgenomic transcripts were produced, each of the spacer-IRES-CAT replicons expressed significant amounts of CAT protein (Fig. 4B). These data indicate that the IRES element in each spacer-IRES-CAT replicon efficiently programmed cap-independent translation.

Mouse immunization with different spacer-IRES replicons

As shown in Fig. 4B, the level of CAT expression from the different IRES replicons, as determined in cell culture assays, was dependent on the spacer element located upstream of each IRES element. To determine whether in vitro expression levels correlated with in vivo immunogenicity and efficacy, the C-terminal portion of the heavy chain of botulinum neurotoxins A and B (BoNT/HcA and BoNT/HcB, respectively) was cloned into spacer-IRES replicons. The relative expression levels for the BoNT/Hc proteins were determined for each spacer-IRES replicon by densitometric analysis of Western blots probed with BoNT/Hc specific antibodies and compared to the expression level of the same genes expressed directly as a subgenomic transcript without a spacer-IRES cassette. The results of each comparison, normalized to the expression from the non-spacer-IRES replicon, are shown in Table 1. Replicons containing the EV71 IRES generally expressed the BoNT/Hc genes at a higher level as compared to the replicons that did not contain the spacer-IRES cassette. In contrast, replicons containing the EMCV IRES uniformly expressed at a lower level as compared to a non-spacer-IRES replicon expressing the same BoNT/Hc gene (Table 1).

Spacer IRES BoNT/Hc replicons, with different protein expression profiles as determined in cell culture, were selected and evaluated for immunogenicity and protective efficacy in a murine BoNT challenge model. One EMCV and one EV71 spacer replicon were selected for both BoNT/HcA and BoNT/HcB.

VRP were generated for each replicon, and mice were immunized twice at a 4-week interval by two routes of administration, subcutaneous (sc) and intramuscular (im). Serum collected from the immunized animals was analyzed by ELISA, and the mice were challenged 4 weeks after the boost with 1000 LD50 of BoNT A or BoNT B. The results of the ELISA analysis and protection from lethal BoNT challenge are summarized in Table 2. Animals immunized with the spacer-IRES BoNT/HcA replicon that showed the lowest level of expression in cultured cells produced the lowest anti-BoNT A antibody responses (a geometric mean titer (GMT) of ~3 logs) and were only partially protected from challenge (Table 2). Only 1 of 10 subcutaneously inoculated mice and 4 of 10 intramuscularly inoculated mice survived challenge. However,
animals inoculated with the spacer-IRES BoNT/HcA replicon that showed the highest level of expression in vitro produced higher anti-BoNT A antibody responses (GMT $\sim 5$ logs) and were completely protected from challenge. In contrast, animals vaccinated with either the low or high level expressing spacer-IRES BoNT/HcB replicons produced similar immune responses (GMT $\sim 5$ logs) and were completely protected from a lethal BoNT B challenge.

Discussion

Here we describe a system for examining the activity of IRES elements using an alphavirus replicon vector. These constructs make use of the alphavirus subgenomic promoter to produce mRNA transcripts containing IRES elements that allow for the analysis of cap-independent translation of a GOI. Furthermore, the incorporation of spacer elements with IRES elements provides the ability to modulate protein expression from the replicon system.

In addition to their use as expression and vaccine vectors, these new alphavirus replicon vectors provide an attractive alternative to the standard dicistronic DNA vectors or in vitro translation systems currently used to analyze putative IRES elements. The possible presence of either cryptic promoters or splice acceptor sites present in putative IRES elements has caused concern about the use of traditional dicistronic DNA vectors (Han and Zhang, 2002; Kozak, 2001a, 2001b; Liu et al., 2005; Wang et al., 2005), and the replicon system offers a way to eliminate such concerns for IRES analyses. Because the replicon is RNA and does not have a DNA stage during replication, there is no concern with respect to cryptic promoters. In addition, replication of the replicon RNA occurs entirely in the cytoplasm of cells therefore nuclear splicing events cannot occur. Although in vitro translation systems and
transfection of non-replicating dicistronic RNAs into cells in culture can also obviate the concerns described above, the sensitivity of these assays is limited by the amount of input RNA (Han and Zhang, 2002; Thompson and Sarnow, 2003). It has been reported that members of the Alphavirus genus (Sindbis and Semliki Forest virus) activate protein kinases involved in the cellular responses to stress factors (Ventoso et al., 2006). Activation of these kinases catalyzes the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α) which in turn down-regulates cellular protein synthesis. It is possible that alphavirus replication, by virtue of stimulating cellular stress responses which down-regulate normal protein synthesis, may provide an excellent environment to detect IRES activity. However, the replicon system may not be suited for studying IRES elements if a non-stress cellular environment is required.

Subgenomic transcription from the 26S promoter amplifies the number of transcripts containing the IRES sequence, thereby increasing the chance of detecting IRES activity and potentially allowing for the study of RNA–RNA or RNA–protein interactions. Greater than 70,000 viral transcript copies per cell have been detected in alphavirus infected cells (Powers et al., 1996). The large number of transcripts containing the IRES of interest should readily allow biochemical studies involving association of initiation factors with IRES elements to direct ribosome binding, as has been done with other IRES elements (Holcik and Korneluk, 2000; Kieft et al., 2001; Kim et al., 2001, 2003; Lomakin et al., 2000; Mitchell et al., 2001, 2003; Otto et al., 2002; Pestova et al., 1998; Pickering et al., 2003; Spahn et al., 2001). In addition, concerns over the small differences between background expression measured for the negative control and putative IRES activity may be addressed.

Table 1
Relative expression levels for BoNT/Hc IRES replicons

<table>
<thead>
<tr>
<th>BoNT/HcA replicons</th>
<th>Spacer</th>
<th>342</th>
<th>357</th>
<th>383</th>
<th>579</th>
<th>749</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRES</td>
<td>EMCV</td>
<td>EMCV</td>
<td>EMCV</td>
<td>EMCV</td>
<td>EMCV</td>
<td>EMCV</td>
<td>No IRES</td>
</tr>
<tr>
<td>Normalized expression</td>
<td>0.4</td>
<td>0.6</td>
<td>0.6</td>
<td>0.5</td>
<td>0.5</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>IRES</td>
<td>EV71</td>
<td>EV71</td>
<td>EV71</td>
<td>EV71</td>
<td>EV71</td>
<td>EV71</td>
<td>No IRES</td>
</tr>
<tr>
<td>Normalized expression</td>
<td>1.4</td>
<td>0.7</td>
<td>1.1</td>
<td>1.2</td>
<td>1.4</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BoNT/HcB replicons</th>
<th>Spacer</th>
<th>342</th>
<th>357</th>
<th>383</th>
<th>579</th>
<th>749</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRES</td>
<td>EMCV</td>
<td>EMCV</td>
<td>EMCV</td>
<td>EMCV</td>
<td>EMCV</td>
<td>EMCV</td>
<td>No IRES</td>
</tr>
<tr>
<td>Normalized expression</td>
<td>0.7</td>
<td>0.6</td>
<td>0.5</td>
<td>0.6</td>
<td>0.4</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>IRES</td>
<td>EV71</td>
<td>EV71</td>
<td>EV71</td>
<td>EV71</td>
<td>EV71</td>
<td>EV71</td>
<td>No IRES</td>
</tr>
<tr>
<td>Normalized expression</td>
<td>0.7</td>
<td>1.4</td>
<td>1.3</td>
<td>0.8</td>
<td>1.1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

a Cells electroporated with replicon RNAs were analyzed in parallel by IFA using a VEE anti-nsP2 specific antibody to ensure equivalent transfection efficiency among the different constructs.

b Spacer fragment size in base pairs.

c BoNT/Hc-specific Western blot analysis and subsequent densitometric examination were carried out on protein lysates generated from electroporated cells. BoNT/Hc expression from a replicon with no IRES or spacer was set at a value of 1.0. The BoNT/Hc expression detected from spacer IRES replicons is shown as relative values compared to the BoNT/Hc expression detected from a replicon with no IRES or spacer present.

Table 2
Serological responses and protection of mice vaccinated with IRES BoNT/Hc replicons

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Relative expression level</th>
<th>Route of immunization</th>
<th>ELISA GMT</th>
<th>Survivor/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>342/EMCV/BoNT/Hc A</td>
<td>Low</td>
<td>sc</td>
<td>11,143</td>
<td>1/10</td>
</tr>
<tr>
<td>342/EV71/BoNT/Hc A</td>
<td>High</td>
<td>sc</td>
<td>409,600</td>
<td>10/10</td>
</tr>
<tr>
<td>342/EMCV/BoNT/Hc A</td>
<td>Low</td>
<td>im</td>
<td>8,445</td>
<td>4/10</td>
</tr>
<tr>
<td>342/EV71/BoNT/Hc A</td>
<td>High</td>
<td>im</td>
<td>382,170</td>
<td>10/10</td>
</tr>
<tr>
<td>Control (BoNT A challenge)</td>
<td>na</td>
<td>sc</td>
<td>129</td>
<td>0/5</td>
</tr>
<tr>
<td>Control (BoNT A challenge)</td>
<td>na</td>
<td>im</td>
<td>49</td>
<td>0/5</td>
</tr>
<tr>
<td>342/EMCV/BoNT/Hc B</td>
<td>Low</td>
<td>sc</td>
<td>89,144</td>
<td>10/10</td>
</tr>
<tr>
<td>357/EV71/BoNT/Hc B</td>
<td>High</td>
<td>sc</td>
<td>89,144</td>
<td>10/10</td>
</tr>
<tr>
<td>342/EMCV/BoNT/Hc B</td>
<td>Low</td>
<td>im</td>
<td>102,400</td>
<td>10/10</td>
</tr>
<tr>
<td>357/EV71/BoNT/Hc B</td>
<td>High</td>
<td>im</td>
<td>95,543</td>
<td>10/10</td>
</tr>
<tr>
<td>Control (BoNT B challenge)</td>
<td>na</td>
<td>sc</td>
<td>84</td>
<td>0/5</td>
</tr>
<tr>
<td>Control (BoNT B challenge)</td>
<td>na</td>
<td>im</td>
<td>48</td>
<td>0/5</td>
</tr>
</tbody>
</table>

a Animals were vaccinated twice with 10^7 IU of each VRP at 4-week intervals.
b Relative BoNT/Hc protein expression level was determined by Western blot analysis.
c Routes of immunization: sc, subcutaneous; im, intramuscular.
d GMT, reciprocal geometric mean titers just prior to BoNT challenge.
e Control groups received irrelevant VRP.
f na: not applicable.
using an alphavirus system (Kozak, 2001b, 2003). Given the large difference in expression between null (spacer-CAT) replicons and spacer-IRES-CAT replicons demonstrated in this report (>50-fold in several examples, Fig. 4B), it may be possible to study IRES elements with weak activity. This approach may also have particular utility in the study of putative cellular IRES elements. Indeed, the stress environment created by replication of the replicon RNA is not unlike the environment in which many cellular IRES elements have been identified (Cornelis et al., 2000; Pyronnet et al., 2000; Coldwell et al., 2001). That is, most cellular IRES elements have been identified under conditions where cap-dependent translation has been interdicted. In addition, use of the replicon system may eliminate spurious results due to cryptic promoters and splice acceptors that have been shown to be present in some previously described cellular IRES elements (Han et al., 2003; Liu et al., 2005; Wang et al., 2005). Moreover, alphaviruses replicate in many cell types, ranging from mammalian to insect (Corsini et al., 1996). IRES containing replicon RNAs can be introduced into these cell types either directly by electroporation of the replicon RNA or by infection with VRP. In this way, IRES function may be analyzed in a wide array of cell types that were previously refractory due to the lack of cell type specific DNA promoters to drive dicistronic vector transcription.

The presence of a spacer region upstream of an IRES element on a subgenomic transcript resulted in a cap-independent translation of the CAT reporter gene. The null replicons (spacer-CAT) resulted in limited CAT expression via cap-dependent translation, while replicons with spacer sequences upstream of the IRES elements (spacer-IRES-CAT) efficiently expressed CAT protein. Northern blot analysis indicated that the robust expression from spacer-IRES-CAT replicons was not due to production of a larger amount of subgenomic transcripts. In fact, in most instances, the CAT expression detected from spacer-IRES-CAT replicons was translated from fewer transcripts than were present for the matched null replicons. Although possible, it is unlikely that the CAT expression detected in these experiments was the result of translation off nicked replicon RNAs. The RNAs resulting from a nicking event, that retain the CAT gene, would not be capped and as a result would not be translated in a cap-dependent manner. In addition, nicked RNAs would not be amplified because they would lack the 5′ non-coding region required for replication. Therefore, nicked RNAs would not be expected to add any significant expression of CAT protein. To demonstrate that little if any CAT expression results from translation of replicon RNA that is not replicated, uncapped spacer-IRES replicon RNAs were electroporated into Vero cells and CAT protein expression was analyzed. No CAT expression was detected by ELISA (data not shown). For these reasons, we conclude that the CAT expression detected in these experiments is due to subgenomic transcription of the replicon RNA rather than spurious translation from nicked replicon RNAs.

Interestingly, replicons with the same IRES element but with different spacer sequences expressed the GOI at different levels, thus providing a new mechanism for controlling gene expression from alphavirus vectors. One possible explanation for the range of expression may be that the spacer sequence influences (both positively and negatively) the ability of the IRES elements to recruit initiation factors by altering the secondary structure of the IRES element itself. In addition, the sequence of a GOI may also affect the function of an IRES element. This is suggested by the results that showed that the same spacer-IRES combination did not always result in the highest levels of expression for each gene examined (i.e., the CAT, BoNT/Hc A and BoNT/Hc B genes). Examples of mRNA secondary structure affecting translation efficiency are well documented (Jaffrey et al., 1993; Kozak, 1986, 1990, 1991; Ray et al., 1983; Sonenberg, 1994). It is also possible that the presence of upstream initiation codons (AUG) in the spacer sequence may interfere with ribosome scanning which in turn may enhance cap-independent translation via the IRES element (Arrick et al., 1991; Kozak, 1989). The number of AUG codons present in each spacer is as follows: 342 spacer (6 AUG), 357 spacer (3 AUG), 383 spacer (4 AUG), 579 spacer (9 AUG) and 749 spacer (16 AUG). The number of AUG codons present in each spacer sequence did not uniformly predict the expression level, but the 749 spacer-IRES replicons (containing 16 AUG codons) did express higher levels of those genes examined here. It is likely that the combination of secondary structure and the presence of initiation codons in each spacer sequence results in the different expression levels demonstrated in these studies. Analysis of predicted secondary structure for each spacer-IRES-GOI combination may help optimize each combination for efficient cap-independent translation.

The VEE replicon system has been shown to be an effective means of immunizing animals against a wide variety of antigens (Balasuriya et al., 2000, 2002; Davis et al., 2000; Hevey et al., 1998; Lee et al., 2001, 2002, 2003; Pushko et al., 2001; Wilson et al., 2001; Wilson and Hart, 2001). Upon inoculation, VRP infection of cells leads to efficient expression of the antigenic protein. VRP have also been shown to target antigen-presenting dendritic cells (MacDonald and Johnston, 2000) and are capable of inducing a broad array of Th-1 biased immune responses to the encoded gene product, including cytotoxic T lymphocytes (CTL), lymphoproliferative responses, and neutralizing and protective antibody responses (Balasuriya et al., 2002; Davis et al., 2000; Hevey et al., 1998; Lee et al., 2001, 2002, 2003; Nelson et al., 2003; Pushko et al., 2001; Wilson et al., 2001; Wilson and Hart, 2001). The high protein expression levels attained with alphavirus replicon systems are thought to contribute to its effectiveness (Liljestrom and Garoff, 1991; Pushko et al., 1997). Other groups have shown, using DNA expression systems, that increasing the level of antigen expression can increase the immunogenicity of that antigen in animal models (Deml et al., 2001; Ko et al., 2005; Narum et al., 2001; Qiu et al., 1999; Sasaki et al., 2004; Stratford et al., 2000; zur Megede et al., 2000). Utilizing the various spacer-IRES replicons that express the GOI at different levels, we were able to address a similar question. When animals were immunized with a low-expressing BoNT/HcA replicon (as measured by expression in Vero cells), the vaccine elicited lower ELISA responses and only 5 out of 20 animals were protected from a lethal BoNT A challenge. However, animals immunized with the high-expressing BoNT/HcA replicon were completely
protected against a similar challenge. In contrast, both the low and the high expressing BoNT/HcB replicons provided complete protection to the inoculated animals and stimulated similar antibody responses in those animals, perhaps due to inherent immunogenicity differences among these two expressed products. However, it is clear that, depending on the antigen tested, relatively small differences in in vitro expression level may result in large differences in the immune response observed in vivo. Selecting a BoNT/HcA replicon with an intermediate expression level from the two examined in this study may allow insight into the minimum amount of expression required to impart a completely protective BoNT A immune response in mice.

There are a number of advantages to having the ability to control the level of protein expression from a replicon vector. First, as indicated above for BoNT/HcA, higher expression resulted in the stimulation of a more protective immune response. Second, reduced expression could be advantageous for the expression of genes where high levels of the expressed products are toxic to cells or in which only low levels of expressed protein are desirable, as in the co-expression of immunomodulators. Because the gene of interest encoded in the replicon vector is expressed in the cells initially used to produce the VRP, low expression of a toxic gene may increase VRP yields, and in these cases a compromise must be selected between VRP yield and in vivo immune response desired. Third, it may be advantageous to modify the expression of two proteins from a single replicon or combine replicons that express proteins at different levels to tailor an immune response in the context of a multivalent vaccine.

In conclusion, we demonstrate that alphavirus replicon vectors can be used to examine the cap-independent translation imparted by both type I (EV71) and type II (EMCV) IRES elements (Hellen and Sarnow, 2001; Thompson and Sarnow, 2003), and the RNA nature of alphavirus systems helps to eliminate two frequent criticisms associated with IRES analyses imparted by both type I (EV71) and type II (EMCV) IRES elements. Furthermore, we have shown that the use of an IRES in combination with varying spacer sequences can alter the level of expression of that gene. The ability to design alphavirus vectors that express GOI at varying levels should allow selection of replicon-based vaccines that are both immunogenic and produce efficient VRP yields in a manufacturing setting.

Materials and methods

Construction of transfer cloning vectors

A transfer vector (pCDNA3.3) was prepared for accepting IRES and GOI sequences. Plasmid pCDNA3.1(+) (Invitrogen, Carlsbad, CA) was digested with restriction enzyme BamHI and treated with T4 DNA polymerase to create blunts ends that eliminated the unique BamHI restriction site, resulting in generation of pCDNA3.2. The pCDNA3.2 DNA was further digested with restriction enzyme XbaI and also treated with T4 DNA polymerase to remove the unique XbaI restriction site, resulting in generation of pCDNA3.3.

An intermediate cloning vector containing the multiple cloning site (MCS) from a VEE replicon vector was prepared by ligating an ∼250 bp ApaI/NotI MCS fragment into ApaI/NotI linearized pBluescript KS+ (Stratagene, La Jolla, CA) DNA, generating pKS-rep2. The EMCV IRES (a type II IRES element) was digested from pD1+2+3 (Kaminski et al., 1995) with restriction enzymes EcoRI and BamHI and ligated into EcoRI and BamHI linearized pKS-rep2 DNA, generating pKS-rep2/EMCV. The EMCV IRES and MCS sequence from the pKS-rep2/EMCV vector was PCR amplified using primers EMCVF (Ascl).2 and EMCVR (Ascl).1 (Table 3). The EMCV-MCS PCR product was digested with Ascl restriction enzyme and ligated into Ascl linearized VEE replicon (pERK) vector DNA, generating pERK/EMCV. To complete the transfer cloning vector, pERK/EMCV DNA was digested with EcoRV and NotI restriction enzymes and the 862 bp EcoRV/NotI fragment was isolated and then ligated into EcoRV and NotI digested pCDNA3.3 DNA, generating pCDNA3.3/EMCV. The sequence of the EMCV IRES and associated multiple cloning sites was confirmed in the pCDNA3.3/EMCV vector before preparing additional constructs.

Table 3

<table>
<thead>
<tr>
<th>Primer name</th>
<th>5' primer sequence 3'</th>
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<tr>
<td>EMCVF (Ascl).2</td>
<td>TGGCGCGCCGCTCGGAATTTCCTCCCTCCCTCC</td>
</tr>
<tr>
<td>EMCVR (Ascl).1</td>
<td>AAGCCGCCCTTCTATGGAAGCCTGCTGCC</td>
</tr>
<tr>
<td>F'-CAT (BamHI)</td>
<td>GCTGGATCCATGGGAAAGAAAAATACCTGAGA</td>
</tr>
<tr>
<td>R'-CAT (XbaI)</td>
<td>CGATCTAGATTACGCCCCGCTGCCACCTCA</td>
</tr>
<tr>
<td>CAT-F (Ascl)</td>
<td>GGGCCGCCCACTAGGAGAAAAAAAATACCTGAGA</td>
</tr>
<tr>
<td>CAT-R (Ascl)</td>
<td>GCGGCGCGCTTACGCCCCGCCGCTGCCACCTCA</td>
</tr>
<tr>
<td>dc/MS (EcoRI) F</td>
<td>CGAATTTCTTTAAACAGGTGTTGGTTGA</td>
</tr>
<tr>
<td>dc/MS (BamHI) R</td>
<td>CCGGGATCCGCTCAACTGTATTGAGGTATTAATATAAG</td>
</tr>
<tr>
<td>ΔEV71-F</td>
<td>CCCAGTAAACCAACCTAGGAAGGTTTTGCTGCACGCTCCTCCCC</td>
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<tr>
<td>ΔEV71-R</td>
<td>GGCTCATTCCTACCTCCTCACCACAAAAAGAGGACGTGAGAGGGGG</td>
</tr>
<tr>
<td>luc BoNR</td>
<td>TTTCGCCATACCACCGGCGAGTTCATGTAAGCAGC</td>
</tr>
<tr>
<td>luc Pmel F</td>
<td>GGGAAGTTAAAAACATGGGAGGGCCAAAAACATGGGAAAG</td>
</tr>
</tbody>
</table>

Materials and methods

Construction of transfer cloning vectors

A transfer vector (pCDNA3.3) was prepared for accepting IRES and GOI sequences. Plasmid pCDNA3.1(+) (Invitrogen, Carlsbad, CA) was digested with restriction enzyme BamHI and treated with T4 DNA polymerase to create blunts ends that eliminated the unique BamHI restriction site, resulting in generation of pCDNA3.2. The pCDNA3.2 DNA was further digested with restriction enzyme XbaI and also treated with T4 DNA polymerase to remove the unique XbaI restriction site, resulting in generation of pCDNA3.3.

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The human enterovirus 71 (EV71) IRES element (a type I IRES element) (Thompson and Sarnow, 2003) was also cloned into the pCDNA3.3 transfer vector. The EV71 IRES element (strain 7423/MS/87) was PCR amplified from pde/MS DNA (Thompson and Sarnow, 2003) using primers dc/MS (EcoRI) F and dc/MS (BamHI) R (Table 3). The EV71 IRES PCR product was digested with EcoRI and BamHI restriction enzymes and ligated into pCDNA3.3/EMCV digested with EcoRI and BamHI to replace the EMCV sequence with the EV71 sequence, generating pCDNA3.3/EV71. The EV71 IRES region, in pCDNA3.3/EV71, was sequenced to verify that no nucleotide changes were introduced during the PCR amplification.

Construction of inactivated IRES intermediate vectors

An inactivated EV71 IRES (ΔEV71) was produced by site directed mutagenesis using primers ΔEV71-F and ΔEV71-R (Table 3) and a QuikChange mutagenesis kit (Stratagene) to delete five bases, from position 279 to position 283. The pCDNA3.3/ΔEV71 vector was sequenced to confirm the absence of the essential bases.

Construction of spacer-IRES replicon constructs

The CAT reporter gene was cloned into each of the pCDNA3.3 transfer vectors. The CAT gene was amplified using primers F′-CAT (BamHI) and R′-CAT (XbaI) (Table 3). The PCR product was digested with BamHI and XbaI restriction enzymes and ligated into BamHI and XbaI linearized pCDNA3.3/EMCV, pCDNA3.3/EV71 and pCDNA3.3/ΔEV71 plasmids. The following constructs were produced in this manner pCDNA3.3/EMCV/CAT, pCDNA3.3/EV71/CAT and pCDNA3.3/ΔEV71/CAT. The CAT gene was sequenced in each construct to ensure that no errors had been introduced during PCR amplification.

To generate a replicon vector with a functional EMCV/CAT cassette downstream of the 26S promoter, pCDNA3.3/EMCV/CAT DNA was digested with AscI restriction enzyme to release a 1303 bp EMCV/CAT fragment. The AscI digested EMCV/CAT fragment was then ligated into AscI linearized pERK vector DNA, generating pERK/EMCV/CAT. Random DNA fragments were cloned between the EMCV IRES sequence and the VEE subgenomic promoter at a unique EcoRV site located in the pERK vectors (Fig. 4A). The random DNA fragments cloned between the 26S promoter and the EMCV IRES were generated by digesting pCDNA3.1(−) DNA (Invitrogen) with AluI restriction enzyme. The AluI restriction enzyme cuts frequently within the pCDNA3.1(−) plasmid resulting in blunt ended fragments ranging in size from 6 bp to 706 bp. The AluI digested pCDNA3.1(−) fragments were ligated into EcoRV linearized pERK/EMCV/CAT. Individual clones were analyzed by restriction analysis to demonstrate the presence of a spacer sequence, and selected clones were sequenced to determine the size of the spacer fragment in each new replicon vector.

Spacer replicon constructs containing the EV71 IRES were produced by digesting the pCDNA3.3/EV71/CAT plasmid with AscI restriction enzyme and ligating the EV71/CAT AscI fragments in place of the AscI fragment removed from the pERK/EMCV/CAT spacer vectors described above.

Null (spacer-CAT) replicons were constructed in two steps. First the CAT gene was PCR amplified with CAT-F (AscI) and CAT-R (AscI) primers (Table 3) and cloned into pCR2.1 TOPO (Invitrogen), generating pCR2.1/CAT. The CAT gene was sequenced to ensure that no errors were introduced during amplification. To generate null replicons, each spacer replicon was digested with AscI restriction enzyme to remove the IRES-CAT fragment. The CAT gene was digested out of pCR2.1/CAT with AscI restriction enzyme and ligated into each AscI linearized spacer replicon. Restriction analysis was carried out, and clones with the CAT gene in the correct orientation were selected.

Construction of spacer-IRES BoNT/Hc replicon vectors

Human codon-optimized BoNT A/Hc and BoNT B/Hc genes were synthesized (Blue Heron, Bothell, WA) and cloned into the spacer replicons in two steps. First the genes were cloned into transfer vector pCDNA3.3/EV71 or pCDNA3.3/EMCV. Each gene was cloned as a BamHI/XbaI fragment into BamHI and XbaI digested pCDNA3.3/EV71 or pCDNA3.3/EMCV DNA, downstream of the IRES element, generating pCDNA3.3/EMCV/BoNT/AHc and pCDNA3.3/EMCV/BoNT/BHc. The CAT gene was amplified with AscI to release a IRES-BoNT/Hc-gene cassette. The IRES-BoNT/Hc-gene AscI fragments were then ligated into AscI linearized spacer replicons and the orientation of the inserts determined by restriction analysis. Clones with BoNT/Hc-genes in the sense orientation were selected and sequenced to ensure that no errors were introduced during the cloning process.

Construction of dicistronic replicon vectors

Replicon constructs, with no spacer inserts, containing either the EV71/CAT or ΔEV71/CAT cassettes were produced by digesting the pCDNA3.3/EV71/CAT and pCDNA3.3/ΔEV71/CAT plasmids with AscI restriction enzyme. The EV71/CAT and ΔEV71/CAT AscI fragments were then ligated into AscI linearized pERK DNA, generating pERK/EV71/CAT and pERK/ΔEV71/CAT vectors. To generate dicistronic replicon vectors that contain the LUC gene in the 5′ (cap-dependent) position and the CAT gene in the 3′ (cap-independent) position, the firefly luciferase (LUC) gene was PCR amplified from pde/MS DNA (Thompson and Sarnow, 2003) using PCR primers luc BamR and luc Pmel F (Table 3). The PCR product was digested with BamHI and then treated with T4 DNA polymerase to produce a blunt end. The LUC gene was then further digested with Pmel restriction enzymes and ligated into EcoRV linearized pERK/EV71/CAT and pERK/ΔEV71/CAT DNAs. The orientation of the LUC gene was determined by restriction analysis, and clones with the LUC gene in the correct orientation were selected, generating pERK/LUC/EV71/CAT and pERK/LUC/ΔEV71/CAT dicistronic replicon vectors.
RNA transription, electroporation and VRP production

Vero cells were maintained at 37 °C in an atmosphere containing 5% CO₂. The cells were grown in Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS, HyClone, Logan, UT), MEM non-essential amino acid solution (Invitrogen) and antibiotic–antimycotic (GIBCO). Capped replicon RNAs were in vitre transcribed using a T7 RiboMax kit (Promega, Madison WI) following the manufacturer’s instructions, supplemented with 7.5 mM CAP analog (Promega), from NotI linearized replicon plasmid. RNAs were purified using RNEasy purification columns (Qiagen, Valencia, CA) following the manufacturer’s instructions. For replicon RNA only electroporation, 3 × 10⁷ Vero cells suspended in PBS (GIBCO) were combined with 30 μg of RNA in 0.2 cm cuvettes. The cells were pulsed 4 times using a BIO-RAD Gene Pulser (BIO-RAD, Hercules, CA) with the instrument set at 290 V and 25 μF. After electroporation, the cells were suspended in 35 ml of OptiPro media (Invitrogen), 10 ml of each cell suspension was seeded into 25 cm² flasks and the flasks were incubated at 37 °C in 5% CO₂. The cells were grown in Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 5% FBS, 5% CO₂, and antibiotics and incubated at 37 °C for an additional 24 h. VRP were purified by affinity column chromatography using Cellufine sulfate resin (Chisso Corporation, Tokyo, Japan) and stored at −80 °C until used.

Analysis of luciferase and CAT expression from dicistronic replicon vectors

The expression of luciferase and CAT protein was analyzed from dicistronic replicon vectors. Vero cells were electroporated with 30 μg of replicon RNA (as described above). Luciferase and CAT expression was quantified by luciferase assay and CAT ELISA, using electroporated cell lysates and commercially available Luciferase and CAT ELISA kits (Boehringer Mannheim, Indianapolis, IN) following the manufacturer’s instructions. Luciferase activity was used to normalize the CAT expression level between the two replicon vectors.

Analysis of CAT expression from IRES and null replicon vectors

The expression of CAT protein from spacer-IRES-CAT replicons and null (spacer-CAT) replicons was compared. Vero cells were infected (MOI=3) with VRP generated with each spacer-IRES-CAT or null (spacer-CAT) replicon. Cell lysates were prepared ~20 h post-infection, and CAT ELISA (Boehringer Mannheim) analysis was carried out according to the manufacturer’s instructions. Total cellular RNA was collected using an RNAwiz extraction kit (Ambion, Inc., Austin, TX) following the manufacturer’s instructions from cells infected in parallel with each VRP. Northern blot analysis was carried out on each sample using probes specific for CAT and 12S ribosomal RNAs. After chemiluminescent processing of Northern blots, the densitometry function of the Labworks Analysis Software associated with an Epi Chemi II Darkroom (UVP) was used to quantitate the relative quantity of both subgenomic CAT and 12S ribosomal RNA transcripts for each sample.

BoNT Western blot analysis

For Western blot analysis, cells were lysed in TX100 lysis buffer (10 mM Tris, 1 mM EDTA, 0.25 M NaCl, 1% Triton X-100) for 10 min at 4 °C. After lysis, nuclei were removed by microcentrifugation (12,000 RPM (12,800×g) for 10 min at 4 °C), and the total cell protein concentration for each sample was determined using a BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer’s instructions. Equal amounts of sample protein were combined with sodium dodecyl sulfate (SDS) gel loading buffer and heated for 10 min at 95 °C before electrophoresis through a 4–12% NuPAGE Bis–Tris polyacrylamide gel (Invitrogen). The separated proteins were transferred to Immun-Blot PVDF membranes (BIO-RAD) using x-cell II blotting system and NuPage buffer (Invitrogen). Proteins were detected using goat anti serum specific for BoNT/HeA and BoNT/HeB proteins, alkaline phosphatase (AP)-conjugated anti-goat antibody (Sigma-Aldrich, St. Louis, MO) and AP-specific color development solution (BIO-RAD) for visualization. Relative amounts of detected proteins were compared using the densitometry function of the Labworks Analysis Software associated with the Epi Chemi II Darkroom (UVP, Inc., Upland, CA).
Vaccination and challenge of mice

Mice were inoculated by the sc or im route with 10^7 IU of each VRP diluted in 200 μl of PBS on day 0 and day 28 and were challenged intraperitoneally on day 56 with 1000 median lethal doses (MLD50) of BoNT in diluted in 100 μl PBS containing 0.2% gelatin.

BoNT/Hc ELISA

The quantity of antibody present in the serum of vaccinated animals just before challenge was measured by ELISA as previously described (Lee et al., 2001). Briefly, microtiter plates were coated with purified C. botulinum to express BoNT protein diluted in PBS. Titers for antibodies to BoNT are defined as the reciprocal of the last dilution with an A405 of ≥0.1 after correction for background. Titers below 2.00 log10 and above 5.61 log10 were estimated. Serum from individual animals was assayed in duplicate and used to calculate a geometric mean titer for the group.

Northern blot analysis

Northern blot analysis was carried out on RNA isolated from Vero cells after VRP infection or replicon RNA electroporation. Total cellular RNA was isolated from the Vero cells 20 h post electroporation or infection using an RNAwiz extraction kit (Ambion, Inc., Austin, TX) following the manufacturer’s instructions. The RNAs were quantitated using a UV/VIS spectrophotometer, and 2–3 μg of each RNA was run on a 1% glyoxal agarose gel. The separated RNAs were transferred to a BrightStar-Plus membrane (Ambion) by passive transfer using a TurboBlotter system (Whatman) according to the manufacturer’s protocols. The nucleic acids were then UV cross-linked to the membranes using a HL-2000 HybiLinker (UVP Inc.). The membranes were blocked using UltraHyb solution (Ambion) for 1 h at 68 °C and then probed overnight with UltraHyb solution containing ~100 ng of CAT and 12S ribosomal RNA specific psoralen-biotin probes (Ambion) at 68 °C. The CAT and 12S ribosomal RNA specific psoralen-biotin probes were generated according to the manufacturer’s instructions (Ambion). After overnight hybridization, the blot was processed for chemiluminescent RNA detection using a BrightStar BioDetect kit (Ambion) according to the manufacturer’s instructions and visualized by exposing the blots to film.

Acknowledgments

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References


