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IRES-dependent replication of Venezuelan equine encephalitis virus makes it highly attenuated and incapable of replicating in mosquito cells

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Abstract

The development of infectious cDNA for different alphaviruses opened an opportunity to explore their attenuation by extensively modifying the viral genomes, an approach that might minimize or exclude the reversion to the wild-type, pathogenic phenotype. Moreover, the genomes of such alphaviruses can be engineered to contain RNA elements that would be functional only in cells of vertebrate, but not insect, origin. In the present study, we developed a recombinant VEEV that is more attenuated than TC-83 and capable of replicating only in vertebrate cells. This phenotype was achieved by rendering the translation of the viral structural proteins, and ultimately viral replication, dependent on the internal ribosome entry site of encephalomyocarditis virus (EMCV IRES). This recombinant virus was viable, but required additional, adaptive mutations in nsP2 that strongly increased its replication rates. In spite of efficient replication in cultured vertebrate cells, the genetically modified VEEV demonstrated a highly attenuated phenotype in newborn mice, and yet induced protective immunity against VEEV infection.

Introduction

The *Alphavirus* genus in the *Togaviridae* family contains a number of important human and animal pathogens. These viruses are widely distributed on all continents, except for the Antarctic region, and represent a significant public health threat (Griffin, 2001; Strauss and Strauss, 1994). Under natural conditions, most of the alphaviruses are transmitted by mosquitoes, in which they cause a persistent, life-long infection that has little effect on the biological functions of the vector. In vertebrates infected by mosquitoes during their blood meal, alphaviruses cause an acute infection, characterized by a high-titer viremia that is a prerequisite of infection of new mosquitoes and virus circulation in nature.

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Venezuelan equine encephalitis virus (VEEV) is one of the most pathogenic members of the alphavirus genus. It continuously circulates in South, Central and North America and causes sporadic epidemics and epizootics that involve humans, horses and other domestic animals. During the most recent major outbreak in Venezuela and Colombia (1995) involving subtype IC VEEV, about 100,000 human cases occurred, with over 300 fatal encephalitis cases estimated (Rivas et al., 1997). During VEEV epizootics, equine mortality due to encephalitis can reach 83%, and, while the overall mortality rate is low in humans (<1%), neurological disease, including disorientation, ataxia, mental depression, and convulsions, can be detected in up to 14% of infected individuals, especially children (Johnson and Martin, 1974). The human disease caused by VEEV is characterized as a febrile illness with chills, severe headache, myalgia, somnolence and pharyngitis. Young and old individuals develop a reticuloendothelial infection with severe lymphoid depletion, followed by encephalitis. The result of the CNS infection is an acute meningoencephalitis that leads to the death of neuronal cells (Dal Canto and Rabinowitz, 1981). The neurologic signs appear within 4-10 days of the onset of illness and include seizures, paresis, behavioral changes and coma.

In spite of the continuous threat of VEEV epidemics, no safe and efficient vaccines have been designed for this virus. The attenuated TC-83 strain of VEEV was developed more than four decades ago by serial passage of a highly virulent Trinidad donkey (TRD) strain of VEEV in guinea pig heart cells (Berge, Banks, and Tigertt, 1961). Presently, TC-83 is still the only available vaccine for laboratory workers and military personnel. Over 8,000 people have been vaccinated (Alevizatos, McKinney, and Feigin, 1967; Burke, Ramsburg, and Edelman, 1977; Pittman et al., 1996), and the cumulative data unambiguously demonstrate that nearly 40% of all vaccinees develop a disease with some symptoms typical of those in cases of natural VEE, including fever, systemic illness and other adverse effects (Alevizatos, McKinney, and Feigin, 1967). This TC-83 strain universally kills newborn, but not adult, mice after i.c. and s.c. inoculation (Paessler et al., 2003), and thus provides a good starting point for further attenuation and study of the effects of the mutations on viral pathogenesis.

The VEEV genome is a nearly 12-kb-long, single-stranded RNA molecule of positive polarity that mimics the structure of cellular mRNAs. The genome RNA contains both a 5′ methylguanylate cap and a 3′ polyadenylate tail (Kinney et al., 1989), features which allow translation of viral proteins by host cell machinery immediately after release of the genome RNAs from the nucleocapsids. The 5′ two-thirds of the genome is translated into nonstructural proteins (nsPs) that comprise the viral components of the replicative enzyme complex required for replication of the viral genome and transcription of the subgenomic RNA. The subgenomic RNA corresponds to the 3′ third of the genome. It is synthesized from the subgenomic promoter and translated into the viral structural proteins. The attenuated phenotype of the VEEV strain TC-83 is the result of two mutations in the strain TRD genome: one of them replaced an amino acid at position 120 in E2 glycoprotein, and the second changed nt 3 in the 5′UTR (Davis et al., 1991; Kinney et al., 1993; Kinney et al., 1989; White et al., 2001). Thus, because of the very high mutation rate of the alphaviruses, the reversion of TC-83 to a pathogenic phenotype remains a great concern in the event that the appropriate selective conditions, such as virus passage in vivo, would occur. Moreover, VEEV TC-83 is capable of replicating in mosquito cells, and infecting mosquitoes following vaccination (Pedersen, Robinson, and Cole, 1972); therefore, its transmission by mosquitoes remains possible. Ideally, live arbovirus vaccine strains should not be transmissible by arthropod vectors, because circulation among reservoir hosts could lead to unforeseen changes that might include increased virulence. This is especially true for attenuated strains, produced from wild-type viruses that rely on small numbers of attenuating mutations that may be subject to reversion, or for genetically modified strains that might evolve in unanticipated ways, if they undergo vector-borne circulation. The former risk was underscored by detection of the VEEV TC-83 vaccine strain in mosquitoes collected in Louisiana during 1971 (Pedersen, Robinson, and Cole, 1972), outside the

epizootic/epidemic area that was restricted to Texas. It was also unambiguously demonstrated that point mutations in alphavirus genome can dramatically increase the alphavirus epidemic potential (Brault et al., 2004; Tsetsarkin et al., 2007).

The development of infectious cDNA for alphaviruses opened an opportunity to explore their attenuation by extensively modifying the viral genomes, an approach that might minimize or exclude the reversion to the wt, pathogenic phenotype. Moreover, the genomes of such alphaviruses can be engineered to contain RNA elements that would be functional only in cells of vertebrate, but not insect, origin. Thus, such extensive mutations could prevent transmission of the genetically modified viruses by mosquito vectors.

In the present study, we developed a recombinant, attenuated VEEV, which can only replicate in cells of vertebrate origin. This phenotype was achieved by causing the translation of the viral structural proteins and, ultimately, viral replication to depend on the internal ribosome entry site of encephalomyocarditis virus (EMCV IRES). The previously published data strongly indicated that this IRES suppose to function very inefficiently in driving translation in insect cells (Finkelstein et al., 1999; Woolaway et al., 2001). The designed recombinant VEEV was viable, but additional, adaptive mutations in VEEV nsP2 strongly increased its replication rates. In spite of efficient replication in cultured vertebrate cells, the genetically modified VEEV demonstrated a highly attenuated phenotype in newborn mice. Thus, the designed strategy of genome modification appears to be applicable to other alphaviruses for development of new vaccine candidates.

Results

Recombinant VEEV TC-83- based viruses

The rationale of this study was to develop alphaviruses capable of efficient replication in vertebrate cells, but not those of mosquito origin. Therefore, replication of such viruses had to depend on proteins or RNA sequences that function only in vertebrate, but not in insect, cells. To achieve this, we made the expression of the alphavirus structural proteins dependent on the EMCV IRES. The designed IRES did not contain the poly(C) sequence, but retained the first 4 codons of EMCV polyprotein to achieve the most efficient translation of VEEV TC-83 structural genes. In later experiments, we confirmed that these additional amino acids had no negative effect on virus replication, but had a detectable positive effect on the translation of viral structural proteins (data not shown). In one of the constructs, VEEV/IRES, the IRES sequence was cloned into the subgenomic RNA downstream of the nt 30 of the intact 5′UTR (Fig. 1A). Therefore, such virus was expected to be capable of subgenomic RNA synthesis. In another recombinant, VEEV/mutSG/IRES, the subgenomic promoter was inactivated by 13 synonymous point mutations (Figs. 1A and B), which were expected to prevent reversion to an active SG RNA promoter. To promote synthesis of the VEEV structural proteins, the IRES sequence was cloned to replace the 5′UTR in the subgenomic RNA.

The genome RNAs of VEEV/IRES, VEEV/mutSG/IRES and unmodified VEEV TC-83 were synthesized in vitro and transfected into BHK-21 cells. In the infectious center assay, the VEEV/IRES RNA demonstrated the same infectivity as did the RNA of VEEV TC-83, and developed plaques of a uniform size (Figs. 1A and C). This was a strong indication that no additional, adaptive mutations were required for productive replication of the designed virus. VEEV/IRES replicated to titers exceeding 10^9 PFU/ml, but these final titers and virus replication rates were significantly slower than those of VEEV TC-83 (Fig. 1D). BHK-21 cells transfected with another recombinant viral genome, VEEV/mutSG/IRES, which had a mutated subgenomic promoter, produced infectious virus very inefficiently (Fig. 1D). In the infectious center assay, this construct developed mainly pinpoint plaques, and their number was difficult to estimate. Surprisingly, this virus demonstrated further evolution upon serial passage and

rapidly developed variants that produced larger plaques (Fig. 1C and data not shown). The growth curve presented in Fig. 1D represents the release of both small and large plaque-forming viruses.

Thus, the results of these experiments indicated that, at least in the context of the VEEV/IRES genome, EMCV IRES could produce structural proteins at levels sufficient for VEEV replication. The construct with a mutated subgenomic promoter, VEEV/mutSG/IRES, produced a defective-in-replication virus that could evolve for more efficient replication.

Analysis of adaptive mutations in VEEV/mutSG/IRES

The evolution of VEEV/mutSG/IRES to a large plaque phenotype suggested an accumulation of additional mutations in the viral genome. The reversion to the wt genome sequence was an impossible event due to the large number of introduced point mutations, so the location of adaptive mutations was difficult to predict. To identify the mutations, we randomly selected 5 plaques of VEEV/mutSG/IRES from the samples harvested at 24 h post electroporation, and sequenced the entire genomes (including the 3′ and 5′UTRs) of two plaque-purified variants. The list of the mutations identified is presented in Fig. 2A. The majority of them were synonymous and were not present in the known cis-acting RNA elements. Thus, their effect on virus replication was very unlikely. However, the genomes of both plaque isolates contained the same mutation in the nsP2 protein, Y_{370}→C, and one of the genomes had the next encoded aa changed as well (K_{371}→Q).

To test the effect of the mutations on virus replication, we cloned Y_{370}→C and both Y_{370}→C and K_{371}→Q into the original VEEV/mutSG/IRES construct (Fig. 2B) and compared the RNA infectivity, virus replication rates and plaque sizes with those of the original VEEV/mutSG/IRES and other constructs. The same mutations were also cloned into the VEEV TC-83 genome to test their effect on the replication of this parental virus. The IRES-encoding genome RNAs with either one or both mutations in the genome, VEEV/mutSG/IRES/1 and VEEV/mutSG/IRES/2, demonstrated the same infectivity in the infectious center assay as did VEEV TC-83 RNA, and rescued viruses formed uniform plaque, having sizes similar to those of the VEEV TC-83 (data not shown). They also demonstrated a strong increase in growth (Figs. 2C), but the effect of the second mutation was barely detectable. Thus, taken together, the data indicated that the Y_{370}→C mutation in the nsP2 had a strong positive effect on virus replication, while the second mutation did not noticeably improve it. When introduced into the VEEV TC-83 genome, the same mutations did not have any detectable effect on the rates of virus replication or on final titers (Fig. 2C), which suggests that the replication enhancement was specific to the VEEV/mutSG/IRES variant.

The identified aa changes (Y_{370}→C and K_{371}→Q) could represent only a fraction of possible mutations leading to the efficient replication of the VEEV/mutSG/IRES. Therefore, in parallel experiments, we sequenced nt 2161-2959 in the other three plaque purified variants, isolated from the samples, harvested at 24 h post transfection of the in vitro-synthesized RNAs, and in 5 plaque-purified viruses, isolated from the virus stock after additional 3 passages in Vero cells. (The latter cells are usually used in vaccine development and production.) We anticipated that such passaging would lead to the selection of the most efficiently replicating viruses. The list of the identified mutations is presented in Fig. 3A. Sequencing was performed directly from the RT-PCR-derived DNA fragments; therefore, the presented mutations represent the consensus sequences in the plaque-derived virus population.

All of the isolates contained mutations in the sequence, corresponding to the carboxy terminal fragment of the RNA helicase domain of nsP2, and all of the altered amino acids were located between aa 348-424. Moreover, the most common mutation, both in the original virus stock, generated after electroporation, and in the passaged pool, was Y_{370}→C. This was an indication

that it likely has one of the most prominent effects on replication; therefore, the above-described variant with this particular mutation, VEEV/mutSG/IRES/1 (Fig. 2B), was used in the experiments outlined in the following sections.

Effect of the nsP2 Y_{370}→C mutation on virus replication

Identification of the adaptive mutation in the carboxy terminal fragment of the nsP2-associated RNA helicase was surprising and did not suggest any obvious explanation for the increase in VEEV/mutSG/IRES replication. This mutation could possibly have a stimulatory effect either on RNA replication, or the viral structural proteins translation, or viral particle formation, or replicative complexes compartmentalization, etc. However, the most expected effect was an increase in viral RNA synthesis. Therefore, we transfected BHK-21 cells with in vitro-synthesized genomes of different VEEV variants, metabolically labeled the newly synthesized viral RNAs with [^{3}H]uridine in the presence of ActD for 4 h beginning 4.5 h post electroporation, and then analyzed the RNA by electrophoresis in agarose gels (Fig. 4A). As expected, VEEV/IRES was capable of subgenomic RNA synthesis, which indicated to us that the IRES, introduced at the 3′ end of the subgenomic RNA 5′UTR, did not interfere with the subgenomic promoter activity. VEEV/mutSG/IRES and its variants with adaptive mutations in nsP2 produced no detectable SG RNAs. Thus, 13 mutations introduced into the promoter sequence of these genomes abolished the transcription of the subgenomic RNA. Surprisingly, the adaptive mutations in the nsP2 did not have a noticeable effect on RNA genome replication, and VEEV/mutSG/IRES/1 and VEEV/mutSG/IRES/2 genome RNAs replicated as efficiently as did the originally designed VEEV/mutSG/IRES genome. Moreover, the genome RNA replication of all of the variants was very similar to that of VEEV TC-83. No effect of these mutations was detected in the context of the original VEEV TC-83 RNA as well (see lanes corresponding to VEEV/1 and VEEV/2). This finding strongly suggested that adaptation did not result in an increase in RNA replication.

The synthesis of viral structural proteins was evaluated at 12 h post electroporation (Fig. 4B). By that time, VEEV/IRES- and VEEV/mutSG/IRES-specific capsid and likely other structural proteins were synthesized ~2-fold less efficiently than in the cells transfected with VEEV TC-83 RNA. This reasonably small difference does not explain the more than 4 and 7 orders of magnitude lower infectious titers of VEEV/IRES and VEEV/mutSG/IRES viruses, respectively (compared to the titers of VEEV TC-83), detected in samples harvested at 12 h post transfection (Fig. 1D). Moreover, in this and other experiments, we did not detect strong difference between the synthesis of viral proteins in BHK-21 cells containing the original VEEV/mutSG/IRES genomes versus VEEV/mutSG/IRES/1 and VEEV/mutSG/IRES/2, which had adaptive mutations in the nsP2 (Fig. 4B). The labeled proteins in cells infected with the IRES-encoding viruses were distinguished by their the noticeably slower processing of p62 protein and in presence of two additional bands, identified by mass spectrometry as heat-shock proteins Hsp90 and Hsp72. The biological significance of their induction is not yet clear, but might result from abnormalities in viral structural protein(s) folding, leading to stress development in the cells with viral structural proteins expressed from the IRES. The differences in accumulation of viral structural proteins were more obvious. By 12 h post transfection with VEEV/IRES and VEEV/mutSG/IRES RNAs, cells accumulated 4- and 8-fold less capsid, respectively, than did those transfected with VEEV TC-83 RNA (Fig. 4C). The adaptive mutations in nsP2 led to a detectably higher (2-3-fold) accumulation of viral capsid and envelope proteins in the cells, containing VEEV/mutSG/IRES/1 and VEEV/mutSG/IRES/2 variants, compared to those having original VEEV/mutSG/IRES. The difference between synthesis and accumulation of structural proteins might be an indication of their higher degradation rates. However, it is still difficult to understand how 2-3-fold higher levels of protein expression at 12 h post transfection (Fig. 4C) lead to ~1000-fold higher titers of viruses with mutated nsP2, compared to the titers of VEEV/mutSG/IRES (Fig. 2C).

In additional experiments, we assessed the intracellular distribution of the viral glycoproteins in cells infected with VEEV TC-83, VEEV/mutSG/IRES and VEEV/mutSG/IRES/1, and analyzed the presence of these proteins on the cell surface by staining with VEEV-specific antibodies. No noticeable difference in the distribution of the glycoproteins was identified. We also tested the possibility that the adaptive mutations caused the formation of an additional packaging signal in the viral genome; the mutation-containing fragment (corresponding to nt 2533-2950 of the VEEV genome) was cloned into the 3′ UTR of the VEEV/mutSG/IRES genome, and the recombinant, in vitro-synthesized RNA was tested in the infectious center assay. No increase was detected in plaque size or virus titers, compared to those of the original VEEV/mutSG/IRES (data not shown). In another variant, we cloned a subgenomic promoter and a VEEV capsid-coding sequence into the 3′UTR of VEEV/mutSG/IRES genome to test whether the additional capsid expression from the subgenomic RNA would increase the efficiency of virus replication. This modification also did not have any positive effect on virus titers (data not shown).

Thus, taken together, the above-described complex analysis did not point to obvious mechanistic explanations for the very inefficient replication of the original VEEV/mutSG/IRES or for the positive effect of the detected mutations in VEEV nsP2 on the virus replication. However, the main aim of the study was in the development of the VEEV variants, whose replication depends on the EMCV IRES function, and both VEEV/IRES and VEEV/mutSG/IRES/1 appeared to meet this goal.

Replication of the IRES-dependent VEEV variants in the mosquito cells and mosquitoes

The accumulated data about alphavirus replication unambiguously demonstrate these viruses' genetic instability and high rate of evolution, resulting in the deletion of any heterologous genes (Gorchakov et al., 2007; Thomas et al., 2003), particularly if they have a negative effect on virus replication. Therefore, one of the critical questions of the study was whether the designed EMCV IRES insertions would be stable and render the viruses incapable of replication in mosquito cells. To test this, we infected $C_7$10 mosquito cells with VEEV/IRES and VEEV/mutSG/IRES viruses harvested at 24 h post electroporation of the in vitro-synthesized RNAs into BHK-21 cells. VEEV/mutSG/IRES was used instead of the above-described VEEV/mutSG/IRES/1, with an adaptive mutation Y_{370}→C in the nsP2, to test the entire library of the variants, released after the RNA transfection, for the ability to establish replication in mosquito cells. Notably, in this library, ~50% of the variants likely contained an indicated Y_{370}→C mutation (Fig. 3A).

On the first passage, at 48 h post infection of $C_7$10 cells, the titer of VEEV/IRES approached 1.5×10^{10} PFU/ml, and a similar titer was detected in the stock, harvested after the second passage (Figs. 5A and B). The titers of VEEV/mutSG/IRES, in contrast, were 150 PFU/ml after the first passage, which likely reflected residual virus used for infection rather than nascent virus produced in the mosquito cells, and below the detection limit after the second passage (Figs. 5B). In additional experiments, we attempted to passage in $C_7$10 cells the plaque-purified variants of VEEV/mutSG/IRES that contained adaptive mutations in nsP2, used for design VEEV/mutSG/IRES/1. No infectious virus was ever recovered after two blind passages (data not shown). In another set of experiments, VEEV TC-83, VEEV/IRES, VEEV/mutSG/IRES and VEEV/mutSG/IRES/1 RNAs were directly electroporated into C_7/10 cells, and titers of the released viruses were tested at different times post transfection. Initially, VEEV/IRES demonstrated a strong delay in replication, but ultimately replicated as efficiently as did VEEV TC-83 (Fig. 5C). No infectious virus was recovered from the cells transfected with VEEV/mutSG/IRES and VEEV/mutSG/IRES/1 RNAs (Fig. 5C).

In a parallel experiment, *Ae. aegypti* mosquitoes were intrathoracically inoculated with ca. 10^5 PFU of VEEV TC-83 and the VEEV/mutSG/IRES/1 variant (see Materials and Methods

for details). None of the 17 mosquitoes inoculated with the IRES mutant exhibited detectable virus replication, whereas 17 of 17 mosquitoes inoculated with the VEEV TC-83 parent strain demonstrated virus replication with a mean titer of over 10^6 PFU/mosquito. Thus, the IRES-encoding VEEV variant VEEV/mutSG/IRES/1 was incapable of replicating in mosquito cells both in vitro and in vivo.

To explain the high titers of VEEV/IRES (capable of producing the subgenomic RNA variant) after passaging in mosquito cells, we randomly selected two individual plaque-purified variants from the stock, harvested after passaging in mosquito cells, and sequenced the IRES-containing fragment of the genome. In both isolates, the IRES sequence was no longer present in the viral genomes, and only 13 and 15 residual nucleotides of the original IRES were found (Fig. 5C).

Thus, replication of VEEV/IRES variant genome in mosquito cells led to an accumulation of the IRES-negative variants, and VEEV/mutSG/IRES (having the inactivated subgenomic promoter) did not develop mutants capable of replicating efficiently in the mosquito-derived cell line.

VEEV/mutSG/IRES/1 variant demonstrates an attenuated phenotype

This study was aimed at development of VEEV variants incapable of replicating in cells of mosquito origin (and, correspondingly, in mosquito vectors), but demonstrating a more attenuated phenotype in vertebrates than the parental VEEV TC-83. The slower replication rates of the VEEV/mutSG/IRES/1 variant raised a concern that this virus might be incapable of replicating in vertebrate cells with intact IFN-α/β production and signaling. However, this was not the case. The results of the experiments, presented in Fig. 6, demonstrate that VEEV/mutSG/IRES/1 replicated in the NIH 3T3 cells, which have no defects in IFN-α/β secretion and signaling, to the titers above 10^9 PFU/ml. Its replication caused a more efficient IFN-α/β induction (Fig. 6), but apparently the IFN release did not abrogate the already established virus replication. As shown in BHK-21 cells (Fig. 2C), replication of VEEV/mutSG/IRES/1 was less efficient than that of the VEEV TC-83 (Fig. 6), which suggested that the IRES-dependent mutant might be attenuated in vivo. Indeed, after the i.c. inoculation of 6-day-old mice with ca. 10^6 PFU, 86% survived the infection and did not develop signs of encephalitis; in contrast, 92% of the mice were killed by the same dose of VEEV strain TC-83 (Fig. 7). Taken together, these data indicate that genetically modified, IRES-dependent VEEV was more attenuated than the parental VEEV TC-83.

Nevertheless, the VEEV/mutSG/IRES/1 variant remained immunogenic in both neonatal and adult mice. Of the twelve 6-day-old mice that survived i.c. inoculation with VEEV/mutSG/IRES/1, 10 survived s.c. challenge with 10^4 PFU of wild-type VEEV strain 3908 administered 5 weeks later (data not shown); in contrast, of 12 sham (PBS)-infected mice challenged in the same manner, none survived. The VEEV/mutSG/IRES/1 was also immunogenic in adult mice; one s.c. immunization with ca. 10^6 PFU protected 80% of mice against s.c. challenge 3 weeks later with 10^4 PFU (~10^4 LD_{50}) of VEEV strain 3908 (Fig. 8). Neutralizing antibody titers ($PRNT_{80}$) were <1:20 in all of these mice immediately before challenge, which suggested that the incomplete protection after one vaccination was likely a result of a lower level of IRES-containing virus replication in vivo. Thus, its high level of attenuation confers a high degree of safety, but repeated vaccinations will likely be required.

Discussion

The development of the infectious cDNA clones of Sindbis and other alphaviruses (Davis et al., 1989; Kuhn et al., 1996; Rice et al., 1987) opened the opportunity not only for reverse genetics experiments aimed at studying different aspects of virus biology and pathogenesis, but also for the development of new recombinant vaccines. Attenuation of the viruses by

passaging either in tissue culture or in chicken embryos (Murphy and Chanock, 2001) generally results from accumulation of small numbers of point mutations in the structural and nonstructural genes, and in the cis-acting elements of viral genomes. For example, the VEEV TC-83 vaccine strain relies on only 2 point mutations for its attenuation, and the high degree of reactogenicity (34) probably reflects the instability of this attenuation mechanism. This raises a concern about the possible reversion to the wt pathogenic phenotype during virus replication in vaccinated individuals. The number of mutations can be additionally increased by chemical mutagenesis (Morrill et al., 1991), but this procedure also does not make the introduced changes irreversible. The genetic manipulations with infectious cDNA clones of the RNA viruses open great possibilities for stronger modification of viral genomes, and provide an opportunity to introduce either extensive deletions that would make it impossible to revert to the wt genome sequence (Blaney et al., 2006; Blaney et al., 2005; Davis et al., 1995; Hart et al., 2000), or additional genetic material that might enhance the immunogenicity of the variants.

There is also a concern that genetically altered arboviruses might be introduced into the natural circulation, mediated by mosquito vectors, and may demonstrate further evolution during long-term replication, either in mosquitoes or during viremia development in vertebrate hosts. An example is the use of VEEV TC-83, which is capable of producing levels of viremia in equids sufficient for infecting mosquitoes. The isolation of TC-83 from naturally infected mosquitoes collected in Louisiana (Pedersen, Robinson, and Cole, 1972) during the 1971 Texas epidemic demonstrated the risk of transmission of the attenuated alphaviruses. Therefore, in designing a new generation of live vaccine strains, it is prudent to make arboviruses not only highly attenuated, but also capable of replicating only in cells of vertebrate origin. This can be achieved by cloning cell-specific RNA elements into viral genomes. The use of an EMCV IRES opens such an opportunity. In contrast to the cricket paralysis virus IRES (Jan and Sarnow, 2002), the EMCV-specific element was expected to function very inefficiently in arthropod cells (Finkelstein et al., 1999; Woolaway et al., 2001). Therefore, its application in expressing viral nonstructural and/or structural proteins could restrict virus replication in mosquito cells and mosquitoes.

In this study, we cloned EMCV IRES into the VEEV TC-83 genome to make the translation of viral structural genes IRES-dependent. One of the genomes contained a functional subgenomic promoter and the IRES in the 5′UTR of the subgenomic RNA. This virus was viable, but its ability to produce the subgenomic RNA promoted further evolution, which resulted in IRES deletion and reversion, most likely, to a standard, cap-dependent translation of the structural proteins. The latter deletions made it capable of replicating in mosquito cells. The second variant with multiple mutations in the subgenomic promoter was stable in terms of its inability to revert to a cap-dependent translation. Because such reversion would require not only the IRES deletion, but also the restoration of the subgenomic promoter, which we inactivated by 13 mutations, direct reversion of these mutations probably represents a negligible risk. However, this variant developed an interesting way to evolve to a more efficiently replicating phenotype by accumulating additional, adaptive mutations in the nsP2 gene. These mutations did not noticeably change the level of viral RNA replication, synthesis of the viral structural proteins, or their compartmentalization in the cells. The detected mutations also did not create an additional signal that could increase the efficiency of the genome packaging. Thus, the mechanism of their functioning remains to be determined. However, the accumulating published data suggest that the packaging of the genomes of the RNA viruses is strongly determined by the replicative complexes, and the genomes need to be presented by the functional nsPs to the structural proteins for particle formation (Khromykh et al., 2001; Nugent et al., 1999). Our working hypothesis is that the helicase domain of the nsP2 might be involved in the viral genome presentation for its packaging into the nucleocapsids,

and, thus, the identified mutations could have a positive effect on the efficiency of this process. However, this hypothesis certainly needs additional experimental testing.

Our study was not the first attempt to transform the subgenomic RNA-producing positive-strand RNA genome virus into an EMCV IRES-dependent variant that lacks a subgenomic promoter. The rubella virus with IRES-containing genome was also viable (Pugachev, Tzeng, and Frey, 2000) and, most likely, also further evolved, because a two-orders-of-magnitude increase in virus titers was detected after 5 passages in Vero cells (Pugachev, Tzeng, and Frey, 2000). Unfortunately, the genomes of the variant(s) demonstrating more efficient replication were not sequenced, but one might speculate that EMCV IRES-containing rubella, a distant relative of alphaviruses such as Sindbis virus or VEEV (Strauss and Strauss, 1994), had a similar mechanism of adaptation to more efficient replication in tissue culture.

The new characteristic features of the designed VEEV/mutSG/IRES/1 virus, which include a lack of the subgenomic promoter, IRES-dependent translation of the structural genes, and an inability to replicate in mosquito cells, raise the interesting question of whether this virus can be considered an alphavirus. At present, it is probably best to accept the view that it is, because of the structure of the virion and synthesis of the nonstructural and structural polyprotein from different cistrons in the RNA genome. Another issue is that the IRES-encoding VEEV variants have now to deal with clearing the ribosomes from viral genomes to synthesize the negative-strand intermediate. This is an important and intensively studied process in poliovirus replication (Barton, Morasco, and Flanegan, 1999). However, in our experiments, the IRES-encoding VEEV genomes replicated with an efficiency similar to that of the VEEV TC-83. Thus, the ribosome dislodging from viral RNA templates may be not critical for VEEV RNA replication. However, based on the lower replication rates of both VEEV/IRES and VEEV/mutSG/IRES/1 variants, the ribosome clearing may be important in packaging the genomes into the viral particles.

It should be noted that the main goal of the study was not in reaching an understanding of the newly implied functions of VEEV nsP2 (which we certainly continue to investigate), but rather to develop VEEV variants that are incapable of replicating in arthropod vectors and demonstrate a stable, more attenuated phenotype. Slower growth of the designed VEEV/mutSG/IRES/1 variant in both IFN-α/β-competent and IFN signaling-deficient BHK-21 cells, its ability to induce higher levels of IFN-α/β in tissue culture, its greatly reduced ability to kill newborn mice even after i.c. inoculation, and its inability to replicate in mosquito cells suggest that this variant might meet those requirements. Its immunogenicity will be further investigated in different animal models. Moreover, we believe that other encephalogenic alphaviruses can be attenuated by using a similar, EMCV IRES-based strategy, which can be applied in combination with other approaches that we and other research groups have developed within the recent years (Aguilar, Weaver, and Basler, 2007; Garmashova et al., 2007a; Garmashova et al., 2006; Garmashova et al., 2007b; Paessler et al., 2003).

Materials and Methods

Cell cultures

The BHK-21 cells were kindly provided by Dr. Paul Olivo (Washington University, St. Louis, Mo), and the Vero cells by Dr. Charles Rice (Rockefeller University, New York, NY). The NIH 3T3 cells were obtained from the American Type Tissue Culture Collection (Manassas, VA). These cell lines were maintained at 37°C in alpha minimum essential medium (αMEM) supplemented with 10% fetal bovine serum (FBS) and vitamins. Mosquito $C_7$10 cells were obtained from Dr. Henry Huang (Washington University, St. Louis, Mo) and propagated in DMEM supplemented with 10% heat-inactivated FBS and 10% tryptose phosphate broth (TPB).

Plasmid constructs

Standard recombinant DNA techniques were used for all plasmid constructions. Maps and sequences are available from the authors upon request. The original plasmid with VEEV TC-83 genome under the control of SP6 RNA polymerase promoter, pVEEV TC-83, was described elsewhere (Petrakova et al., 2005). pVEEV/IRES contained EMCV IRES with the first 4 codons of the EMCV polyprotein. This sequence was cloned into the VEEV subgenomic RNA-coding sequence between the end of the 5′UTR (nt 30) and the initiating AUG (Fig. 1A). pVEEV/mutSG/IRES encoded the VEEV TC-83 genome, in which the subgenomic promoter was inactivated by clustered point mutations, which did not modify the amino acid sequence of the carboxy terminus of nsP4 (Figs. 1A and B). This viral genome had the 5′UTR of the subgenomic RNA deleted. Thus, VEEV TC-83 nonstructural and structural proteins were expected to be synthesized from the same genomic RNA. The adaptive mutations were introduced into pVEEV/mutSG/IRES-encoded nsP2 by PCR amplification of the fragments of interest, followed by replacement of the corresponding fragment in the original genome. The same PCR-based technique was used for synthesis cloning of different fragments into the SphI site in the 3′UTR of the VEEV/mutSG/IRES genome. All of the cloned fragments were sequenced before further virus-rescue experiments.

RNA transcriptions

Plasmids were purified by centrifugation in CsCl gradients and linearized by MluI digestion. RNAs were synthesized by SP6 RNA polymerase (Ambion) in the presence of cap analog (New England Biolabs). The yield and integrity of transcripts were analyzed by gel electrophoresis under non-denaturing conditions. RNA concentration was measured on a FluorChem imager (Alpha Innotech), and transcription reactions were used for electroporation without additional purification.

RNA transfections

In all of the experiments, the transfections were performed by electroporation of the in vitro-synthesized RNA using previously described conditions (Liljeström et al., 1991). To rescue the viruses, 1 μg of in vitro-synthesized viral genome RNA was electroporated into the cells (Liljeström et al., 1991), and then they were seeded into 100-mm dishes and incubated until cytopathic effects were observed. Virus titers were determined using a standard plaque assay on BHK-21 cells (Lemm et al., 1990). To assess the RNA infectivity, 10-fold dilutions of electroporated BHK-21 cells were seeded in 6-well Costar plates containing subconfluent, naïve cells. After 1 h incubation at 37°C in a 5% CO_2 incubator, cells were overlaid with 2 ml of MEM-containing 0.5% Ultra-Pure agarose (Invitrogen) supplemented with 3% FBS. Plaques were stained with crystal violet after 2 days incubation at 37°C, and infectivity was determined in plaque-forming units (PFU) per μg of transfected RNA.

Sequencing of viral genomes

Large plaques were randomly selected during titering of viral stocks (without staining with neutral red). Viruses were extracted from the agarose plugs into MEM, and aliquots of the latter media were used to infect BHK-21 cells in 35-mm dishes. After development of profound CPE, virus stocks were harvested for further characterization, and RNAs were isolated from the infected cells by TRizol reagent according to the instructions of the manufacturer (Invitrogen). ~1000 nt-long, overlapping fragments were synthesized using standard RT-PCR techniques, purified by agarose gel electrophoresis and sequenced. Sequencing of the 5′UTR was performed by using a FirstChoice RLM-RACE Kit (Ambion) as described elsewhere (Gorchakov et al., 2004).

Viral replication analysis

One-fifth of the electroporated cells were seeded into 35-mm dishes. At the times indicated in the figures, media were replaced, and virus titers were determined by plaque assay on BHK-21 cells (Lemm et al., 1990). Alternatively, BHK-21, NIH 3T3 or $C_7$10 cells were seeded into 35-mm dishes and infected at the MOIs indicated in the figures. Media were replaced by fresh media, and virus titers in the harvested samples were determined by plaque assay on BHK-21 cells.

Analysis of protein synthesis

BHK-21 cells were electroporated with 4 μg of the indicated RNAs, and one-fifth of the electroporated cells were seeded into six-well Costar plates. At 12 h post transfection, proteins were metabolically labeled by incubating for 30 min in 0.8 ml of DMEM medium lacking methionine, supplemented with 0.1% FBS and 20 μCi/ml of [^{35}S]methionine. After this incubation, they were scraped into the media, pelleted by centrifugation and dissolved in 100 μl of standard protein loading buffer. Equal amounts of proteins were loaded onto sodium dodecyl sulfate (SDS)-10% polyacrylamide gels. After electrophoresis, gels were dried and autoradiographed.

RNA analysis

To analyze the synthesis of virus-specific RNAs, cells were electroporated with 4 μg of the in vitro-synthesized viral RNAs, and one-fifth of the cells were seeded into 35-mm dishes. At 4.5 h post transfection, medium in the wells was replaced by 1 ml of αMEM supplemented with 10% FBS, ActD (1 μg/ml) and [^{3}H]uridine (20 μCi/ml). After 4 h of incubation at 37°C, total cellular RNAs were isolated by Trizol (Invitrogen) according the manufacturer's protocol, then denatured with glyoxal in dimethyl sulfoxide and analyzed by agarose gel electrophoresis using the previously described conditions (Bredenbeek et al., 1993). Gels were impregnated with 2,5-diphenyloxazole (PPO), dried and autoradiographed.

IFN-α/β assay

The concentrations of IFNα/β in the media were measured by a previously described biological assay (Trgovcich, Aronson, and Johnston, 1996). Briefly, L929 cells were seeded in 100 μl of complete media at a concentration of 5×10^4 cells/well in 96-well plates and incubated at 37° C for 6 h. Samples of media harvested from infected NIH 3T3 cells were treated with UV light for 1 h, and serially diluted in two-fold steps directly in the wells with L929 cells. After incubation for 24 h at 37°C, an additional 100 μl of media with 2×10^5 PFU of vesicular stomatitis virus (VSV) was added to the wells and incubation continued for 36-40 h. Then cells were stained with crystal violet, and the end point was determined as the concentration of IFNα/β required for protecting 50% of the cells from the VSV-induced CPE. The IFN-α/β standard for normalization of the results was purchased from the ATCC.

Evaluation of virus replication in mosquitoes

To assess replication competence in mosquitoes in vivo, intrathoracic inoculations of *Aedes aegypti* (a colony originating in Galveston, Texas) mosquitoes using 10^5 PFU in a 1 μL volume were used. Intrathoracic inoculation was selected over oral exposure because nearly any culicine mosquito is highly susceptible to intrathoracic infection by any alphavirus, while oral susceptibility is highly variable and much less sensitive (Weaver, 1997). Following inoculation using a glass pipette, mosquitoes were incubated for 10 days at 27°C and then triturated individually in 1 mL of MEM supplemented with 20% FBS and Fungizone. A 100 μL volume of each trituturated mosquito was then added to a Vero cell monolayer on a 24-well plate, and cells were observed for 5 days for cytopathic effects to detect infection. Assay included the TC-83 parent virus and the IRES mutant.

Immunization and challenge with virulent VEEV

Six-day-old NIH Swiss mice were inoculated intracerebrally (i.c.) with VEEV TC-83 strain or the designed mutant at a dose of ca. 10^6 PFU in a total volume of 20 μl of PBS. After infection, each cohort of 8-10 animals was maintained for 2 months without any manipulation. For 21 days, mice were observed daily for signs of illness (ruffled fur, depression, anorexia and/or paralysis) and/or death.

Eight-week-old female NIH Swiss mice were vaccinated s.c. at a dose of ca. 10^6 PFU/mouse using VEEV TC-83 or the recombinant virus, then challenged subcutaneously 4 weeks later with ca. 10^4 PFU of highly virulent VEEV strain 3908. For 21 days, mice were observed twice daily for signs of illness (ruffled fur, depression, anorexia and/or paralysis) and/or death.

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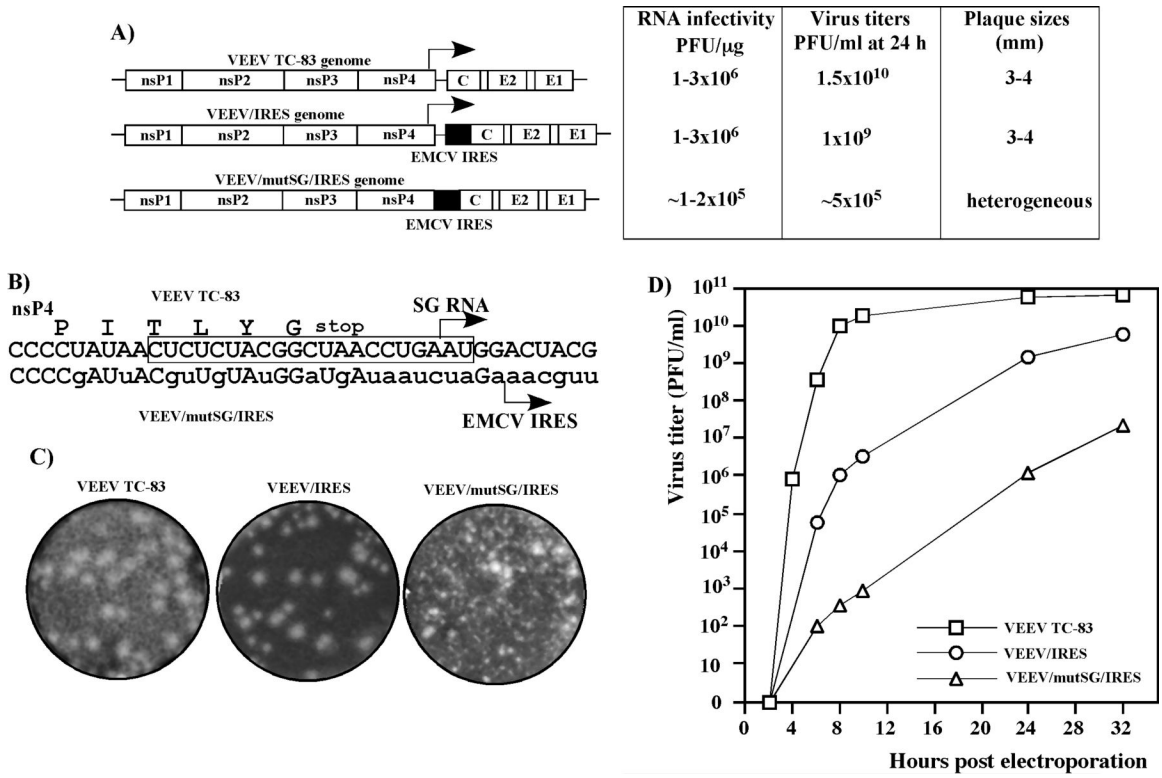

RNA infectivity PFU/μg	Virus titers PFU/ml at 24 h	Plaque sizes (mm)
1-3x10^6	1.5x10^{10}	3-4
1-3x10^6	1x10^9	3-4
~1-2x10^5	~5x10^5	heterogeneous

Fig. 1.

Replication of the recombinant, EMCV IRES-encoding, VEEV TC-83-derived viruses in BHK-21 cells. (A) Schematic representation of the designed viral genomes, infectivities of the in vitro-synthesized RNAs in the infectious center assay, virus titers at 24 h post transfection of 1 μg of the in vitro-synthesized RNAs into BHK-21 cells, and sizes of the plaques, formed by indicated viruses in BHK-21 cells at 48 h post transfection. Arrows indicate functional subgenomic promoters. Filled boxes indicate positions of EMCV IRES. (B) Alignment of the subgenomic promoter-containing fragment of the VEEV TC-83 genome and the corresponding fragment of the VEEV/mutSG/IRES. The position of the promoter is indicated by open box. The start of the subgenomic RNA in the VEEV TC-83 genome and the beginning of the EMCV IRES are indicated by arrows. The mutations, introduced into the VEEV/mutSG/IRES genome are shown in lower case letters. (C) Plaques, formed in BHK-21 cells by viruses, harvested at 24 h post transfection. (D) Replication of the viruses after transfection of 1 μg of the in vitro-synthesized RNAs into BHK-21 cells.

A)

Mutation	Isolate 1	Isolate 2	aa change
$A_{1613} \rightarrow G$	+	+	No
$C_{1616} \rightarrow A$	+	+	No
$T_{1619} \rightarrow C$	+	+	No
$T_{2176} \rightarrow A$	+	-	No
$A_{2758} \rightarrow G$	+	+	$Y_{370} \rightarrow C$
$A_{2760} \rightarrow C$	+	-	$K_{371} \rightarrow Q$
$T_{9702} \rightarrow C$	+	+	No
$T_{10299} \rightarrow G$	+	+	No

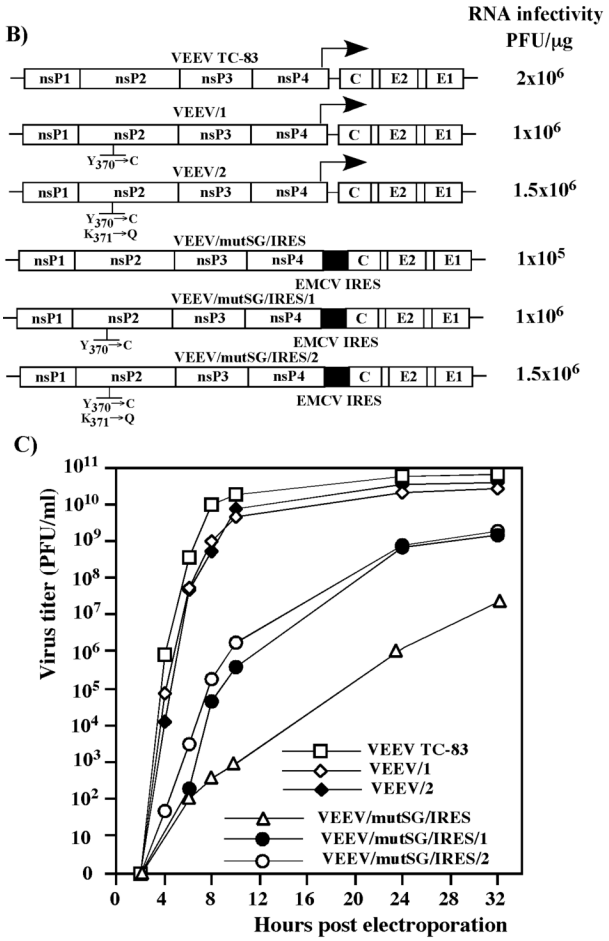

Fig. 2.

Mutations found in the plaque-purified VEEV/mutSG/IRES variants, which demonstrated more efficient replication in BHK-21 cells, and the effect of the defined adaptive mutations on VEEV TC-83 and VEEV/mutSG/IRES replication. (A) The list of the mutations, found in the genomes of plaque isolates, compared to published sequence of VEEV TC-83 (Kinney et al., 1989). (B) The schematic representation of the VEEV TC-83 and VEEV/mutSG/IRES genomes, having either one or two of the identified mutations, and the infectivity of the in vitro-synthesized viral RNAs in the infectious center assay. Functional subgenomic promoters are indicated by arrows, and EMCV IRES by filled boxes. (C) Replication of the designed viruses in BHK-21 cells after transfection of 1 μg of the in vitro-synthesized viral genomes.

A)

Plaque isolates	$P_{349} \rightarrow T$	$Y_{370} \rightarrow C$	$K_{371} \rightarrow Q$	$D_{418} \rightarrow A$	$K_{423} \rightarrow T$
Orig. pl 1		+			
Orig. pl 2		+	+		
Orig. pl 3		+			
Orig. pl 4	+				
Orig. pl 5					+
Pass. pl 1		+			
Pass. pl 2		+			
Pass. pl 3		+			
Pass. pl 4				+	
Pass. pl 5					+

B)

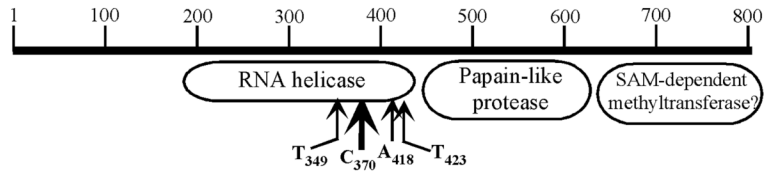

Fig. 3.

Mutations identified in the nsP2 protein of VEEV/mutSG/IRES variants demonstrating a large-plaque phenotype. (A) List of the mutations identified in the genomes of the plaque-purified isolates from virus stock, harvested at 24 h post transfection of the in vitro-synthesized RNA (Orig.), and in the genomes of isolates from the stock that was additionally passaged three times in Vero cells (Pass.). (B) Localization of the defined mutations in the VEEV nsP2. The positions of currently known functional domains in alphavirus nsP2 (Russo, White, and Watowich, 2006; Strauss and Strauss, 1994) are indicated.

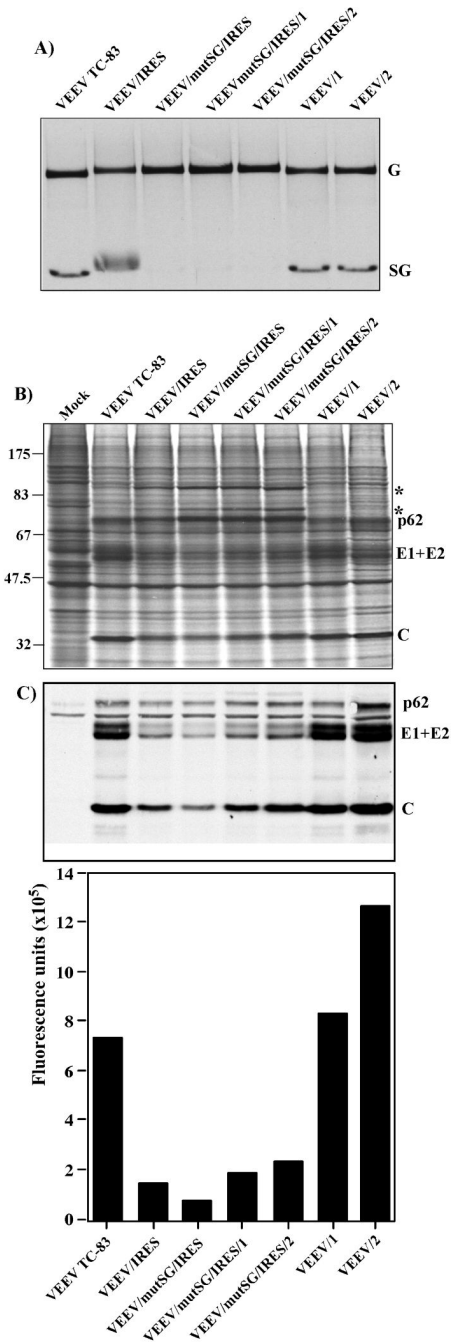

Fig. 4.

Analysis of protein and RNA synthesis in BHK-21 cells transfected with the in vitro-synthesized recombinant viral RNAs. Cells were electroporated with 4 μg of the indicated RNAs and seeded into 35-mm dishes as indicated in Materials and Methods. (A) At 4.5 h post transfection, medium in the wells was replaced by 1 ml of αMEM supplemented with 10% FBS, ActD (1 μg/ml) and [^{3}H]uridine (20 μCi/ml). After 4 h of incubation at 37°C, RNAs were isolated and analyzed by agarose gel electrophoresis as described in the Materials and Methods. The positions of viral genomic and subgenomic RNAs are indicated by G and SG, respectively. The VEEV/IRES-specific subgenomic RNA forms a more diffuse band than do other, subgenomic RNA-producing, viruses, because, in the gel, it co-migrates with the ribosomal

28S RNA. (B) At 12 h post transfection, proteins were metabolically labeled with [^{35}S] methionine and analyzed on a sodium dodecyl sulfate-10% polyacrylamide gel as described in the Materials and Methods. The positions of molecular weight markers (kDa) are indicated at the left side of the gel. The positions of viral structural proteins: C, E1, E2 and p62 (the precursor of E2) are shown at the right side of the gel. Asterisks indicate the positions of cellular proteins (the heat-shock proteins), induced by replication of the IRES-encoding viruses (see text for details). Noticeable differences in capsid mobility indicate the presence of additional 4 amino acids, which were cloned into IRES-containing viral genomes (see Materials and Methods for details). (C) The same samples of the proteins were analyzed by Western blotting. Membranes were developed by VEEV-specific mouse antibody and anti-mouse IRDye 800 secondary antibody. The intensity of the capsid-specific signals was evaluated on LI-COR imager. The positions of viral structural proteins: C, E1, E2 and p62 (the precursor of E2) are shown at the right side of the gel.

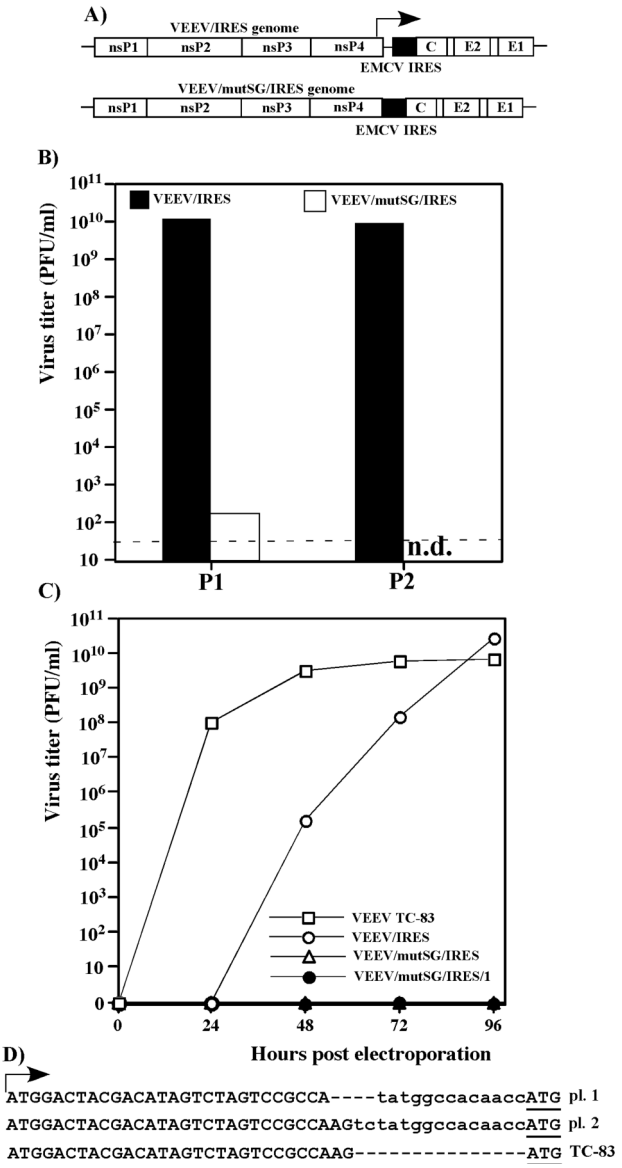

Fig. 5.

Passaging of the recombinant, EMCV IRES-encoding VEEV variants in $C_7$10 cells. (A) The schematic representation of viral genomes. Arrow indicates the position of the functional subgenomic promoter. Filled box indicates the position of EMCV IRES. (B) Titers of the recombinant viruses after passaging in $C_7$10 cells. Cells in 35-mm dishes were infected with 400 μl of virus samples harvested either at 24 h post transfection of BHK-21 cells with the in vitro-synthesized RNA (P1) or 48 h post infection of $C_7$10 cells (P2). Samples were harvested at 48 h post infection, and titers were determined by plaque assay on BHK-21 cells. Dashed line indicates the limit of detection. n.d. indicates that titer was below the detection limit. (C) Replication of the indicated viruses after transfection of 5 μg of the in vitro-synthesized RNAs into $C_7$10 cells. Titers were determined by plaque assay on BHK-21 cells. (D) The deletions of the IRES-specific sequence identified in the plaque-purified VEEV/IRES variants, demonstrating efficient replication in $C_7$10 cells. The residual EMCV IRES-specific sequences are indicated by lower case letters.

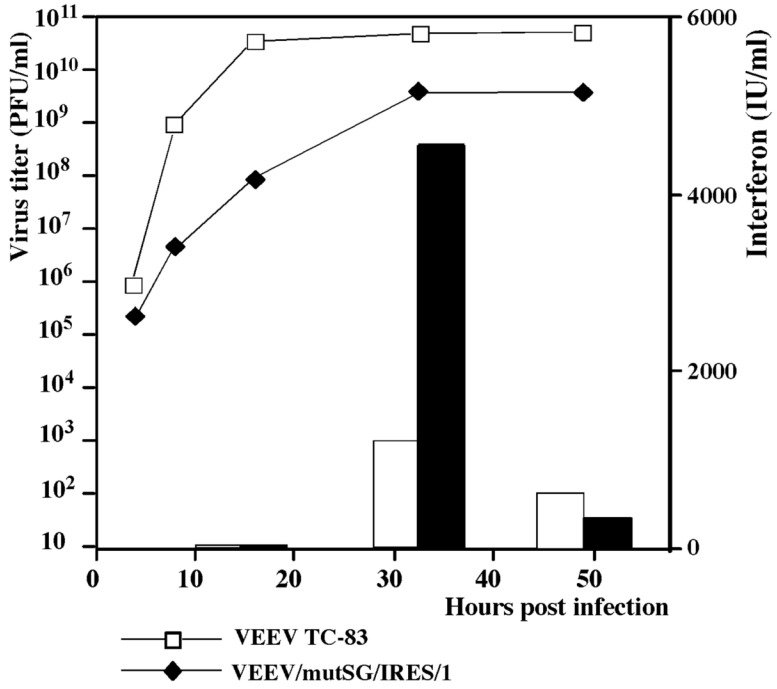

Fig. 6.

Replication of VEEV/mutSG/IRES/1 and VEEV TC-83 in the NIH 3T3 cells. Cells were infected at an MOI of 10 PFU/cell. Media were replaced at the indicated time points, and virus titers were measured by plaque assay on BHK-21 cells. The same samples were used to measure IFN-α/β release in biological assays, as described in Materials and Methods. Concentrations of released IFN-α/β are presented in international units (IU) per ml.

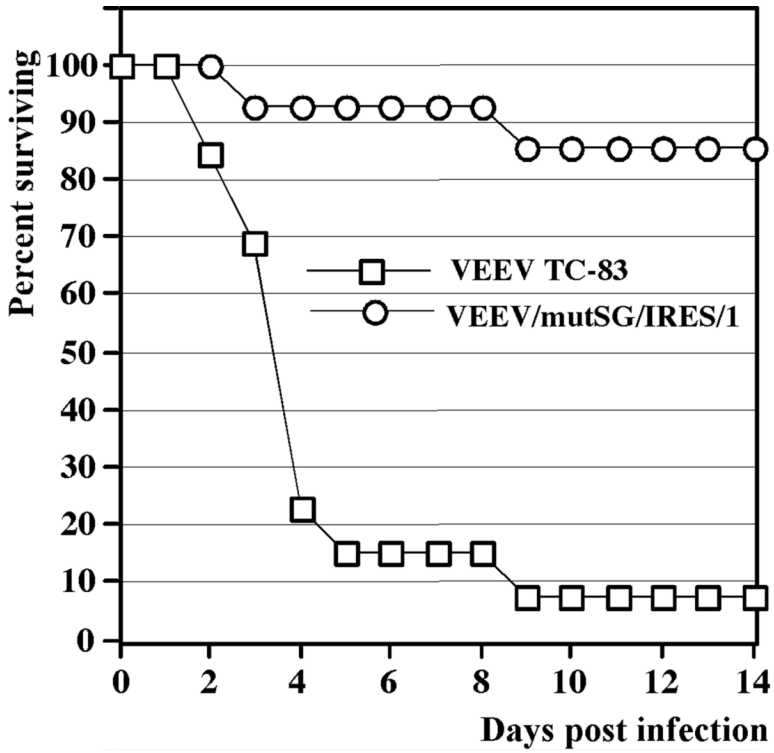

Fig. 7.

Survival of mice infected with VEEV TC-83 and VEEV/mutSG/IRES/1 viruses. Six-day-old NIH Swiss mice were inoculated i.c. with ca. 10^6 PFU of the indicated viruses. Animals were monitored for two months. No deaths occurred after day 9 post-infection in these experiments.

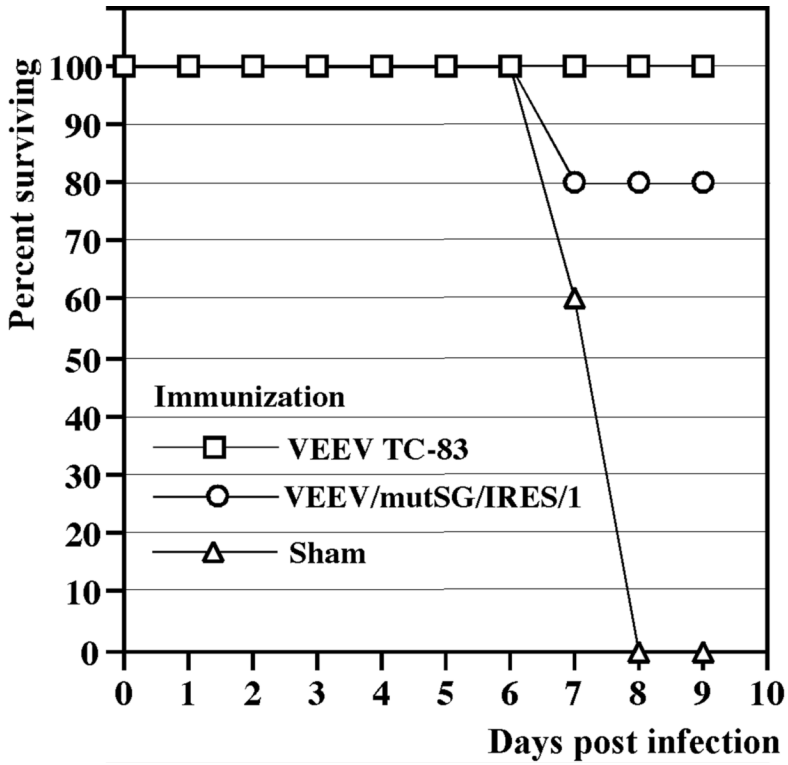

Fig. 8.

Survival following vaccination and challenge of adult mice. Five-to-6-week-old female NIH Swiss mice were immunized s.c. with VEEV strain TC-83 or the recombinant virus at a dose of ca. 10^6 PFU. Three weeks after immunization, mice were challenged s.c. with ca. 10^4 PFU of VEEV strain 3908, and mortality was recorded.