A sindbis virus mRNA polynucleotide vector achieves prolonged and high level heterologous gene expression in vivo

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ABSTRACT
The direct Intramuscular delivery of naked plasmid DNA has been demonstrated to allow expression of encoded heterologous genes in the target myocytes. The method has been employed to elicit Immunization based upon delivery of antigen encoding plasmid DNA. For application in the context of achieving anti-tumor immunization against antigenic transforming oncoproteins, delivery of plasmid DNAs encoding these molecules would create significant potential safety hazards. As an alternative to DNA polynucleotide vectors, we explored the utility of mRNA vehicles for Inducing foreign gene expression in muscle cells in vivo. Synthetic reporter-gene encoding mRNA transcripts were derived for this analysis. The sindbis virus vector was also used to derive luciferase mRNA transcripts which possessed self-replication capacity. In these studies, it could be shown that the replicative vector was capable of directing significantly elevated levels of reporter gene expression in myocytes compared to a non-replicative mRNA species. In addition, the replicative species was capable of achieving significantly prolonged levels of in vivo gene expression compared to non-replicative mRNA. Both of these characteristics will make replicative mRNA vectors of utility for polynucleotide-based immunization protocols.

INTRODUCTION
Direct intramuscular injection of plasmid DNA encoding specific antigens has been employed as a means to achieve highly specific immunization. This polynucleotide vaccine strategy has elicited humoral and cellular immune responses to a variety of infectious agents including influenza, hepatitis B, HIV and others (1,2,3). In this context, polynucleotide immunization has attracted broad interest due to the following advantages: (i) it elicits both humoral and cell-mediated immune responses without adjuvants; (ii) intracellular synthesis of the antigen favors MHC class I peptide display, considered pivotal to the generation of cytolytic T cells (4); and (iii) large quantities of purified DNA for vaccination can be prepared and standardized with relative ease compared to protein purification techniques. To examine the ability of polynucleotide immunization to achieve specific anti-tumor immunity, we have constructed a plasmid DNA encoding the full-length cDNA for human carcinoembryonic antigen (CEA) under transcriptional regulatory control of the cytomegalovirus early promoter/enhancer (5). This plasmid can function as a polynucleotide vaccine to elicit CEA-specific humoral and cellular immune responses as well as protection against syngeneic, CEA-expressing colon carcinoma cells (5–7). These effects were comparable to the immune response and immunoprotection achieved with a recombinant vaccinia virus encoding CEA (5).

In considering the application of this technology to additional tumor-associated antigens, it is noteworthy that many recently cloned tumor-associated antigens consist of the protein products of proto-oncogenes such as mutated Ras or non-mutated proto-oncogenes such as Her2/neu (8–10). In this regard, prolonged cellular expression of mutated Ras or over expression of Her2/neu have been associated with malignant transformation (11–13). Thus, use of plasmid DNA encoding such proto-oncogenes for polynucleotide immunization would pose the risk of malignant transformation of host cells consequent to long-term proto-oncogene expression. As a strategy to circumvent this theoretical risk, and facilitate the use of oncogene derived tumor-associated antigens, we have examined the utility of mRNA as a polynucleotide vehicle for immunization. Since host cells lack endogenous reverse transcriptase required to convert mRNA to DNA, mRNA cannot integrate into the host genome. Thus, polynucleotide immunization with mRNA transcripts encoding oncogenic sequences would produce self-limited expression of the immunogen without the potential for malignant transformation of host cells. To achieve this end, we have generated mRNA transcripts which are capped, polyadenylated and stabilized by human ß-globin 5' and 3' untranslated regions (14). These constructs encoding firefly luciferase directed luciferase expression in muscle. In addition, such transcripts

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encoding carcinoembryonic antigen 'primed' an immune response to CEA (14).

In an effort to improve the efficiency of mRNA polynucleotide immunization, we have considered the use of a self-replicating RNA vector system. As a prototype, we selected the sindbis virus expression vector. In this system the gene of interest is cloned into a plasmid DNA vector that serves as a template for in vitro synthesis of translation-competent, replicative RNA (15,16). This RNA construct is of positive polarity and encodes the sindbis virus non-structural proteins required for RNA replication and transcription. Thus, when transfected into the cytoplasm of cells, the sindbis virus genome drives its own replication and transcription of foreign sequences (15,16). For this study, we replaced the structural protein-encoding region in cDNA clones of sindbis virus with the firefly luciferase gene to derive a vector with a high level of analogy to the system described by Huang (15). In this report, we examine the feasibility of this strategy utilizing non-infectious, self-replicating mRNA transcripts encoding firefly luciferase, including in vitro characterization of the vector and in vivo expression following intramuscular delivery.

MATERIALS AND METHODS

Cell culture

BHK21 is a Syrian hamster kidney cell line obtained from the ATCC. These cells were maintained in Eagle's minimum essential medium (EMEM) supplemented with 1x non-essential amino acids, 10% fetal bovine serum (FBS) (PAA Laboratories, Inc., Linz, Austria) and antibiotics. The human ovarian adenocarcinoma cell line SKOV3 was obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics. The murine colonic adenocarcinoma cell line MC38 was provided by Dr S. A. Rosenberg (Bethesda, MD) and maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1x non-essential amino acids and antibiotics. The human adenocarcinoma cell line MDA-MB-435 was provided by Dr J. E. Price (Houston, TX) and maintained in DMEM 10% FBS with antibiotics.

Synthesis of mRNA transcripts

Gene transfer studies employed synthetic mRNA transcripts derived by in vitro synthesis using modifications of the methods of Kreig and Melton (17). Plasmid DNA templates for the DNA-directed mRNA transcription reaction were first constructed. The plasmid pGT28 was employed for the production of non-replicative firefly luciferase mRNA transcripts. This plasmid has been previously described (18). Transcripts derived from this plasmid contain the luciferase coding region flanked by 5' and 3' untranslated regions derived from human β-globin mRNA, as well as a synthetic polyA region encoded by the pSP64 parent plasmid (Fig. 1). The firefly luciferase gene was also cloned into the sindbis virus vector TLXN to produce the plasmid pTLXN-Luc. TLXN was kindly provided by Dr Henry V. Huang (St Louis, MO). The plasmid DNA pTLXN-Luc contains the SP6 RNA polymerase promoter followed by sindbis virus non-structural genes required for RNA replication, a subgenomic promoter sequence, the luciferase coding region and a poly(A) sequence. This plasmid was employed for the derivation of firefly luciferase transcripts capable of self-replication. The pGT28 and pTLXN-Luc plasmids were linearized for run-off transcription by digestion with BamHI and SphiI, respectively. DNA templates were then purified employing methods described by Malone (19). Synthetic mRNA transcripts incorporating the OTP cap analog were derived using an in vitro transcription kit (Ambion, Austin, TX) according to the manufacturer's instructions. Following the RNA synthesis reaction, DNA templates were removed by digestion with RNase-free DNase I. Transcripts were then extracted twice with phenol (pH 5.2)/chloroform and once with chloroform and precipitated with 5 M ammonium acetate and 2-propanol. The non-replicative and replicative luciferase mRNA species were then analyzed by electrophoresis in a 1% (w/v) agarose Tris/Borate/EDTA (TBE) gel containing 1 μg/ml ethidium bromide. Size was determined employing commercial RNA standards (Promega, Madison, WI).

Liposome-mediated mRNA transfection

In vitro transfection of cell lines was accomplished using a cationic liposome vector employing previously described techniques (19). Briefly, cells were first seeded into 35 mm 6 well tissue culture plates at a concentration of 2.5 × 10^5 cells per well. After 24 h, the cells were transfected employing lipofectin–mRNA complexes. To accomplish this, the liposome–mRNA complexes were formed by the addition of 4.0 μg of mRNA transcript to 20 μg of lipofectin reagent (Gibco-BRL) in 400 μl phosphate buffered saline, pH 7.4, and incubated for 10 min at 4°C. The liposome–mRNA mixture was then added directly to cells devoid of tissue culture medium and incubation carried out for 10 min at room temperature. After this step, 1 ml of OPTI-MEMI medium (Gibco-BRL) was added to each well and incubation carried out at 37°C in a 5% CO2 humidified environment for the indicated time interval. Transfected cells were harvested and lysates analyzed for luciferase gene expression using the luciferase assay system kit (Promega, Madison, WI) under conditions recommended by the manufac-
turer. Briefly, cell lysates were normalized for total protein content of 20 µg and analyzed for luciferase activity using a Lumat LB950 (Berthold) luminometer counted over a 30 s interval.

**In vivo injection of RNA and DNA polynucleotide vectors**

Six to eight week old female C57BL/6 mice (Charles River Laboratories, Raleigh, NC) were anesthetized with ketamine and xylazine by intraperitoneal injection. For intraglottal injections, tongues were pulled out of the mouth gently with forceps to allow a 28-gauge needle to penetrate the bulk of the tongue muscle. All polynucleotide injections employed 50 µg of mRNA or DNA in 50 µl of RNase-free sterile saline. Preliminary studies and others have demonstrated that tongue injections of this volume do not impair the ability of animals to eat or drink following recovery from anesthesia (5–7,20). The tongue was selected to allow direct visualization of the strated muscle without necessitating a surgical procedure. For quadriceps (rectus femorus) injections, a 1 cm incision in the skin was made to allow direct visualization of the underlying muscle. Injections employed a 28 gauge needle and the skin was closed with polypropylene suture (Ethicon, Inc., Somerville, NJ).

**In vivo characterization of mRNA transcript expression in muscle**

The non-replicative and replicative luciferase mRNA species were analyzed for their ability to direct luciferase expression in tongue or quadriceps muscle. Groups of five mice received single 50 µg doses of either non-replicative luciferase mRNA (derived from pGT28) or replicative luciferase mRNA (derived from pTLXN-Luc) in either the tongue or quadriceps muscle. Muscles were harvested 16 h post-injection based upon available data regarding the time course of mRNA expression following intramuscular injection (21). The entire muscle was removed and minced with scissors into a 17 x 100 mm polypropylene tube containing 400 µl of homogenizer buffer (50 mM potassium phosphate, pH 7.8; 1 mM EDTA; 1 mM DTT and 10% glycerol), containing several protease inhibitors (10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 µg/ml aprotinin) to minimize proteolysis of luciferase. A tissue homogenizer (OMNI International, Gainesville, VA) was used to prepare a homogenous suspension. Luciferase activity was quantitated using the luciferase assay kit (Promega, Madison, WI). Muscle extracts were normalized to 50 µg of total protein and luciferase activity was analyzed over a 30 s interval. A positive result was defined as exceeding 2 SD above the mean of normal control tongues and quadriceps injected with water.

To examine the time course and relative magnitude of luciferase expression in muscle, groups of five mice received single 50 µg injections of either non-replicative luciferase mRNA, replicative luciferase mRNA, or luciferase plasmid DNA (pCLuc4) in the tongue followed by sacrifice at various intervals up to 28 days to analyze luciferase activity within the tongue muscle. To examine the effect of replicative mRNA dose on the magnitude of luciferase expression, groups of five mice received single intraglottal injections of 1, 10 or 50 µg of replicative luciferase mRNA with sacrifice 24 h or 7 days post-injection to analyze luciferase activity within the tongue muscle. The tongue was selected as the site for these studies based upon ease of administration and superior luciferase expression compared to the quadriceps muscle 16 h following replicative or non-replicative luciferase mRNA injection.

**RESULTS**

**Derivation of firefly luciferase-encoding mRNA transcripts**

To accomplish synthesis of functional mRNA transcripts encoding the firefly luciferase gene, DNA directed RNA transcription reactions were carried out employing as templates the plasmids pGT28 and pTLXN-Luc (Fig. 1). The plasmid DNA pGT28 was constructed by insertion of the luciferase gene into the plasmid pSP64T as previously described (18). DNA directed RNA transcription of this template yielded the luciferase gene flanked by 5' and 3' non-coding regions derived from the human β-globin transcript to provide mRNA stabilization. This mRNA species would be predicted to be 1.8 kb in size. The plasmid pTLXN-Luc was constructed by insertion of the luciferase gene into the sindbis virus vector TLXN. This vector encoded sindbis virus non-structural proteins required for self-replication of the resultant recombinant mRNA transcript. However, due to deletion of all three sindbis virus structural protein genes, the transcripts were not packaged into mature virions. DNA-directed RNA transcription of this plasmid DNA would be predicted to yield a transcript of 8.0 kb. This transcript would encode sindbis virus non-structural proteins to provide RNA self-replicative functions as well as the firefly luciferase gene. The two luciferase mRNA species would thus be predicted to differ functionally in that the pGT28 derived transcript would be non-replicative whereas the pTLXN-Luc transcript would be capable of self-replication. In vitro transcription reactions employing the template DNA pGT28 and pTLXN-Luc yielded mRNA transcripts of the size predicted (Fig. 2). Gel analysis confirmed the absence of any residual DNA template in both instances (data not shown). Thus, species of both 'non-replicative' and 'replicative' firefly luciferase mRNA transcripts may be derived employing the vectors pGT28 and pTLXN-Luc, respectively.

**Augmented gene expression in vitro deriving from replicative mRNA transcripts**

To determine the relative functional capacities of the two luciferase encoding mRNA transcripts, in vitro transfection was accomplished. For this analysis liposome–mRNA complexes
were formed and delivered to cells employing techniques which we have previously described (19). The target cell for these initial studies was BHK21. Previous studies by Huang et al. had demonstrated the capacity of sindbis viral vectors to replicate efficiently in this cellular target. Preliminary experiments in our laboratory demonstrated that the optimal liposome vector for this cellular target was lipofectin and the optimal ratio of mRNA to cationic lipid for complex formation was 4 μg mRNA to 20 μg of lipofectin reagent (data not shown). Liposome–mRNA complexes were thus formed as described above and delivered to the BHK21 cells with harvest for analysis of the induced luciferase activity at various times post-transfection. In this analysis, it could be seen that both the non-replicative and replicative luciferase-encoding mRNA species efficiently induced expression of the reporter gene in BHK21 cell lysates (Fig. 3). For the non-replicative luciferase mRNA, peak levels of expression occurred between 8 and 24 h post-transfection. Luciferase levels thereafter rapidly extinguished such that levels were not significantly above background 5 days post-transfection. For the replicative mRNA luciferase-encoding species, peak levels of expression were also noted between 8 and 24 h post-transfection. The levels of the reporter gene expression obtained with the replicative mRNA species, however, were approximately two orders of magnitude greater than for the non-replicative species. It should be noted that this experiment involved delivery of comparable masses of the two types of mRNA transcripts. Given the larger size of the replicative transcript, fewer luciferase gene copies were actually delivered in this instance. In addition to the higher peak levels of gene expression noted with the luciferase encoding replicative mRNA species, persistence of gene expression at elevated levels was significantly greater for the replicative, as compared to non-replicative mRNA. In this regard, 7 days post-transfection the replicative mRNA species transfectants exhibited levels of luciferase activity that were >10-fold over background. In contrast, non-replicative mRNA transfectants exhibited only background levels 5 days post-transfection. The employment of the sindbis vector thus allows the achievement of both higher levels of reporter gene expression, and an extended period of reporter gene expression in BHK21 cells transfected in vitro.

The known permissivity of BHK21 for sindbis virus replication predicted the efficacy of the replicative compared to the non-replicative mRNA species. Sindbis virus has a relatively broad host range and thus the utility of the self-replicative mRNA vector might likewise be predicted to be general. Thus, to extend these observations, a similar analysis was carried out in a series of cellular targets. As before, complexes were formed by combination of the cationic liposome vector lipofectin and either the replicative or non-replicative luciferase-encoding mRNA species. Cells were transfected as before and analyzed for luciferase activity at 16 h post-transfection (Fig. 4). Each of the cellular targets transfected with either the non-replicative or replicative luciferase mRNA demonstrated luciferase activity above background levels. For the non-replicative mRNA, however, substantial differences in reporter gene expression were noted in the various cellular targets, likely reflecting the relative transducibility of the target cells with the cationic liposome vector. Of note, however, the differences in luciferase gene expression achieved by the non-replicative and replicative mRNA for a given cell line differed significantly. In this regard, whereas the replicative mRNA species achieved luciferase levels nearly three orders of magnitude greater than the non-replicative species for BHK21, for other cell lines such as SKOV3, the differential observed was less than an order of magnitude. In the other cellular targets studied, the differential of observed gene expression achieved by the replicative compared to the non-replicative mRNA was highly variable. As the transfection conditions were standard for the various cell lines, these differences likely reflect the intrinsic ability of the sindbis virus vector to replicate in the various target cells. Thus, whereas the sindbis replicative mRNA vector is capable of achieving significantly augmented levels of gene expression compared to non-replicative mRNA vectors, this effect is highly dependent on the cellular target, likely reflecting cell-specific factors relevant to sindbis virus replicative capacity.

Augmented gene expression in vivo deriving from replicative mRNA transcripts

We next examined in vivo expression of non-replicative and replicative mRNA transcripts in tongue or quadriceps muscle using the luciferase reporter gene (Fig. 5). Injection of non-replicative mRNA transcripts into the tongue or quadriceps muscle produced luciferase activity 16 h later which was not significantly above background levels. Replicative mRNA transcripts injected into the tongue or quadriceps muscle generated luciferase activity which was 83- or 6-fold that of uninjected control muscle, respectively. Thus, reporter gene expression following injection of naked mRNA transcripts was more readily detectable in the tongue than in the quadriceps muscle. Furthermore, replicative mRNA transcripts directed 24-fold more luciferase expression 16 h following intraglossal injection than did non-replicative mRNA transcripts.

Based upon these observations, we next evaluated the time-course of luciferase expression following injection of naked mRNA transcripts into the tongue muscle (Fig. 6). A single 50 μg
Figure 4. *In vitro* transfection of various cell lines employing liposome–rRNA complexes containing replicative or non-replicative luciferase mRNA. Target cells were transfected as before with lipofectin–rRNA complexes and cell lysates analyzed 16 h post-transfection for luciferase activity. Data represents the mean ± SE of the mean for quadruplicate observations. The dash line at 800 indicates the threshold for a positive result defined as exceeding the mean ± 2 SD for a panel of eight untransfected cell lines.

Figure 5. *In vivo* gene expression mediated by replicative and non-replicative luciferase mRNA species delivered by direct intramuscular injection. Luciferase mRNA derived from pGT28 or pTLXN-Luc (50 μg) was delivered by direct intramuscular injection to myocytes within the quadriceps or the tongue. At 16 h post-injection, tongues and quadriceps were harvested and analyzed for luciferase expression. Data demonstrates the mean ± SE of the mean for groups of five mice. The dashed line indicates background luciferase levels observed in uninjected control muscles.

Figure 6. Temporal pattern of luciferase activity *in vivo* induced by luciferase mRNA transcripts. Replicative and non-replicative luciferase mRNA transcripts (50 μg) were delivered by intraglossal injection and reporter gene expression evaluated at various points post-delivery. Plotted data represents the mean ± SE of the mean for five mice. The dashed line represents background luciferase levels in uninjected control muscles.

Non-replicative luciferase mRNA
Replicative luciferase mRNA

Light units

MDA-MB-435 MC38 SKOV3 BHK21

Cell lines

Non-replicative luciferase mRNA
Replicative luciferase mRNA

Light units

Quadriceps 7 Day 1 Day 2 Day 3 Day 4 Days 5 Day 6 Day 7 Day
Post-Injection

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We next evaluated the effect of replicative mRNA dose on the magnitude of luciferase expression following intraglossal injection (Fig. 8). Single doses of 1, 10 or 50 μg of replicative luciferase mRNA produced luciferase activity 24 h post-injection which was 49-, 93- or 117-fold that of uninjected control muscle respectively. Thus, a 50-fold decrease in the dose of replicative luciferase mRNA produced only a 2.4-fold decrease in luciferase activity.

DISCUSSION

For transient production of heterologous mRNAs and proteins, the Sindbis virus expression system offers several potential advantages: (i) a broad range of susceptible host cells including those of insect, avian and mammalian origin; (ii) high levels of mRNA and protein expression within the host cell cytoplasm; (iii)
inhibition of host cell protein synthesis and (iv) ease of manipulation of recombinant mRNA molecules through use of a sindbis virus cDNA clone from which infectious RNA transcripts can be generated by in vitro transcription (23,24). In this regard, Huang's group has shown that replacement of the sindbis structural protein-coding region with the bacterial chloramphenicol acetyltransferase (CAT) gene resulted in a self-replicating mRNA producing $10^7$-10$^8$ polypeptides per cell of active CAT, corresponding to 3% of total cell protein (23). This is comparable to the level of recombinant protein production achieved with vaccinia virus vectors.

Based upon these advantages, recombinant sindbis virus has been proposed as a vaccine vector. In this context, intraperitoneal delivery of encapsidated sindbis virions encoding influenza hemaglutinin primed influenza-specific cytolytic T cell responses in mice (24). Furthermore, simliki forest virus (SFV), a similar single-stranded RNA alphavirus, has shown promise as a vaccine vector. Recombinant SFV RNA encoding influenza nucleoprotein delivered intravenously as encapsidated infectious particles or intramuscularly as naked RNA transcripts elicited nucleoprotein-specific humoral and cellular immune responses (25). These studies did not characterize the replicative mRNA directed protein expression in vivo. Pursuant to the use of self-replicative recombinant sindbis mRNA as a nucleic acid vaccine, we sought to characterize heterologous protein expression in vivo following intramuscular injection of naked, replicative mRNA transcripts. With regard to our prior studies of DNA based polynucleotide immunization, such in vivo expression data has proven essential to the rational design and implementation of immunization studies (5–7).

For employment in the context of polynucleotide immunization, we hypothesized that the replicative capacity of the sindbis virus vector would potentially allow elevated and sustained levels of expression of the immunizing gene construct in target myocytes. In this regard, whereas we have previously utilized CEA mRNA to achieve anti-CEA immunization by the direct intramuscular injection route, the observed levels of elicited immunologic response were quantitatively less than achieved with a naked plasmid DNA vector. Initial studies employing the sindbis virus polynucleotide vector were designed to define the parameters of heterologous gene expression achieved by the replicative mRNA in vivo. After construction, the ability of the replicative mRNA vector to achieve elevated levels of gene expression was verified using BHK21 cells, a line previously demonstrated to allow replication of a CAT-encoding sindbis virus vector mRNA construct. Not unexpectedly, we confirmed the ability of the luciferase-encoding sindbis virus vector to likewise achieve elevated levels of in vitro reporter gene expression. When we extended these studies to a series of additional cell lines, the observed differential of gene expression achieved by replicative versus non-replicative mRNA transcripts was highly variable; for certain cell types this differential was considerably less than that observed than for the BHK21. This observation likely reflects the intrinsic capacity of the vector to replicate in a given target. It was thus not apparent a priori if the level of gene expression in muscle would reflect the ability of the vector to replicate in this cellular target. When analyzed, however, it could be shown that the replicative mRNA vector achieved a markedly elevated level of gene expression compared to the non-replicative vector. In addition, the levels of gene expression observed were sustained for 10 days compared to 2 days with the non-replicative mRNA vehicle. Thus, the permissivity of myocytes for replication of the sindbis virus mRNA may be exploited to achieve levels of gene expression of utility for our polynucleotide immunization strategies.

There was no evidence of replicative mRNA directed protein expression 28 days post-injection. Three hypotheses could explain the attenuation of gene expression following replicative mRNA delivery: (i) immune response to the heterologous protein (luciferase) or sindbis virus non-structural proteins, (ii) inhibition
of host cell protein synthesis with resultant cytopathogenicity or (iii) loss of mRNA through a shift in the balance between viral replicase and endogenous ribonuclease activities. In support of an immunologic mechanism, Griffin's group has shown in a mouse model of sindbis virus encephalitis that host production of antibody acts to down regulate virus replication in the infected neurons by a non-cytolytic mechanism and clears infectious virus from the central nervous system (26,27). In the context of naked DNA transduction, Acsadi et al. has suggested an immunologic mechanism for the attenuation of reporter gene expression following naked plasmid DNA injection into rodent myocardium (28). In this report, luciferase expression in muscle was significantly prolonged in immunocompromised rats such as nude rats or normal rats receiving cyclosporin (28). Similar studies are underway to better characterize the role of immune response in cessation of replicative mRNA directed protein expression in myocytes.

In support of the second hypothesis, previous authors have demonstrated that infection of BHK21 cells by sindbis virus replicons, in which the LacZ reporter gene replaces all of the viral structural protein genes, still produces shut-off of host cell protein synthesis and cytopathic effects within 40 h (29). It would not be unexpected that post-mitotic myocytes might be less dependent on protein synthesis and take longer to succumb to this viral directed cytopathicity. With regard to the third hypothesis it is also likely that host cell factors may negatively impact on the stability of the heterologous mRNA transcript. In this regard, the prolonged expression of naked plasmid DNA within myocytes is related to its ability to persist within the nuclei of host cells in an intact and functional form. For mRNA to allow prolonged expression the nucleic acid would be required to persist within the myocyte cytosol. Whereas heterologous plasmid DNA persistence within the post-mitotic nucleus is not inconsistent with the physiologic fate of its host cell counterpart, mRNA transcripts possess an intrinsic instability, based upon normal endogenous break-down and turnover, as a regulatory mechanism of gene expression. This suggests that whereas the mRNA may be intrinsically less capable of long term persistence within myocytes than DNA, manoeuvres known to stabilize mRNA transcripts may be likewise of benefit in the context of the sindbis virus replicative transcript. Thus, this report provides in vitro and in vivo data demonstrating that a sindbis virus based replicative RNA vector can enhance cellular protein expression as compared to non-replicative RNA and that substantial levels of heterologous protein expression (luciferase) were documented following intraglossal injection of such constructs for a self-limited interval of at least 7–10 days. These characteristics would seem to be ideal for an RNA polynucleotide vaccine strategy particularly applicable to potentially oncogenic proteins. Such studies are underway in regards to immune response and anti-tumor effects.

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