Virology 390 (2009) 368–373

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

A high capacity *Alphavirus* heterologous gene delivery system

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ARTICLE INFO

Article history:
Received 14 April 2009
Returned author for revision
11 May 2009
Accepted 25 May 2009
Available online 16 June 2009

Keywords:
Alphavirus
Gene delivery
Morphological variants

ABSTRACT

A novel replication competent *Sindbis virus* based gene delivery vector has been developed for the introduction of genetic cargo into cell lines *in vitro* and potentially, animal models *in vivo*. This delivery system expands the previous uses of *Sindbis virus* as a gene delivery system in that no replicons are required and the resulting cargo containing virus particles are infectious. The heterologous vector is based on a morphological mutant in C, Ser180/Gly183 which produces larger than the normal size $T=4$ virus particles of 70 nm in size. This mutant produced particles up to 205 nm in size equal to a triangulation number of 36. It was postulated that because the Ser180/Gly183 mutant was capable of assembling such large particles, that increasing the size of the RNA genome incorporated into this mutant capsid protein would favor the assembly of larger than $T=4$ wild type sized virions. The first generation prototype larger vehicle, described here, carries a ~18 kb cDNA insert, however it is conceivable that RNA as large as 32 kb could be transcribed and packaged. The large variant produces a high virus titer of ~10^9 pfu/ml from either mammalian or insect cells in culture. Multiple passages of the virus show no loss of the inserted genetic material.

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Introduction

Sindbis virus is the archetypal representative of the genus *Alphavirus* in the family *Togaviridae*. The alphaviruses are also members of a group of viruses which are vectored in nature by insects, arboviruses (*ar*thropod *bo*rne *virus*) (Brown and Condreay, 1986). In nature, these viruses are propagated via a complex cycle of infection involving an insect vector, a mammalian or avian reservoir with incidental dead-end hosts. Many members of this genus are non pathogenic. In the insect vectors, the virus establishes a persistent infection and is produced in large quantities in the salivary glands with little to no apparent pathology to the insect. Infected mosquitoes transfer the virus through the saliva when taking a blood meal from mammals or birds. These infected hosts will in turn transmit the infection to a naïve mosquito during a subsequent blood meal. In mammals, infections with arboviruses take a distinct course. Non pathogenic viruses will quickly succumb to the innate immune response displaying little to no symptoms. Pathogenic viruses however, can produce arthralgia or encephalitis. The particular life cycle of the alphaviruses has endowed this genus with a large host range including cultured cells of insect, mammalian and avian origin.

The alphavirus Sindbis is encoded by a single + stranded RNA containing 11,703 nucleotides (Strauss et al., 1984) not including a polyA tail of variable size. The virion is simple, composed of three structural proteins E1, E2 and capsid (C) (Jones et al., 1974). The glycoproteins E1 and E2 are embedded into a lipid bilayer of host cell origin which encloses the nucleocapsid composed of the RNA genome condensed with capsid protein to form the inner core. *Sindbis virus* displays $T=4$ icosahedral symmetry in which the proteins E1, E2 and C are associated in a 1:1:1 stoichiometric ratio (Paredes et al., 1993). The outer glycoprotein shell is held together with the inner core through non covalent interactions of the E2 endodomain with the nucleocapsid (Lee et al., 1996). *Sindbis virus* infections are initiated with transcription of the 49S genome into the negative strand RNA which is the template for replicated genomic RNA and a 26S subgenomic RNA. The subgenomic RNA is the template for the translation of the structural proteins in the sequence 5′ C-PE2-6K-E1 3′ while the genomic RNA produces the nonstructural proteins nsP 1, 2, 3 and 4. Only genomic RNA however, contains the encapsidation signal to form nucleocapsids. Assembled nucleocapsids are organized in $T=4$ geometry and confer this geometry to the outer shell during the process of envelopment (Ferreira et al., 2003). The final product of the infection is a virus structure which is highly symmetrical displaying a high degree of stability producing particle to pfu ratios approaching unity.

Viral gene delivery vehicles have been in development over the past 20 years having demonstrated a strong potential to treat various diseases. Viral vectors such as Adeno-associated virus (AAV), Herpes virus, and Retroviral vectors have been used for gene delivery in human trials (Schaffer et al., 2008). Alphaviruses have also shown great promise for application to gene therapy (Agapov et al., 1998; Frolov et al., 1996; Huang, 1996; Lundstrom, 1999, 2002, 2005; Polo et al., 1999). To be an effective gene delivery vehicle, a viral vector must meet certain criterion. For ease of genomic manipulation,

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0042-6822/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2009.05.026

simplicity of the viral system is advantageous. Other characteristics include efficiency of gene transfer, the ability to carry and deliver a large transgenic load, and the growth of the chimeric vector to high titer. The viral vector should also encode the ability to target specific cells which are proliferating or non-proliferating. Infectious vectors should not be human pathogens. Alphavirus vectors used in gene delivery can be mutagenized to limit cytopathology induced by the infection (Agapov et al., 1998; Garmashova et al., 2006; Huang, 1996) and are poised to meet these criteria.

We have developed a method for increasing the capacity of an alphavirus to deliver heterologous genetic material. In its current design, the prototypic vector produced is self replicating and requires no helper virus sequences. This eliminates the necessity to use replicons as in the case in the typical alphavirus gene delivery system although it is conceivable that this strategy could be incorporated into the replicon system. The largest of these virus particles suggest that the only limit in its capacity is the size of RNA that can be managed in the laboratory.

Results

Production of the Sindbis virus Tandem and Tandem-GFP DNA clones

The Tandem and Tandem-GFP clones were constructed as described in Fig. 1 and Methods. The final products of the cloning process are two distinct virus populations. 1). Tandem virus which contains two copies of the virus structural genes each with its own promoter and each containing the Ser180/Gly183 mutations described previously (Ferreira et al. 2003). 2).Tandem-GFP which contains two copies of the virus structural genes with the gene for GFP inserted between the two clusters of structural genes. The first set of structural genes and the *GFP* gene both have their own 26S promoter to insure the independent synthesis of GFP. In this construct a stop codon is inserted at the end of the *GFP* gene and the second copy of the structural genes has no promoter. The 3′ terminal untranslated region (UTR) which is required for minus strand RNA synthesis (Hardy, 2006; Hardy and Rice, 2005; Ou et al., 1982a, 1981, 1983, 1982b; Strauss et al., 1983) is attached downstream from the second structural gene cluster. The construct was made in this way to insure that the larger genome could be efficiently encapsidated, producing high titers of progeny virus of increased size without the need for additional structural protein synthesis (which is provided in the tandem mutant, described above, by the addition of a second set of structural gene sequences with an active promoter) as would be the case for the expression of larger heterologous genes. The RNA of these mutants was transfected into BHK-21 cells, the resulting virus was plaque purified and amplified in BHK-21 cells by three passages and the resulting virus served as "stock virus" for the experiments described below.

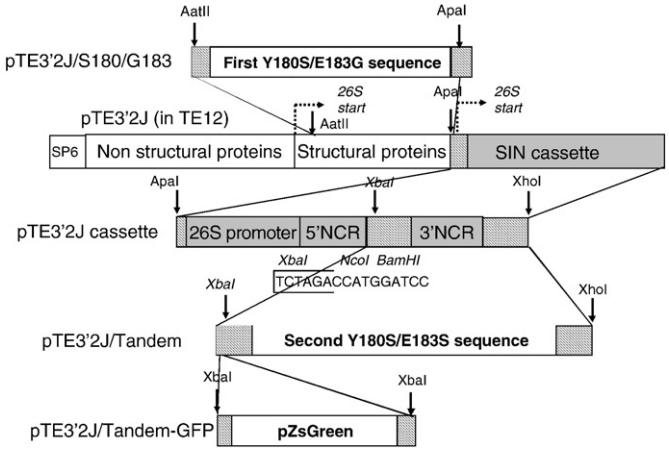

Fig. 1. Construction of pTE3′2J/Ser180/Gly183 Tandem clone and the Tandem clone with GFP cargo. See Methods for complete description.

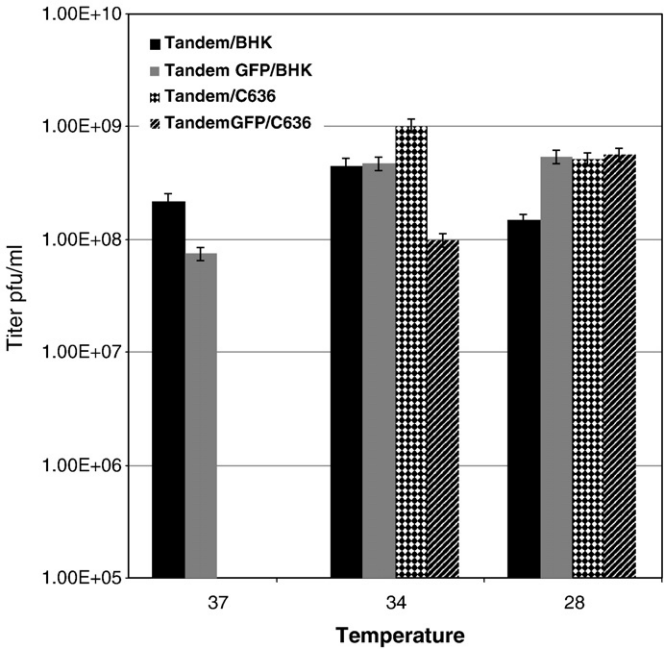

Fig. 2. Titers of the gene delivery virus vectors. Mutant viruses were grown at 3 different temperatures (37, 34 and 28 °C) in BHK cells (solid colors) and 34 and 28° in mosquito C6/36 cells (hatched bars). Virus was collected at 24 h for BHK cells and 48 h for *Aedes albopictus* cells. At each temperature the data presented are from left to right Tandem virus, Tandem-GFP, Tandem virus, Tandem-GFP. The figure shows the average of three experiments and the variability was ± 10–15%

Growth of the Tandem virus mutants in insect and mammalian cells

Transcripts of the Tandem virus and Tandem virus containing the GFP protein produced as described in Fig. 1 and Methods were transfected into BHK-21 cells to produce stock virus populations as described in Methods. These stock viruses were used to infect monolayers containing roughly equal numbers of BHK-21(mammalian) or *Aedes albopictus* C6/36 (mosquito) cells at temperatures of 28, 34 and 37 °C. Virus production was determined by titration of the supernatant of the infected cells at 48 h for mosquito cells or 24 h for the BHK cells, after infection. We have previously shown that these times represent the point of maximum virus production in these two cell types (Gliedman et al., 1975; Renz and Brown, 1976). The result of this experiment is presented in Fig. 2. The Tandem virus and the Tandem-GFP virus grow to high titers in both mammalian and mosquito cells at 28 and 34 °C. Some difference is seen in virus production in that the Tandem-GFP virus grows to slightly lower titer in mosquito cells at 28 °C and the Tandem virus grows to slightly lower titer in mammalian cells at 34 °C. At 37 °C the Tandem and Tandem-GFP grow to titers in mammalian cells somewhat lower than at the lower temperatures. Thus the Tandem and the Tandem-GFP grow to high titer in both insect and mammalian cells without the requirement for helper viruses or helper virus sequences as is the case in the replicon systems.

Structure of virus produced by the Ser180/Gly183 mutant and the Tandem-GFP virus

We have previously shown that virus produced by *Sindbis virus* containing the mutation Ser180/ Gly183 produces morphological variants when infecting BHK cell monolayers (Ferreira et al., 2003; Lee and Brown, 1994). These variants were shown to fall into size classes consistent with class $P=1$ icosahedra displaying triangulation

numbers from $T=4$ to $T=36$ (Ferreira et al., 2003). These particles range in size from a diameter of 70 nm to over 200 nm. Preliminary experiments indicated that these larger particles were infectious (Ferreira et al., 2003). In these previous experiments the smallest sized $T=4$ virus represented the vast majority of the total virus population. This suggested that assembly of the mutant favored the $T=4$ virion because this structure encapsidated the normal virus genome of 11,703 nucleotides (plus polyA tail). It has been demonstrated that virus capsid protein can assemble into capsid structures in the presence of small oligonucleotides of RNA or DNA (Tellinghuisen et al., 1999; Wengler et al., 1982). These observations suggested that the creation of larger RNA genomes containing the virus packaging signals would assemble with the virus capsid protein containing the Ser180/Gly183 mutant favoring the formation of larger T number capsids and hence larger T number virions. This hypothesis was tested by infecting BHK or C6/36 cells with either the Ser180/Gly183 or the Tandem-GFP mutant. In each case the infection was conducted using virus which had been plaque purified and passaged three times in BHK-21 cells (see Methods). Virus produced in these infections was purified by density gradient centrifugation on linear potassium tartrate gradients. Virus bands were collected and examined in the electron microscope by negative contrast staining with uranyl acetate. Typical fields are shown in Fig. 3. Fig. 3A shows virus produced by the Ser180/Gly183 mutant described by Ferreira et al. (2003). The image is similar to that published previously (Ferreira et al., 2003) with the normal $T=4$ virions making up most of the virus population with a few larger putative $T=9$ particles seen. Viruses purified from cells infected with the Tandem-GFP mutant are shown in Fig. 3B. The Tandem-GFP mutant was found to produce larger than normal virus. Although the critical point drying experiments described previously (Ferreira et al., 2003) which allowed estimation of triangulation numbers was not done, these particles have negative stain diameters consistent with T numbers of $T=9$ and $T=16$. A few particles having diameters expected of $T=4$ virions were seen (not shown) however we are unable to determine if this is the result of loss of part of the RNA genome in some replicating forms or cores from the larger particles. These particles were few in number and we were unable to detect normal size virus RNA upon analysis of this virus population or infected cells (see Fig. 4). Because the capsid protein

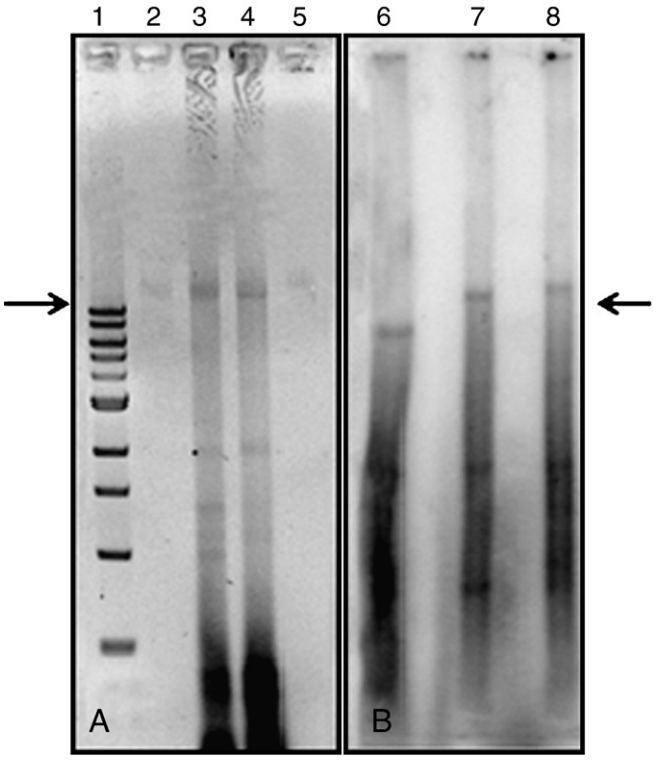

Fig. 4. RNA from pTE3′2J parent virus, Tandem and Tandem-GFP virus. In (A) is shown RNA extracted from virus purified by isopycnic gradient centrifugation or from infected cell supernatants. Lane 1. 1 kb marker ladder. Lane 2. Tandem RNA extracted from purified virus. Lane 3. Tandem virus RNA from extracted cell supernatant. Lane 4. Tandem-GFP RNA from extracted supernatant. Lane 5. Tandem RNA extracted from purified virus. Note that the smaller RNA molecules are not present in the purified virus indicating that the small RNA are degradation products from contamination in the supernatant. In (B) are shown RNAs extracted from pTE3′2J virus, Tandem virus and Tandem with GFP from supernatant in a second experiment including the pTE3′2J parent virus. Lane 6. RNA from pTE3′2J parent virus, note that virus from the supernatant of the wild type infection displays the same sized fragments as those seen in the Tandem virus lanes. Lane 7. RNA from Tandem virus. Lane 8. RNA from Tandem virus with GFP. The arrows show the position of the 10 kb DNA ladder fragment. Images are shown in their inverted color format rendered in black on white to improve visualization of the fainter bands.

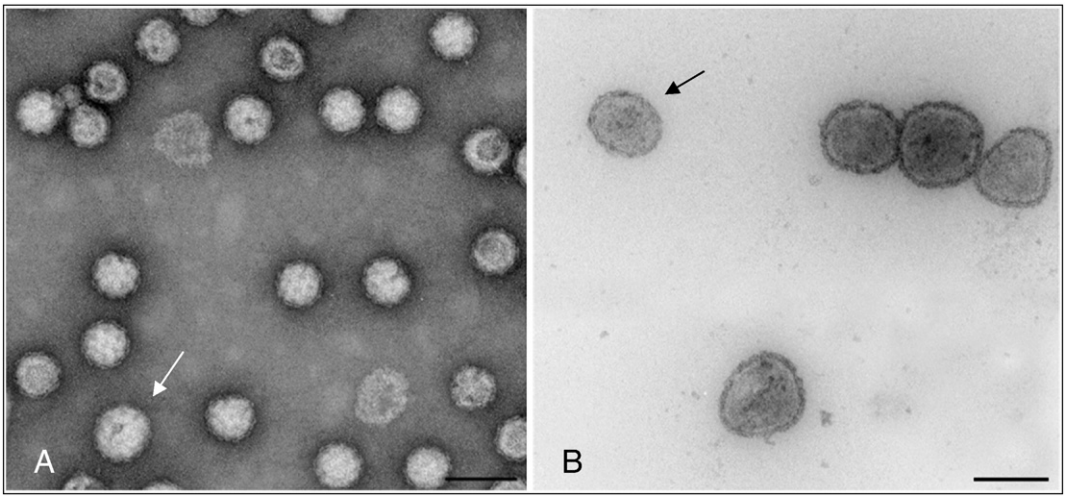

Fig. 3. Electron microscope images of negative stains of morphological variant virus strains. In (A) is shown the parent virus Ser180/Gly183 showing a field of $T=4$ virus with the arrow pointing to a possible $T=9$ virion. In (B) is shown the Tandem virus with GFP cargo. These particles are estimated to be $T=9$ (arrow) and $T=16$ sized particles based on published data (Ferreira et al., 2003). The Tandem virus with the *GFP* gene contains ~18,500 bases of RNA. The magnification bar in B is 100 nm and is scale for both A and B images.

produced is the same in both the Tandem and the Tandem-GFP infections we conclude that the preponderance of larger particles produced by this mutant is the result of incorporation of ~18,500 bases (plus polyA tail) of RNA making up the Tandem-GFP genome into the developing virus capsid pushing the assembly of capsid protein into the larger *T* number structures.

Size of RNA genomes incorporated into Tandem and Tandem-GFP virions

Evidence that the RNA incorporated into the large virions was of the size predicted for the Tandem-GFP genome was strongly suggested by the fact that the virus replicates efficiently (see above). Had either the 3′ or 5′ UTR of the molecule been lost the components of the UTR required for replication would have been deleted and no virus would be produced. We wished to ensure that the RNA incorporated into the larger virus particles was indeed the anticipated size of the Tandem virus (17,547 bases plus polyA) and tandem-GFP (18,471bases plus polyA). To determine this, RNA was prepared from virus purified on continuous potassium tartrate equilibrium gradients and directly from the media of infected cells as described in Methods. To ensure that the viral RNA was of the expected size it was initially extracted from infected cell supernatants as shown in Fig. 4 lanes 3 and 4, and lanes 6, 7 and 8 from a second experiment. This virus however, was not of sufficient purity to assure that the smaller bands visible in the gel were nicked products and not smaller RNA. Although the sizes of the smaller RNAs are within the sizes published for some DI RNAs we consider the possibility that these forms represent replication competent DI RNAs unlikely for the following reasons; 1: DI RNA production requires multiple undiluted passages of Sindbis at high multiplicity. Up to 15 to 20 such passages are required in most cases. Reports show the earliest appearance of these RNAs after 5 high multiplicity passages. The virus employed in these studies was from 3 consecutive passages after plaque purification in which the multiplicity of infection did not exceed 10. 2: in Fig. 4B lane 6, the parent virus also shows nicked RNA, where this infection was not from serially passaged virus and produced no DI particles. In the wild type infection the genome is known not to be unstable. 3: the previous work published by Ferreira (Ferreira et al. 2003) showed that the mutant Ser180/Gly183 in standard Sindbis produced larger than the normal $T=4$ particles but that the overwhelming majority of particles were $T=4$ structures. These observations lead to the hypothesis that larger RNAs would favor the production of the larger virions which is tested in this study. If significant amounts of self replicating, packaging competent small RNAs were produced we should have seen many $T=4$ particles in the electron microscope (as the smaller RNA would favor the formation of $T=4$ virions) and there should have appeared as a second band in density gradient centrifugation which was not seen. 4: the titers of virus produced by the Tandem and Tandem-GFP virus is high as shown in Fig. 2. This would not be expected in the presence of interfering RNAs.

For comparison, in Fig. 4 lanes 1 and 5 RNA from purified Tandem virus or Tandem-GFP is shown in which no visible nicking was observed. Note that the Tandem-GFP RNA runs slightly above the tandem RNA as is expected. In lane 4 showing tandem-GFP from supernatant, a double band of RNA is seen at the top of the gel, one of which runs with the RNA from the purified virus shown in lane 5 indicating that the additional bands seen for the unpurified virus are from nicked RNA. In Fig. 4B is shown the mobility of extracted RNA including the parent virus pTE3′2J relative to a 1 kb DNA ladder (lane 1) and as indicated by the arrow to the right of the gel. The top band of the DNA ladder is 10 kb.

These results confirm that the size of the RNA molecules is that predicted by the cDNA sequence.

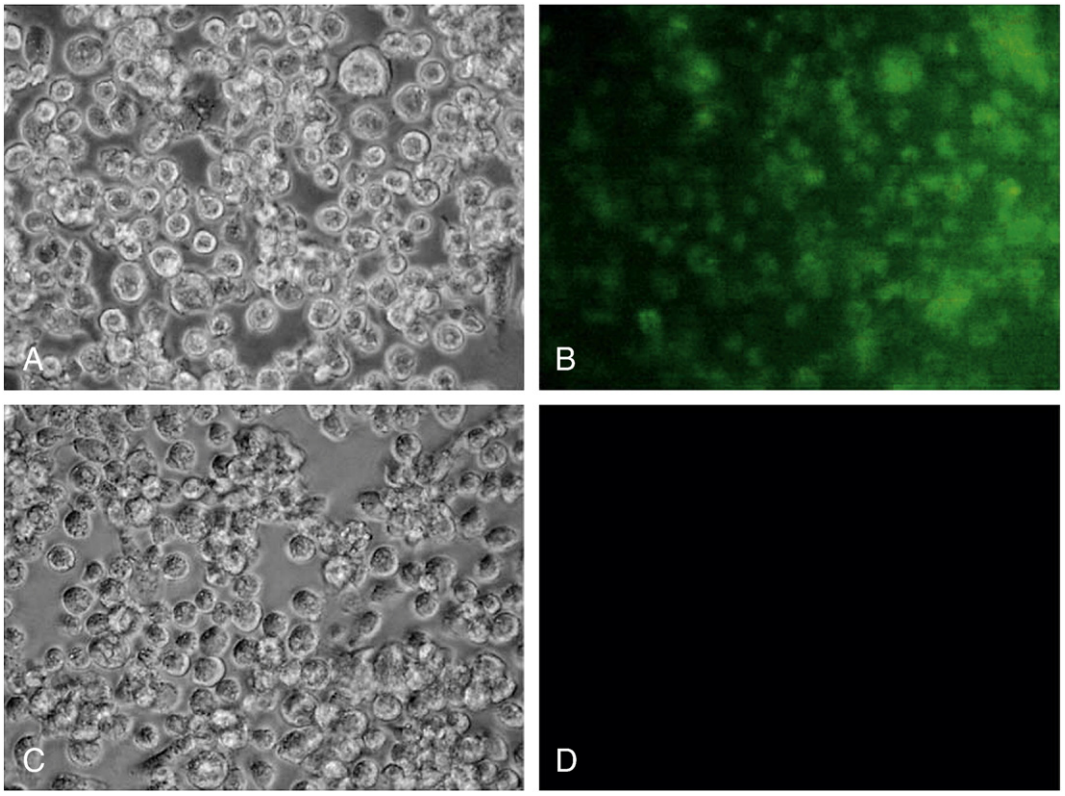

Fig. 5. Expression of *GFP* cargo gene in C6/36 cells. C6/36 cells were infected with Tandem virus or Tandem with GFP (MOI 10) and incubated for 24 h at 28 °C. In (A) is shown the monolayer using phase contrast to visualize cells infected with Tandem with GFP virus and in (B) is shown the same field under green fluorescence excitation (exciter filter 450–490 nm). In (C) is shown a monolayer infected with the Tandem virus without the *GFP* gene and the same field in (D) with excitation showing no detectable fluorescence.

Delivery of, and expression of, GFP in tissue cultured cells by Tandem-GFP virus

To test the ability of the Tandem virus to deliver a heterologous gene in its expressible form, C6/36 insect cells were infected with either the Tandem virus or the Tandem-GFP virus. Infected cells were incubated at 28 °C for 48 h and then examined and photographed by light and fluorescence microscopy. The result of this experiment is shown in Fig. 5. The cells infected with the Tandem-GFP virus are clearly seen to express the GFP protein and nearly all of the cells seen by normal white light microscopy are infected and expressing GFP when viewed by fluorescence (Figs. 5A and B). The cells infected with the Tandem virus without GFP are clearly negative for fluorescence (Figs. 5C and D).

Discussion

The alphaviruses have become popular vectors for the delivery of foreign (heterologous) genes to tissue cultured cells and the tissues of animals and insects (Agapov et al., 1998; Polo et al., 1999). These vectors have been used in experiments investigating the effects of expression of particular genes on fundamental biological processes. These vectors have also shown promise as platforms for vaccine development by expressing epitopes of proteins capable of inducing an immune response. Alphavirus vector platforms are typically a two component system composed of an RNA containing the nonstructural genes with their associated packaging signal and the structural proteins removed and replaced with heterologous gene sequences. The helper RNA contains the virus structural and nonstructural proteins without the packaging signal. These two component, replicon, based systems are limited in the amount of RNA they can package by the volume of the virus capsid. The production of Sindbis virions containing additional sequence has been reported by Hahn et al. (1992). These investigators demonstrated that additional RNA of up to 2 kb can be incorporated and expressed in wild type *Sindbis virus*. These authors found that larger inserts were much less stable and lost the ability to express the heterologous gene (*β-galactosidase*) immediately after the initial transfection. The mutations described above increase the capacity of alphaviruses to deliver heterologous genes by increasing the volume of the virus capsid. In the experiments presented above the size of the virus genome was increased by about 40% by increasing the size of the message from 11,703 nucleotides to ~18,000 nucleotides. The increased RNA was incorporated into larger virus particles. Negative staining of the resulting virus suggested an increase in triangulation number from $T=9$ to $T=16$ based on the work of Ferreira et al. (2003). We are presently confirming this change in morphology by cryo-electron microscopy in collaboration with Dr. Angel Paredes (University of Texas Health Sciences Center). A GFP reporter gene was incorporated as part of this additional RNA to show that additional cargo delivery was successful. These virus vectors grow to high titers of 10^8 to 10^9 pfu/ml compared to titers of 10^6 to 10^7 pfu/ml typically seen in the two component vector systems employing the wild type capsid gene and the $T=4$ core (Polo et al., 1999). This may imply an increased stability of the Tandem virus due to its increased volume. The stability of these mutant virions is underscored by the fact that the mutant RNA was transfected into cells, the resulting virus was plaque purified and the virus produced from the plaque amplified to produce a stock virus which was then used to produce the infections described in this report. This constitutes 3 passages of the mutant virus without loss of phenotype. The mutations in the capsid protein resulted in self replicating cargo containing virions which do not require helper functions for assembly as is the case in the two component system. The two component vector system has distinct advantages over that described here in cases where safety of a self replicating vector is a concern. We anticipate that these same mutations can be incorporated into the capsid sequence of the two component, replicon based gene delivery system increasing the capacity of that system in cases where propagation of the gene delivery system as progeny virus is not desirable. Though we have only looked at GFP under the control of a Sindbis promoter, it is very likely that any of the other reporter/promoter combinations (for example described by Polo et al. 1999) will express as well in this system as they do in any of the other vectors.

This research and the research described previously (Ferreira et al., 2003) suggest a prominent role for virus RNA in determining the structure of the maturing virus particle. The mutations involved in the development of the virus particle are in the capsid protein while the sequences of the envelope proteins are wild type. In the absence of the additional RNA described here the virus produces larger *T* number virions but the particles are predominately wild type $T=4$ geometry (Ferreira et al., 2003). It appears that the additional RNA sequence favors the formation of the larger *T* numbers resulting in few normal size particles recovered.

Methods

Cell culture, plaque assay and virus purification

BHK-21 and U4.4 mosquito cell culture, plaque assay, and virus purification are described in detail in the Chapter; Sindbis virus: propagation, quantization and storage (Hernandez, 2006). C6/36 cells were adapted to growth in MEM-E supplemented with 10% FBS (Hyclone, lot # 18779, Logan, UT), 5% tryptose phosphate broth and 5 mM glutamine as described previously (Renz and Brown, 1976; Scheefers-Borchel et al., 1981).

Construction of the pTE3′2J/Ser180/Gly183 Tandem clone

Full length recombinant *Sindbis virus* cDNA clone (pTE3′2J) (Hahn et al., 1992) was a gift of Charles Rice (Rockefeller University) and was derived from the plasmid pTE12, a variant of Toto1101 (Lustig et al., 1988). This cDNA contains a unique ApaI site at nucleotide (nt) 11,386 not found in the Toto1101 cDNA. Downstream of this restriction site a second 26S subgenomic promoter (nt −109 to 49 relative to the 26S subgenomic RNA start site) was also installed. Immediately downstream of the second promoter a multiple cloning site (Xba I, Nco I, Bam HI, 5′TCTAGACCATGGATCC 3′) is available for the insertion of foreign genes. This site is immediately followed by the viral 3′ NCR and polyA tail followed by the original Xho I site from Toto 1101 (nt 11,749) used for linearization and the generation of transcripts. The structural region of pTE3′2J containing the wild type capsid gene was replaced with the Ser180/Gly183 mutation (Lee and Brown, 1994) by an exchange of the pTE3′2J fragment from AatII (nt 8000, pTE3′2J numbering) through the entire structural protein coding sequence through the ApaI site (nt 11,386) using a PCR generated fragment from the Ser180/Gly183 template. One primer was designed to anneal upstream of the AatII site in the template; AatII-(sense):5′ GCAGGCGCCCAAGCAACC3′ and a second primer designed to incorporate the ApaI site downstream of E1, ApaI (antisense) 5′CGGATCATTGGGCCCTAGCGGTCATC3′. Standard PCR was employed using *Pfu* DNA polymerase (Stratagene) to construct the insert. This plasmid was designated pTE3′2J/Ser180/Gly183. A second set of mutant capsid, E1 and E2 structural genes was installed downstream of the second subgenomic promoter in pTE3′2J/Ser180/Gly183, producing a Tandem Ser180/Gly183 mutation. This insertion also served to increase the size of the viral genome from 13.8 kb (pTE3′2J) to 17.5 (pTE3′2J Tandem) and finally 18.5 kb (pTE3′2J Tandem-GFP, described below) which was predicted to favor the assembly of the $T=9$ virus particles. Primers were designed for amplification of the second set of structural genes through the use of the XbaI and XhoI ends. The existing XhoI site of the template was incorporated into the tandem construct using the primer 3′NC11,817 XhoI (antisense): 5′

GGTTCCGCGCACATTTCCCC3′ which anneals downstream of the XhoI site in the pTE3′2J/Ser180/Gly183 template. A forward primer incorporating the XbaI site just upstream of the 26S promoter: 5′ NC7636-XbaI-(sense): 5′GCTCTAGAACCATGAATAGAGG3′ was used to produce a PCR product using the method described above.

Construction of the pTE3′2J/Ser180/Gly183 Tandem clone with GFP cargo

The plasmid pZsGreen containing the green fluorescent protein from a variant of wild type *Zoanthus* sp. was purchased from Clonetech (Clonetech, Mountain view, CA). The pZsGreen coding sequence was amplified by PCR as described above using the primer XbaI Zsgreen (sense) 5′ GCTCTAGAACCATGGCTCAGTCAAAGC 3′ which installed an upstream XbaI site 2 nt from the initiation ATG of the GFP. The primer pZsGreen 12,250 (antisense) 5′ GCATCCG-GATCTGCATTGCCC 3′ primed downstream of the GFP coding sequence unique XbaI site and terminus which together produced a PCR product of 743 nt containing two XbaI ends. The resulting PCR product was inserted into the Tandem pTE3′2J/Ser180/Gly183 at the unique XbaI site (Fig. 1) downstream of the second promoter and the resulting cDNAs were screened for the proper orientation (see Fig. 1). The insertion of the *GFP* gene increased the virus genome to 18.5 kb and this cDNA was designated pTE3′2J/Ser180/Gly183Green or Tandem-GFP for simplicity. The cDNA was transcribed (Hernandez, 2006) and the RNA used to transfect BHK cells for the production of GFP expressing Tandem-GFP virus using conditions described previously (Hernandez, 2006).

RNA extraction and transcript production

RNA extraction and RNA transcript production was done as described in Hernandez et al. (2000) without modifications. Extracted RNA from twice purified virus or infected cell supernatants was run on 1% agarose gels for analysis and is shown in Fig. 4.

Acknowledgments

This research was supported by the Foundation for Research, Carson City NV and by the North Carolina Agricultural Research Service.

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