

Research article

## Development of dengue virus replicons expressing HIV-1 gp120 and other heterologous genes: a potential future tool for dual vaccination against dengue virus and HIV

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### Abstract

**Background:** Toward the goals of providing an additional vector to add to the armamentarium available to HIV vaccinologists and of creating a bivalent vaccine effective against dengue virus and HIV, we have attempted to create vectors which express dengue virus non-structural proteins and HIV immunogens. Previously we reported the successful construction of dengue virus replicons which lack structural genes necessary for virion release and spreading infection in culture but which can replicate intracellularly and abundantly produce dengue non-structural proteins. Here we attempted to express heterologous genetic material from these replicons.

**Results:** We cloned into a  $\Delta$ pre-M/E dengue virus replicon genes for either green fluorescent protein (GFP), HIV gp160 or HIV gp120 and tested the ability of these constructs to express dengue virus proteins as well as the heterologous proteins in tissue culture after transfection of replicon RNA.

**Conclusions:** Heterologous proteins were readily expressed from these constructs. GFP and gp120 demonstrated minimal or no toxicity. Gp160 expressing replicons were found to express proteins abundantly at 36 hours post transfection, but after 50 hrs of transfection, few replicon positive cells could be found despite the presence of cellular debris positive for replicon proteins. This suggested that gp160 expressed from dengue virus replicons is considerably more toxic than either GFP or gp120. The successful expression of heterologous proteins, including HIV gp120 for long periods in culture suggests this vector system may be useful as a vaccine vector, given appropriate delivery methods.

### Background

Despite tremendous progress in developing anti-retroviral drugs to combat HIV, there remains a need for an effective HIV vaccine. This need is particularly pressing in third world countries, where demographics and economics make drug therapy difficult to deliver. Although HIV

infection elicits neutralizing antibodies and a cellular immune response against the virus [reviewed in [1] & [2]] and there exist "exposed uninfected" (EU) individuals that appear to have acquired resistance to infection by HIV [3,4], the hallmark of HIV infection is the almost universal inability of humans to mount an immune re-

sponse that can prevent the eventual development of AIDS.

An effective vaccine will require not only the design of effective immunogens, but also the design of optimized protocols of immunogen delivery. As a live, attenuated vaccine for HIV is considered difficult to test and dangerous to implement [1,2,5-9], various alternatives to HIV could be considered as potential "live" vectors for HIV immunogens, including enteric bacteria, poxviruses (vaccinia and canarypox), small RNA viruses (e.g. poliovirus and Semliki Forest virus), Rhabdoviruses (e.g. vesicular stomatitis virus), DNA viruses (e.g. adenovirus and adeno-associated viruses) and even naked DNA to achieve expression in living host cells [2,10].

Dengue possesses several advantages which favor its choice as a vector for HIV immunogens. As a flavivirus, it replicates entirely in the cytoplasm through RNA directed RNA polymerization and is incapable of integrating into the host genome. Flavivirus replicons can replicate inside cells and achieve prolonged expression of high levels of virally encoded proteins with minimal toxicity [11,12] and are unable to recombine or mutate to produce infectious HIV particles. Finally, by eliciting an immune reaction against the dengue non-structural proteins remaining in replicons, dengue virus replicons may induce a protective immunity against dengue which would not predispose vaccinated individuals to DHF. Properly administered, dengue virus replicons expressing HIV epitopes might thus serve as dual vaccines, conferring protection against dengue virus as well as HIV.

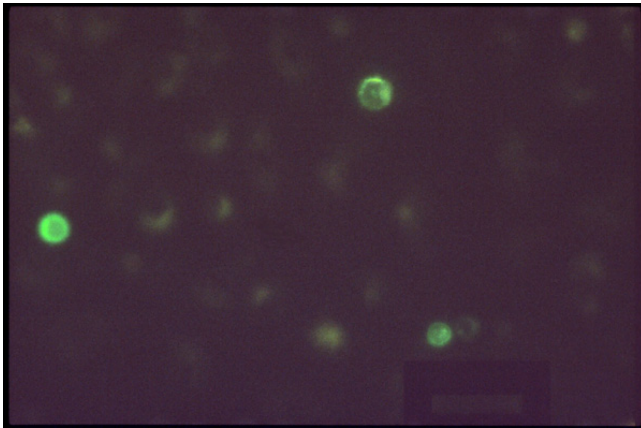
The challenges in developing a safe and effective HIV vaccine are many and varied. Choice of immunogen is clearly problematic. Critical epitopes may be masked by glycosyl groups and/or tertiary structure [13-15] and [16]. The extensive genetic variability of HIV complicates immunogen choice and the high rate of mutation increases the likelihood of the rapid development of resistance. Furthermore, the method of immunogen delivery (e.g. purified subunits or inactivated virus vs. various forms of "live" expression) can determine the relative nature and extent of humoral and cell mediated immunologic responses. Priming with various types of "live" expression followed by boosting with purified subunits is currently favored as a method to obtain stronger immunologic responses that either method alone [reviewed in [1] and [2]]. Previously acquired immunity to a viral vector such as vaccinia may influence its efficacy in inducing immunity against heterologous proteins being delivered [16-19] and it may be wise to provide physicians with HIV vaccines based on a variety of vectors to handle a variety of clinical situations.

The use of live dengue as a vaccine or as a vector for heterologous immunogens has historically been considered problematic because of the pathologies associated with dengue infection. Although dengue fever (DF) is usually self limited, dengue hemorrhagic fever (DHF) is considerably debilitating and frequently fatal [20]. However, DHF is unlikely to result from or be promoted by the vectors reported here. The enhanced replication of virus seen in dengue hemorrhagic fever is generally seen upon reinfection by dengue virus of a serotype different from previous infections and is believed to be mediated by antibodies against viral structural proteins: so called antibody dependent enhancement of infection, or ADE. These cross reacting antibodies actually promote viral uptake by macrophages [21,22]. The main challenge in using live dengue in humans is thus avoiding the development of antibody dependent enhancement (ADE) of infection by antibodies against the pre-M and E proteins of one dengue strain which weakly cross react with the pre-M and E of a second infecting dengue strain. Since the replicons reported here lack the major viral structural protein genes, they are not only incapable of sustaining a spreading infection but also are incapable of eliciting antibodies against the missing structural proteins. They should neither induce DF nor promote DHF.

Dengue virus has a typical flavivirus genome structure, as described in Figure 1. The structural proteins, C, pre-M (M) and E, are involved in packaging, export and subsequent entry. The non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 include an RNA-directed RNA polymerase and a protease function involved in cleaving certain positions of the long viral polyprotein which contains all the viral genes [23,24]. The four serotypes of dengue virus ("1" through "4") share approximately 60%-74% amino acid residue identity with one another in the E gene [25] and induce cross-reacting antibodies [26].

Two strategies suggest themselves for circumventing the problem of ADE from dengue vaccination. One strategy is to immunize with multiple strains of dengue virus to elicit high affinity, neutralizing antibodies against the multiple dengue serotypes. At least one vaccine to do this (using dengue vaccine candidates, DEN-1 PDK13, DEN-2 PDK53, DEN-3 PGMK 30/F3, and DEN-4 PDK48) has been in clinical trials [27,28]. A second strategy is to induce immunity only to viral proteins other than pre-M and E. Several studies have shown that the nonstructural glycoprotein NS1 can play an important role in protection against dengue. Mice immunized with purified dengue-2 NS1 protein injected intramuscularly and boosted after 3 days and two weeks were protected from developing lethal dengue encephalitis upon subsequent challenge with dengue 2 virus. [29]. Similarly, mice





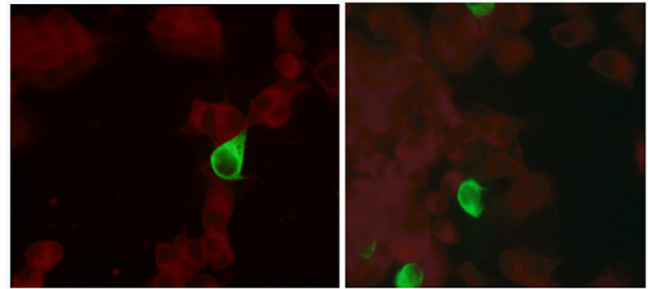
**Figure 2**  
Expression of green fluorescent protein (GFP) by  $\Delta$ pre-M/E-GFP 48 hours post transfection.

immunized with recombinant vaccinia virus expressing authentic NS1 [30] were protected against the development of dengue-4 virus encephalitis when challenged by intracerebral injection. Inoculation of mice with specific combinations of monoclonal antibodies (Mabs) directed against dengue-2 NS1 [31] also protects against lethal virus encephalitis upon intracerebral dengue-2 challenge. Other nonstructural proteins are also immunogenic and may participate in eliciting protection [32].

Previously we have reported the successful construction of several dengue virus replicons which replicate intracellularly without the pre-M and E proteins required to form infectious virions, including one replicon which can be expressed from transfected DNA [12]. Towards the goal of devising a "live" dual vaccine based on only non-structural dengue proteins and heterologous HIV material, we report here that these replicons can be harnessed to express heterologous genes, including HIV gp160 and gp120. Upon introduction into a host's cells, these subgenomic fragments should replicate intracellularly and support prolonged expression of dengue and heterologous immunogens without producing the deleted dengue structural proteins and without forming infectious virions.

## Results

In various previous attempts to express heterologous genes in full length, wild type dengue virus, we experienced a very poor success rate, despite attempts to clone heterologous material into various positions of the genome (data not shown). Our first efforts to determine whether or not heterologous material could be readily expressed in dengue replicons was to clone the comparatively tractable green fluorescent protein (GFP) into the  $\Delta$ pre-M/E replicon, into the position from which the pre-

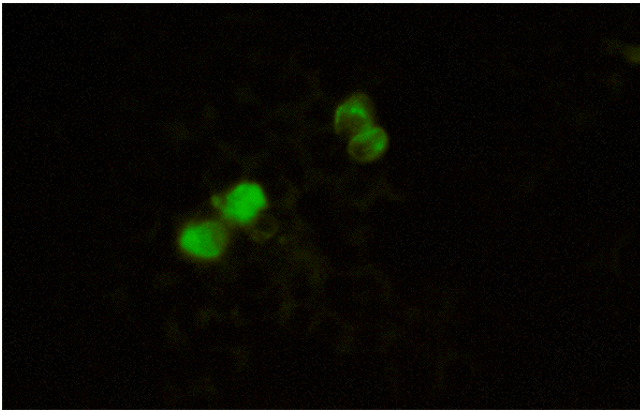


**Figure 3**  
Expression of proteins by  $\Delta$ pre-M/E-gp120 50 hours post transfection. Left and right frames are two independent fields. Anti-dengue serum was used in these experiments.

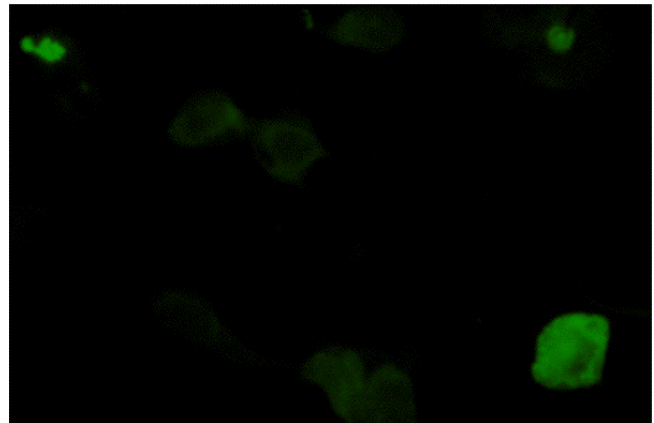
M and E genes had been deleted (Figure 1). GFP was readily visualized in cultures 48 hours post transfection with  $\Delta$ pre-M/E-GFP, as seen in Figure 2.

Encouraged by the success with GFP, we next looked at  $\Delta$ pre-M/E replicons with HIV-1 env material cloned into the position of the deleted pre-M and E genes. We analyzed two clones,  $\Delta$ pre-M/E-gp120 and  $\Delta$ pre-M/E-gp160, expressing HIV-1 gp120 and gp160 respectively (Figure 1). Expression of genes in the  $\Delta$ pre-M/E-gp120 replicon was reproducibly visualized at 48–50 hours post transfection (Figures 3 & 4), at a level of approximately 1% of the cells, but in many experiments, the corresponding cultures transfected with the gp160 replicon,  $\Delta$ pre-M/E-gp160, either no fluorescence could be visualized (not shown), or only fluorescent cells with a bizarre morphology (characterized by debris and/or degenerative appearance) could be visualized (Figure 5). However, when we harvested cultures earlier, at 36 hours post transfection with  $\Delta$ pre-M/E-gp160, intact, fluorescing cells were readily found, though the morphology still appeared atypical compared to either that of cultures transfected with wild type dengue virus and dengue replicons [12] or the  $\Delta$ pre-M/E-gp120 replicon (see Figure 6).

To serve as effective vaccines, it is preferable, if not necessary, that expression systems be capable of expressing immunogens for longer than a couple of days. Although we knew from previous experiments [12] that dengue replicons could survive for at least 7 days in culture, the limited durability of cells transfected with gp160-expressing replicons raised the question of whether or not cells transfected with  $\Delta$ pre-M/E-gp120 replicons could survive for similarly long times in culture. When cultures transfected with  $\Delta$ pre-M/E-gp120 were trypsinized and replated on day 7 post transfection and then analyzed on day 9 post transfection, fluorescent cells were readily visualized (Figure 7). In comparison to cultures that were not trypsinized on day 7 post transfection however, these



**Figure 4**  
Expression of proteins by  $\Delta$ pre-M/E-gp120 48 hours post transfection. Left and right frames are two independent fields. Anti-HIV serum was used in these experiments.



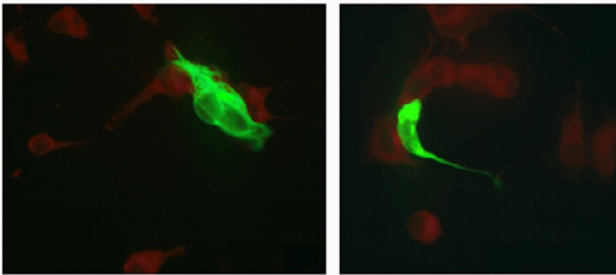
**Figure 5**  
Expression of gp160 by  $\Delta$ pre-M/E-gp160 48 hours post transfection. Left and right frames are independent fields. Anti-HIV serum was used in these experiments.

cultures had fewer intact fluorescent cells and more debris (not shown). Although this suggests that gp120 expression from a dengue replicon stresses cells, we did find fields with adjacent, gp120 positive cells, suggesting that at least one cell division between day 7 and day 9 had occurred in a cell successfully transfected with  $\Delta$ pre-M/E-gp120 (Figure 7, right panel). A 9 days of culture post transfection with  $\Delta$ pre-M/E-gp120, only about 0.1% of the cells or less were positive (not shown), which represents a considerable decrease from 48 hours post transfection.

In the experiments described above and in Figures 3 through 7, expression of dengue replicons with heterologous material from HIV was followed either using anti-HIV sera or anti-dengue sera, depending on the experiment. To demonstrate that the same cells were expressing both dengue proteins and HIV proteins, we used a double label technique, with FITC detecting HIV proteins and rhodamine detecting dengue proteins. Figure 8 demonstrates the concordance of dengue virus protein and gp120 expression in cultures 4 days post transfection with  $\Delta$ pre-M/E-gp120, (Figure 8). The more extensive background of auto fluorescence encountered when visualizing the rhodamine fluorescence makes low levels of specific rhodamine fluorescence more difficult to discern, but clearly all intact cells positive for HIV are also positive for dengue proteins. The rhodamine-positive spot in the lower left of the panel is cellular debris and is also positive for dengue proteins, but the FITC fluorescence was not well reproduced by digital photography, though it still may be visualized on certain monitor/computer combinations. Similar results were obtained at 7 days post transfection (Figure 9).

## Discussion

Our finding that dengue virus replicons can express heterologous genes, including HIV envelop, for prolonged periods of time in cell culture without selection represents a significant step in developing a new vector system potentially capable of delivering immunogens to any host in whose cells the dengue replicons can replicate. Flavivirus replicons have previously been demonstrated to express heterologous genes for up to 41 days in tissue culture in Kunjin [11]. However, these experiments were done in the presence of selection for the heterologous genes cloned into the replicons. We have demonstrated heterologous gene expression in the absence of selection for up to at least 9 days post transfection with chimeric dengue replicons. Although we have formally demonstrated expression, not replication, our previous demonstration of the replication [12] of dengue replicons lacking heterologous material suggests that the replicons described here, which contain heterologous material, are indeed replicating. Evidence that cells continue to replicate and express replicon proteins in both daughter cells after transfection with these chimeric replicons further supports the implication of chimeric replicon replication. Ideally, to serve as dual vaccines against dengue as well as against other pathogens, the replicons should express the dengue NS1 protein [29–32]. So far, attempts to visualize NS1 production by Western blots have failed, presumably because of the low transfection efficiencies. However, we have previously argued that the replication of dengue replicons could not take place in the absence of the essential non-structural gene, NS1, which implies that NS1 is being made. The frequencies and fluorescence intensities of replicon positive cells seen in the experiments reported here are comparable to those seen for dengue replicons lacking heterologous material [12],



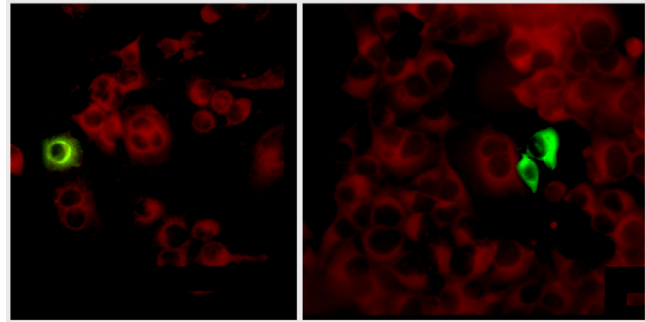
**Figure 6**  
Expression of gp160 by  $\Delta$ pre-M/E-gp160 36 hours post transfection. Anti-dengue Serum was used in these experiments. Left and right panels are independent fields.

suggesting that replication of the  $\Delta$ pre-M/E replicons containing heterologous material is occurring as well. The finding of at least one closely apposed pair of cells expressing high levels of replicon proteins on day 9 post transfection, two days after trypsinization and replating (Figure 7, right panel), not only implies replicon replication, but also implies the expression of NS-1 protein as well. The definitive demonstration of effective NS-1 production will have to await studies of the immune response in animals immunized with these replicons.

Choice of immunogen remains problematic for these vectors. Clearly, HIV-1 gp160 is too toxic for prolonged expression. Even the gp120-expressing replicon seems mildly toxic in that the frequency of gp120 positive cells declines with time in culture post transfection with  $\Delta$ pre-M/E-gp120 (not shown). However, as noted above, we have seen at least one instance of putative cellular division at least 7 days after being successfully transfected by  $\Delta$ pre-M/E-gp120 (Figure 7, right panel). Experiments are now in progress to determine the feasibility of long term expression of other HIV-1 immunogens, including gag and tat.

### Conclusion

Demonstration of long term protein expression by a gp120-expressing replicon alone, of course, does not demonstrate that the chimeric dengue replicons constitute an effective vaccine. However, at the very least they add to the potential armamentarium available to the vaccinologist. It is highly likely that a successful HIV vaccination protocol will involve multiple immunogens and delivery protocols. For instance, mice immunized with attenuated Friend leukemia virus (FLV) develop an immune response whose efficacy is dependent on the additive effects of at least three separable spleen cell populations [33]. By analogy, it may be necessary to devise multiple strategies to obtain a similarly complex and effective immune response in humans against HIV. In



**Figure 7**  
Expression of proteins by  $\Delta$ pre-M/E-gp120 9 days post transfection. Cells were trypsinized and replated on day 7 post transfection and harvested for immunofluorescence two days later. Left and right frames are two independent fields. Anti-dengue serum was used in these experiments.

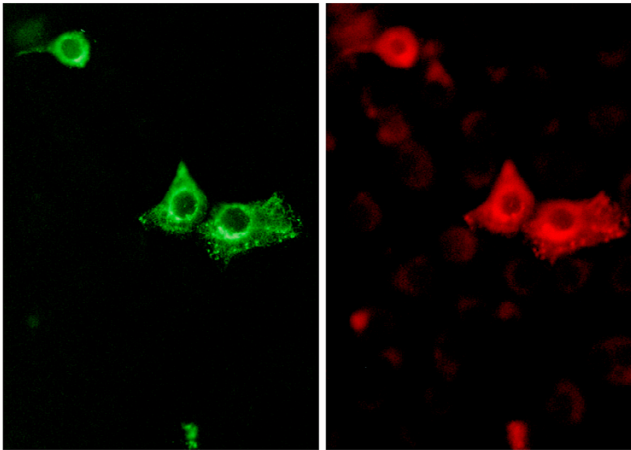
animal models of HIV, different immunogens and modes of immunization can induce different modes of protection with varying degrees of effectiveness [34–40]. Harnessing multiple immune responses may be the answer to designing an effective HIV vaccine [1] and the availability of multiple vectors may facilitate the harnessing of multiple responses.

Much work obviously remains to be done to develop dengue replicons as effective vectors for HIV immunogens. More work needs to be done on determining the ability of dengue replicons to express other potential immunogens, including HIV-1 gag, tat (reviewed in [41]) and env genes with deleted hypervariable regions [see [42]]. Animal studies in our lab are currently being initiated to determine the nature of the immune response such vectors can induce in mice and these studies will presumably need to be extended to primates. Finally, effective delivery systems will need to be devised. Although our successful development of a plasmid which can express a dengue replicon from transfected DNA facilitates delivery by DNA vaccination [12], the development of packaging cell lines which can package these replicons into virions would be a major step forward towards a vaccine which could be conveniently administered in typical clinical situations. Experiments are currently underway in our laboratory to develop such packaging cell lines.

### Materials and methods

#### Culturing of dengue Virus

Dengue virus strains DEN1/WP and DEN2/NGC, kindly provided by Dr. Lewis Markoff, [43,44] were passaged in monkey LLC-MK2 cells at 37°C in a humidified incubator under 5% CO<sub>2</sub>, using Medium 199 plus 10% fetal bovine serum (FBS) and 50 ug of Gentamicin per ml. The cells were trypsinized a day before virus infection and

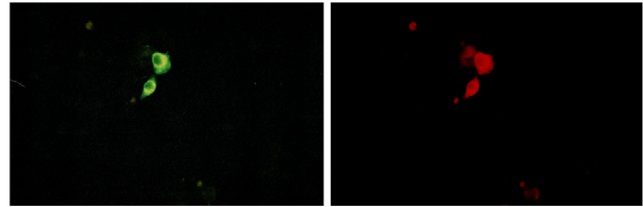


**Figure 8**  
Simultaneous expression of HIV and dengue proteins by  $\Delta$ pre-M/E-gp120-transfected cells 4 days post transfection. Left frame: FITC detection of HIV proteins. Right frame: Rhodamine detection of dengue proteins.

plated to reach approximately 80% confluence on the day of infection. Infections were typically at an MOI of 0.01 PFU/cell in Medium 199 plus 2% FBS.

#### ***In vitro mutagenesis***

Heterologous genes were cloned into the previously described  $\Delta$ pre-M/E replicon [12], into the position previously occupied by the pre-M/E genes. DNA fragments used for desired regions of heterologous genes (see Figure 1) were synthesized by polymerase chain reaction (PCR) from short overlapping primers. For the Green Fluorescent Protein (GFP) gene, the 5' primer was 5'CGAAAAAAGGCGAGAAATACGCCTTTCAATATGCTGAAACGCGAGAGAATGGTGAGCAAGGGCGAGGAGCTG3' and the 3' primer was 5'AAGGTCAAATTTCAACAGCTGCTTGTACAGCTCGTCATGCC3'. For HIV-1 gp120 gene, the 5' primer was 5'ATCATTATGCTGAATCCAACAGTGATGGCGTTCCATTTACCACACGTAACATGAGAGTGATGGGGATCAGGAG3' and the 3' primer was 5'AAGGTCAAATTTCAACAGCTGGGTGGGTGCTAATCTAATGGTTC3'. GFP and HIV-1 gp120 were fused with FMDV/2A self cleaving protein sequence to replace natural cleavage sites in the dengue polyprotein. These sites seem to lose their activity when juxtaposed with heterologous material (data not shown). PCR was used to amplify DNA coding for the FMDV/2A self cleaving protein. The 5' primer was 5'CAGCTGTTGAATTTTGACCTTCTTAAGCTTGC GGGA GACGTCGAGTCCAACCCTGGCCCC3' and the 3' primer was 5'ATACAGCGTCCAGACTCCCACCAATACTAGTGACACAGACAGTGAGGTGCTGGGGCCAGGGTTGGACTC-GAC.



**Figure 9**  
Simultaneous expression of HIV and dengue proteins by  $\Delta$ pre-M/E-gp120-transfected cells 7 days post transfection. Left frame: FITC detection of HIV proteins. Right frame: Rhodamine detection of dengue proteins.

Dengue-2 virus cDNA cloned in the yeast shuttle vector pRS424, linearized by excision of a short Bam H1 fragment was transfected into competent [45] *S. cerevisiae* YPH857 (kindly provided by Barry Falgout, (CBER/FDA), along with the appropriate PCR fragment spanning the desired deletion. Yeast colonies which grew on tryptophan minus plates represented vectors which had recircularized by homologous recombination with these PCR fragments [35]. DNA from these colonies was transformed into *E. coli* STBL 2 cells (Life Technologies, Inc.) to make sufficient quantities of dengue recombinant, genomic-length DNAs for characterization and analysis.

#### ***Expression of virus & replicons in cells***

The full length virus and replicon cDNA plasmids isolated from STBL 2 cells were linearized with Sac I, purified by Qiagen chromatography, and eluted by RNAase-free water in preparation for transcription. The transcription reaction mixtures contained  $\mu$ g of linearized DNA; 0.5 mM(each) ATP, CTP, and UTP; 0.1 mM GTP; 0.5 mM cap analog (NEBL); 10 mM DTT; 40 U of Rnasin (Promega); 30 U of SP6 RNA polymerase; and 1  $\times$  SP6 RNA polymerase buffer (Promega) in a volume of 30  $\mu$ l. The reaction mixtures were incubated at 400°C for 2 hr. Aliquots (12.5  $\mu$ l) of the reaction mixtures, containing full length viral RNA, were used to transfect approximately  $2 \times 10^6$  Monkey LLC-MK2 cells in phosphate-buffered saline (PBS) by electroporation in a 0.4 cm gap electroporation cuvette. Each cuvette was pulsed at 200 V, 950  $\mu$ F using a BioRad Genepuls electroporator. The cells were then resuspended in growth medium and plated on the appropriate tissue culture dish.

After electroporation, cells were either plated directly on multiwell plates for harvest at short time periods (typically 4 days or less) or on tissue culture dishes for trypsinization and seeding onto multiwell plates one or two days before final harvest for longer time periods.

### Immuno-histochemical methods

For immunofluorescent detection of dengue-specific proteins, cells growing on chamber slides were rinsed in room-temperature PBS and then fixed in cold acetone for 10 min at -20°C. After being air dried, each chamber was covered with 50 µl of a 1:50 dilution of DEN2-specific hyperimmune mouse ascitic fluid (HMAF, American Type Culture Collection) in PBS plus 2% normal goat serum and incubated at room temperature for 1 h in a humidified atmosphere and then rinsed twice in PBS. After washing, cells were subsequently incubated with a 1:100 dilution of fluorescein isothiocyanate-labeled goat anti-mouse antibodies (Kirkegaard and Perry Laboratory) and rinsed twice in PBS. For detection of HIV-specific proteins, the same protocol was used except that cells were initially incubated with human HIV-1 serum from Waldheim Pharmazeutika Ges.m.b.H. Neufeld-Vienna, Austria and then subsequently incubated with fluorescent-labeled goat anti-human antibody. Cells in some, but not all experiments were counterstained with 0.02% Evans Blue.

For dual labeling, the first antibodies were a 1:50 dilution of dengue type 2 specific hyperimmune mouse ascitic fluid (HMAF, American type culture collection) and a 1:100 dilution of human HIV positive serum in PBS plus 2% normal goat serum. The second antibodies were a 1:100 dilution of FITC-labeled goat anti-human antibodies (Waldeim Pharmazeutika) and a 1:50 dilution of goat anti-mouse IgG-L-Rhodamine (Boehringer Mannheim Biochemicals).

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