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13. For affinity selection, 200 mg of streptavidin agarose beads (Pierce) were washed once with WB-50 buffer [20 mM tris (pH 7.6), 0.01% NP-40, 1.5% $NaN_3$, 50 mM NaCl, and 2 mM $MgCl_2$]. Beads were preblocked with glycogen (0.1 mg/ml), bovine serum albumin (1 mg/ml), and tRNA (0.1 mg/ml) in WB-50 buffer for 20 min at 4°C, then washed four times with 1 ml of WB-50 and once with WB-250 (WB-50 brought to 250 mM NaCl). After resuspension in an equal volume of WB-250, 50-mg aliquots of beads were gently rocked for 1 hour at 4°C with 25 μl of a standard splicing reaction containing 60% v/v HeLa nuclear extract but no substrate or, in later experiments, with either 250 μl of each glycerol gradient fraction. Beads were washed four times with 0.5 ml WB-250 buffer, and bound RNA was purified by digestion with proteinase K, phenol extraction, and ethanol precipitation. Affinity selection on a monomeric avidin column was performed according to the manufacturer (Pierce) with WB-500 as the wash buffer. The three RNA oligonucleotides used in Fig. 4 were 5′-CAGGUAAGUAdT-3′ (5′ SS), 5′-CAUACUUAUUCCdU-3′ (BP), and 5′-CCCUUUUUUCCACAGCUdC-3′ (Py-3′ SS). The sequence of α-Py is 5′-GGAAAAAAGGG-3′.
14. BU5Ae interacts efficiently with the accessible subpopulation of U5 snRNP, although only 7 ± 3% of total U5 snRNP is retained on the streptavidin column after extensive washing to reduce background binding (Fig. 2C). In a typical experiment, 25 to 50% of total U5 snRNP in nuclear extract can be cleaved by RNase H and the corresponding DNA oligonucleotide spanning the same positions as BU5Ae (*8*). Pretreatment of nuclear extract with BU5Ae completely blocks RNase H cleavage (*15*), implying that BU5Ae interacts quantitatively with the entire cleavable subpopulation of U5 snRNP. Trace amounts of U2 selected by BU5Ae (Fig. 2A, left) presumably reflect the known U1/U2 association [D. L. Black, B. Chabot, J. A. Steitz, *Cell* **42**, 737 (1985)] because trace amounts of U2 snRNP (<0.8%) were also retained by a biotinylated RNA oligonucleotide complementary to U1 positions 64 to 75 ($BU1_{64-75}$), which selects U1 almost exclusively (Fig. 2A, right).
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24. We thank J. A. Steitz for many useful suggestions, oligonucleotides, and Y12 antibody; members of her laboratory for suggestions; A. D. Bailey and T. A. Pavelitz of our laboratory for discussions and critical reading of the manuscript; and M. Moore for the gift of p220 antibody. G.A. was supported by a Human Frontiers Science Program Organization Long-Term Fellowship. This work was funded in part by NIH award GM31073 to A.M.W.

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# Genetically Engineered Resistance to Dengue-2 Virus Transmission in Mosquitoes

K. E. Olson, S. Higgs, P. J. Gaines, A. M. Powers, B. S. Davis, K. I. Kamrud, J. O. Carlson, C. D. Blair, B. J. Beaty

The control of arthropod-borne virus diseases such as dengue may ultimately require the genetic manipulation of mosquito vectors to disrupt virus transmission to human populations. To reduce the ability of mosquitoes to transmit dengue viruses, a recombinant Sindbis virus was used to transduce female *Aedes aegypti* with a 567-base antisense RNA targeted to the premembrane coding region of dengue type 2 (DEN-2) virus. The transduced mosquitoes were unable to support replication of DEN-2 virus in their salivary glands and therefore were not able to transmit the virus.

DEN viruses (serotypes 1 to 4; Flaviviridae) contain a positive sense RNA genome (11 kb) that encodes three structural and seven nonstructural proteins (*1*). The viral genome is the only viral mRNA found in infected cells and is translated as a single polyprotein (350 kD), which is cleaved by both host cell and virus-encoded proteases to generate individual proteins (*2*). The natural cycle involves only mosquitoes, which develop a lifelong, persistent, noncytocidal infection, and humans, who may manifest either DEN fever (DF), an acute, debilitating illness, or DEN hemorrhagic fever (DHF), a severe disease that usually results from sequential infections by different viral serotypes (*3*, *4*). Female *Ae. aegypti* acquire DEN viruses by biting and taking a bloodmeal from an infected human. The virus enters the midgut, replicates, and disseminates to other mosquito organs, including the salivary glands. After further replication of the virus in salivary glands, the virus in mosquito saliva can be transmitted to humans during the ingestion of the next bloodmeal (*5*).

DEN viruses are a major health concern to urban populations throughout tropical regions of the world (*3*, *4*, *6*). Conventional measures to limit the proliferation of the disease have failed in part because of the lack of effective vaccines and the collapse of health care and mosquito control infrastuctures in many developing countries (*4*, *6*). Novel control strategies are now being considered, including the genetic alteration of mosquito vectors, to control the rapid proliferation of arthropod-borne pathogens.

A potentially powerful control strategy is to prevent replication of DEN viruses in the midgut or salivary glands of the mosquito by expressing part of the virus's own genetic material in those organs and establishing intracellular immunity to the virus (*7*). Engineering pathogen refractoriness into arthropod vectors has been difficult because of a lack of efficient DNA expression systems (*8*). Viral transducing systems derived from infectious complementary DNA (cDNA) clones of the mosquito-borne RNA virus Sindbis (SIN; Togaviridae) have recently been developed that achieve efficient, long-term gene expression in mosquitoes and can be used to rapidly assess promising gene-based approaches for pathogen control (*9*). The TE/3′2J double subgenomic SIN (dsSIN) transducing viruses contain a second subgenomic promoter, between the end of the structural protein coding region and the viral 3′ noncoding region. These dsSIN viruses transcribe one genomic and two subgenomic mRNAs in infected cells, and heterologous proteins may be translated from the second subgenomic mRNA (*9*). The dsSIN viruses replicate in the cytoplasm of infected mosquito cells, which obviates potential problems such as mRNA splicing, mRNA transport, and poorly characterized DNA promoters in mosquitoes. We have demonstrated that dsSIN viruses can efficiently express heterologous proteins and antisense RNAs in the head and salivary gland tissues of infected *Ae. triseriatus* mosquitoes more than 20 days after infection (*10*). Previous studies also have demonstrated that dsSIN viruses can transduce cultured mosquito cells with RNAs complementary to specific RNA sequences of unrelated arthropod-borne viruses, such as La Crosse (Bunyaviridae) and DEN-2 viruses, and can establish intracellular immunity to those viruses (*11*–*13*).

A dsSIN virus, designated D2prMa, has been generated that expresses a 567-base antisense RNA targeted to the premembrane

Arthropod-Borne and Infectious Diseases Laboratory (AIDL), Department of Microbiology, Colorado State University, Fort Collins, CO 80523, USA.

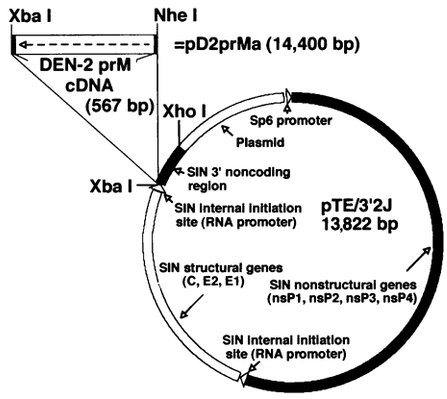


**Fig. 1.** Plasmid map showing construction of pD2prMa from the parent dsSIN construct pTE/3′2J. To insert the antisense sequence into pTE/3′2J, we synthesized primers to amplify DEN-2 prM cDNA by polymerase chain reaction from p30-VD2 (*24*), which contained cDNA of the structural coding region of DEN-2 (Jamaica) virus. Amplified prM cDNA was TA-cloned (*25*) into a modified Bluescript II SK+ plasmid (Stratagene) that contained an Nhe I site in the polylinker. The prM cDNA was excised with Nhe I and Xba I restriction endonucleases and inserted at the Xba I site downstream of the second RNA promoter of pTE/3′2J. Insert orientation was confirmed by sequence analysis. The dsSIN DNA templates were linearized at the Xho I site and transcribed in vitro from the bacteriophage SP6 promoter (*26*). RNA products were electroporated (BTX, San Diego, CA) into BHK-21 cells at 500 V, 100 μF, and 720 ohms for a duration of approximately 0.8 ms. The dsSIN viruses were harvested from the medium 20 hours later and titrated in BHK-21 cells with the use of an end-point assay (*27*).

**Table 1.** Interference of transmission of DEN-2 virus in *Ae. aegypti* by transduction of mosquitoes with an antisense RNA targeted to the prM coding region of DEN-2 virus. The number of saliva samples positive for DEN-2 virus after injection of saliva into mosquitoes is indicated as the first number in the second column; the total number of saliva samples injected into mosquitoes, by the second number. In trial 1, one mosquito injected with saliva from a mosquito transduced with prM antisense RNA in trial 1 was weakly positive for DEN-2 virus (fourth column); ND, not done. The total number of saliva samples assayed was produced from three separate trials. Mosquitoes were injected with L-15 medium and DEN-2 virus or co-injected with D2prMa or TE/3′2J virus and DEN-2 virus as described (*16*). Fourteen days after infection, saliva samples were collected from mosquitoes for detection of DEN-2 virus.

| | Saliva samples from mosquitoes infected with | | |
|---|---|---|---|
| Trial | DEN-2 virus + L-15 medium | DEN-2 + TE/3′2J viruses | DEN-2 + D2prMa viruses |
| 1 | 8/8 | 7/9 | 1/8 |
| 2 | ND | 7/10 | 0/10 |
| 3 | ND | 6/7 | 0/8 |
| Total | 8/8 | 20/26 | 1/26 |

(prM) coding region of the DEN-2 virus genome (Fig. 1). The prM coding region was targeted for two reasons. The prM gene product is an essential component of DEN virion assembly (*2*, *14*); also, because the prM coding region is near the 5′ end of the DEN virus genome, antisense RNA inhibition of translation would affect the synthesis of the eight viral proteins downstream of the prM. The expression of prM antisense RNA was characterized by Northern (RNA) blot analysis of viral RNAs isolated from *Ae. albopictus* (C6/36) cells infected with either D2prMa virus or a control dsSIN virus (TE/3′2J) lacking the antisense insert. The size of the virus genomic and two subgenomic mRNAs in cells infected with D2prMa virus was larger than the TE/3′2J RNAs by the size of the inserted DEN-2 sequence (*15*) (Fig. 2).

To test if D2prMa virus could establish intracellular immunity, we intrathoracically co-injected into *Ae. aegypti* mosquitoes $10^{5.0}$ median tissue culture infectious doses ($TCID_{50}$'s) of D2prMa or TE/3′2J and $10^{3.0}$ $TCID_{50}$'s of DEN-2 (strain 16681, Thailand) virus (*16*). Intrathoracic inoculations ensured that all of the mosquitoes were infected. Eleven days later, their salivary glands and midguts were dissected for immunofluorescence analysis. Tissues were assayed for SIN envelope (E1) and DEN-2 envelope (E) proteins.

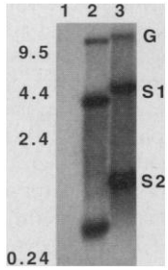


**Fig. 2.** Northern blot analysis of D2prMa virus RNA in C6/36 cells. The blot was hybridized with a TE/3′2J-specific, $^{32}P$- labeled oligonucleotide. The lanes show profiles of dsSIN RNA from C6/36 cells infected with no virus (lane 1), TE/3′2J virus (lane 2), or D2prMa virus (lane 3). Abbreviations are as follows: G, genomic RNA; S1, first subgenomic RNA; and S2, second subgenomic RNA. Size markers in kilobases are on the left.

Mosquitoes injected with TE/3′2J virus supported DEN-2 virus replication in the salivary glands (Fig. 3, A and B), and $10^{2.8}$ $TCID_{50}$'s of virus was detected per salivary gland pair. Mosquitoes injected with D2prMa virus did not permit DEN-2 virus replication in salivary glands, and no DEN-2 virus could be detected either by immunofluorescence (Fig. 3, C and D) or by titration analysis. Densitometric analysis of slot blots of RNA isolated from salivary gland pairs of individual *Ae. aegypti* inoculated with $10^{5.0}$ $TCID_{50}$'s of D2prMa virus showed that approximately $4 \times 10^8$ copies of the antisense sequence were present 14 days after infection (*17*). The midgut was not resistant to DEN-2 virus because dsSIN and DEN-2 viruses exhibited different tropisms when delivered intrathoracically, with dsSIN viruses infecting midgut-associated nerve and muscle tissues and DEN-2 infecting midgut epithelial cells (Fig. 3, E and F). The dsSIN viruses displayed a similar tropism in the midguts of *Ae. triseriatus* 11 days after intrathoracic inoculation (*10*).

We next investigated the effect of prM antisense RNA expression on DEN-2 virus transmission. Female *Ae. aegypti* mosquitoes were co-injected with D2prMa and DEN-2 viruses as described above. Saliva was collected from each of 26 mosquitoes 14 days after infection (*18*). Each saliva sample was treated with an SIN polyclonal antibody to neutralize the D2prMa virus (*19*); each saliva sample was then intrathoracically inoculated into groups of five *Ae. aegypti* (*20*). This inoculation was done because it is the

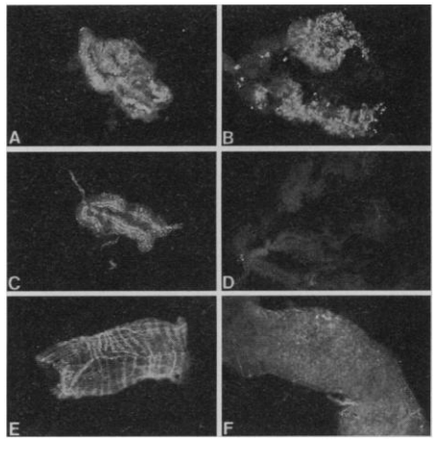


**Fig. 3.** Detection of SIN E1 and DEN-2 E proteins in *Ae. aegypti* by an indirect immunofluorescence assay (IFA) 11 days after infection. Salivary glands from mosquitoes co-injected with TE/3′2J and DEN-2 virus were analyzed for the presence of SIN (**A**) (20× magnification) and DEN-2 (**B**) (40× magnification) antigens. (**C** and **D**) Salivary glands from mosquitoes co-injected with D2prMa and DEN-2 virus analyzed for the presence of SIN (C) (20× magnification) and DEN-2 (D) (40× magnification) antigens. (**E** and **F**) Midguts from mosquitoes co-injected with D2prMa and DEN-2 virus analyzed for the presence of SIN (E) (20× magnification) and DEN-2 (F) (20× magnification) antigens. Mosquitoes were co-injected with either TE/3′2J or D2prMa virus and DEN-2 (16681) virus and incubated at 28°C for 11 days. Tissues were subjected to IFA with the use of either monoclonal antibodies 30.11 (anti-SIN E1) (*28*) or 813 (anti-flavivirus E) (*29*) as the primary antibodies and a biotinylated sheep antibody to mouse (Amersham, Arlington Heights, IL) as the secondary antibody. Fluorescence produced by bound fluorescein-streptavidin (Amersham) was viewed with an Olympus BH-2 epifluorescence microscope. Tissues positive for DEN-2 E antigen were considered positive for DEN-2 virus replication.

most sensitive technique for detecting infectious DEN viruses (*16*). After 7 days, mosquito heads were examined by immunofluorescence for the presence of DEN-2 virus. Expression of D2prMa virus efficiently inhibited the biological transmission of DEN-2 virus because, of the 26 saliva samples tested, only one was weakly positive for DEN-2 infection (Table 1). In contrast, 20 of 26 saliva samples obtained from mosquitoes co-infected with TE/3′2J and DEN-2 viruses were positive for DEN-2 virus when inoculated into mosquitoes (Table 1). Saliva samples were scored positive for the presence of virus if at least one of the five saliva-injected mosquitoes was positive for DEN-2 antigen by immunofluorescence.

Both DEN-2–derived sense and antisense prM RNA have been shown to interfere with DEN-2 virus replication in mosquito cells. This interference is specific because there is no inhibition of replication of DEN-3 and DEN-4 viruses (*13*). Future work will focus on using dsSIN viruses to transduce mosquito cells with antisense RNAs that will target the genomes of all four DEN virus serotypes. The dsSIN transducing viruses are well suited for delivering anti-virus molecules to mosquito tissues where arbovirus replication occurs. These results show that antisense RNA can be used to ablate flavivirus transmission from *Aedes aegypti*. Ultimately, mosquito genomes will need to be manipulated with DNA-based transformation techniques to ensure both heritability and transcriptional control of the desired anti-virus agent in the mosquito (*21*).

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15. C6/36 cells were infected at a multiplicity of infection of 10 with the dsSIN viruses and incubated at 28°C for 48 hours. Total RNA was extracted by the acid guanidinium technique (*22*) from infected cells, fractionated on a formaldehyde denaturing gel (*23*), and transferred to a nylon membrane (Nytran Plus, Schleicher & Schuell, Keene, NH). An oligonucleotide probe (5′-gctggtcggatcattggggcg-3′) complementary to the dsSIN 3′ nontranslated region was end-labeled with [$\gamma$-$^{32}$P]deoxyadenosine triphosphate with the use of T4 polynucleotide kinase (Promega). The hybridization solution contained 10% dextran sulfate, 1% SDS, 4.8% NaCl (w/v), and 200 $\mu$g/ml of denatured salmon sperm DNA. The blot was hybridized at 50°C for 12 hours with $5 \times 10^5$ cpm of probe (specific activity $>10^8$ cpm/$\mu$g).
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19. The dsSIN viruses in the saliva were neutralized with a mouse SIN polyclonal antibody (MB92) diluted 1:50. Viruses were neutralized for 1 hour at 4°C before each mosquito was intrathoracically injected with 0.5 $\mu$l of saliva.
20. Seven days after inoculating mosquitoes with saliva (*16*), we analyzed the mosquito heads for the presence of DEN-2 E antigen by indirect immunofluorescence assay using a DEN-2–specific mouse polyclonal antibody. The presence of DEN-2 antigen in the mosquitoes indicated the presence of DEN-2 virus in the saliva that escaped interference.
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# Two Genetically Separable Steps in the Differentiation of Thymic Epithelium


Michael Nehls,* Bruno Kyewski, Martin Messerle,† Ralph Waldschütz, Kerstin Schüddekopf,‡ Andrew J. H. Smith,§ Thomas Boehm||



The development of the thymus depends initially on epithelial-mesenchymal and subsequently on reciprocal lympho-stromal interactions. The genetic steps governing development and differentiation of the thymic microenvironment are unknown. With the use of a targeted disruption of the *whn* gene, which recapitulates the phenotype of the athymic nude mouse, the WHN transcription factor was shown to be the product of the *nude* locus. Formation of the thymic epithelial primordium before the entry of lymphocyte progenitors did not require the activity of WHN. However, subsequent differentiation of primitive precursor cells into subcapsular, cortical, and medullary epithelial cells of the postnatal thymus did depend on activity of the *whn* gene. These results define the first genetically separable steps during thymic epithelial differentiation.


The primary function of the thymus is to generate and select a highly diverse repertoire of T cells that exhibit self tolerance and restriction to self major histocompatibility complex (*1*). The importance of the thymic microenvironment in shaping the T cell repertoire has long been recognized (*2*). Development of a functionally competent thymus depends on a series of epithelial-mesenchymal and subsequent lympho-stromal interactions (*3*), which implies the existence of developmentally restricted control points in the differentiation of thymic epithelium (*4*). Here, we have taken a genetic approach to identify the WHN transcription factor as an early regulator of thymic epithelial differentiation.

The *whn* gene (*5*) is mutated on mouse and rat nude alleles (*5*, *6*), which suggests that the WHN transcription factor is encoded by the *nude* locus. We used a targeted


M. Nehls, B. Kyewski, M. Messerle, R. Waldschütz, K. Schüddekopf, T. Boehm, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany.
A. J. H. Smith, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK.

*Present address: Zentrum der Inneren Medizin-Molekulare Kardiologie, Universität Frankfurt, 60590 Frankfurt, Germany.
†Present address: Institut für Virologie, Universität Heidelberg, Heidelberg, Germany.
‡Present address: Institut für Humangenetik, Universität Hamburg, 22529 Hamburg, Germany.
§Present address: Center of Genome Research, University of Edinburgh, Edinburgh EH9 3JQ, UK.
||To whom correspondence should be addressed. E-mail: t.boehm@dkfz-heidelberg.de