Large-scale recoding of an arbovirus genome to rebalance its insect versus mammalian preference

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The protein synthesis machineries of two distinct phyla of the Animal kingdom, insects of Arthropoda and mammals of Chordata, have different preferences for how to best encode proteins. Nevertheless, arboviruses (arthropod-borne viruses) are capable of infecting both mammals and insects just like arboviruses that use insect vectors to infect plants. These organisms have evolved carefully balanced genomes that can efficiently use the translational machineries of different phyla, even if the phyla belong to different kingdoms. Using dengue virus as an example, we have undone the genome encoding balance and specifically shifted the encoding preference away from mammals. These mammalian-attenuated viruses grow to high titers in insect cells but low titers in mammalian cells, have dramatically increased LD_{50}s in newborn mice, and induce high levels of protective antibodies. Recoded arboviruses with a bias toward phylum-specific expression could form the basis of a new generation of live attenuated vaccine candidates.

codon pair bias | arbovirus | dengue | vaccine

Insects and mammals are separated by about 1 billion years of evolution (1), and besides obvious differences at the organismal level, they differ also in many biochemical processes (2–5). They have subtle differences in how they encode proteins: for instance, their genes have different codon preferences (6, 7), different dinucleotide frequencies (8), and different codon pair preferences (see below). That is, the gene expression and protein synthesis machineries of these phyla have different preferences for the way proteins are encoded. There are a large number of viruses, the arthropod-borne viruses [arboviruses (9–12)], that infect both insects and mammals. Similarly, there are viruses that infect both insects and plants (12, 13). A virus is constrained to use the protein synthesis machinery of its host: our work here is partly motivated by the question of how a single virus can find homes in two phyla with different encoding biases.

Codon pair preference, or codon pair bias (CPB), is a phenomenon in which certain pairs of adjacent codons are used more frequently or less frequently than expected after accounting for use of individual codons (14–16). Every codon pair has a codon pair score (CPS) (16), which is the natural logarithm of the ratio of the observed frequency of the codon pair to its expected frequency [i.e., CPS = ln(observed/expected)] (16). A negative CPS denotes that a pair is underrepresented (16), possibly suggesting that it is unfavorable for the organism, whereas a positive CPS may be preferred. Indeed, recoding a segment of poliovirus with underrepresented codon pairs yielded a dead virus (16) even though the recoded region contained the same synonymous codons and translated into exactly the same protein as wild type. The phenomenon of codon pair bias is related to dinucleotide bias; for instance, in mammals, CpG and UpA dinucleotides occur less frequently than expected (17–19), as do the codon pairs with a central xxCpGxx or xxUpAxx (20). The mechanistic reasons for this underrepresentation of dinucleotides are not well understood.

Whole viral genome synthesis (21) has made it possible to study the effects of selectively altered codon pair bias on gene expression by large-scale recoding. We have been designing genes and viruses containing hundreds of unfavorable codon pairs, a process we call synthetic attenuated virus engineering. Recoded viral genomes are typically attenuated but strongly immunogenic in animals (16, 22, 23).

In this study, we focus on the effects of altered codon pair bias in a particular arbovirus, dengue virus, which infects both mosquitoes and humans. We find this an interesting example because more than a third of the world's population lives in areas at risk for dangerous dengue infections, and there is currently no vaccine.

Results

Codon Pair Bias Is Different Between Insects, Mammals, and Plants. Our analysis revealed that codon pair preferences in insects and mammals are very different and almost uncorrelated with each other (compare Fig. 1*A* with Fig. 1*B*). For example, in mammals the CPS of GCG GGC (Ala Gly) is +0.655, whereas in insects it is −0.651; in contrast, the CPS of CTT CCC (Leu Pro) in mammals is −0.021, whereas in insects it is +0.615 (Dataset S1). Because viruses depend on host protein synthesis machinery and because a powerful negative effect of unfavorable codon pair bias on viral replication has been shown (16, 22), how can an arbovirus successfully replicate in both phyla? The answer may be that these arboviruses use a limited subset of carefully selected codon pairs that are relatively good in both phyla and so permit efficient use of both insect and mammalian translational machineries.

Significance

Arboviruses (arthropod-borne viruses), a large group of RNA viruses, replicate in insects that transmit them to mammals, their second host. Insects and mammals have evolved different protein encoding strategies (codon pair bias); hence, arboviruses must delicately balance their encodings between two phyla. Using dengue virus (DENV), the most important human arbovirus pathogen, as a model, we have, by computer design and chemical synthesis, undone this balance in codon pair bias in favor of insects. Recoded DENVs grow well in insect cells but are highly attenuated in mammalian cells and in suckling mice. This unique approach offers a previously unidentified possibility to rapidly develop new vaccine candidates against DENV and perhaps against many different human arboviruses.

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Conflict of interest statement: C.B.S., S.M., and E.W. are affiliated with Codagenix.

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Data deposition: The sequences reported in this paper have been deposited in GenBank (accession nos. KP161064, KP161065, KP161066, and KP161067).

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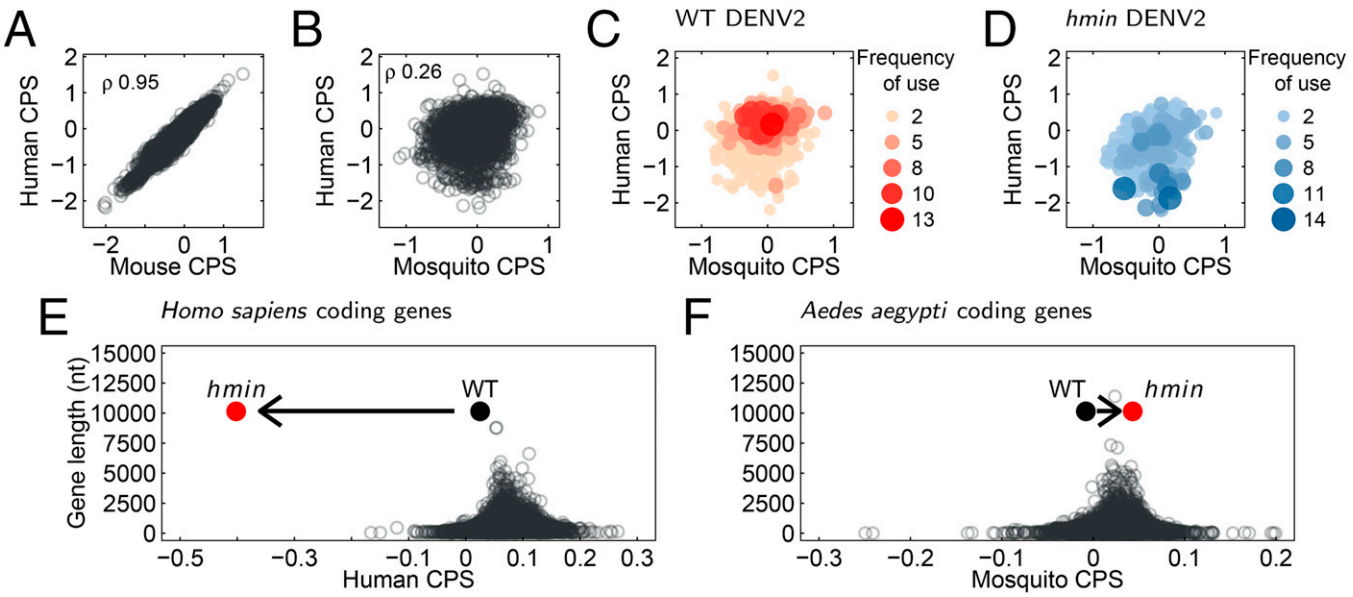

Fig. 1. Codon pair bias in humans and mosquitoes. (*A*) Codon pair preferences are well-correlated (Spearman rho = 0.95) between humans and mice. Human codon pair preferences were calculated as described before (16); those for insects were calculated using genomic sequences of *Aedes aegypti*. (*B*) Codon pair preferences are poorly correlated (Spearman rho = 0.26) between humans and mosquitoes. Each circle represents one of the 3,721 possible codon pairs. (*C*) Codon pairs actually used by natural wild-type dengue virus, type 2 (16681) (red dots). The more times a particular codon pair is used by the virus, the bigger and more red the dot. (*D*) Codon pairs used by an in silico recoded dengue virus designed to have a good codon pair score in mosquitoes but a bad (negative) codon pair score in humans (blue dots). "*hmin*" signifies a human minimized virus. (*E*) The average human codon pair score of the in silico *hmin* virus (red dot) compared with WT (black dot) and the average codon pair scores of >14,000 human coding genes. (*F*) The same two viruses as in *E* (red and black dots) evaluated using mosquito codon pair scores and compared with all *Aedes aegypti* mosquito coding genes. The in silico *hmin* virus demonstrates that it is possible to design a synthetic dengue virus drastically deoptimized in humans but optimized in mosquitoes.

To investigate this possibility, we looked at the genome of Rift Valley fever virus (RVFV), a negative-stranded RNA virus of *Bunyaviridae*, which infects mosquitoes and sheep (and other mammals) (24). We found that the codon pairs used by RVFV are strongly biased toward the relatively small set of codon pairs that have high codon pairs scores (i.e., which are preferred) in both sheep and mosquitoes (Fig. S1 *A* and *B*). We then specifically looked at dengue virus (DENV), a positive-stranded RNA virus of *Flaviviridae*, which infects mosquitoes and humans (25, 26). Again, its genome has a bias toward codon pairs with high scores in both hosts (Fig. 1 *A*–*C*), although in this case the bias toward the high insect scores was somewhat less strong than with RVFV.

The term "arboviruses" is commonly used for viruses that infect humans and/or other mammals via insect vectors (9), but insect-borne transmission of viruses is widespread also in the Plantae kingdom (12). An example is Maize fine streak virus (MFSV), a nucleorhabdovirus that infects leafhoppers (an insect) and is transmitted to *Zea mays* (corn, a plant) and other plants (27, 28). Again, the codon pairs present in the MFSV genome were strongly biased toward those pairs having high codon pair scores in both leafhoppers and corn (Fig. S1 *C* and *D*).

Although these results suggested that arboviruses do use a restricted, balanced set of codon pairs to compromise between their hosts, it is not clear how important this is. Over evolutionary time, even a tiny selective pressure could modify codon pair use. Therefore, to measure the significance of this balance, we have undone this evolutionary selection by recoding the genome of the dengue virus (DENV) so that it uses codon pairs favorable for insects but at the same time unfavorable for mammals. We illustrate this principle in Fig. 1 *E* and *F* with an in silico recoded DENV. Here *hmin* combines a minimized human CPS (see next section) with a greater than wild-type mosquito CPS. (Note that we did not synthesize this virus; the viruses synthesized were constrained to have mosquito CPS equal to or less than WT.)

CPB Recoded Dengue Viruses Prefer Insect to Mammalian Cells. DENV infections are the leading cause of arthropod-borne diseases in the world (26), leading to illnesses ranging from self-limiting dengue fever to life-threatening dengue shock syndrome and dengue hemorrhagic fever. It has been estimated that 2.5 billion people worldwide are at risk for DENV infections (26). There is currently no vaccine.

DENV, a virus encoding a single polyprotein (Fig. 2*A*), occurs in four serotypes (25). We synthesized the 10,723-nt-long infectious cDNA for DENV type 2, strain 16681 (accession no. U87411), designed to contain 26 silent nucleotide changes for convenient restriction sites and watermarks every ∼1 kb (*SI Materials and Methods*). Replication of this synthetic virus, designated "D2-syn," is indistinguishable from that of the natural DENV2 strain (16681) in monkey LLC-MK2 and mosquito C6/36 cells (Fig. 3 *A* and *B*). We then designed three synthetic, recoded dengue viruses using a simulated annealing heuristic (*SI Materials and Methods*) in which existing synonymous dengue codons were rearranged to replace existing codon pairs with pairs that are unfavorable in humans but acceptable in insects (Fig. 2*A* and Table 1). These recodings altered neither the encoded protein nor the codon use.

We changed the coding sequence of three dengue proteins, E, NS3, and NS5 (Fig. 2*A* and Table 1), that play multiple roles in the replicative cycle of DENV (25). The E glycoprotein functions in viral attachment, entry, and membrane fusion; NS3 is a multifunctional enzyme with serine protease/helicase/NTPase activity; and NS5 is the RNA-dependent RNA polymerase crucial for viral genome replication that also harbors methyltransferase activity (25). The three recoded ORFs each harbor more than 300 nucleotide changes and have strongly negative human codon pair scores but are similar to wild type with respect to mosquito codon pair scores (Fig. 2*A* and Table 1). Each of the three recoded segments was synthesized and cloned, separately, into the synthetic wild-type D2-syn to create three new viruses,

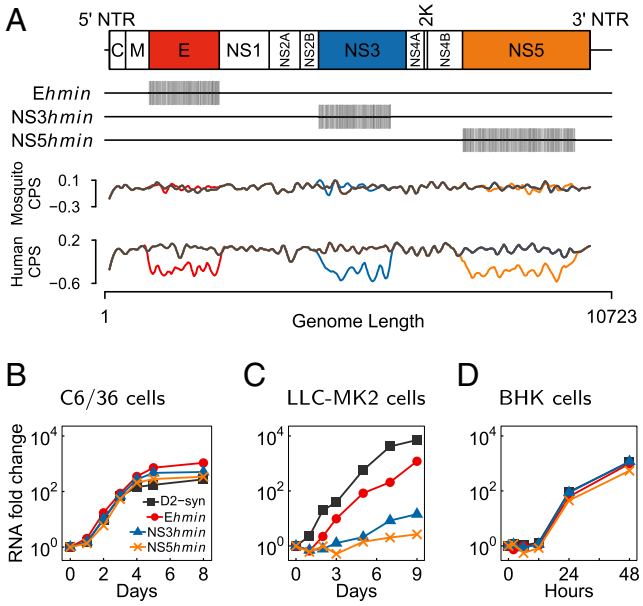

Fig. 2. Design and growth kinetics of WT (D2-syn) and three *hmin* dengue viruses in mammalian and mosquito cell lines. (*A*) (*Top*) Diagram of the DENV2 genome marks the polyprotein coding region and the coding regions of polypeptides before proteolytic processing. The color-coded regions indicate regions recoded in the three novel *hmin* viruses. Full length genomes of the three *hmin* viruses (E^{hmin}, $NS3^{hmin}$, and $NS5^{hmin}$) are aligned to the WT (D2-syn) sequence, and point mutations generated by codon pair recoding are indicated by a barcode diagram. The two CPS line plots (*Bottom*) show how the codon pair score changes along the length of the genome for each virus relative to the mosquito and human CPBs. There are four overlapping loess curves (see *SI Materials and Methods*): E^{hmin} is in red, $NS3^{hmin}$ is in blue, $NS5^{hmin}$ is in yellow, and D2-syn is in gray. (*B–D*) Virus growth curves in different cell lines produced by measuring the fold change in virus RNA concentration from time 0.

referred to as E^{hmin}, $NS3^{hmin}$, and $NS5^{hmin}$, where "*hmin*" signifies a human minimized codon pair score, whereas the codon pair score for insect mRNA is maintained. The precise mechanism of attenuation by poor codon pair score has not been solved, so it is unclear whether these three separate recodings of the genome specifying different polypeptides of the polyprotein (Fig. 1*A*) should cause gene-specific effects or, alternatively, whether each affects the whole polyprotein.

Cultured C6/36 mosquito cells (29) were transfected with the various synthetic transcripts. Viruses were isolated and used to infect C6/36 mosquito cells, and viral replication was followed using quantitative RT-PCR (Fig. 2*B*) or using plaque-forming units (PFUs) (Fig. 3*A*). In agreement with our hypothesis that replication in insect cells correlates with a good codon pair score for mosquitoes, the three human deoptimized viruses E^{hmin}, $NS3^{hmin}$, and $NS5^{hmin}$ all grew with similar kinetics to the D2-syn virus. Preliminary results suggest that all four viruses also grow similarly to each other in a different mosquito cell line, Aag-2 (30).

In contrast, when the human deoptimized viruses E^{hmin}, $NS3^{hmin}$, and $NS5^{hmin}$ were cultured in mammalian LLC-MK2 cells (31), they were strongly attenuated. This was true whether growth and replication was assayed by RT-qPCR (to monitor RNA abundance) (Fig. 2*C*) or by PFUs (Fig. 3 *B* and *C*). For these three *hmin* viruses, the ability to form a focus (suggesting virion formation and spread) was also attenuated in at least two other mammalian cell lines, Vero E6 and A549 cells (Fig. 3*D*).

Perhaps surprisingly, the three *hmin* viruses did not seem attenuated in BHK-21 (baby hamster kidney) cells as measured by qPCR (Fig. 2*D*) and were only modestly attenuated as measured by focus-forming ability (Fig. 3*D*). BHK cells have a defect in RIG-I signaling (32), leading to imperfections in both IFN production and innate immune response. Perhaps because of these deficiencies, BHK cells are highly sensitive to dengue (33). This may explain why the three human deoptimized viruses grew well in this cell type; that is, we imagine that despite being attenuated, the viruses are still able to grow well in this highly susceptible cell line, which is unable to defend against the virus. Indeed, all D2-syn dengue variants gave relatively clear, well-defined plaques on BHK cells allowing us to use BHK cells in an assay to determine titers in PFUs.

To test the idea that defective innate immunity of BHK-21 cells allows susceptibility to the three *hmin* viruses, we partially mimicked this situation in LLC-MK2 cells by inhibiting the IFN response with 10 μM Jak inhibitor I (34) (*SI Materials and Methods*) (Fig. 3*E*). Indeed, the *hmin* viruses grew relatively better in LLC-MK2 cells with inhibitor treatment than without, with $NS3^{hmin}$ growth stimulated by as much as 100-fold. $NS5^{hmin}$, however, could not be recovered after 7 d even after Jak inhibitor I treatment.

The *hmin* Viruses Are Attenuated in Newborn Mice and Confer Protective Immunity. Dengue is a disease of primates, and no other good animal model exists. However, neonatal mammals are more susceptible than adult mammals to infections (35–37).

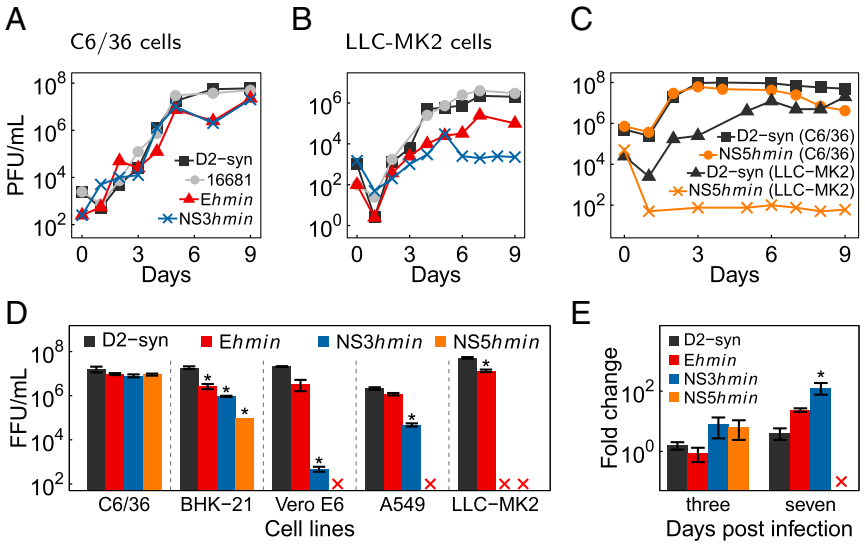

Fig. 3. Growth curves of viruses in different cells. (*A*) Insect C6/36 cells or (*B*) mammalian LLC-MK2 cells were infected with virus variants at a multiplicity of infection (MOI) of 0.001. Virus titer was measured by plaque assay on BHK cells. (*C*) $NS5^{hmin}$ growth kinetics in different cells. C6/36 or LLC-MK2 cells were infected with $NS5^{hmin}$ at a MOI of 1. Virus titer was measured by plaque assay on BHK cells. (*D*) Virus titers, measured by focus forming assays in C6/36, BHK-21, Vero E6, A549, or LLC-MK2 cell lines. (*E*) Effect of Jak inhibitor 1 treatment on virus titer. LLC-MK2 cells were pretreated with Jak inhibitor 1, and the fold change in virus titer relative to untreated cells was measured by a 50% tissue culture infectious dose ($TCID_{50}$) assay at three and seven days postinfection. Significant differences from D2-syn in *D* and *E* are marked by *P value < 0.05 by Wilcox rank sum test.

Table 1. Human and mosquito average codon pair scores for all virus constructs and the number of nucleotide changes

Design	Mosquito CPB	Human CPB	ΔNT
WT (D2-syn)	−0.008	0.0253	26/10,173*
E^{hmin}	−0.018	−0.36	334/1,485
$NS3^{hmin}$	−0.015	−0.379	337/1,854
$NS5^{hmin}$	−0.019	−0.37	505/2,700

*Number of bases in coding region of D2-syn.

Newborn mice have often been used for studies of viral virulence for which proper animal models were not available [for example, coxsackie viruses (38) or DENV (39, 40)]. We therefore analyzed D2-syn^{hmin} variants for attenuation by the assay of intracranial injection into newborn ICR mice (mice and humans have almost identical CPB; Fig. 1*A*).

Groups (n = 5–12, depending on litter size) of 1- to 2-d-old ICR mice were injected intracranially with 10-fold dilutions of each virus (D2-syn, E^{hmin}, $NS3^{hmin}$, and $NS5^{hmin}$) or PBS (Fig. 4; additional doses not shown). D2-syn was highly virulent in these neonatal ICR mice, with an LD_{50} of 5 PFU. Dramatic attenuation was observed with the codon pair deoptimized viruses (Fig. 4 *A* and *B*), revealing 100-fold ($NS3^{hmin}$), 200-fold ($NS5^{hmin}$), and 2,000-fold (E^{hmin}) increases in LD_{50} compared with D2-syn (Fig. 4*C*). Considering the order of virulence in tissue culture cells, the different order of virulence in the animals ($NS3^{hmin}$ > $NS5^{hmin}$ >> E^{hmin}) was a big surprise.

All three deoptimized viruses induced high levels of neutralizing antibodies in the adult survivors that had been vaccinated as newborns (Table 2).

To see if vaccinated mothers could pass these antibodies on to offspring, female CD-155tg mice (*SI Materials and Methods*) were vaccinated as neonates with 1×10^5 FFU D2-Syn or E^{hmin} and then boosted at 3 wk of age intranasally with the same dose. Their offspring were analyzed at 21 d for maternal antibodies using a plaque reduction neutralization test 50% ($PRNT_{50}$) assay, and high levels of neutralizing activity were found (Fig. 4*D*).

Because adult ICR mice are not susceptible to DENV infection, we could not directly assay whether adults vaccinated as neonates were protected against lethal challenge. However, because vaccinated females passed their antibodies on to offspring (Fig. 4*D*), this provided an alternative assay. We challenged naïve, newborn offspring of mothers that had been vaccinated as newborns with wild-type virus delivered by intracranial injection. Ninety-three percent of these naïve newborns survived the challenge with 200 LD_{50} of wild-type (D2-syn) virus (Table 3). This demonstrates that intracranial injection with attenuated virus induces neutralizing antibodies in newborn mice, and after these mice mature, these antibodies can be transmitted to offspring and protect against DENV. The choice of the $NS3^{hmin}$ variant in this experiment was based on available virus. Preliminary results with E^{hmin} and $NS5^{hmin}$ have yielded results similar to those with $NS3^{hmin}$. This strategy for assaying protective antibodies against DENV may be particularly useful when tetravalent DENV live vaccine candidates are to be developed (DENV types 1, 2, 3, and 4). It may also be applicable to the development of live attenuated vaccines candidates protecting against other arboviruses.

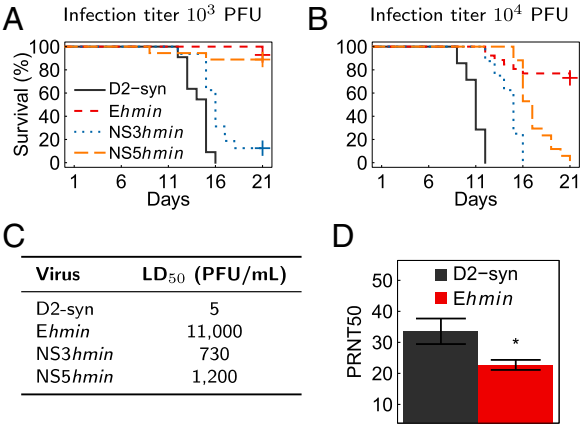

Fig. 4. Survival curves showing attenuation of the *hmin* viruses in newborn mice. (*A* and *B*) Attenuation of *hmin* viruses after intracerebral infection. Groups of newborn ICR mice (1–2 d old) were infected intracerebrally with 10^3 (*A*) or 10^4 PFU (*B*) of (D2-syn) or *hmin* viruses, respectively. (*C*) Median lethal dose (LD_{50}) values in newborn mice after intracerebral infection. (*D*) Maternal antibody $PRNT_{50}$ titer in juvenile mice born to mothers vaccinated (when they were newborn animals) with D2-syn or E^{hmin} (*P value < 0.05 by Wilcox rank sum test).

Discussion

Arboviruses that proliferate in cells of different phyla or even kingdoms have evolved a balanced codon pair bias that compromises between hosts. There may well be similar balances in other encoding biases (e.g., codon preference). These viruses evolved because there must be an advantage in their skill to proliferate successfully in very different hosts. However, maintaining this balance decreases positive selection for either host alone and reduces the rate of nonsynonymous substitutions, ultimately constraining RNA virus evolution (41, 42).

In our experiments we have unbalanced dengue virus encoding by computer-aided changes, yielding viral genomes in which insect codon pair bias was favored. This resulted generally in reduced proliferation of the D2-syn^{hmin} variants in mammalian cells compared with insect cells. Concomitantly, the D2-syn^{hmin} variants expressed a dramatic attenuation phenotype in mice.

The precise mechanism by which poor codon pairs cause attenuation in D2-syn^{hmin} variants is still unknown. It could relate to the synthesis of the polyprotein, its processing, misfolding, and/or stability, all parameters that have yet to be investigated. A second issue is the frequency of CpG and UpA dinucleotides in the altered viral genomes. It has long been known that genomes of RNA viruses have low CpG and UpA dinucleotide frequencies (18, 19), as do mammalian coding regions generally (17), a phenomenon which must be advantageous for viruses. Indeed, if the CpG or UpA content is raised in the poliovirus polyprotein by encoding it with synonymous rare CpG- and UpA-rich codons, the resulting variants are either debilitated in growth (43, 44) or nonviable (45). We note that the recoded segments within the D2-syn^{hmin} genomes also present with an increased frequency of CpG and UpA dinucleotides (Table S1). However, because only existing codons are used in codon pair deoptimization, the additional dinucleotides map between the newly formed codon pairs as in xxCpGxx and xxUpAxx (Table S1). It has been proposed that increased CpG may activate in some tissue culture cells the innate immune response, thereby

Table 2. Induction of neutralizing antibodies by the hmin viruses

Dose,* PFU	D2-syn	E^{hmin}	$NS3^{hmin}$	$NS5^{hmin}$
10^1	533 ± 107	—	—	—
10^2	—	427 ± 107	160	53 ± 13

*Newborn mice were injected intracranially with the indicated dose of each virus. Serum collected from surviving mice was then assayed for neutralizing antibodies by measuring focus forming unit reduction on Vero E6 cells. Titers are presented as the reciprocal of serum dilution (e.g., 500 indicates a 1/500 dilution of serum) ± SEM.

Table 3. Induction of protective antibodies by NS3^{hmin}

Mother	Percent survival*
Naïve	0 (0/6)
NS3^{hmin}	93 (13/14)

*Percent survival of newborn mice born from unvaccinated naive mothers, or from mothers infected as newborns with NS3^{hmin}, was measured after challenge with 200 LD_{50} of wild-type D2-syn.

reducing viral replication (43, 44). This is supported by data shown in Fig. 3*E* in which the replication of NS3^{hmin} and NS5^{hmin} is partially rescued by the Jak inhibitor I. Tulloch et al. (46) tried to distinguish dinucleotide and codon pair effects using virus "Min-H." Unfortunately, this virus had not only increased CpG and UpA dinucleotides, but also (contrary to the text of ref. 46) increased frequency of very disfavored codon pairs, and so does not distinguish the two effects.

As shown in Figs. 2 and 3, the fitness of replication of the D2-syn^{hmin} variants in tissue culture cells dramatically decreases in the order E^{hmin} > NS3^{hmin} > NS5^{hmin}. Therefore, we expected that virulence in newborn mice would follow the same pattern. Remarkably, the opposite is the case (Fig. 4). The LD_{50} values of NS3^{hmin} or NS5^{hmin} are ~10-fold lower than that for E^{hmin} (Fig. 4*C*) even though the NS3^{hmin} and NS5^{hmin} variants have a greater number of xxC_3G_1xx dinucleotides compared with E^{hmin} (Table S1). The reason for this paradox is not yet known but reinforces the accepted fact that results in tissue culture cells do not (necessarily) translate to experiments in animals.

Despite strong attenuation of virulence, the recoded viruses induced high levels of neutralizing antibodies in mice (Table 2), mirroring previous results with poliovirus (16) and influenza (22, 23). Remarkably, female mice vaccinated as newborn animals passed these antibodies on to their offspring; we consider it likely that the DENV-specific antibodies then protect the animals from lethal challenge (Table 3). We note that despite recoding, the *hmin* viruses produce proteins that are 100% wild type in sequence, helping to explain the strong protection. Passage of antibodies from suckling mice to suckling mice via breeding respective mothers was observed very recently also by Ng et al. (47).

Recoding can be tailored not only within a single coding sequence but also by combining distinct coding sequences within the viral genome, allowing different degrees of attenuation. This could be particularly important for the development of a dengue vaccine, which is complicated by the phenomenon of antibody-dependent enhancement (ADE) (48). Because of ADE, a successful dengue vaccine must simultaneously protect against all four serotypes. Successful production of such a tetravalent vaccine may be aided by the ability to fine-tune the attenuation of each individual component. Because numerous nucleotides are changed in the course of codon pair deoptimization, reversion to virulence through numerous passages is not very likely. This needs to be confirmed.

The fact that phylum-specific attenuation allows us to separate growth in insect cells from growth in mammalian cells allows another possibility, which is to extend the deoptimization and attenuate the virus to nonviability in mammalian cells. Large amounts of the virus nonviable in mammalian cells could still be grown in insect cells. Such a virus would be morphologically perfect, presumably highly immunogenic, and yet innocuous in mammals. It would combine features of live attenuated viruses with features of inactivated viruses, perhaps to great advantage.

Materials and Methods

See *SI Materials and Methods* for full protocols.

Design of Codon Pair-Deoptimized Sequences. A simulated annealing heuristic was implemented. Each codon is swapped with a randomly chosen synonymous codon with a certain configured probability of retaining the change even if an increase in CPS occurs during deoptimization to reach a global minimum CPS. This process is iterated several hundred thousand times over a particular sequence. For details, see *SI Materials and Methods*.

Cells and Experimental Animals. See *SI Materials and Methods*.

D2-Syn, E^{hmin}, NS3^{hmin}, and NS5^{hmin} Infections and Viral Titrations. Virus infections and titrations were described in *SI Materials and Methods*. Serum neutralizing antibody titers were measured in Vero cells using a modified $PRNT_{50}$ using focus forming units (FFU) as described in *SI Materials and Methods*.

Intracranial Challenge of Newborn Mice. Neonatal ICR mice (groups of 5–12) were infected via the intracranial route with each D2-Syn variant to determine LD_{50}. Two groups of four female mice each were vaccinated with D2-Syn and E^{hmin} (vaccinated with 10^5 PFU i.p. at 1–2 d old and boosted with 10^5 PFU i.n. at 3 wk old). After 3 mo postvaccination, the females were bred, and the offspring were challenged with 10^4 (2,000 LD_{50} PFU). Some neonates from each mother ($n = 4$) were mock-challenged and sera collected to test for maternal antibodies via $PRNT_{50}$. All animal studies were conducted at the Division of Laboratory Animal Services in Stony Brook University under approved institutional protocols and in accordance with the guidelines established by Stony Brook University's Institutional Animal Care and Use Committee. Further details are in *SI Materials and Methods*.

Quantitative PCR and Statistical Analyses. Quantitative PCR and statistical analyses are described in *SI Materials and Methods*.

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