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Determinants in the Envelope E Protein and Viral RNA Helicase NS3 That Influence the Induction of Apoptosis in Response to Infection with Dengue Type 1 Virus

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One mechanism by which dengue (DEN) virus may cause cell death is apoptosis. In this study, we investigated whether the genetic determinants responsible for acquisition by DEN type 1 (DEN-1) virus of mouse neurovirulence interfere with the induction of apoptosis. Neurovirulent variant FGA/NA d1d was generated during the adaptation of the human isolate of DEN-1 virus strain FGA/89 to grow in newborn mouse brains and mosquito cells *in vitro* [Desprès, P. Frenkiel, M.-P. Ceccaldi, P.-E. Duarte Dos Santos, C. and Deubel, V. (1998) *J. Virol.,* 72: 823–829]. Genetic determinants possibly responsible for mouse neurovirulence were studied by sequencing the entire genomes of both DEN-1 viruses. Three amino acid differences in the envelope E protein and one in the nonstructural NS3 protein were found. The cytotoxicity of the mouse-neurovirulent DEN-1 variant was studied in different target cells *in vitro* and compared with the parental strain. FGA/NA d1d was more pathogenic for mouse neuroblastoma cells and attenuated for human hepatoma cells. Changes in virus replicative functions and virus assembly may account, in a large part, for the differences in the induction of apoptosis. Our data suggest that identified amino acid substitutions in the envelope E protein and viral RNA helicase NS3 may influence DEN-1 virus pathogenicity by altering viral growth.

Key Words: dengue virus; envelope protein; RNA helicase; mouse neuron; human hepatocyte; mosquito; pathogenicity; genetic determinant of virulence; apoptotic cell death.

INTRODUCTION

Dengue (DEN) is the most important vector-borne viral disease in tropical countries (Henchal and Putnak, 1990; Gubler and Trent, 1994). It is caused by four serotypes of the dengue virus (DEN-1, -2, -3, and -4), a member of the *Flavivirus* genus (family *Flaviviridae*), transmitted to humans by *Aedes (Ae) aegypti* mosquitoes. It causes a spectrum of illnesses, ranging from a flu-like disease (DEN fever) to DEN hemorrhagic fever (DHF), a fulminating illness that can progress to DEN shock syndrome (DSS) and death (Henchal and Putnak, 1990; Marianneau *et al.,* 1998a; Rigau-Pérez *et al.,* 1998). The clinical features of severe DEN disease include hemorrhagic diathesis, liver involvement, and encephalopathy (Rigau-Pérez *et al.,* 1998; Salomon *et al.,* 2000). The major pathophysiological change that determines the severity of disease in DHF is the leakage of plasma. The pathogenesis of DHF is not well understood. Cytokine production and T-lymphocyte activation appear to be important in the pathogenesis of DHF (Rothman and Ennis, 1999; Green *et al.,* 1999). The severity of the disease may also be due to the intrinsic biological properties of the infecting strains of DEN virus (Rico-Hesse *et al.,* 1997, 1998; Leitmeyer *et al.,* 1999).

The DEN virion is composed of three structural proteins, designated C (core protein), M (membrane protein), and E (envelope protein) (Chambers *et al.,* 1990; Rice, 1996). Protein E, which is exposed on the surface of the virus particle, is responsible for the main biological functions of the virion, including virus attachment and virus-specific membrane fusion in acid pH endosomes (Chambers *et al.,* 1990; Rice, 1996). The genomic RNA is translated to give rise to a large polyprotein precursor, which is cotranslationally processed by host-cell and virus-specified proteases to yield the individual viral proteins. The structural proteins are C, prM (the glycosylated precursor of the M protein), and E, and the nonstructural (NS) proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Chambers *et al.,* 1990; Rice, 1996). Flavivirus replication occurs within the cytoplasm of infected cells (Mackenzie *et al.,* 1999). A model for the

The nucleotide sequences reported in this study have been submitted to the GenBank Data Library under accession numbers AF 226686 (FGA/NA d1d) and AF 226687 (FGA/89).

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TABLE 1

Positions of Amino Acid Differences between FGA/89 and FGA/NA d1d

Virus	E	NS3
FGA/89	Met_{196} ATG Val_{365} GTC Thr_{405} ACC	Leu_{435} TTA
FGA/NA d1d	Val_{196} G•• Ile_{365} A•• Ile_{405} •T•	Ser_{435} •C•

formation of the replication complex (RC) of flaviviruses has been recently proposed (Khromykh *et al.,* 1999a, b). The first steps of DEN virus assembly take place in association with the membranes of the endoplasmic reticulum (ER) (Chambers *et al.,* 1990; Rice, 1996). The DEN virion is first assembled as an immature particle that contains prM noncovalently associated with E in a heterodimeric complex (Chambers *et al.,* 1990; Desprès *et al.,* 1996; Rice, 1996; Courageot *et al.,* 2000). Proteolysis of prM leads to the formation of homodimeric forms of E in virus particles before their release from the cell (Rice, 1996).

Recombination between different subtypes of DEN virus has been suggested to produce new, biologically successful strains (Holmes *et al.,* 1999; Worobey *et al.,* 1999). South American DEN-1 virus strain FGA/89 isolated in 1989 from a woman with DF appeared to be a descendant of a single recombinant ancestor produced by genetic exchanges between DEN-1 viruses of two different subtypes (Desprès *et al.,* 1993; Holmes *et al.,* 1999). It is assumed that the intrinsic biological properties of the infecting virus strains could contribute to the pathogenicity of DEN virus (Desprès *et al.,* 1993, 1996, 1998; Edelman *et al.,* 1994; Kinney *et al.,* 1997; Bhamarapravati and Yoskan, 1997; Bray *et al.,* 1998; Mangada and Igarashi, 1998). Low-passaged DEN-1 virus isolate FGA/89 was initially selected on the basis of its biological properties and neurovirulence for newborn mice (Desprès *et al.,* 1996, 1998).

There is growing evidence that DEN-1 virus infection triggers apoptotic cell death (Desprès *et al.,* 1996, 1998; Marianneau *et al.,* 1997, 1998b, 1999; Couvelard *et al.,* 1999). Recently, we reported that the infection of mouse neuroblastoma and human hepatoma cell lines with DEN-1 virus strain FGA/89 induced cell death typical of apoptosis (Desprès *et al.,* 1996, 1998; Marianneau *et al.,* 1997, 1998b). It has also been suggested that the induction of apoptosis in human hepatocytes infected with DEN virus is a major cause of hepatic failure in cases of DHF-DSS (Marianneau *et al.,* 1997, 1998a, b; Couvelard *et al.,* 1999).

DEN virus infection of the mouse central nervous system (CNS) has been used as a model system for the characterization of genetic determinants and viral factors involved in the pathogenicity of DEN-1 virus (Desprès *et al.,* 1996, 1998; Lee *et al.,* 1997; Bray *et al.,* 1998). Serial passaging of FGA/89 in mouse brain and a mosquito cell line selected a highly neurovirulent variant, FGA/NA d1d, which replicates more efficiently in the CNS (Desprès *et al.,* 1998). Apoptosis has been shown to be the mechanism by which FGA/NA d1d infection causes neuronal cell death in the mouse CNS (Desprès *et al.,* 1998). Here, we used FGA/89 and its mouse-neurovirulent variant, FGA/NA d1d, to identify the determinants that may be relevant to DEN-1 virus pathogenicity.

RESULTS

Genetic relationships between FGA/89 and its mouse neurovirulent variant

Complete sequencing of the genome of the mouse-passaged, neurovirulent variant of FGA/89 allowed identification of the amino acid changes that are potentially responsible for acquisition of mouse neurovirulence. The 5′ and 3′ untranslated regions (UTRs) of FGA/NA d1d and FGA/89 are identical. The amino acid sequences of the C and prM proteins of the two DEN-1 virus variants are also identical (Desprès *et al.,* 1998).

FGA/NA d1d E protein has three amino acid substitutions: at positions 196, 365, and 405 (Table 1; Desprès *et al.,* 1998). Only the Ile-for-Thr difference at position E405 introduces a significant change in the character of the residue. To investigate the structural consequences of the amino acid substitutions, a three-dimensional model for the ectodomain of FGA/89 E protein was derived by homology to the known high-resolution structure of protein sE (lacking the stem anchor region of E) from tick-borne encephalitis (TBE) virus (Rey *et al.,* 1995). As in the structure of the TBE-sE protein, the polypeptide chain of DEN-1-sE is predicted to fold into three distinct domains: the central domain I, the dimerization domain II, and the C-terminal module domain III (Rey *et al.,* 1995). The conservative amino acid substitution at E196 maps to the interface of domains I and II (Fig. 1A, yellow and red domains) in a region that has been proposed to act as a hinge during the fusogenic conformational change of the protein (Rey *et al.,* 1995). The conservative amino acid substitution at E365 maps to the interface between domains I and III (Fig. 1A, red and blue domains). The Ig-like domain III (Fig. 1A, blue domain) has been implicated in receptor recognition (Rey *et al.,* 1995). The non-conservative amino acid substitution at position E405 maps to a predicted amphipathic α-helix 1 ($H1^{pred}$; amino

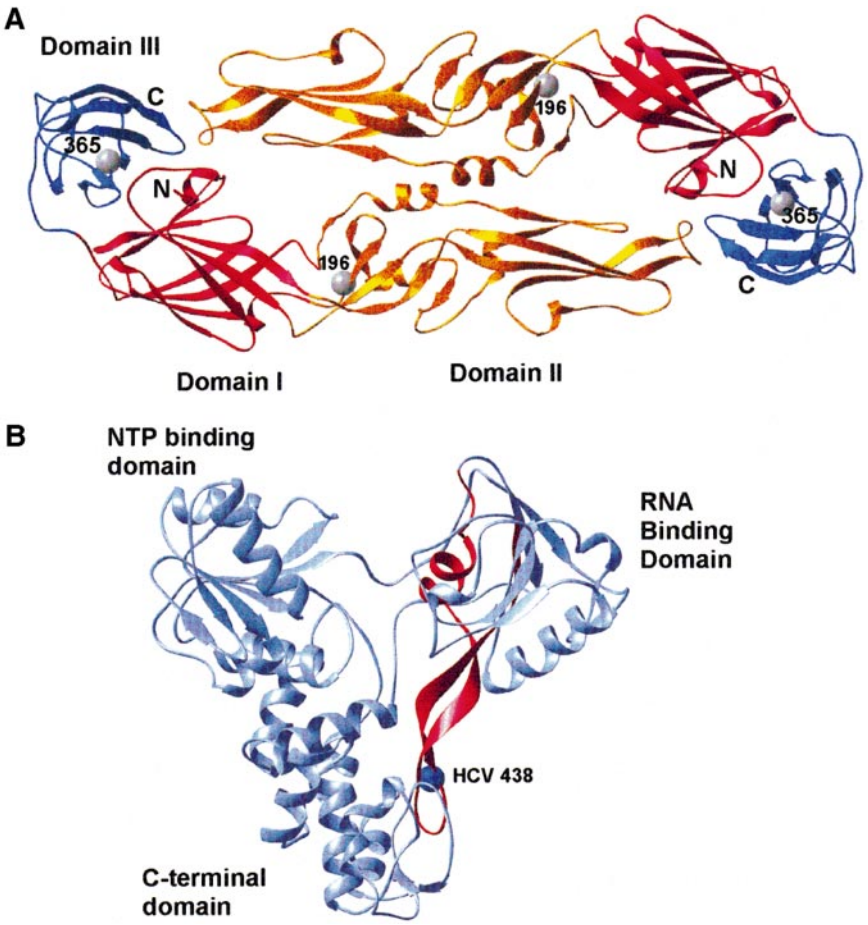

FIG. 1. Diagrammatic representation of the structures of the DEN-1-sE protein and the HCV RNA helicase domain. (A) The E protein was modeled based on the high-resolution crystal structure of the TBE-sE protein (Rey *et al.,* 1995). The central domain (I) is shown in yellow, the dimerization domain (II) is shown in red, and the C-terminal domain (III) is shown in blue. The gray spheres indicate the locations of two of the amino acid substitutions in FGA/NA d1d E protein. N and C label the N- and C-termini of the polypeptide chain, respectively. (B) The RNA helicase domain of HCV NS3. The long β-hairpin emerging from the RNA binding domain is shown in red. The blue sphere indicates the location of the amino acid at position 438 in HCV NS3 protein (equivalent to NS3-435 in DEN-1 virus).

acids 399–412) (Allison *et al.,* 1999) of the stem region (adjacent to the membrane-spanning segment), which is not present in the TBE-sE fragment. The $H1^{pred}$ in the stem region has been shown to be required for the conversion of the E protein from homodimers to homotrimers (Allison *et al.,* 1999), a process that also takes place during the acid pH-triggered fusogenic conformational change of E.

Complete sequencing of the NS1–NS5 protein-coding genes identified only one amino acid substitution in the nonstructural protein NS3 (Table 1). NS3 has several activities associated with virus replication, including NTPase, helicase, and triphosphatase, which are located within a large C-terminal domain (Kapoor *et al.,* 1995; Chen *et al.,* 1997; Li *et al.,* 1997; Cui *et al.,* 1998). The nonconservative Leu_{435}-to-Ser substitution maps to the helicase domain of NS3. The available crystal structure of the hepatitis C virus (HCV) NS3 helicase domain (Yao *et al.,* 1997; Kim *et al.,* 1998; Cho *et al.,* 1998) was used to position the amino acid change in FGA/NA d1d NS3 protein in the context of the folded protein. The identity between the NS3 proteins of DEN-1 virus and HCV is about 25% for a segment of roughly 500 amino acids encompassing the respective NTPase–helicase domain. The alignment is facilitated by the fact that there are conserved sequence elements scattered throughout the NS3 proteins. The Leu_{435}-to-Ser change in FGA/NA d1d NS3 protein (NS3-438 in HCV) is located between the conserved ^{423}VID tripeptide (^{425}VID in HCV) and sequence ^{454}SAAQRRGRIGR (^{457}SxxQRRGRxGR in HCV), encompassing motif VI of superfamily II RNA helicases (Gorbalenya and Koonin, 1993; Kadare and Haenni, 1997; Grassman *et al.,* 1999). Interestingly, the intervening segment between these two elements forms a long β-hairpin that emerges from the RNA-binding domain (Fig. 1B, red segment). The tip of this long β-hairpin contains hydrophobic residues that pack against the core of the adjacent α-helical domain (Fig. 1B).

Fusogenic properties of DEN-1 virus variants

Substitutions in DEN-1 virus E protein during mouse brain tissue–specific adaptation may change the affinity of binding to receptors on target cells or affect the entry of virions by altering the fusion-regulating structural change within the virus particle (McMinn *et al.,* 1996; Lee *et al.,* 1997). We therefore investigated whether the substitutions in the E protein of FGA/NA d1d affect the membrane fusion properties of the virion (Desprès *et al.,* 1993). Infection of AP61 cells with FGA/89 and FGA/NA d1d showed no cytopathic effects (data not shown). DEN viruses can cause fusion of erythrocytes at mildly acidic pH, and hemagglutination (HA) activity reflects the activity of the E protein (Desprès *et al.,* 1993). We found that both FGA/89 and FGA/NA d1d virions expressed HA activity in a range of pH values from 6.4 to a maximum at pH 6.0 (data not shown). These results suggest that fusogenic properties of FGA/NA d1d and FGA/89 do not differ significantly.

DEN virus growth in simian cells and mosquito cells

It has been reported that certain mouse-adapted variants of DEN-1 virus replicate less efficiently than the parental virus in nonhuman primate cells (Bray *et al.,* 1998). The capacity of FGA/NA d1d to infect the simian Vero cell line, a common substrate for DEN virus growth, did not differ from that of FGA/89 (data not shown). We examined whether the amino acid substitutions in the E and NS3 proteins affected viral growth: the mouse-neurovirulent DEN variant replicated as efficiently as the parental strain in Vero cells (Fig. 2A).

Serial passage of DEN virus in mouse CNS or mammalian cell cultures can yield variants with altered infectivity for mosquito vectors (Bhamarapravati and Yoksan, 1997; Jirakananakit *et al.,* 1999). To determine whether this was the case, FGA/89 and FGA/NA d1d were studied for their abilities to infect mosquito vectors via the oral route and to replicate (Vazeille-Falcoz *et al.,* 1999). Female *Ae. aegypti* mosquitoes were fed blood meals containing various doses of highly purified FGA/NA d1d or FGA/89. DEN-1 antigens were detected in mosquito tissues only after virus doses higher than 10^5 $_{AP61}$FFU/ml of meal (data not shown). At a dose of 10^6 $_{AP61}$FFU/ml of blood meal, the infection rate of FGA/NA d1d was significantly higher than that of FGA/89 ($P = 0.02$) (Fig. 2B). *In vitro* analysis showed that the infectivity of FGA/NA d1d in mosquito cell line AP61 was similar to that of FGA/89 (data not shown). Cells were infected at a multiplicity of infection (m.o.i.) of 10 FFU/cell to compare the abilities of DEN-1 virus trains to produce infectious particles. Virus production was higher in AP61 cells infected with FGA/NA d1d than after infection with FGA/89 ($P < 0.001$, 48 h postinfection) (Fig. 2A). These results indicate that the mouse-neurovirulent variant replicates as efficiently as the parent DEN-1 virus in simian and invertebrate cells.

DEN virus-induced apoptosis in Neuro 2a cells

Previous studies have shown that apoptotic cell death in mouse neuroblastoma cell line Neuro 2a was more pronounced after infection with mouse neurovirulent variant FGA/NA d1d than after infection with the parental strain (Desprès *et al.,* 1998). To monitor the induction of apoptosis in DEN virus-infected Neuro 2a cells, the release of histone-associated cytoplasmic DNA generated by the internucleosomal degradation of genomic DNA was analyzed by immunoassay. Neuro 2a cells were infected with FGA/89 or variant FGA/NA d1d at an m.o.i. of 400 $_{AP61}$FFU/cell, which results in optimal infection (80% of Neuro 2a cells 24 h postinfection) (Desprès *et al.,* 1996, 1998). Oligonucleosomal DNA fragments were detected in FGA/NA d1d-infected Neuro 2a cells as early as 27 h postinfection, whereas no significant apoptotic DNA degradation was observed in FGA/89-infected Neuro 2a cells during this period (Fig. 3). By 30 h of infection, the amount of the histone-associated cytoplasmic DNA was not significantly different between FGA/NA d1d and FGA/89 (Fig. 3). It appeared, therefore, that the apoptotic process in Neuro 2a cells progressed more rapidly during FGA/NA d1d infection.

DEN virus protein expression in Neuro 2a cells

The infection of mouse neuroblastoma cells with FGA/89 triggers apoptotic cell death after viral proteins accumulate in intracellular membranes (Desprès *et al.,* 1996). We therefore investigated whether the higher efficiency of FGA/NA d1d to induce apoptosis in Neuro 2a cells could be attributed to differences in viral protein production. Previous studies showed that the rate of DEN-1 virus protein synthesis in mouse neuroblastoma cells reached a maximum 20 h after infection and then decreased by 25 h, when apoptotic cell death occurred (Desprès *et al.,* 1996). Viral protein synthesis in Neuro 2a cells infected with FGA/NA d1d and FGA/89 was monitored by radioimmunoprecipitation (RIP) assay 20 and 25 h postinfection (Desprès *et al.,* 1996). To study the processing of structural and nonstructural proteins, DEN proteins were immunoprecipitated with anti-DEN-1 virus hyperimmune mouse ascites fluid (HMAF) or with prM- and E-specific monoclonal antibodies (mAbs) directed against DEN-1 virus (Desprès *et al.,* 1993, 1996; Courageot *et al.,* 2000).

Viral protein expression in FGA/89-infected Neuro 2a cells was clearly detected 20 h postinfection and peaked by 25 h (Desprès *et al.,* 1996; Fig. 4, lanes 8/9). Immunoprecipitation analysis of cell lysates revealed that viral protein expression levels were significantly lower in FGA/NA d1d-infected Neuro 2a cells at all time points studied (Fig. 4, lanes d1d). Indeed, viral protein synthesis

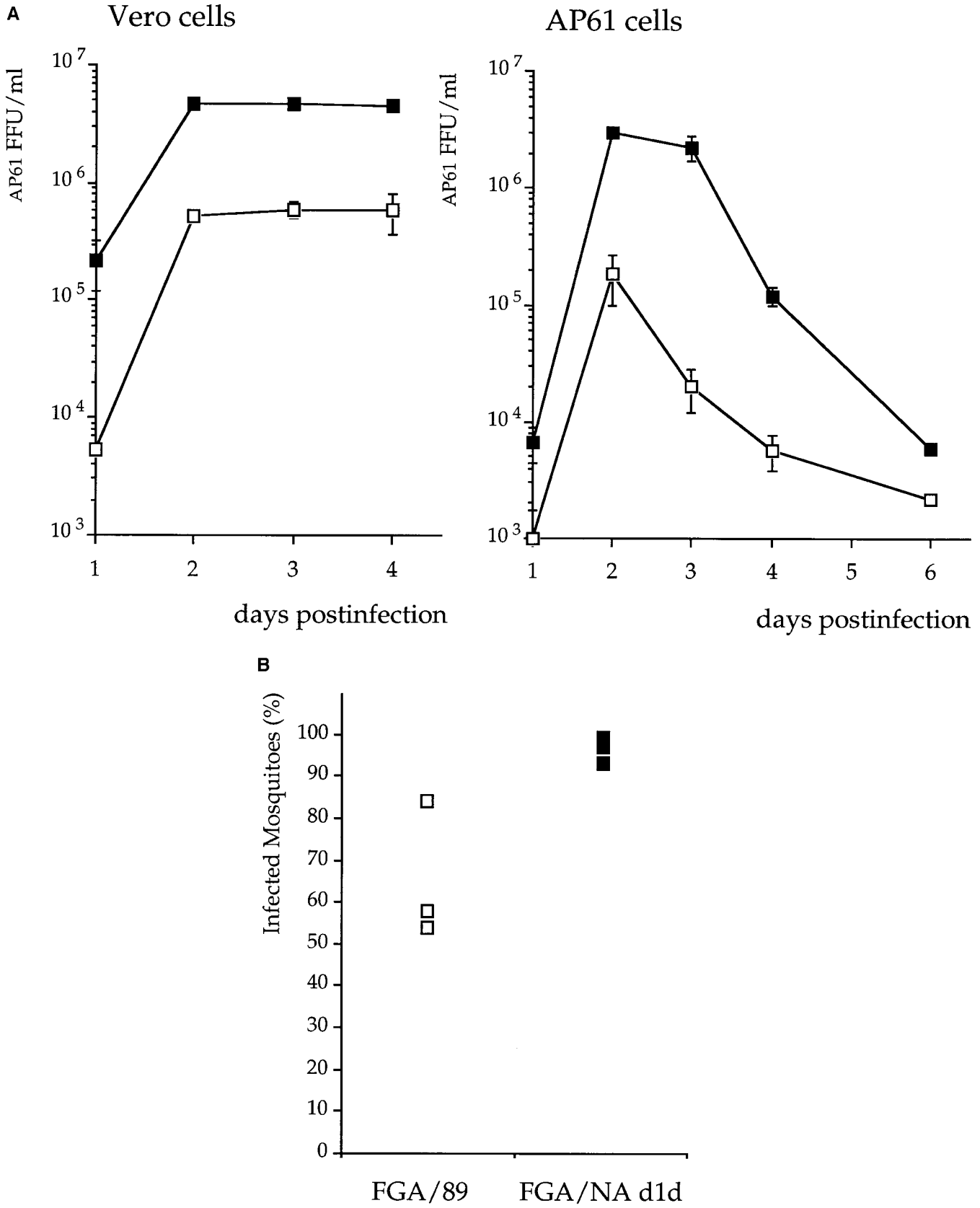

FIG. 2. Growth curves in infected primate and mosquito cells. (A) Vero and AP61 cells were infected with DEN-1 virus FGA/89 □ or FGA/NA d1d ■ at MOI of 10 $_{AP61}$FFU/cell. At various times postinfection, virus particles produced in supernatants were tritered on AP61 cells. Each point represents the mean for three determinations. (B) Three independent groups of 100 female *Ae. aegypti* mosquitoes were fed with 10^6 $_{AP61}$FFU of DEN virus/ml of blood meal. The proportion of infected mosquitoes in each group was determined by IF analysis.

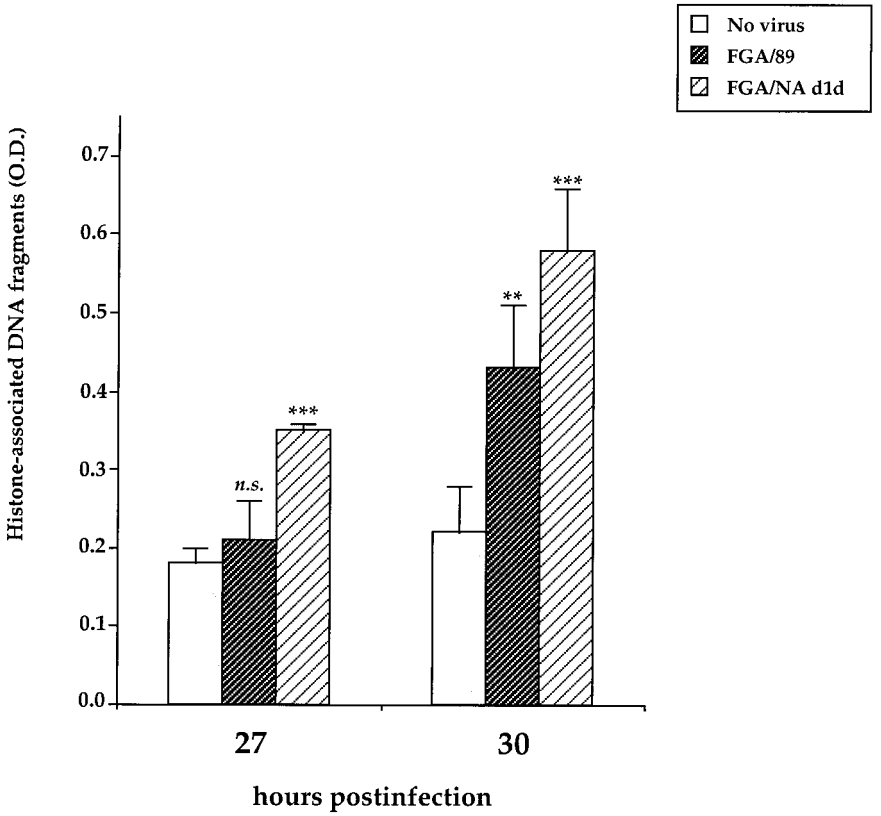

FIG. 3. Kinetics of DEN virus-induced apoptosis in Neuro 2a cells. Cells were infected with FGA/89 or FGA/NA d1d or mock-infected (no virus). They were lysed at various times postinfection, and the oligonucleosomal DNA fragments released in the cytoplasmic fractions were quantified with a cell death detection ELISA kit. Optical density was measured at 405 nm. Statistical analysis for DEN virus versus no virus: not significant (n.s., $P > 0.05$) or significant (**$P < 0.01$; ***$P < 0.001$).

only started in FGA/NA d1d-infected Neuro 2a cells as late as 25 h postinfection. It appeared, therefore, that the induction of apoptosis in FGA/NA d1d-infected Neuro 2a cells at 27 h postinfection paralleled the start of viral protein synthesis. Conversely, FGA/89-infected Neuro 2a cells accumulated viral proteins as early as 20 h postinfection but did not exhibit signs of cell death before 30 h (Fig. 3).

Viral RNA production in Neuro 2a cells

We investigated whether differential expression of FGA/NA d1d proteins was a result of different patterns of viral RNA synthesis in Neuro 2a cells. The intracellular virus-specific RNAs were studied by slot-blot hybridization, as a measure of plus-strand RNA synthesis. The overall amount of virus-specific RNA was approximately 10-fold lower in FGA/NA d1d-infected Neuro 2a cells than in their FGA/89-infected counterparts at 25 h postinfection ($P < 0.001$), calculated relative to the quantities of genomic RNA extracted from purified virions (Fig. 5). Thus the lag phase of viral RNA production may account in large part for the reduced FGA/NA d1d protein expression in Neuro 2a cells.

Processing of FGA/NA d1d envelope glycoproteins in Neuro 2a cells

A causal relationship between the efficiency of DEN-1 virus morphogenesis and the induction of apoptosis seems likely (Desprès *et al.,* 1996; Marianneau *et al.,* 1997, 1998a).

To assess whether the amino acid substitutions in FGA/NA d1d may affect the productive folding pathways of viral envelope glycoproteins in Neuro 2a cells, we studied the processing of FGA/NA d1d prM and E proteins that leads to the formation of enveloped viral particles. Previous studies have shown that the time required for half-maximal ($T_{\frac{1}{2}}$) FGA/89 prME heterodimer formation was 60 min, indicating that this process is slow (Courageot *et al.,* 2000). Pulse-chase analysis 24 h postinfection was used to analyze the folding and the heterodimerization of prM and E in FGA/NA d1d-infected Neuro 2a cells. The folding of protein E and its assembly with prM was assessed using mAbs that correctly recognized folded protein E (anti-E mAb 4D2) and the prME heterodimer (anti-prM mAb 15H5) (Desprès *et al.,* 1996; Courageot *et al.,* 2000). RIP assay with anti-DEN virus HMAF showed that the prM, E, and NS1 glycoproteins

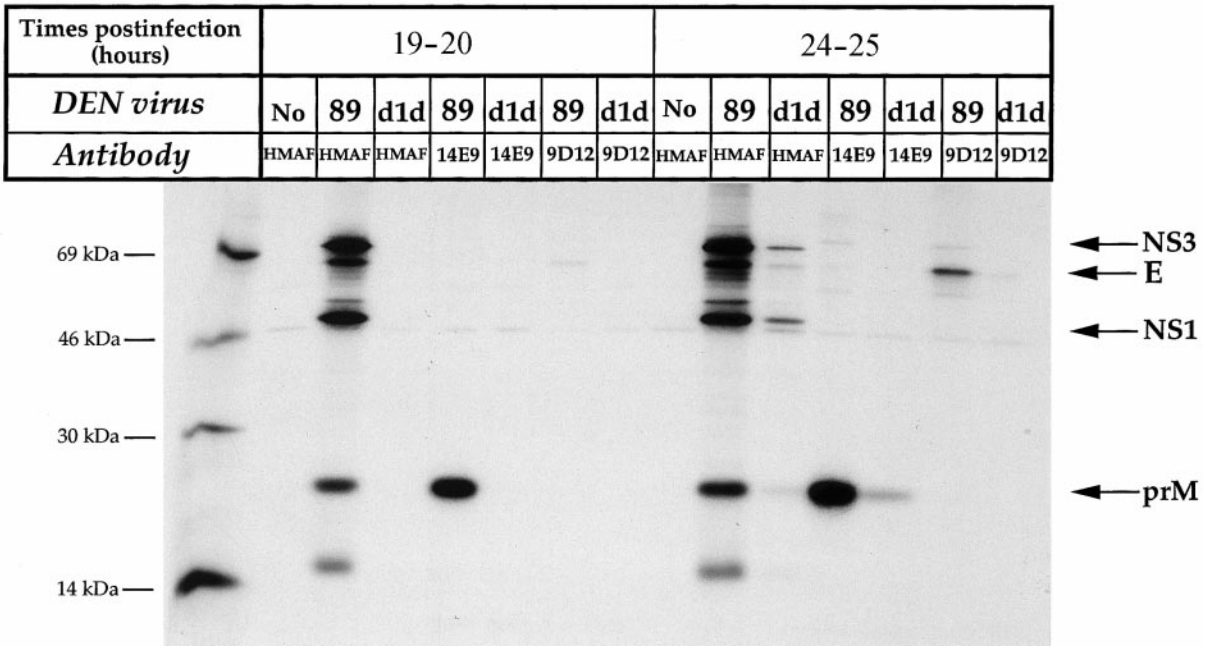

FIG. 4. Kinetics of DEN virus protein production in Neuro 2a cells. Cells were infected with FGA/89 (89) or FGA/NA d1d (d1d) at an m.o.i. of 400 AP61FFU/cell or mock-infected (No). Proteins in cells were labeled for 1 h at various times postinfection. Cells were lysed, and viral proteins from cell lysates were immunoprecipitated with anti-DEN-1 virus HMAF (HMAF), anti-prM mAb 14E9 (14E9), or anti-E mAb 9D12 (9D12). Samples were analyzed by SDS–15% PAGE under reducing conditions.

were correctly processed in FGA/NA d1d-infected Neuro 2a cells (Fig. 6, panel HMAF). The $T_{\frac{1}{2}}$ of formation of the conformation-dependent 4D2 epitope was more than 60 min (Fig. 6, panel 4D2). The $T_{\frac{1}{2}}$ of the association between prM and E was 60 min (Fig. 6, panel 15H5). Thus the kinetics of the folding and heterodimerization of prM and E in FGA/NA d1d-infected Neuro 2a cells did not differ significantly from those of their counterparts in FGA/89-infected Neuro 2a cells (Courageot *et al.,* 2000). The greater efficiency of FGA/NA d1d at inducing apoptosis in Neuro 2a cells was not linked to an obvious defect in the first steps of viral morphogenesis.

DEN virus growth in HepG2 cells

The presence of viral antigens inside human hepatocytes indicates that these cells may be susceptible to infection by DEN virus (Marianneau *et al.,* 1996; Couvelard *et al.,* 1999). DEN-1 virus strain FGA/89 has been shown to replicate in human hepatoma HepG2 cells (Marianneau *et al.,* 1996). We examined whether FGA/89 and its mouse-neurovirulent variant differed in their capacity to grow in HepG2 cells. The efficiencies of infection of both DEN-1 viruses were first examined by immunofluorescence (IF) assay. HepG2 cells were infected at an m.o.i. of 10–80 AP61FFU/cell. Inoculation with 40 AP61FFU/cell was needed to infect 50% of HepG2 cell monolayers with FGA/89 as monitored by immunostaining of viral antigens 25 h postinfection (Fig. 7A). In contrast, no more than 20% of FGA/NA d1d-infected HepG2 cells were positive for viral antigens at the highest m.o.i. tested (Fig. 6A). Thus FGA/89 and its mouse-neurovirulent variant differed in their efficiency of infection of human hepatoma cells. The difference may be due to a lag phase before viral protein production. To determine whether this was the case, HepG2 cells were infected at an m.o.i. of 40 AP61FFU/cell, and viral protein production was monitored by IF staining at 25 and 30 h postinfection. The number of FGA/89-infected HepG2 cells remained constant between 25 and 30 h postinfection, whereas the proportion of cells positive for FGA/NA d1d antigens increased from 15% to 35% (Fig. 7B). These results suggest that FGA/NA d1d growth was delayed in HepG2 cells.

Viral RNA synthesis in HepG2 cells

Viral protein synthesis in HepG2 cells progressed more slowly after infection with FGA/NA d1d. To investigate whether the delay was due to altered viral RNA synthesis, the production of viral RNA was examined in HepG2 cells infected with FGA/NA d1d and FGA/89 at an m.o.i. of 40 AP61FFU/cell at various times postinfection. The overall amount of viral RNA, and therefore presumably RNA synthesis, was significantly lower in HepG2 cells infected with FGA/NA d1d than in those infected with FGA/89 within the first 25 h of infection ($P < 0.05$) (Fig. 8A). The rate of viral RNA synthesis in FGA/NA d1d-infected HepG2 cells was significantly increased between 25 and 30 h postinfection (Fig. 8A) ($P < 0.05$).

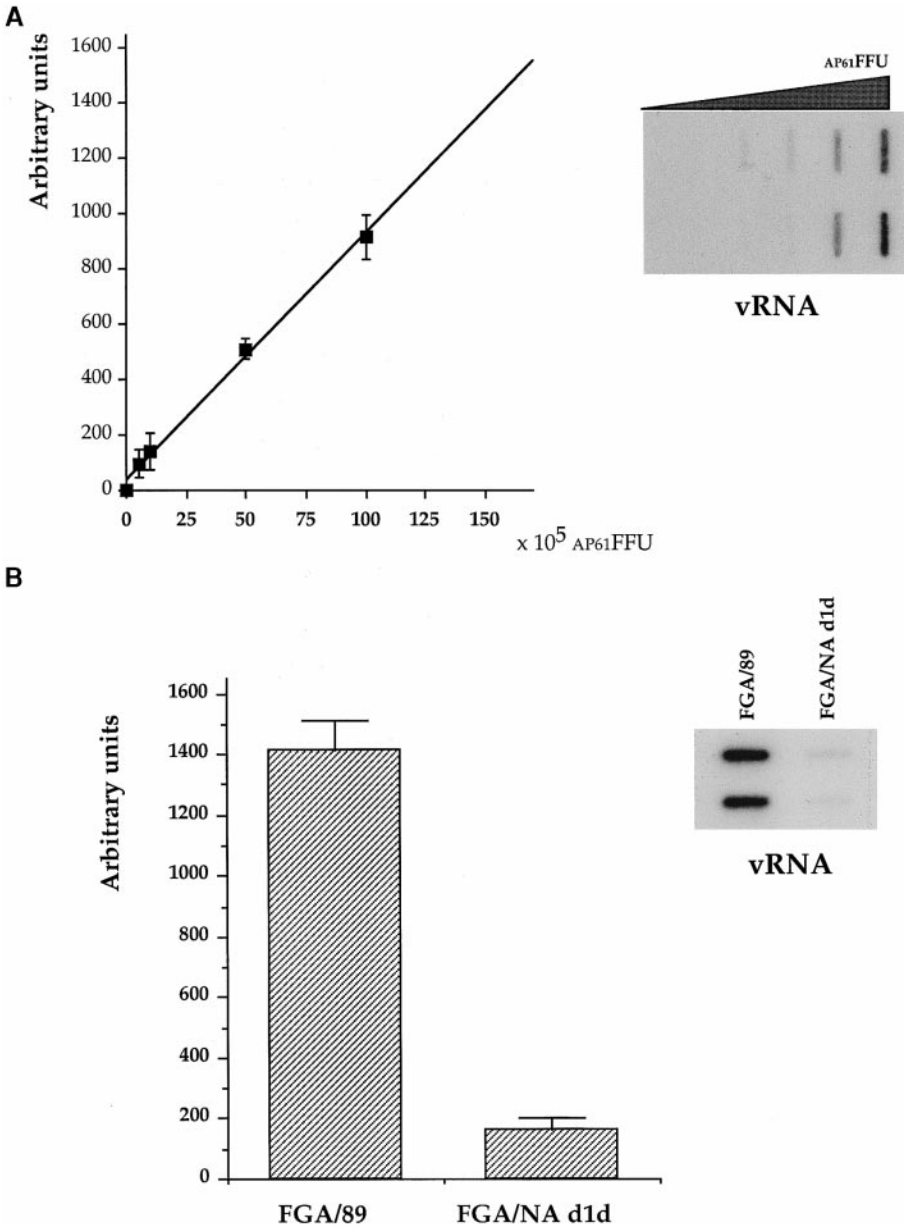

FIG. 5. Viral RNA production in Neuro 2a cells. Slot-blot hybridization of FGA/89 genomic RNA (A) or virus-specific RNAs from DEN virus-infected Neuro 2a cells at 25 h postinfection (B). Viral RNA were hybridized with a ^{32}P-labeled PCR product specific for DEN-1 virus (right) and then quantitated with a PhosphorImager (left). (A) Two independent samples of viral RNA extracted from various amounts of purified infective particles (AP61FFU). (B) Two independent samples of total RNA extracted from infected cells.

These results suggest that viral RNA synthesis was delayed in FGA/NA d1d-infected HepG2 cells. The cytopathic effects in FGA/89-infected HepG2 cells may contribute to the lower amount of viral RNA 30 h postinfection (Fig. 8A; Marianneau *et al.,* 1997, 1998b).

DEN virus-induced apoptosis in HepG2 cells

Infection of the human hepatoma cell line HepG2 with DEN-1 virus strain FGA/89 results in extensive apoptosis after 40 h of infection (Marianneau *et al.,* 1997, 1998b). We examined whether FGA/89 and its mouse-adapted neurovirulent variant differed in their efficiency to induce apoptosis in hepatoma cells. HepG2 cells were infected with FGA/89 or FGA/NA d1d at an m.o.i. of 40 AP61FFU/cell, and apoptotic DNA degradation was quantitatively assayed by immunoassay. The amount of histone-associated cytoplasmic DNA fragments was significantly higher in FGA/89-infected HepG2 cells than in FGA/NA d1d-infected HepG2 cells 45 h postinfection ($P = 0.001$) (Fig. 8B). This suggests that apoptosis was much less pronounced after infection with FGA/NA d1d than with the parental strain. FGA/NA d1d induced cytopathic

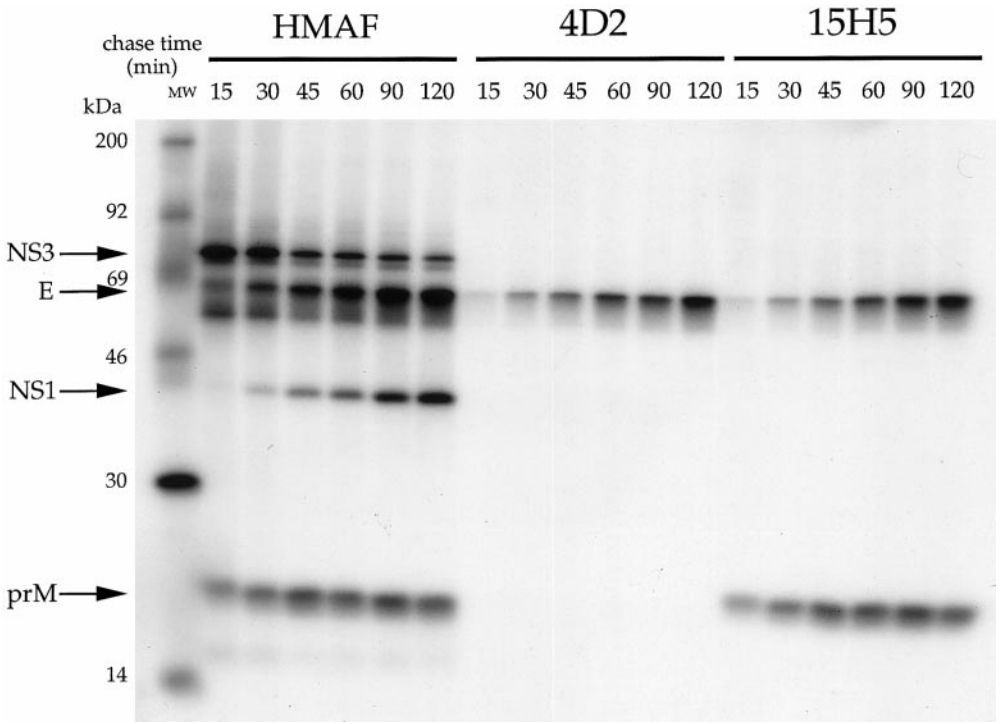

FIG. 6. Kinetics of FGA/NA d1d envelope glycoprotein processing in Neuro 2a cells. Cells infected with FGA/NA d1d were pulse-labeled for 10 min at 24 h postinfection and chased for various times. Cells were lysed, and viral proteins from cell lysates were immunoprecipitated with anti-DEN-1 virus HMAF (HMAF), anti-prM mAb 15H5 (15H5), or anti-E mAb 4D2 (4D2). Samples were analyzed by SDS–15% PAGE under nonreducing conditions.

changes in HepG2 cells only 72 h after infection (data not shown).

DEN virus production in HepG2 cells

The production of infectious particles in DEN virus-infected human hepatoma cells is very low, suggesting that viral morphogenesis is an inefficient process in this cell type (Marianneau *et al.,* 1996, 1998b). We examined whether the lower cytotoxicity of FGA/NA d1d for HepG2 cells results in an increased viral yield. HepG2 cell monolayers (2.5×10^5 cells/well) were infected with FGA/89 or FGA/NA d1d at an m.o.i. of 20 $_{AP61}$FFU/cell, and the release of infective particles was assayed at various times postinfection. Progeny virus production in FGA/89-infected HepG2 cells was similar at 24 and 48 h postinfection ($6.5\ [\pm 2.5] \times 10^3$ $_{AP61}$FFU/ml), whereas infective particles accumulated in FGA/NA d1d-infected HepG2 cells during the same interval to $2.0\ (\pm 0.2) \times 10^4$ $_{AP61}$FFU/ml at 48 h ($P < 0.01$).

DEN virus protein expression in HepG2 cells

We examined whether the differences in DEN-1 virus production in HepG2 cells were also due to differences in viral protein processing. Viral protein production in HepG2 cells infected with FGA/NA d1d and FGA/89 was monitored by RIP assay at various times postinfection. The major DEN proteins were clearly detected at 25 h of infection (Fig. 9, HMAF). No obvious differences were observed in viral protein processing between FGA/89 and FGA/NA d1d. Detailed RIP analysis showed that anti-DEN-1 virus HMAF immunoprecipitated a small amount of labeled protein E (Fig. 9, HMAF). This suggests that E production from DEN-1 structural polyprotein was significantly impaired in HepG2 cells.

To study the processing of DEN-1 virus E protein in HepG2 cells, samples were immunoprecipitated with the anti-E mAbs 9D12 and 8C2. mAb 9D12 recognizes a conformation-independent epitope within the domain III that is accessible in intracellular forms of E and in DEN-1 virions (Mégret *et al.,* 1992; Després *et al.,* 1993; Courageot *et al.,* 2000). The immunoreactivity of mAb 9D12 with FGA/89 and FGA/NA d1d E proteins did not differ significantly at 30 h postinfection (Fig. 9, 9D12). However, discrete labeled polypeptides migrating faster than protein E were clearly detected (Fig. 9, 9D12), suggesting a possible degradation of DEN E glycoprotein in HepG2 cells. Anti-E mAb 8C2 binds to a linear epitope within the domain II of DEN-1 virus E protein and recognizes incompletely folded species of protein E (Mégret *et al.,* 1992; Després *et al.,* 1993, 1996; Courageot *et al.,* 2000). RIP analysis with the anti-E mAb 8C2 showed that a large amount of prM interacted with E to form a heterocomplex (Fig. 9, 8C2). This unexpected finding suggests that newly synthesized DEN envelope proteins may aggregate in HepG2 cells.

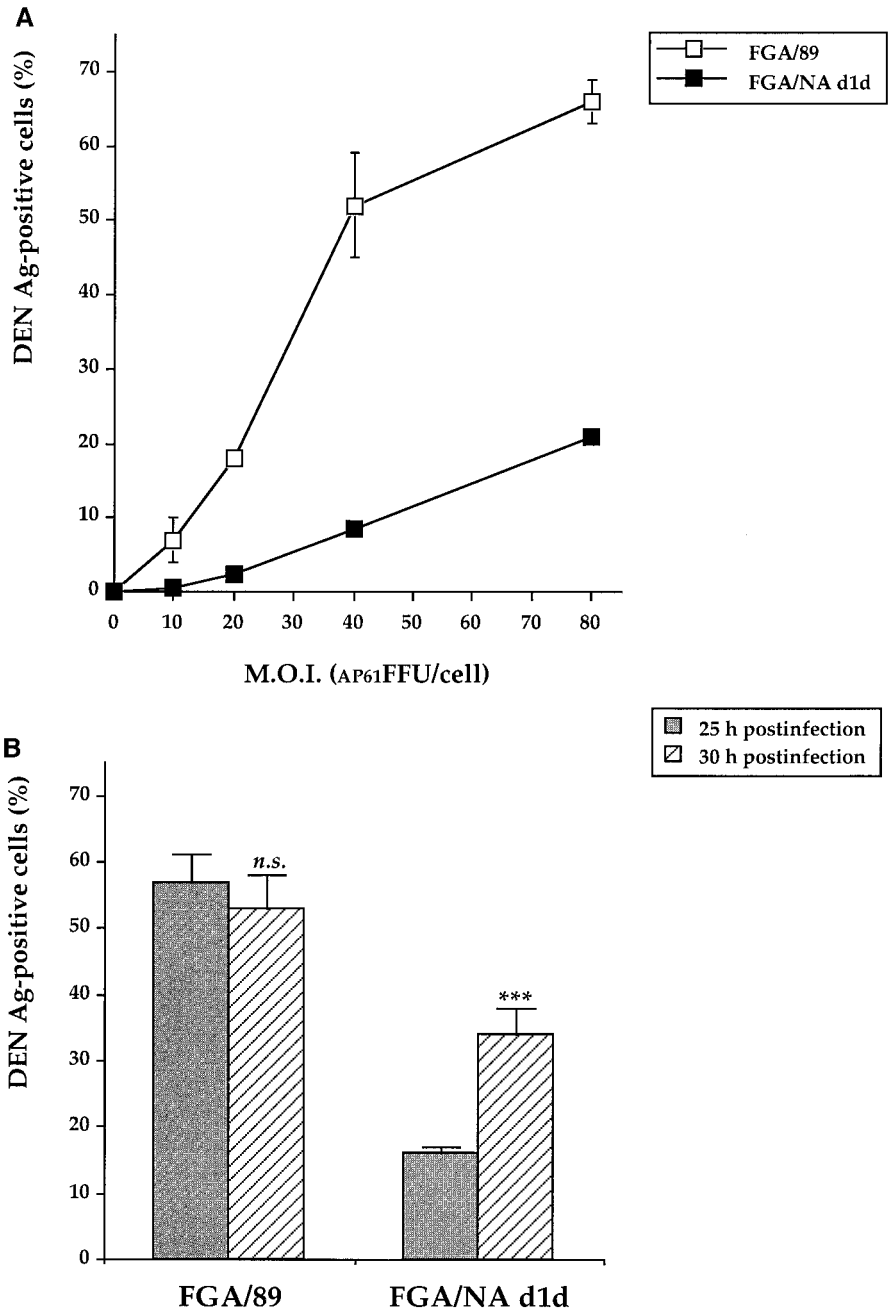

FIG. 7. Viral growth in infected HepG2 cells. Cells infected with DEN-1 virus at various m.o.i.s (A) or an m.o.i. of 40 AP61FFU/cell (B) were analyzed for viral protein production. Viral antigens were visualized by an IF assay with anti-DEN-1 virus HMAF. The proportion is shown of DEN antigen-positive cells in three sets of cell monolayers 25 h postinfection (A) or at various times postinfection (B). Statistical analysis 30 h versus 25 h: not significant (n.s., $P > 0.05$) or significant (***$P < 0.001$).

The productive assembly of DEN envelope glycoproteins was assessed using anti-prM mAb 14E9, which recognizes the prME heterodimer. The rate of prM production in DEN virus-infected HepG2 cells increased slightly during the first 30 h of infection (Fig. 9, compare panels 25 and 30, 14E9). RIP assay with the anti-prM mAb 14E9 revealed that prM and E heterodimerized in FGA/NA d1d-infected HepG2 cells within 30 h of infection (Fig. 9, lanes d1d, 14E9). In contrast, only a very small amount of FGA/89 E protein coprecipitated with prM (Fig. 9, lanes 89, 14E9). This suggests that the formation of DEN-1 envelope glycoprotein complexes was more efficient in HepG2 cells infected with FGA/NA d1d than in those infected with the parental strain.

DISCUSSION

Neurovirulent variant FGA/NA d1d was generated during the adaptation of the human isolate of DEN-1 virus strain FGA/89 to grow in newborn mouse brain and

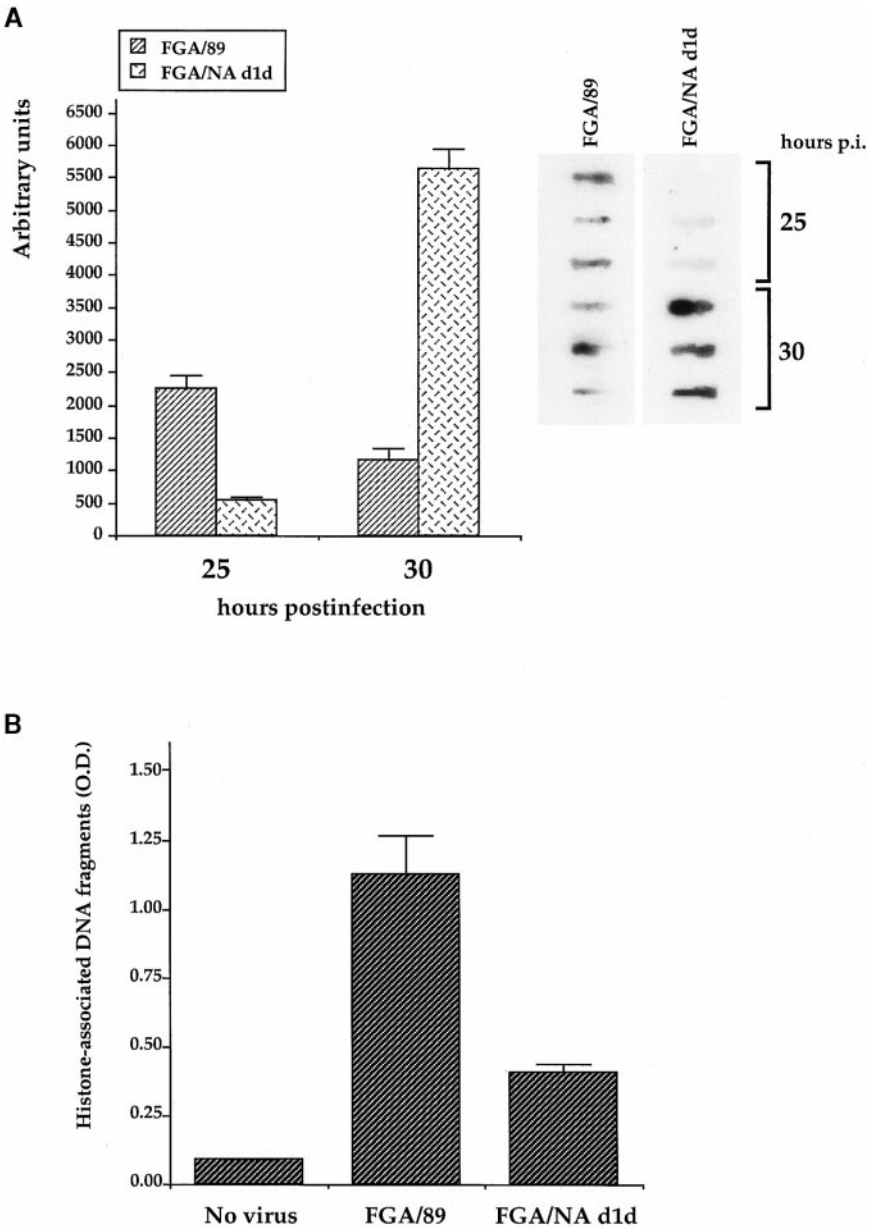

FIG. 8. Viral RNA synthesis and DEN virus-induced apoptosis in HepG2 cells. Slot-blot hybridization of virus-specific RNA at various times postinfection (A) and release of histone-associated DNA fragments 45 h postinfection (B). (A) Three independent samples of total RNA extracted from infected cells. Experiments were performed as described in the legend to Fig. 3. RNAs were hybridized with ^{32}P-labeled plasmid pCI/prM-E. (B) Three sets of HepG2 cell monolayers infected with DEN-1 virus or mock-infected (No virus) were analyzed for apoptotic cell death. The release of apoptotic DNA fragments was quantified as described in the legend to Fig. 2.

mosquito cells *in vitro* (Desprès *et al.,* 1998). We identified the amino acid changes in the mouse-passaged, neurovirulent variant of FGA/89 that are potentially responsible for the acquisition of mouse neurovirulence. FGA/NA d1d has three amino acid substitutions in the envelope E protein and one in the viral RNA helicase NS3. It is interesting to note that for both viral proteins, the identified amino acid substitutions map to the interfaces between structural domains (Fig. 1).

The conservative amino acid substitutions at positions E196 and E365 map to the interfaces of domains I and II and of domains I and III, respectively (Fig. 1A). Previous reports have shown that mutations in the interfaces between structural domains of the E protein may influence the pathogenicity of flaviviruses (Rey *et al.,* 1995). The nonconservative amino acid substitution at position E405 maps to $H1^{pred}$ next to the membrane anchor region (Allison *et al.,* 1999). It has been suggested that formation of DEN envelope glycoprotein complexes is mediated by domains in the C-terminus of E (Allison *et al.,* 1999; Wang *et al.,* 1999). The Thr_{405}-to-Ile substitution alters the hepta-repeat pattern of hydrophobic and polar residues

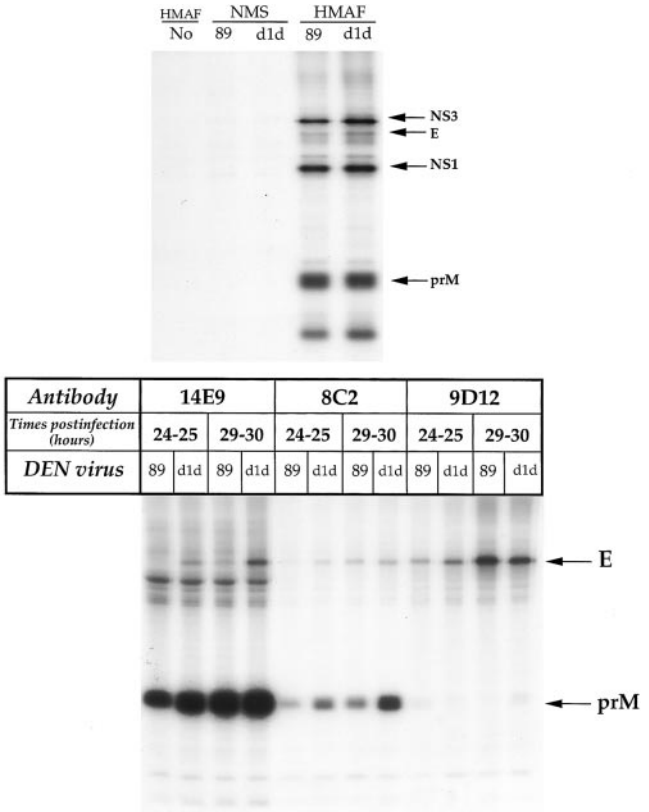

FIG. 9. DEN-1 virus protein expression in HepG2 cells. Cells were infected with FGA/89 (89) or FGA/NA d1d (d1d) at an m.o.i. of 40 $_{AP61}$FFU/cell or mock-infected (No). Proteins in cells were labeled for 1 h at 25 h postinfection (top) or various times postinfection (bottom). Cells were lysed, and viral proteins were immunoprecipitated with normal mouse serum (NMS), anti-DEN-1 virus HMAF (HMAF), anti-prM mAb 14E9 (14E9), or anti-E mAbs 8C2 (8C2) and 9D12 (9D12). Samples were analyzed by SDS–15% PAGE under reducing conditions.

that are important for interactions between α-helices in the stem region of protein E.

The active flavivirus RC is composed of the NS1, NS2A, NS3, NS4A, and NS5 proteins and viral RNA template (Chen *et al.,* 1997; Khromykh *et al.,* 1999a, b; Mackenzie *et al.,* 1999), and cell proteins that interact with the terminal stem-loop of the 3′ UTR of genomic RNA may also be involved (Blackwell and Brinton, 1997). The composition of the RCs yielding new viral RNAs had been suggested to be similar early and late in infection (Khromykh *et al.,* 1999b). The viral RNA helicase activity of NS3 is regulated by the RNA-dependent RNA polymerase NS5 in the flavivirus RC (Kapoor *et al.,* 1995; Chen *et al.,* 1997; Rice, 1996; Cui *et al.,* 1998; Khromykh *et al.,* 1999a, b). The nonconservative Leu-to-Ser change at position NS3-435 in FGA/NA d1d virus has not been previously described in any DEN-1 virus strains. We have used the known structure of the helicase domain of protein NS3 from HCV to localize the amino acid substitution in DEN-1 NS3 protein in the context of three-dimensional structure of the folded polypeptide (Fig. 1B). The amino acid substitution in FGA/NA d1d NS3 protein maps to the tip of the β-hairpin emerging from the RNA binding domain to contact the C-terminal, α-helical domain. The connection between the two domains via the β-hairpin may be important for the NTPase/helicase activity of the NS3, which is thought to change conformation on ATP hydrolysis to unwind viral RNA. It is possible that the radical Leu_{435}-to-Ser change at the interface of the RNA binding and C-terminal, α-helical domains may affect the FGA/NA d1d NS3 protein activities that are required in DEN-1 virus replicative functions. Interestingly, this amino acid substitution was also found in a second mouse-passaged, neurovirulent FGA/89 variant, FGA/NA a5c (Desprès *et al.,* 1998).

All four amino acid substitutions in FGA/NA d1d virus lie at regions that are likely to be important for conformational changes during the virus life cycle. The amino acid changes at positions E196, E405, and NS3-435 were also identified in FGA/NA a5c (Desprès *et al.,* 1998). Which amino acid contributes to the acquisition of mouse neurovirulence is not yet clear. We speculate that the identified amino acid changes in the ectodomain of DEN-1 E protein may account for the differences in the pathogenesis of virus infection observed *in vivo.* A major determinant of DEN-2 virus mouse neurovirulence was identified at position E126 (Gualano *et al.,* 1998). Two amino acid substitutions in domain II result in the acquisition of mouse neurovirulence of DEN-2 virus strain New Guinea C (Bray *et al.,* 1998). Furthermore, the Phe_{402}-to-Leu amino acid change in DEN-4 E protein confers the mouse neurovirulence phenotype to a non-neurovirulent virus strain (Bray *et al.,* 1998). These findings support the notion that genetic determinants in DEN E protein play an essential role in increasing virulence *in vivo.* A full-length infectious cDNA for DEN-1 virus strain FGA/89 with the identified amino acid substitutions is needed to provide direct evidence for their contribution to the pathophysiology of newborn mice in the response to DEN virus infection.

We have examined the contribution of the E and NS3 determinants in DEN-1 virus pathogenicity *in vitro.* The rate of viral protein production in mouse neuroblastoma cells infected with FGA/NA d1d was much lower than that in cells infected with the parental strain. The differences in viral protein production were essentially due to differences in the rate of viral RNA production. Because 5′ and 3′ UTRs of genomic RNA are identical in FGA/89 and FGA/NA d1d, presumably the change at position NS3-435 alters the activity of RCs in mouse neuroblastoma cells. It has been shown that, for the bovine viral diarrhea virus (*Flaviviridae*), the NS3 NTPase/helicase activity is essential for the synthesis of minus-strand RNA (Gu *et al.,* 2000). One way of identifying the role of NS3-435 in the functional activity of RC is to develop a functionally active DEN-1 RC from replicon RNA (Khromykh *et al.,* 1999a, b).

The titers of progeny virus in mouse neuroblastoma cells infected with FGA/89 and FGA/NA d1d were similar

(Desprès *et al.,* 1998), despite viral protein production being different. Our data suggest that the productive assembly pathways of FGA/NA d1d envelope glycoproteins were more efficient than those for the parental strain. It has been proposed that domain II and the stem anchor of protein E influence the efficiency of oligomerization of the two viral envelope glycoproteins (Rey *et al.,* 1995; Allison *et al.,* 1999). Thus amino acid changes at positions E196 and E405 could facilitate the oligomeric assembly of DEN-1 envelope glycoproteins in mouse neuroblastoma cells. The involvement of the amino acid changes in E could be confirmed through the use of stable cell clone N2aprM+E expressing recombinant FGA/89 envelope glycoproteins under the control of an inducible, exogenous promoter (Courageot *et al.,* 2000).

An important finding was that the mouse neurovirulent DEN-1 virus variant induced apoptosis more rapidly than the parental strain in mouse neuroblastoma cells, albeit yielding much lower amounts of viral proteins. Consistent with the notion that amino acids substitutions in FGA/NA d1d E protein facilitate viral morphogenesis in the ER, a more efficient assembly of virus particles might promote the induction of apoptosis by causing a more severe stress than that due to infection with the parental strain. Accumulation of DEN envelope glycoprotein complexes in the ER membranes has been suggested to cause a stress activating the apoptotic pathway (Desprès *et al.,* 1996; Marianneau *et al.,* 1998a).

The amino acid substitutions in FGA/NA d1d E and NS3 proteins neither affected its capacity to replicate in primate cells nor caused lower infectivity for mosquito vectors. We postulate that the mouse neurovirulent variant has the ability to be transmitted to primates by the vector mosquitoes *Ae. aegypti.* It has been suggested that the acquisition of mouse neurovirulence of DEN virus is directly or indirectly associated with attenuation in primates (Bray *et al.,* 1998). We therefore examined whether FGA/89 and its mouse neurovirulent variant differed in their capacity to grow in human hepatocytes. We observed that the kinetics of FGA/89 and FGA/NA d1d virus replication were different. FGA/NA d1d infection was characterized by the late accumulation of intracellular antigens. Altered viral growth may account for the lower efficiency of FGA/NA d1d virus infection in human hepatoma cells. The rate of viral RNA synthesis was low in FGA/NA d1d virus-infected hepatoma cells during the first 25 h of infection and then increased abruptly by 30 h. In contrast, viral RNA synthesis was maintained at a constant level in FGA/89-infected hepatoma cells during the same period. Thus, a Leu_{435}-to-Ser amino acid change in FGA/NA d1d NS3 protein could modulate the efficiency of RCs to catalyse the synthesis of new RNA molecules in human hepatoma cells, as already observed in mouse neuroblastoma cells.

Analysis of the viral proteins suggested that most DEN-1 E protein was degraded in human hepatoma cells. However, DEN-1 virus envelope glycoproteins may aggregate, leading to a nonproductive pathway. It has recently been shown that HCV glycoproteins E1 and E2 produced in human hepatoma cells aggregate in ER-like structures (Choukhi *et al.,* 1999). The aggregation of DEN-1 envelope glycoproteins might involve incorrect disulfide linkages in the ER (Molinari and Helenius, 1999). The conversion of soluble proteins into insoluble aggregates may have a significant effect on cell functions (Welch and Gambetti, 1998). The formation of DEN-1 glycoprotein aggregates may account for the induction of apoptosis in human hepatoma cells.

FGA/NA d1d has a greater capacity than the parental strain to produce virus progeny in human hepatoma cells. This difference paralleled the higher level of FGA/NA d1d prME heterodimers, probably due to a greater capacity of FGA/NA d1d E protein to associate with prM as a consequence of amino acid substitutions. The extent of apoptosis was much lower in FGA/NA d1d-infected human hepatoma cells than in cells infected with the parental strain. A greater efficiency of folding and oligomeric assembly of DEN-1 envelope glycoproteins may be largely responsible for the lower cytotoxicity of FGA/NA d1d in restricting the overloading of DEN-1 glycoprotein aggregates in the ER membranes. It is also possible that altered viral growth accounts for the lower cytotoxicity of FGA/NA d1d for human hepatoma cells.

In conclusion, the identified substitution in DEN-1 RNA helicase NS3 might alter the efficiency of viral RNA production by RCs during the first stages of virus replication. The amino acid substitutions at the interfaces of domains I, II, and III and in the $H1^{pred}$ of the E protein may account for the differences in DEN-1 progeny titers by facilitating virus assembly. Previous reports indicate that DEN virus mutations that affect viral growth may contribute to attenuation in humans (Bhamarapravati *et al.,* 1987; Edelman *et al.,* 1994; Bray *et al.,* 1998). Our data suggest that differences in virus life cycle influence the pathogenicity of DEN-1 virus. Altered viral growth coupled with a greater efficiency of viral morphogenesis triggered a rapid induction of apoptosis in mouse neurons but caused attenuation in human hepatocytes *in vitro.* The discrepancy in DEN-1 virus pathogenicity may also be related to particular apoptotic pathways that are activated in response to the viral replicative functions (Roulston *et al.,* 1999). Indeed, DEN-1 virus infection of human hepatoma cells activates the transcription factor NF-κB, which in turn, promotes the induction of apoptosis (Marianneau *et al.,* 1997). We are currently investigating whether the lower cytotoxicity of FGA/NA d1d for human hepatoma cells is the result of variations in the levels of NF-κB activity. An understanding of the specific mechanisms by which DEN virus replication induces apoptosis in mouse neurons and human hepatocytes will

give new insights into the contribution of the E and NS3 determinants in DEN-1 virus pathogenicity.

MATERIALS AND METHODS

Cells and DEN-1 viruses

The mosquito *Ae. pseudoscutellaris* cell line AP61, the mouse neuroblastoma cell line Neuro 2a, the human hepatoma cell line HepG2, and the nonhuman primate cell line Vero were propagated as described previously (Desprès *et al.,* 1993, 1996; Marianneau *et al.,* 1996).

The derivation of the DEN-1 viruses FGA/89 and FGA/NA d1d has been previously reported (Desprès *et al.,* 1993, 1998). The production and purification of DEN virus from AP61 cell monolayers and virus titration on AP61 cells by focus immunodetection assay (FIA) were performed as previously described (Desprès *et al.,* 1993). Virus titers are expressed as AP61FFU/ml.

IF assay

For IF assay, cells were fixed with 3% paraformaldehyde in PBS for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. The fixed cells were incubated for 20 min with a 1:100 dilution of anti-DEN-1 virus HMAF. FITC-conjugated goat anti-mouse immunoglobulin (Sigma Chemical Co., St. Louis, MO) was used as the second antibody.

Virus infectivity in *Ae. aegypti*

The Paea strain of *Ae. aegypti* provided by Institut Louis Malarde (Tahiti, French Polynesia) and reared in Paris since 1994 was used for the oral infection experiments. The mosquitoes were maintained in the laboratory at 28 ± 1°C with 80% relative humidity with a 16/8-h photoperiod. Larvae were fed with yeast tablets, and adults were fed with 10% sucrose solution.

The oral susceptibility of females was tested by a feeding protocol as described elsewhere (Vazeille-Falcoz *et al.,* 1999). Briefly, 5- to 7-day-old females were deprived of sucrose solution 1 day before the infectious meal and then allowed to feed for 20 min through a chicken skin membrane covering an apparatus containing the feeding mixture maintained at 37°C. The infectious meal consisted of 70% washed rabbit erythrocytes, 30% virus suspension, and ATP (as a phagostimulant) at a final concentration of 5×10^{-3} M. Only fully engorged females were transferred to small cardboard containers and maintained at 28 ± 1°C for 14 days. Surviving females were killed and tested for the presence of DEN-1 virus by IF assay of head squashes as previously described (Kuberski and Rosen, 1977).

Viral RNA analysis

For analysis of viral RNA, the cDNA nucleotide region 2025–2325 of the DEN-1 virus coding sequence (Desprès *et al.,* 1993) was amplified from the plasmid pCI/prM-E by PCR (Desprès *et al.,* 1998). The PCR product containing DEN-1 virus sequence and plasmid pCI/prM-E was radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol) using the Nick Translation Kit (Roche Molecular Biochemicals, Mannheim, Germany). Genomic RNA extracted from purified DEN virus and total RNA from infected cells were prepared with RNA-*PLUS* reagent (Quantum Bioprobe, Montrevil, France) according to the manufacturer's instructions. RNA samples were denatured at 65°C in MOPS buffer containing 50% formamide and 30% formaldehyde. Viral RNA was analyzed by slot-blot hybridization on a Hybond-N nylon membrane (Amersham Pharmacia/Biotech, Orsay, France) according to the manufacturer's instructions and then quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Viral protein analysis

Cells were starved for 1 h in DMEM without methionine and cysteine (ICN Biomedicals, Inc., Irvine, CA) and then labeled for 1 h with 100 μCi/ml Tran^{35}S-label (ICN Biomedicals Inc.). Cells were lysed with ice-cold RIP assay buffer, and an RIP assay was performed as previously described (Desprès *et al.,* 1993, 1995, 1996). For pulse-chase analysis, cells were pulse-labeled with 500 μCi/ml Tran^{35}S-label and chased as previously described. Cells were lysed with ice-cold 1% Triton X-100 in TNI buffer, and an RIP assay was performed as previously described (Desprès *et al.,* 1995; Courageot *et al.,* 2000). Samples were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

Apoptotic cell death detection

To detect apoptotic cell death, oligonucleosomal DNA fragments in cell lysates were quantified by ELISA (Cell Death Detection ELISA^{PLUS} kit; Roche Molecular Biochemicals) as previously described (Desprès *et al.,* 1998). Optical density was measured at 405 and 492 nm as described in the manufacturer's manual.

Nucleotide sequence by RT-PCR

Monolayers of AP61 cells were used to prepare highly purified DEN-1 virus stocks from which genomic RNA was extracted. The nucleotide sequences of the structural protein genes in FGA/89 and FGA/NA d1d have been determined previously (Desprès *et al.,* 1993, 1998).

Genomic RNA was extracted from purified DEN-1 virus using a standard phenol–chloroform method. Two overlapping cDNA products were prepared from the purified RNA using Expand Reverse Transcriptase (Roche Molecular Biochemicals) as described in the manufacturer's manual. The cDNA nucleotide regions 1–5277 and 5125–10735 were amplified according the manufacturer's instructions using the Expand High Fidelity PCR system

(Roche Molecular Biochemicals). The PCR products were purified by High Pure PCR Product Purification Kit (Roche Molecular Biochemicals). Both strands of the cDNA/PCR products were sequenced using the Thermo Sequanase Kit (Amersham, Arlington Heights, IL). Sequences from the 5′ and the 3′ noncoding regions were obtained after uncapping and RNA ligation as described elsewhere (Mandl *et al.,* 1991).

Three-dimensional structures of the E and NS3 proteins

The E protein from the related TBE virus (Rey *et al.,* 1995) which shares about 40% sequence identity, was used as the reference structure. The DEN-1-sE (strain FGA/89) and TBE-sE (strain Neudorfl) sequences were aligned using the program SIM (Huang and Miller, 1991) and modified by hand so that all insertions and deletions would lie within predicted loop regions. Modeling was done with the program MODELLER (Sali and Blundell, 1993). For comparative modeling, MODELLER derives a set of spatial constraints from the reference structure or structures and then applies these restraints to the target sequence, based on the sequence alignment. The resulting structure is displayed using the program RIBBONS (Carson, 1987).

The NS3 protein from HCV (Protein Data Bank accession code 1a1v), which shares about 25% sequence identity, was used as the reference structure (Kim *et al.,* 1998). Program PSI-BLAST (Altschul *et al.,* 1997) was used to obtain an alignment of the NS3 helicase region using the available sequences of members of the *Flaviviridae* (SwissProt database). No attempt was made to model the actual sequence of DEN-1 virus onto the HCV sequence. Several stretches of conserved sequence elements scattered throughout the polypeptide (some common to nucleic acid helicases in general and others restricted to the *Flaviviridae*) are convincing landmarks to predict the position of DEN-1 NS3-435 in the NS3 helicase region of HCV (Phe_{438}).

Statistical analysis

Data for groups or samples were compared by Fisher's and Yates' t tests, and $P < 0.05$ was considered statistically significant.

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