JOURNAL OF VIROLOGY, Apr. 1997, p. 3244–3249 Vol. 71, No. 4
0022-538X/97/$04.00+0


# NOTES

## Dengue Virus Replication in Human Hepatoma Cells Activates NF-κB Which in Turn Induces Apoptotic Cell Death

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Received 3 September 1996/Accepted 10 January 1997

**The severe outcome of the dengue (DEN) virus infection known as DEN hemorrhagic fever-DEN shock syndrome (DHF-DSS) is, in some cases, accompanied by liver injury. Councilman bodies observed in liver biopsies of DHF-DSS cases may correspond to hepatocytes in apoptosis. We show here that infection of the hepatoma cell line HepG2 with DEN type 1 virus induced cell death typical of apoptosis late in the virus cycle. The transcription factor NF-κB was activated concomitantly with viral protein synthesis and thus before the appearance of apoptotic cells. Inhibition of apoptosis was observed when DEN virus-infected cells were treated with NF-κB decoys, indicating the involvement of this transcription factor in induction of cell death. Thus, infected hepatocytes appear to be subject to apoptosis in vitro, and this may be a key element in the pathophysiology of hepatic failure associated with DHF-DSS.**

Dengue (DEN) viruses (serotypes 1 to 4) are mosquito-borne flaviviruses that cause significant morbidity in tropical areas. Classic DEN fever is an acute, debilitating, although rarely fatal illness. DEN hemorrhagic fever (DHF) is a severe disease characterized by a variable degree of hemoconcentration and thrombocytopenia often leading to fatal shock, known as DEN shock syndrome (DSS) (22). The pathogenesis of DHF-DSS is not well understood. Thus, whether the pathogenesis of severe DEN disease is due mainly to viral virulence or to host susceptibility is unclear. However, the number and type of infected cells in target tissues and organs, the interaction between virus replication and cell function, and the nature of the immune responses to DEN virus infection are likely to govern the outcome of the disease.

Although monocytic cells have been considered to be the targets of the infection (22), attention has been drawn to the involvement of the liver in DHF by the detection of viral antigens in hepatocytes and Kupffer cells and by virus recovery from liver biopsies (5, 21, 48). Injury of the liver has also been revealed, as in other forms of viral hepatitis, by an increase in the transaminase level (31) and by impaired synthesis of factor XII (11). Cell degeneration in centrilobular and midzonal areas of the liver with Councilman bodies resembling those found in yellow fever cases has been described (3, 26). Such bodies have been reported as hepatocytes in an apoptotic state (29, 32). There have been few studies of the interactions between DEN virus and liver cells, which are potential targets of DEN pathogenesis in humans (1, 40). Recently, we showed that DEN type 1 (DEN-1) virus replicated in human hepatoma cell line HepG2 and that infectious particles were released into the culture medium (40). Furthermore, apoptosis has been implicated as a mechanism of cell damage in response to DEN virus replication in vitro (10). Several viruses have been shown to induce programmed cell death (apoptosis), which often contributes directly to their cytopathogenic effects in host cells (see for a review, see reference 51) (12, 28, 30, 35, 42, 51, 56). This led us to investigate whether apoptotic cell death of human hepatoma cells was triggered by DEN-1 virus infection.

The Oster human isolate of DEN-1 virus was produced in mosquito cell line AP61 and purified on sucrose gradients (40). To study the susceptibility of HepG2 cells to DEN virus, cytopathogenic effects in cells infected with this Oster strain were examined. DEN virus-infected HepG2 cell death was apparent 48 h postinfection, and mortality was maximum at 80 h as determined by trypan blue dye exclusion. By immunofluorescence assay (10), we observed that HepG2 cells replicating DEN virus displayed characteristics typical of cells undergoing apoptosis with membrane blebbing and chromatin condensation (data not shown). Apoptotic DNA degradation in DEN virus-infected HepG2 cells was assessed by a DNA fragmentation assay (39). Internucleosomal DNA cleavage in infected cells 60 h postinfection was similar to that obtained in HepG2 cells treated with the apoptosis inducer menadione (25) (Fig. 1A and B). Apoptotic DNA fragmentation was blocked in infected cells treated with the nuclease inhibitor aurintricarboxylic acid (100 μM) after 48 h of infection (data not shown). These results suggested that infection of human hepatoma cells with DEN virus induced apoptotic processes.

To determine the time course of apoptotic cell death, DEN virus-infected HepG2 cells were examined at different times postinfection by an immunofluorescence assay using anti-DEN virus immune mouse serum and the DNA intercalator propidium iodide (PI) (10). DEN virus-infected cells presented extensive condensation of chromatin 40 h postinfection. The progression of apoptotic cell death was slow: at least 40% of cells replicating virus exhibited the characteristics typical of cells undergoing apoptosis 3 days postinfection (Table 1).

To determine whether there is a causal relationship between DEN virus replication and apoptosis, HepG2 cells were in-

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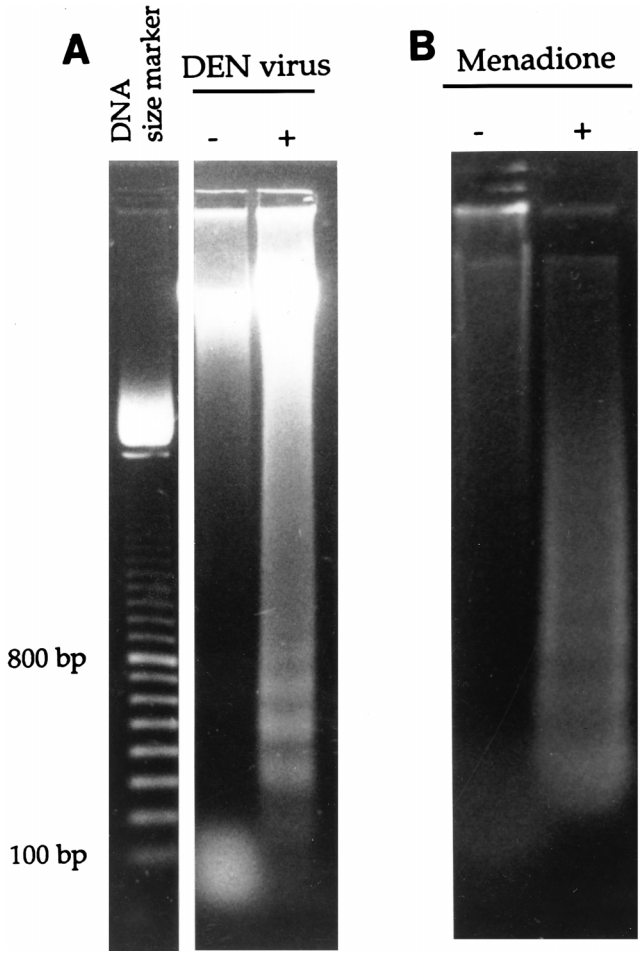



FIG. 1. Apoptotic nuclear DNA degradation in human hepatoma HepG2 cells. (A) HepG2 cells were infected with DEN-1 virus (+) or mock infected (−) for 60 h. (B) HepG2 cells were treated with 2 μM menadione (+) or mock treated (−) for 5 h. Soluble DNA was extracted from HepG2 cell lysates and separated on a 1% agarose gel in Tris-borate-EDTA buffer. The sizes of DNA markers are indicated.

fected at various multiplicities of infection and apoptotic DNA degradation was monitored by a modified terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) technique based on the detection of radiolabeled DNA breaks. Briefly, DEN virus-infected HepG2 cells were fixed 48 h postinfection with paraformaldehyde and the TUNEL assay was performed essentially as previously described (13), except that DNAs end labeled with biotin-dUTP were incubated with $^{125}$I-labeled streptavidin (Amersham). The $^{125}$I-radiolabeled DNA breaks were counted in a β-imager 2000 (Biospace; Paris, France) (6). The amount of radioactivity incorporated into the nuclei increased in a virus dose-dependent manner (Fig. 2). The rate of specific radioactivity incorporation into DNA breaks correlated with the number of apoptotic nuclei in infected cell monolayers as determined by PI staining (Fig. 2).

We sought to identify the mechanisms by which DEN virus infection induces apoptotic cell death in human hepatoma cells. The pathways leading to apoptosis are very varied (20) and depend on the cell type. Because apoptosis is an active process often requiring protein synthesis, transcriptional control of specific "death genes" probably plays a crucial regulatory role (50). To test transcription factor DNA binding activity in DEN virus-infected HepG2 cells, total cell lysates were prepared at various times postinfection and DNA-protein complexes were detected by an electrophoretic mobility shift assay (EMSA) using different consensus binding sites as probes (10, 45). EMSA with consensus binding sites for transcription factors CREB, AP-1, and SRE as probes did not reveal any activation of these factors in DEN virus-infected HepG2 cells (data not shown). We observed activation of transcription factor NF-κB in DEN virus-infected HepG2 cells. The DNA binding activity of NF-κB was first observed 24 h postinfection in infected cells and was higher at 32 h (Fig. 3). Thus, activation of NF-κB preceded apoptosis in HepG2 cells replicating DEN virus.

NF-κB is a crucial transcription factor present in an inactive, cytoplasmic form in almost all cell types (52). Upon stimulation, the NF-κB dimer is released from its inhibitory subunit I-κB and translocated to the nucleus (55). NF-κB is involved in a number of different cellular processes, such as immune cell activation, B- and T-cell development, multiple stress response, and the control of some apoptotic processes (2, 19, 36, 53). Various viruses have been shown to activate NF-κB. They include cytomegalovirus (7), human immunodeficiency virus type 1 (9, 14, 15), human T-lymphotropic virus type 1 (24, 37, 43), hepatitis B virus (8), Epstein-Barr virus (23), influenza virus (45), and Sindbis virus (36). However, only the activation of NF-κB by Sindbis virus is associated with apoptosis (36). Furthermore, NF-κB seems to play an important role in hepatic processes since it has been shown that hepatocyte gene regulation may be modulated by a regulatory pathway including NF-κB activation in the acute-phase response to hepatic injury (16).

To identify the NF-κB subunits detected by EMSA in DEN virus-infected HepG2 cells, total cell extracts were incubated with subunit-specific antibodies and subjected to EMSA supershift analysis. The activated complex in infected cells was supershifted by anti-p50 and anti-p65 sera but not by anti-c-Rel serum. Furthermore, a mixture of anti-p50 and anti-p65 sera completely supershifted the complex, indicating that the major complex activated in infected cells included a p50-p65 heterodimer (Fig. 4).

Double-stranded phosphorothioated oligodeoxynucleotides containing NF-κB binding sites are able to inhibit competitively binding of NF-κB to native DNA binding sites (18). These modified NF-κB oligonucleotides used as transcription factor decoys (TFDs) have been shown to prevent apoptotic cell death triggered by Sindbis virus infection (36). To assess the role of NF-κB in DEN virus-induced apoptosis, TFDs were added to culture media of HepG2 cells immediately after DEN virus infection and after 20 h of infection. A TFD concentration as high as 30 μM had no effect on the viability of mock-

TABLE 1. Percentages of DEN virus-infected HepG2 cells in apoptotic state at various times postinfection

| H postinfection[a] | Avg % of cells in apoptotic state[b] ± SD |
|---|---|
| 24 | 3 ± 0.5 |
| 40 | 7 ± 1 |
| 48 | 14 ± 2.5 |
| 64 | 35 ± 4 |
| 72 | 40 ± 5 |

[a] HepG2 cells were infected with DEN virus at a multiplicity of infection of 100, and at least 80% of the cells were infected within 1 day as determined by immunofluorescence assay.

[b] The percentage of apoptotic cells was determined by immunofluorescence assay and PI staining.

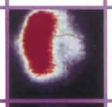
| | Radioactive TUNEL | | Immunofluorescence |
|---|---|---|---|
| multiplicity of infection | ß-Im. | Emission [ x million counts (± SD)] | Cells in Apoptotic State (%) |
| 500 FFU/cell |  | 1.00 ± 0.25 | 35 |
| 250 FFU/cell |  | 0.60 ± 0.15 | 20 |
| 50 FFU/cell |  | 0.25 ± 0.15 | 10 |
| No virus |  | < 0.05 | 5 |

FIG. 2. Detection of apoptotic DNA degradation in HepG2 cells by a radioactive TUNEL assay and PI staining. The radioactive TUNEL assay was performed essentially as previously described (10). Incorporated biotin-dUTP in fragmented DNA was detected by using 25 μCi of $^{125}$I-labeled streptavidin per ml. Emission from the radioactive cell monolayer in each microchamber was visualized and counted in a β-imager (β-Im). The same samples were also subjected to DNA staining with PI, and the nuclei in the apoptotic state were counted. FFU, focus-forming units.

infected HepG2 cells and on viral replication as assessed by the percentage of infected cells and by titration of infectious virus (data not shown). The proportion of TFD-treated HepG2 cells undergoing apoptotic DNA degradation was evaluated 48 h postinfection. TFD treatment at a concentration as low as 1 μM completely blocked the apoptotic process in DEN virus-infected cells as measured by β-imager detection (Table 2). At a lower concentration (500 nM), the percentage of infected HepG2 cells in the apoptotic state was reduced to 15% (Table 2). Treatment of DEN virus-infected HepG2 cells by NF-κB TFDs delayed the apoptotic process by about 24 h (data not shown). The ability of NF-κB TFDs to inhibit NF-κB DNA binding activity in DEN virus-infected HepG2 cells was verified in total cell extracts by EMSA. The relative amounts of

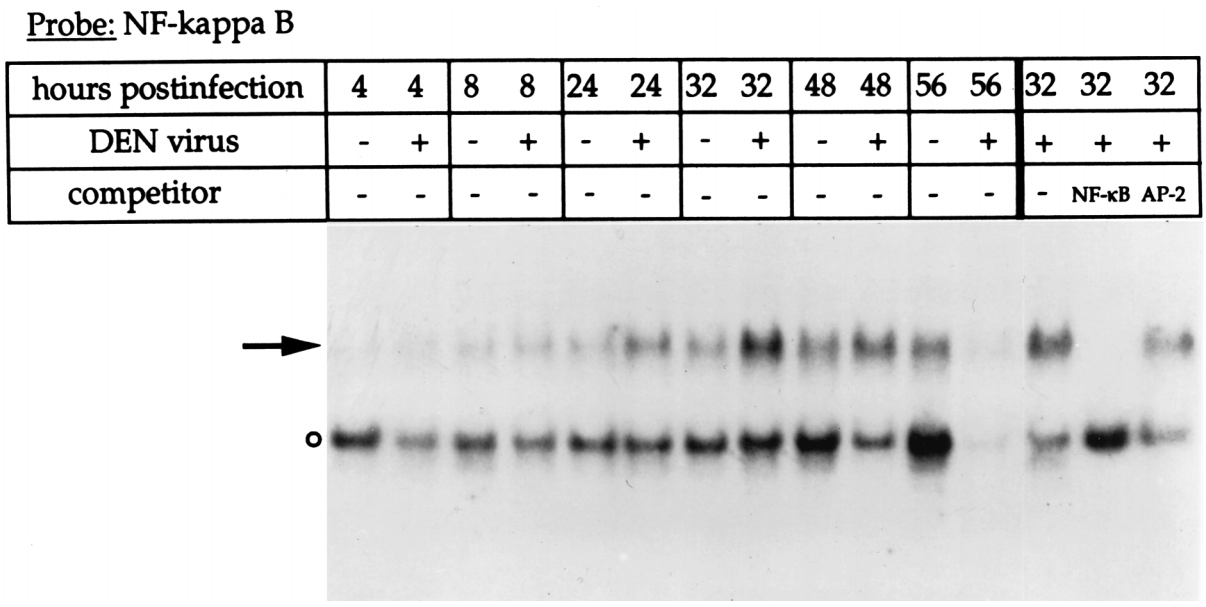



FIG. 3. Activation of NF-κB in DEN virus-infected HepG2 cells. HepG2 cells were infected with DEN virus at a multiplicity of infection of 100 or mock infected. Cells were lysed in a high-salt detergent buffer (35). EMSAs were performed with total extract containing 10 μg of protein by using a $^{32}$P-labeled oligonucleotide with a consensus κB sequence (5′-AGTTGAGGGGACTTTCCCAGGC-3′). The specificity of the retarded complexes was assessed by preincubating an extract with a 50-fold excess of unlabeled NF-κB or an unrelated AP-2 probe. The slower-migrating band indicated by the arrow disappeared completely when competitor unlabeled NF-κB was included, indicating that this complex is specific for the NF-κB probe. The faster-migrating band indicated by the circle is not specific for the NF-κB probe.

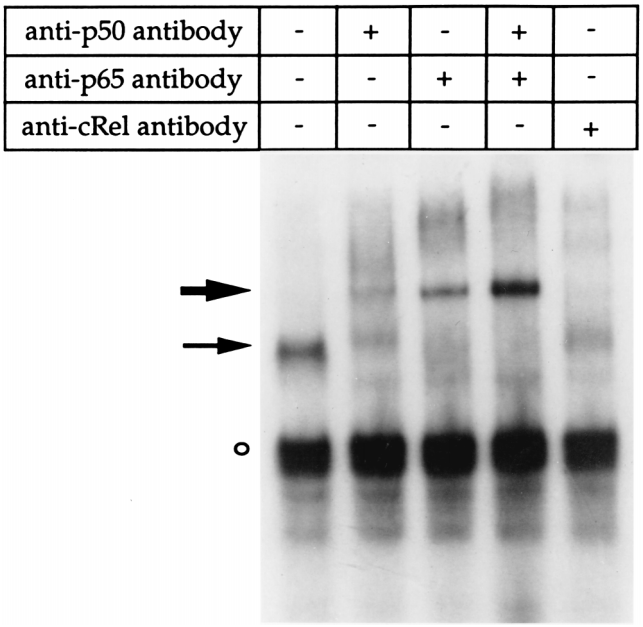



FIG. 4. Identification of the major NF-κB complex induced by DEN virus infection in HepG2 cells. Anti-p50, anti-p65, or anti-c-Rel antibodies were added at a dilution of 1/40 to the total binding mixture described in the legend to Fig. 3. The small arrow points to the NF-κB complex. The large arrow shows the complex supershifted by antibody binding. The circle indicates the nonspecific complex.

TABLE 2. Effects of TFD treatment on apoptosis in DEN virus-infected HepG2 cells at 48 h postinfection[a]

| TFD concn (nM) | Radioactive TUNEL result | | | % of cells in apoptotic state[b] |
|---|---|---|---|---|
| | No. of untreated cells | No. of treated cells | Treated/untreated cell ratio | |
| 0 | 27,371 | 578,129 | 21.1 | 35 |
| 125 | 30,162 | 602,252 | 19.9 | 35 |
| 500 | 20,402 | 146,847 | 7.2 | 15 |
| 1,000 | 17,514 | 17,632 | 1 | 0 |

[a] HepG2 cells grown in a microchamber and infected with DEN virus at a multiplicity of infection of 100 were treated with TFD or mock treated and subjected to a radioactive TUNEL assay and PI staining as described in the legend to Fig. 2.
[b] Determined by immunofluorescence assay.

DNA–NF-κB complexes were measured with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). NF-κB DNA binding activity was lower in infected or mock-infected cells treated with TFDs (Fig. 5). TFD treatment of DEN virus-infected cells reduced the DNA binding activity of NF-κB threefold at 32 h postinfection and fivefold at 48 h. However, we cannot exclude the possibility that NF-κB binding to a radiolabeled oligonucleotide may be reduced due to the presence of residual unlabeled TFDs copurified with the total cell extract. These experiments suggest that NF-κB activation is necessary to induce apoptosis in infected HepG2 cells and that apoptosis can be diminished in a dose-dependent manner and delayed by TFDs.

NF-κB activating stimuli generally seem to use oxidative stress as a common signal transduction pathway to elicit their response (41, 49). Oxidative stress can result from the gener-

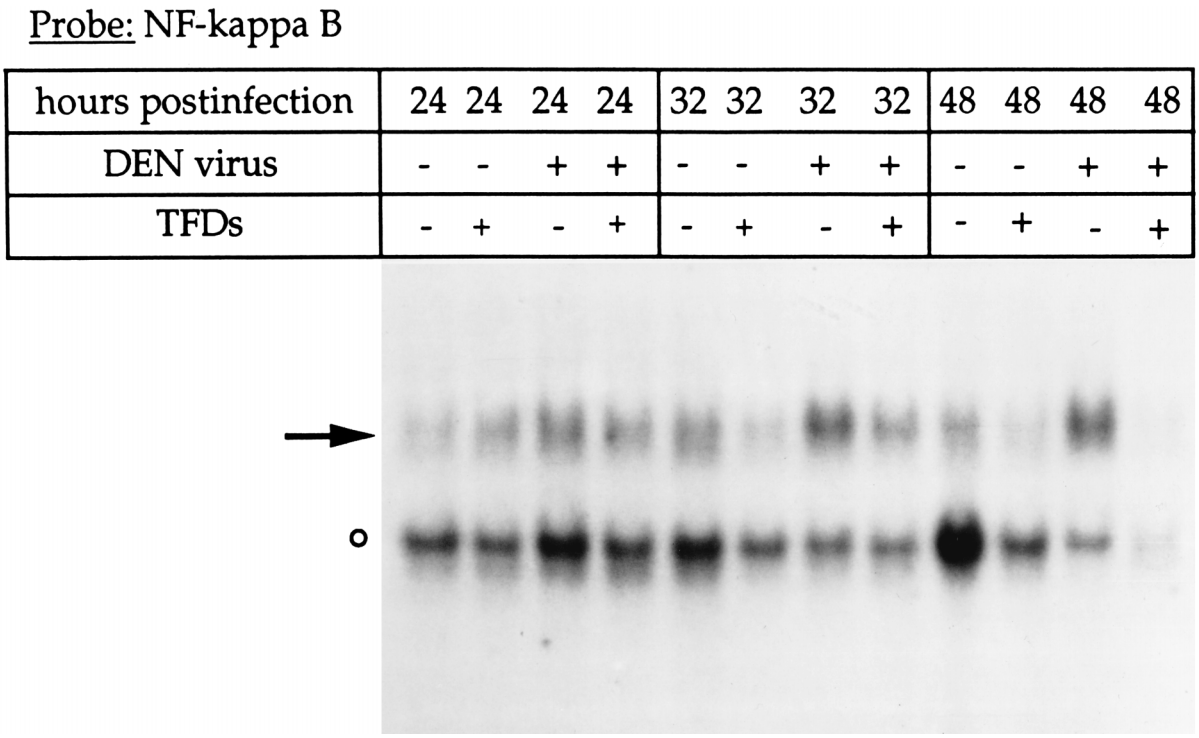



FIG. 5. Analysis of the inhibition of NF-κB binding activity by double-stranded phosphorothioate competitors used as TFDs. TFDs contain three copies of the consensus κB sequence and were prepared as previously described (28). TFDs were added to cell medium to a concentration of 1 μM immediately and 20 h after DEN virus infection. Total cell lysates were prepared at various times postinfection and subjected to EMSA as described in the legend to Fig. 3. The arrow and circle indicate the NF-κB complex and the nonspecific complex, respectively.

ation of reactive oxygen species or from the loss of antioxidant defenses (4). Apoptotic cell death triggered by menadione, a redox-cycling quinone that generates reactive oxygen species in the presence of oxygen (54), was associated with NF-κB activation in HepG2 cells. This indicates that human hepatoma cells are very susceptible to oxidative stress (data not shown). Virus infection may also cause increased production of oxygen radicals which, in turn, act as apoptotic stimuli in the host cells (34, 36, 47, 51). Intracellular synthesis of viral proteins seems to be required to activate apoptotic pathways in the host cells (33, 38, 44, 57). It has been suggested that NF-κB is activated by stress from ER due to protein accumulation in intracellular membranes (46). In mouse neuroblastoma cells, apoptotic cell death triggered by DEN virus infection may be linked to the endoplasmic reticulum stress due to viral protein accumulation (10). To determine whether the time course of DEN protein synthesis was similar to that of NF-κB activation, infected HepG2 cells were pulse-labeled at various times postinfection and viral proteins were analyzed by a radioimmunoprecipitation assay. DEN proteins were detected 20 h after infection and peaked at 30 h (data not shown). Thus, NF-κB was activated when DEN proteins began to accumulate in HepG2 cells. We are therefore now analyzing whether accumulation of DEN proteins in human hepatoma cells generates oxidative stress which may activate NF-κB. However, other mechanisms may be responsible for activating NF-κB and should be further investigated.

It remains to be established which apoptotic genes under the control of NF-κB are responsible for apoptosis of HepG2 cells replicating DEN virus. It has been demonstrated that p53 and c-Myc play an important role in the apoptosis signalling pathway in HepG2 cells induced by divergent stimuli (27) and could also be implicated in the apoptotic process triggered by DEN virus.

This report shows that replication of DEN virus in human hepatoma cells led to activation of the transcription factor NF-κB which, in turn, induced apoptotic cell death. To our knowledge, this is the first report suggesting that replication of a member of the *Flaviviridae* family in a human hepatoma cell line may activate NF-κB-dependent apoptotic pathways. The finding is important because apoptotic cell death is considered to be a major feature of viral hepatitis (17).

We are grateful to Rajiv Ratan (The Johns Hopkins University School of Medicine, Baltimore, Md.) for helpful suggestions. We thank M.-T. Drouet for technical expertise in cell culture and virus purification. We also thank Sylvie Memet (Institut Pasteur, Paris, France) for technical advice and Benoit Viollet (Institut Cochin de Génétique Moléculaire, Paris, France) for helpful discussions and constructive comments.

P.M. is supported by a grant from the Centre National de la Recherche Scientifique. This work was supported by grants from INSERM (93/BCR/683) and DRET (95.1378).

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