TABLE I. Base Line and Clinical Features of Chronic Liver Disease Patients in Pakistan

Features	Total (n = 189)	Non-HCC (n = 107)	HCC (n = 82)	*P*-value
Age 'Yrs' (Mean ± SD)	47.6 ± 13.0	41.3 ± 11.5	55.8 ± 9.9	<0.0001
Sex (M/F)	125/64	62/45	63/19	<0.001
ALT, U/L median (range)	55.5 (12–1,030)	60 (15–769)	53 (12–1,030)	NS
ALP, U/L median (range)	141 (32–643)	122 (32–472)	145 (32–643)	<0.0005
T. Bil., mg/dl median (range)	1.9 (0.3–66.5)	1.4 (0.3–33.6)	2.17 (0.5–66.5)	NS
Anti-HCV positive	158/189 (83.6)	93/107 (87.0)	65/82 (79.2)	NS
HCV RNA positive	129/158 (81.6)	71/93 (76.3)	58/65 (89.2)	NS
HCV RNA, log copies/ml (Mean ± SD)	6.5 ± 1.7	4.7 ± 1.1	6.8 ± 1.8	NS
Anti-HBc positive	146/189 (77.2)	85/107 (79.4)	61/82 (74.3)	NS
Anti-HBs positive	72/189 (38)	45/107 (42)	27/82 (32.9)	NS
HBsAg positive	23/189 (12.1)	12/107 (11.2)	11/82 (13.4)	NS
HBV DNA positive	16/23 (69.5)	7/12 (58.3)	9/11 (81.8)	NS
Anti-HDV positive	7/23 (30.4)	6/12 (50)	1/11 (9)	NS
HDV RNA positive	3/7 (42.8)	3/6 (50)	0	ND
Patterns of mono or coinfection				
HCV only	153/189 (80.9)	91/107 (85)	62/82 (75.6)	NS
HBV only	13/189 (6.9)	5/107 (4.7)	8/82 (9.7)	NS
HCV + HBV	3/189 (1.6)	1/107 (0.9)	2/82 (2.4)	NS
HBV + HDV	5/189 (2.6)	5/107 (4.7)	0	NS
HCV + HBV + HDV	2/189 (1)	1/107 (0.9)	1/82 (1.2)	NS

the HCC or non-HCC group was positive by serological screening for HIV. Based on the serological findings, patients were categorized into five groups, HCV only (80.9%), HBV only (6.9%), HCV/HBV (1.6%), HBV/HDV (2.6%), and HCV/HBV/HDV (1%). None of these groups reached statistical significance when compared for the severity of clinical disease (Table I).

The clinical features among patients with chronic liver disease infected with HCV were also compared by first dividing these patients into three categories based on the severity of disease, that is, chronic hepatitis, liver cirrhosis and HCC (Table II). It was found that the mean age of the patients was significantly higher in patients with liver cirrhosis and patients with HCC as compared to chronic hepatitis ($P < 0.001$). Patients with HCC were also of significantly older in age than patients with liver cirrhosis ($P < 0.001$). Male gender was predominant in HCC as compared to chronic hepatitis ($P < 0.01$) but the difference did not reach statistical significance between HCC and cirrhosis. Serum AFP was respectively higher in chronic hepatitis and HCC, that is, $P < 0.02$ and <0.001 as compared to cirrhosis (Table II). High mean viral titer was observed for chronic hepatitis and patients with HCC compared to cirrhosis ($P < 0.0001$). The prevalence of anti-HBc was high among all three groups with no statistically significant difference among them (Table II).

HCV Genotypes

HCV genotyping was carried in both structural and non-structural parts of HCV genome by two independent methods, using type-specific primers of the core, the NS5B region and phylogenetic analysis based on the nucleotide sequences in NS5B or E1 region. No discrepancy was observed between the results obtained by each method, however 10.8% cases were unclassified by the NS5B region (Table III). Overall, HCV-3a was the predominant genotype (81.4%) in Pakistan, followed by 3b (9.3%), 3k (2.3%), 4a (2.3%), 1a (1.5%), 1c (1.5%), 1b (0.8%), and 2a (0.8%). All potential forms of recombination were also excluded by matching the results of genotyping based on both structural and non-structural genomic parts (Table III). Genotype 3a was the most

TABLE II. Comparison of Base Line and Clinical Features Among Chronic Liver Disease Patients Infected With HCV

Features	Total (n = 158)	Chronic hepatitis[a] (n = 67)	Liver cirrhosis[b] (n = 26)	HCC[c] (n = 65)	*P*-value
Age 'Yrs' (Mean ± SD)	47.3 ± 12.5	38.9 ± 9.7	48.3 ± 11.8	55.9 ± 9	<0.001[a,b,c]
Sex (M/F)	102/56	35/32	16/10	51/14	<0.01[c]
ALT, U/L median (range)	60 (15–769)	67 (15–200)	40 (18–769)	60.5 (22–548)	NS
ALP, U/L median (range)	148 (44–643)	126 (32-472)	112.5 (44–234)	184 (68–643)	<0.01[c]; <0.02[a]
T. Bil., mg/dl median (range)	2 (0.3–16.8)	1 (0.3–4.4)	1.8 (0.5–8.4)	2.1 (0.5–16.8)	NS
HCV RNA positive	128/158 (81)	46/67 (68.6)	24/26 (92.3)	58/65 (89.2)	NS
HCV RNA, LOG copies/ml (Mean ± SD)	6.5 ± 1.7	7.3 ± 1.2	4.7 ± 1.1	6.8 ± 1.8	<0.0001[a,b]
Anti-HBc positive	118/158 (74.7)	53/67 (79.1)	19/26 (73)	46/65 (70.7)	NS

[a]Chronic hepatitis versus liver cirrhosis.
[b]Liver cirrhosis versus HCC.
[c]Chronic hepatitis versus HCC.

TABLE III. HCV Genotypes as Determined by the NS5B, E1, and 5′UTR Regions

Classification based on the Core and/or E1 genes	Classification based on the NS5B gene							
	3a	3b	3k	4a	1a	1c	ND	n
3a	97						8	105
3b		9					3	12
3k			3					3
4a				3				3
1a					1		1	2
1c						2		2
1b							1	1
2a							1	1
n	97	9	3	3	1	2	14	129

ND, not determinable.

prevalent in HCC 44/58 (75.8%) as well as in patients without HCC 61/71 (85.9%) (Table IV).

Phylogeny and Historical Analysis of HCV Population by Coalescent Theory

All sequences generated in this study (the NS5B region nucleotide from 8,278 to 8,618) were subjected to phylogenetic analysis together with all published sequences retrieved from DDBJ/EMBL/GenBank data base. The majority of the Pakistan strains belonged to genotype 3, forming a distinct phylogenetic cluster of 33 sequences within HCV-3a (Fig. 1). This cluster was subjected further to maximum-likelihood-based phylogenetic analysis with enforced molecular clock as described previously [Pybus et al., 2001; Tanaka et al., 2002]. Figure 1 shows the phylogenies of the HCV strains obtained in this study along with closely related sequences and representatives of other genotypes.

The epidemic history of the population sampled showing the effective number of HCV infections through time is shown in Figure 2. The estimates represent the epidemic history from the time of divergence of the viruses sampled, year 1920 to the time of sampling year, 2006–2007. The estimates of the effective number of HCV infections showed the appearance of HCV genotype 3a in this region around the 1920s, and a rapid exponential growth in the 1950s (Fig. 2). Genetic distances were also estimated among Pakistan HCV-3a strains for all synonymous and non-synonymous nucleotide positions and compared to the data obtained for other countries, as reported previously [Tanaka et al., 2006]. It was found that the genetic distance among Pakistan sequences lay between the ones from Egypt and Spain, but was comparatively smaller than that in Japan. The data indicates that HCV-3a population in Pakistan is younger than the Japanese HCV-1b population, but older in age than 1b in Spain, 5a in South Africa, 1a in USA, 3a in Russia, and the 6a population in Hong Kong. Therefore, a high rate of HCC due to HCV may be expected in Pakistan during the coming decades.

TABLE IV. HCV Genotypes Stratified With Clinical Groups

Genotypes	Non-HCC (n = 71)	HCC (n = 58)	*P*-value
3a	61 (85.9)	44 (75.8)	NS
3b	5 (7)	7 (12)	NS
3k	1 (1.4)	2 (3.4)	NS
4a	2 (2.8)	1 (1.7)	NS
1c	1 (1.4)	1 (1.7)	NS
1a	1 (1.4)	1 (1.7)	NS
1b	0	1 (1.7)	NS
2a	0	1 (1.7)	NS

Numbers in brackets represent percentage.

DISCUSSION

The present study was carried out on 189 patients with chronic liver disease, where 83.6% of patients were found to be infected with HCV and 12.1% with HBV. Infection with HCV was found in 79% of the patients with HCC, while only 13.4% of these were infected with HBV. Anti-HBc was the leading seropositive marker in the cohort studied (77.2%), although it did not present statistically significant differences between HCC and non-HCC. These results are in accordance with the most recent studies in Pakistan showing relatively high prevalence of anti-HCV and low prevalence of HBsAg among chronic liver disease patients [Butt et al., 1998; Khokhar, 2002; Khokhar et al., 2003; Raza et al., 2007]. A decreasing trend in hospital admissions due to HBV infection and increasing trend due to HCV infection has also been reported in Pakistan [Hamid et al., 1999].

While most of the studies in Pakistan suggest a high prevalence of HCV genotype 3 (3a), some studies also show genotypic mixtures or isolates that were untypeable [Afridi et al., 2008; Idrees and Riazuddin, 2008]. These studies may have been limited by the use of less informative or less specific genotyping assays. Direct sequencing is the most accurate method for HCV-genotyping, but again the genotyping of the 5′UTR is less informative, since sequence variation between genotype and/or subtypes is greatest in NS5, less in the envelope and the core, and least in the 5′UTR [Cook et al., 2006]. HCV-3a was found as the predominant genotype (81.4%) in the cohort studied, followed by 3b (9.3%), 3k (2.3%), 4a (2.3%), 1a (1.5%), 1c (1.5%), 1b (0.8%), and 2a (0.8%). There were no cases that were untypeable or that had genotype mixtures.

Chronic HCV infection with genotype 1b is reportedly associated with a more severe liver disease and a more aggressive course than in infection with other genotypes [Silini et al., 1995; Zein et al., 1996b]. However, there are some reports which refute this association [Lau et al., 1996; Benvegnu et al., 1997], suggesting that the length of the course of the disease may be associated with the increasing likelihood of the development of HCC [Zein and Persing, 1996; Zein et al., 1996a; Zein, 2000; Tanaka et al., 2006]. Although 76% of HCC cases were infected with HCV-3a, due to very few patients with non-3a genotypes, the role of genotypes in the development of

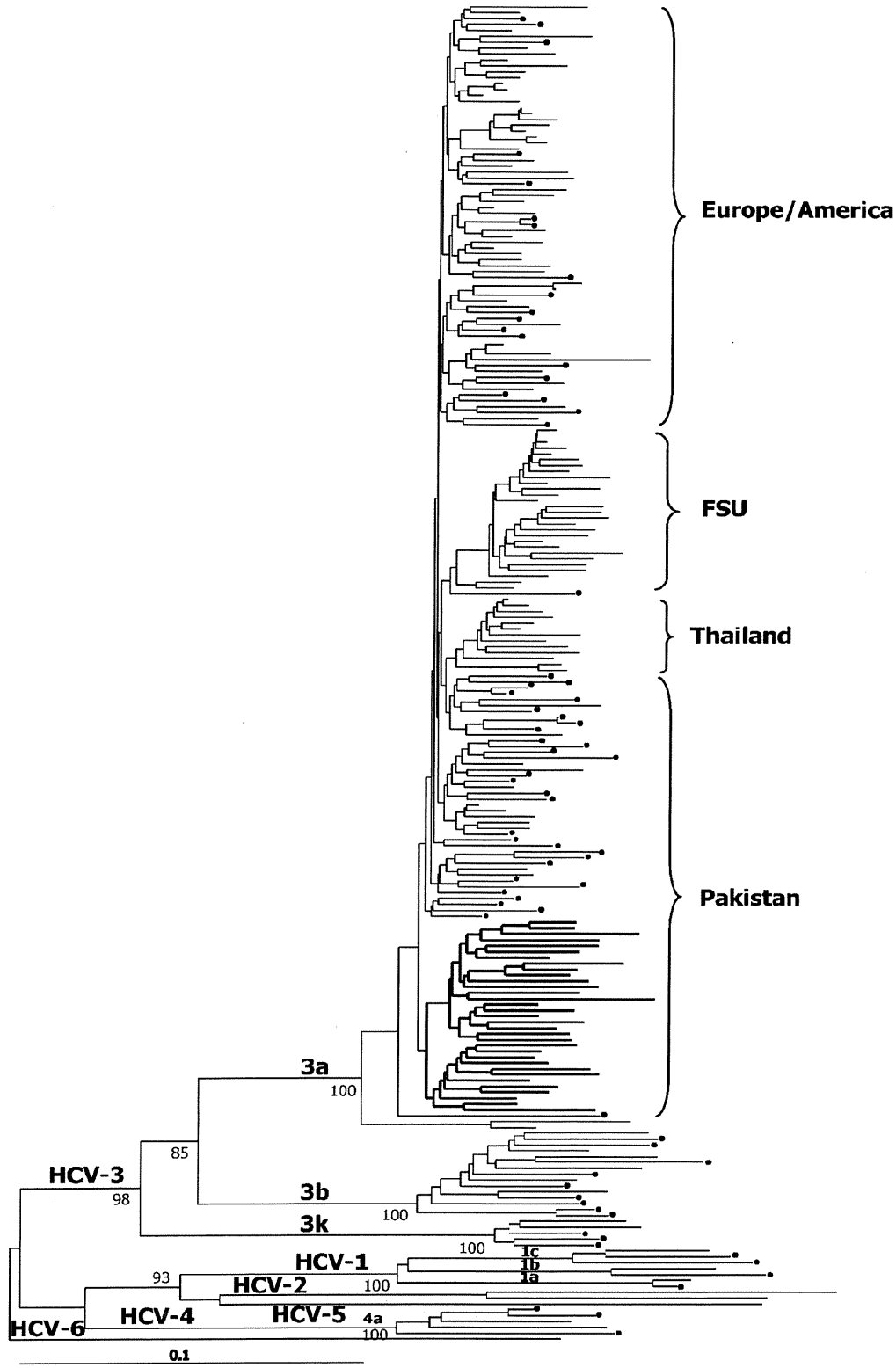

Fig. 1. Phylogenetic tree constructed in NS5B region that includes * marked Pakistan isolates and the reference sequences retrieved from databases (DDBJ/EMBL/Gene Bank). The numbers in the tree indicates bootstrap reliability by the interior branch test. Exceptional strains are indicated according to their area of origin. Significant cluster obtained for Pakistan was subjected to population history analyses.

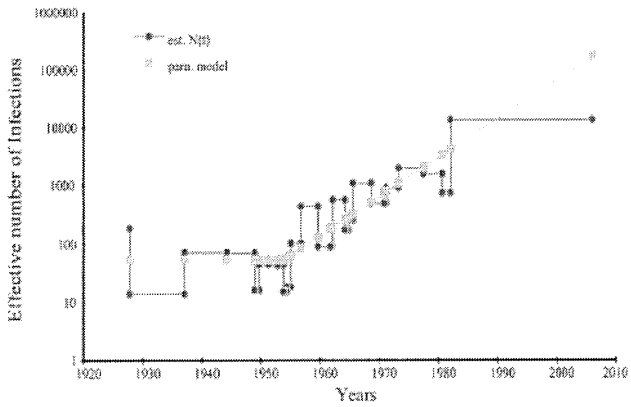

Fig. 2. The maximum-likelihood estimates of nonparametric functions of the effective number of infections with HCV-3a in Pakistan. The parametric model is indicated by the gray line, and stepwise plots are indicated by the black line that represents corresponding nonparametric estimates (number as a function of time). Genetic distances are transformed into a time scale of years using estimates of the molecular clock in the NS5B region. (Nt); effective population size at time t in the past.

HCC cannot be inferred. However the variation in outcome within the HCV-3a population may be because of the duration of the infection, that is, the older patients might have been infected with this genotype for a longer time developed HCC. Since patients with HCC were significantly older than the patients without HCC ($P < 0.0001$), this indicates that the course of infection is an important factor in the development of HCC.

A distinct Pakistan specific phylogenetic cluster of HCV-3a was found in this study. As the past population dynamics of a virus can be inferred from viral gene sequences using the coalescent theory approach [Tanaka et al., 2002, 2006; Pybus et al., 2003, 2005] this model was used to investigate the population history of HCV genotype 3a in Pakistan. The period of epidemic growth of this subtype in the region appears to be the 1920s and 1930s, as was also estimated by Pybus et al. [2005]. As the 1940s approached, an increase in the estimated effective population size of this subtype was observed, which may be related to the population movement as a result of the partition of the Indian Subcontinent. A period of estimated exponential growth of HCV infections during the 1950s coincides with the phase when Pakistan was establishing its health care system and launched the first 5-year health care action plan [EMRO, 2007]. The new health care system, with poorly trained medical practitioners, therapeutic injections, reuse of syringes, contaminated surgical and dialysis equipment, and other percutaneous procedures may have been the cause for this exponential growth [Sharma, 2000; Hamid et al., 2004; Raja and Janjua, 2008]. Transmission surged during the period of universal small pox vaccination 1964–1982 [Aslam et al., 2005].

The recreational use of drugs has existed throughout human history. Recreational use of opium was once common in Asia, and from there it spread to the West. The region being wedged between the Golden Crescent, the name given to the opium producing regions of Afghanistan, Iran, and Pakistan and the Golden Triangle, the name given to the opium producing regions of Myanmar (Burma), Laos, Vietnam, and Thailand, serves as a transit point for opiates from Asia to worldwide [Buxton, 2006]. Its use peaked in the nineteenth century, when the opium trade was legalized in the Indian Subcontinent (http://psychology.wikia.com/wiki/Recreational_drug_use) with an increase of the intravenous drug user population in the Indian Subcontinent. This may be the time when HCV-3a originated. Since wars proved to nurture drug production and trade, the political conflicts in Afghanistan from 1978-onwards accounted for the large opium influx into Pakistan. The large opium influx and the increase in intravenous drug population led to the transmission of the virus to the new local populations. Drug traffic and migration of drug addicts and intravenous drug users to industrialized nations led to subsequent transmission of this genotype worldwide [Pybus et al., 2005]. Until 1990s, when Pakistan introduced health-related information programs, the HCV transmission had already reached a stage, whose affects are now appearing in Pakistan with the surge of cases of severe liver complications and high rate of HCV associated HCC.

Previous estimates of the epidemic history of HCV have also indicated exponential growth periods for subtypes 1a and 1b globally, subtypes 1a and 3a in intravenous drug users, and subtype 4a in Egypt [Pybus et al., 2001, 2003, 2005; Tanaka et al., 2004a, 2006]. Taking the results of this study into account, it is reasonable to assume that there has been a common ancestral HCV genotype 3a population in the Indian Subcontinent and that the virus has spread from this pool to other parts of the world through population movements. Political conflicts, drug trafficking, and laborers traveling from Asia to other continents led to the dissemination of HCV-3a worldwide. It can be estimated that, since the HCV-3a population is older in Pakistan than in other countries, an increased mortality rate due to HCC may be expected in the future.

Investigation of the different circulating genotypes and their evolution is not only crucial for epidemiological and clinical analysis but might be helpful for the improvement of diagnostic tests and treatment regimens. The clustering of HCV strains permit tracing the transmission of such a genotype in a region. HCV appears to be a major etiological agent for chronic liver disease and HCC in Pakistan. The epidemic spread of HCV-3a having occurred earlier in Pakistan than in other countries is related to the increasing incidence of HCC.

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Genetic Variability of Hepatitis C Virus in South Egypt and Its Possible Clinical Implication

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Egypt is one of the countries with very high rates of hepatitis C virus (HCV) related morbidity and mortality. However, little is known about geographical and clinical differences in genetic variability of HCV in Egypt. Using direct sequencing and phylogenetic analysis of partial core/E1 and NS5B regions of the HCV genome, HCV genotype/subtype was determined in 129 HCV-infected patients residing in three governates in south Egypt: Assuit, Sohag, and Qena. According to clinical stage of infection, patients were categorized into four groups: asymptomatic carriers, n = 16; chronic hepatitis C patients, n = 36; liver cirrhosis, n = 54; and hepatocellular carcinoma (HCC), n = 23. Genotype 4a was detected in 80.6%, whereas 1g, 4l, 4n, 4o, 4f, and 4m were identified in 7.7%, 4.7%, 3.9%, 1.6%, 0.8%, and 0.8% of cases, respectively. The prevalence of 4a differed regionally; from 88.5% (in Sohag) to 64% (in Assuit, $P = 0.002$). Genotypes 4l and 4n had a higher prevalence in Assuit (12.8%, 10.3%) than Sohag (0%, 0%; $P \leq 0.011$). Difference in clinical features of determined genotypes/subtypes was observed; more carriers of non-4a variants (4l and 4n, 4f, or 4m) had chronic hepatitis compared to carriers of 4a (53.3% vs. 23.1%, $P = 0.025$), while more patients with 4a had liver cirrhosis (45.2% vs. 13.3%, $P = 0.023$). Two HCV-4o strains were isolated in this study, both from patients with HCC. In conclusion, geographical diversity of HCV was revealed in this study in southern Egypt. A further case–control study is required to confirm the trends of differential pathogenicity of HCV subtypes, indicated by this study. ***J. Med. Virol. 81:1015–1023, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: HCV; Egypt; hepatocellular carcinoma; genotype 4o; epidemiology

INTRODUCTION

Hepatitis C virus (HCV) is a positive single stranded enveloped RNA virus. The HCV genome consists of >9,500 bp [Choo et al., 1991]. Infection with hepatitis C is associated closely with chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [Saito et al., 1990; Purcell, 1997].

Based upon phylogenetic analysis of genomic regions and the complete genome of HCV, six genotypes (HCV genotype types 1–6) have been described and subclassified into numerous subtypes (e.g., HCV subtype 1a, 1b) [Simmonds et al., 2005]. Molecular epidemiological studies have indicated a geographical restriction for some HCV genotypes (e.g., genotype 4 to the Middle East, genotype 5a to South Africa, and genotype 6 to Southeast Asia) [Simmonds et al., 1993] while others are distributed globally, for example, 1a, 1b, 2a, 3a [Smith

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et al., 1997]. HCV genotype is an important predictor of response to interferon-alpha and ribavirin combination therapy [Hnatyszyn, 2005]. The route of transmission and serological reactivity are other factors which may differ among different HCV genotypes [Dhaliwal et al., 1996]. However, it is unclear, whether or not HCV genotypes differ in their pathogenicity [Zein, 2000].

One hundred seventy million people worldwide estimated to be infected with HCV are at a risk of developing progressive liver disease and death due to infection with HCV [WHO, 1999]. According to different reports, the prevalence of HCV in Egypt ranges from 11% to 14% of the general population, with 5–7 million people with active infection (have detectable HCV RNA) [Abdel-Wahab et al., 1994; Arthur et al., 1997; Abdel-Aziz et al., 2000]. By these estimates, Egypt is considered to be the country with the highest prevalence rate of HCV in the world with a majority of genotype 4 [Frank et al., 2000].

Few data are available regarding the molecular epidemiology of HCV in south Egypt. The aim of this study was to extend the mid-core and NS5B database for HCV strains isolated from HCV-infected patients residing in south Egypt with respect to the clinical and virological characteristics of the isolates examined.

PATIENTS AND METHODS

Patients

Serum samples were collected from August to October 2007 from 151 consecutive chronic liver disease patients seen in affiliated hospitals of Sohag University Hospital and South Egypt Cancer Institute. Among the 151 patients with hepatitis, 30 were assigned into asymptomatic carrier group, 86 into a liver cirrhosis group, and 35 into an HCC group. Anti-HCV was detected in a total of 126/151 (83.4%); 25/30 (83.3%) in the asymptomatic carrier group, 68/86 (79.1%) in the liver cirrhosis group, and 33/35 (94.3%) in the HCC group. In addition to the specimens collected consecutively, sera were collected also from 41 patients with diagnosed chronic hepatitis C. The clinical classification of the patients infected with HCV was based upon (1) measurement of serum alanine aminotransferase (ALT), (2) ultrasound examination, and (3) detection of a serological tumor marker (alpha fetoprotein for the diagnosis of HCC). The asymptomatic carrier group include patients with anti-HCV with normal liver enzymes for more than 6 months with minimal or no symptoms. Patients with liver cirrhosis were diagnosed clinically by the presence of splenomegaly, ascites, and other peripheral signs of portal hypertension together with ultrasonographic findings such as a shrunken, coarse texture liver with enlarged portal and splenic veins. A total of 167 anti-HCV-positive samples belonged to patients from three governates in south Egypt, 93 samples from the Sohag governate (467 km from Cairo), 45 samples from the Assuit governate (375 km south of Cairo), and 29 samples from the Qena governate (600 km south of Cairo).

Serological Methods

Serum samples were examined for anti-HCV, hepatitis B surface antigen, anti-HBc, and anti-HBs by chemiluminescence enzyme immunoassay using commercial assay kits (Fujirebio, Inc., Tokyo, Japan). Hepatitis C core antigen (HCVcAg) was measured using enzyme immunoassay (Fujirebio, Inc.) [Aoyagi et al., 1999].

Detection of the HCV RNA

Viral RNA extraction was carried out with a Sepa-Gean RV-RN Nucleic acid extracting kit (Sanko Junyaku Co. Ltd., Tokyo, Japan) following the manufacturer's protocol. The extracted RNA was reverse transcribed into cDNA using SuperScript II RNAse H Reverse Transriptase (Invitrogen Corp., Carlsbad, CA) and random hexamer primers (Takara Shuzo, Co. Ltd., Tokyo, Japan) as described previously [Ohno et al., 1997]. Confirmation of HCV RNA was performed by amplifying partial genome of the 5-non-coding region according to the protocol described previously [Takeuchi et al., 1999].

Sequencing and Phylogenetic Analysis

For genotyping the HCV isolates, PCR was used to amplify parts of both the structural (core/E1) and non-structural (NS5B) coding regions of the HCV [Tanaka et al., 2002]. The sequencing reaction of the amplified products was performed with the Prism Big Dye (Pekrin-Elmer Applied Biosystems, Foster City, CA) in an ABI 3100 DNA automated sequencer according to the manufacturer's protocol. Sequences were aligned with the CLUSAL X software program [Thompson et al., 1994]. The phylogenetic trees were constructed by neighbor joining method with Tamura-Nei distance correction model using online tools in the HCV database [Shin-I et al., 2008]. Bootstrap values were determined on 1,000 resampling tests in the HCV database. The sequences of other HCV isolates used for the phylogenetic analysis were retrieved from the DDBJ/EMBL/GenBank sequence database and are indicated by their accession number in the phylogenetic tree. The nucleotide sequence data reported in this article will appear in the DDBJ/EMBL/GenBank sequence databases with accession numbers: AB470005–AB470069, AB470243–AB470255, and AB470103–AB470215. Statistical analysis was performed with Fisher's exact probability test and an independent t-test for continuous variables using the SPSS software package (SPSS, Chicago, IL). P-values (two-tailed) less than 0.05 were considered statistically significant.

RESULTS

Demographic and Clinical Characteristics of the Patients With Chronic Liver Disease

Figure 1 summarizes the detected rates of hepatitis markers and coinfection patterns among the clinical

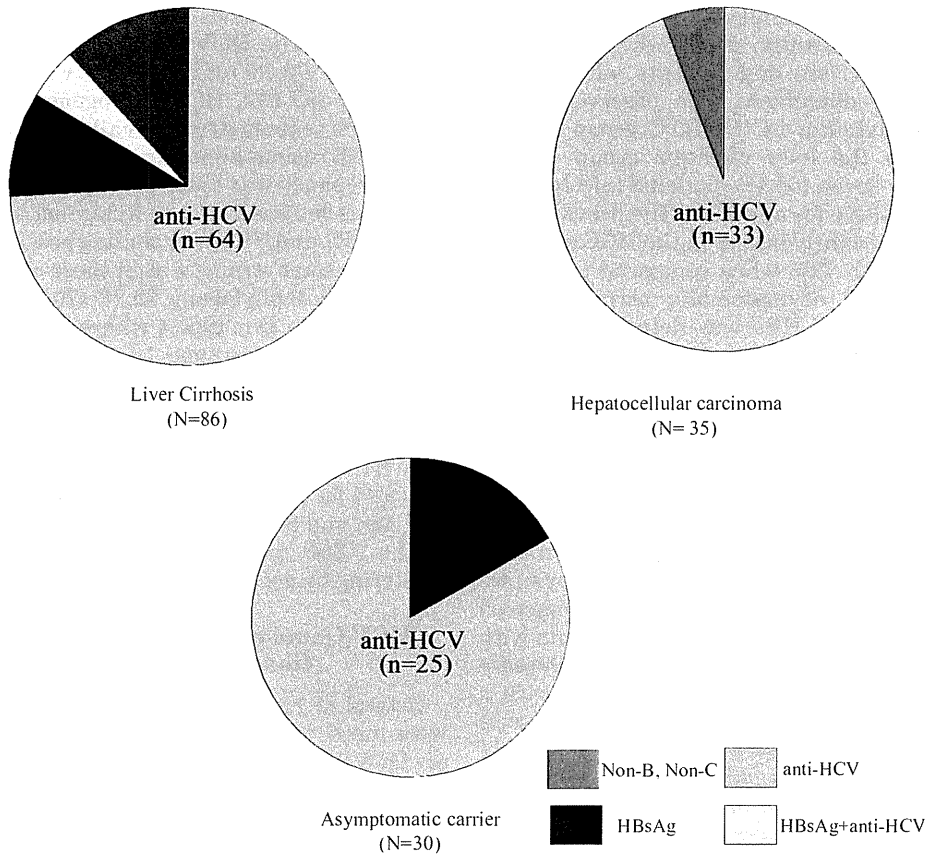

Fig. 1. Incidence of hepatitis markers among patients with progressive liver diseases.

groups studied; asymptomatic carriers (n = 30), liver cirrhosis (n = 86), and HCC (n = 35). Anti-HCV and HBsAg were detected in 25/30 (83.3%) and 5/30 (16.7%) of the asymptomatic carriers, respectively. In the liver cirrhosis group, anti-HCV was detected in 64/86 (74.4%), HBsAg was detected in 8/86 (9.3%), and simultaneous presence of both markers was detected in 4/86 (4.7%). Ten patients (11.6%) in the liver cirrhosis group were negative serologically for both anti-HCV and HBsAg and were designated as non-B hepatitis and non-C hepatitis, respectively. In the HCC group, anti-HCV was detected in 33/35 (94.3%), whereas the remaining two patients (5.7%) were negative for both anti-HCV and HBsAg and were designated as non-B hepatitis and non-C hepatitis.

Table I summarizes the baseline features of the 167 patients infected with HCV subdivided into four clinical groups: 25 asymptomatic carriers, 41 chronic hepatitis, 68 liver cirrhosis, and 33 patients with HCC. Patients in the HCC group were older than patients in

TABLE I. Description of the Patients Infected With HCV Stratified by Their Clinical Characteristics

	Total (n = 167)	Asymptomatic carrier (n = 25)	Chronic hepatitis (n = 41)	Liver cirrhosis (n = 68)	HCC (n = 33)	*P* value
Age[a]	47.5 ± 11.8	29.8 ± 7.0*	43.4 ± 6.7	52.5 ± 7.8	59.0 ± 8.6*	<0.006
Gender (male)	116 (69.4)	16 (64)	32 (78)	41 (60.2)	27 (81.8)	NS
ALT (IU/L)[a]	42.7 ± 28.5	28.4 ± 16.2*	42.3 ± 17.1	42.8 ± 29.6	70.8 ± 42.1*	<0.005
AST (IU/L)[a]	64.9 ± 46.0	29.6 ± 9.8*	63.5 ± 34.2	75.4 ± 53.8	86.1 ± 47.2*	<0.0001
HCVcAg (fmol/L)[b]	434.2 (0.1–19,654)	463 (0.1–12,549.8)	1,162.5 (5.0–19,654)	409.6 (0.1–13,219.2)	741.6 (50.7–7,703.8)	NS
HCV RNA[c]	139 (83.2)	19 (76)	38 (92.7)	56 (82.4)	26 (78.8)	NS
HBsAg[c]	5 (3.0)	0	1 (2.4)	4 (5.9)	0	NS
Anti-HBs[c]	33 (19.8)	6 (24)	9 (22)	10 (24.4)	8 (24.2)	NS
Anti-HBc[c]	97 (58.1)	12 (48)	20 (48.8)	49 (72.1)	24 (72.7)	0.023

[a]Mean + SD.
[b]Median (range).
[c]Positive number (%).
*$P < 0.05$.

the other clinical groups. Similarly, patients with liver cirrhosis were significantly older when compared to asymptomatic carriers and patients with chronic hepatitis. Male predominance was observed in all groups with a maximum in the HCC group (81.8%) and minimum in the liver cirrhosis group (60.2%, $P = 0.041$). Ninety-seven (58.1%) patients infected with HCV were also positive for anti-HBc. The prevalence of anti-HBc was significantly higher in the HCC and liver cirrhosis groups with 72% when compared to chronic hepatitis 48.8% and asymptomatic carriers 58.1% ($P < 0.05$, Table I). Anti-HBs was detected in 19.8% (33/167) of patients infected with HCV with no significant difference found between the studied groups.

Detected HCV viremic cases tended toward a higher rate in the chronic hepatitis group 92.7% (38/41) compared to the asymptomatic carrier group (92.7% vs. 76%, $P = 0.072$), and a similar trend was observed in the HCVcAg level when the clinical groups were compared. To investigate the association between viral markers and progression of liver disease, 38 asymptomatic carrier and chronic hepatitis cases were compared to 38 age-sex-matched liver cirrhosis and patients with HCC (Table II). The prevalence of anti-HBc was the only significant difference between the two groups, with a higher rate in patients with progressive liver diseases ($P = 0.038$). HCV genotypes were determined in 65/76 of the age-sex-matched groups. Although no significant difference in the prevalence of HCV genotypes was found between the non-progressive and progressive liver disease groups, 4m and 4o were detected only in the latter group, and 1g prevalence was higher also in the latter, that is, patients with progressive liver disease.

Phylogenetic Analysis of the Core/E1 and NS5B Regions and Determination of the HCV Genotype/Subtype

Among the 167 anti-HCV-positive cases, HCV RNA positivity was examined by 5′UTR-targeted PCR (detection limit 1,000 copies/ml); in total 139 (83.2%) cases were positive and subjected to determination of HCV genotype by amplification, direct sequencing, and phylogenetic analysis based on the NS5B and/or the E1 regions. The HCV genotype was determined in a total of 129 (92.8%) of the 139 HCV RNA-positive cases. In 75/129 (58.1%), HCV phylogenetic analyses of the nucleotide sequences were available in both the core/E1 and NS5B regions simultaneously. In these 75 patients, no discrepancy was observed regarding the genotyping results as obtained by the phylogenetic analysis of both regions (E1 and NS5B), excluding possibility of presence of recombinant strains within these cases (Table III).

In the NS5B phylogeny, 89.7% (70/78) of samples were clustered with genotype 4 whereas 10.3% (10/78) were clustered with genotype 1g. Bootstrap values for all subtypes within genotype 4 was more than 75%, being lowest in the subtype 4a (BV = 79%). Eight samples were clustered with genotype 1g strains of Egyptian origin with BV of 100% (Fig. 2A). In the core/E1 phylogeny, BV was 99% for genotype 4a; 100% for genotypes 4n, 4l, 4o, and 4m; and 99% for genotype 1g (Fig. 2B). Inspection of the NS5B and core/E1 trees indicated no specific clustering by geographic regions in Egypt.

HCV Genotypes/Subtypes Among Different Governates in South Egypt

A total of 129 HCV samples which were genotyped were used to analyze the geographical distribution of HCV variants. Seventy-eight isolates belonged to the population studied in the Sohag governate, 39 isolates were from the Assuit governate population, and 12 isolates belonged to patients from the Qena governate. In south Egypt, the predominant genotype was HCV-4a (104/129, 80.6%), followed by genotype 1g (7.8%), genotype 4l (4.7%), then 4n (4.1%), and 4o (1.6%). Genotypes 4m and 4f were rarely found and were detected in 0.8% for each genotype. HCV genotype 4a was the predominant genotype in the Sohag governate and was detected in 88.5% (69/78) of the population studied, followed by genotype 1g (6.4%). Sporadic cases of infection with subtypes of genotype 4 (other than genotype 4a) were also observed including genotype 4o (2.6%), genotype 4f (1.3%), and genotype 4m (1.3%).

In the Assuit governate, HCV genotype 4a was observed in 64% (25/39), that is less frequently than in

TABLE II. Comparison Between Non-progressive and Progressive Liver Disease in Age and Gender-Matched Groups

	Total (n = 76)	Non-progressive liver disease (n = 38)	Progressive liver disease (n = 38)	*P*-value
Age[a]	45.6 ± 4.8	44.9 ± 5.4	46.3 ± 4.1	Matched
Gender (male)[b]	50 (65.8)	29 (76.3)	21 (55.3)	Matched
Asymptomatic carrier/chronic hepatitis/liver cirrhosis/HCC	6/32/30/8	6/32/0/0	0/0/30/8	
Anti-HBc[b]	40 (52.6)	15 (39.5)	25 (65.8)	0.038
Schistosoma Ab[b]	57 (80.3)	34 (89.5)	23/33 (70)	NS
HCV-RNA[b]	69 (90.8)	36 (94.7)	33 (86.8)	NS
HCV genotype[b]				
4a	50/65 (76.9)	26/35 (74.3)	24/30 (80)	NS
4n	4/65 (6.2)	4/35 (11.4)	0	NS
4l	4/65 (6.2)	4/35 (11.4)	0	NS
4m	1/65 (1.5)	0	1/30 (3.3)	NS
4o	2/65 (3.1)	0	2/30 (6.6)	NS
1g	4/65 (6.2)	1/35 (2.9)	3/30 (10)	NS

[a]Mean + SD.
[b]N (%).

TABLE III. Genotyping Results as Determined by Sequence and Phylogenetic Analysis of the E1 and NS5B Regions

	Classification based on NS5B								
Classification based on E1	1g	4a	4n	4l	4o	4m	4f	ND	Total
1g	8							2	10
4a		59						32	91
4n			2					2	4
4l				4				1	5
4o					1			1	2
4m						1			1
4f								1	1
ND		13	1	1					15
Total	8	72	3	5	1	1		39	129

the Sohag governate, genotype 1g found in 12.8%, genotype 4l in 10.3%, and genotype 4n in 12.8%. Only two genotypes were detected in patients from the Qena governate and they were genotype 4a (10/12, 83.3%) and genotype 4l (2/12, 16.7%) (Fig. 3). A significant difference in HCV genotypes distribution was observed between patients from the Sohag and Assuit governates, where incidence of infection with HCV genotype 4a was higher in the Sohag governate ($P = 0.002$). Infection with genotypes 4l and 4n was significantly higher in the population studied from the Assuit governate compared to the Sohag governate ($P = 0.003$, 0.011 respectively).

Clinical and Virological Characteristics of the Determinant HCV Genotypes/Subtypes

Clinical and virological characteristics were compared between patients infected with different variants of HCV; 104 cases infected with genotype 4a, 15 cases infected with genotype non-4a (including six cases with 4l, five cases with 4n, two cases with 4o, and one case with 4f, one with 4m), and 10 cases infected with HCV genotype 1g (Table IV). The mean age was similar between patients infected with different HCV genotypes. More patients among those infected with HCV-4a had liver cirrhosis than among patients infected with non-4a. More patients among those infected with (non-HCV-4a) had chronic hepatitis than among patients infected with HCV-4a. Interestingly, two cases were found to be infected with genotype 4o and both cases were patients with HCC. A significantly higher level of HCVcAg was observed in patients infected with HCV genotype 4a (median, range; 939.5, 24.7–13,219.2 fmol/L), compared to patients infected with genotype 1g strains (median, range; 203.1, 5.0–924.4 fmol/L) ($P < 0.0001$).

DISCUSSION

Genotype analysis of HCV within a defined population is an important issue in the study of the evolution of HCV infection in different geographical regions besides its importance for the development of an effective vaccine [Pybus et al., 2001; Cantaloube et al., 2005]. In addition, evidence supporting the differential pathogenicity of HCV subtypes has emerged in many studies. To the best of our knowledge, this is the first study concerned with the genetic diversity of HCV, and the clinical and virological characteristics of HCV infection in south Egypt.

Genotype 4 strains belonged to different subtypes including 4l, 4n, 4o, with solitary isolates of 4f and 4m together with the predominant genotype 4a in the population studied from south Egypt. Studies on the epidemic history of HCV in Egypt have indicated an exponential spread of the infection, occurring from the 1940s through 1980s; a period coinciding with mass campaigns of parenteral treatment for schistosomiasis [Tanaka et al., 2004, 2006]. This explosive epidemiological spread of HCV was also responsible for the simultaneous dissemination of multiple lineages of genotypes 4 and 1 [Pybus et al., 2003]. Genovese et al. [2005] indicated also the unexpected diversity of genotype 4 in a population studied in Alexandria in north-central Egypt compared to another study [Simmonds, 2004; Genovese et al., 2005]. However, the second most frequent subtype in the Alexendria study population was 4m (11% of cases), while that in the present study in patients from south Egypt was 4l (5% of HCV genotype 4), and genotype 4m was the less observed genotype in this cohort study. Genotype 1 was less frequent and only HCV genotype 1g was detected and observed in a considerable prevalence of the genotyped samples examined from this cohort. In a previous study where 68 blood donor specimens were selected from geographically distinct governates and analyzed phylogenetically genotype 1g was detected in 5/68 (7.4%), three of these five patients were from south Egypt [Ray et al., 2000].

The HCV prevalence throughout Egypt is associated directly with the amount of intravenous tartar emetic used to control schistosomiasis in the period, 1950–1980. The lowest rates were observed in Cairo and Alexendria (<8%), the highest in rural areas of the Nile Delta [Lower Egypt (>15%)], and intermediate prevalence (8–16%) in rural areas along the Nile south of Cairo (Middle and Upper Egypt) [Abdel-Aziz et al., 2000; Nafeh et al., 2000] which is the geographical area representing the samples collected in the current study. Geographical differences in the distribution of the HCV subtype was observed also between the Assuit and Sohag governates as represented by the presence of a high proportion of HCV subtypes 1g, 4l, and 4n in the former governates. The difference in the variability between the two governates might be explained by the fact that the Assuit governate, which is the largest town

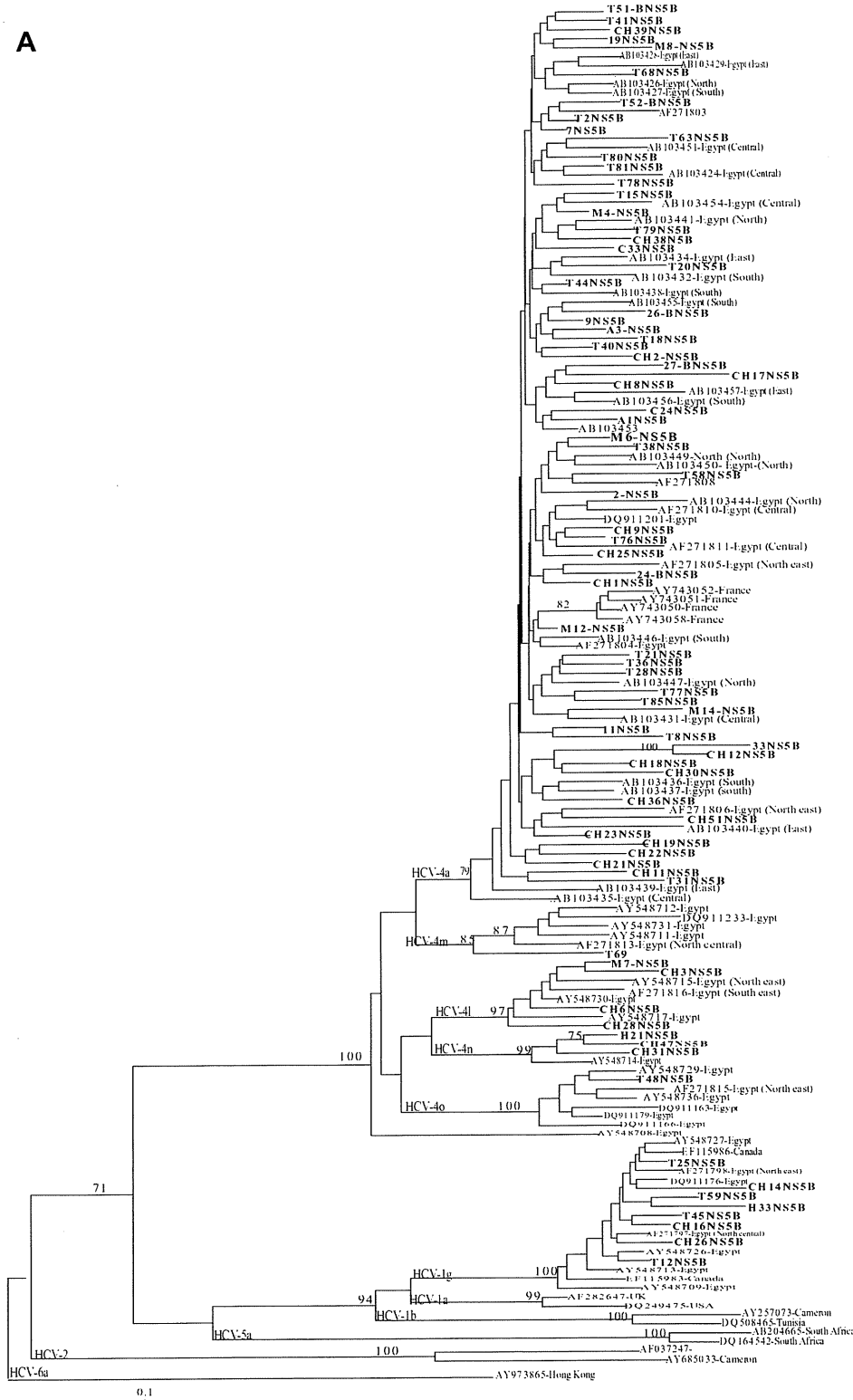

Fig. 2. Phylogenetic tree of the HCV (**A**) partial NS5B region sequences and (**B**) partial core/E1 region sequences isolated from patients infected with HCV in south Egypt (are indicated in bold) and a panel of reference strains retrieved from DDBJ/EMBL/GenBank identified by their accession number. The origins of reference strains are indicated. Data regarding the regional origin of HCV reference sequences of Egyptian origin are also indicated in parentheses when available in the GenBank database. Boot strap values are indicated in the tree root.

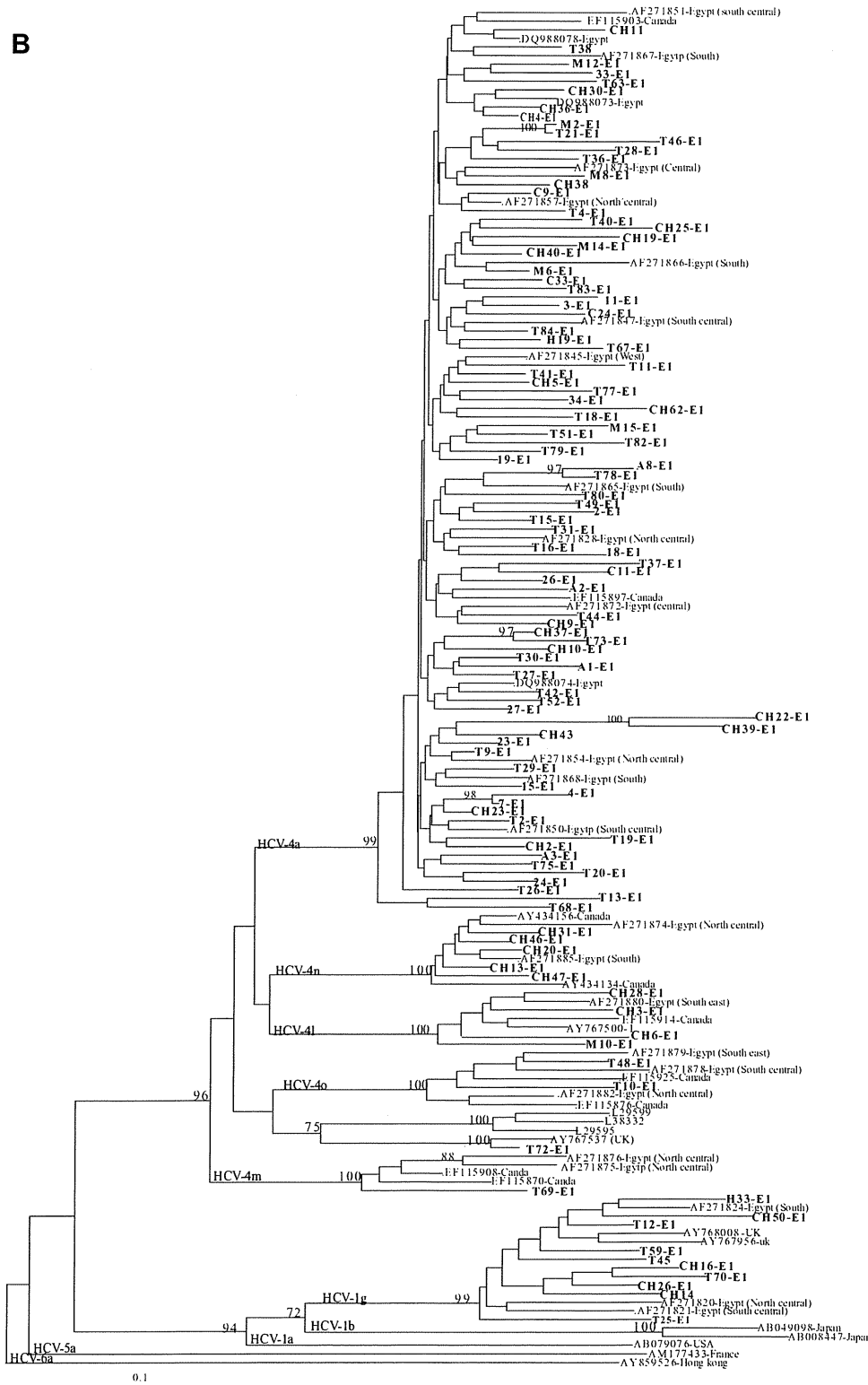

Fig. 2. (*Continued*)

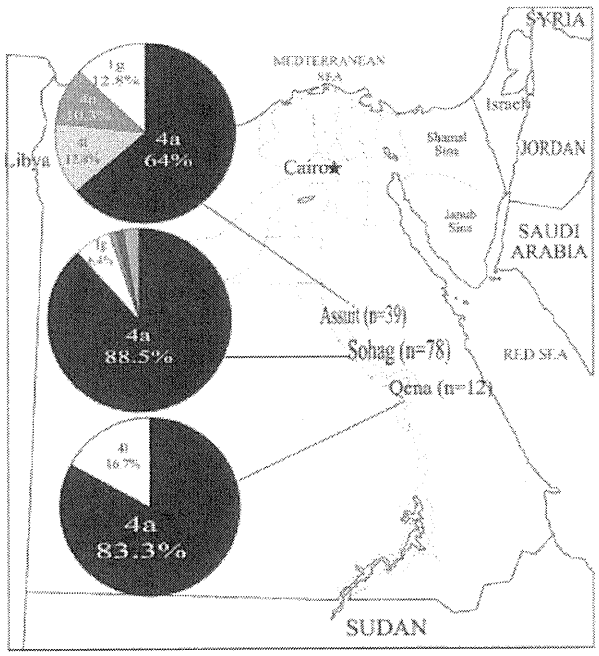

Fig. 3. Distribution of HCV genotypes in the population studied from different governates in south Egypt.

in southern Egypt, lies geographically in the Middle Egypt, while the Sohag governate is in the Upper Egypt. In Middle and Lower Egypt, intravenous tartar emetic was used more extensively and for several years longer to treat Schistosomiasis than it was in Upper Egypt [Arthur et al., 1997; Frank et al., 2000]. The high level of viral heterogeneity may be related to the high level of exposure to reinfection [Argentini et al., 2000].

Examining the association between HCV subtypes and liver diseases, a higher incidence of cirrhosis was observed in patients with HCV-4a (45.2%) than HCV-non-4a (13.3%), whereas HCV genotypes 4l and 4n, 4f, and 4m (non-4a) were more prevalent in patients with chronic hepatitis. Genotype 4o circulates in regions geographically distant from each other in Egypt [Ray et al., 2000]. In this study, genotype 4o was detected in 2/129 (1.6%). Interestingly both cases with genotype 4o were included in the HCC group. Abdel-Hamid et al. [2007] was the first to report the novel association between HCC and cluster subtype 4o as indicated by the significantly higher frequency of HCC in patients infected with subtype 4o than those infected with other subtypes. This novel association is not explained by the older age as the mean age in the two groups HCC with 4o versus HCC with the non-4o subtype was not different [Abdel-Hamid et al., 2007]. The data associating the HCV genotype with clinical and histological characteristics within HCV-infected patients are conflicting. The present study revealed a significant association between infection with HCV genotype 4n/4l and chronic hepatitis. Definite evidence for a differential pathogenicity of HCV genotypes has been limited by several factors, including the naturally long duration of HCV-related liver disease and the cohort effects in the circulation of viral types. However, a recent case–control study established a definite association between chronic infection with HCV genotype 1b and the high risk of cirrhosis [Osella et al., 2001]. Another report also indicated that all patients who developed cirrhosis in <10 years were infected with genotype 1b [Kurbanov et al., 2003].

Measurement of HCVcAg is specific, sensitive, and suitable for the detection of viremia in HCV-infected subjects. In the present study, there was a significantly lower level of HCVcAg in patients infected with genotype 1g compared to those infected with genotype 4a. The same result was also observed even after adjusting the compared clinical groups. In a previous report, a lower level of HCVcAg was observed in specimens with genotype 4 compared to genotype 1 [Agha et al., 2004]. A possible explanation for this discrepancy between the present study and previous study was the difference in the studied groups, as the previous study included only blood donors with genotype 1b when the HCVcAg level was compared. On the other hand, the mean level of HCVcAg in genotype 4 was similar to that in the previous study [Agha et al., 2004].

In conclusions, genetic diversity in HCV was detected in the present cohort study in south Egypt. Increasing evidence of differences in the clinical and virological

TABLE IV. Clinical Characteristics of the Patients Infected With HCV Stratified by the HCV Genotypes

		Genotype 4			
	Total (n = 129)	4a (n = 104)	Non-4a (n = 15)	1g (n = 10)	*P*-value
Age[a]	49.1 ± 11.2	49.1 ± 11.4	49.2 ± 10.7	48.4 ± 9.9	NS
Gender (male)[b]	91 (70.5)	70 (67.3)	12 (80)	9 (90)	NS
ALT (IU/L)[a]	43.4 ± 28.9	40.8 ± 27.8	60.1 ± 30.8	43.0 ± 31.0	0.049[d]
AST (IU/L)[a]	68.5 ± 48.3	62.1 ± 43.3	105 ± 64.3	73.6 ± 46	0.035[d]
HCV-cAg (fmol/L)[c]	897.2 (5.0–19,654)	939.5 (24.7–13,219.2)	2,354.8 (59.4–19,654.2)	203.1 (5.0–924.4)	<0.0001[e]
Asymptomatic carrier[b]	16 (12.4)	16 (15.4)	0	0	NS
Chronic hepatitis[b]	36 (27.9)	24 (23.1)	8 (53.3)	4 (40)	0.025[d]
Liver cirrhosis[b]	54 (41.9)	47 (45.2)	2 (13.3)	5 (50)	0.023[d]
HCC[b]	23 (17.8)	17 (16.3)	5 (33.3)	1 (10)	NS

[a]Mean ± SD.
[b]N (%).
[c]Median (range).
[d]Patients infected with HCV genotype 4a versus patients infected with HCV-4 non-subtype 4a.
[e]Patients infected with HCV genotype 4a versus patients infected with 1g.

characteristics of the isolated strains between different genotypes/subtypes has been introduced in the present study. The data highlight the need for further studies exploring the HCC mortality burden within different districts in Egypt; a country with the highest HCV prevalence in the world. There is also a need for a case–control study to investigate the trends in the association of HCC development with infection by HCV genotype 4o.

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TANK-binding kinase 1 (TBK1) controls cell survival through PAI-2/serpinB2 and transglutaminase 2

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The decision between survival and death in cells exposed to TNF relies on a highly regulated equilibrium between proapoptotic and antiapoptotic factors. The TNF-activated antiapoptotic response depends on several transcription factors, including NF-κB and its RelA/p65 subunit, that are activated through phosphorylation-mediated degradation of IκB inhibitors, a process controlled by the IκB kinase complex. Genetic studies in mice have identified the IκB kinase-related kinase TANK-binding kinase 1 (TBK1; also called NAK or T2K) as an additional regulatory molecule that promotes survival downstream of TNF, but the mechanism through which TBK1 exerts its survival function has remained elusive. Here we show that TBK1 triggers an antiapoptotic response by controlling a specific RelA/p65 phosphorylation event. TBK1-induced RelA phosphorylation results in inducible expression of plasminogen activator inhibitor-2 (PAI-2), a member of the serpin family with known antiapoptotic activity. PAI-2 limits caspase-3 activation through stabilization of transglutaminase 2 (TG2), which cross-links and inactivates procaspase-3. Importantly, *Tg2*$^{-/-}$ mice were found to be more susceptible to apoptotic cell death in two models of TNF-dependent acute liver injury. Our results establish PAI-2 and TG2 as downstream mediators in the antiapoptotic response triggered upon TBK1 activation.

apoptosis | tumor necrosis factor signaling | transcriptional regulation | posttranslational protein modification

Apoptosis is a highly regulated cell death process that controls cellular homeostasis and prevents survival of injured, damaged, or transformed cells (1). Apoptosis depends on a proteolytic cascade involving intracellular proteases, known as caspases, that are activated in response to cell-intrinsic and -extrinsic insults (2). The proinflammatory cytokine TNF can trigger apoptosis through its main cell surface receptor, TNF receptor (TNFR) type 1 (TNFR1) (3, 4). Engagement of TNFR1 results in assembly of multiprotein signaling complexes around its cytoplasmic death domain, leading to activation of caspase-8, which in turns activates the executioner caspase-3 that ultimately mediates cell death (3, 4). TNF-induced apoptosis is prevented by rapid activation of the IκB kinase (IKK) complex and subsequently NF-κB. NF-κB antagonizes apoptosis and maintains cell survival through induction of antiapoptotic genes encoding factors that tightly control caspase activation, such as members of the cellular inhibitor of apoptosis family (cIAP), the cellular FLICE inhibitory protein, and Bfl-1/A1, a member of the prosurvival Bcl-2 family (5, 6). Besides this intrinsic survival pathway, additional autocrine cascades activated through release of cytokines, such as TGF-α, provide additional prosurvival signals, suggesting the existence of yet unexplored feedback loops controlling cell-fate decisions (7).

TANK-binding kinase 1 (TBK1; also called NAK or T2K) was proposed to serve as an NF-κB activator (8, 9). TBK1 was originally found to interact with the TNFR- associated factor (TRAF) binding protein TANK and form, with TRAF2, a ternary complex able to activate NF-κB in a kinase-dependent manner (8). In addition, TBK1 was found to be recruited to TNFR1 upon TNF binding (10). Mice deficient in TBK1 die during embryonic development from massive liver apoptosis (11, 12), a phenotype also exhibited by mice lacking the NF-κB subunit RelA/p65 or critical components (IKKβ and IKKγ/NEMO) of the IKK complex (13). Embryonic lethality in *Tbk1*$^{-/-}$ mice was confirmed to be TNF-dependent, as these mice survive upon deletion of TNFRI (11). However, characterization of *Tbk1*$^{-/-}$ mice failed to explain how TBK1 contributes to NF-κB–dependent gene expression (11), and the mechanism by which TBK1 controls NF-κB activity to exert its antiapoptotic function has remained elusive.

In the present study, we have readdressed the function of TBK1 in TNF-mediated NF-κB activation by using a systematic approach aimed at identifying and characterizing NF-κB–dependent genes activated in a TBK1-dependent manner that encode potential antiapoptotic factors. Defective expression of such genes may account, at least in part, for TNF-induced liver failure in *Tbk1*$^{-/-}$ embryos. We now show that TBK1 controls the expression of plasminogen activator inhibitor-2 (PAI-2), a member of the ov-serpin family (14). PAI-2 maintains survival of TNF-stimulated cells through the protein modifier transglutaminase 2 (TG2), a pleiotropic enzyme able to cross-link procaspase-3 into inactive dimers. Our data support an unexplored regulatory mechanism in the TNF-activated pathway.

Results

TBK1 Controls TNF-Mediated NF-κB Activation Through RelA Ser^{534} Phosphorylation. We used a pool of spontaneously immortalized mouse embryonic fibroblasts (MEFs) derived from WT and *Tbk1*$^{-/-}$ mice to understand how TBK1 prevents apoptosis. *Tbk1*$^{-/-}$ cells were more sensitive to TNF-induced apoptosis than WT cells when challenged for 24 h, and this was further enhanced in the presence of the protein synthesis inhibitor cycloheximide (CHX) (Fig. 1*A*). Enhanced TNF-induced apoptosis

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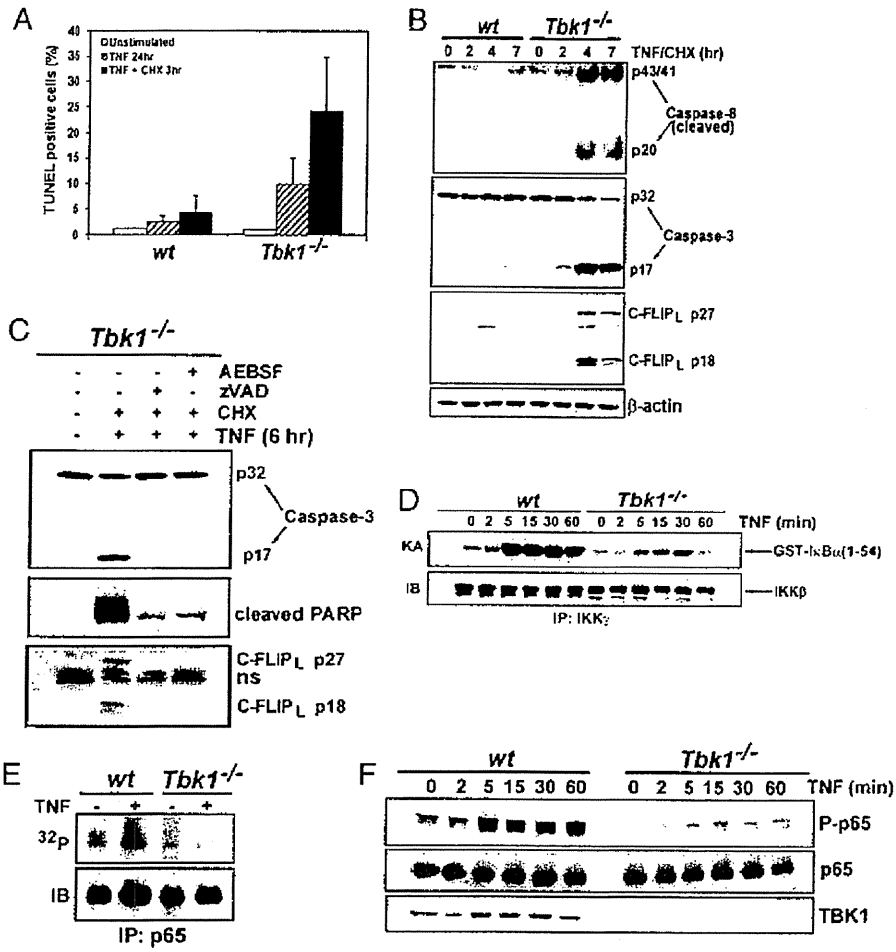

Fig. 1. TBK1 protects MEFs from TNF-induced apoptosis and modulates IKK-dependent phosphorylation of RelA/p65. (*A*) Wt and *Tbk1*$^{-/-}$ MEFs were treated for 24 h with TNF (25 ng/mL) alone or for 3 h with TNF in the presence of CHX (10 μg/mL). Apoptotic cells were detected by TUNEL assays. The bars represent averages ± SD of three different experiments. Approximately 1,200 cells were counted in each experiment. (*B*) MEFs treated with TNF and CHX for the indicated times were analyzed for caspase-8 and caspase-3 activation by immunodetection of their cleaved forms. (*C*) Cell extracts prepared from *Tbk1*$^{-/-}$ MEFs treated for 6 h with TNF and CHX in the presence of z-VAD-FMK (20 μM), AEBSF (0.5 mM), or DMSO (vehicle) were analyzed by immunoblotting for caspase-3 activation and PARP cleavage. (*D*) IKK activity in TNF-treated MEFs was measured by immunocomplex kinase assay using GST-IκBα(1-54) as a substrate (19). (*E*) RelA phosphorylation was examined after its immunoprecipitation from [^{32}P]orthophosphate-labeled and TNF-stimulated MEFs. Phospho-RelA was detected by autoradiography and the total amount of RelA by immunoblotting (IB). (*F*) Phosphorylation of RelA was examined by immunoblotting using a phospho-specific huRelA(Ser^{536}) antibody.

was associated with elevated caspase-8 and caspase-3 activation in *Tbk1*$^{-/-}$ MEFs (Fig. 1*B*). Caspase-dependent cleavage of cFLIP$_L$, the cellular inhibitor of caspase-8, was also observed in *Tbk1*$^{-/-}$, and not in WT, MEFs (Fig. 1*B*), but cFLIP expression, which depends on NF-κB, remained unchanged (Fig. 2*C* and Fig. S1). TNF-induced apoptosis as determined by cleavage of poly(ADP-ribose) polymerase (PARP), caspase-3, and cFLIP$_L$, was prevented by treatment of *Tbk1*$^{-/-}$ MEFs with the pan-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (z-VAD-FMK) or the serine protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride, AEBSF (Fig. 1*C*). Amounts of several other antiapoptotic proteins, including c-IAP1, c-IAP2, Bcl-2, and Bcl-X$_L$, were not substantially reduced in *Tbk1*$^{-/-}$ MEFs (Fig. S1).

In addition to NF-κB and caspases, TNF activates MAPKs c-Jun NH$_2$-terminal kinase (JNK), p38, and ERK (15). Instead of being strongly enhanced as in RelA- or IKKβ-deficient cells (16), JNK and p38 activities were somewhat reduced in *Tbk1*$^{-/-}$ MEFs (Fig. S2*A*). However, activation of the JNK-regulated transcription factors c-Jun and ATF-2 through phosphorylation and nuclear translocation was not affected by TBK1 ablation (Fig. S2*B*). Phosphorylation of nuclear CREB, a target for p38, was also normal. Interestingly, IKK activation was reduced in TNF-stimulated *Tbk1*$^{-/-}$ MEFs compared with WT cells (Fig. 1*D*). This is consistent with our previous observation that TBK1 could activate IKK in vitro (9). However, in agreement with a previous report (11), IκBα and IκBβ were phosphorylated and degraded normally in TNF-stimulated *Tbk1*$^{-/-}$ MEFs (Fig. S3*A*), but delayed IκB resynthesis was observed, suggesting a possible defect in NF-κB–directed transcription. Nuclear accumulation of RelA, p50, and c-Rel appeared to be normal in *Tbk1*$^{-/-}$ MEFs (Fig. S3 *B* and *C*), and so was the binding of RelA to its target gene promoters (Fig. S3*D*).

TBK1 can phosphorylate RelA in vitro (17, 18). Therefore, we examined the role of TBK1 in RelA phosphorylation in living cells. Endogenous RelA, immunoprecipitated from untreated and TNF-stimulated WT MEFs, was labeled with [^{32}P]orthophosphate. TNF stimulated phosphorylation of RelA in WT cells (Fig. 1*E* and Fig. S4*A*), but both basal and induced RelA phosphorylations were diminished in *Tbk1*$^{-/-}$ MEFs (Fig. 1*E*). Experiments performed in MEFs defective for individual IKK

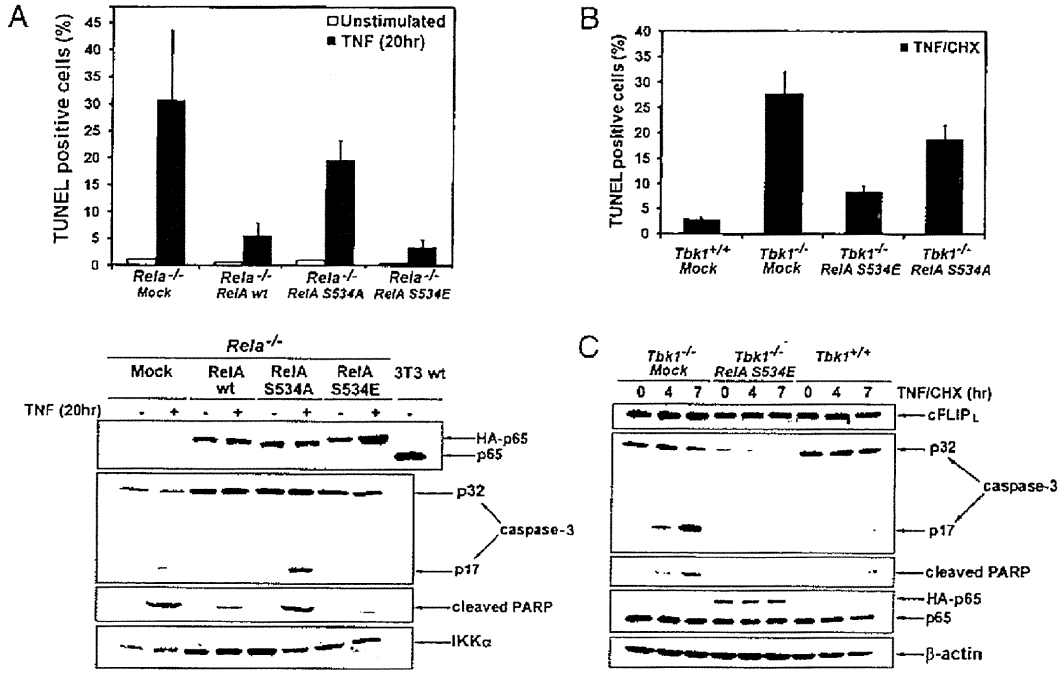

Fig. 2. Phospho-RelA protects MEFs from TNF-induced apoptosis. (*A*) *Rela*$^{-/-}$ MEFs infected with an empty retrovirus (mock) or retroviruses expressing HA-tagged wt RelA, RelA(S534A), or RelA(S534E) were left unstimulated or were treated with TNF (20 h). Apoptotic cells were detected by TUNEL staining. Represented are averages ± SD of three separate experiments. Cell lysates were analyzed for caspase-3 activation, PARP cleavage, and RelA expression by immunoblotting. (*B*) *Tbk1*$^{-/-}$ MEFs expressing RelA(S534A) or RelA(S534E) were treated for 3 h with TNF in the presence of CHX. Apoptotic cells were detected by TUNEL assay and quantified as described earlier. (*C*) Lysates were prepared from cells in *B* that were stimulated with TNF plus CHX for the indicated times. Caspase-3 activation and protein expression were determined by immunoblotting as in *A*.

subunits indicated that a functional IKK complex was also required for TNF-induced RelA phosphorylation (Fig. S5*A*). These results are consistent with our early suggestion that TBK1 stimulates NF-κB activity through the canonical IKK complex (9). By using an established peptide mapping strategy (19), the TNF-inducible phosphorylation sites were localized to a 4.6-kDa CNBr peptide located in the C-terminal transactivation domain of RelA (Fig. S4 *B* and *C*). The major TNF-inducible phosphorylation site was identified as Ser^{534} of mouse RelA (corresponding to Ser^{536} of human RelA; Fig. S4*D*). Reduced Ser^{534} phosphorylation in *Tbk1*$^{-/-}$ MEFs was confirmed by immunoblotting performed with phospho-specific RelA antibody (Fig. 1*F*). RelA Ser^{534} phosphorylation was also impaired in cells defective in both IKK catalytic subunits (Fig. S5*B*). Ser^{534} phosphorylation took place in the cytosol, as it was dramatically reduced in *IκBα*$^{-/-}$ MEFs, in which most of RelA is constitutively nuclear (Fig. S5*C*).

Ser^{534} Phosphorylation Regulates RelA Antiapoptotic Function. To examine whether phosphorylation of RelA at Ser^{534} was required to protect MEFs from TNF-induced apoptosis, a retroviral vector was used to stably express WT RelA, RelA(S534A), or a phospho-mimic RelA(S534E) mutant in *Rela*$^{-/-}$ MEFs. Expression levels were similar to that of endogenous RelA in WT MEFs (Fig. 2*A*), and all the expressed proteins underwent nuclear translocation. Whereas WT RelA and RelA(S534E) protected *Rela*$^{-/-}$ MEFs from TNF-induced apoptosis, expression of RelA(S534A) provided little protection (Fig. 2*A*). Similar results were obtained when caspase-3 activation and cleavage of PARP were examined: WT RelA and RelA(S534E) prevented caspase-3 activation, whereas RelA(S534A) did not (Fig. 2*A*). Expression of RelA(S534E) but not RelA(S534A) also prevented apoptosis (Fig. 2*B*), PARP cleavage, and caspase-3 activation (Fig. 2*C*) in *Tbk1*$^{-/-}$ MEFs. Surprisingly, the amount of procaspase-3 was dramatically reduced in *Tbk1*$^{-/-}$ MEFs expressing RelA(S534E) (Fig. 2*C*). This reduction in procaspase-3 protein amount appeared to be specific, as other proteins such as cFLIP$_L$ (Fig. 2*C*) and XIAP (Fig. 3*G*) were not affected.

TBK1 Controls TNF-Induced Expression of Survival Gene *Pai-2/serpinB2*. Genes that mediate the survival function of TBK1 were identified by microarray analysis of RNAs isolated from resting and TNF-stimulated WT and *Tbk1*$^{-/-}$ MEFs. Selected data were validated by quantitative real-time PCR (qRT-PCR). Induction of most previously described (20) NF-κB–dependent genes was not affected by TBK1 deficiency (Fig. S6). As expected, induction of genes that require activation of IFN regulatory factors in addition to NF-κB, such as *IP-10*, *Rantes*, or *Irf9* was impaired in TNF-stimulated *Tbk1*$^{-/-}$ MEFs (Fig. 3*A* and Fig. S6). Among well established antiapoptotic genes, induction of *c-Iap-1*, *c-Iap-2*, *Xiap*, and *BclX$_L$* was observed in *Tbk1*$^{-/-}$ MEFs, whereas *c-Flip* expression was constitutive and not TNF-inducible in MEFs (Fig. S6). Further examination of less studied NF-κB target genes with a documented survival function identified *Pai-2/serpinb2*, a gene encoding PAI-2, a serine protease inhibitor previously shown to inhibit TNF-induced apoptosis in cancer cells (21, 22), as a potential candidate (Fig. 3*A*). Both basal (Fig. 3*A*, *Inset*) and TNF-induced expressions of *Pai-2* were dramatically reduced in *Tbk1*$^{-/-}$ MEFs (Fig. 3*A*). However, *Pai-2* expression was restored in *Tbk1*$^{-/-}$ MEFs upon expression of RelA(S534E), whereas *IP-10* induction remained defective (Fig. 3*B*). Induction of other NF-κB–dependent genes such as *IκBα* and *iNos* was not affected (Fig. 3*B*). Reconstitution of *Tbk1*$^{-/-}$ MEFs with RelA(S534E) also increased PAI-2 protein expression (Fig. 3*C*).

The antiapoptotic function of ectopic PAI-2 reintroduced into *Tbk1*$^{-/-}$ MEFs was examined by retroviral transduction. Recon-

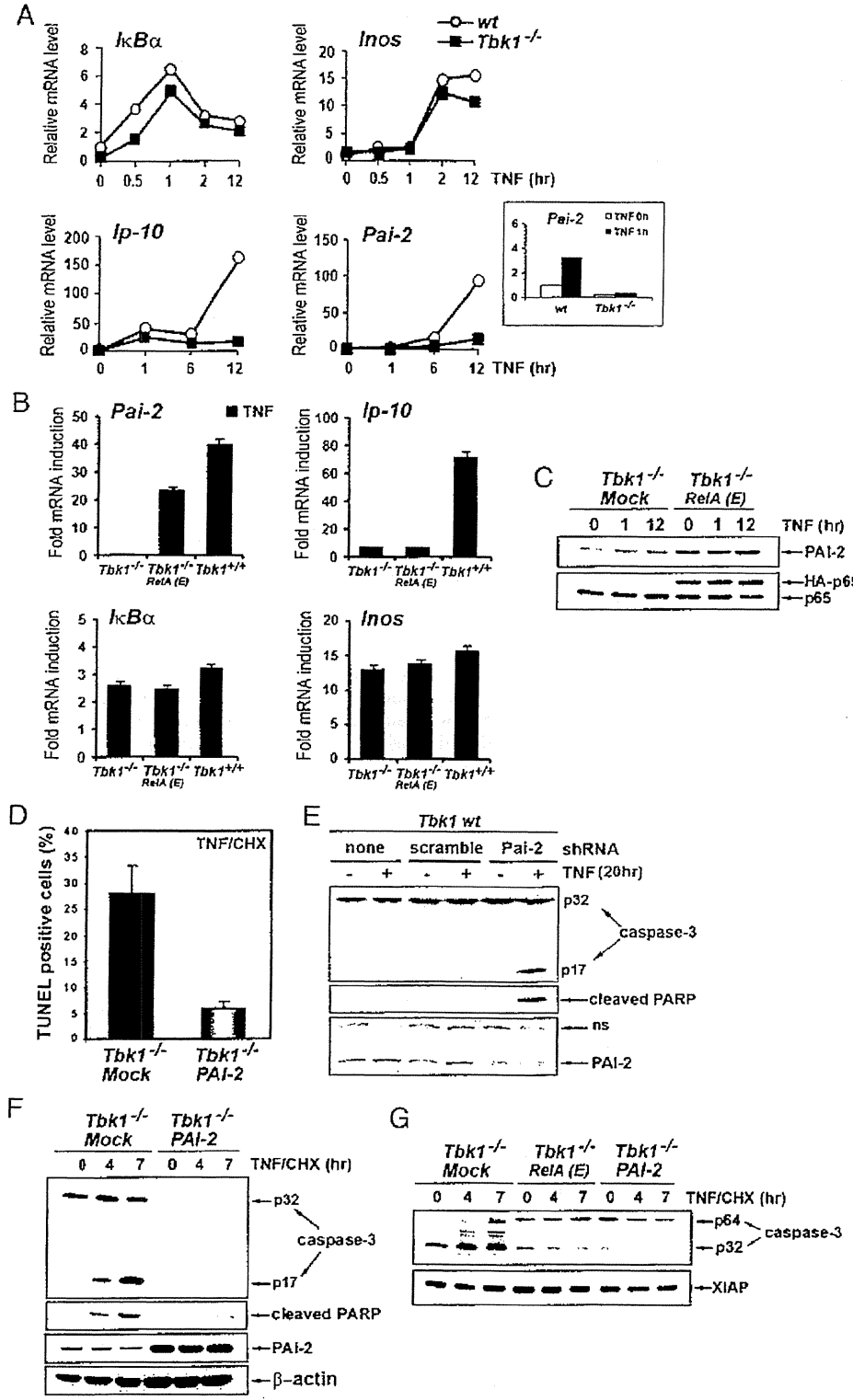

Fig. 3. TBK-1 controls NF-κB–dependent expression of the survival factor PAI-2. (*A*) Induction of NF-κB target genes was determined by qRT-PCR amplification of mRNAs prepared from WT and *Tbk1*$^{-/-}$ MEFs that were stimulated with TNF for the indicated times. *Inset*: Short time course (0 and 1 h) of *Pai-2* mRNA induction in both cell types. (*B*) Relative mRNA induction was analyzed by qRT-PCR amplification of RNAs prepared from WT (*Tbk1*$^{+/+}$), *Tbk1*$^{-/-}$, and *Tbk1*$^{-/-}$ MEFs expressing RelA(S534E), depicted as RelA(E) that were stimulated with TNF for 2 h (*IκBα*), 6 h (*Pai-2* and *Inos*), or 14 h (*Ip10*). (*C*) Expression of endogenous PAI-2 and RelA was examined by immunoblotting in extracts prepared from cells that were stimulated with TNF for the indicated times. (*D*) Apoptotic cell death, determined by TUNEL assay as described earlier, was quantified in *Tbk1*$^{-/-}$ MEFs and in cells stably expressing PAI-2 that were stimulated for 3 h with TNF in the presence of CHX. (*E*) WT MEFs expressing a *Pai-2* shRNA or a scrambled shRNA were treated with TNF for 20 h. Caspase-3 activation, PARP cleavage, and protein expression were determined by immunoblotting. (*F* and *G*) Caspase-3 activation, PARP cleavage, and protein expression were determined by immunoblotting in extracts prepared from the indicated cells that were stimulated with TNF in the presence of CHX for the indicated times.

stituted cells were protected from TNF-induced apoptosis (Fig. 3*D*) or activation of caspase-3 (Fig. 3*F*). By contrast, down-regulation of *Pai-2* expression by stably expressing a *Pai-2*-specific shRNA, but not a scrambled shRNA, potentiated TNF-induced caspase-3 activation and apoptosis in WT MEFs (Fig. 3*E*). Similarly to what was observed in $Tbk1^{-/-}$ MEFs expressing RelA(S534E), the amount of procaspase-3 was dramatically reduced in $Tbk1^{-/-}$ MEFs expressing PAI-2, and a slow-migrating form of procaspase-3 with an apparent molecular weight of 64 kDa (depicted as p64) was observed in $Tbk1^{-/-}$ MEFs expressing either RelA (S534E) or PAI-2 (Fig. 3*G*). A similar slow-migrating caspase-3 isoform was previously described as a cross-linked procaspase-3 in thapsigargin-treated HCT116 cancer cells and in tumor cells exposed to hypoxia (23, 24). In both cases, caspase-3 cross-linking into nonfunctional dimers or multimers is mediated by TG2, a multifunctional enzyme that induces posttranslational protein modifications by transamidation (25). TG2 was reported to have antiapoptotic function not only in vitro (23, 24) but also in vivo, as TG2-deficient mice show increased sensitivity to apoptosis induced by activation of the CD95/Fas receptor (26), a molecule related to TNFR1.

TG2 Is a TBK1-Regulated Antiapoptotic Factor. Endogenous TG2 activity was analyzed in WT and $Tbk1^{-/-}$ MEFs stimulated with TNF by examining incorporation of biotinylated pentylamines into cellular proteins (27). TNF induced transamidation in WT MEFs but not in $Tbk1^{-/-}$ MEFs (Fig. 4*A*). TNF also failed to stimulate *Tg2* gene expression in $Tbk1^{-/-}$ MEFs, whereas expression of *Tg1* was somehow increased in these cells (Fig. 4*B*). TG2 protein could hardly be detected in MEFs because of the poor quality of available antibodies combined with its very low level of expression. However, we could observe that basal and TNF-induced endogenous TG2 protein amounts were increased in $Tbk1^{-/-}$ MEFs expressing RelA(S534E) (Fig. 4*C*, *Upper*). Interestingly, basal TG2 expression was also increased in $Tbk1^{-/-}$ MEFs constitutively expressing PAI-2, suggesting that PAI-2 might stabilize TG2 (Fig. 4*C*, *Lower*). Similarly, MEFs treated with the proteasome inhibitor MG132 contained more TG2 protein (Fig. S7*A*), suggesting the existence of a tight post-translational control that down-regulates TG2 protein expression. Consistent with PAI-2's involvement in stabilization of TG2, an interaction between PAI-2 and TG2 was observed by coimmunoprecipitation in MEFs expressing HA-PAI-2 and human TG2 (Fig. 4*D*). We next examined whether the activity responsible for cross-linking of procaspase-3 was associated with PAI-2. An in vitro transamidation assay was performed with PAI-2 immunoprecipitates from $Tbk1^{-/-}$ MEFs stably expressing untagged PAI-2 that were left untreated or were stimulated with TNF, and recombinant HA-procaspase-3 generated by in vitro translation was used as a substrate. Caspase-3 cross-linking activity was present in PAI-2 immunoprecipitates but not in control immunoprecipitates, and it was strongly elevated in TNF stimulated cells (Fig. S7*B*). Similarly, TG2 immunoprecipitated from MEFs expressing TG2 and PAI-2 showed inducible transamidation activity toward procaspase-3 (Fig. 4*E*). Assays performed by using recombinant PAI-2 or TG2 further confirmed that the transamidating activity was carried out by TG2 and not by PAI-2 (Fig. 4*E*, *Right*).

We next examined if the protective effect of PAI-2 and TG2 was indeed mediated through inhibition of caspase-3 without affecting alternative (i.e., mitochondrial-mediated) pathways. A slight increase in BID cleavage after stimulation with TNF and CHX was observed in $Tbk1^{-/-}$ MEFs relative to WT cells (Fig. S8*A*). Expression of PAI-2 in these cells was unable to prevent BID cleavage (Fig. S8*B*), suggesting that PAI-2 is not a bona fide antiapoptotic factor. We also analyzed TNF-induced apoptosis in cells defective in caspase-3. In agreement with previous studies (28, 29), we found that caspase-3–deficient ($Casp3^{-/-}$) MEFs were highly resistant to TNF-induced apoptosis (Fig. S8*C*), highlighting that the absence of caspase-3 does not generate a compensatory cell death pathway (e.g., TNF-induced BID cleavage) in MEFs and that caspase-3 is indeed critical to mediate the TNF response in these cells. Taken together, these experiments clearly indicated that PAI-2 and TG-2 act specifically on TNF-induced, caspase-3–dependent apoptosis.

To examine the antiapoptotic function of TG2, we first used the irreversible TG2 inhibitor KCC009 (30). As shown in Fig. 4*F*, treatment with KCC009 strongly potentiated TNF-induced caspase-3 activation and apoptosis in WT MEFs. To further confirm the antiapoptotic function of TG2, primary MEFs derived from $Tg2^{+/+}$ and $Tg2^{-/-}$ littermate embryos (31) were examined for their sensitivity to TNF-induced apoptosis. Apoptosis (PARP cleavage) and caspase-3 activation were detected by immunoblotting (Fig. 4*G*). Caspase-3 activation was observed in $Tg2^{-/-}$ MEFs treated with TNF without the need for inhibition of de novo protein synthesis, indicating that these cells have an intrinsic defect in the antiapoptotic response to TNF. The extent of cell death was quantified by staining with propidium iodide (PI) (Fig. 4*H*) and FACS analysis after annexin V and PI staining confirmed that $Tg2^{-/-}$ MEFs showed increased susceptibility to TNF-induced apoptosis (Fig. S9).

TG2 Is Essential for Prevention of TNF-Dependent Liver Injury. To assess the antiapoptotic function of TG2 in vivo, we used two models of TNF-induced liver injury in mice. WT and $Tg2^{-/-}$ mice were first injected intraperitoneally with TNF and actinomycin D (Act D). $Tg2^{-/-}$ mice showed clear signs of liver failure such as elevated serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels (Fig. 5*A*), massive infiltration with neutrophils, and presence of numerous apoptotic bodies (Fig. 5*B*). The presence of a high amount of TUNEL-positive cells at the injury sites indicated that cell death mainly occurred through apoptosis (Fig. 5*B*). Caspase-3 activation was also observed in liver extracts from $Tg2^{-/-}$ mice injected with TNF and Act D (Fig. 5*C*). To rule out that apoptosis occurred in endothelial cells, staining with anti-CD31 (PECAM-1), which detects endothelial cells, was performed on sections consecutive to those used for histological evaluation and TUNEL assays. The absence of TUNEL-positive cells in the CD31-positive population, combined with histological evaluation, indicated that hepatocytes were the major cell type undergoing apoptosis in TNF/Act D-challenged $Tg2^{-/-}$ mice (Fig. 5*B*). $Tg2^{+/+}$ mice also experienced liver damage, but the response was much weaker than in $Tg2^{-/-}$ mice (Fig. 5*A*) and no caspase-3 activation could be detected (Fig. 5*C*). Thus, $Tg2^{-/-}$ mice are more susceptible to TNF-induced liver injury than WT mice. $Tg2^{-/-}$ mice were also highly sensitive to T cell-mediated liver injury induced by i.v. injection of Con A, which is TNF-dependent. Liver damage indicated by ALT release was present in both genotypes but was more severe in $Tg2^{-/-}$ mice (Fig. 5*D*). Massive hepatocyte cell death detected by TUNEL staining was observed in $Tg2^{-/-}$ liver sections 8 h after Con A injection (Fig. 5*E*), and caspase-3 activation indicated that cell death in $Tg2^{-/-}$ mice occurred through apoptosis (Fig. 5*F*). Liver destruction was clearly visible 24 h after Con A injection in $Tg2^{-/-}$ mice, and the apoptotic cell remnants could no longer be detected by TUNEL reaction. Only few apoptotic hepatocytes were found in liver sections from $Tg2^{+/+}$ mice (Fig. 5*E*), and caspase-3 activation was not observed (Fig. 5*F*). These data strongly support the physiological relevance of TG2 as an inhibitor of TNF-induced apoptosis.

Discussion

The role of TBK1 in mediating the innate immune response to viruses and dsDNA through induction of type I IFN is well documented. By contrast, the role of TBK1 in IKK–NF-κB signaling has been debated, because of the observation that, al-