

Circulating Hepatitis B Virus Nucleic Acids in Chronic Infection: Representation of Differently Polyadenylated Viral Transcripts during Progression to Nonreplicative Stages

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ABSTRACT

Purpose: Beside the established maturation of hepatitis B virus (HBV) transcripts at a polyadenylation signal downstream of the HBV x protein open reading frame, maturation at an internal polyadenylation signal has been observed in the chronically infected liver. In the present study, it was the aim to identify the respective circulating full-length and truncated transcripts in plasma/serum of carriers.

Experimental Design: Nucleic acids extracted from sera were analyzed using established PCR and reverse transcription-PCR procedures targeted to HBV x protein gene regions. Amplification products were cloned and sequenced.

Results: Base substitution patterns were determined, which indicated infection stages advanced to different degrees regardless of the transcript type analyzed. HBV full-length RNA (fRNA) showed a high correlation with hepatitis B e antigen and viral DNA, indicative for a replicative infection. In contrast, truncated RNA (trRNA) appeared to be independent of hepatitis B e antigen and showed only a weak association with circulating viral DNA. No correlation was observed between the levels of trRNA and the apparent liver damage as reflected by alanine transaminase levels. An age-dependent representation of fRNA and trRNA was observed: fRNA decreased progressively to low levels, whereas trRNA remained at comparably high values. trRNA and RNA not polyadenylated at either of the two polyadenylation signals were detected even in the absence of any other

conventional HBV seromarker, including viral DNA. This was shown for patients with cryptogenic cirrhosis and hepatitis C virus carriers.

Conclusions: The identification of HBV RNA in human serum has a diagnostic potential for apparent and for inapparent infection stages.

INTRODUCTION

Conventionally, chronic infection with HBV² is defined via the lasting presence of HBs in the serum for at least 6 months. The prevalence of HBs carriers is estimated to be 6% of the total world population. In regions endemic for the virus, >60% of the total population have a history of an infection (1–3). The risk for individuals exposed to the virus to become chronic carriers inversely correlates with age. It is reported to be high (>50%) for infants and low (3–8%) for adults (3–7).

Chronic infection proceeds from replicative to nonreplicative stages (8) and may be asymptomatic for many years or result in slight liver damage. Alternatively, it leads to progressive liver disease. Regardless if causing progressive liver disease or not, the chronic infection appears to be linked to the development of HCC, although to a different extent (1, 9). New cases of HCC (>400,000) are found each year worldwide with a 5-year survival rate <3% (10). HBV is considered to be the main causative factor of liver cancer, possibly accounting for 60% of cases worldwide and ~70% of cases in endemic areas (11). Although vaccination programs in several countries have been successful (2, 3, 12, 13), for those already infected, it is important to develop discriminative screening procedures as a basis for targeted therapy of critical infection stages. The present study introduces circulating viral transcripts as a potential parameter.

The four overlapping ORFs on viral DNA, *i.e.*, PreC/C, polymerase, PreS1/PreS2/S, and X, are transcribed into genomic and subgenomic RNA molecules. The translation product of the smallest viral transcript, HBx, is regarded as a hepatocarcinogenic factor. Properties assigned to HBx include the stimulation of transcription and signal transduction (14, 15), interference with DNA repair (16, 17), induction of apoptosis (18–21), and malignant transformation both *in vitro* and *in vivo* (22–26).

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² The abbreviations used are: HBV, hepatitis B virus; ALT, alanine transaminase; Dig, digoxigenin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBc, hepatitis B core antigen; HBs, hepatitis B e antigen; HBs, hepatitis B surface antigen; HBx, hepatitis B virus x protein; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; RT-PCR, reverse transcription-PCR; fRNA, full-length RNA; trRNA, truncated RNA; oligo(dT), oligodeoxythymidylic acid; CI, confidence interval; ORF, open reading frame; Eb, ethidium bromide; Hyb, hybridization.

Although the mechanism of HBV-associated hepatocarcinogenesis is not established, integration of viral DNA into chromosomes of hepatocytes (27–29), preferential preservation of HBx ORF sequences (30), and selective, albeit weak expression of HBx in preneoplastic (31) and neoplastic human hepatocytes (29, 31–33) may be critical.

During replication of HBV, all viral transcripts mature at a unique poly(A) signal downstream of the HBx ORF, namely a UAUAAA motif at position 1789 (34, 35). Collectively, these transcripts are referred to as full-length transcripts in this study. Chromosomally integrated DNA usually does not allow generation of full-length transcripts. Truncations affecting the 3'-end region of the HBx ORF are frequent (28, 29, 36–38) and lead to fusion of HBx sequences with adjacent cellular sequences. Respective HBx/cell hybrid transcripts may direct the synthesis of functionally active HBx/cell fusion proteins (25, 36, 39–41). Other transcripts, designated as truncated, use for maturation a cryptic poly(A) signal, a CAUAAA motif within the HBx ORF (position 1661). They are present in HCC and surrounding liver parenchyma (36). The existence of respective truncated HBx proteins with growth-regulating properties has been postulated (42). In a recent study, using liver tissue samples from HCC patients, trRNA has been shown to be a frequent marker of chronic infection, the representation of which increases with advancing age (43).

Although it cannot be excluded that free forms of viral DNA contribute to the pool of trRNA, a substantial part of it is likely to be transcribed from chromosomally integrated HBV DNA, which accumulates during chronic infection. Hence, truncated transcripts are attractive markers not only for monitoring progression from replicative to nonreplicative stages but also for recognizing inapparent infection stages. Usually, for analyzing viral RNA, liver tissue samples from biopsies or resection material are required. In the present study, we propose that it is also possible to use serum samples to detect and describe viral RNA related to chronic infection. The feasibility of detecting transcripts from a DNA virus in serum has been demonstrated previously for the EBV (44).

MATERIALS AND METHODS

Serum Samples and Extraction of Nucleic Acids. Sera from a total of 437 individuals with and without HBV infection were studied. Samples from HBV-infected individuals were taken before antiviral therapy. Sera (118) from HCC patients were obtained as follows: 71 from the Tangdu Hospital in Xi'an, China; 25 from the Tianjin Cancer Hospital in Tianjin, China; 16 from the Shaanxi Cancer Hospital in Xi'an; and 8 from the Takegoshi Clinic in Takaoka, Japan. Sera (168) from chronic HBs carriers without detectable HCC were obtained as follows: 167 from the Tangdu Hospital and 1 from the Takegoshi Clinic. Sera of 84 patients negative for HBs were obtained from Tangdu Hospital. Of these, 21 were cases of extra-hepatic, non-neoplastic liver diseases, 9 were cases of cryptic cirrhosis, and 54 were cases of nonhepatic neoplasms. The non-neoplastic cases comprised 22 thoracic cancers, 15 gliomas/meningiomas, 10 gastric/colon carcinomas, and 7 other tumors. Sera (37) from apparently healthy local urban inhabitants of Xi'an receiving routine physical and laboratory checkups were used as a reference group. A

second reference group included 30 samples from healthy blood donors (Lübeck, Germany). The collection of samples was approved by the Medical Ethics Commission of The Fourth Military Medical University in Xi'an. All serum samples were separated as soon as the blood was coagulated and stored at -80°C in RNase-free tubes. Isolation of nucleic acids was performed using the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany). The supplied poly(A) RNA carrier was replaced by 16S and 22S rRNA (Roche). For each isolation, 200 μl of serum were used resulting in 50 μl of nucleic acid extract. ALT values were obtained using a kit of ALT Liquid Reagents (Changzheng-Kangren Corp. Ltd., Shanghai, China). The detection limit of this assay is 2 units/liter (0.03 $\mu\text{cat/liter}$). This assay covers a range of 7–420 units/liter. The HBV seromarkers were assayed with a one-step ELISA kit (Free Biotech, Beijing, China) with a detection limit for HBs at a concentration of 1 ng/ml.

Primers and Probes. Upstream primers: txs3 (1434; Ref. 45): TCT CAT CTG CCG GAC CGT GT; txs1 (1454): GCA CTT CGC TTC ACC TCT GC; txs (1445): GGA CCG TGT GCA CTT CGC TT; and GAPDH1 (3755; Ref. 46): CAT CTC TGC CCC CTC TGC TG. Downstream primers: txas5 (1683): (T)₁₅ GCT GG; Rxas2 (1808): (T)₁₅ GAA GC; Rxas4 (1806): (T)₁₅ AGC TC; xas1 (1668): AAT TTA TGC CTA CAG CCT CC; xas2 (1634): TTA ACC TAA TCT CCT CCC CC; and GAPDH2 (4344): GGA TGA CCT TGC CCA CAG CCT. Probes for polarity determination: 1561+: GAC CGA CCT TGA GGC ATA CTT CAA AGA CTG and 1590-: CAG TCT TTG AAG TAT GCC TCA AGG TCG GTC.

PCR for X-region DNA. For amplification of X-region DNA, 2 μl of nucleic acid extract, 75 ng each of txs3 and xas1 (Fig. 1), deoxynucleotide triphosphate (0.2 mM each; Life Technologies, Inc., Gaithersburg, MD), and 1.25 units of Taq DNA polymerase (Life Technologies, Inc.) were incubated in a reaction volume of 50 μl as specified by the supplier. Cycles (35) were performed (40 s denaturation at 90°C , 50 s annealing at 56°C , and 40 s elongation at 70°C). Amplification efficacy and specificity were demonstrated by positive controls, using plasmids pMT9T40A (200 μg) and pMT9T41A (200 μg) carrying cloned cDNAs of full-length and truncated HBV transcripts, respectively (36), and by a negative control (target sequences replaced by water).

RT-PCR for X-region RNA. Viral RNA spanning positions 1434 and 1668 (*Xho*I coordinates) was collectively designated as HBx RNA. The composition of this RNA may vary (see below). HBx RNA was detected through an RT-PCR procedure after pretreatment of nucleic acid extracts with DNase I. To this end, 6 μl each were digested for 30 min at 25°C in a reaction volume of 20 μl containing DNase I (Amplification Grade; Life Technologies, Inc.). The digestion product (6 μl) was subjected to RT-PCR using the Titan One-Tube RT-PCR System (Roche) in a reaction volume of 50 μl containing deoxynucleotide triphosphate (0.2 mM each), 150 ng of each primer (txs3 and xas1; Fig. 1). Cycles (35) were performed as described in the reaction for viral DNA after reverse transcription (20 min at 50°C). Samples containing cDNA of the plasmids pMT9T40A and pMT9T41A, before and after digestion with DNase I, served as positive and negative controls. In addition, water controls were included. rRNA (1 ng), used as a

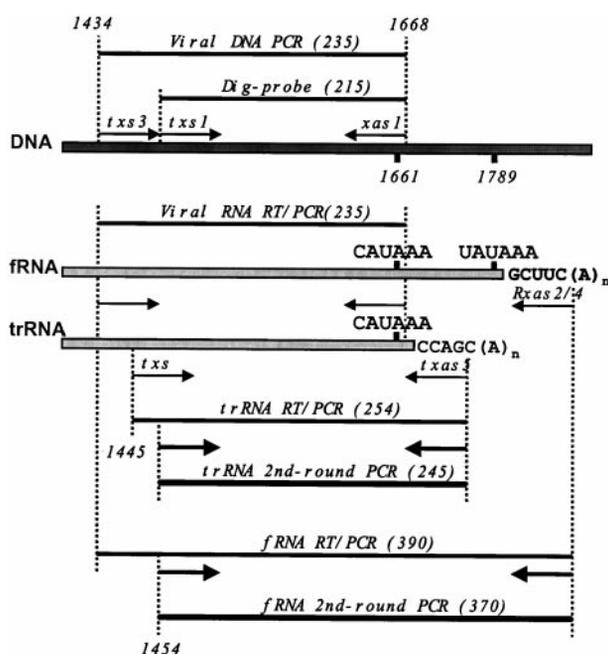


Fig. 1 Location of primers in relation to HBx DNA and RNA. Numbers indicate positions on viral DNA (*XhoI* coordinates). Primers carrying the respective designations used in the text are symbolized by arrows. Amplification products are depicted as bold lines with sizes in parenthesis. *Dig-probe*, a Dig-labeled Hyb probe. CAUAAA and UAUAAA are the signals at which trRNA and fRNA are polyadenylated. GCUUC(A)_n and CCAGC(A)_n symbolize the target sequences for the anchored oligo(dT) primers (as described in "Materials and Methods").

carrier during the nucleic acid extraction described above, served as an additional negative control. To ensure RNA specificity, the described PCR procedures were performed with and without a reverse transcriptase reaction. RT-PCR for detection of GAPDH transcripts using the primers GAPDH1 and GAPDH2 (46) served as controls for the presence and integrity of RNA in the sera.

Identification of Full-length and Truncated HBV Transcripts via a Semi-nested PCR After Reverse Transcription. As anchored oligo(dT) primers ensure selective amplification of RNA (43, 45), pretreatment of samples with DNase I was not required when studying 3'-end structures. Nucleic acid extracts, 10 μ l each, were used for the first-round PCR (anchored RT-PCR) with a one-tube system in a reaction volume of 50 μ l as described above. The upstream primer (150 ng; txs3 for fRNA and txs for trRNA; Fig. 1) and 300 ng of the downstream anchored oligo(dT) primer (a Rxa2/Rxa4 mix for fRNA and txas5 for trRNA; Fig. 1) were used for the first-round PCR. Amplification products, 2 μ l each, were then subjected to the second-round PCR with txs1 as the upstream inner primer for both reactions (full-length and truncated transcripts), while maintaining the anchored oligo(T) primers used in the respective first-round PCR (Fig. 1). Amplification conditions were the same as described above except for the reduction of the annealing temperature to 53°C (43, 45).

Aside from water controls, in a series of ~15 samples,

efficacy and specificity of the reaction for full-length transcripts were assessed using 50-pg plasmid pMT9T40A as a positive control and 50-pg plasmid pMT9T41A as a negative control. Inversely, for truncated transcripts, plasmid pMT9T41A was used as a positive control, and plasmid pMT9T40A was used as a negative control. The inclusion of the positive controls also allowed to assess signal intensities at identical staining conditions. In some cases, RNA extracted from HBV-infected liver shown previously to contain only full-length transcripts (43) and another extract from HCC tissue containing only the truncated transcripts (43) were included as positive controls. In addition, 1 ng of rRNA was used as a negative control. Special attention was paid to avoiding contamination of the samples, as described by Kwok and Higuchi (47). Assays were carried out at least twice in independent series.

DNA Probe Preparation. A DNA probe for Hyb was prepared by PCR with Dig-11-dUTP (Roche), pMT9T40A as a template, and the primers txs1 and xas1 (Fig. 1). A low-incorporation ratio Dig-11-dUTP:dTTP = 1:20 was used to verify visible (Eb) DNA products as HBx specific rather than to increase the sensitivity.

Electrophoresis, Southern Blotting, and Hyb. Amplification products (15 μ l) were separated on a 2% agarose gel in the presence of Eb (0.2 μ g/ml) and visualized under UV light. Subsequently, the gels were blotted onto Hybond-N⁺ nylon membranes (Amersham, Buckinghamshire, England) and hybridized to the Dig-labeled DNA probe. Hyb signals were visualized via consecutive application of a sheep anti-Dig antibody (Roche) and alkaline phosphatase-labeled rabbit antishoep IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). PCR involving the anchored oligo(dT) primers usually yielded two products, one with the predicted migration behavior and a faster migrating one. The strand composition of each product was determined via Hyb with ³²P-labeled probes of opposing polarity, 1651+ and 1590-.

Cloning and Sequencing of Amplified cDNA. Selected amplification products of full-length and truncated transcripts were ligated into the pCR2.1-TOPO vector (Invitrogen, Groningen, the Netherlands). Sequencing of both strands was performed by the Sanger di-deoxy-chain-termination-method.

Statistical Analysis. Cases positive for HBV seromarkers were analyzed for correlation between age, conventional seromarkers (determined at a 1:200 dilution of the serum), viral DNA, fRNA, and trRNA. The following marker combinations were used to establish subgroups: HBs-positive/HBe-positive, HBs-positive/HBe-negative, HBs-negative/viral nucleic acid-positive, and negative for all markers. Statistical evaluations were carried out using the software S-PLUS (48) and StatXact 3 for Windows (Cytel Software Corp., Cambridge, MA). Pearson's correlation coefficient (*r*) was used to describe relations between circulating viral transcripts or DNA and immunological seromarkers for HBV infection, serum ALT values, or ages of donors, within a 95% CI. To compare ages of different patient groups, the Kruskal-Wallis rank-sum test was used for an overall difference, and the Wilcoxon's rank-sum test (49) was used for pairwise comparisons. Fisher's exact test (two-sided; Ref. 50) was applied to compare frequencies of viral transcripts and DNA, in sera positive and negative for anti-HBc and in cases with or without HCC. Each respective contingency table was

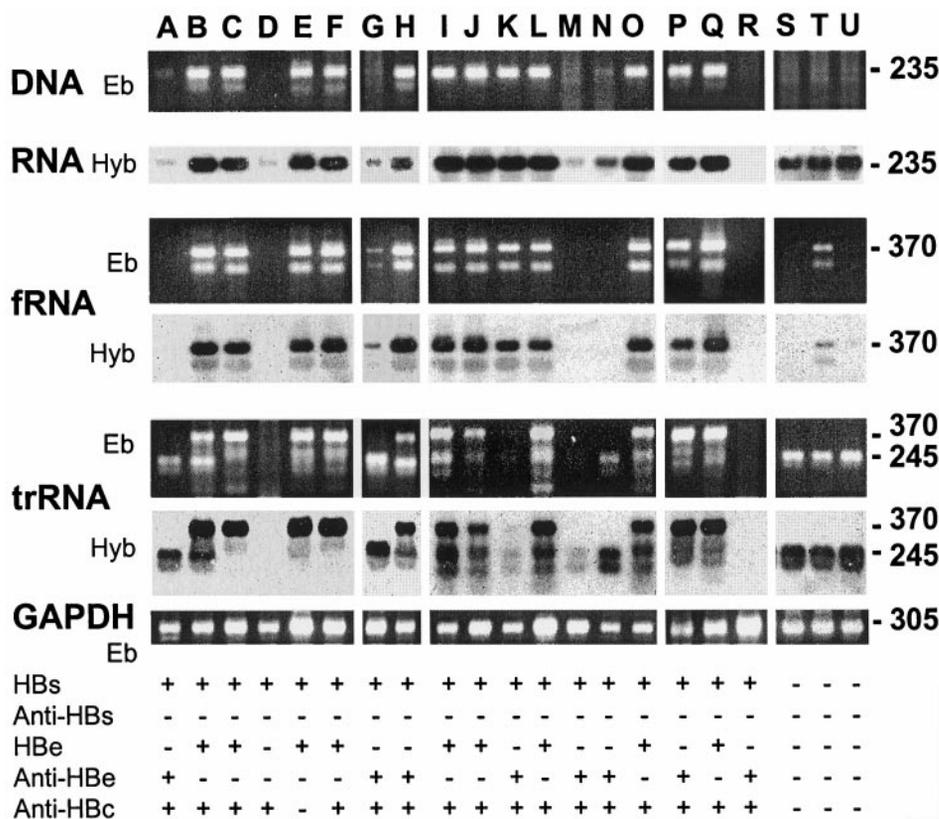


Fig. 2 PCR patterns of serum viral nucleic acids from HBV carriers. Capital letters (top) indicate individual patients. Reaction products were visualized with Eb and through Hyb. Expected positions for amplification products of HBx-region DNA and RNA, fRNA, trRNA, and of GAPDH RNA are indicated together with their sizes in bp (235, 370, 245, and 305 bp, respectively). Amplification products of cases A–H were analyzed in one assay series; cases I–O, P–R, and S–U were analyzed in separate ones. Conventional seromarkers are indicated at the bottom.

assessed for heterogeneity or equivalently for dependency of the two factors generating the table using Fisher’s exact test in the case of smaller samples ($n < 50$). In the case of larger samples ($n \geq 50$), the χ^2 test or an approximate version of StatXact software was used. All correlations are given as absolute (positive) values in this paper. $P < .05$ was regarded as being statistically significant.

RESULTS

Detection of Viral Transcripts and DNA in Sera. Nucleic acids extracted from sera of patients positive or negative for HBs and with or without HCC were analyzed via PCR for viral DNA and RNA with homology to the HBV X-gene region. Primarily, it was examined if this simple approach yields similar transcription and polyadenylation patterns as observed previously for RNA extracted from tissue samples (43).

Proceeding from top to bottom, Fig. 1 outlines HBx target sequences for PCR analyses: (a) PCR for the detection of DNA carrying sequences between map positions 1434 and 1668; (b) RT-PCR for detection of RNA spanning the same sequence; (c) RT-PCR recognizing the junction structure at the poly(A) addition site of full-length transcripts [GCUUC(A)_nI]; and (d) RT-PCR with anchored oligo(dT) primers recognizing the corresponding structure [CCAGC(A)_n] on truncated transcripts. In the first-round amplification for fRNA, the sense primer txs3 (1434) was used, and for trRNA, the sense primer txs (1445) was used. In the second-round PCR, uniformly the sense primer txs1 (1454) was used. A Dig-labeled probe spanning map po-

sitions 1454 and 1668 was used to confirm the HBx-sequence specificity.

RNA specificity of the RT-PCR for X-region transcripts was ensured via parallel PCR testing of samples with and without DNase I pretreatment, as well as with and without reverse transcription. RNA specificity of reactions involving anchored oligo(dT) primers for full-length and truncated transcripts has been demonstrated previously (43, 45). In this study, fRNA and trRNA were identified even in the absence of detectable viral DNA (Fig. 2, cases M and S–U and Fig. 3, cases B, D, and H). These data provide further evidence for the RNA specificity of the reactions conducted. For all 437 sera examined, a GAPDH amplification product of 305 bp was obtained, as expected for GAPDH transcripts, but not of 590 bp, as expected for GAPDH-DNA amplification (46).

Figs. 2 and 3 illustrate representative PCR patterns. In the case of viral DNA and GAPDH mRNA, only the Eb-stained gels are shown; in the case of HBx RNA, the Hyb data are shown; and in addition, in the case of fRNA and trRNA, the Eb-stained gels are shown. HBx RNA signals (Figs. 2 and 3, RNA) are present in most of the cases selected but displayed a wide range of intensities. fRNA signals visualized both by Eb staining and Hyb appear as a double band, with the upper one migrating at the expected position of 370-bp DNA and a faster migrating lower band, which represented single-stranded minus-strand DNA generated by asymmetric amplification as revealed by strand-polarity analysis. A similar tendency for asymmetric amplification was also observed for trRNA (data not shown).

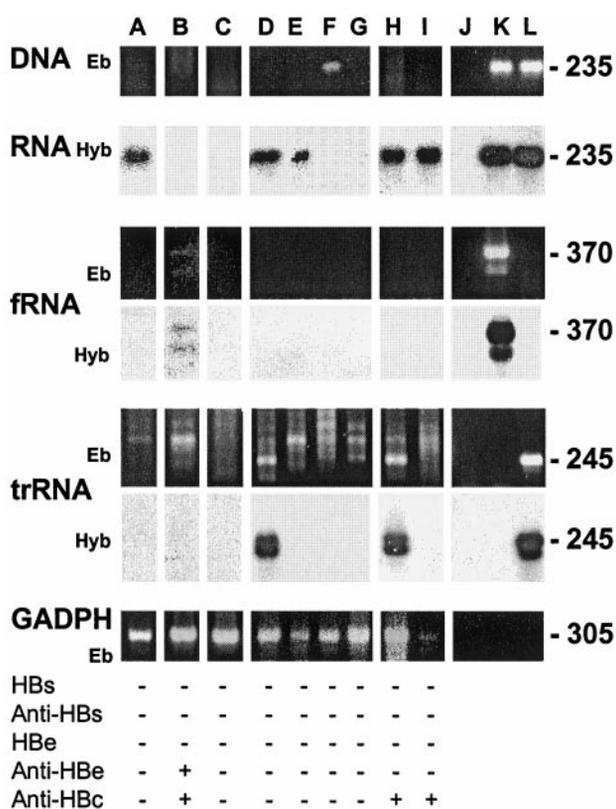


Fig. 3 PCR patterns of HBV nucleic acids from sera of HCV carriers with HCC. Capital letters (*top*) indicate patients (A–I) and control reactions: water (J), full-length cDNA of pMT9T40A (K), and truncated cDNA of pMT9T41A (L). The RT-PCR amplification products of A, B and C, and D–L were analyzed within three series. For GAPDH and conventional seromarkers, see Fig. 2.

The amplification of the 3'-end region of trRNA gave rise to the expected 245-bp product. In all cases positive for the fRNA, an additional 370-bp amplification product was obtained. Cloning and sequencing of this 370-bp cDNA segment confirmed its identity with that of the authentic fRNA amplification product, except for its very 3'-end on which the txas5 anchor was connected to positions ranging from 1803 to 1806. The presence of the trRNA-specific 245-bp product was independent of the presence or absence of the 370-bp product (Fig. 2, compare cases A–C). Apparently, the RT-PCR procedure adapted for the analysis of trRNA from sera recognized both fRNA and trRNA.

PCR signal intensities for fRNA and trRNA obtained with a nucleic acid equivalent of 40 μ l of serum were graded into weak (1+), moderate (2+), and strong (3+) and related to values obtained with 1 fg, 10–100 fg, and \geq 1 pg of cDNA (pMT9T41A). These estimations do not take into account the reduction in sensitivity by background RNA, as described by Schutz *et al.* (45), and, therefore, represent minimum values.

On the basis of presence and intensities of signals for full-length and truncated transcripts, five patterns were distinguished: (a) fRNA dominance at high (3+; Fig. 2, cases B, C, E, F, H–J, O, and Q) and (b) at low levels (2+ or 1+; Fig. 2, case K and Fig. 3, case B); (c) trRNA dominance (Fig. 2, cases

G and T); (d) trRNA exclusively (ranging from 1+ to 3+; Fig. 2, cases A, M, N, S, and U and Fig. 3, cases D and H); and (e) absence of both RNA types (–/–; Fig. 2, case D and R and Fig. 3, cases A, E, and I).

Sequence Variation on the HBx-ORF Segment Amplified by RT-PCR Involving Anchored Oligo(dT) Primers. Amplification products obtained in RT-PCR assays were cloned and sequenced. Table 1 presents base substitution patterns for individual cases of fRNA and trRNA predominance, respectively. Positions different from a putative wild-type sequence (at least in two cases) are indicated by bold letters. Considering 1635^T/1637^A (*Xho*I coordinates; corresponding to *Eco*RI coordinates 1762/1764), 1526^T, and 1626^C or ^G as indicators of an elapsed period of chronicity (reviewed by Kramvis and Kew in Ref. 51), trRNA (Table 1, trRNA) can be assigned to conserved as well as less conserved templates of viral DNA. Hence, trRNA originates early and late during chronic infection. Similar base substitution patterns were gained for fRNA (Table 1, fRNA), although fRNA tended to be less conserved than trRNA.

Viral Transcripts in HBs-positive Sera. Signals for HBV transcripts were identified in the majority of the 237 HBs-positive samples analyzed. Overall, full-length and truncated transcripts were detected in 60% of the cases (Table 2). An HBx RNA signal, *i.e.*, a signal for sequences shared by both fRNA and trRNA, was obtained for almost all samples with full-length and/or truncated viral transcripts. If present, intensities of the HBx RNA signal were in accordance with those for full-length transcripts (Fig. 2); in cases with dominance or exclusive presence of truncated transcripts, HBx RNA signals (one round of RT-PCR) usually appeared weaker than those for truncated transcripts after the seminested (double round) RT-PCR (Fig. 2, cases A, G, N, and S). In some of the HBs-positive samples in which neither full-length nor truncated transcripts were identified (39 of 237), a weak signal was obtained for HBx RNA (Fig. 2, case D), indicating the presence of transcripts not polyadenylated at either of the two viral poly(A) signals.

Full-length Transcripts Are Related to Replicative Processes. Some of the sera with viral nucleic acids were HBs negative. Together with the HBs-positive sera, they were subjected to Pearson's correlation test to assess a relationship between the level of the full-length or truncated viral transcripts and that of viral DNA. As shown in Fig. 4A, a close correlation existed between the levels of full-length transcripts and viral DNA ($r = 0.79$, $P < .05$). It is noteworthy that subgroups with full-length transcripts levels 1+–3+ were found in the group showing the highest levels of viral DNA (3+). In HCC patients, the combination of high DNA levels with full-length transcripts at lower levels appeared to be more frequent compared with HCC-free carriers (data not shown). In summary, most of the sera with high levels of full-length transcripts contained high amounts of viral DNA. By contrast, the correlation between truncated transcripts and circulating viral DNA was very weak ($r = 0.14$, $P < .05$; Fig. 4B).

HBe and HBs Seroconversion Is Correlated with a Decrease in Full-Length Transcripts. The average ages of HBs carriers, HBe positive and HBe negative, were 28.7 and 42.0 years, respectively (difference 28.7/42.0 years; $P < .0001$). The average age of HBs-negative patients positive for viral nucleic acids cases was 52.7 years (difference 42/52.7 years:

Table 1 Sequence variation on fRNA and HBV RNA trRNA^a

fRNA (17) ^b	Position ^c								trRNA (16) ^b
	N Cases	1484	1486	1526	1592	1600	1619	1626	
0	G	G	C	T	A	G	T	AG	1
1	G	G	C	T	A	G	T	AG	0
0	C	G	C	T	G	G	T	AG	2
0	G	G	C	T	G	C	T	AG	2
0	G	G	T	T	G	G	T	AG	1
0	G	A	C	T	G	G	T	AG	1
1	G	G	C	T	G	G	T	AG	2
1	G	G	C	G	A	G	T	AG	0
1	G	G	C	G	C	G	T	AG	0
0	G	A	C	T	A	G	T	TA	1
1	G	A	C	T	A	G	C	TA	0
1	G	A	T	T	A	G	T	TA	1
2	G	G	C	T	A	G	C	TA	0
0	G	G	C	T	A	G	T	TA	1
2	G	G	T	T	A	G	T	TA	2 ^d
0	G	G	T	G	G	G	T	TA	1
1	G	G	C	G	G	G	C	TA	0
1	G	G	C	T	G	G	G	TA	0
0	G	A	C	T	G	G	G	TA	1
1	G	G	C	T	G	G	T	TA	0
2	G	G	C	G	A	G	C	TA	0
1	G	A	T	G	A	G	T	TA	0
1	G	A	C	G	T	G	T	TA	0

^a cDNA clones established via direct (before separation on agarose) ligation into the pCR 2.1-TOPO vector of amplification products. Positions different from a putative wild-type sequence are indicated by bold letters.

^b Representative cDNA clones established from sera of 17 patients displaying full-length RNA dominance and of 15 patients displaying truncated RNA dominance. Additionally, truncated RNA was cloned and sequenced from one of the patients displaying full-length RNA dominance (see^d).

^c Substitutions on a sequence segment representing both full-length and truncated HBV RNA. *XhoI* coordinates (1484 *XhoI* = 1611 *EcoRI*).

^d In one case, both full-length and truncated RNA were determined.

Table 2 Prevalence of HBV transcripts and DNA in sera positive or negative for HBs from individuals with or without HCC

Donors	N Cases	HBx-DNA and/or transcripts ^a	HBx DNA ^a	Transcripts ^{a,b}		
				HBx RNA	fRNA	trRNA
HBs + ^b	237	220 (92.8)	165 (69.6)	211 (89.0)	143 (60.3)	142 (59.9)
HBs + HCC	69	62 (89.9)	58 (84.1)	56 (81.2)	46 (66.7)	34 (49.3)
HBs + HCC-free	168	158 (94.0)	107 (63.7)	155 (92.3)	97 (57.7)	108 (64.3)
HBs - HCC ^c	49	30 (61.2)	10 (20.4)	19 (38.8)	9 (18.4)	14 (28.6)
HBs - anti-HBVAg +/HCC ^d	26	19 (73.1)	6 (23.1)	12 (46.2)	7 (26.9)	10 (38.5)
HBs - anti-HBVAg -/HCC	23	10 (43.5)	3 (13.0)	7 (30.4)	2 (8.7)	4 (17.4)
HBs - other patients ^e	84	16 (19.0)	1 (1.7)	7 (8.3)	6 (7.1)	11 (13.1)
Healthy Chinese ^f	37	4 (10.8)		3 (8.1)		2 (5.4)
Healthy German ^g	30					

^a Number of cases positive, in parentheses, percentage of cases examined. HBx DNA and HBx RNA were tested via single-round PCR and fRNA, as well as trRNA via a semi-nested two-round PCR procedure (as described in "Materials and Methods").

^b Figures add up to >100% because of an overlap of subgroups.

^c Sera of 42 Chinese (3 anti-HCV +) and of 7 Japanese patients (6 anti-HCV +).

^d 21 anti-HBc +, 7 with and 14 without anti-HBs or anti-HBe; 5 only anti-HBs +.

^e 33 anti-HBVAg + (11 anti-HBc +, 13 anti-HBs +, 1 anti-HBe +, 8 anti-HBc +, and anti-HBs +) and 42 anti-HBVAg -. In addition, 9 patients with cryptogenic cirrhosis: 7 anti-HBVAg + (2 anti-HBc +, 3 anti-HBs +, and 2 anti-HBc +, and anti-HBs +) and 2 anti-HBVAg -.

^f Routine physical and laboratory checkups, city residents of Xi'an, China.

^g Blood donor applicants, from Lübeck, Germany.

$P < .001$). The level of serum viral DNA was high in most HBe-positive cases, greatly reduced after HBe seroconversion and further decreased after HBs seroconversion ($r = 0.58$, $P < .05$; Fig. 5A). A similar pattern was observed for full-length viral transcripts ($r = 0.60$, $P < .05$; Fig. 5B). By contrast, no significant change was observed for trRNA during progression to HBs-negative stages ($r = 0.008$, $P > .05$; Fig. 5C). It can be

concluded that there is a sequential change in the ratio of the two forms of viral transcripts.

Viral DNA and Full-Length Transcripts Are Related to ALT Levels. It is conceivable that the presence of viral RNA in sera is related to hepatocyte damage. If so, the assay system used in this study would only be of relevance for patients with a chronic hepatitis of measurable activity. Serum ALT values

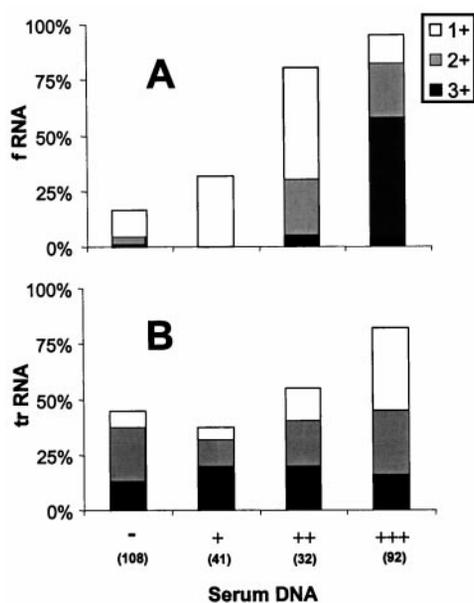


Fig. 4 Correlation of serum DNA and RNA levels. The fraction of cases positive for fRNA (A) and for trRNA (B) are given for groups of patients without (-), low (+), moderate (2+), and high levels (3+) of serum DNA. Case numbers for each group are within parenthesis (abscissa). The respective amounts of RNA are indicated by different shadings (top right). fRNA (A): $r = 0.79$, $P < .05$, 95% CI = 0.73–0.85; trRNA (B): $r = 0.14$, $P < .05$, 95% CI = 0.03–0.26.

reflecting liver damage then would be correlated to circulating viral transcripts. To address this question, 171 HBs-positive cases with serum ALT values (IU/liter) available were divided into groups with normal range (0–40 IU/liter), slight (41–80 IU/liter), or marked (>80 IU/liter) elevation. The fraction of sera with full-length transcripts was increased concomitantly with the elevation of ALT values ($r = 0.28$, $P < .05$; Fig. 6B) and in parallel with the levels of the viral DNA ($r = 0.39$, $P < .05$; Fig. 6A). However, the levels of truncated transcripts were not significantly correlated with ALT values ($r = 0.04$, $P > .05$; Fig. 6C). Apparently, the presence of truncated transcripts is not related to liver damage, whereas this applies to a certain degree for viral DNA and fRNA.

Age-related Progression to Nonreplicative Stages and Persistence of trRNA. Most of the chronic carriers in endemic areas are believed to have been infected at neonatal stage or early childhood (1, 2). Therefore, viral RNA and DNA patterns in sera from 168 HBs-positive and 20 viral nucleic acid-positive carriers of different ages were compared. Data from sera of patients with HCC were excluded from this analysis. At a young age, the full-length transcripts were found at high levels. They dropped in later decades ($r = 0.32$, $P < .05$; Fig. 7B) in parallel with a decline of viral DNA ($r = 0.31$, $P < .05$; Fig. 7A). An age-dependent decline of truncated transcripts was not found ($r = 0.07$, $P > .05$). In the first decade (samples from only 9 patients were available), its level was lower than observed for full-length transcripts. Sustained high levels were observed starting with the second decade (Fig. 7C). In the age group 30–50 years, fRNA dominance at high levels gradually decreased to dominance at lower levels. This was mainly found

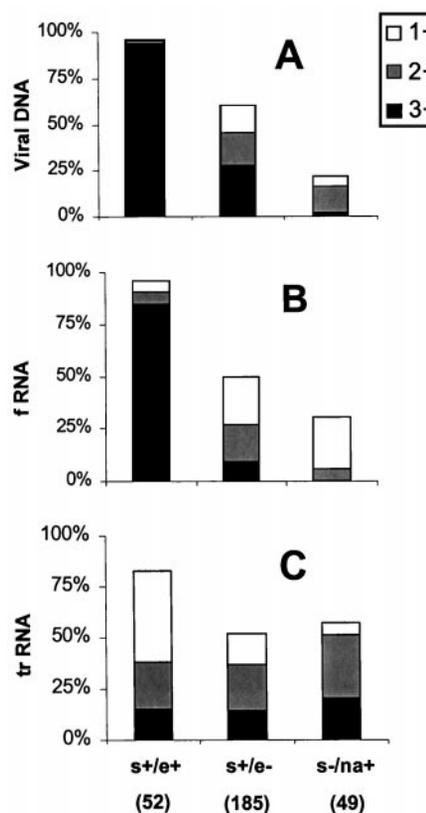


Fig. 5 Representation of viral nucleic acids at HBe and HBs seroconversion. Cases positive for viral DNA (A), fRNA (B), and trRNA (C) are in HBs-positive groups before and after HBe seroconversion (s+/e+ and s+/e-, respectively) and in an HBs-negative group carrying viral nucleic acids (s-/na+). Case numbers are in parenthesis (abscissa). Viral DNA (A): $r = 0.58$, $P < .05$, 95% CI = 0.51–0.64; fRNA (B): $r = 0.60$, $P < .05$, 95% CI = 0.53–0.67; trRNA (C): $r = 0.008$, $P > .05$, 95% CI = -0.12–0.11.

in cases seronegative for HBe with high levels of viral DNA (see Fig. 5, A and B, middle columns). In summary, a gradual decrease in fRNA dominance accompanied by a relative increase in the fraction of trRNA was observed (Fig. 7D).

The mean age of patients with fRNA dominance at high levels (27.5 years) was significantly lower ($P < .005$) than that of patients with fRNA dominance at low levels (36.6 years), with trRNA dominance (34.6 years), with trRNA only (39.2 years), and with both RNA types being absent (40.7 years).

Viral Transcripts in HBs-negative Sera from HCC Patients. Even in HBs-negative HCC patients (Table 2), serum HBV nucleic acids were detected (29 of 49, 49.2%). Their prevalence was higher in sera positive (19 of 26, 73.1%) than in sera negative (10 of 23, 43.5%) for anti-HBc and anti-HBs ($P < .05$). Among the 29 positive cases, 20 were positive only for transcripts, 5 were positive for both transcripts and viral DNA, and 4 showed a weak signal only for viral DNA.

Among the HBs-negative sera from HCC patients, 9 were HCV positive. Only 3 of these were anti-HBc positive, whereas 7 were shown to be positive for the HBV viral nucleic acids. Among the 7 cases, 5 were positive for HBx RNA with 2 being

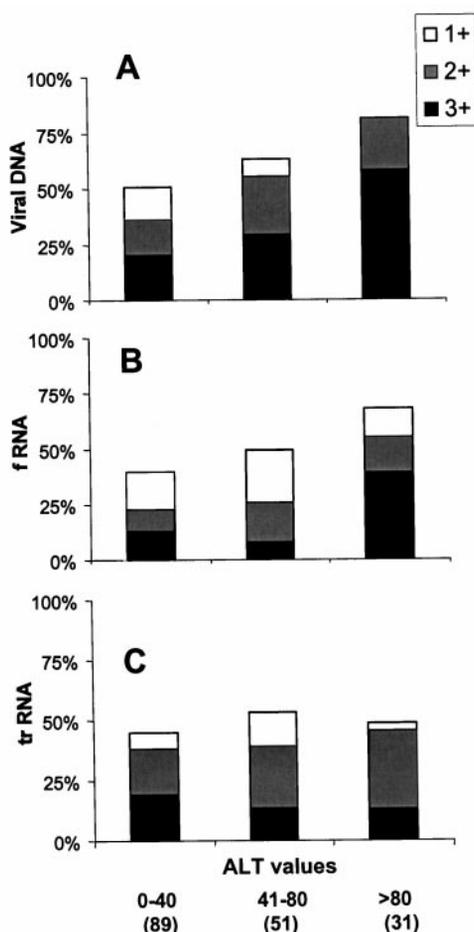


Fig. 6 Viral nucleic acids in sera with different ALT activities. Levels of viral DNA (A), fRNA (B), and trRNA (C) are in patient groups with distinct ranges in ALT activities (IU/liter). Case numbers are in parenthesis (abscissa). Viral DNA (A): $r = 0.39$, $P < .05$, 95% CI = 0.24–0.55; fRNA (B): $r = 0.28$, $P < .05$, 95% CI = 0.11–0.46; trRNA (C): $r = 0.04$, $P > .05$, 95% CI = 0.20–0.13.

positive for both HBx RNA and truncated transcripts. One case of 7 showed only full-length transcripts, one only viral DNA (Fig. 3).

Viral Transcripts in HBs-negative Sera from Patients with Diseases Apparently Not Related to HBV. HBs-negative sera from 9 patients with cryptogenic cirrhosis and 75 patients with extrahepatic chronic diseases were examined as a reference group (Table 2). Of the cirrhosis patients, 5 were positive for viral RNA (3 for truncated, 2 for full length), whereas no case was positive for viral DNA. Of the patients with extrahepatic diseases, 11 were positive for viral RNA. Among these, transcripts were polyadenylated exclusively (7 cases; Fig. 2, cases S and U) or predominantly (2 cases; Fig. 2, case T) at the cryptic poly(A)-signal in 2 cases only at the major poly(A)-signal.

Viral Transcripts in HBs-negative Sera from Apparently Healthy Individuals. Samples from 37 urban inhabitants from Xi'an, China and 30 blood donors from Lübeck, Germany served as reference groups. In no case was viral DNA

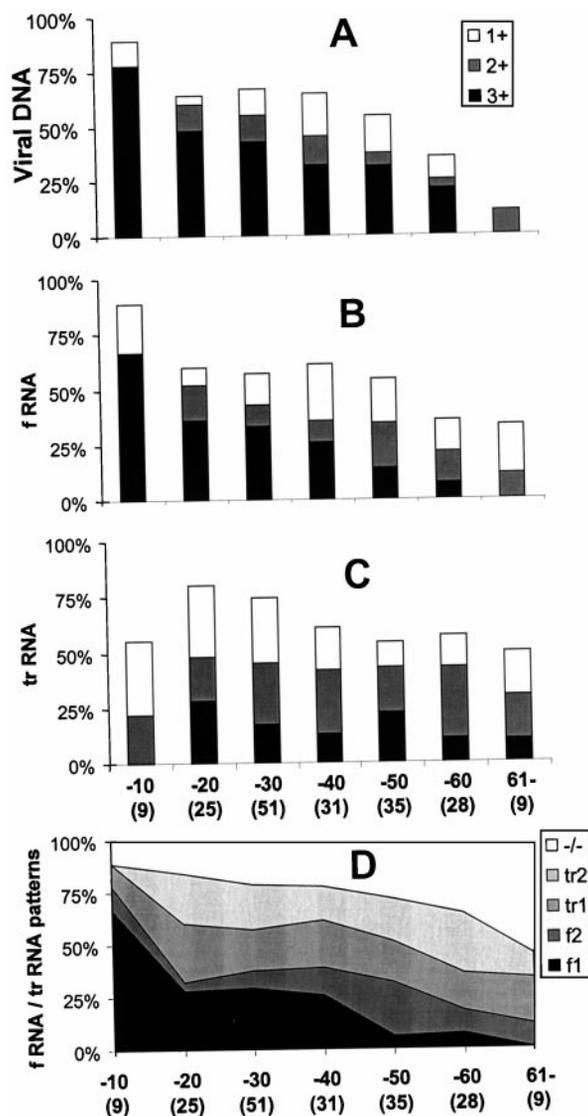


Fig. 7 Age-related changes in the representation of viral nucleic acids in sera. Levels of serum DNA (A), fRNA (B), and trRNA (C) are shown in patients with increasing ages (≤ 10 years, from 11 to 20 years, etc.). Case numbers are in parenthesis (abscissa). D, representation patterns of f/trRNA for the same age groups. -/-, absence of both RNA types; tr2, only trRNA; tr1, trRNA dominance; f1, fRNA dominance at high levels; f2, fRNA dominance at low levels. Viral DNA (A): $r = 0.31$, $P < .05$, 95% CI = 0.17–0.44; fRNA (B): $r = 0.32$, $P < .05$, 95% CI = 0.20–0.45; trRNA (C): $r = 0.07$, $P > .05$, 95% CI = 0.07–0.22; f/trRNA patterns (D): $r = 0.31$; 95% CI = 0.18–0.43.

present (Table 2). In the Chinese group, 4 cases (10.8%) were positive for HBx transcripts. In the German group, no transcript signal was obtained.

DISCUSSION

In endemic areas, infection with HBV occurs perinatally or in early childhood and is linked to a high risk for chronicity (1–7). Initially, the infection is accompanied by high replication levels reflected in the serum by HBe and viral DNA. Mutations affecting the PreC region or its promoter and the gradual de-

crease in replication levels lead to a loss of these markers (reviewed by Brunetto *et al.*; Ref. 52). Loss of HBs (53) and development of anti-HBe characterize later stages.

Thus far, it is generally accepted that, after HBs clearance, viral DNA cannot be detected in serum (54). However, viral DNA has been repeatedly detected in the liver (29, 33, 55–58). In addition, viral RNA, specifically X-region RNA, has been found in hepatic tissues including HCC from HBs-negative patients (29, 33, 59–61). Transcription from integrated DNA (28–30) and low-level replication as observed after HBs seroconversion (57, 62–65) may contribute to the pool of viral RNA. Superinfection with HCV (66, 67) has been related to an inhibition of HBs production, possibly creating conditions where viral RNA is found in the absence of seromarkers indicating replicative processes. Studies on RNA from tissues of chronically infected liver have provided insights into discernible species of HBV transcripts (36, 43, 59). Less is known about changes in the patterns of HBV transcripts during chronic infection. A release of RNA into the blood as observed by Chen *et al.* (68) for telomerase RNA in breast cancer patients should facilitate studies addressing this subject.

In the present study, X-region RNA was identified in 90% of sera from HBs carriers. In line with its preferential maintenance in liver tissue (59), X-region RNA was also present in 40% of sera from HCC patients negative for HBs. Our findings thus render HBx-region RNA a potential seromarker for late nonreplicative carrier states.

Full-length transcripts were closely related to serum DNA and HBe and also to the injury of liver parenchyma as reflected by ALT values. In the course of chronic infection, levels of the full-length transcripts declined in parallel to those of viral DNA. These observations confirm the link between full-length viral transcripts and virus replication, as suggested by Kairat *et al.* (43) on the basis of the analysis of tissue samples.

No pronounced association was observed between circulating truncated viral transcripts and replication parameters or ALT levels. Of all HBV seromarkers examined, trRNA appeared to be the most persistent one (see Fig. 7). Its fraction of total X-region RNA (HBx RNA) increased with increasing age. Similarly, the fraction of HBx RNA polyadenylated at neither the standard nor the cryptic terminating site (HBx RNA found in the absence of full-length and truncated transcripts) increased during chronic infection, whereas DNA is absent in serum of many cases. It is tempting to assume that these X-region RNA species represent virus/cell RNA hybrid molecules and that they are cotranscribed with trRNA from chromosomally integrated HBV DNA at late stages of infection.

The existence of viral RNA in serum is difficult to explain in view of the known presence of RNases (69). Capsids as the principal protecting elements are not very likely, because viral RNA is found also in the absence of replication markers. Furthermore, nucleocapsids are not released from hepatocytes before hydrolysis of the RNA pregenome during its transcription into +strand DNA (70). A possible release of viral RNA not mediated by a capsid structure is further indicated by the absence of HBc-region RNA in cases with a high representation of X-region RNA observed previously for hepatic tissues (43). However, it cannot be totally excluded that there exists some association between viral RNA and circulating virions (71). It

could be conceived that plasma membrane vesicles shed from hepatocytes (72, 73) contain and protect viral RNA; however, other protective elements may exist.

Viral transcripts in serum constitute new parameters to recognize and differentiate apparent and inapparent stages of HBV infection. They are detectable even in apparently healthy individuals without a history of overt infection. For a more accurate estimation of prevalence of these transcripts and assessing their significance, it is necessary to screen large numbers of sera collected from individuals in areas of high and low endemicity. The clinical relevance of HBV infection in the absence of serological markers has been recognized in liver transplantation where the entry of HBV infection into an acute stage is not rare (74).

The sustained expression of HBx for several decades, even after reaching nonreplicative stages, may expose the host to an increased risk for HCC and may be more important for the induction of the tumor than for its continuous growth (29, 43, 59). There are reports on the presence of X-region DNA and RNA in liver tissue of HBs-negative HCV-infected patients (55, 58, 61, 75), indicating also for these cases a role of HBx in HCC development. This notion is further supported by prospective studies which indicate that liver tissue HBx DNA and RNA and the serum marker anti-HBc confer an elevated risk to develop HCC (61, 76). In the present study, we identified HBx RNA in sera of a large number of patients with inapparent HBV infection, including HCV-infected HCC patients. Thus, serodetection of HBV RNA potentially substitutes tissue analysis requiring liver biopsies and may even be more informative and representative.

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