A Short Region in the Genome of Hepatitis B Virus Is Critical for Maintenance of High Transcript Levels

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INTRODUCTION

Exposure to hepatitis B virus (HBV) often leads to persistent viral infection of the liver, cirrhosis, and hepatocellular carcinoma. HBV is a DNA virus that replicates asymptomatically through reverse transcription of an RNA intermediate. HBV has a 3.2-kb partially double-stranded DNA genome from which four major classes of transcripts are synthesized. The 3.5-kb pregenomic RNA not only serves as template for reverse transcription, but also contains coding regions for the nucleocapsid protein and the reverse transcriptase. A subclass of this transcript with a slightly longer 5’ end codes for the precore protein, which, after processing, is secreted as HBV e antigen (HBeAg). The 2.4-kb RNA encompasses the preS1 open reading frame (ORF) that encodes the large surface (L) protein. The 2.1-kb RNA contains the preS2 and S ORFs that encode the middle (M) and small (s) surface proteins, respectively. The smallest transcript (approximately 0.9 kb) codes for the X protein. A common polyadenylation site is believed to be utilized by all transcripts.

Mutations and deletions in the HBV genome have frequently been detected during persistent viral infection. In this regard, studies have emphasized the potential pathogenic role of mutations in the precore and core promoter regions, the precore and core ORFs, and the pre-S region (Gerken et al., 1991; Melegari et al., 1997; Okamoto et al., 1987; Tran et al., 1991). For example, HBV variants exist in certain patients that carry deletions in the promoter region for the M and s proteins and therefore are unable to direct the synthesis of transcripts coding for these proteins. This phenomenon may influence the pathogenesis of HBV infection.

Little is known about determinants governing the intracellular stability and translocation of HBV transcripts. Despite numerous well-conserved regions in the HBV genome that represent consensus sequences for splice donor and acceptor sites, spliced transcripts of HBV have been detected only at low levels (Chen et al., 1989; Rosmorduc et al., 1995; Su et al., 1989a; 1989b; Suzuki et al., 1989, 1990; Terre et al., 1991; Wu et al., 1991), and neither a function for splicing in viral replication nor a role for splice transcripts and associated novel proteins in the pathogenesis of chronic hepatitis has been firmly established. It appears that the majority of HBV transcripts are not spliced during the viral life cycle, which raises the question why several splice donor and acceptor sites are conserved on HBV transcripts. In particular, the genome region between nt 450 and 500 of the HBV genome appears to be rich in such sequences. In this study we deleted a short 30-nt sequence between a conserved splice donor site at HBV genome position 462 and a splice acceptor site at position 491, thus deleting the surface/polymerase open reading frames by 10 amino acid residues. At the transcriptional level, this deletion led to >99% reduction of the 2.1-kb class of subgenomic transcripts in transfected cells. Nuclear run-on experiments revealed that the transcription rate of the deleted 2.1-kb transcript is unchanged when compared with the wildtype, suggesting a posttranscriptional mechanism for the downregulation of the deleted transcript. In addition, experiments with a replication-competent HBV mutant containing the 30-nt deletion showed that the corresponding 10-amino-acid sequence within the reverse transcriptase domain of the polymerase protein appeared to be nonessential.
from the 3.5-kb class of transcripts (Rosmorduc et al., 1995; Su et al., 1989a; Suzuki et al., 1990; Terre et al., 1991; Wu et al., 1991).

The present study demonstrates that the short 30-nt sequence between the splice donor site at HBV genome position 462 and the splice acceptor site at HBV genome position 491 appears to be essential for the posttranscriptional maintenance of the 2.1-kb class of subgenomic transcripts. Deletion of this sequence also had modulatory effects on the levels of 3.5- and 2.4-kb transcripts. However, experiments with a HBV mutant containing the 30-nt deletion show that the corresponding 10-amino-acid sequence within the reverse transcriptase (RT) domain of the polymerase protein appears to be nonessential for viral DNA synthesis.

RESULTS

Splicing of HBV at genome position 462

For the study of certain regions of the HBV genome for use as antisense and sense RNAs, a subgenomic fragment of the HBV genome (HBV 288-919; G of the EcoRI site is position 1) was inserted in the antisense and, for control purposes, in the sense orientation (relative to the orientation in the HBV genome) into the eukaryotic expression vector pC-GR N-795 (Rusconi et al., 1990) under control of the cytomegalovirus immediate-early (CMV-IE) promoter. In the vector pC-GR N-795, sequences from the rabbit $\beta$-globin-like gene cluster, including the 573-nt-long intron 2, are located 3' to its unique BamHI cloning site to facilitate nucleocytoplasmic export of transcribed and subsequently spliced RNAs, thereby enhancing the number of transcripts available for translation (see Fig. 1A). In order to analyze expression of the antisense and sense RNAs, the constructs 288-919 antisense and 288-919 sense were transiently transfected in HuH-7 hepatoma cells. Analysis of total cellular RNA on Northern blot and hybridization with a HBV-specific probe revealed that the HBV 288-919 antisense transcript had the expected size of approximately 1300 nt including a poly(A) stretch of an estimated length of 200 to 300 nt (Fig. 1B, lane 1). However, the HBV 288-919 sense transcript appeared to be 800 nt in length (lane 2), much shorter than the expected size of 1300 nt.

This finding was unexpected because several other antisense/sense RNA pairs studied in similar experiments were of the same apparent length (data not shown). It was hypothesized that the smaller size of the
HBV 288-919 sense transcript was due to an aberrant splicing event involving, on the 5' end, certain sequences within the HBV portion of the transcript and on the 3' end portions of the rabbit β-globin intron 2. As will be described elsewhere in more detail (zu Putlitz et al., manuscript in preparation), this possibility was investigated by performing RT-PCR from total cellular RNA of HBV 288-919 antisense- and HBV 288-919 sense-transfected HuH-7 cells. Sequence analysis of subcloned PCR fragments revealed that the smaller HBV 288-919 sense RNA was indeed spliced at a putative splice donor (5') site within the HBV genome, located at position 462. It was noteworthy that this splice donor site, which is highly conserved among all genotypes of HBV (see Fig. 2), was located exactly 30 nt upstream of an also highly conserved and well-characterized splice acceptor (3') site (at position 491) previously described in the context of generation of spliced transcripts of HBV (Rosmorduc et al., 1995; Su et al., 1989a; Suzuki et al., 1990; Wu et al., 1991). Thus, splicing of a heterologous transcript was observed at the splice donor site position 462 that was located in close proximity to the splice acceptor site at position 491. Both sites lie within a genome region of HBV that encodes the surface antigens and the reverse transcriptase domain of viral polymerase.

A 30-nt deletion in the reverse transcriptase region of the polymerase gene does not affect viral DNA synthesis, but abolishes surface antigen production.

The observed high degree of conservation between genotypes with respect to splice consensus sequences was noteworthy because most HBV transcripts are not spliced. We hypothesized that the clustered splice consensus sequences played some unknown important functional role(s) in the life cycle of HBV. This possibility was first investigated by generation of a 30-nt deletion between genome positions 462 and 491 to lead to 10-aa in-frame deletions (boxed) in both reading frames. This deletion position is a previously identified HLA-A2-restricted CTL epitope located in the polymerase ORF (Rehermann et al., 1995).
pregenomic RNA incorporating the described 30-nt deletion, termed pTC-HBV-D30, was generated. The 30-nt deletion in pTC-HBV-D30 was predicted to lead to 10-aa in-frame deletions in all three surface proteins and the reverse transcriptase domain of viral polymerase (see Fig. 2B). The parental construct, pTC-HBV, contains endogenous promoter regions for all subgenomic HBV transcripts and therefore gives rise to virus particles when transfected into hepatoma cells. pTC-HBV-D30 and its wildtype control, pTC-HBV, were transiently transfected into HuH-7 hepatoma cells. pTC-HBV-FS645, a CMV-IE promoter-driven frameshift mutant of HBV that was encapsidation- and replication- incompetent (unpublished data), was used as negative control in these experiments. First, encapsidation of pregenomic transcripts into nucleocapsids was investigated. For this purpose, transfected cells were lysed and nucleocapsids were precipitated with PEG (Tong et al., 1992) and digested with proteinase K. Using this procedure, only the portion of pregenomic RNA that is encapsidated into nucleocapsids (and is therefore available for replication) will be detected on Northern blot (Fig. 3A), whereas all unpackaged nucleic acids are eliminated by prior DNase and RNase digestion. As demonstrated in Fig. 3A, lane 1, a strong signal corresponding to pregenomic RNA (length 3.5 kb) was observed when WT HBV DNA was transfected. The same result was obtained in the case of pregenomic RNA transcribed from the plasmid pTC-HBV-D30 (lane 3), while no signal representative of encapsidated pregenomic RNA was detectable in the HBV frameshift mutant HBV-FS645. These data suggested that the deleted pregenomic RNA could be efficiently encapsidated into nucleocapsids.

Next, viral DNA synthesis of WT HBV and the D30 mutant was explored. Transient transfections with plasmids pTC-HBV, pTC-HBV-D30, and pTC-HBV-FS645 were performed as described above. Four days after transfection, nucleocapsid-associated replication products of HBV were analyzed on Southern blot with a HBV-specific probe. As demonstrated in Fig. 3B, lane 4, the D30 mutant exhibited a pattern of hybridization similar to the WT pattern (lane 2). All HBV products indicative of virus replication, namely, relaxed-circular (RC), double-stranded linear (DS), and single-stranded (SS) DNA, were observed. This result suggested that the D30 mutant was competent for DNA synthesis in transfected cells, despite a 10-aa de-
ulation in the reverse transcriptase domain of the viral polymerase. As expected, no replication was detected with the mutant HBV-FS645 (lane 3). Cell culture supernatants from transfected cells were assayed by radioimmunoassay for the presence of the viral surface antigen (HBsAg) and the core antigen (HBeAg) (Fig. 3B). HBeAg was detectable in supernatants from D30-, WT-, and FS645-transfected cells. However, quantitation of HBeAg revealed 80% reduction of HBeAg levels in the case of HBV-D30 and 50% reduction in HBV-FS645 (data not shown). Of note, no HBsAg was detectable in cell culture supernatants from HBV-D30-transfected cells, either by monoclonal radioimmunoassay or by immunodetection of surface antigens on Western blot with a polyclonal anti-HBs antiserum (data not shown). Transient transfections with recircularized HBV monomers were performed to assess the impact of the 30-nt deletion in the absence of any heterologous regulatory elements. For this purpose, the construct adw-R9-D30 was generated. HBV monomers were generated from this construct and the parental plasmid, adw-R9. Wildtype HBV and deleted monomers were religated and transfected into HuH-7 HCC cells. As demonstrated in Fig. 3B, lane 7, the D30 mutant exhibited a pattern of hybridization similar to the WT pattern (lane 6), confirming the results obtained with the CMV promoter bearing constructs. Cell culture supernatants from transfected cells were assayed by radioimmunoassay for the presence of HBsAg and HBeAg. HBeAg was detectable in supernatants from both adw-R9- and adw-R9-D30-transfected cells. No HBsAg was detectable in supernatants from adw-R9-D30-transfected cells. Taken together, these results demonstrated that the deletion of 10 aa in the reverse transcriptase domain of polymerase does not affect the competence of the viral enzyme for DNA synthesis but that the deletion of the corresponding 10 aa in the surface antigen ORF leads to undetectable HBsAg levels.

Transcript analysis

The possibility that transcription or translation of surface proteins was altered in the D30 mutant was investigated next. In one set of experiments, cells were transfected with the CMV promoter bearing constructs pTC-HBV (Fig. 4, lane 1), pTC-HBV-FS645 (lane 2), or pTC-HBV-D30 (lane 3). In a second set of experiments, cells were transfected with recircularized HBV monomers derived from the construct adw-R9 (Fig. 4, lane 5) or recircularized, deleted HBV monomers derived from the construct adw-R9-D30 (lane 6). Lysates from transfected cells were analyzed for the synthesis of HBV transcripts by Northern blot with a HBV-specific probe (Fig. 4, top). For loading control purposes the same membranes were stripped and reprobed with a probe specific for the acidic ribosomal phosphoprotein PO (Krowczynska et al., 1989) (Fig. 4, bottom). The transcription efficiencies as determined by human growth hormone (hGH) assay were similar in all individual experiments. For WT HBV, three major classes of transcripts, designated 3.5 kb (pregenomic RNA), 2.4 kb (the transcript encoding the L protein), and 2.1 kb (the transcript encoding the M and s proteins) were detectable (Fig. 4, lane 1). Because transcription of the 3.5-kb class of transcripts was driven by the heterologous CMV-IE promoter, the relative amount of this class of transcripts was elevated compared to transcription driven by the endogenous viral promoter. A somewhat lower level of all three transcripts was observed in the case of the nonreplicating mutant (lane 2). It is currently unclear why HBV-FS645 gives rise to reduced levels of HBV transcripts in transfected cells. In the case of the D30 mutant, levels of the 3.5- and 2.4-kb classes of transcripts carrying the 30-nt deletion were markedly reduced (lane 3). Strikingly, the class of 2.1-kb transcripts was absent. Densitometric analysis revealed that the levels of the deleted 3.5- and 2.4-kb classes of transcripts were reduced by 80% (lane 3) when compared with the WT levels (data not shown). Similar results were obtained when the constructs described

![FIG. 4. Reduction of HBV transcripts by the D30 mutant. (Top left) Northern blot analysis of total cellular RNA isolated 3 days after transfection of DNA constructs pTC-HBV (WT), pTC-HBV-D30 (D30 deletion mutant) and pTC-HBV-FS645 (frameshift mutant at HBV genome position 645) in HuH-7 HCC cells and detection with a HBV-specific probe. Transfection efficiencies were comparable in all cases. Transcripts corresponding to pregenomic RNA (3.5 kb), L protein-encoding RNA (2.4 kb), and M and s protein-encoding RNA (2.1 kb) are visible in the case of WT-HBV (lane 1) and HBV-FS645 (lane 2). Note that the 2.1-kb class of transcripts is not detectable and 3.5- and 2.4-kb transcript levels are reduced in the case of HBV-D30 (lane 3). NC, negative control (mock-transfected HuH-7 HCC cells; lane 4). (Top right) Northern blot analysis of total cellular RNA isolated 3 days after transfection of recircularized HBV monomers derived from adw-R9 (WT, lane 5) or adw-R9-D30 (D30 deletion mutant, lane 6) in HuH-7 HCC cells. The 2.1-kb class of transcripts is not detectable in the case of the D30 deletion mutant, whereas the 3.5- and 2.4-kb transcript levels are similar to that of WT. (Bottom) Rehybridization of the same membranes with a labeled probe specific for the acidic ribosomal phosphoprotein PO (ARPP PO) (Krowczynska et al., 1989) (a kind gift from Robert Hurford) for loading control purposes. 28 S and 18 S, relative position of human 28S and 18S rRNAs.
above were transfected into HEK 293 human embryo kidney cells (Graham et al., 1977) (data not shown). When recircularized WT or deleted HBV monomers were transfected, there was no reduction of the deleted 3.5- and 2.4-kb classes of transcripts (Fig. 4, lane 6) compared to the WT (lane 5). However, the 2.1-kb class of transcripts was undetectable in the case of the deleted monomer, confirming the results of transfection experiments with the CMV promoter bearing constructs. Taken together, these data suggested that HBsAg is absent from supernatants of cells transfected with the HBV-D30 mutant because of the lack of the corresponding class of transcripts.

Detection of deleted 2.1-kb transcripts

The absence of the 2.1-kb class of transcripts carrying the 30-nt deletion was further evaluated by transfection experiments using a construct that encoded the 2.1-kb class and 0.9-kb class of transcripts only. The construct HBV PreS/X (Mclachlan et al., 1987), which contained HBV nucleotides 2837–1989 (a kind gift from Alan McLachlan), included HBV sequences corresponding to the HBV S promoter, the complete S ORF, and the HBV poly(A) recognition sequence but lacked the preS promoter. A derivative of this construct carrying the 30-nt deletion from position 462 to 491, HBV PreS/X-D30, was generated. HuH-7 cells were transfected with the WT plasmid, HBV PreS/X, or the mutation-bearing plasmid HBV PreS/X-D30, and lysates from transfected cells 3 days posttransfection were analyzed by Northern blot with a HBV-specific probe. As illustrated in Fig. 5A, a signal corresponding to the 2.1-kb class of HBV transcripts was detected after transfection of the WT plasmid (lane 1). In contrast, no signal was obtained after transfection of the mutation-carrying plasmid (lane 2). Similar results were obtained when the constructs described above were transfected into HEK 293 human embryo kidney cells (data not shown).

To test the possibility that Northern blot analysis was not sensitive enough to detect trace amounts of the 2.1-kb class of transcripts synthesized by the D30 mutant, a sensitive RT-PCR assay was developed. HBV primers that were able to specifically amplify the 2.1-kb class of transcripts in this experimental setting were utilized, and RT-PCR was performed from the same lysates previously analyzed on Northern blot (see Fig. 5A). To avoid coamplification of the 0.9-kb class of HBV transcripts that were also encoded by the construct HBV PreS/X, one PCR primer (1966-1988) was derived from a HBV genome region that was positioned 5’ to the putative transcription initiation site for this class of transcripts, whereas the second PCR primer (2830-2837) was derived from a region shared by both classes of transcripts. PCR products were visualized by agarose gel electrophoresis (Fig. 5B, top) as well as by Southern blot using a HBV-specific probe (Fig. 5B, bottom). As demonstrated in Fig. 5B, lane 1, a HBV-specific PCR product corresponding to the 2.1-kb class of transcripts was observed in lysates of cells transfected with the WT construct, HBV PreS/X. Of note, a weaker HBV-specific signal was also detected in lysates of cells transfected with the construct carrying the 30-nt deletion, HBV PreS/X-D30 (lane 3); control reactions carried out in the absence of reverse transcriptase were negative in both cases (lanes 2 and 4, respectively). These results suggest that introduction of the 30-nt deletion did not completely abolish the production of the 2.1-kb class of transcripts, i.e., that 2.1-kb transcripts carrying the 30 nt deletion were indeed produced in transfected cells, albeit at very low levels. However, it is important to note that the PCR assay used here cannot discriminate between a nuclear and a cytoplasmic localization of the deleted 2.1-kb transcript. It is therefore possible that the PCR product we detected corresponds to deleted 2.1-kb transcripts that were located in the nucleus of transfected cells.
FIG. 6. Nuclear run-on assay. Transcription rates of mRNAs from HuH-7 HCC cells transfected with the HBV PreS/X and HBV PreS/X-D30 constructs. Labeled transcripts were hybridized to corresponding plasmid DNA that had been dot blotted on nylon membranes. Labeled transcripts were also incubated with hGH DNA for assessment of variations in transcription efficiencies, with ARPP-PO DNA as endogenous control and with pUC13 DNA as negative control. Signal intensities were quantified by densitometry using a phosphoimager system (not shown). Strong signals corresponding to hGH and PreS/X transcripts are visible in the cases of the HBV PreS/X (left) and the HBV PreS/X-D30 transcript (right). The strength of the PreS/X and PreS/X-D30 signals in comparison with their respective hGH signal intensities is similar, indicating that the transcription rate of the deleted HBV 2.1-kb transcript is similar to its wildtype counterpart. Results are representative of three independent experiments.

Nuclear run-on assay

To compare the transcription rates of transcripts derived from HBV PreS/X and HBV PreS/X-D30, a nuclear run-on assay was performed using nuclei isolated from transiently transfected cells. Labeled transcripts synthesized by isolated nuclei were hybridized to plasmid DNA that had been dot blotted on nylon membranes. Labeled transcripts were incubated with PreS/X DNA, with hGH DNA for assessment of variations in transcription efficiencies, with ARPP-PO DNA as endogenous control, and with pUC13 DNA as negative control. Signal intensities were quantified by densitometry using a phosphoimager system. As demonstrated in Fig. 6, strong signals corresponding to hGH and PreS/X transcripts were observed in the cases of the HBV PreS/X and the HBV PreS/X-D30 transcripts. The calculation of signal ratios (PreS/X to hGH) revealed comparable values in both instances, indicating that relative signal intensities were similar. A statistical analysis of signal ratios revealed no significant difference between the relative strengths of RNA signals obtained with HBV PreS/X and HBV PreS/X-D30 (data not shown). These data suggest that the transcription rate of the deleted HBV 2.1-kb transcript is similar to its wildtype counterpart and provide indirect evidence for a posttranscriptional mechanism leading to reduced levels of the transcript with the 30-nt deletion.

DISCUSSION

The present study describes the occurrence of an aberrant splicing event in a transcript incorporating 635 nt of HBV sequence from the polymerase/surface antigen region located upstream from rabbit β-globin gene sequences. Splicing in this transcript was shown to involve a splice donor site at HBV genome position 462 (Cattaneo et al., 1984; Graef et al., 1994; Simonsen and Levinson, 1983; Wang et al., 1992) and a splice acceptor site of the rabbit β-globin intron 2 that is positioned 1048 nt downstream. The genome region of HBV between nt 450 and 500 appears to be rich in splice consensus sequences. A splice acceptor site in the HBV genome that has been well characterized by several investigators (Rosmorduc et al., 1995; Su et al., 1989a; Suzuki et al., 1989; Wu et al., 1991) is positioned at HBV genome position 491, exactly 30 nt downstream from the splice donor site at position 462. The multiple sequence alignment of 24 HBV genomes (see Fig. 2) in this region revealed that a high degree of conservation among genotypes exists with respect to these splice consensus sequences. This was puzzling considering the fact that the majority of HBV transcripts are not spliced. It was therefore hypothesized that the clustered splice consensus sequences in the genome region between nt 450 and 500 played an important functional role in the life cycle of HBV that was not at all or only indirectly associated with splicing. This possibility was investigated by elimination of the 30 nt positioned between the splice donor site at position 462 and the splice acceptor site at position 491 in a HBV genome capable of replication in transfected cells. Deleted transcripts synthesized from this construct, termed HBV-D30, would have been able to serve as templates for the translation of all three known members of the surface antigen family as well as the polymerase protein, each of them with a 10-aa in-frame deletion.

The characterization of the D30 mutant in transiently transfected hepatoma and HEK 293 kidney cells in comparison with the WT revealed that the class of deleted 2.1-kb transcripts appeared to be present in transfected cells at very low levels. Consistent with the loss of 2.1-kb transcripts, no deleted M and s proteins were detectable in transfected cells, and no (deleted) HBsAg could be detected in cell culture supernatants. When CMV promoter bearing constructs were transfected, the levels of the 2.4-kb transcripts and 3.5-kb pregenomic transcripts were also substantially reduced, and consequently, the amount of deleted L protein and core antigen were found to be reduced to a similar extent (data not shown). When recircularized HBV monomers bearing the D30 deletion were transfected, the levels of the 2.4- and 3.5-kb transcripts appeared to be similar to the WT. Importantly, the 2.1-kb class of transcripts appeared to be absent, confirming the results obtained with the CMV promoter bearing constructs. In summary, the deleted HBV genome region was found to be critically important for the maintenance of high 2.1-kb transcript levels. Depending on the type of construct transfected, the levels of all major
HBV transcripts except the 0.9-kb class of (X protein-encoding) transcripts appeared to be differentially modulated by the occurrence of the 30-nt deletion. Analysis of transcription rates of the deleted 2.1-kb transcript in comparison with the WT revealed no reductions, suggesting that the modulatory effect on 2.1-kb transcript levels is mediated posttranscriptionally.

The precise mechanism of the 2.1-kb transcript destabilization requires further investigation. In this regard, Liang and co-workers (personal communication) have shown that point mutations at the potential splice donor site HBV position 462 and the splice acceptor site HBV position 491 were associated with decreased levels of the 2.1-kb class of transcripts if a genetic element essential for high-level expression of HBsAg (the HBV posttranscriptional regulatory element (PRE)) (Huang and Li-ang, 1993; Huang and Yen, 1994) is also present on the same transcript. The presence of the PRE appears to be important for the nucleocytoplasmic export of unspliced HBV transcripts. In view of these observations, a model was proposed in analogy to the human immunodeficiency virus type 1 (HIV-1) protein Rev and its binding sequence on viral RNA, the Rev response element (RRE). Rev appears to increase the transport and utilization of all viral mRNA species that contain the RRE by inhibiting the splicing process. For HBV, it was proposed that an interaction of RNA and/or RNA-binding proteins in the region of the PRE with components of the spliceosome that potentially associate with the putative splice donor (position 462) and/or acceptor sites (position 491) may lead to inhibitory effects on splicing and enables high copy numbers of unspliced (subgenomic) transcripts to be translocated to the cytoplasm from the nucleus. This hypothesis suggests that transcripts carrying the 30-nt deletion studied here might be deficient for the association with protein factors that play a role in efficient nucleocytoplasmic transport.

It was observed that the pregenomic RNA containing the deletion was encapsidated into core particles and subsequently reverse transcribed in a manner indistinguishable from the wildtype. Therefore, this study defines a 10-aa stretch (aa 459 to 468) within the RT domain (aa 336–680) of HBV polymerase that appears to be nonessential for the function of reverse transcription of pregenomic RNA. The deletion in the RT domain of HBV polymerase is positioned between the A domain (aa 423 to 439 in HBV adw2) and the B domain (aa 510 to 537) that are believed to be critical for RT function (Poch et al., 1989). Interestingly, a protein sequence alignment (not illustrated) between HBV and DHBV polymerase revealed that HBV polymerase amino acid sequences positioned between the A and the B domains that is absent in DHBV polymerase, and the data presented here show that partial deletion of this protein loop does not disrupt viral DNA synthesis. It will be of interest to analyze polymerase deletion mutants in this region in more detail with respect to their function. Finally, it is also noteworthy that the 10-aa deletion in the polymerase protein of the D30 mutant eliminated the C-terminal 5 aa of a 9-aa-long, well-characterized HLA-A2.1-restricted CTL epitope (Pol455–463) located in the RT domain (Rehermann et al., 1995).

The deletion of 30 nt in the HBV genome between positions 462 and 491 leads to a polymerase mutant that is capable of reverse transcription of viral RNA and to a deleted L protein whose function appears to be unaltered when compared with the wildtype (data not illustrated). However, the D30 mutant lacks the capability to synthesize detectable amounts of M and s proteins. These properties are reminiscent of other types of previously described defective HBV genomes that have frequently been found to exist in viral particles derived from patients (Melegari et al., 1997). In such HBV variants, the deficiency to synthesize M and s proteins is due to deletions in the S promoter region of the 2.1-kb class of transcripts but may possibly be complemented in trans by surface proteins produced from wildtype genomes. These observations raise the possibility that variant HBV genomes incorporating the D30 deletion described here might occur in certain patients. Therefore, a sensitive PCR-based assay is under development to detect viral genomes carrying the D30 deletion in patient sera, and preliminary data obtained from a small group of patients with chronic hepatitis B suggest that variant HBV genomes with the D30 deletion indeed occur in some cases.

In conclusion, a short region in the genome of HBV was found to be critical for maintenance of high transcript levels, especially in the case of the 2.1-kb subgenomic transcript. Polymerase translated from a mutant devoid of this region is competent for viral DNA synthesis in vitro, but M and s proteins are strongly reduced due to loss of the corresponding transcripts. Further study of this region of the HBV genome will yield insights into the regulation of HBV transcript levels in infected cells. In addition, structure/function relationships in the viral polymerase may be elucidated. The possible occurrence of the D30 mutant in patients may have implications for the study of virus–host interactions with respect to the persistence of viral genomes within hepatocytes and the pathogenesis of this disease.

MATERIALS AND METHODS

DNA constructs

A head-to-tail dimer of wildtype HBV, subtype adw (GenBank Accession No. X02763; (Blum et al., 1991)), was digested with EcoRI and BglII to yield HBV DNA monomers (3.2 kb), which were subsequently digested at
multiple sites with Sau3AI. The resulting subgenomic fragments of HBV were inserted into the BamHI site of the eukaryotic expression vector pC-GR N-795 (Rusconi et al., 1990). Antisense and sense RNAs transcribed from this plasmid (the orientation is given according to the direction of transcription from the HBV genome) contained 75 nt of CMV-IE promoter-derived sequence upstream of inserted HBV sequences and 379 nt of rabbit \( \beta \)-globin-like gene cluster-derived sequence (Margot et al., 1989) downstream from the HBV sequences. The replication-competent construct pTC-HBV (von Weizsacker et al., 1996) (kindly provided by Stefan Wieland) contains a more-than-one-genome-length copy of the HBV genome subtype adw2 (3337 nt; from nt 1822 to nt 1822 (3221 nt; G of EcoRI site is position 1) and continued to nt 1937) under transcriptional control of the CMV-IE promoter. In this construct, transcription of the HBV pre-genomic (3.5 kb) RNA is driven by the heterologous CMV-IE promoter, whereas transcription of all subgenomic transcripts is controlled by their respective endogenous promoters. A derivative of pTC-HBV, pTC-HBV-D30, containing a 30-nt in-frame deletion in the surface antigen/polymerase reading frame (nt 462 to 491 in HBV adw2) was constructed in this study by overlap-extension PCR (Vallejo et al., 1995). The replication-competent construct adw-R9 (Blum et al., 1991) contains a more-than-one-genome-length copy of the HBV subtype adw2 (3393 nt; from nt 1413 (AatII site) to nt 1413 and continued to nt 2182) in a pGEM7 7Zf(+) vector. Similar to the construction of pTC-HBV-D30, a derivative of adw-R9 (adw-R9-D30) containing a 30-nt in-frame deletion in the surface antigen/polymerase reading frame (nt 462 to 491 in HBV adw2) was constructed. adw-R9 and adw-R9-D30 were used to generate linear HBV DNA monomers by digestion with AatII, which, after recircularization with T4 DNA ligase (New England Biolabs, Beverly, MA), were transfected into cells.

For all PCR amplifications performed for cloning purposes, the Expand High Fidelity PCR System (Boehringer Mannheim, Indianapolis, IN) was used according to the instructions supplied by the manufacturer, typically using a reaction volume of 100 \( \mu l \). Reaction conditions for hot-start PCR were denaturation at 94°C for 1 min, 80°C for 1 min (addition of enzyme), 94°C for 1 min; followed by 25 cycles at 94°C for 1 min, 60°C for 30 s, 72°C for 30 s, and a final elongation step at 72°C for 10 min. Two overlapping fragments of HBV that incorporated the 30-nt deletion were amplified using 40 pmol each of primers HBV-XBA (20mer, 5'-GCAGAATCTAGCTGCTGTT-3') and D30-2 (33mer, 5'-GTTGTGTTGTGTGATCTTGATAAATCAGAAGAACCC-3') in the first reaction and primers D30-1 (33mer, 5'-CTGGATTACAAGATGAAACAACACGATTCAGC-3') and HBV-SPE (21mer, 5'-AATGGCCTAGTAACCTGACC-3') in the second reaction. In addition to 1/10 vol of 10X Expanded High Fidelity reaction buffer (final concentration of MgCl\( _2 \) was 1.5 mM), the reaction mix-
MgCl₂ and 1 U of DNase I, RNase-free (Boehringer Mannheim) in a final volume of 20 μl and was performed for 30 min at 37°C, followed by an enzyme inactivation step of 5 min at 75°C. After addition of 200 U SuperScript II RNase H⁻ reverse transcriptase, the RT reaction was incubated for 1 h at 42°C, followed by an enzyme inactivation step of 15 min at 70°C. After addition of 2 U of Escherichia coli RNase H, the reaction was finally incubated for 20 min at 37°C and stored at −40°C. For negative control purposes, individual reactions were performed in parallel without addition of reverse transcriptase. A 1/10 vol of each RT reaction (2 μl) was used in subsequent PCR detections of HBV subgenomic transcripts, which were performed with upstream primer 1966+ (27mer, 5'-TGCCATTGTTCAGTGTCTCGTAGG-GC-3') and downstream primer 2830- (26mer, 5'-CCGGCAGATGAGAAGGCACAGACGG-3') (Kock et al., 1996) at 20 pmoI each in a reaction volume of 100 μl. In addition to 1/10 vol of 10× PCR assay buffer (100 mM Tris–HCl (pH 8.3), 500 mM KCl, 15 mM MgCl₂, 0.01% gelatin; Fisher Scientific), the reaction mixture contained dNTPs at 200 nM each and 1 μl (5 U) of Taq DNA polymerase. The reaction conditions for PCR were denaturation at 94°C for 3 min, followed by 25 cycles at 94°C for 1 min, 64°C for 1 min, 72°C for 1 min and a final elongation step at 72°C for 10 min. PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining and Southern blotting.

Cells and transfections

The human HCC cell line HuH-7 (Nakabayashi et al., 1982) was grown in modified Eagle's minimal essential medium (MEM; Cellgro Mediatech, Washington, DC), supplemented with 10% fetal calf serum (FCS), 1% nonessential amino acid solution (Life Technologies), and 1% penicillin/streptomycin stock solution (Cellgro Mediatech). This cell line supports a complete viral replication construct for human growth hormone, pTKGH, was linearized, single-stranded plasmids (10 μg each) HBV PreS/X-D30. The expression construct for human growth hormone, pTKGH, was cotransfected in each case. Nuclei were prepared by lysis with NP-40 lysis buffer after 24 h and stored in glycerol buffer (50 mM Tris–HCl, pH 8.3, 40% glycerol, 5 mM MgCl₂, 0.1 M EDTA) at −80°C. Nuclei from one transfected 100-mm cell culture plate (100 μl) were then incubated with 100 μl reaction buffer (10 mM Tris–HCl, pH 8.0, 5 mM MgCl₂, 300 mM KCl, 5 mM DTT) containing 0.5 mM each of ATP, CTP, and GTP, 100 μCi of [α-32P]UTP (10 mCi/ml), and 1 U/μl RNasin (Promega). After incubation at 30°C for 60 min, the reaction was extracted with phenol/chloroform, precipitated, dissolved in 50 μl TE, and finally hybridized to nylon membranes onto which linearized, single-stranded plasmids (10 μg each) HBV PreS/X, pTKGH, mARPP-PO, and pUC13 had been spot-
Enzymatic and immunological assays

For the determination of transfection efficiencies, a commercially available assay for secreted human growth hormone (Tandem-R HGH; Hybritech, San Diego, CA) was used. Hepatitis B virus surface antigen (HBsAg), core antigen (HBcAg), and e antigen (HBeAg) levels in cell culture supernatants were determined by commercially available immunoassays (Abbott Laboratories, North Chicago, IL).

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