

## Correspondence

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## **Antisense RNAs transcribed from the upstream region of the precore/core promoter of hepatitis B virus**

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**The bidirectional activity of the precore/core promoter of hepatitis B virus (HBV) has been demonstrated in cultured cell lines. However, HBV antisense transcripts (asRNAs) have not been demonstrated *in vivo*. In the present study using liver tissue from patients with chronic hepatitis, an anchored 5'RACE mapping the 5' ends at position 1680/1681, 1655 or 1609/1602 was carried out. In limited cases, RLM-3'RACE detected asRNAs to terminate at four or five consecutive dT residues in the 0.7 kb downstream region. PCR of oligo(dT)-primed cDNA did not amplify a typical polyadenylated asRNA. RT-PCR using various primers did not detect any spliced forms. Competitive RT-PCR estimated the copy numbers of the asRNAs to be 0.05–0.4 % of total sense RNAs. All sequenced asRNAs had ORF6 but, in one patient, the asRNA initiating at position 1680/1681 had additional initiation and termination codons in front of ORF6. Therefore, asRNAs are transcribed by RNA polymerase III at a low level, encompass a dispensable ORF6 gene and might be retained in the nucleus. The endogenous asRNAs complementary to the common ends of all sense RNAs suggest antisense-mediated self-regulation of hepadnavirus.**

## INTRODUCTION

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Hepatitis B virus (HBV) ORF6, located with an antisense orientation in the X gene, has been proposed to encode a 10.5 kDa protein (Galibert *et al.*, 1979), and several research groups have looked successively for the protein product and its mRNA.

Strandring *et al.* (1983) demonstrated an RNA polymerase III-dependent antisense transcript (asRNA) of cloned HBV DNA in an *in vitro* transcription system using a HeLa cell extract. The non-polyadenylated transcript was initiated at position 1631/1632 or 1628 and terminated at a (dT)<sub>4</sub> tract in the cloning plasmid. It has not been proven whether these polymerase III transcripts are present *in vivo*.

Using Northern blotting, Zelent *et al.* (1987) found several polyadenylated asRNAs in a murine cell line carrying integrated HBV genomes. S1 nuclease analysis revealed that the transcripts initiated at position 1861 and terminated at position 2381. It was speculated that one of the asRNAs was 2.8 kb in length. Chen *et al.* (1993) looked for asRNAs of woodchuck hepatitis virus (WHV) and its coding ORF6 protein in the livers of acutely or chronically infected woodchucks, but their study yielded negative results. Using a luciferase assay of HepG2 cells, Velhagen *et al.* (1995) observed antisense promoter activity of the HBV X gene at positions 1375–1575, which was similar to that of the X gene promoter. They also found extremely low antisense promoter activity in the *StyI* fragment of the precore/core (preC/C) promoter at positions 1645–1885. This activity was one-tenth that of the sense orientation. Shimoda *et al.* (1998) demonstrated bidirectional promoter activity within the X gene of WHV by luciferase assay in human and woodchuck hepatoma cell lines. Initiation sites were mapped near the upstream region of ORF6 and the antisense promoter activity was comparable to that of the S gene promoter in the absence of an enhancer. Since these reporter genes have a polyadenylation signal, these findings suggest RNA polymerase II promoter activity in the antisense orientation. However, in human patients, it has yet to be determined whether asRNAs are expressed and transcribed by RNA polymerase II.

In this study, we examined liver tissue from humans with HBV infection and found a low level of asRNAs expression. We determined both ends of the asRNAs as well as their copy numbers by PCR. The 0.7 kb asRNAs *in vivo* were not polyadenylated and presumed to be transcribed by RNA polymerase III. We discuss the hypothetical 10.5 kDa protein encoded by the transcript as well as the significance of the asRNA in the negative regulation of transcription and translation of the virus.

## METHODS

**Patients and liver tissue.** Samples were obtained from five patients with chronic hepatitis B who underwent surgical resection of hepatocellular carcinoma. All patients were positive for the HBV surface antigen (HBsAg). Patients 2 and 5 were positive for the e antigen, HBeAg, whereas patients 1, 3 and 4 were negative. All patients were negative for serological markers of hepatitis C virus, human T lymphotropic virus type 1 and human immunodeficiency virus. Immediately after resection of the cancerous region, non-tumour tissue was collected from the periphery of the tumour. Samples were frozen immediately in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**Liver DNA and RNA preparation.** Tissue DNA was extracted from each 20 mg liver sample. The sample was transferred to 500  $\mu\text{l}$  lysis buffer (Qiagen) with 1 mg proteinase K  $\text{ml}^{-1}$  and incubated at  $56^{\circ}\text{C}$  for 2 h. DNA was purified using silica columns from the QIAamp DNA Mini kit (Qiagen). Total RNA for anchored-5'RACE, RLM-3'RACE, oligo(dT)-primed 3'RACE and quantitative RT-PCR was prepared using an RNaseasy Mini kit (Qiagen). Total RNA (40  $\mu\text{g}$ ) was treated with 1 U DNaseI (Promega) in 50  $\mu\text{l}$  appended buffer (Moriyama, 1997) at  $37^{\circ}\text{C}$  for 1 h. PCR for amplifying positions 1524–2067 with primers B105 and B108 was done before cDNA synthesis to confirm the complete elimination of undigested HBV DNA.

**Nucleotide sequencing of tissue HBV DNA.** The entire 3.2 kb genome was amplified by PCR with KOD polymerase (Toyobo) and 20 pmol of each of the HB-P1 and HB-P2 primers (Gunther *et al.*, 1995). PCR was done at  $94^{\circ}\text{C}$  for 2 min (for initial denaturation of DNA and inactivation of anti-KOD polymerase antibody), with 30 cycles of  $94^{\circ}\text{C}$  for 15 s,  $60^{\circ}\text{C}$  for 15 s and  $68^{\circ}\text{C}$  for 1 min. PCR products were digested with *NotI* and cloned into pBluescriptKS+ (Stratagene). The nucleotide sequences of the inserts were determined by a Dideoxynucleotide Sequence kit (ABI) using the primers T7Pro (T7 promoter sequence), T3Pro (T3 promoter sequence), B2500, AS11 and B202 (Table 1). For each patient, nucleotide sequences of three clones were determined.

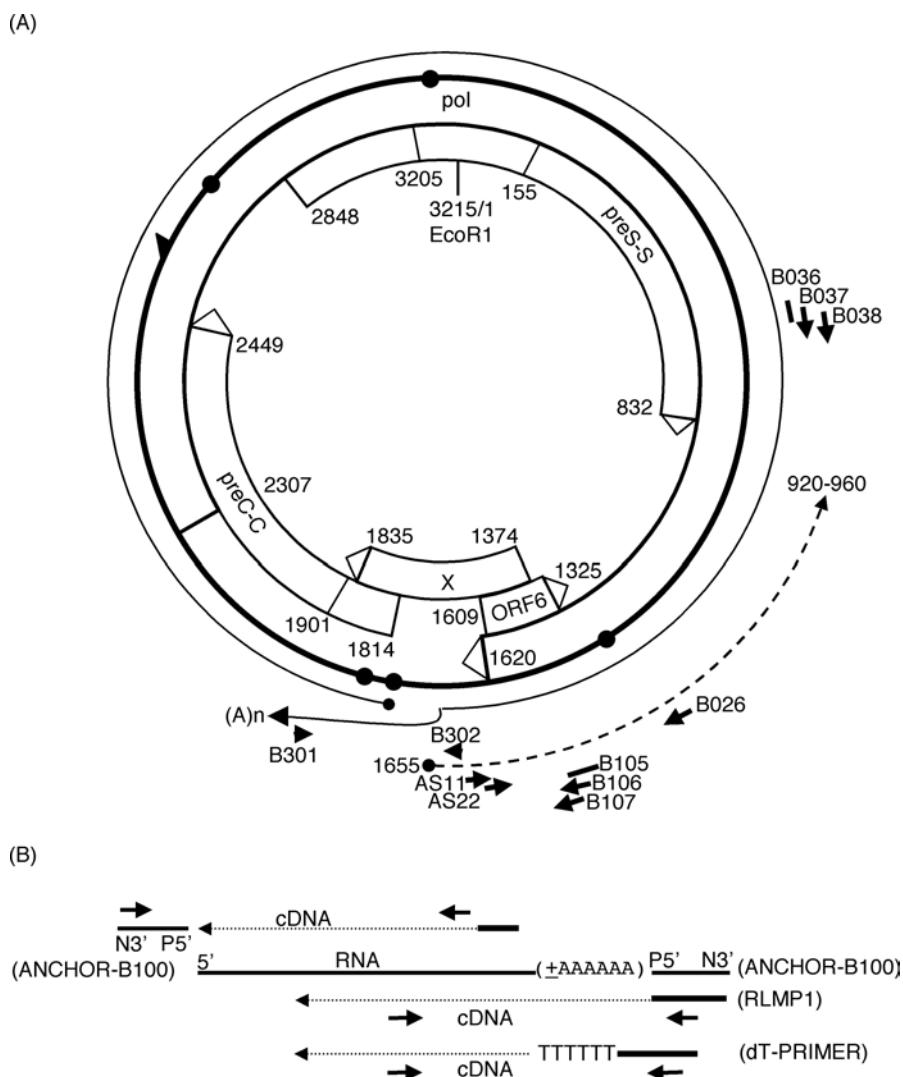
**Table 1.** Synthetic deoxyoligonucleotide primers

Primer	Sequence (5'→3')	Position
ANCHOR-B100	PO-GCACTTGACTAGGATCCACTGAATTCCTTTAGTGAGGGTTAATTGCC-NH <sub>2</sub>	
ANCHOR-OUTER	GGCAATTAACCCTCACTAAAG	
ANCHOR-INNER	AAAGAATTCAGTGGATCCTAG	
dT-PRIMER	GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTVN	
PRIMER-OUTER	GCGAGCACAGAATTAATACGACT	
PRIMER-INNER	CGCGGATCCGAATTAATACGACTCACTATAGG	
AS11	GTGAAGCGAAGTGCACACGG	1594–1575
AS22	CAGATGAGAAGGCACAGACG	1579–1550
RLMP1	TAAAGAATTCAGTGGATCCTAGTCAAGTGC	

RLMP3	GTGGATCCTAGTCAAGTGC	
HB-P1	ATAAGCATGCGGCCGCTCTTCTTTTTACCTCTGCCTAATCA	1821–1841
HB-P2	ATAAGCATGCGGCCGCTCTTCAAAAAGTTGCATGGTGCTGG	1825–1806
T3Pro	AATTAACCCTCACTAAAGGG	
T7Pro	TAATAGCACTCACTATGGGCGA	
B026	CCGATCCATACTGCGGAACTCC	1262–1282
B036	GATGATGTGGTATTGGGGGCC	742–762
B037	GATGTGGTATTGGGGGCAAG	745–765
B038	GGTATTGGGGGCAAGTCTG	750–7769
B105	GCGCACCTCTCTTTACGCGG	1524–1543
B106	GTGCCTTCTCATCTGCCGGAC	1555–1575
B107	ACAGAATTCTTCTCATCTGCCGGACCGTG	1558–1579
B108	CTGAGTGCAGTATGGTGAGG	2067–2048
B202	CCCAAGAATATGGTGACC	2838–2821
B301	GGAAAGAAGTCAGAAGGCAA	1974–1955
B302	CACCTCTGCCTAATCATC	1825–1843
B2500	CTGTACCTGTCTTTAATCC	2509–2528
GAPDH-F	TGATGACATCAAGAAGGTGGTGAAG	
GAPDH-R	TCCTTGGAGGCCATGTGGGCCAT	
GAPDHpBSF1	GATGACATCAAGAAGGTGGTGAAGCTAGAACTAGTGGATCCC	
GAPDHpBSR2	TCCTTGGAGGCCATGTGGGCCATGAACGACCGAGCGCAGCG	
AS11pBSF	GTGAAGCGAAGTGCACACGGCTAGAACTAGTGGATCCC	
B026pBSR	CCGATCCATACTGCGGAACTCCGAACGACCGAGCGCAGCG	
B301pBSR	GGAAAGAAGTCAGAAGGCAAGAACGACCGAGCGCAGCG	
B302pBSF2	CACCTCTGCCTAATCATCCTAGAACTAGTGGATCCC	

**Anchored-5'RACE.** The 5' ends of the asRNAs were mapped by anchored-5'RACE (Fig. 1). For the first anchored-5'RACE, the primer B105 was mixed with 1  $\mu$ g RNA and cDNA was synthesized using reverse transcriptase (Superscript II, Invitrogen) and appended buffer. B105 (positions 1524–1543) anneals with the upstream region of the preC/C promoter (Fig. 1A). After removal of RNA by incubating with 0.1 vol. 2 N NaOH at 50 °C for 30 min, the reaction mixture was neutralized with 0.1 vol. 2 N acetic acid. Excess primers and dNTPs were removed by passage through an agarose column (Chromaspin-100, Clontech). The 3' end of the cDNA was ligated with the 5'-phosphorylated and 3'-aminated anchor deoxyoligonucleotide ANCHOR-B100 using T4 RNA ligase (Takara) and 20 % PEG 2000 in 20  $\mu$ l appended buffer. After a 24 h ligation reaction at 15 °C, 1  $\mu$ l of the mixture was used for the first PCR with the primers ANCHOR-OUTER and B106 (20 pmol each), which anneal with the anchor and cDNA, respectively. The PCR was started with KOD polymerase by incubation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 15 s, 60 °C for 15 s and 68 °C for 1 min. The nested PCR was performed using the primers ANCHOR-INNER and B107 employing the same thermal cycles as those above. PCR products were precipitated in 70 % ethanol and 0.1 M ammonium acetate in order to inactivate the KOD polymerase. The pellets were suspended and incubated with 1 U *Taq* polymerase in buffer containing 0.2 mM dNTP at 72 °C for 20 min to add an overhang adenosine at both ends of the PCR products. After

running in agarose gels, bands were excised and ligated with the pGEM-T vector (Promega). After transfection of *Escherichia coli* DH5 $\alpha$ , nucleotide sequences of the cloned inserts were determined. For the second anchored-5'RACE, the primer B036 was used to synthesize cDNA. B036 anneals with the region 0.9 kb downstream of the initiation sites of asRNA. The cDNA was ligated with ANCHOR-B100, the first PCR was done with the primers ANCHOR-OUTER and B037 and the nested PCR was performed using the primers ANCHOR-INNER and B038.



**Fig. 1.** Map of HBV form I DNA in the cell nucleus. (A) The thick circle represents dsDNA. The large arrows in the sense orientation represent the recognized functional ORFs. The thin line indicates preC/C and pregenome RNA. The small solid circles indicate the transcriptional initiation sites of three major transcripts, all of which terminate identically near the polyadenylation signal at positions 1916–1921. The filled triangle indicates the polyadenylation signal in the antisense orientation. Thick bars indicate primers for cDNA synthesis. Small arrows are the PCR primers. Dashed lines represent the asRNAs detected in the experiments. (B) Schematic diagram of the anchored amplification of cDNA 3' ends to determine 5' ends of RNA (anchored-5'RACE), RNA ligase-mediated amplification of cDNA 5' ends to determine 3' ends of RNA (RLM-3'RACE) and the oligo(dT)-primed amplification of cDNA 5' ends to determine cleavage sites of polyadenylated RNA [oligo(dT)-primed 3'RACE].

**RLM-3'RACE.** The 3' ends of total RNA (1 µg) were ligated with the 5' ends of ANCHOR-B100 (80 pmol) (Fig. 1B). The RNA and anchor were mixed and heated at 90 °C for 2 min. Ligation was done in a 50 µl volume of a mixture containing 50 mM HEPES (pH 7.5), 20 mM MgCl<sub>2</sub>, 5 mM DTT, 10 % DMSO, 10 µg BSA ml<sup>-1</sup>, 1 unit RNase inhibitor ml<sup>-1</sup> (Promega), 240 pmol ATP and 50 U T4 RNA ligase at 15 °C for 6 h (Liu & Gorovsky, 1993). cDNA was synthesized with primer RLMP1, whose sequence is complementary to that of ANCHOR-B100. After incubation at 65 °C for 5 min, reverse transcription was done with Superscript II in 20 µl appended buffer at 42 °C for 40 min. The first PCR was done with the primers ANCHOR-OUTER and AS11. Nested PCR was done with the primers RLMP3 and AS22. Addition of an overhang adenosine at both ends of the products, cloning and nucleotide sequencing were done in the same manner as that for 5'RACE.

**Southern blot of RLM-3'RACE products.** Five microlitres of 500-fold diluted RLM-3'RACE products were run in 1 % agarose gel and electroblotted onto a nylon membrane (Hybond-N<sup>+</sup>, Amersham). Hybridization was carried out with cloned one-genome-length HBV DNA labelled with alkaline phosphatase in 6× SSC and 0.25 % non-fat dried milk at 55 °C for 8 h. The filter was washed under highly stringent conditions and specific bands were visualized with CDP-Star Chemiluminescence Detection Reagent (Amersham).

**Oligo(dT)-primed 3'RACE.** cDNA was synthesized with 1 µg total RNA and 100 pmol oligo(dT) primer (12 dT residues). After incubation at 65 °C for 5 min, reverse transcription was done with Superscript II in 20 µl appended buffer at 42 °C for 40 min. RNaseH (1 U, Invitrogen) was added and incubated at 37 °C for 15 min to eliminate template RNA. Excess primers and dNTPs were removed by passage through an agarose column Chromaspin-100. The first PCR was done with the primers PRIMER-OUTER and AS11. Nested PCR was done with the primers PRIMER-INNER and AS22. PCR, cloning and nucleotide sequencing were done in the same manner as those for anchored-5'RACE.

**Quantitative RT-PCR.** cDNA was synthesized with 0.25 µg total RNA in 20 µl of reaction mixture containing a primer mixture of 2.5 pmol of each of B026, B301 and GAPDH-R, and 1 U of Superscript II. The reaction mixture was then diluted with 120 µl 10 mM Tris, pH 8.4, and stored at -20 °C. Each 4 µl was amplified by competitive PCR in the presence of each 10-fold- or 2-fold-diluted competitor DNA Comp-AS (430 bp). For competitor DNA template Comp-AS, the 430 bp region of pBluescriptKS<sup>+</sup> was amplified with the primers AS11pBSF and B026pBSR. PCR primers B301pBSR and B302pBSF2 were used for making the competitor DNA Comp-PC. Primers GAPDHpBSF1 and GAPDHpBSR2 were used for making the competitor DNA Comp-GAPDH. Both the 5' and 3' ends of these competitor DNAs have annealing sites for primer sets AS11 and B026, B301 and B302 or GAPDH-F and GAPDH-R. Each PCR product was passed through a Chromaspin-100 column to eliminate residual dNTPs and primers. The

purified competitor was assayed for DNA concentration by a UV spectrophotometer and normalized by adding 10 mM Tris, pH 8.0. A 4 µl sample of the cDNA product was mixed with 2 µl of serially diluted competitor DNA Comp-PC, Comp-AS or Comp-GAPDH. Then, the mixture was reacted with 20 pmol of each primer pair using *Taq* polymerase in 50 µl appended buffer for 30 cycles of 94 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s. The cDNA of asRNA was amplified with the primers AS11 and B026. The cDNAs of preC/C, pregenome, preS/S and X RNA were amplified with the primers B301 and B302, and the cDNA of GAPDH mRNA was amplified with the primers GAPDH-F and GAPDH-R. After agarose gel electrophoresis, the lane showing a density equal to that of the objective band and the competitor product was determined and the concentration of each cDNA was deduced from the copy number of the competitor.

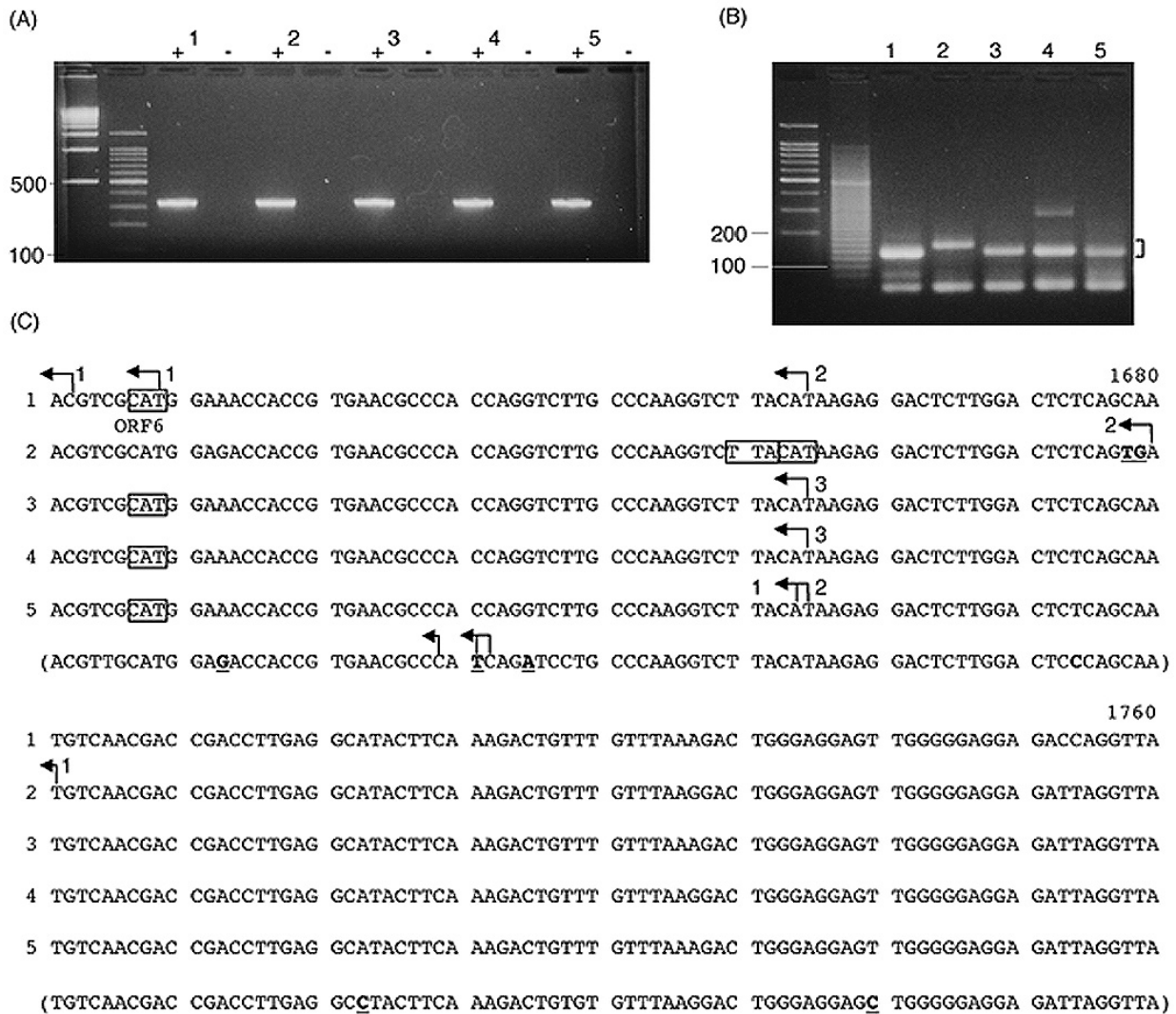
## RESULTS

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### Detection of asRNAs transcribed from the X gene

RT-PCR was performed to determine whether any asRNAs are transcribed from the preC/C promoter region. Total RNA obtained from patients with chronic hepatitis was treated with DNaseI and cDNA was synthesized using primer B026, which anneals to the upstream region of the preC/C promoter (positions 1262–1282) (Fig. 1A and Table 1). PCR for positions 1282–1575 of the cDNA of all five cases yielded bands of the expected size by agarose gel electrophoresis (Fig. 2A).

To confirm the results of the RT-PCR for asRNAs and to determine their 5' ends, we performed anchored-5'RACE and nucleotide sequencing of the products. First, we used the primer B105 (positions 1524–1543) to synthesize cDNA (Fig. 1A). After ligating the 3' ends of the cDNAs with the 5' end of ANCHOR-B100, we amplified the joined regions by PCR (Fig. 1B). The resulting PCR products were run in agarose gels and their sizes were found to be approximately 120 bp (Fig. 2B), corresponding to the asRNA 5' ends initiating around position 1650. Attempts to amplify longer transcripts, i.e. asRNAs initiating from a region further upstream, by prolonging the polymerization time at 68 °C were unsuccessful. We cloned and sequenced the 120 bp products shown in Fig. 2(B) and compared the results with the HBV DNA sequences from each of the patients. The 5' ends of asRNAs corresponded to position 1602, 1609, 1655, 1680 or 1681 (Fig. 2C). In four patients, position 1655 appeared to be the major initiation site. In patient 2, only asRNA starting 24/25 nucleotides upstream at position 1680/1681 was amplified. A database search using the NCBI BLAST program showed that T<sup>1678</sup> and G<sup>1679</sup> from patient 2 were unique. This asRNA has an initiation and a termination codon 46 nucleotides upstream of ORF6.



**Fig. 2.** Detection of asRNAs transcribed from the preC/C promoter region and determination of their 5' ends. (A) Agarose gel electrophoresis of RT-PCR products of asRNAs in the livers of five patients. Total RNA was isolated from liver and treated with DNase I. cDNA was synthesized with the primer B026, which anneals with the upstream region of the preC/C promoter (positions 1262–1282). PCR was done with primers B026 and AS11, which amplify positions 1282–1575. PCR templates made without reverse transcriptase were used as negative controls and are indicated by '-'. (B) Agarose gel electrophoresis of nested PCR products of anchored-5'RACE. cDNA in the sense direction was synthesized with the primer B105 (annealing at positions 1524–1543) and its 3' end was ligated with the anchor oligonucleotide ANCHOR-B100. The first PCR was done with primers ANCHOR-OUTER and B106 (position 1555–1575) and the second PCR with primers ANCHOR-INNER and B107 (position 1558–1579). The molecular mass markers are 20 and 100 bp dsDNA ladders. (C) Nucleotide sequences of HBV DNA. Transcription initiation sites of asRNA were determined by nucleotide sequencing of plasmid clones harbouring a single molecule of the 5'RACE product shown in (B). Numbers on arrows indicate numbers of 5'RACE clones sequenced. Potential initiation and termination codons are boxed. Transcriptional initiation sites of RNA polymerase III in HeLa cell extracts determined by Standring *et al.* (1983) are shown in parentheses. The negative regulatory element (Lo & Ting, 1994) is located at positions 1611–1634 and the core upstream regulatory sequence (Yuh *et al.*, 1992) is located at positions 1643–1742.

Next, we used primer B036 (positions 742–762) to synthesize the cDNA (Fig. 1A). It was considered that 5'RACE would detect the asRNA 5' ends initiating downstream from the former cDNA primer B026. However, under several conditions, we did not detect any 5'RACE products containing the viral nucleotide sequences.

### **Determination of 3' ends of asRNAs by RLM-3'RACE**

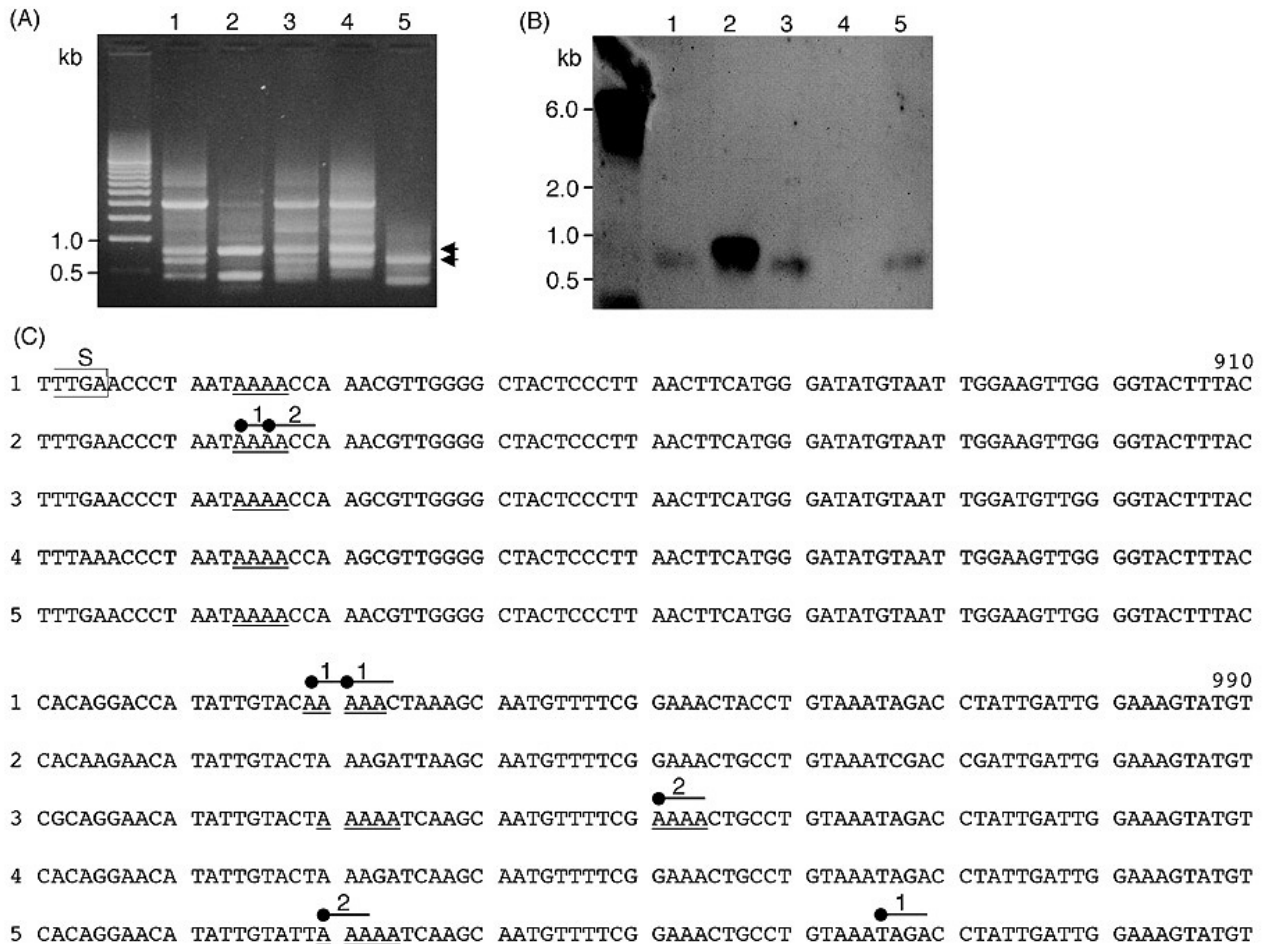
To determine the 3' ends of asRNAs, we performed RLM-3'RACE. We ligated the 3' ends of total RNA with ANCHOR-B100 and synthesized the cDNA using the primer RLMP1, which anneals with the anchor (Fig. 1B). We used the first and nested PCR primers AS11 and AS22 to anneal with the region near the 5' ends of the asRNAs. PCR of the joined region produced several fragments (Fig. 3A). Therefore, we performed a Southern blot of the 500-fold diluted PCR products using HBV DNA as a probe (Fig. 3B). It was found that only the 0.7 kb products contained the viral sequence. Amplification of the 0.7 kb product from patient 4 was unsuccessful. Cloning and sequencing of the 0.7 kb products revealed that the asRNAs ended at position 844/846, 929/930/931, 951 or 966. All of them, except for one clone from patient 5, were located in the (dT)<sub>4</sub> or (dT)<sub>5</sub> tracts that can be termination cleavage signals for RNA polymerase III (Fig. 3C). The procedure did not detect a polyadenylated asRNA.

### **Failure to detect the polyadenylated asRNAs by oligo(dT)-primed 3'RACE**

We looked further for the asRNAs by oligo(dT)-primed 3'RACE (Fig. 1B), which synthesizes cDNA more efficiently than RLM-3'RACE. We synthesized the cDNA using the primer dT-PRIMER and used the counter primers AS11 and AS22 located near the 5' end of the asRNA. Nested PCR amplified several products in some cases. However, nucleotide sequencing revealed that the ends of the amplified products were the (dA)<sub>3</sub> or (dA)<sub>4</sub> stretch of the viral sequence (data not shown).

### **Search for spliced forms and quantification of asRNA**

Anchored-5'RACE, RLM-3'RACE or oligo(dT)-primed 3'RACE failed to reveal any nucleotide sequences compatible with the spliced forms. Therefore, to find any spliced forms of asRNAs, we performed further RT-PCRs of the region encompassing positions 1282–1650. However, only non-spliced products were obtained consistently and some smaller products were observed in a limited number of cases. We cloned and sequenced the RT-PCR products smaller than 0.7 kb but their deleted sequences lacked the intron GT or AG at both ends; they were made by homologous recombination or polymerase jumping in PCR (data not shown). Therefore, it is likely that the 0.7 kb non-polyadenylated asRNAs lack introns.



**Fig. 3.** Determination of the 3' ends of asRNAs by RLM-3'RACE. (A) Agarose gel electrophoresis of RLM-3'RACE products. 3' ends of RNA were ligated with ANCHOR-B100 and the cDNA was synthesized using primer RLMP1 annealing with ANCHOR-B100. Arrows indicate PCR products found to be bearing nucleotide sequences specific for HBV. The molecular mass marker is a 500 bp dsDNA ladder. (B) Southern blot of RLM-3'RACE products. PCR products diluted 500-fold were run in a 1 % agarose gel and blotted onto a nylon membrane. The HBV-specific sequence was detected by alkaline phosphatase-labelled HBV DNA. (C) Nucleotide sequences of HBV DNA from five patients. The HBV-specific RLM-3'RACE products were cloned in plasmids and the nucleotide sequences were determined. The 3' ends of the asRNAs are indicated by filled circles. Numbers on the lines indicate the number of clones sequenced. Antisense (dT)<sub>4</sub> and (dT)<sub>5</sub> tracts are underlined.

We estimated the amounts of HBV transcripts by competitive PCR. We synthesized the cDNAs using primer B026, which anneals at positions 1262–1282. To the same reaction mixture, we added primers B301 and GAPDH-R to reverse transcribe the sense RNA of the virus as well as GAPDH mRNA. We then amplified the cDNA by PCR in the presence of either a 10-fold- or a 2-fold-diluted competitor DNA. The amounts of asRNAs and sense RNAs varied among the five liver tissue samples examined and their ratios were estimated to be approximately 1:250–1:2000 (Table 2).

**Table 2.** Copy numbers of asRNA, sense RNA (snRNA) and spliced GAPDH mRNA (GAPDH) in the livers of study patients, as determined by competitive PCR

Patient	asRNA	snRNA	GAPDH
1	50*	$12.5 \times 10^3$	$2.5 \times 10^5$
2	12.5	$2.5 \times 10^4$	$2.5 \times 10^5$
3	50	$12.5 \times 10^3$	$2.5 \times 10^5$
4	25	$6.25 \times 10^3$	$2.5 \times 10^5$
5	25	$12.5 \times 10^3$	$2.5 \times 10^5$

\*Unit is copy number of competitor cDNA added to 50  $\mu$ l PCR and corresponds to the copy number of asRNA cDNA synthesized from 14 ng total RNA.

## DISCUSSION

As reported previously for WHV, but unlike previous transfection experiments, the expression levels of asRNAs examined in this study were extremely low *in vivo*. Northern blotting using a sense-strand ribonucleotide probe corresponding to positions 679–1700 failed to detect any signals specific for HBV (data not shown). Only RT-PCR was able to detect asRNAs. The copy numbers of the asRNA were estimated to be 0.4–0.05 % of those of sense RNAs by our quantitative PCR. In addition, two rabbit antisera raised against a synthetic peptide of the deduced amino acid sequence of ORF6 yielded negative results in Western blots of patients' liver tissue (data not shown). This negative result seems reasonable if we consider the extremely low copy numbers of asRNAs, even though they could be the coding transcripts.

Anchored-5'RACE amplified the transcripts initiating at position 1655 in four cases. A database search showed that the co-existence of T<sup>1678</sup> and G<sup>1679</sup> in patient 2 was a rare event and appeared to have altered the initiation site of the asRNA from 1680/1681. This suggests that this region is, in fact, an antisense promoter region.

Position 1655 lies near the position that was reported previously to be the initiation site of RNA polymerase III at positions 1629, 1631 or 1632 (Fig. 2C) (Standring *et al.*, 1983). There is no typical TATA, CAAT or GC box around this region and the asRNAs terminated at (dT)<sub>4</sub> or (dT)<sub>5</sub> tracts (Fig. 3C). Therefore, it is possible that asRNAs are transcribed by a polymerase III system *in vivo* (Geiduschek & Tocchini-Valentini, 1988; Willis 1993) and the difference between our results and those of Standring *et al.* (1983) may be due to the different nucleotide sequences of this region (Fig. 2C).

asRNAs detected in our patients had ORF6, capable of encoding a protein of 95 amino acids with a predicted molecular mass of 10.5 kDa. It has been demonstrated that an RNA transcribed from a strong polymerase III promoter of a plasmid construct in transfected HeLa cells produces the coding protein (Gunnery & Mathews, 1995). Thus, human cells can utilize polymerase III transcripts as functional mRNAs and neither a cap nor a poly(A) tail is essential for translation. However, there is no known natural case of translation of RNAs made by polymerase III. One asRNA initiating at position 1680/1681 in patient 2 had the initiation and adjacent termination codons in an area 40 nucleotides upstream of ORF6. In addition, the most widely reported HBV subtype *adw* has an upstream initiation codon at positions 1631–1629. Moreover, the length and amino acid sequence of the carboxy-terminal region varies among reported strains. Therefore, ORF6 and its product are not required for virus proliferation.

asRNAs can regulate the transcription of sense mRNA by annealing with the complementary sequences of DNA templates (reviewed by Kumar & Carmichael, 1998). asRNAs can also hybridize with complementary sense RNAs. The dsRNA molecules in the nuclear compartment can be a substrate for enzymes that deaminate adenosine to inosine. Extensively modified RNAs are either degraded rapidly or retained within the nucleus. Transcripts with few modifications may be transported to the cytoplasm, where they serve to produce altered proteins. Therefore, asRNAs can cause amino acid alterations of polymerase and X proteins. dsRNA molecules in the cytoplasm can trigger sequence-non-specific interferon synthesis. Minced small molecular dsRNAs in the cytoplasm can also induce the Dicer pathway (Ahlquist, 2002). Since this cycle does not require a large amount of trigger RNA, the small amount of cytoplasmic asRNA, if any, might be sufficient to inactivate any molar excess of sense RNAs. Therefore, the existence of endogenous asRNAs complementary to the common ends of all sense RNAs of HBV suggests an antisense-mediated self-regulation of hepadnavirus.

## ACKNOWLEDGEMENTS

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