A trans-activator function is generated by integration of hepatitis B virus preS/S sequences in human hepatocellular carcinoma DNA

(trans-activation/truncation/pSV2CAT reporter plasmid)

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ABSTRACT The X gene of wild-type hepatitis B virus or integrated DNA has recently been shown to stimulate transcription of a variety of enhancers and promoters. To further delineate the viral sequences responsible for trans-activation in hepatomas, we cloned the single hepatitis B virus insert from human hepatocellular carcinoma DNA M1. The plasmid pM1 contains 2004 base pairs of hepatitis B virus DNA subtype adr, including truncated preS/S sequences and the enhancer element. The X promoter and 422 nucleotides of the X coding region are present. The entire pre/C gene is deleted. In transient cotransfection assays using Chang liver cells (CCl 13), pM1 DNA exerts a 6- to 10-fold trans-activating effect on the expression of the pSV2CAT reporter plasmid. The trans-activation occurs by stimulation of transcription and is dependent on the simian virus 40 enhancer in the reporter plasmid. Deletion analysis of pM1 subclones reveals that the trans-activator is encoded by preS/S and not by X sequences. A frameshift mutation within the preS2 open reading frame shows that this portion is dispensable for the trans-activating function. Initiation of transcription has been mapped to the S1 promoter. A comparable trans-activating effect is also observed with cloned wild-type hepatitis B virus sequences similarly truncated. These results show that a transcriptional trans-activator function not present in the intact gene is generated by 3' truncation of integrated hepatitis B virus DNA preS/S sequences.

Strong epidemiological evidence closely links the development of hepatocellular carcinoma (HCC) with chronic hepatitis B virus (HBV) infection (1). Consistently integrated HBV DNA sequences are found in HCC tissue of more than 90% of hepatitis B surface antigen (HBsAg)-seropositive patients (2). The integration of HBV DNA is thus considered to be a pathogenic factor for tumor development.

In human HCCs cis-activation of oncopgenes by HBV promoter or enhancer insertion cannot be regarded as a general mechanism of transformation, although a case is reported where a cellular gene related to a known oncopgene might have been activated by integrated HBV DNA (3). On the other hand, HBV like many other human DNA viruses (4) bears trans-activational potential. The HBV X open reading frame (ORF) encodes a transcriptional trans-activator that stimulates transcription from its own and many heterologous promoters in transient transfection assays (5–7). The trans-activating function of a tumor-derived HBV subclone was conserved, even when X sequences had been truncated by integration (8). To extend these observations and to delineate the viral sequences responsible, we cloned a single HBV DNA insert from human hepatoma tissue. Here we provide evidence for a transcriptional trans-activator encoded within the preS/S region of HBV DNA whose activity is newly generated by dislocation from its downstream sequences.

MATERIALS AND METHODS

Tissue of the hepatoma designated M1 was obtained by partial hepatectomy from a 46-year-old Chinese man with a histologically confirmed HBsAg-positive HCC. DNA Extraction and Southern Blot Hybridization. Ten micrograms of high molecular weight genomic DNA M1 DNA was digested with restriction endonucleases, electrophoresed in 0.8% agarose gels, and transferred to nitrocellulose membranes (9). Hybridization was carried out in 50% (vol/vol) formamide, 5x SSC (1x SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), and 1% sarcosyl at 42°C by using DNA probes labeled with 32P by random priming (10). Filters were autoradiographed for 4–24 hr at ~70°C.

Genomic Library Construction. HindIII-digested M1 DNA was ligated into the HindIII arms of λ vector Charon 28. Recombinant bacteriophages were packaged and adsorbed to Escherichia coli 490-A cells. The library was screened with 32P-labeled HBV DNA, and the DNA from a plaque-purified positive clone was recloned in the pUC19 vector to create plasmid pM1.

Plasmid Construction and Sequencing. All plasmids were cloned in the pUC19 vector by using standard methods unless otherwise stated. The nucleotide sequences of pM1 and its subclones were determined by dyeoxy sequencing (11). The computer software package of the University of Wisconsin Genetics Computer Group (UWGCG) and the nucleotide sequences of the European Molecular Biology Laboratory (EMBL) Data Library (release 20.0) were used for comparison of sequence homologies.

Transient Expression of Plasmid DNAs. Chang cells (ATCC CCL 13) originally derived from nonmalignant human liver cells (12, 13) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) fetal calf serum. Five picomoles of the respective plasmid DNA was transfected by the calcium phosphate technique (14). Cells were harvested 12–18 hr later and lysed in guanidinium isothiocyanate. Total cellular RNA was purified by CsCl gradient centrifugation (15) and treated with RNase-free DNase. Poly(A)+ RNA was isolated through oligo(dT)-cellulose columns. One microgram of poly(A)+ RNA was electrophoresed in agarose gels with 20 mM 4-morpholinepropanesulfonic acid (Mops), 5 mM sodium acetate, and 0.1% EDTA, blotted to nylon membranes, and hybridized as described above.

Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40; ORF, open reading frame; HBsAg, hepatitis B surface antigen.

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**RESULTS**

**Structural Organization of Clone pM1.** Southern blot hybridization of HindIII-digested genomic DNA from HCC tissue M1 revealed a single 4.8-kilobase (kb) fragment that hybridized to HBV DNA (Fig. 1A). An M1 genomic library was constructed and screened with 32P-labeled HBV DNA. DNA from a plaque-purified positive clone was cloned in the pUC19 vector, yielding plasmid pM1. The nucleotide sequence of pM1 was determined by dideoxy sequencing (11). The insert in clone pM1 consists of 2004 base pairs (bp) of HBV DNA with an overall homology of 97.9% to the HBV subtype adr (18). The integrated HBV DNA contains a truncated preS/S gene (nucleotides 2821–423 and fragments were analyzed on 6% polyacrylamide/8 M urea gels (16). Autoradiography was performed at −70°C.

**Chloramphenicol Acetyltransferase (CAT) Assay.** One microgram of the reporter plasmid pSV2CAT (17) was cotransfected with a 10-fold molar excess of test plasmid into CCL 13 cells. Forty to 48 hr after transfection, the cells were lysed by sonication. Fifty micrograms of total protein was incubated at 37°C for 90 min in the presence of 4 mM acetylcoenzyme A and 75 nCi (1 Ci = 37 GBq) of [32P]chloramphenicol. TLC of the reaction products was performed as described (17). All experiments were repeated four to six times with at least two different preparations of DNA. Acetylation was quantified by cutting out the appropriate portions of the TLC plate and determining the radioactivity in a liquid scintillation counter.

**In Vitro Transcription and RNase Protection Mapping.** The appropriate template plasmid was used to synthesize [32P]-UTP-labeled riboprobes complementary to the mRNA transcribed from the transfected test plasmids with SP6 polymerase. About 1.2 × 10⁶ CCL 13 cells were cotransfected with 2 μg of pSV2CAT and a 10-fold molar excess of test plasmid. Thirty micrograms of total cellular RNA was hybridized to 1 × 10⁶ cpm of the riboprobe for 12–15 hr in 50% (vol/vol) deionized formamide, 40 mM Pipes (pH 6.6), and 400 mM NaCl at 42°C. After treatment with RNase A (20 μg/ml) and RNase T1 (5000 units/ml) for 1 hr at 37°C, the protected

![Diagram](image-url)

**Fig. 1.** (A) Southern blot analysis of human HCC M1 DNA revealed a single HBV band of 4.8 kb and no episomal HBV DNA. (B) Northern blot analysis of poly(A)⁺ RNA isolated from pM1-transfected CCL 13 cells showed a viral-cellular read-through transcript of 2.5 kb, which hybridized with total HBV (lane 1) and preS/S-specific probes (nucleotides 2844–127, lane 2). An X gene-specific probe (nucleotides 1400–1984, lane 3) failed to hybridize. Molecular size markers (in kb) are provided on the right.

![Diagram](image-url)

**Fig. 2.** Structural organization of clone pM1. The indicated nucleotide positions refer to the HBV insert. Boxes represent integrated HBV DNA. The 905-bp deletion covering the C gene is depicted. The triangle indicates a 1-bp deletion that shifted the X ORF. Solid bars represent the flanking cellular regions. The transcriptional start sites at the X, C, S1, and S2 promoters; the sizes of respective viral-cellular fusion transcripts; and the location of a cellular poly(A) site are shown. A physical map of the HBV genome is also shown. P, promoter; E, enhancer; DRI/II, direct repeat I/II; AUG start codon.
717–832; nomenclature of Galibert, ref. 19) with the S1 (20) and S2 (20, 21) promoters. The viral enhancer element (22) and the X promoter are conserved. Twelve 3' nucleotides (1797–1808) of the X ORF are missing. The recently identified ORF 5 (24) is intact. Deletion of the entire preC/C gene fused preS sequences directly to the X ORF. The 5' viral integration site maps to nucleotide 717 and the 3' viral end maps to nucleotide 423, both located in the single-stranded gap region within the S gene, which is one of the preferred sites for recombination (ref. 25; Fig. 2). Computer analysis of the sequenced 5' and 3' flanking cellular DNA revealed no homology with any known human genes.

**Transcriptional Trans-Activating Function of preS/S Sequences.** In transient cotransfection assays using the CCL 13 cell line, pM1 DNA stimulated the expression of pSV2CAT DNA 6- to 10-fold as compared to the pUC19 vector DNA alone (Fig. 3). The reporter plasmid pSV2CAT (17) contains the CAT gene under the control of the simian virus 40 (SV40) early promoter and enhancer, whereas pSV1CAT is lacking the SV40 enhancer (17). No activation of the CAT gene expression was detectable when pSV1CAT was used. These data suggest that the SV40 enhancer element is necessary for the effect.

Initially assuming that the activation was due to X sequences, we constructed subclone pM35 (Fig. 3) containing HBV DNA from nucleotides 948–1796 (i.e., eliminating all but the X ORF and ORF 5 sequences and their regulatory elements). pM35 did not stimulate the CAT gene expression significantly higher than the controls. This might be due to a 1-bp deletion at position 1725 that shifts the reading frame and results in a translational stop at nucleotide 1758. The plasmids pFr and pFl (Fig. 3) covering the 5' and 3' cellular flanking sequences, respectively, exerted no activating function either (Fig. 3). To test the remaining HBV sequences of pM1 for trans-activational properties, subclones pM18 (nucleotides 717–1236) and pM27 (nucleotides 1372–1796/2703–247) were constructed (Fig. 3). Whereas plasmid pM18 showed no stimulatory effect on CAT gene expression, pM27, comprising the X gene without its 5' regulatory elements and the preS/S region, exerted a similar activating effect (average of 6.3-fold stimulation) as the entire pM1 clone (Fig. 3). Subclone pM56, which contains the preS/S sequences (nucleotides 1764–1796 and 2703–217) and both S promoters stimulated CAT gene expression 7.1-fold with respect to the pUC19 control (Fig. 3). A frameshift mutant pM56fsXhoI constructed by cutting the DNA at the XhoI site (nucleotide 127) within the preS2 ORF lost the trans-activating function (Fig. 3). These data suggest that the stimulatory effect on CAT gene expression is mediated by means of a trans-acting gene product of preS/S specificity and that the preS2 portion is dispensable for trans-activation. The truncated protein would contain the known myristoylation site (26) and the N-terminal amino acids of the large S protein (27), both of which have been suggested to inhibit the secretion of the S protein. On the other hand, the presence of 14 of 15 amino acids of the membrane translocation signal (amino acids 8–22 of the major S protein, ref. 28) and the loss of the membrane stop signal (amino acids 80–98 of major S protein, ref. 28) could result in secretion of the trans-activator protein. However, with standard HBsAg RIA's (Austria II, Abbott), no cross-reactive antigens were detectable so far in supernatants of CCL 13 cells transfected with pM1.

To investigate the mode of the trans-activation, CAT-specific transcripts were measured by RNase protection analysis after cotransfection of CCL 13 cells with pSV2CAT and various preS/S-containing test plasmids. Transfection with plasmids pM1, pM27, or pM56 led to elevated levels of

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**Fig. 3.** CAT assays for trans-activation by pM1, subclones of pM1, and wild-type HBV DNA. The top line represents pM1. Solid lines below the map symbolize pM1 subclones. The inverted triangle in clone pM56fsXhoI indicates the position of the frameshift mutation. Wild-type HBV clones are depicted at the bottom: pVPS (nucleotides 2352–217) contains 3' truncated preS/S DNA; pHBS (nucleotides 2434–833) contains the full-length preS/S sequence. The autoradiogram shows trans-activation of pSV2CAT expression by preS/S-containing test plasmids in transiently transfected CCL 13 cells. The factor of activation was calculated in relation to the acetylation obtained by the pUC19 control. CM, unreacted chloramphenicol; 1-Ac-CM, 1-acetylchloramphenicol; 3-Ac-CM, 3-acetylchloramphenicol.
CAT mRNA as reflected by the intensity of the 150-nucleotide RNase-resistant band (Fig. 4). The 8-fold increase of CAT mRNA as determined by densitometry corresponded to a 7- to 8-fold activation in CAT assays. We therefore conclude that trans-activation by integrated preS/S sequences occurs at the transcriptional level.

Since both S gene promoters were contained in all trans-activating clones, we performed RNase protection mapping of preS/S-specific mRNAs to determine the transcription start sites. Analysis of mRNA isolated from CCL 13 cells transfected with pM27 or pM56 revealed two protected RNAs initiating at the S1 promoter (Fig. 5). One transcript most likely started at the previously described initiation site (20) and a second one started about 30 nucleotides upstream. Transcripts arising from the S2 promoter were not detected.

**Transient Expression of a Viral–Cellular Fusion Transscript by pM1 DNA.** Northern blotting of total and polyadenylated RNA isolated from CCL 13 cells transiently transfected with pM1 DNA showed a distinct transcript of 2.5 kb that hybridized to total HBV (Fig. 1B, lane 1) and preS/S (Fig. 1B, lane 2) but not to X DNA (Fig. 1B, lane 3). The size of the transcript suggested a read-through from viral to flanking 3' cellular sequences and probably termination at a sequenced poly(A) site identified 1700 bp downstream of the viral–cellular junction.

**Trans-Activation by Cloned Wild-Type HBV preS/S Sequences.** To test for the trans-activational potential of the corresponding cloned wild-type HBV sequences, plasmid pVPS was constructed. It comprises the HBV nucleotides 2512–217 (i.e., the complete preS region and the S sequences truncated at exactly the same position as in the integrated HBV DNA in pM56). When tested for CAT activity, pVPS exerted a 5- to 8-fold activating effect that equaled the level of stimulation by the integrated counterpart (Fig. 3). In contrast, cloned full-length wild-type preS/S sequences contained in clone pHBS (nucleotides 2434–833) did not stimulate CAT gene expression (Fig. 3; refs. 6 and 30). Thus, even in the unintegrated state, the preS/S gene exerts a trans-activating function only when 3' truncated.

**DISCUSSION**

We provide evidence for the presence of a previously unreported transcriptional trans-activator within the preS/S region of HBV. The single-copy HBV DNA insert of a human

![Fig. 4. RNase protection analysis of CAT mRNA. (A) Schematic illustration. In vitro transcription of EcoRI-digested plasmid pSPTKCAT (29) by SP6 polymerase gives rise to an antisense CAT riboprobe of 298 nucleotides (nts). Hybridization to the 5' portion of CAT mRNA initiating at the SV40 early promoter of pSV2CAT results in a 150-nucleotide RNase-resistant fragment. TK, thymidine kinase. (B) Autoradiograph showing the relative increase in intensity of the 150-nucleotide CAT fragment after cotransfection of preS/S comprising subclones pM1 (lane 3), pM27 (lane 4), and pM56 (lane 5) is about 8-fold with respect to the pUC19 control (lane 2). Lane 1, radiolabeled molecular size markers (in nucleotides).](image)

![Fig. 5. RNase protection analysis of preS/S mRNA. (A) Schematic illustration. The blunt-ended insert of plasmid pM56 (nucleotides 1764–1776 and 2703–218) was cloned into the Sma I site of plasmid pGEM3Z (Promega) generating plasmid pGEM3Z56. In vitro transcription of EcoRI-digested plasmid pGEM3Z56 by SP6 polymerase gives rise to an antisense preS/S probe of 750 nucleotides. Hybridization to the 5' portion of preS/S mRNAs results in RNase-resistant fragments of 620 and 650 nucleotides (nts). (B) Autoradiograph. The presence of two protected RNA fragments of 620 and 650 nucleotides from CCL 13 cells transfected with pM27 (lane 2) and pM56 (lane 3) indicates that transcription of preS/S sequences initiated at the known and an additional unreported start site of the S1 promoter. Lanes: 1, pUC19 control; 4, 1000 cpm of preS/S antisense riboprobe (750 nucleotides); 5, radiolabeled molecular size markers (in nucleotides).](image)
HCC was cloned. The clone, pM1, contains truncated preS/S and X sequences. A major deletion of 905 bp (HBV nucleotides 1799-2702) led to the elimination of the C gene.

Several instances of truncated preS/S sequences have been identified in various HCCs or hepatoma cell lines (40-42); thus, trans-activation by integrated truncated preS/S sequences may have implications in tumor development in HBV-infected hepatocytes.

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