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Regulatory Sequences of Duck Hepatitis B Virus C Gene Transcription

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The regulatory elements involved in transcription of the C gene of duck hepatitis B virus (DHBV) were investigated. Several DHBV DNA fragments were assayed for C gene promoter, enhancer, and silencer activity by using a chloramphenicol acetyltransferase (CAT) reporter gene and transfection of established liver and nonliver cell lines. A major transcript initiating at nucleotide positions 2532 and 2533 and three minor transcripts initiating at positions 2453/2454 and 2461 were identified in cells containing these constructs. These positions correspond to the 5′ end of the C mRNA and were close to that of the pre-C mRNAs, respectively, found in infected livers. The pre-C mRNAs were only detected when sequences located between the initiation sites of the pre-C and C mRNAs were deleted. These sequences downregulated, in an orientation-independent fashion, a heterologous promoter and were found to contain a consensus motif common to negative transcriptional regulatory elements previously characterized in other cellular and viral genes. C gene promoter activity was only observed in highly differentiated liver cells and was dependent on a short DHBV DNA fragment containing an enhancer core consensus motif. These data indicate that transcription of the DHBV C gene is regulated by positive, negative, and differentiation factor-responsive elements.

The C gene of all hepadnaviruses encodes the nucleocapsid protein and, in most hepadnaviruses, a precursor protein (precore) for a secreted polypeptide, designated e antigen, which is not associated with virus particles (for review, see reference 24). In mammalian hepadnaviruses, only two mRNAs appear to be necessary for expression of C gene-encoded proteins: a minor mRNA (pre-C mRNA) with heterogeneous 5′ ends initiating upstream of the C gene, which serves as a template for translation of the precore protein, and a major mRNA (C mRNA) initiating between the first and second AUG of the C gene (36), from which the nucleocapsid protein is translated by initiation of translation at the second AUG of the C gene (18). The C mRNA also serves as pregenome, which is encapsidated into viral core particles and reverse-transcribed within these particles into viral DNA (31). In contrast, the pre-C mRNA is selectively excluded from encapsidation, and this is mediated by ribosomes engaged in translation of the pre-C region, which prevent recognition of the encapsidation signal (19).

Regulation of C gene transcription has been studied so far only for hepatitis B virus (HBV). A promoter region directing several heterogeneously initiating mRNAs was identified, and its activity was shown to be regulated by several cellular factors, a virus-encoded transcriptional transactivator protein, designated HBx, and at least one enhancer element (for review, see reference 22). Related avian hepadnaviruses (duck hepatitis B virus [DHBV] and heron hepatitis B virus [HHBV]; for review, see reference 25) do not encode an HBx-related transactivator protein, and a putative enhancer has so far been localized only by sequence homology, not by functional analysis (29).

DHBV-infected Pekin ducks are a most convenient animal model which has been used extensively and successfully to elucidate various aspects of the life cycle of hepadnaviruses (for review, see reference 25). DHBV, like HBV, also encodes a major RNA (C mRNA) that is transcribed from the C gene of DHBV and serves as a translation template for synthesis of the nucleocapsid (DHBV c antigen) and the P proteins (6). A very minor pre-C mRNA which serves for precore and DHBV e antigen synthesis has only recently been identified and was found in only a few infected livers in very low concentrations (23). It is currently unclear whether the variable and low amounts of the DHBV pre-C mRNA are due to differential regulation of a weakly active pre-C mRNA-specific promoter. In addition, regulatory sequences involved in DHBV pre-C and C mRNA transcription have not been examined so far.

We have therefore studied DHBV C gene transcription to identify regulatory sequences involved in synthesis of pre-C and C mRNAs. We demonstrate that positive, negative, and cell differentiation-specific factors regulate DHBV C gene transcription.

MATERIALS AND METHODS

Plasmid constructions. All cloning procedures were performed as described elsewhere (20). DNA fragments used for cloning were isolated by electroelution from agarose or polyacrylamide gels, and plasmids were prepared by an alkaline lysis method and purified by CsCl gradient centrifugation. For dideoxy sequencing of the vector-insert junctions of plasmids containing DHBV DNA and a CAT gene, a CAT gene-specific synthetic oligonucleotide (5′-CTTTAC GATGCCATTGGG-3′) and a dideoxy sequencing kit (Sequenase; US Biochemicals, Cleveland, Ohio) were used.

The longest DHBV DNA fragment used in the CAT gene-containing plasmids (Fig. 1A and B) were derived from plasmid pMc5-8 (30) carrying a full-length *Eco*RI fragment of the DHBV26 genome (3,024 bp long [25]), into which a single-nucleotide mutation was introduced by oligonucleotide-directed mutagenesis, changing the first ATG of the C gene to TTG (plasmid pDHBV26TTG [23]). This mutation was introduced to eliminate this ATG upstream of the CAT

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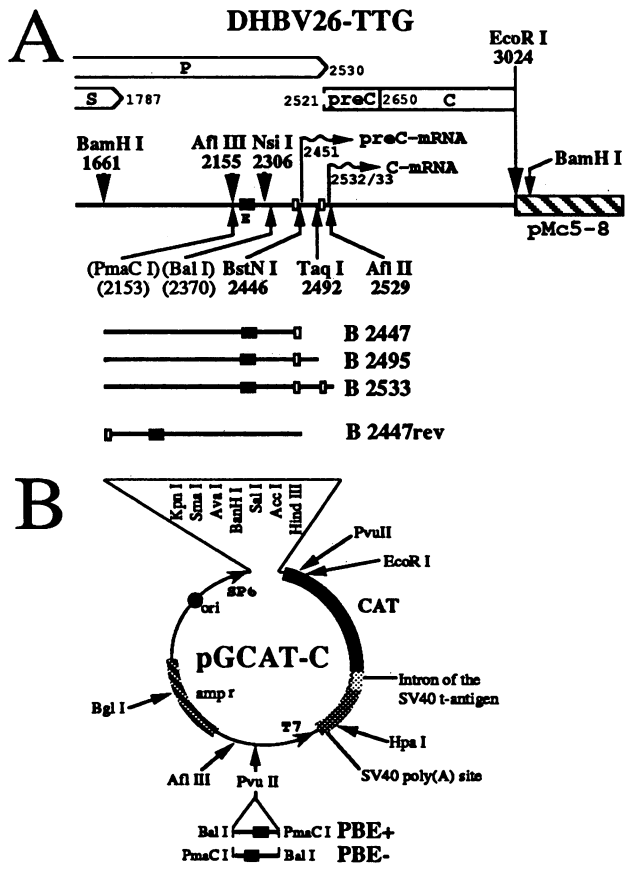

FIG. 1. (A) DHBV genome region and C gene promoter fragments analyzed. Restriction enzyme cleavage sites (numbers denote nucleotide sequence positions) used for cloning and the transcription initiation sites of the C and pre-C mRNAs (wavy lines with arrows) as mapped in vivo are indicated. The enhancer core sequence motif is drawn as a black box, and the two TATA boxes presumably involved in C and pre-C mRNA transcription initiation are indicated as small open boxes. Restriction enzyme recognition sites in parentheses correspond to those of the DHBV3 sequence used for cloning of the enhancer region. (B) CAT vector used to test promoter and enhancer (PBE) fragment activity. Indicated are restriction sites used for cloning and functional elements important for cloning and expression.

gene translation initiation codon in some DHBV DNA–CAT gene–containing plasmids. The longest DHBV DNA fragment tested for promoter activity in the pGCAT-C plasmid (Fig. 1B) (12) spanned the DHBV region from nucleotide position 1661 at the *Bam*HI site to 2533 (plasmid pB2533) and contained the putative enhancer region, both TATA boxes, and the transcription initiation sites of the C and pre-C mRNAs (Fig. 1A), as determined previously (6, 23, 29). Plasmid pB2533 was obtained by first isolating a DHBV DNA *Bam*HI fragment with a few nucleotides derived from the vector at its 3′ end from plasmid pDHBV26TTG (Fig. 1A), inserting this fragment into the *Bam*HI site of pGCAT-C (Fig. 1B), and then deleting an *Afl*II-*Sal*I fragment (Fig. 1A) by restriction enzyme digestion. The ends were made blunt ended by a fill-in reaction with T4 polymerase and ligated.

Plasmid pB2495, which differs from pB2533 by deletion of DHBV sequences at the 3′ end (up to nucleotide position 2495), was obtained by ligating a 0.9-kb *Bam*HI-*Taq*I DHBV fragment (Fig. 1A) with vector pGCAT-C linearized by *Bam*HI and *Acc*I (Fig. 1B). Plasmids pB2447 and pB2447rev were obtained by isolation of a 0.8-kb *Bam*HI-*Bst*NI DHBV DNA fragment from pDHBV26TTG (Fig. 1A), which was then blunt-ended with Klenow polymerase, modified by addition of *Bam*HI linkers, and cloned into the *Bam*HI-linearized pGCAT-C vector (Fig. 1B). Correct insert orientation and the DHBV-vector junction were determined by sequencing with CAT gene- and SP6 promoter-specific primers (data not shown).

To delimit the pre-C/C promoter region further, several mutants of plasmids pB2447 and pB2533 were created in which 5′-terminal sequences of the corresponding DHBV DNA fragment were deleted up to the *Afl*III (plasmids pA2447 and pA2533) or *Nsi*I (plasmids pN2447 and pN2533) restriction site (for restriction sites, see Fig. 1A). Deletion of DHBV sequences up to the *Nsi*I site was achieved by removal of the *Kpn*I-*Nsi*I fragment of the respective plasmid and religation after blunt ending with T4 DNA polymerase. For DHBV-CAT plasmids with 5′ deletions in the DHBV fragment up to the DHBV *Afl*III site, the second *Afl*III site (Fig. 1B) in pB2447 and pB2533 first had to be destroyed. This was achieved by first isolating a *Bgl*I-*Hpa*I fragment derived from the pGCAT-C vector spanning the T7 promoter (Fig. 1B), in which the previously unique *Afl*III restriction site was destroyed by restriction enzyme digestion with *Afl*III and blunt-ending by a fill-in reaction with T4 DNA polymerase. This fragment was then exchanged for the analogous *Bgl*I-*Hpa*I fragment of plasmids pB2447 and pB2533, leaving a unique *Afl*III site in the DHBV DNA fragment. Now the 5′ DHBV DNA sequences could be deleted by *Ava*I-*Afl*III digestion, blunt-ending by a fill-in reaction with T4 DNA polymerase, and ligation at low DNA concentration.

To test the enhancer of a DHBV fragment with a core consensus motif of known eukaryotic enhancers (29), a 0.22-kb *Pma*CI-*Bal*I DHBV3 DNA fragment (restriction sites indicated on the DHBV26 genome in Fig. 1A) of plasmid pSPD3 (28) containing this motif was inserted downstream of the CAT coding region of vector pGCAT-C into the *Pvu*II site (Fig. 1B). This required three cloning steps. First, one of the two *Pvu*II sites of the pGCAT-C vector had to be destroyed, which was achieved by deletion of the small *Eco*RI fragment spanning the polylinker region. Second, into the unique *Pvu*II site of this pGCAT-C derivative, the *Pma*CI-*Bal*I DHBV3 DNA fragment (Fig. 1A) was inserted by blunt-end ligation in both orientations. Third, the *Bgl*I-*Hpa*I fragments containing the *Pma*CI-*Bal*I DHBV3 fragments of both of these plasmids were ligated with the *Bgl*I-*Hpa*I fragment of pN2447 or pN2533 (see above). The four plasmids thus obtained (pN2447PBE+, pN2447PBE−, pN2533PBE+, and pN2533PBE−, with the enhancer fragment in authentic (+) or inverse (−) orientation, allowed us to test the enhancer function of the DHBV *Pma*CI-*Bal*I fragment on two DHBV pre-C/C promoter fragments (spanning nucleotides 2306 to 2447 and 2306 to 2533, respectively) and to test the enhancer function in an orientation-independent manner and in a heterologous sequence context, separated from the pre-C/C promoter region by unrelated sequences.

To test the putative silencer function of a DHBV DNA fragment, two plasmids were constructed by inserting this fragment into an expression unit consisting of the Rous sarcoma virus (RSV) promoter linked to a CAT gene. This expression unit was obtained from plasmid pRSV-CAT (15) by linearization by *Nde*I digestion, blunt-ending the sticky

ends by a fill-in reaction with Klenow polymerase, and release of the fragment by *Hin*dIII digestion. The fragment was purified and inserted into pGCAT-C linearized by *Hin*dIII and *Sma*I digestion, resulting in plasmid pRSV CAT-C. Into the *Hin*dIII site of this plasmid, the putative silencer element was inserted between the transcription initiation site of the RSV promoter and the coding region of the CAT gene by using appropriate synthetic oligonucleotides: S+, 5′-AGCTAGCACAAACCGCCTGATTGGACG GCTGTTACATACACCCCTCTCTCGA-3′; S−, 5′-AGCT TCGAAGAGGGGTGTATGTAACAGCCGTCCAAT CAGGCGGTTTGTGCT-3′. Correct insertion and orientation of the oligonucleotides were determined by sequencing. As a control, instead of the putative DHBV silencer sequence a polylinker sequence of almost the same length and a similar purine-pyrimidine ratio (5′-AGCTTGCATGCCAG CAGGTCGACTCTAGAGAGGATCCCCGGGTACCGAG CTC-3′) was inserted into the same *Hin*dIII site of pRSV CAT-C.

Mammalian cell lines. The following cell lines were used: HepG2, a highly differentiated human hepatoblastoma cell line (1); Hep2.2.15, a derivative of HepG2 transfected with cloned human HBV DNA and producing infectious virus (26); PLC/PRF/5, a highly differentiated human hepatocellular carcinoma cell line which carries chromosomally integrated copies of truncated and mutated fragments of HBV DNA (2); CCL13, a cell line derived from nonmalignant human liver tissue (8) but with HeLa cell markers (information from American Type Culture Collection); SK-Hep, a dedifferentiated human liver cell line (11); HeLa, a cell line derived from a human cervical carcinoma (14); and NIH 3T3, a cell line derived from mouse fibroblasts (17). All cell lines were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 2 mM sodium pyruvate, 2× nonessential amino acids, 100 U of penicillin and 100 μg of streptomycin per ml, and 7 mM L-arginine and kept under a 5% CO_2 atmosphere.

Transfection of mammalian cells. Plasmid DNAs used for transfection of cells were prepared by alkaline lysis and purified twice by CsCl gradient centrifugation and extensive dialysis against water (20). Cell monolayers were transfected at 40 to 60% confluency with 10 μg of plasmid DNA per 5-cm dish by the $Ca_3(PO_4)_2$ coprecipitation technique (16). After 12 to 14 h, the cells were shocked with dimethyl sulfoxide (10% in DMEM, 2 min at 37°C). The dimethyl sulfoxide-containing medium was removed, the cells were washed three times with phosphate-buffered saline (PBS; 12 mM NaCl, 17 mM Na_3PO_4, 2.5 mM KH_2PO_4 [pH 7.3]), and new medium was added. After incubation of the cells for 48 h, they were washed twice with PBS, scraped off with a rubber policeman into 1 ml of PBS, and pelleted for 10 min at 4°C. The pellet was resuspended in 200 μl of 250 mM Tris-HCl (pH 7.5) and frozen at −70°C until used in the CAT assay.

CAT assay. After the frozen cells were thawed for 4 min at 37°C, protein extracts were prepared by sonification of the cells three times on ice for 5 s each (intensity level 6, 60% duty; Branson cell disruptor B15). Cell debris was removed by centrifugation at 4°C for 10 min, and the extracts were stored at −20°C until use. The CAT assay was carried out as described before (15) with 30 μg of protein, 10 μl of [^{14}C]chloramphenicol (57 mCi/mmol in 250 mM Tris-HCl [pH 7.5]; Amersham-Buchler, Braunschweig, Federal Republic of Germany [FRG]), and 20 μl of 4 mM acetyl coenzyme A (Pharmacia, Freiburg, FRG) in a final reaction volume of 100 μl. After incubation for 2 h at 37°C, the reactions were stopped, and acetylated and nonacetylated forms of chloramphenicol were isolated and analyzed as described before (15).

RNA analysis. For isolation of RNA from transfected cells, a modified version of an established technique (3) was used. For each construct (pB2447, pB2495, pB2533, and pRSV), eight 10-cm dishes of Hep2.2.15 cells were transfected as described above with 20 μg of plasmid per dish. Then, 24 h after the dimethyl sulfoxide shock, the cells were washed twice with ice-cold PBS, scraped off into 1 ml of PBS, and pelleted for 5 min at 4°C. The pellets were pooled and resuspended in 1 ml of PBS and 1 ml of 0.2 M Tris-HCl (pH 7.5) containing 25 mM EDTA, 0.3 M NaCl, and 2% sodium dodecyl sulfate. After the suspended pellets had been squeezed several times through a 20-gauge needle, 50 μl of proteinase K (20 mg/ml) was added, and the mixture was incubated at 37°C for 30 min. After phenol-chloroform extraction (1:1, vol/vol), the aqueous phase was mixed with an equal volume of 4 M LiCl, and the RNA was precipitated overnight at 4°C. The RNA was then pelleted for 15 min at 16,800 × *g* at 4°C and washed with 80% ethanol. To remove residual plasmid DNA, the RNA isolated was digested with RQ1 DNase (RNase-free DNase; Promega, Heidelberg, FRG), following the manufacturer's protocol. After phenol-chloroform extraction and ethanol precipitation, polyadenylated [poly(A)$^+$] RNA was prepared by oligo(dT)-cellulose chromatography (20). The primer extension reaction was performed as described before (6) with a 5′-labeled synthetic CAT gene-specific oligonucleotide (spanning nucleotide positions 41 to 58 of the CAT coding region) as a primer. For each primer extension reaction, 1×10^5 to 5×10^5 cpm of labeled CAT primer (sequence, 5′-CTTTACGATGCCAT TGGG-3′; specific activity, 1×10^6 to 3×10^6 cpm/pmol) was used. The reaction products were analyzed on 60-cm-long 6% polyacrylamide–7 M urea sequencing gels. The relative amounts of different primer extension products were determined by densitometric scanning of the autoradiograms.

RESULTS

Delimitation of the C gene promoter region. The 3′ end of the C gene promoter region was defined by transfection of liver and nonliver cells with plasmids containing the coding region for CAT as a reporter gene and DHBV DNA fragments with various 3′ ends close to the C gene and a fixed 5′ end at the *Bam*HI site (nucleotide position 1661) located at the end of the envelope (S) gene (Fig. 1A). All DHBV DNA fragments tested included a short sequence (indicated by a black box and designated E in Fig. 1A) which was speculated to contain an enhancer element (29). The longest DHBV DNA fragment tested (designated B2533 below) included, in addition to the putative enhancer element, two TATA boxes (indicated as open boxes in Fig. 1A) at appropriate distances from the in vivo transcription initiation site of the pre-C (23) and C (6) mRNAs. The 3′ end of this fragment (nucleotide position 2533) coincides with the initiation site of the C mRNA and is separated by 54 nucleotides of polylinker sequences from the AUG codon of the CAT gene (Fig. 1B).

Transfection of liver and nonliver cell lines with the plasmid containing this fragment resulted in transient CAT enzyme synthesis in liver cells at levels similar to those obtained with two other strong heterologous promoters (simian virus 40 [SV40] early [data not shown] and RSV long terminal repeat promoter; for a summary of data obtained in several experiments and with different cell lines, see Table

TABLE 1. C gene promoter activity in different cell types transfected with various plasmids[a]

Plasmid	Relative CAT activity (% conversion)						
	Hep2.2.15	HepG2	PLC/PRF/5	NIH 3T3	CCL13	HeLa	SK-Hep 1
pRSV	97 ± 1	94 ± 3	97 ± 0	95 ± 5	98 ± 0	98 ± 0	93 ± 2
pB2447rev	1 ± 0	2 ± 0	3 ± 1	<1	<1	2 ± 0	<1
pB2447	15 ± 2	15 ± 3	31 ± 11	<1	<1	<1	<1
pB2495	43 ± 22	NT[b]	26 ± 20	<1	<1	<1	<1
pB2533	78 ± 12	24 ± 3	75 ± 11	<1	<1	<1	<1

[a] C gene promoter activity was measured in human liver and nonliver and mouse cells transfected with plasmids containing DHBV C gene promoter fragments and the RSV promoter, each linked to a CAT reporter gene. Relative CAT enzyme activities are given as percent conversion of unacetylated chloramphenicol into acetylated forms obtained with 30 μg of cellular protein extract when incubated for 2 h. Data represent average CAT enzyme activities obtained in three or more independent transfection experiments and with two plasmid DNA preparations.

[b] NT, not tested.

1). The highest levels of CAT enzyme activity were obtained in the productively HBV-infected Hep2.2.15 cell line and in PLC/PRC/5 cells, containing chromosomally integrated fragments of HBV; lower levels were expressed in the non-HBV-producing HepG2 cells; and no expression was found with human and mouse nonliver cells (HeLa and NIH 3T3), with CCL13 cells, which are believed to represent dedifferentiated human liver cells, or with another dedifferentiated human liver cell line, SK-Hep. These data indicate that fragment B2533 harbors strong promoter elements driving C gene transcription which are dependent on liver cell- and differentiation-specific factors and are affected by expression of HBV.

The second DHBV fragment tested (designated B2495 below) differed from fragment B2533 only at its 3′ end by a deletion of 38 nucleotides, including the most proximal TATA box of the C gene (Fig. 1A). Transfection of Hep2.2.15 and PLC/PRF/5 cells with the plasmid containing this fragment resulted in a two- to threefold reduction in CAT activity compared with fragment B2533 (Table 1), suggesting that DHBV sequence 2495 to 2533 harbors positive regulatory sequences for C gene promoter activity. Human and mouse nonliver cells and cell lines CCL13 and SK-Hep did not show C gene promoter activity, demonstrating that a 3′-truncated C gene promoter region also exhibits highly differentiated liver cell-specific promoter activity. In comparison to fragment B2495, fragment B2447, which differs from it by a further deletion of 48 nucleotides at its 3′ end, showed promoter activity similar to that of B2495 in PLC/PRF/5 cells and only approximately 30% of its activity in Hep2.2.15 cells (Table 1). Again, no promoter activity was detected with NIH 3T3, CCL13, HeLa, and SK-Hep cells. From these data alone, DHBV sequence 2447 to 2495 appears to have no significant effect on C gene promoter activity in PLC/PRF/5 cells, dedifferentiated liver cells, and nonliver cells but appears to stimulate its activity in Hep2.2.15 cells. The finding of no significant promoter activity with fragment B2447 in the reverse orientation (Table 1) suggests that no promoter reading in the opposite direction is present on this fragment and demonstrates the specificity of the C gene promoter assay system used.

Plasmids containing 5′-truncated versions of DHBV fragments B2533 and B2447 were constructed to narrow down the 5′ end of the C gene promoter region and to elucidate the role of the DHBV sequence with enhancer core sequence similarity. For this purpose, four different plasmids were constructed with 5′ deletions of the fragments up to the *Afl*III (A2447 and A2533) and *Nsi*I (N2447 and N2533) restriction sites (Fig. 1A). Four further plasmids were constructed in which the putative DHBV enhancer sequence was cloned in both orientations downstream of the CAT gene (Fig. 1B) to test for the orientation- and location-independent function on two DHBV C gene promoter fragments (B2447 and B2533) (Fig. 1A). Transfection of Hep2.2.15 cells with a plasmid containing DHBV fragment A2447 or A2533 (related to fragments B2447 and B2533 but with DHBV *Bam*HI-*Afl*III sequences deleted [Fig. 1A]) yielded CAT enzyme levels similar to those with the parental plasmids with the B2447 and B2533 DHBV fragments (Fig.

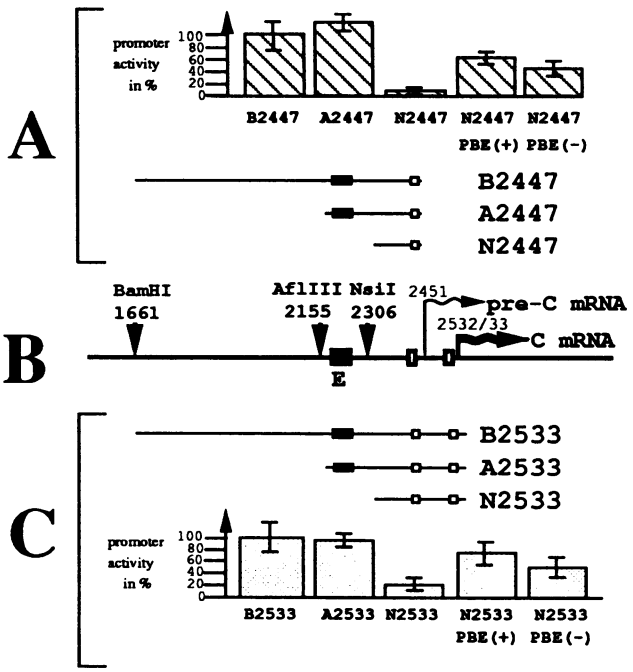

FIG. 2. Delimitation of the 3′ end of the C promoter region and characterization of the DHBV enhancer. (A) Promoter activity of DHBV fragments B2447, A2447, and N2447 and restoration of promoter activity of fragment N2447 by insertion of fragment PBE in both plasmids in either orientation (PBE+, authentic orientation; PBE−, inverse orientation). Data are from three independent transfection experiments. (B) Genomic DHBV region analyzed. Restriction enzyme sites, the enhancer core consensus motif (black box) and TATA boxes (open boxes), and the transcription initiation sites of the C/pre-C mRNAs as mapped in vivo are indicated. (C) Promoter activity of DHBV fragments B2533, A2533, and N2533 and restoration of promoter activity of fragment N2533 by insertion of fragment PBE in both plasmids in either orientation (PBE+ and PBE−). Data are from three independent transfection experiments.

2). This indicates that sequences between nucleotide positions 1661 and 2155 do not contribute significantly to C gene promoter activity. In contrast, when DHBV fragments A2447 and A2533 were further truncated at the 5′ end up to the *Nsi*I site (fragments N2447 and N2533), a dramatic drop in CAT enzyme expression was noted (Fig. 2). This drop in transient CAT activity indicates that DHBV sequences spanning nucleotides 2155 to 2306 and including the putative enhancer element E are essential for C gene promoter activity.

To determine whether this sequence functions as a true enhancer, independent of orientation, DHBV-CAT plasmids containing DHBV fragment N2447 or N2533 upstream of the CAT gene and also the *Pma*CI-*Bal*I (Fig. 1A) DHBV fragment (designated PBE and including the enhancer core consensus motif) cloned in both orientations downstream of the CAT gene (Fig. 2) were used. The very low promoter activity of DHBV fragments N2447 and N2533 was strongly upregulated in all plasmids containing the PBE fragment downstream of the CAT gene, and this upregulation was virtually independent of the orientation of the PBE fragment (Fig. 2A and C, compare PBE+ and PBE−). This result demonstrates that the PBE fragment has orientation- and location-independent enhancer activity.

Mapping of the core gene promoter transcripts. To determine whether transcription initiation and efficiency are identical in virus-infected livers and in cultured cells transfected with C gene promoter fragments, the 5′ ends of the pre-C and C mRNAs produced in cells transfected with plasmids containing C gene promoter fragments were analyzed by primer extension analysis. For these studies and for further analysis, we used Hep2.2.15 cells as plasmid recipients because they exhibited the lowest background of CAT enzyme levels and the highest activity of the C gene promoter. Four plasmids with the CAT gene as a reporter for promoter activity were used for this analysis; three plasmids contained DHBV fragment B2447, B2495, or B2533, and in one plasmid (used as a control), the CAT gene was linked to the RSV promoter. Poly(A)$^+$ RNA was isolated from Hep2.2.15 cells 48 h after transfection, and a 5′-end ^{32}P-labeled synthetic oligonucleotide complementary to the 5′ end of the CAT gene sequence was used as a primer.

Only two primer extension products differing by one nucleotide in length were observed with fragment B2533 (Fig. 3, lane 1). Their sizes (113 and 114 nucleotides; 58, 54, and 1 or 2 nucleotides derived from the CAT coding region, the polylinker region of the vector, and DHBV sequences, respectively) indicate transcription initiation at nucleotides 2532 and 2533, and these positions coincide with the 5′ ends of the C mRNA, as determined with RNA from infected livers (Fig. 4). A signal corresponding to the 5′ end of a pre-C mRNA, as recently identified in infected livers (23) (Fig. 4), was not observed (Fig. 3, lane 1). Surprisingly, with fragment B2447, three primer extension products were observed (115, 122, and 123 nucleotides long; comprised of 58 nucleotides of the CAT coding region and 57, 64, and 65 nucleotides, respectively, from the polylinker region of the vector) that indicated transcription initiation at nucleotide positions 2453, 2454, and 2461 (Fig. 3, lane 3). All of these positions are upstream of the C gene and close to a TATA box (Fig. 4) which may be involved in pre-C mRNA initiation in vivo (23). None of these positions coincide precisely with the 5′ end of the pre-C mRNA, as determined with RNA from infected livers (23). In addition, densitometric evaluation of the autoradiogram of the RNA analysis of fragment B2533 (data not shown) suggests a C/pre-C mRNA ratio of 4:1, much lower than that found in vivo. However, a similar ratio is suggested by comparing the promoter activity measurements obtained with different constructs in the CAT assay (Table 1).

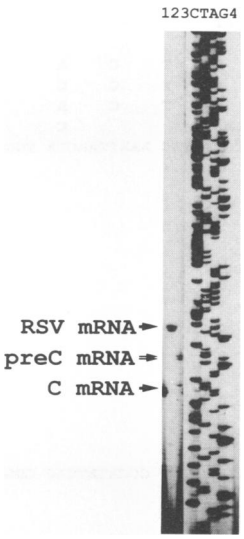

FIG. 3. Primer extension analysis with poly(A)$^+$ RNA from Hep2.2.15 cells transfected with plasmid pRSV (lane 2), pB2447 (lane 3), or pB2533 (lane 1) or mock transfected (lane 4) and an oligonucleotide primer homologous to sequences of the 5′ end of the CAT enzyme-coding region. Lanes CTAG represent sequencing products obtained by primer extension performed with a synthetic RNA and the same oligonucleotide as used in lanes 1 to 4.

The specificity and the relative efficiency of the primer extension reaction were determined with RNA from cells transfected with the pRSV control plasmid or with RNA from mock-transfected cells. With pRSVCAT, a primer extension product of the expected size (131 nucleotides long, composed of sequences from the CAT gene, the vector, the oligonucleotide, and the RSV long terminal repeat [40, 37, 18, and 36 nucleotides, respectively]) and of strong intensity (Fig. 3, lane 2) was observed, whereas no product was seen in the mock-transfected control (Fig. 3, lane 4). Together, these data indicate that the C gene promoter fragments tested in vitro harbor transcription elements that direct correct initiation of the C mRNA and imprecise but more efficient initiation of pre-C mRNAs than in the in vivo situation. Moreover, the efficient synthesis of pre-C mRNAs from fragment B2447 only and the lack of synthesis of a C mRNA from the same fragment indicate that sequences spanning nucleotides 2495 to 2447 can downregulate pre-C mRNA transcription.

Characterization of a DHBV sequence which downregulates pre-C mRNA synthesis. To address the question of whether DHBV sequence 2495 to 2447 acts as a negative regulatory element for pre-C mRNA synthesis, we first searched for sequence similarities with negative regulatory sequences and silencer elements of cellular and viral genes. A computer-based sequence similarity search revealed a short consensus sequence motif present in the negative regulatory elements of 11 viral and cellular genes, including HBV (Fig. 5), and thus supported our suspicion that DHBV sequence 2495 to 2447 acts as a negative regulatory element. To confirm this experimentally, this DHBV sequence was cloned in a corresponding position (downstream of the transcription initiation site) of a heterologous promoter (RSV), but in both orienta-

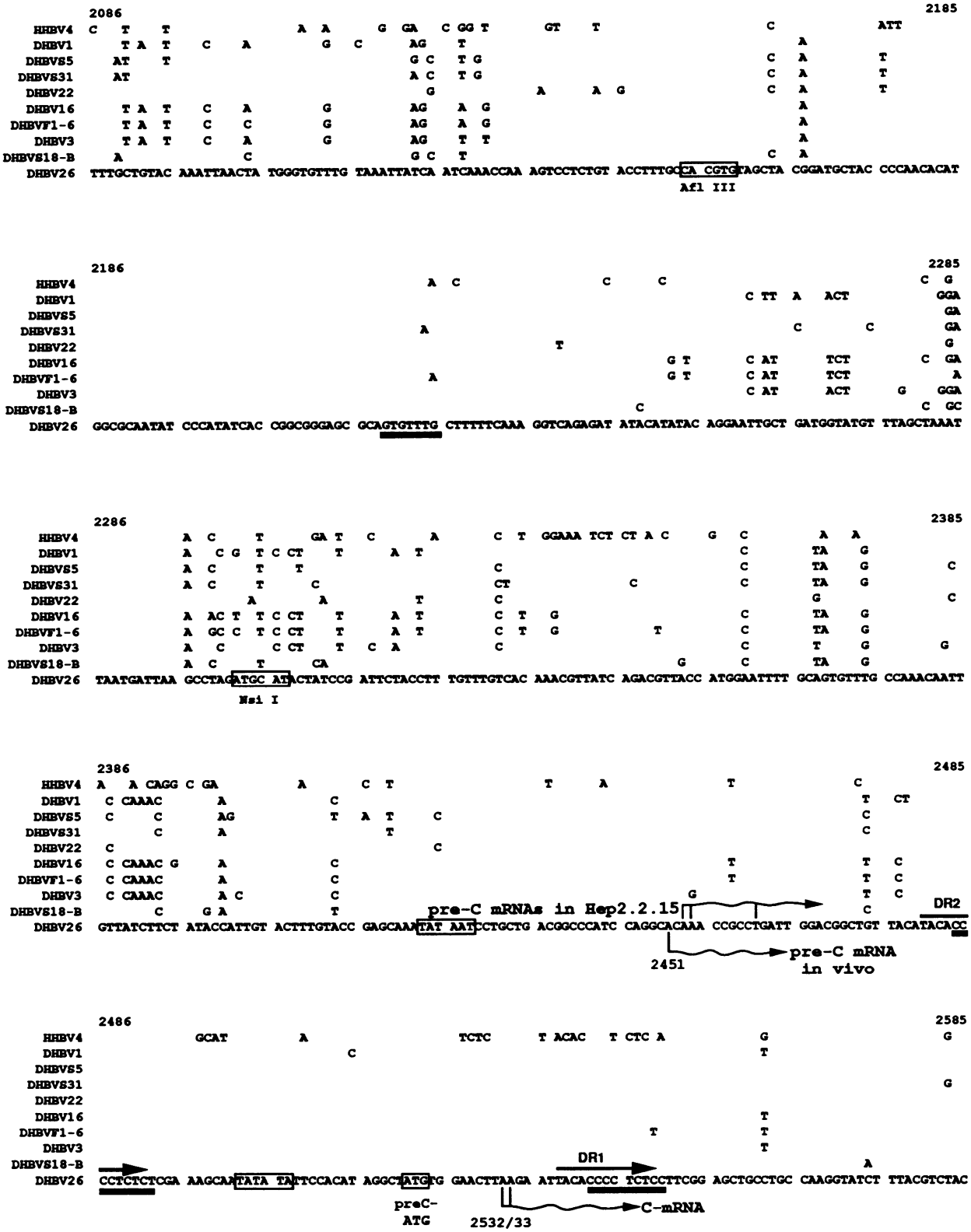

FIG. 4. Comparative sequence analysis of the C gene promoter region of all DHBV isolates that have been sequenced so far. The sequence of the DHBV26 isolate is shown; nucleotides that differ from this sequence are shown for the other isolates. The infectivity of several of the isolates was previously experimentally proven; DHBV3 (16, 28); all others (35 and references therein); DHBV1 and DHBV22 genomes are not infectious (35); DHBVS5 and DHBVS31 (32) have not yet been tested for infectivity. HHBV (27) and DHBV1 are replication competent and produce core protein in transfected liver cell lines (35; for sequences, see reference 25). Some restriction enzymes used for DHBV-CAT gene constructions are indicated. The enhancer core sequence motif is underlined, and the direct sequence repeats DR1 and DR2 involved in viral replication are indicated with arrows. TATA boxes upstream of the C and pre-C mRNAs and the translation initiation codon of the precore protein are boxed. The DHBV silencer core sequence motifs are underlined. The C and pre-C mRNA transcription initiation sites mapped in vitro and in vivo are indicated by wavy lines with arrows.

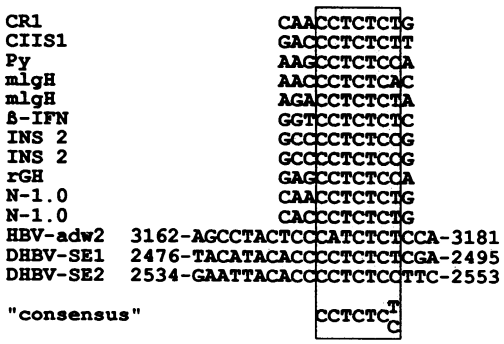

FIG. 5. Negative regulatory elements of transcription of viral and cellular genes and sequence similarity with a functionally similar DHBV and HBV element, as well with a DHBV element of unknown importance. The consensus sequence for all these elements is boxed (5; reference 21 and references therein). DHBV-SE1 and DHBV-SE2 are DHBV sequences. Abbreviations: CR1 and CIIS1, rat collagen II gene; Py, polyomavirus; mlgH, mouse immunoglobulin heavy chain enhancer; β-IFN, human beta interferon gene; INS 2, rat insulin 1 repetitive element; rGH, rat growth hormone gene; N-1.0, chicken lysozyme gene.

tions to test its potential for orientation-independent function (Fig. 6). As a control, an unrelated polylinker sequence of similar length and purine-pyrimidine composition (see Materials and Methods) was tested in parallel (Fig. 6, RSV PL). Hep2.2.15 cells transfected with the RSV-CAT plasmid without the test insert exhibited a high level of CAT enzyme activity, as expected (Fig. 6). Irrespective of orientation, the RSV-CAT plasmids that contained the putative negative regulatory element (RSV SE+ and RSV SE−) showed a 50% reduction in CAT activity. In contrast, insertion of the unrelated polylinker sequence had no significant effect on CAT enzyme expression (Fig. 6, RSV PL). These data demonstrate that DHBV sequence 2447 to 2495 is a transcription-regulating element which can suppress the activity of a promoter from a position downstream of its transcription initiation site in an orientation-independent manner.

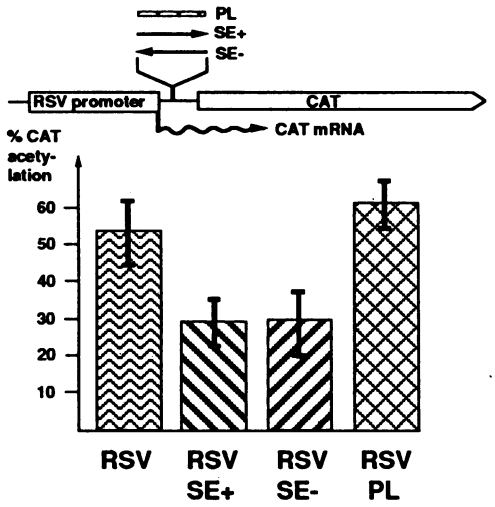

FIG. 6. Characterization of the silencer element of DHBV. (Top) Construction used for characterization. The DHBV silencer was inserted in both orientations (SE+ and SE−) into the untranslated region of a CAT gene, the expression of which was under the control of the RSV promoter. As a control, an unrelated sequence of similar length and nucleotide composition (PL) was inserted into the same vector in the same position. (Bottom) CAT enzyme activity with 5 μg of protein extract from Hep2.2.15 cells transfected with the plasmids described above. Error bars indicate standard deviations of six different CAT assays from three independent transfection experiments.

DISCUSSION

In this study we have delimited and characterized the promoter region of the DHBV C gene. C gene promoter activity was shown to be liver cell specific, active only in highly differentiated liver cells, and dependent on a DHBV enhancer element. Additionally, synthesis of a pre-C mRNA was shown to be downregulatable by a negative regulatory sequence element.

The promoter region, including the enhancer, was narrowed down to nucleotide positions 2155 to 2533 (Fig. 1A). The 5′- and 3′-terminal ends of this region are highly conserved among DHBV genomes of proven and unknown infectivity, among defective DHBV genomes, and in a closely related HHBV genome (29, 35), whereas the central region is quite variable in sequence (Fig. 4). The high degree of sequence conservation may be due at least in part to the presence of sequence elements that are essential for viral replication and to its function as the coding region for the RNase H enzyme. The 3′-terminal half of this region (starting at nucleotide position 2306) was inactive in our assays. It was, however, strongly active when the 5′-terminal half of the promoter region (nucleotide positions 2155 to 2306), which harbors the enhancer element, was inserted elsewhere in the plasmid, regardless of its orientation. It is therefore possible that the 3′-terminal half of the promoter region contains most, if not all, of the regulatory elements for C gene transcription except the enhancer.

Transcription initiation of the C mRNA on the DHBV DNA fragments tested mapped at the same positions as found in vivo. This indicates that all sequences necessary for correct transcription initiation for the C mRNA are present within the shortest fragment, B2533. In contrast, none of the transcription initiation sites of the pre-C mRNA coincided with the one mapped with RNA from infected livers (23). Since the pre-C mRNAs were only seen in vitro after deletion of sequences of the pre-C region, an obvious explanation for the discrepancy between the in vivo and in vitro data could be a role of these sequences in controlling precise transcription initiation. Discrepancies between in vivo and in vitro RNA mapping data were also observed in related studies with the S promoter of HBV (7) and many other promoters (for one very similar example, see reference 4). They could be due to cell type-specific factors, which may be quantitatively and qualitatively different in individual cell lines and in authentic liver cells in vivo, and they could be affected by the HBV-encoded or -induced gene products in Hep2.2.15 cells.

The level of promoter activity was dependent on the cell lines used and roughly paralleled the stage of liver-specific differentiation. The highest activity was observed in Hep2.2.15 and PLC/PRF/5 cells. These two cell lines are not only highly differentiated but also produce all or a subset of HBV proteins. The higher activity of the DHBV C gene promoter in Hep2.2.15 than in HepG2 cells suggests that a virus-encoded and/or a virus-induced cellular factor(s) may enhance promoter activity.

Sequences located between nucleotide positions 2155 and 2306 behaved like a typical eucaryotic enhancer-responsive

element (34) because they functioned in a relatively orientation- and position-independent manner. The DHBV enhancer region as defined in this study has no significant sequence similarity to either of two previously described HBV enhancer regions (10, 33, 37) except for a common enhancer core consensus motif (GTGT/AT/A/T/AG) found in many eucaryotic enhancers (10, 33, 34, and references therein). By sequence comparison (Fig. 4), this region has no striking sequence similarity to previously identified HBV enhancer region-associated protein-binding sites (for a review, see reference 22). One of the two known HBV enhancers (enhancer I), located approximately 600 nucleotides upstream of the C mRNA transcription initiation site, appears to have a broad type of cell specificity (10); the other (enhancer II), located close to the HBV C gene promoter (33, 37), is strongly active only in highly differentiated liver cells. In this study, the DHBV C gene promoter was shown to be inactive in all cell lines tested when the enhancer was deleted. Therefore, our studies do not allow us to answer whether the enhancer region or the truncated promoter region confers cell type-specific DHBV C gene promoter activity. The combination of both renders the promoter activity liver specific, and this is probably one reason why DHBV propagates most efficiently in highly differentiated liver cells (9, 13).

A negative transcriptional regulatory element located downstream of the pre-C mRNA transcription site which selectively suppresses pre-C mRNA transcription was also identified. This sequence element functions in an orientation-independent fashion, a property common to known silencer elements (for references, see legend to Fig. 5). Strong sequence similarities to part of this region (designated below as the silencer core sequence motif) are found in several well-studied sequence elements, and this suggests a similar mechanism of action. Whether the DHBV silencer element also functions independently of position and only in liver cells remains to be tested. Several silencer elements located downstream of transcription initiation sites and suppressing the activity of the corresponding promoters have been identified in other genes (5, 38). The complete conservation of this negative regulatory sequence element in all DHBV isolates sequenced so far and in the related HHBV (Fig. 4) and the low expression of pre-C mRNA in infected livers (23), however, make a biological function for this sequence likely.

Interestingly, a sequence similar to the DHBV silencer core motif occurs a second time a few nucleotides downstream of the 5′ end of the C mRNA (Fig. 4 and 5). The presence of this sequence does not, however, prevent efficient synthesis of the C mRNA despite the fact that it is in an analogous position. It is therefore conceivable that the silencer core motif is not sufficient for its function as a silencer and depends on sequence context and other factors.

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