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Expression of Hepatitis B Viral Core Region in Mammalian Cells

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We studied the expression of the core region of the hepatitis B virus genome in mammalian cells with recombinant plasmid vectors. Stably transformed rat fibroblast cell lines were established by transfection with vectors containing subgenomic and genome-length hepatitis B virus DNA, followed by G418 selection. The RNA transcripts directed by the core region were characterized by Northern blot hybridization and S1 nuclease mapping. Using the chloramphenicol acetyltransferase gene expression system, the promoter activity located upstream of the core open reading frame was confirmed. The synthesis of core and e polypeptides was studied with a commercial radioimmunoassay. These studies show that partial deletion of the precore sequences abolished secretion of the e antigen, but there was pronounced synthesis of the core antigen in transfected cells.

The sequence analysis of cloned hepatitis B virus (HBV) DNA has resulted in the identification of at least four open reading frames (ORFs): S/pre-S, C, X, and P (21, 32). The *S* gene has been efficiently expressed in cultured cells (5, 23, 24, 27), and the transcriptional control elements have been defined (5, 23a, 27). Expression of the core (C) region with native transcriptional control elements, however, has proven to be more difficult, probably reflecting the strict host and tissue tropism of the virus. Recent studies have indicated that the promoter preceding the C ORF is dependent on a highly specific upstream enhancer sequence which is active only in human hepatocytes (14).

The C ORF contains an in-frame ATG located 87 base pairs (bp) downstream of the first ATG of this ORF. The C region is responsible for the expression of polypeptides with distinct antigenic determinants designated core (HBcAg) and e (HBeAg) (19). Both of these antigens are found associated with the icosahedral core of the viral particle (28) derived from intact virions and infected hepatocytes; HBeAg is also found in the serum of infected individuals (16). Takahashi et al. (29) have shown that HBeAg from the serum of chronic carriers and HBcAg from virions react with anti-HBe or anti-HBc, respectively, in their native conformations, but share antigenicity in their denatured forms.

Studies on the expression of the C ORF have used recombinant vectors employing heterologous control elements such as the simian virus 40 promoters (34) or have relied on in vitro transcription (7, 20) to determine the basic structure of C ORF transcripts. The in vitro studies have indicated that the 5′ end of the C ORF-specific RNA maps at about nucleotide (nt) 1689 (20), located about 130 bp upstream from the first ATG (nt 1820). Other studies have shown greater-than-genome-length RNA species, both in transfection with a head-to-tail multimer of the HBV genome (10) and in animals infected with HBV and related viruses (4, 6, 9). It has been suggested that this large RNA species could serve as a message for the C region. A 1.8-kilobase (kb) subgenomic fragment of HBV, however, has been shown to direct the synthesis of C ORF-related polypeptides (36).

In the present study we initially focused on the in vivo expression of the C ORF in stably transformed rat cell lines, directed by genomic and subgenomic fragments containing native transcriptional control elements. We examined the C ORF-related mRNA species from cloned cell lines, which express and secrete HBeAg into the culture medium. We also demonstrated the promoter activity of the region upstream of the C ORF by using the easily assayable chloramphenicol acetyltransferase (CAT) gene and by S1 nuclease mapping of the CAT mRNA. Identification of the HBV enhancer, which appears to be functionally active in human liver cells (14, 22), led us to transiently express the C ORF in a human hepatoma cell line. These studies demonstrate that the entire C ORF under the influence of native transcriptional control elements directs the synthesis and secretion of only HBeAg. By deleting the first ATG and flanking sequences, also called the precore region, detectable secretion of HBeAg is abolished, but the antigen is found intracellularly. Transfection with this construct also resulted in the synthesis of large amounts of HBcAg.

MATERIALS AND METHODS

Construction of plasmid vectors. Recombinant vectors were constructed from a cloned subtype adw HBV genome described previously (25). Plasmids pML and pMLneo were obtained from M. Botchan (University of California, Berkeley), and plasmid pSV2CAT was from B. Howard (National Institutes of Health, Washington, D.C.). All enzymes were obtained either from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) or New England BioLabs, Inc. (Beverly, Mass.). For construction of pNEC (Fig. 1A), HBV DNA was digested to completion with restriction enzyme *Bam*HI, and the termini were blunted with T4 DNA polymerase. The 1.8-kb fragment, containing the entire C ORF with 400 bp of upstream sequences and 800 bp of downstream sequences, was purified. After *Eco*RI linkers were added, the fragment was ligated with *Eco*RI-digested pMLneo by standard methods (17). Plasmid pNER (Fig. 1A) was constructed in a similar manner, with HBV DNA linearized with *Eco*RI.

For the construction of pCEN (Fig. 1B), HBV DNA was digested with *Hpa*I and *Eco*RI, and the 2.3-kb fragment was isolated and cloned into pML between the *Cla*I (blunted with T4 DNA polymerase) and *Eco*RI restriction sites. Plasmid pSCΔPC (Fig. 1B) contained HBV sequences similar to

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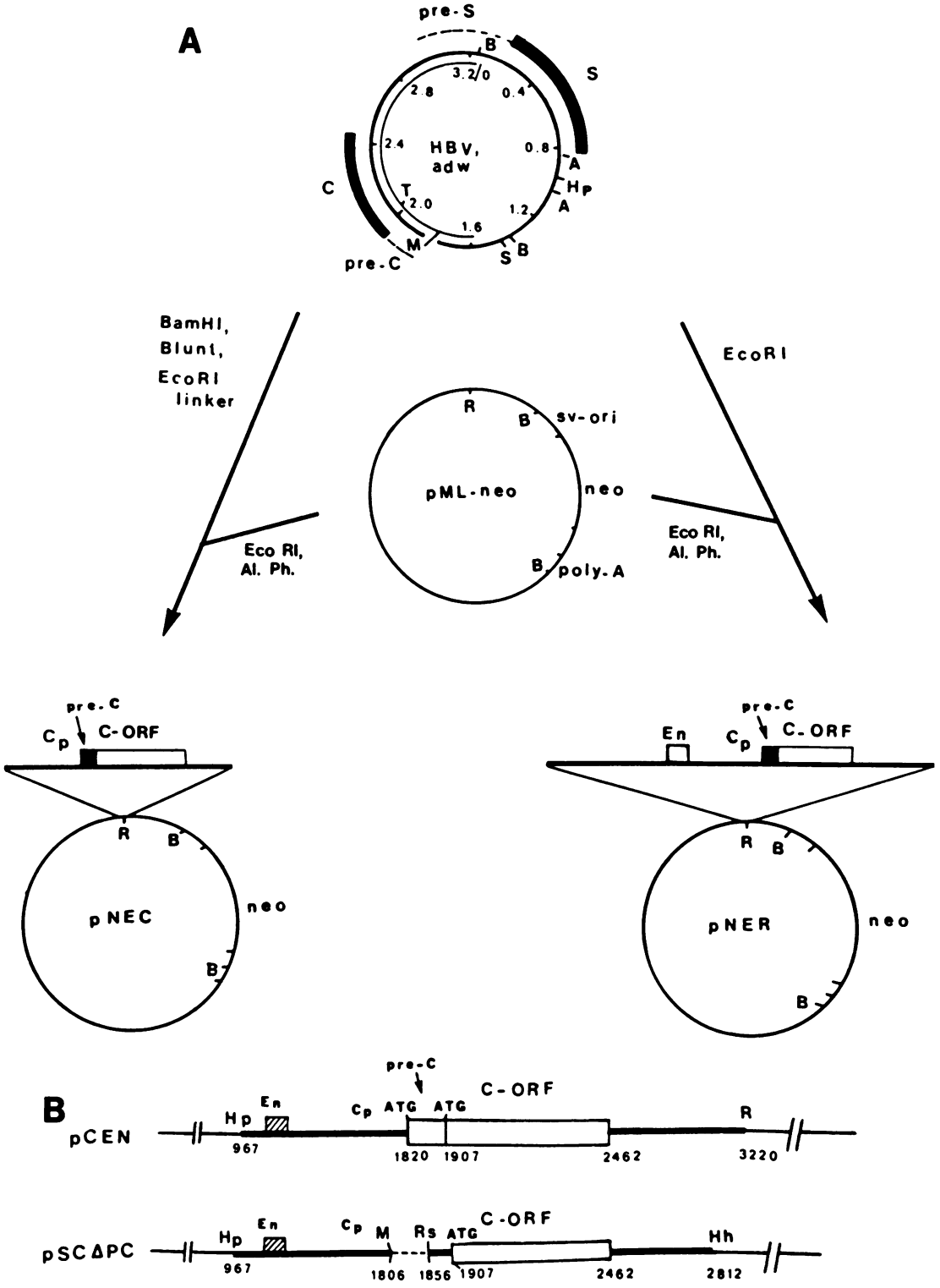

FIG. 1. Construction of recombinant plasmid vectors. (A) pNEC and pNER construction. (B) Schematic representation of HBV sequences in pCEN and pSCΔPC. The sequences between the *Mst*I and *Rsa*I sites have been deleted in pSCΔPC. Pre-S, S, pre-C, and C refer to the ORFs of HBV. Abbreviations used: A, *Acc*I; B, *Bam*HI; R, *Eco*RI; Hh, *Hha*I; Hp, *Hpa*I; M, *Mst*I; Rs, *Rsa*I; S, *Sst*II; T, *Taq*I; Al. Ph., calf alkaline phosphatase; Cp, C promoter; En, enhancer sequences.

those of pCEN except with a deletion of the sequences between restriction sites *Mst*I (nt 1806) and *Rsa*I (nt 1856). This resulted in the removal of the first ATG at map position 1820 and about 50 flanking nucleotides.

The two HBV fragments used to assay for C promoter activity were inserted between the *Acc*I and *Hin*dIII sites of the promoterless plasmid pSV0CAT (11) by blunt-end ligations, using T4 DNA polymerase and T4 DNA ligase (see Fig. 3A). The HBV fragment in pCPCAT contained nucleotide sequences from map positions 1076 (*Acc*I) to 1991 (*Bgl*II) and included both the ATGs in the C ORF. The HBV fragment in pCMCAT, from nt 1076 (*Acc*I) to 1806 (*Mst*I), lacked the two ATGs and downstream sequences. Both fragments however, contained the HBV enhancer element (14, 22). Plasmid pCPCAT-ΔE (see Fig. 3A) was generated by removal of the HBV enhancer element (*Acc*I to *Sst*II, nt 1076 to 1458) from pCPCAT. CAT activity was assayed at 48 h posttransfection by the method of Gorman et al. (11).

Transfection and establishment of cell lines. Rat fibroblast cells (R. Erikson, Harvard University, Cambridge, Mass.) were transfected, and permanent cell lines were established by G418 selection according to methods described previously (12, 26). Clones resistant to G418 were screened by RNA dot-blot analysis for the expression of C-specific RNA. NEC-4 is a cell line established by pNEC transfection, and NER-41 was established with pNER. For transient expression in Hep-G2 cells (kind gift of B. Knowles, Wistar Institute, Philadelphia, Pa. [14]), 2 pmol of plasmid DNA was used to transfect (12). Plasmid pBR322 DNA was used to keep the DNA concentration constant at 12 μg/100-mm dish. Cells were transfected at about 60% confluency (5×10^6 to 7×10^6 cells per dish), with a transfection efficiency of 10 to 16%.

Analysis of poly(A)$^+$ RNA. Cytoplasmic RNA was isolated by a modification of the method of Chirgwin et al. (8). Confluent cells from tissue culture dishes were scraped off and lysed in Nonidet P-40 lysis buffer (10 mM Tris, 1 mM $MgCl_2$, 100 mM NaCl, 0.5% Nonidet P-40). Nuclei were removed by centrifugation, and the cleared lysates were mixed with 6 M guanidine isothiocyanate. The mixture was pelleted through a 1.2-ml cushion of 5.7 M CsCl by centrifugation in an SW-55 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 44,000 rpm for 18 h. RNA pellets were suspended in STE (0.1 M NaCl, 0.5 M Tris [pH 7.5], 1 mM EDTA) and precipitated with ethanol. Poly(A)$^+$ RNA was purified by chromatography through an oligo(dT)-cellulose column (1). For Northern blot hybridization (30), 20 μg of poly(A)$^+$ RNA was subjected to electrophoresis through a 1% agarose-formaldehyde gel (17). The blotted RNA was hybridized with a 1-kb (nt 1806 to 2812) radiolabeled negative-strand RNA generated in vitro by the SP6 system (18). S1 nuclease mapping was by the methods of Weaver and Weissman (32). The 5′-end-labeled probe used in S1 mapping of the C ORF-specific transcripts contained HBV DNA sequences from nt 1406 to nt 2020 (*Bam*HI to *Taq*I).

Analysis of HBcAg and HBeAg. Culture supernatants (8 ml/100-mm dish) were collected 48 h posttransfection for transient expression assays and at cell confluency for permanent cell lines. They were then clarified at 12,000 × *g* for 30 min and concentrated 35- to 40-fold with an Amicon YM2 filter. Samples (200 μl) of concentrated material were used for radioimmunoassay (HBe RIA; Abbott Laboratories, North Chicago, Ill.). Culture medium from a stable cell line, NET 22 (23a), which does not contain an intact C ORF was used as a control. To assay the cell lysates, confluent cells in 100-mm tissue culture plates were harvested, and the cells were lysed by sonication for two 5-s bursts in a Branson cell disruptor 185 at maximum output. To distinguish between HBeAg and HBcAg, we used purified human anti-core immunoglobulin fraction which was free from HBeAg and anti-HBe reactivity (see Table 2) to block Abbott HBe RIA activity. After antigen binding according to the directions included with the kit, an additional incubation with anti-HBc was included. Reduction in the positive/negative (P/N) value in the anti-HBc blocking assay indicated the presence of HBcAg; no reduction indicated the absence of HBcAg. All assays were done in a 200-μl volume and represent medium or lysate from one 100-mm dish.

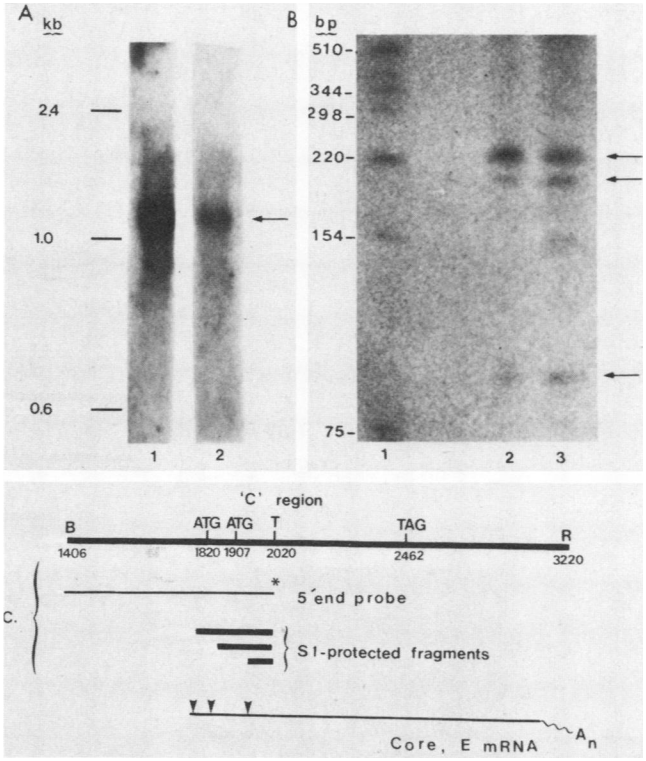

FIG. 2. Characterization of C-region transcripts. (A) Northern blot hybridization of poly(A)$^+$ RNA (20 μg) from cell lines NER-41 (lane 1) and NEC-4 (lane 2). Transcripts generated in vitro by the SP6 system were used as molecular weight standards. A 1-kb (nt 1806 to 2812) SP6 radiolabeled negative-strand RNA was used as a probe. (B) S1 nuclease mapping analysis of poly(A)$^+$ RNA from NEC-4 (lane 2) and NER-41 (lane 3). NEC-4 or NER-41 poly(A)$^+$ RNA (5 μg) was hybridized with the probe shown in panel C. Molecular weight standards in lane 1 are from end-labeled *Hin*fI-digested pBR322 DNA. (C) Schematic representation of S1 nuclease analysis. Abbreviations used: B, *Bam*HI; R, *Eco*RI; T, *Taq*I.

RESULTS

Analysis of C gene-related transcripts. Northern blot analysis (Fig. 2A) of poly(A)$^+$ RNA from NEC-4 and NER-41 cell lines revealed a band with a migration pattern consistent with a size of about 1.1 kb. This band probably contained more than one species of RNA transcripts. At least 20 μg of poly(A)$^+$ RNA and 1 week of exposure were required to visualize this band, indicating the low abundance of the mRNA species. The sizes of the RNAs were assessed by using RNA species of various molecular sizes, generated in vitro by the SP6 system (18), as markers.

We used S1 nuclease analysis to map the initiation sites of the C ORF transcripts. Poly(A)$^+$ RNA from NEC-4 or

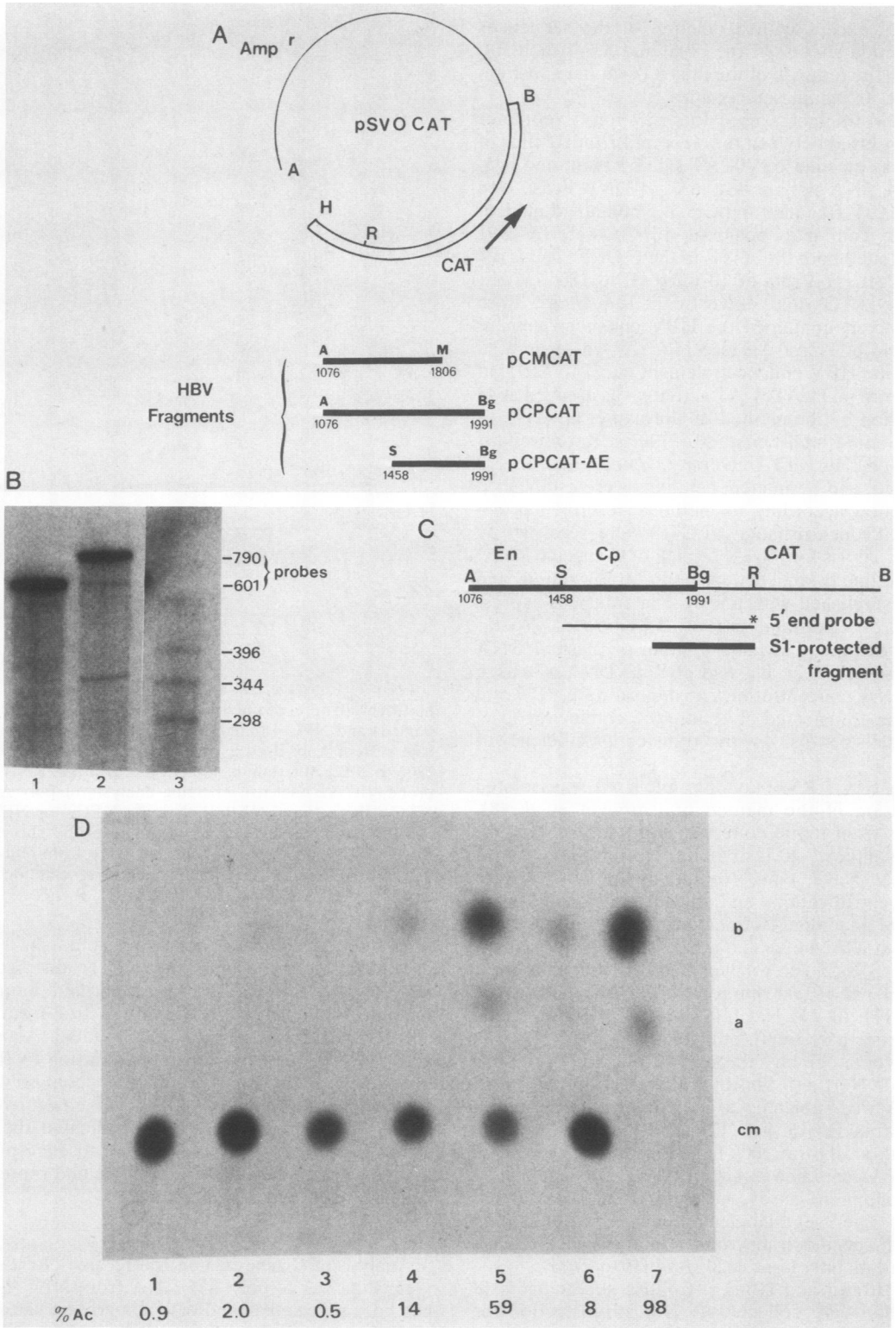

FIG. 3. Analysis of C promoter-enhancer-directed expression. (A) Construction of CAT plasmids. Solid bars represent HBV DNA sequences which were inserted into pSV0CAT at the *Acc*I and *Hin*dIII sites. (B) S1 nuclease analysis of RNA transcripts from pCMCAT (lane 1)- or pCPCAT (lane 2)-transfected Hep-G2 cells. Poly(A)$^+$ RNA (1.5 μg) from pCMCAT or pCPCAT was hybridized with the appropriate probes described in the text. Molecular weight markers were 5′-end-labeled fragments from a *Hin*fI digestion of pBR322. (C) Schematic representation of S1 nuclease analysis. (D) Expression of CAT activity in HeLa cells (lanes 1 to 3) and Hep-G2 cells (lanes 4 to 6) owing to plasmids pCMCAT (lanes 1 and 4), pCPCAT (lanes 2 and 5), and pCPCAT-ΔE (lanes 3 and 6). Lane 7 is a positive control with CAT enzyme. Each assay included extract equivalent to 0.5 mg of protein and was carried out for 2 h. After separation of chloramphenicol (cm) and its acetylated forms, 1-acetate (a) and 3-acetate (b), by thin-layer chromatography (0.2-mm silica), the percent acetylation (%Ac) was determined by scintillation counting. Abbreviations used are: A, *Acc*I; B, *Bam*HI; H, *Hin*dIII; M, *Mst*I; Bg, *Bgl*II; S, *Sst*II; Cp, C promoter; En, enhancer sequences.

NER-41 cells was annealed with a 5'-end-labeled DNA fragment (*Bam*HI to *Taq*I, nt 1406 to 2020; Fig. 2C). S1-protected fragments were fractionated through a 6% urea–polyacrylamide gel (Fig. 2B). A major S1-protected band of about 230 nt was seen which corresponded with a 5' end at nt 1790 on the HBV map. Two additional less abundant S1-protected fragments of about 190 and 100 nt, corresponding to nt 1830 and 1920 on the HBV map, were also resolved on the gel. RNA from the NER-41 cell line contained an additional band not seen in the NEC-4 RNA. This corresponded to an RNA initiation site at about nt 1870 (150-bp band). It is clear from these results that at least one species of C-specific RNA does not include the first ATG at nt 1820 and that the C ORF RNA transcripts seem to be heterogeneous, similar to other HBV and related virus transcripts (9, 23a, 30).

Assay for core promoter by CAT expression. The expression of the C ORF by our recombinant vectors is controlled by the native promoter sequences as shown by S1 nuclease mapping data (Fig. 2B). To independently test the promoter activity in sequences upstream of the C ORF, we utilized the bacterial CAT gene system. Construction of the CAT plasmids is discussed in Materials and Methods. Plasmid pCPCAT showed about 60% acetylation of chloramphenicol in Hep-G2 cells (Fig. 3D, lane 5), a human hepatoma cell line negative for HBV sequences (14). This activity was markedly reduced when assayed under identical conditions in HeLa cells (Fig. 3D, lane 2) or several other nonhepatic or nonhuman (or both) cell lines (14). Plasmid pCPCAT-ΔE (Fig. 3A), in which the upstream enhancer sequences were deleted, was also unable to efficiently express CAT activity in Hep-G2 cells (Fig. 3D, lane 6), indicating the dependence of the core promoter on the enhancer sequences.

Plasmid pCPCAT contains HBV sequences bound by the restriction sites *Acc*I (nt 1076) and *Bgl*II (nt 1991). This includes the two ATGs as well as about 85 bp of the C ORF. In view of the model for eucaryotic systems that the first ATG is preferentially used for translational initiation (15), we felt that the two C ORF ATGs in addition to the ATG of the CAT gene in pCPCAT might be interfering with CAT expression. We therefore constructed plasmid pCMCAT (Fig. 3A) containing an HBV fragment bound by the restriction sites *Acc*I (nt 1076) and *Mst*I (nt 1806), removing the two ATGs as well as other C ORF sequences while retaining the promoter and enhancer region. Surprisingly, pCMCAT was unable to efficiently express CAT activity in Hep-G2 cells (Fig. 3D, lane 4). This suggests that sequences downstream of the *Mst*I site (nt 1806) contain important regulatory signals.

TABLE 1. HBcAg and HBeAg synthesis in stably transformed rat cell lines[a]

Cells	Source	Anti-HBc[b]	P/N[c]
NEC-4	Medium	−	5.8 ± 0.5
NEC-4	Medium	+	5.7 ± 0.3
NER-41	Medium	−	7.1 ± 0.6
NER-41	Medium	+	7.3 ± 0.2
NEC-4	Lysate	−	2.3 ± 0.6
NEC-4	Lysate	+	2.3 ± 0.5
NER-41	Lysate	−	4.0 ± 1.6
NER-41	Lysate	+	3.4 ± 0.6

[a] Assayed by Abbott HBe RIA.
[b] +, Additional incubation with anti-HBc (see Table 2) before incubation with ^{125}I-labeled antibody; −, no anti-HBc used.
[c] P/N, Positive/negative. Negative values were obtained with a cell line which does not contain an intact C gene. A P/N greater than 2 is considered positive. All values are normalized to a positive control and represent two values.

TABLE 2. Analysis of human anti-HBc[a]

Test[b]	Sample	N/P[c]	P/N[d]
CORAB	+Control[e]	44.7	
CORAB	Anti-HBc	67.3	
Anti-HBe	+Control	3.6	
Anti-HBe	Anti-HBc	0.28	
HBe RIA	+Control		3.7
HBe RIA	Anti-HBc		1.1

[a] Gamma globulin fraction of human sera positive for anti-HBc.
[b] All tests were performed with the indicated kits from Abbott Laboratories.
[c] Antibody assays are expressed as negative over positive. Negative control in all cases was normal human serum. For the CORAB assay, an N/P value greater than 5 is considered positive. For anti-HBe, an N/P value greater than 2 is considered positive.
[d] Antigen assays are expressed as positive over negative. A P/N value greater than 2 is considered positive.
[e] All positive controls are positive human serum samples provided with Abbott kits.

We further carried out S1 nuclease mapping of CAT RNA transcribed from pCPCAT and pCMCAT (Fig. 3B and C). A 5'-end-labeled probe was generated from the respective plasmids by digestion with *Sst*II (HBV nt 1458) and *Eco*RI (230 bp 3' to the CAT gene ATG). The probes contained either 540 bp (pCPCAT) or 351 bp (pCMCAT) of HBV sequences and 250 bp of CAT gene sequences. Poly(A)$^+$ RNA from Hep-G2 cells transiently transfected with pCPCAT or pCMCAT was annealed with the respective probes. pCPCAT produced CAT transcripts whose initiation sites mapped at two positions upstream of the C ORF region (Fig. 3B, lane 2). The smaller S1-protected fragment (about 350 nt) places the initiation site at about map position 1890 on the HBV map, between the two ATGs. The larger S1-protected fragment (about 600 nt) corresponds to an RNA start site at approximately 1640 on the HBV map, upstream of the two ATGs. An RNA start site in this region has been described earlier in an in vitro transcription study (20). The absence of an S1-protected fragment in pCMCAT-transfected cells (Fig. 3B, lane 1) further suggests that important regulatory signals were deleted in this construct. The low level of CAT activity seen in the CAT assay in pCMCAT-transfected Hep-G2 cells (Fig. 3D, lane 4), even though no mRNA was detectable, is probably due to the greater sensitivity of detection of the CAT assay, as compared with S1 nuclease analysis, or the instability of CAT mRNA (S. Jameel, unpublished data), or both.

Analysis of antigen production from stably transformed cell lines. The NEC-4 and NER-41 cell lines were further analyzed for the production of the core and e antigens (Table 1). The clarified culture supernatants were concentrated 35- to 40-fold with an Amicon YM2 filter and assayed for HBeAg-HBcAg reactivity with a commercial RIA kit (Abbott Laboratories). Since the Abbott RIA kit used to assay for HBeAg utilizes human anti-HBe, which is always found in conjunction with anti-HBc, this system detects HBcAg as well as HBeAg (34). To distinguish between the core and e polypeptides produced in our cell lines, we utilized human

TABLE 3. Effect of enhancer on HBeAg synthesis during transient expression in Hep-G2 cells[a]

Plasmid	P/N[b]
pCEN	8.6
pNEC	1.2
pNER	3.3

[a] See Table 1, footnote *a*. Assays were done with concentrated culture media.
[b] P/N, Positive/negative. Negative control was untransfected Hep-G2 cells. A P/N value greater than 2 is considered positive.

anti-HBc in a blocking assay. The anti-HBc was analyzed by several commercial RIA kits from Abbott Laboratories: the CORAB assay detects anti-HBc, the HBe RIA detects HBeAg and HBcAg, and the anti-HBe detects antibody to HBeAg. The results of this analysis (Table 2) clearly show that the anti-HBc antibody is free of anti-HBe and HBeAg.

By blocking with anti-HBc we showed that the polypeptides secreted into the culture supernatants of the NEC-4 and NER-41 cell lines bear only e antigenic determinants (Table 1); we could not detect any HBcAg in the concentrated culture supernatants. Detectable levels of HBeAg were also found in the cell lysates, but no HBcAg was detected (Table 1). These results, taken together, indicate that the predominant C ORF protein product in these cells and in the culture supernatants is HBeAg.

Effect of enhancer sequences on HBe-HBcAg production. Recent studies by Jameel and Siddiqui (13) clearly demonstrate that the HBV enhancer element is necessary to regulate transcription from the putative core promoter. Since the enhancer element exhibits tissue and host tropism, i.e., it is functionally active only in human cells of hepatic origin, we studied the transient expression of the C ORF in the human hepatoma cell line Hep-G2 under the control of the native regulatory elements. Plasmids pNEC, pNER, and pCEN (Fig. 1) were used for the transient expression of HBeAg-HBcAg in Hep-G2 cells. Culture medium was assayed as described above, and the results are described in Table 3. Transient expression of pCEN, which contains the enhancer element and core promoter region, led to the synthesis of levels of e antigen easily detectable by our assay. Cells transfected with pNEC, which does not contain the enhancer sequences, did not produce detectable levels of antigen. Transfection of Hep-G2 cells with pNER, which also produces HBsAg (data not shown), showed a marked reduction in the expression of HBeAg-HBcAg as compared with that of pCEN-transfected cells. This may reflect a regulatory control exerted by HBsAg on HBeAg-HBcAg synthesis.

Deletion of precore sequences of the C ORF. The first ATG of the C ORF, at map position 1820, is followed by an in-frame ATG located about 87 bp downstream. To determine how the precore sequences (bound by the two ATGs) affect synthesis of the HBcAg and the HBeAg polypeptides, plasmid pSCΔPC was constructed, in which precore sequences upstream of the second ATG were deleted, while the core promoter region and enhancer element were retained (Fig. 1B). We then transiently expressed pCEN and pSCΔPC in Hep-G2 cells and assayed for HBeAg and HBcAg as described above (Table 4). In each assay the lysate or the concentrated culture medium equivalent to one 100-mm dish (approximately 10^7 cells) was used.

The culture medium of the pCEN-transfected Hep-G2 cells contained HBeAg, as it was not significantly blocked with anti-HBc (Table 4). Much less antigen was seen in the cell lysate, indicating that the protein is secreted and that its accumulation in the medium is not due to cell lysis. Transfection of Hep-G2 cells with pSCΔPC resulted in the accumulation of comparatively large amounts of antigen in the culture medium as detected by RIA (Table 4). However, the immunoreactivity of this sample was completely blocked by the addition of anti-HBc, indicating that all of the antigen is HBcAg. In the lysate of these cells anti-HBc reduced the P/N value of the RIA by about 60%, suggesting that both core and e antigenic polypeptides are found within the cells. In the assay of the pSCΔPC-transfected cells, the P/N values for the culture medium and the cell lysate were approximately the same, suggesting that secretion is not taking place but that cell lysis is occurring and releasing antigen into the culture medium. A lactic dehydrogenase assay (35) of medium from transfected cells indicated that about 10% of the cells were lysing as compared with untransfected cells (M. Roossinck, unpublished data).

It should also be noted that HBeAg is not found in the culture medium of pSCΔPC-transfected cells but only in the cell lysate. This may reflect a membrane association of HBeAg within the cells, which would pellet with the cell debris in the clarified medium used for the assay. We obtained similar results in a transiently replicating system with COS cells transfected with appropriate plasmids (unpublished data). We can conclusively say from these results that the deletion of the first ATG of the C ORF abolishes secretion of HBeAg in Hep-G2 cells and greatly augments the synthesis of HBcAg. This increase in HBcAg synthesis suggests a regulatory repression of HBcAg synthesis when the entire sequence of the C region is available.

DISCUSSION

The studies described here dealt with the expression of the HBV C region under the control of native transcriptional signals both by stably transformed cell lines and by transient expression. In summary, our studies demonstrate that: (i) C ORF-specific mRNA can be synthesized under the direction of subgenomic fragments; (ii) the 5′ ends of these transcripts are heterogeneous; (iii) the sequences immediately upstream of the C ORF contain promoter activity, which is dependent on a distant enhancer element; (iv) the synthesis of HBeAg and HBcAg, under the control of HBV transcriptional signals, can be accomplished via recombinant molecules; (v) the intact C ORF can direct the synthesis and secretion of HBeAg; and (vi) the removal of the precore sequences results in the loss of HBeAg secretion and the augmentation of HBcAg synthesis.

TABLE 4. Effect of deletion of precore sequences[a]

Sample[b]	Source	Anti-HBc[c]	P/N[d]
pCEN	Medium	−	7.5
pCEN	Medium	+	6.4
pCEN	Lysate	−	2.8
pCEN	Lysate	+	2.3
pSCΔPC	Medium	−	19.8
pSCΔPC	Medium	+	1.8
pSCΔPC	Lysate	−	22.6
pSCΔPC	Lysate	+	9.0

[a] See Table 1, footnote *a*.
[b] Hep-G2 cells transiently transfected with the indicated plasmid vector.
[c] See Table 1, footnote *b*.
[d] See Table 3, footnote *b*.

In vivo studies have suggested that greater-than-genome-length transcripts are the mRNA for the core and polymerase genes (6, 10). In our expression systems, however, we were able to demonstrate that subgenomic fragments are capable of directing the synthesis of poly(A)$^+$ C-region-related RNA transcripts. The size of the RNA is consistent with the use of a modified polyadenylation signal (AUUAAU) found at nt 2583, which is followed by the sequence CACUGUAAA at nt 2615. Modifications of the AAUAAA sequence are used as polyadenylation signals in about 20% of the known mRNAs (33). The consensus sequence CAYUGNAAA has been proposed as a secondary recognition sequence for polyadenylation, involved in small nuclear ribonucleoprotein binding (2). Further analysis of this poly(A)$^+$ RNA by S1 nuclease digestion revealed heterogeneous 5′ ends mapping before and after the first ATG of the C ORF.

Our analysis of transcriptional signals upstream of the C ORF revealed a promoter sequence which is dependent upon the enhancer sequences located about 350 to 400 bp upstream. The HBV enhancer element, however, is functional only in human hepatoma cell lines (14, 22). This explains the difficulty in achieving detectable levels of C ORF-related expression in other cell lines, as we showed in NEC-4 and NER-41.

Using the commercially available HBe RIA (Abbott Laboratories) we examined the synthesis of the core and e antigen polypeptides. The e antigen appeared to be secreted into the culture medium, and its secretion was abolished by deleting precore sequences. The 29 amino acids encoded by the precore sequences showed a high degree of hydrophobicity, a feature characteristic of signal peptides (3). The association of these sequences with HBeAg secretion seems reasonable in view of the presence of HBeAg in the sera of infected individuals, and has been suggested by others (J. Ou, D. Standring, and W. Rutter, personal communication).

Detectable HBcAg synthesis under the transcriptional control of native regulatory sequences via recombinant vectors has not been previously achieved in cultured cells. In our expression system we found that HBcAg synthesis was accomplished only by the removal of precore sequences. Similar results were also found by using the simian virus 40 late promoter to direct C ORF expression (34). How this relates to the expression of HBcAg in natural infection is unclear.

In the transient expression of pSCΔPC in Hep-G2 cells, HBcAg was synthesized in much greater amounts than the e antigen synthesis observed with pCEN. This dramatic derepression of HBcAg synthesis suggests that the nucleotide sequences that were deleted (*Mst*I to *Rsa*I, nt 1806 to 1856) are important in the regulation of C ORF-encoded proteins. This hypothesis is complicated by the failure of pCMCAT, which contains HBV sequences up to the *Mst*I site, to direct significant CAT expression, whereas pCPCAT, in which all the sequences including the two ATGs are retained, is fully capable of expressing CAT activity. This suggests that the sequences adjacent to the *Mst*I and *Rsa*I sites are important components of the regulation of C-region expression.

A tentative model can be put forward now to account for our observations and those of others. The C ORF promoter region controls the expression of a number of mRNAs with heterogeneous 5′ ends. The differential expression of these mRNAs may account for the different levels of expression of HBeAg and HBcAg seen in the rat fibroblast and Hep-G2 cell lines. Translational regulation, as well as posttranslational processing, may also be important control mechanisms affecting the levels of HBcAg and HBeAg. It is apparent that precore sequences contain a strong regulatory element for the expression of HBeAg and HBcAg in transfected cells. The exact location and function of this element is unknown. It should be noted that the second copy of the direct repeat (31), which may be an important control element of the HBV genome, is located at map position 1830 and is completely deleted in pSCΔPC. Finally, it seems likely that the sequences between the first two ATGs of the C ORF code for a signal peptide which controls the secretion of the HBeAg, since elimination of this sequence abolishes HBeAg secretion but not its synthesis. Further analyses of both the transcripts and the polypeptides expressed by the C ORF are needed to determine the regulatory aspects of HBcAg and HBeAg synthesis.

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