JOURNAL OF VIROLOGY, July 1991, p. 3882–3886
0022-538X/91/073882-05$02.00/0

Vol. 65, No. 7

Identification of a Strong Enhancer Element Upstream from the Pregenomic RNA Start Site of the Duck Hepatitis B Virus Genome

B. CRESCENZO-CHAIGNE,[1] J. PILLOT,[1] A. LILIENBAUM,[2] M. LEVRERO,[3] AND E. ELFASSI[1]*

Unité d'Immunologie Microbienne[1] and SCME,[2] Institut Pasteur, 75724 Paris, France, and Università di Roma La Sapienza, Rome, Italy[3]

Received 23 January 1991/Accepted 28 March 1991

The genome of the duck hepatitis B virus (DHBV) contains an enhancer element. This sequence, of 192 bp, is located in the 3′-terminal coding region of the DNA polymerase gene (nucleotides 2159 to 2351), upstream from the pregenomic RNA start site. This enhancer potentiates a marked increased activity from the heterologous thymidine kinase promoter in an orientation-independent manner and at a proximal, as well as at a distal, location. The DHBV enhancer activates transcription in a relatively cell-type-independent manner. Sequence homologies with the nuclear factor EF-C binding site are located in the DHBV enhancer. By using the HepG2 nuclear extracts and the DHBV enhancer as probes, a complex was observed in mobility shift assays.

Duck hepatitis B virus (DHBV) is a member of the hepadnavirus group, which consists of the human hepatitis B virus (HBV) and the woodchuck and ground squirrel hepatitis B viruses (9). All these small DNA viruses may cause persistent infection in their natural hosts.

DHBV is the most divergent member of the group. Differences include structure and genetic organization of the genome and biological properties. DHBV is the smallest of the hepadnaviruses (3,020 bp). In contrast to mammalian hepadnaviruses, the encapsidated genomic molecules are mostly fully double-stranded DNA. The genome contains only three overlapping genes, designated pre-C/C, pre-S/S, and *pol* (16). The DHBV genome lacks the X gene, and the core gene is larger than that of the mammalian viruses. The X gene product of HBV is a transcriptional transactivator. The X protein increases the amount of both genomic and subgenomic HBV mRNAs in infected cells (4). The HBV X protein is capable of stimulating the expression of genes under the control of the HBV enhancer as well as several RNA polymerase II and III promoters (1, 4, 14). In vivo, DHBV infects not only the liver but also the kidney, pancreas, and spleen, as evidenced by the presence of replicative forms of viral nucleic acids in these organs (12). Infections with DHBV have very little pathologic consequence (9), and, in general, DHBV does not integrate into the cellular genome. Many aspects of the biology of hepadnavirus are derived from the Pekin duck animal model, including the replication via reverse transcription (22). However, little is known about the regulation of viral gene expression, and it is likely that transcriptional control in DHBV differs from that in mammalian viruses. The transcription of HBV is controlled both by at least four promoters and by a transcriptional activator element located in the coding region of the *pol* gene (7, 23, 24). This enhancer has been implicated in the regulation of transcripts for the envelope, core, and X proteins and therefore seems to play a key role in the life cycle of HBV. Enhancer regions in other hepadnaviruses have not yet been identified. We examine the possibility that the most divergent member of the hepadnavirus group, the avian DHBV genome, contains an enhancer, and we compare its location, activity, and host tropism specificity with those of the HBV enhancer.

To determine whether the genome of DHBV contains an enhancer element, we constructed chloramphenicol acetyltransferase (CAT)-based vectors with different DHBV DNA segments positioned upstream and downstream of the thymidine kinase (tk) promoter. The pBLCAT2 plasmid used in this study contains the tk promoter followed by a CAT gene and the simian virus 40 polyadenylation signals, all preceded by a polylinker region (15). We started by inserting six DNA fragments covering the whole 3.0-kb DHBV genome into pBLCAT2 (Fig. 1). Whenever needed, natural restriction sites were converted to convenient cloning sites either by a repair reaction with the Klenow fragment of DNA polymerase I or by insertion of synthetic linkers and subsequent ligation (17). The resulting clones were used to transfect the human hepatoblastoma cell line HepG2 by the calcium phosphate method or by the DEAE-dextran technique (11), and the amount of CAT activity present in extracts of these cells was determined. The protein concentration in the cell lysates was determined, and CAT activity was assayed by using the same amount of total protein for all samples in an individual experiment. The CAT activity was determined quantitatively by measuring the amount of radioactivity associated with the various acetylated forms of chloramphenicol and by expressing this value as the percentage of the total radioactivity associated with the [^{14}C]chloramphenicol substrate (10). As shown in Fig. 1A, only DH6-transfected cells exhibited a large increase (77.5%) of CAT activity as compared with pBLCAT2-transfected cells. The *Bam*HI-*Taq*I fragment (nucleotides 1658 to 2489, 831 bp) is located in the 3′ terminus of the *pol* gene. Sequences within this fragment exhibit enhancer activity since the same fragment, in opposite orientation of the CAT transcription unit (DH6-2), increases the CAT gene expression equally well (73%) (Fig. 1B and 2). From these experiments, we conclude that a strong enhancer element is located within the *Bam*HI-*Taq*I fragment of the DHBV genome.

To further locate the DHBV enhancer element, the *Bam*HI-*Taq*I fragment was digested with *Alu*I and the resulting 403-, 330-, and 98-bp fragments were inserted upstream of the tk promoter and transfected in HepG2 cells. As shown in Fig. 1B, only the DH8-transfected cells exhibited a large

* Corresponding author.

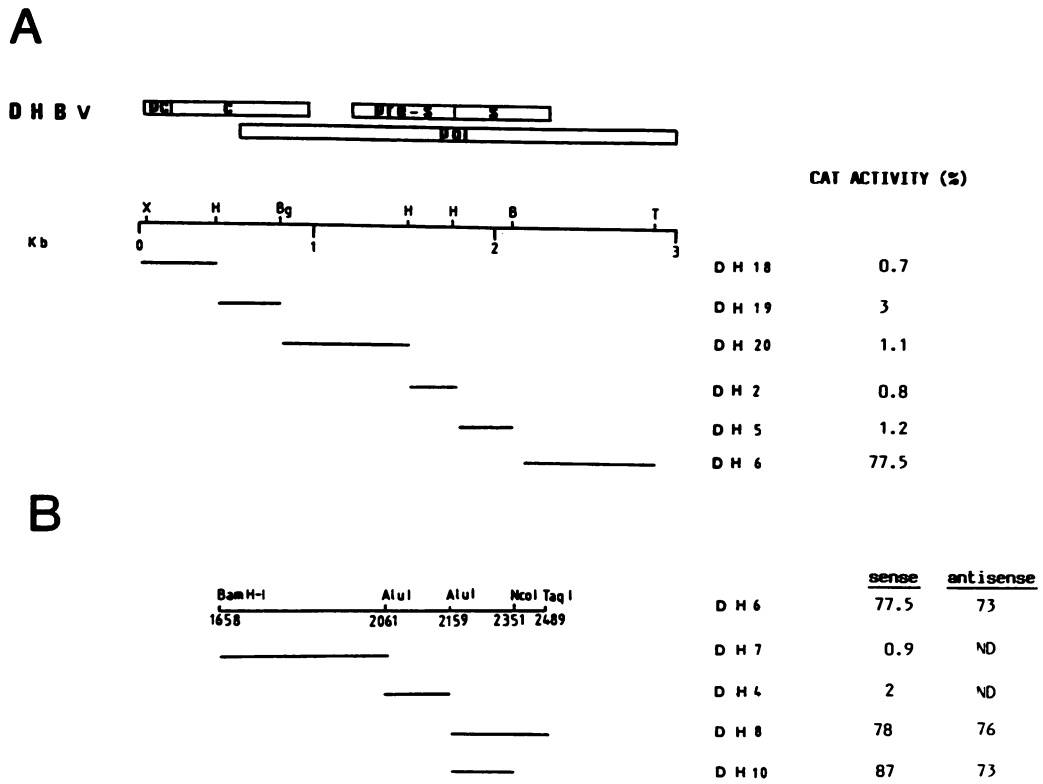

FIG. 1. Structure and enhancer activity of the recombinant DHBV DNA constructions in HepG2 cells. (A) Open reading frames of the DHBV genome are shown at the top of the figure. Recombinant DNA constructions were made by using the available restriction endonuclease cleavage sites shown above the end points. Bars represent the DHBV fragments inserted into pBLCAT2 plasmid. DH18 contains the *Xba*I-*Hin*dIII fragment from nucleotides 2662 to 3020; DH19 contains the *Hin*dIII-*Bgl*II fragment from nucleotides 14 to 391; DH20 contains the *Bgl*II-*Hin*dIII fragment from nucleotides 391 to 1093; DH2 contains the *Hin*dIII-*Hin*dIII fragment from nucleotides 1093 to 1334; DH5 contains the *Hin*dIII-*Bam*HI fragment from nucleotides 1334 to 1658; and DH6 contains the *Bam*HI-*Taq*I fragment from nucleotides 1658 to 2489. (B) Delimitation of the DHBV enhancer. Numbers indicate nucleotide position in the DHBV genome. (16). Plasmids DH7, DH4, DH8, and DH10 are derivatives of DH6. DH7 contains the *Bam*HI-*Alu*I fragment from nucleotides 1658 to 2061; DH4 contains the *Alu*I-*Alu*I fragment from nucleotides 2061 to 2159; DH8 contains the 330-bp fragment from *Alu*I to the *Taq*I site (nucleotides 2159 to 2489); and DH10 contains the *Alu*I-*Nco*I fragment from nucleotides 2159 to 2351. The values are the means of three to five independent experiments. Abbreviations: X, *Xba*I; H, *Hin*dIII; Bg, *Bgl*II; B, *Bam*HI; T, *Taq*I.

increase in CAT activity. The two DH8 plasmids, which contain the 330-bp *Alu*I-*Taq*I (nucleotides 2159 to 2489) fragment in both orientations upstream of the tk promoter, increase CAT gene expression equally well (78 and 76%).

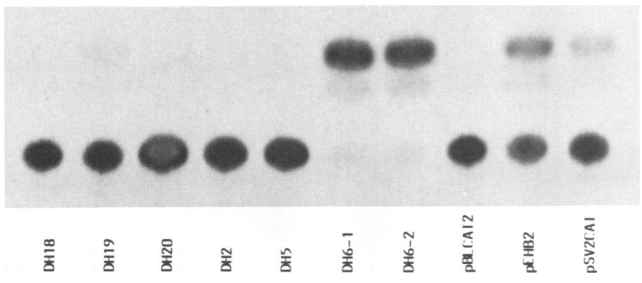

FIG. 2. CAT activity in extracts of HepG2 cells transfected by DHBV CAT plasmids. The percent conversions of [^{14}C]chloramphenicol to the acetylated derivative are shown in Fig. 1A. DH6-1 and DH6-2 contain the *Bam*HI-*Taq*I fragment from nucleotides 1658 to 2489 in the same and opposite orientations, respectively, relative to the CAT transcription unit. pBLCAT2 is an enhancerless plasmid, pEHB2 contains the HBV enhancer (7), and pSV2CAT contains the early simian virus 40 promoter and enhancer sequences.

Plasmid, which contains the same fragment 3′ of the CAT gene, also increases CAT gene expression (41% increase) (data not shown). This 330-bp fragment (nucleotides 2159 to 2489) contains the enhancer, and probably the proximal promoter of the C gene, since the pregenome CAP site is located at nucleotide 2529. To separate these two *cis*-acting regulatory sequences and to avoid any interference, we made a deletion of 138 nucleotides from *Nco*I to *Taq*I (nucleotides 2351 to 2489) in the DH8 fragment. The HepG2 cells transfected with the resulting plasmid (DH10) containing 192 bp of DHBV genome (nucleotides 2159 to 2351) also showed an increase of CAT activity comparable to that obtained with the two DH8 plasmids (Fig. 1B).

All plasmids that contain this region located in the 3′ terminus of the *pol* gene display a higher level of CAT activity in the transfection assays than does the pBLCAT2 plasmid. The level of CAT activity has been normalized to the level of CAT activity directed by the tk promoter regardless of their orientation or their location (proximal or distal) with respect to the promoter. Cotransfection of all plasmids with pRSV-β-gal plasmid, which contains the long terminal repeat of Rous sarcoma virus and the β-galactosidase genes, was also done. No significant variation in β-galactosidase activity was detected between samples.

TABLE 1. DHBV enhancer activity in various cell lines

Cell line[a]	% CAT activity[b] with:		
	pBLCAT2	DH10	pSV2CAT
HepG2	0.9	87	25
HeLa	3	2	15
CV1	0.7	15	41
CHO	1.2	36	24

[a] A total of 10^6 cells of the cell lines listed were cotransfected with 5 μg of the CAT expression plasmid indicated and 1 μg of pRSV-β-gal by the calcium phosphate method or the DEAE-dextran technique (11). At 48 h after transfection, lysates were prepared for CAT and β-galactosidase activity assays. The amount of DNA used in the transfection procedure was within the linear range of the assay.

[b] All experiments were performed at least three times with at least two separate plasmid preparations. Percentages represent the average values from three independent experiments.

The experiments summarized in Fig. 1 serve to localize a sequence capable of stimulating the tk promoter to a region in the *pol* gene, upstream of the C gene (nucleotides 2159 to 2351). The *pol* gene encodes a large polypeptide containing the DNA polymerase and reverse transcriptase and the RNase H catalytic domains separated by a spacer region (21). This gene is expressed by internal translational initiation at the *pol* AUG rather than by ribosomal frameshifting (3). The HBV P products also play an important role in pregenome encapsidation and are required as structural components (2, 13). A smaller DHBV polymerase protein than the corresponding HBV protein can be predicted (16), but, as is the case for HBV, the enhancer of DHBV is located in the 3′ terminus of the *pol* gene.

To determine the effect of the DHBV enhancer on gene expression in cell lines from different species and tissues, we transfected vectors carrying the DHBV enhancer into several cell lines listed in Table 1. These cell lines include the human cervical carcinoma cell line HeLa, the hamster ovary epithelial cell line CHO, and the simian kidney cell line CV-1. The results of transfection of different cell lines with the DH10 plasmid are listed in Table 1. The levels of CAT activity in these cell lines directed by pSV2CAT, which contains the entire early region promoter of simian virus 40, as well as the level of activity of the pBLCAT2 plasmid, are also given.

These results indicate that DHBV sequences contained in this region display a strong enhancer activity in human HepG2 cells and a moderate enhancer activity in CV1 and CHO cells. No increase in the level of CAT activity was detected upon transfection of the HeLa cell line with plasmids that contained the DHBV sequences. These cells were competent for transfection as judged by their level of CAT activity directed by the pSV2CAT plasmid. From these experiments, we conclude that the DHBV enhancer activates transcription, preferentially, but not exclusively, in hepatoma cell lines.

The DHBV enhancer in vitro is not species specific, since the ability of DHBV fragments to exhibit enhancer activity was tested by using the human HepG2 cells. This differentiated human hepatoma cell line has recently been shown to support replication and production of the infectious HBV, as well as DHBV, upon transfection with cloned HBV and DHBV DNA, respectively (8).

One of the properties of enhancers is that they can exhibit tissue type specificity of transcription activation. Since the hepadnavirus are characterized by a high species specificity and relative liver tropism, it has been suggested that tissue-specific expression of HBV was due to the restricted function of the HBV enhancer (6). However, several groups have reported that the HBV enhancer was not specific for liver cells but exhibited activity in a variety of cell types (7, 23, 24). Transfection of the DHBV enhancer in several cell lines resulted in a variable increase in CAT gene expression. These results suggest that the enhancer is not solely responsible for the hepatotropic phenotype of the virus.

The mechanisms by which enhancers function are not known, but these sequences are composed of multiple elements or modules consisting of short conserved-sequence motifs that are binding sites for transcription factors. In the case of the HBV enhancer, multiple factors bind to distinct domains, and most of these factors are ubiquitous. These domains individually exhibit a very inefficient enhancing activity but act synergistically to give a high level of activity (6). This suggests that factor-binding sites in the enhancer act to facilitate the formation of a functional complex. To assess the ability of nuclear extracts to bind to the DHBV enhancer, we performed mobility shift assays by using a ^{32}P-labeled DNA fragment that contained the enhancer region. Nuclear proteins from HepG2 cells were extracted with 0.3 M NaCl and dialyzed, and 3 μg of extract was used for a gel retardation assay, as described previously (5). The DHBV DNA probes were the *Alu*I-*Nco*I fragment (nucleotides 2159 to 2351; 192 bp). Probe DNAs were end labeled at the unique *Nco*I site of the vector with the Klenow fragment of DNA polymerase and [α-^{32}P]dCTP. A prominent complex was detected when nuclear extracts from HepG2 cells were incubated with salmon sperm DNA or poly(dI-dC) homopolymer as the nonspecific competitor and the DHBV

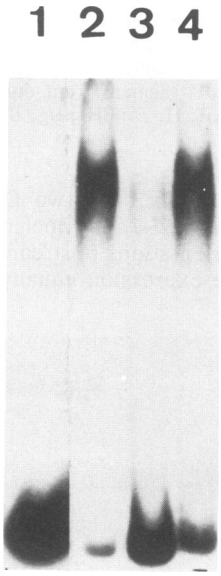

FIG. 3. Binding of the HepG2 nuclear factors to the DHBV enhancer. A probe from the DHBV enhancer (*Alu*I-*Nco*I, nucleotides 2159 to 2351, 192 bp) was incubated with a HepG2 nuclear extract. Lane 1 shows the DHBV probe without nuclear extract. Lanes 2, 3, and 4 contain 3 μg of nuclear extract, 2 μg of salmon sperm DNA, and the DHBV probe. Lane 2 lacks DNA competitor; lane 3 contains 250 ng of cold DNA competitor identical to the probe; lane 4 contains 250 ng of an unrelated cold DNA fragment of 241 nucleotides (DHBV fragment, nucleotides 1093 to 1334). The samples were loaded onto a 5% polyacrylamide gel.

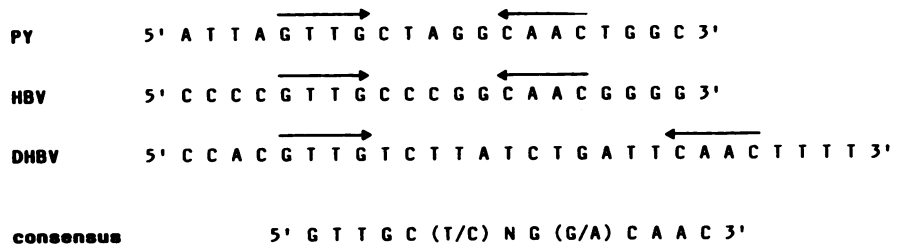

FIG. 4. EF-C binding site. Sequence of the polyomavirus (PY) and HBV EF-C binding sites are shown at the top. Arrows correspond to the inverted repeats. Similar sequences in the DHBV genome are indicated (nucleotides 2294 to 2323). Below the EF-C binding site, the consensus sequence is shown.

enhancer fragment as the probe (see Fig. 4, lane 2). This complex was inhibited by various concentrations of cold DNA, identical to the DHBV enhancer probe, but not by unrelated cold DNA (DHBV fragment, nucleotides 1093 to 1334; 241 bp). The results of representative experiments with 250 ng of cold DNA are shown in Fig. 3; they suggest that nuclear factors in HepG2 cells can bind to the DHBV enhancer.

Recently two cellular factors, C/EBP and EF-C, have been shown to bind to the human HBV enhancer (19, 20). C/EBP binds to at least two different sequence motifs, the CCAAT promoter domain (5'-CCAAT-3') and a core sequence common to many viral enhancers [5'-TGTGG(A/T)(A/T)(A/T)G-3']. C/EBP factor contains a leucine zipper structure important for DNA-binding activity through protein dimerization. C/EBP binds to two sites within the HBV enhancer and can function as a transcriptional repressor (20). EF-C is a nuclear factor that binds to the murine polyomavirus enhancer C element, [5'-GTTGC(T/C)NG(G/A)CAAC-3'] (18). Ostapchuk et al. demonstrated that EF-C binds to the HBV inverted repeat in vitro and is an important functional component of the HBV enhancer region in vivo (19). They also showed that the DHBV genome contained sequence similarities to the inverted repeat, with an additional 9 bp in the spacer region (2294 to 2323). The enhancer region that we identified here contains this inverted repeat sequence homologous with the EF-C site (Fig. 4) and therefore, as is the case for HBV, may play an important role in enhancer function.

It is possible that this 192-bp region (which exhibits enhancer activity) located upstream of the pregenomic RNA start site is composed of distinct domains or enhansons and contains other regulatory sequences. Also, this study does not eliminate the possibility that another enhancer, combined with negative regulatory elements, for example, exists somewhere else in the DHBV genome. In this respect, it is interesting that the DH19 construct (Fig. 1A), which contains a fragment spanning the 3' terminus of the C gene and the 5' terminus of the *pol* gene, caused a low but significant activity. Recently a second enhancer located in the X gene coding region in HBV has been identified (25).

In conclusion, we demonstrate that the DHBV genome contains a strong enhancer located in the 3' terminus of the *pol* gene upstream from the pregenomic RNA start site. This enhancer functions in a relatively cell-type-independent manner. We also show that nuclear factors can bind to the DHBV enhancer. These results suggest that the host specificity of hepadnavirus is not controlled solely by specific transcriptional factors but primarily by events at the cell surface, with a yet undefined host cell receptor.

Comparative analysis of gene expression between the two most distant hepadnaviruses, HBV and DHBV, will provide insight into the mechanisms by which the *cis*-regulatory sequences in hepadnavirus genomes govern the expression, the host tropism, and ultimately the disease process induced by these viruses.

We thank A. Kay for the kind gift of pUKD2 and H. Hamdi for typing the manuscript.

REFERENCES

1. **Aufiero, B., and R. J. Schneider.** 1990. The hepatitis B virus X gene product transactivates both RNA polymerase II and III promoters. EMBO J. **9**:497–504.
2. **Bartenschlager, R., M. Junker-Niepmann, and H. Schaller.** 1990. The P gene product of hepatitis B virus is required as a structural component for genomic RNA encapsidation. J. Virol. **64**:5324–5332.
3. **Chang, L. J., D. Ganem, and H. E. Varmus.** 1990. Mechanism of translation of the hepadnaviral polymerase (P) gene. Proc. Natl. Acad. Sci. USA **87**:5158–5162.
4. **Colgrove, R., G. Simon, and D. Ganem.** 1989. Transcriptional activation of homologous and heterologous genes by the hepatitis B virus X gene product in cells permissive for viral replication. J. Virol. **63**:4019–4026.
5. **Dignam, J. D., R. M. Lebovitz, and R. G. Reodev.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
6. **Dikstein, R., O. Faktor, R. Ben-Levy, and Y. Shaul.** 1990. Functional organization of the hepatitis B virus enhancer. Mol. Cell. Biol. **10**:3683–3689.
7. **Elfassi, E.** 1987. Broad specificity of the hepatitis B enhancer function. Virology **160**:259–262.
8. **Galle, P. R., H. J. Schlicht, M. Fischer, and H. Schaller.** 1988. Production of infectious duck hepatitis B virus in a human hepatoma cell line. J. Virol. **62**:1736–1740.
9. **Ganem, D., and H. E. Varmus.** 1987. The molecular biology of the hepatitis B viruses. Annu. Rev. Biochem. **56**:651–693.
10. **Gorman, C., L. Moffat, and B. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
11. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
12. **Halpern, M. S., J. M. England, L. Flores, J. Egan, J. Neubold, and W. S. Mason.** 1984. Individual cells in tissues of DHBV-infected ducks express antigens crossreactive with those on virus surface antigen particles and immature viral cores. Virology **137**:408–413.
13. **Junker-Niepmann, M., R. Bartenschlager, and H. Schaller.** 1990. A short cis-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. EMBO J. **9**:3389–3396.
14. **Levrero, M., C. Balsano, G. Natoli, M. L. Avantaggiati, and E. Elfassi.** 1990. Hepatitis B virus X protein transactivates the long

terminal repeats of human immunodeficiency virus types 1 and 2. J. Virol. **64**:3082–3086.
15. **Luckow, B., and G. Schütz.** 1987. CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. Nucleic Acids Res. **15**:5490.
16. **Mandart, E., A. Kay, and F. Galibert.** 1984. Nucleotide sequence of a cloned duck hepatitis B virus genome: comparison with woodchuck and human hepatitis B virus sequences. J. Virol. **49**:782–792.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Ostapchuk, P., J. F. X. Diffley, J. T. Bruder, B. Stillman, A. J. Levine, and P. Hearing.** 1986. Interaction of a nuclear factor with the polyomavirus enhancer region. Proc. Natl. Acad. Sci. USA **83**:8550–8554.
19. **Ostapchuk, P., G. Scheirle, and P. Hearing.** 1989. Binding of nuclear factor EF-C to a functional domain of the hepatitis B virus enhancer region. Mol. Cell. Biol. **9**:2787–2797.
20. **Pei, D., and C. Shih.** 1990. Transcriptional activation and repression by cellular DNA-binding protein C/EBP. J. Virol. **64**:1517–1522.
21. **Radziwill, G., W. Tucker, and H. Schaller.** 1990. Mutational analysis of the hepatitis B virus P gene product: domain structure and RNase H activity. J. Virol. **64**:613–620.
22. **Summers, J., and W. S. Mason.** 1982. Replication of the genomes of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell **29**:403–415.
23. **Tognoni, A., R. Cattaneo, E. Serfling, and W. Schaffner.** 1985. A novel expression selection approach allows precise mapping of the hepatitis B virus enhancer. Nucleic Acids Res. **13**:7457–7472.
24. **Vannice, J. L., and A. D. Levinson.** 1988. Properties of the human hepatitis B virus enhancer: position effects and cell type nonspecificity. J. Virol. **62**:1305–1313.
25. **Yee, J.-K.** 1989. A liver-specific enhancer in the core promoter region of human hepatitis B virus. Science **246**:658–661.