Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 1627–1631, March 1986
Biochemistry

Hepatitis B virus DNA contains a glucocorticoid-responsive element

(recombinant DNA/chloramphenicol acetyltransferase expression vectors/hepatitis B virus enhancer/hormone regulation of gene expression)

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Communicated by Hans Popper, November 4, 1985

ABSTRACT **It has recently been shown that hepatitis B virus (HBV) contains a transcriptional enhancer element. In order to determine whether this enhancer responds to glucocorticoids, a series of derivatives of plasmid $pA_{10}CAT_2$ was constructed containing the HBV enhancer and variable lengths of further upstream sequences. Transient expression of chloramphenicol acetyltransferase (CAT) was determined after introduction of these plasmids into PLC/PRF/5, Hep 3B, Hep G2, HeLa, and mouse L cells. Highest CAT activity was noted in the human hepatocellular carcinoma line PLC/PRF/5, which contains integrated HBV DNA sequences. Dexamethasone augmented CAT expression in all cell lines tested with 40% of maximal induction at 10 nM and maximum stimulation (3- to 8-fold) at 1 μM dexamethasone. Dexamethasone augmentation of CAT expression was observed only when constructs contained HBV DNA sequences residing upstream to map position 735 from the *Eco*RI site. This indicates that the glucocorticoid-responsive region is distinct from the previously defined HBV enhancer sequence located at map position 1080–1234. These studies suggest that HBV DNA contains a glucocorticoid-responsive element, which may mediate expression of HBV genes in infected mammalian cells.**

Hepatitis B virus (HBV) is a small double-stranded circular DNA virus that causes acute and chronic hepatitis in man and is strongly associated with development of hepatocellular carcinoma (1), which occurs at a much higher frequency in males than in females (2). It has been suggested that hormonal influences may be responsible for this male preponderance. In addition, corticosteroids increase expression of HBV gene products in two human hepatocellular carcinoma cell lines, PLC/PRF/5 (3) and Mahlavu (4). These findings are consistent with the observations that glucocorticoid therapy in patients with HBV-related chronic hepatitis causes activation of latent infection (5, 6), increased levels of HBV markers (7), and increased severity of liver disease (8).

Hormones are known to induce transcription of cellular and viral genes. One of the best described hormone-dependent viral systems is the mouse mammary tumor virus (MMTV), in which a hormone receptor complex binds to a glucocorticoid-responsive enhancer element, which can then up-regulate expression of both autologous and heterologous genes (9–14). To determine whether the increased expression of gene products by glucocorticoids in HBV-infected cells is due to a direct interaction of the hormone with the HBV transcription unit, we examined the effects of dexamethasone (Dex) on gene expression in recombinant plasmids that contain different segments of the HBV genome. In this study, we demonstrate that glucocorticoids stimulate HBV enhancer activity in various eukaryotic cell lines. This expression is independent of the position or orientation of the enhancer element in relation to the bacterial gene for chloramphenicol acetyltransferase (CAT), which was used to assess enhancer function. A dose–response curve for the effect of various Dex concentrations on HBV enhancer-mediated CAT gene expression in PLC/PRF/5 cells showed 50% induction at 20 nM Dex. Using constructs that contain various fragments of HBV DNA upstream to the enhancer, we demonstrated further that the glucocorticoid-responsive element is distinct from the previously mapped HBV enhancer (15).

MATERIALS AND METHODS

Construction of Plasmids and DNA Preparation. The 1.4-kilobase (kb) *Bam*HI fragment of the HBV genome (16), map position 30–1402, was introduced into the plasmid $pA_{10}CAT_2$ 3′ to the CAT gene in the sense orientation (Fig. 1). The $pA_{10}CAT_2$ plasmid contains the simian virus 40 (SV40) early promoter–enhancer 5′ to the CAT gene, but there is a deletion in the enhancer sequence, so that very little CAT expression is produced in the absence of an added enhancer. The 1.4-kb HBV *Bam*HI fragment was also introduced into $pA_{10}CAT_2$ 3′ to the CAT-encoding gene in the antisense orientation and into the *Bgl* II site of $pA_{10}CAT_2$ 5′ to the CAT-encoding gene in both orientations. pSV_2CAT, which contains both an SV40 promoter and functional enhancer, was used as a control. Other constructs were made by introducing fragments of HBV DNA 5′ to the SV40 early promoter in plasmid $pA_{10}CAT_3$ (15). This plasmid contains a unique *Sph* I site located 5′ to the SV40 promoter and a unique *Pvu* II site within the pBR322 region. The *Sph* I–*Pvu* II fragment was replaced with the HBV DNA fragment from (*i*) the *Bgl* II site at position 2431 to the *Sph* I site at 1234 (pHBΔ3CAT), (*ii*) the *Alu* I site at 2834 to the *Sph* I site at 1234 (pHB1.6CAT), (*iii*) the *Alu* I site at 735 to the *Sph* I site at 1234 (pHB0.5CAT), or (*iv*) the *Alu* I site at 1080 to the *Sph* I site at 1234 (pHB0.15-CAT). All DNA constructs were prepared by standard recombinant DNA techniques (17), and plasmid DNA was purified by centrifugation in CsCl gradients.

Other constructs used as control plasmids were pMMTV-CAT, which contains the MMTV long terminal repeat (LTR) 5′ to the CAT-encoding gene and the SV40 enhancer 3′ to the CAT-encoding gene in plasmid $pCAT_3M$ (18), and $pPrLTR_2$-CAT (19), which contains the Rous sarcoma virus (RSV) LTR 5′ to the CAT-encoding gene in plasmid $pCAT_3M$. Prior to experiments, DNA samples were analyzed on agarose gels

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Abbreviations: HBV, hepatitis B virus; CAT, chloramphenicol acetyltransferase; Dex, dexamethasone; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; SV40, simian virus 40; RSV, Rous sarcoma virus; kb, kilobase(s).

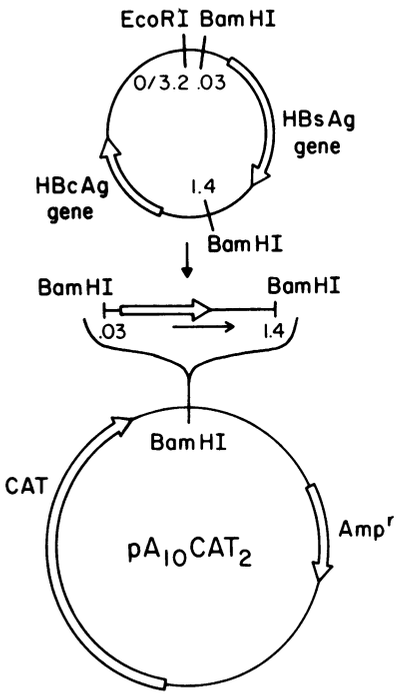

FIG. 1. Structure of the $pA_{10}CAT_2$ plasmid containing inserted HBV DNA sequences. The map of the HBV genome and cleavage sites for *Bam*HI are indicated. The HBcAg (hepatitis B core antigen) and HBsAg (hepatitis B surface antigen) genes are represented by arrows. The smaller *Bam*HI fragment of HBV DNA (map position, 30–1402) was ligated into the *Bam*HI site of $pA_{10}CAT_2$ 3′ to the CAT-encoding sequence in the sense orientation (pHB1.4-3′CAT). Amp^r, ampicillin resistance.

and were used only when >50% of the preparation was in form I configuration.

Cell Lines. The cell lines PLC/PRF/5 and Hep 3B are derived from human hepatocellular carcinomas; both contain integrated HBV sequences and synthesize viral gene products (20–22). Hep G2 is a well-differentiated human hepatoblastoma cell line not containing HBV DNA (23); HeLa is a human cervical cancer epithelial line, and L is a mouse fibroblast line.

Cells were grown in minimal essential medium (PLC/PRF/5 and Hep G2) or Dulbecco's modified Eagle's medium (Hep 3B, HeLa, and L) containing 10% fetal calf serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. One day before transfection, cells were plated at a density of 10^4 cells per cm^2 on 100 mM plates; 3 hr before addition of DNA, cells were refed with fresh medium.

Cell Transfection and Dex Addition. Cells were transfected by the calcium phosphate precipitation method (24) with 5–25 μg of plasmid DNA per 100-mm culture dish. After the linear range was established in terms of the amount of plasmid used for transfection versus expression of the CAT gene (the midrange was 10–15 μg of DNA per dish), comparisons were made between pSV_2CAT, $pPrLTR_2CAT$, pMMTV-CAT, and specific vectors containing HBV DNA sequences. Six hours after addition of DNA, the medium was removed and the cells were fed fresh medium in the presence or absence of Dex (Sigma) at the various concentrations noted.

Assay for CAT Activity. Cells were harvested 48 hr after transfection, and extracts were prepared by sonication and centrifugation. The cell extracts were assayed for CAT activity essentially as described by Gorman *et al.* (25). The percentage of acetylation of ^{14}C-labeled chloramphenicol per 50 μg of protein in cell extracts was determined by TLC and liquid scintillation spectroscopy of acetylated and nonacetylated bands identified by autoradiography of the TLC plates.

RESULTS

Effect of Dex on Transient Expression of CAT. To test the effect of Dex on HBV enhancer activity, we assayed for CAT expression in various cell lines transfected with the recombinant plasmid pHB1.4-3′CAT, which contains the 1.4-kb *Bam*HI fragment of HBV DNA inserted 3′ to the CAT-encoding gene (Fig. 1), and with control plasmids $pA_{10}CAT_2$ and pSV_2CAT. Cells were grown in the presence or absence of 1 μM Dex.

An example of HBV sequence enhancement of CAT expression by pHB1.4-3′CAT and Dex stimulation of this expression in transfected human hepatocellular carcinoma cells (Hep 3B) is shown in Fig. 2. There was no detectable CAT activity in nontransfected Hep 3B cells (lane 5) or in cells transfected with $pA_{10}CAT_2$ (lane 4). However, cells transfected with pHB1.4-3′CAT showed an enhancer effect on CAT activity (lane 1), which was increased 3-fold by addition of 1 μM Dex (lane 2). Lane 3 shows abundant CAT activity obtained in Hep 3B cells transfected with control plasmid pSV_2CAT.

We next determined whether the pHB1.4-3′CAT construct displayed similar expression in other human cell lines of hepatocytic origin. CAT activity was measured in PLC/PRF/5 and Hep G2 cells transfected with pHB1.4-3′CAT and control recombinant plasmids in the presence or absence of 1 μM Dex (Table 1). The plasmid pHB1.4-3′CAT induced CAT activity in both cell lines. Cells transfected with pSV_2CAT showed highest activity, whereas mock-transfected cells or cells transfected with $pA_{10}CAT_2$ showed almost no CAT activity. Dex at 1 μM did not increase CAT expression in PLC/PRF/5, Hep 3B, or Hep G2 cells transfected with various amounts of $pA_{10}CAT_2$ or pSV_2CAT (between 5–25 μg per plate). The highest CAT activity induced by the HBV enhancer was observed in PLC/PRF/5. Addition of 1 μm Dex to the culture medium raised CAT activity 2.6- to 3.5-fold with pHB1.4-3′CAT in the three liver-derived cell lines (Table 1). Analysis of pHB1.4-3′CAT activity and Dex stimulation of CAT expression was also examined in HeLa and L cells. In both cases, CAT activity was present at a reduced level compared to that observed with human liver-

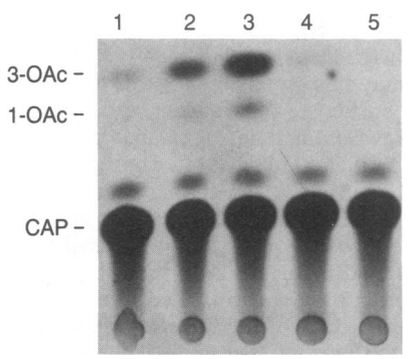

FIG. 2. CAT activity in Hep 3B cells transfected with $pA_{10}CAT_2$, pHB1.4-3′CAT, or pSV_2CAT. Cells were transfected and fed 6 hr later with fresh Dulbecco's modified Eagle's medium containing 10% fetal calf serum in the presence or absence of 1 μM Dex. Cells were harvested 48 hr after transfection, and soluble extracts were prepared. CAT assays were performed as described by Gorman *et al.* (25) with incubation for 1 hr at 37°C. Ethyl acetate-extracted samples (20 μl) were then spotted on silica gel thin-layer plates, and [^{14}C]chloramphenicol (CAP) and its acetylated forms, chloramphenicol 1-acetate (1-OAc) and chloramphenicol 3-acetate (3-OAc), were detected by autoradiography. Lanes: 1, extract of cells transfected with pHB1.4-3′CAT; 2, extract of cells transfected with pHB1.4-3′CAT in the presence of 1 μM Dex; 3, extract of cells transfected with pSV_2CAT; 4, extract of cells transfected with $pA_{10}CAT$; 5, extract of nontransfected cells.

Table 1. Dex stimulation of CAT activity by recombinant plasmid pHB1.4-3'CAT in various cell lines

Cell	Dex (μM)	% acetylation*			Relative CAT activity†		Fold increase by Dex
		$pA_{10}CAT_2$	pSV_2CAT	pHB1.4-3'CAT	pSV_2CAT	$pA_{10}CAT_2$	
PLC/PRF/5	−	0.15	62.0	5.2	8.0	34.0	2.6
	+	0.16	64.0	13.8	21.5	86.0	
Hep 3B	−	0.02	4.0	0.4	10.0	20.0	3.5
	+	0.02	4.2	1.5	35.0	75.0	
Hep G2	−	0.02	26.0	0.4	1.5	20.0	3.4
	+	0.02	27.0	1.4	5.1	70.0	
HeLa	−	0.35	98.0	0.8	0.8	2.3	5.7
	+	0.33	97.0	4.6	4.6	13.9	
L cells	−	0.02	5.0	0.13	2.6	6.5	7.6
	+	0.02	4.4	0.9	20.0	45.0	

CAT activity of pHB1.4-3'CAT, pSV_2CAT, and $pA_{10}CAT_2$ plasmids in eukaryotic cells transfected with these respective plasmids is shown. Equivalent amounts (15 μg) of the various plasmids were transfected into the different cell lines by the calcium phosphate precipitation method (24). Six hours after transfection, the medium was removed and fresh medium was added in the presence or absence of μM Dex. The cells were harvested 48 hr after transfection and analyzed for CAT activity (25). The % acetylation of ^{14}C-labeled chloramphenicol per 50 μg of protein in cell extracts was determined by TLC and liquid scintillation spectroscopy of acetylated and nonacetylated bands identified by autoradiography of the TLC plates (≈500,000–700,000 cpm were applied to each spot on the TLC plate).

*Values represent the average of two or three independent experiments.

†Relative CAT activity is the ratio of the % acetylation of [^{14}C]chloramphenicol in pHB1.4-3'CAT compared to pSV_2CAT × 100 or $pA_{10}CAT$ × 100.

derived cell lines but was stimulated 5.7- to 7.6-fold by 1 μM Dex (Table 1). This indicated that, although the enhancer effect was lower in nonhuman and human non-liver-cell lines, the glucocorticoid-mediated response was maintained. Stimulation of enhancer activity by Dex was also obtained with the 1.4-kb *Bam*HI fragment of HBV DNA inserted either 5' to the CAT gene in the sense or antisense orientation or 3' to the CAT gene in the antisense orientation (data not shown).

Effect of Dex Concentration on CAT Activity. To determine whether Dex stimulation of HBV enhancer function might operate through a mechanism similar to that observed in the glucocorticoid-responsive MMTV system, a concentration–response curve for Dex stimulation was performed with plasmid pHB1.4-3'CAT compared to pMMTV-CAT in PLC/PRF/5 cells (Fig. 3). In the absence of Dex, activity with pMMTV-CAT was very low and comparable to that obtained with $pA_{10}CAT_2$. With both pHB1.4-3'CAT and pMMTV-CAT, there was a progressive increase in CAT activity with increasing concentrations of Dex, 3- to 5-fold above the background. Maximum stimulation was observed between 0.1 μM and 1 μM Dex. No Dex effect was noted in cells transfected with either pSV_2CAT, containing an SV40 enhancer, or $pPrLTR_2CAT$, containing an RSV enhancer (Fig. 3). Similar results were observed in Hep G2 cells transfected with pHB1.4-3'CAT (data not shown).

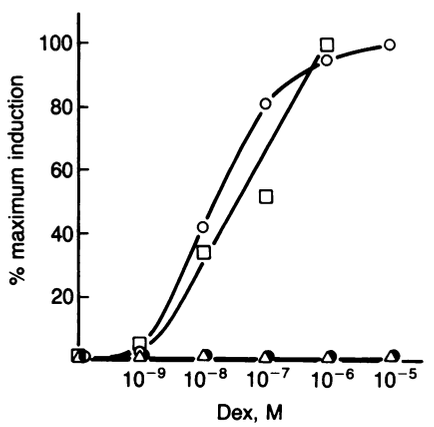

FIG. 3. Stimulation of CAT activity in PLC/PRF/5, cells transfected with pHB1.4-3'CAT, pMMTV-CAT, $pPrLTR_2CAT$, or pSV_2CAT and treated with various concentrations of Dex. Medium was removed 6 hr after transfection, and cells were fed with fresh medium containing 1 nM, 10 nM, 100 nM, 1 μM, or 10 μM Dex. The cells were harvested 48 hr after transfection and analyzed for CAT activity. The percent acetylation of ^{14}C-labeled chloramphenicol was determined as described in Table 1. Data shown are for transfection with 15 μg of plasmid DNA per plate, although no Dex stimulation was noted for pSV_2CAT or $pPrLTR_2CAT$ at 5, 10, 15, or 20 μg of plasmid DNA. ○, pHB1.4-3'CAT; □, pMMTVCAT; △, $pPrLTR_2$-CAT; ●, pSV_2CAT.

Identification of a Glucocorticoid-Responsive Region in HBV DNA. A consensus sequence, GNNACAANNTGTYCT, which functions as a specific glucocorticoid receptor binding site, has been identified in several glucocorticoid-regulated genes, including human growth hormone, human metallothionein IIA, and MMTV 1.3 (26). In order to define the region in HBV DNA responsible for the glucocorticoid effect, we used constructs spanning the enhancer region but also containing various lengths of HBV DNA upstream to the enhancer (Fig. 4). Three of these constructions, pHBΔ3CAT, pHB1.6CAT, and pHB1.4-3'CAT, containing the enhancer region and sequences upstream to map position 735 showed 2.5- to 3.0-fold stimulation of CAT expression in PLC/PRF/5 cells by Dex. In contrast, two constructions, pHB0.5CAT and pHB0.15CAT containing the enhancer element and other sequences from position 735 to 1234, showed no stimulation of CAT expression by Dex (Fig. 4).

DISCUSSION

In various systems, glucocorticoids have been shown to regulate specific gene expression—e.g., human metallothionein (27), human and rat growth hormone (26, 28), chicken lysozyme (29), and MMTV DNA (30). The specific sequences responsible for hormonal induction of MMTV genes are located in the LTR region (11). Studies in L cells lacking thymidine kinase (TK) expression (LTK^- cells) transfected with an MMTV promoter–enhancer element linked to a herpesvirus TK gene showed that glucocorticoids can increase enhancer activity of a linked heterologous gene (31).

In the previous (15) as well as the present study, a fragment of HBV DNA has been shown to contain a transcriptional enhancer element. Highest expression was demonstrated in

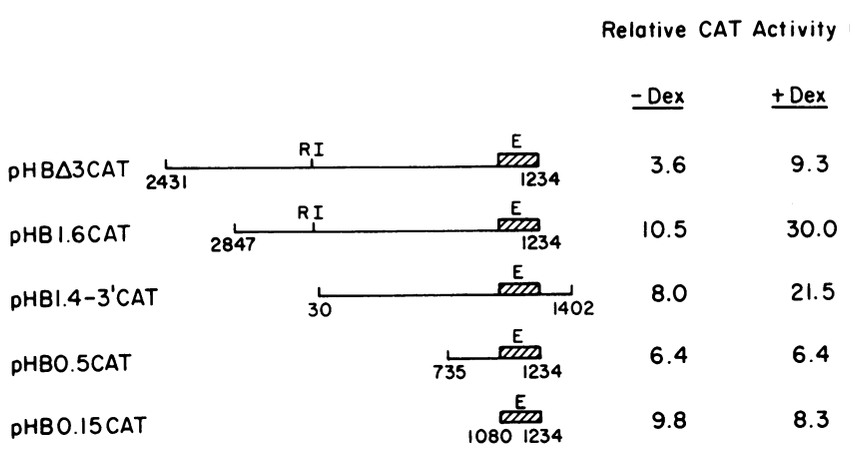

FIG. 4. Comparison of Dex effect on CAT activity induced by plasmids containing various fragments of HBV DNA. Schematic representations of specific HBV sequences present in plasmid constructs are shown on the left (see *Materials and Methods* and ref. 15), and the relative CAT activity produced in PLC/PRF/5 cells transfected with the various plasmids in the absence or presence of 1 μM Dex is shown on the right. RI, *Eco*RI restriction site; E (hatched bars), enhancer region of HBV DNA.
*Relative CAT activity is the ratio of the % acetylation of [^{14}C]chloramphenicol by pHB1.4-3' CAT compared to pSV$_2$CAT × 100.

human liver-derived cell lines and in particular PLC/PRF/5, which contains integrated HBV DNA sequences. Dex stimulated HBV enhancer activity, and this stimulation was noted in a variety of human and nonhuman cells. This is consistent with the observation that all mammalian cells have glucocorticoid receptors. The effect of Dex on HBV enhancer activity was specific, since no Dex stimulation was noted with the SV40 or RSV promoter–enhancer. Lack of responsiveness of pSV$_2$CAT to Dex has also been noted by others (32).

Moore *et al.* (26) have reported a consensus sequence GNNACAANNTGTYCT in the human growth hormone gene that functions as a specific glucocorticoid receptor binding site (map position, 100 to 111). Such sequences are also present in MMTV DNA (map position, −185 to −155) and in the human metallothionein gene (map position −270 to −240). A search of the HBV genome reveals a similar sequence (*NCAANNTGTYCT*) between map positions 351 and 366 in three separately cloned and sequenced HBV genomes (33–35). In MMTV DNA, the sequence TGTTCT occurs four times in the LTR region (36); in the HBV genome, TGT$^{T}_{C}$CT occurs multiple times in the map region 200–500. However, at present, a specific role of this sequence as part of a glucocorticoid-responsive element in HBV DNA has not been established.

The similar dose–response curves for Dex stimulation of pHB1.4-3'CAT and pMMTVCAT suggest a common mechanism involving a glucocorticoid-responsive element. In both cases, we have observed a similar increase in CAT expression with half saturation at 2–5 × 10 nM Dex, which is approximately 1 order of magnitude greater than the concentration required for half-maximal steroid hormone–receptor complex formation (37). However, it should be emphasized that hormone induction of any enzyme represents a separate phenomenon from steroid–protein binding; in previous studies of MMTV mRNA induction, half-maximal effect was also observed at 2–5 × 10 nM Dex (38).

Using constructs that contain the HBV enhancer but different fragments of HBV DNA upstream to this region, we demonstrated that the sequence responsible for glucocorticoid stimulation of enhancer activity is distinct from the previously defined HBV enhancer element. Such a separation of the glucocorticoid receptor binding site from other sequences involved in eukaryotic gene enhancement is consistent with results obtained for the human metallothionein gene, in which DNA sequences responding to the glucocorticoid–receptor complex are located upstream to the Cd^{2+}-responsive site in the metallothionein enhancer element (27). The fact that glucocorticoid-responsive element sequences confer hormone inducibility only in the presence of an enhancer has recently been reported by Overhauser and Fan (39). These investigators observed that the insertion of MMTV sequences into a Moloney murine leukemia virus LTR deleted of its enhancer did not produce biological activity; however, when that enhancer was not deleted, CAT plasmids containing MMTV sequences inserted into the Moloney murine leukemia virus LTR could be induced 2- to 5-fold by Dex (39).

Since the MMTV glucocorticoid-responsive element at map positions −185 to −155 has the ability to stimulate proximal and distal promoters (31) and can function in either 5' or 3' orientation (10), this hormone-responsive region has enhancer properties by itself. Furthermore, it belongs to the category of "conditioned" enhancers (40), which are dependent on exogenous factors, since glucocorticoids appear to activate this glucocorticoid-responsive element by mediating binding of receptor proteins to this specific DNA sequence (36, 41). As indicated above, there was no Dex stimulation of the previously defined HBV enhancer at map positions 1080–1234. However, at this time, we have not determined whether the hormone-responsive region of HBV DNA has enhancer activity independent of the previously defined HBV enhancer.

Results in the present and previous study (15) indicate that the HBV enhancer is functional in a broad spectrum of cell types. However, highest enhancer activity was noted in human liver-derived cell lines (a 34-fold stimulation above pA$_{10}$CAT$_2$ in PLC/PRF/5, 20-fold in Hep 3B and Hep G2, but only 2.3- and 6.5-fold in HeLa and L-cells, respectively). This suggests that HBV enhancer activity might be influenced by a specific host-cell factor(s), a phenomenon that has been reported previously for other enhancers (42–44).

An important remaining question is whether steroid hormones modulate HBV gene transcription by activating enhancer sequences directly or whether the effect of the steroid–receptor–glucocorticoid-responsive element complex is mediated via the known HBV enhancer. In the present study, we demonstrated that expression of HBV DNA might be regulated by glucocorticoids, and it is tempting to speculate that hormonal effects on HBV enhancer activity may in part be associated with changes in HBV replication or expression in patients undergoing glucocorticoid treatment.

Note Added in Proof. Since this study was communicated, Tognoni *et al.* (45) also reported the presence of an enhancer in the HBV

genome located essentially in the same position as that previously reported by Shaul *et al.* (15).

The authors thank Drs. Charles E. Rogler and Luz Teicher for advice and assistance in various aspects of these studies and Drs. M. Kessel and L. Laimins for plasmids pMMTV-CAT and pPrLTR$_2$-CAT. R.T.-K. is the recipient of a John E. Fogarty International Fellowship from the National Institutes of Health and is a visiting Fulbright Scholar on leave from the Department of Medicine A, Hadassah University Hospital, Jerusalem, Israel. This research was supported in part by National Institutes of Health Grants CA-32605, KO8-CA-00983 (R.D.B.), and AM-17702 and the Gail I. Zuckerman Foundation.

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