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Alpha Interferon Suppresses Hepatitis B Virus Enhancer Activity and Reduces Viral Gene Transcription

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Interferons inhibit replication of hepatitis B virus (HBV). The mechanism for this inhibition was investigated by analyzing the effect of interferons on transcription of a chloramphenicol acetyltransferase reporter gene under control of HBV regulatory sequences and by determining the steady-state level of viral mRNAs in permanently HBV-transfected HepG2 cells. Low doses (100 U/ml) of alpha interferon (IFN-α) but not IFN-γ inhibited chloramphenicol acetyltransferase expression in cultured cells transfected with plasmids containing the HBV enhancer linked to either HBV or simian virus 40 promoters. IFN-α also lowered expression of HBV mRNA in HBV-transfected HepG2 cells actively replicating virus, suggesting that IFN-α inhibits HBV replication by reducing transcription of viral genes driven by the HBV enhancer.

Interferons (IFNs) are natural antiviral substances which have been studied for more than 10 years as potential therapeutic agents for persistent hepatitis B virus (HBV) infection (12). Clinical trials have shown that IFN-α causes suppression of virus replication, manifested by a reduction in HBeAg, viral DNA polymerase activity, and serum HBV DNA (6, 13, 14). Seroconversion from HBeAg to anti-HBeAg occurs in ~50% of IFN-treated patients, but disappearance of HBsAg occurs in a much lower percentage (6, 7, 13, 14). However, the mechanism whereby interferon suppresses virus replication has not been fully elucidated, although in RNA viruses, induction of 2′-5′-oligoadenylate synthetase and P1/eIF-2 kinase is thought to be involved (1, 21). IFNs can also restrict the growth of DNA viruses (e.g., simian virus 40 [SV40], adenovirus, vaccinia virus, etc.), but the mechanisms involved remain even less well delineated. In the intact host, immunoregulatory mechanisms influenced by IFNs (e.g., stimulation of natural killer cell activity) may also play a role in eliminating cells persistently infected or actively replicating virus (5).

Since IFNs exert profound regulatory effects on cellular gene expression (8, 9, 22, 23) and have also been shown to play a direct role in modulating viral gene expression (2, 18, 19, 30), we explored the possibility that such modulation is relevant to the mechanism(s) whereby interferon regulates HBV replication. To determine whether IFNs exert any effect on the regulatory sequences of the HBV genome, we examined changes in expression of a reporter gene, chloramphenicol acetyltransferase (CAT), under control of the HBV enhancer linked to various promoters, as well as changes in the level of viral mRNA in a human liver-derived cell line persistently infected with HBV, HepG2 2.2.15 (31). Constructs were prepared by introducing fragments of HBV DNA into plasmid $pA_{10}CAT_2$, which contains the SV40 early promoter 5′ to the CAT gene but also has a deletion of most of the enhancer sequence, so that very little CAT expression is produced in the absence of an added enhancer (20). The sequence in $pA_{10}CAT_2$ between its unique *Sph*I and *Pvu*II restriction sites was excised and replaced with various HBV DNA restriction fragments (Fig. 1). Three plasmids, pHB 0.15 CAT, pHB 0.5 CAT, and pHB 1.4 CAT, contain the HBV enhancer, map position (m.p.) 1080 to 1234, and in the latter two cases, additional HBV upstream sequences linked 5′ to the SV40 promoter (32, 34). pHB E^+C^+ contains HBV sequences from m.p. 963 to 1634, including the HBV enhancer plus the core gene promoter, linked to pSV_0CAT, which has neither an enhancer nor a promoter. pHB E^+S^+ contains the HBV enhancer region (m.p. 963 to 1402) plus a fragment containing the pre-S_1 and -S_2 promoters (m.p. 2408 to 30) linked once again to pSV_0CAT. Control plasmids included pSV_2CAT, which contains both the SV40 early promoter and a functional enhancer, and pGEM-CAT-E, which contains a 0.83-kilobase 5′ fragment of the human 2′-5′-oligoadenylate synthetase E gene, including regulatory sequences inducible by IFN-α, -β or -γ (4) (kindly provided by M. Revel).

The effects of IFN-α and -γ on PLC/PRF/5 or Hep3B cells transfected with pHB 0.15 CAT, pHB 0.5 CAT, or pHB 1.4 CAT are shown in Table 1. Cultured PLC/PRF/5 cells transfected with pHB 0.15 CAT and treated with 100 U of IFN-α per ml showed a four- to fivefold reduction in CAT expression compared with that of untreated cells. IFN-γ at similar doses had no effect on CAT expression. Hep3B cells transfected with pHB 0.5 CAT also showed a fivefold reduction of CAT expression after the culture was treated with 100 U of IFN-α per ml (Table 1). No effect of IFN-α or -γ on CAT expression was observed with control plasmids containing either the SV40 promoter-enhancer region (pSV_2CAT) or the SV40 promoter alone ($pA_{10}CAT_2$) (Table 1) or with a Rous sarcoma virus long terminal repeat promoter-enhancer plasmid (RSVCAT) (data not shown). A similar reduction in CAT expression by IFN-α was also demonstrated with pHB 1.4 CAT, which contains the HBV enhancer region plus additional upstream sequences, including a glucocorticoid-responsive element (34).

Experiments were then performed to determine whether the IFN effect also occurred with plasmids containing natural HBV promoters, such as pHB E^+C^+, a plasmid contain-

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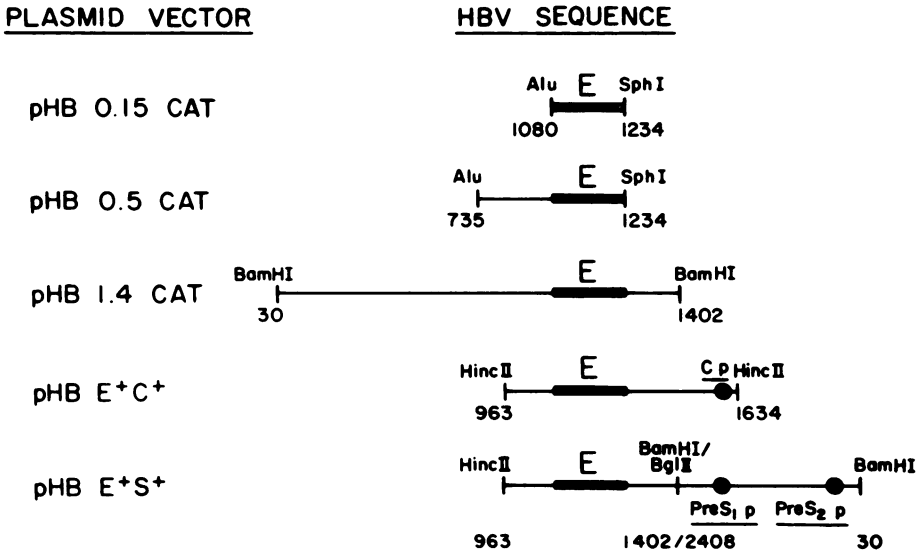

FIG. 1. Schematic diagram of HBV enhancer-CAT plasmids used in the present study.

ing the HBV enhancer and HBV core gene promoter region (m.p. 963 to 1634), or pHB E^+S^+, a plasmid containing the HBV enhancer region linked to a region of the HBV genome containing the pre-S_1 and -S_2 promoters (m.p. 2408 to 30). IFN-α treatment of PLC/PRF/5 cells transfected with either of these constructs reduced CAT activity four- to fivefold (Fig. 2, lanes C and D for results with pHB E^+C^+ and lanes E and F for results with pHB E^+S^+), as compared with no IFN-α-mediated reduction of CAT expression with plasmid pSV_2CAT containing the SV40 enhancer (lanes A and B). As a positive control to show that our preparation of IFN-α could induce gene expression in plasmids under the control of regulatory sequences from an interferon-responsive gene, we used a plasmid containing 5′ regulatory sequences of the 2′-5′-oligoadenylate synthetase E gene linked to CAT (pGEM-CAT-E) (4). Cells transfected with pGEM-CAT-E showed increased expression of CAT after being treated with IFN-α, as indicated by almost total acetylation of the chloramphenicol substrate (Fig. 2, lanes G and H). This demonstrated that the IFN-α-mediated inhibition of CAT expression under the control of the HBV regulatory sequences was not due to toxicity or generalized suppression of cellular gene expression. Similar results were also obtained in HeLa cells (data not shown), so that the interferon effect occurs through a general cellular mechanism that is not specific for hepatocyte-derived cells. Further controls, using Hirt extraction (16) of separate portions of the same cells, showed no differences in the amounts of CAT plasmid DNA present in cells treated or not treated with IFN-α. The measurement of 2′-5′-oligoadenylate synthetase enzyme activity in cell extracts used for CAT expression (kindly performed by L. Shulman, Weizmann Institute of Science, Rehovot, Israel) also showed IFN induction of this enzyme independent of whether CAT activity was increased (pGEM-CAT-E), decreased (pHB E^+C^+), or unaffected (pSV_2CAT). Therefore, IFN-α-mediated suppression of CAT expression in plasmid-transfected cells represents a selective inhibitory effect on HBV gene regulatory sequences, specifically on the HBV enhancer.

Since the HepG2 cell line is known to utilize the HBV enhancer to drive expression of CAT genes under the control of the SV40 early promoter (32, 34), an HBV-transfected derivative of HepG2 (2.2.15) was used to test the influence of IFN-α on endogenous HBV gene expression in persistently infected cells. The addition of 100 U of IFN-α per ml to the culture medium of HepG2 2.2.15 cells reduced the level of

TABLE 1. Effect of IFNs on CAT expression by various plasmids

Plasmid	Cell line	Type of IFN	CAT activity[a]		
			% (−IFN)	Acetylation (+IFN)	Reduction (fold) by IFN
pHB 0.15 CAT_2	PLC/PRF/5	α	10.2 ± 1.5	2.1 ± 1.3	5[b]
pHB 0.15 CAT_2	PLC/PRF/5	γ	9.4 ± 2.1	8.6 ± 0.9	N.C.
pHB 0.5 CAT_2	Hep3B	α	7.2 ± 2.5	1.5 ± 0.7	4.8[b]
pHB 1.4 CAT	PLC/PRF/5	α	8.5 ± 2.2	2.2 ± 1.4	3.9[b]
pSV_2CAT_2	Hep3B	α	18.1 ± 2.0	16.0 ± 1.7	N.C.
$pA_{10}CAT_2$	Hep3B	α	0.2 ± 0.13	0.15 ± 0.07	N.C.

[a] Cells were transfected by the calcium phosphate precipitation method with 5 to 25 μg of plasmid DNA per 100-mm-diameter culture dish. After the linear range for transfecting DNA was established (the mid-range was 10 to 15 μg of DNA per dish), comparisons between the various vectors were made (34). At 6 h after the addition of DNA, the medium was removed and cells were fed with fresh medium in the presence or absence of recombinant IFN-α or -γ (100 U/ml), kindly provided by Hoffmann-La Roche Inc. and Genentech Corp., respectively. At 48 h after transfection, soluble extracts were prepared by sonication and centrifugation of particulate matter at 8,000 × *g* for 10 min and assayed for CAT activity essentially as described by Gorman et al. (11). Results in the absence (−) or presence (+) of IFN represent mean values and standard deviations from at least three independent sets of experiments. N.C., No change in CAT activity.

[b] $P < 0.02$.

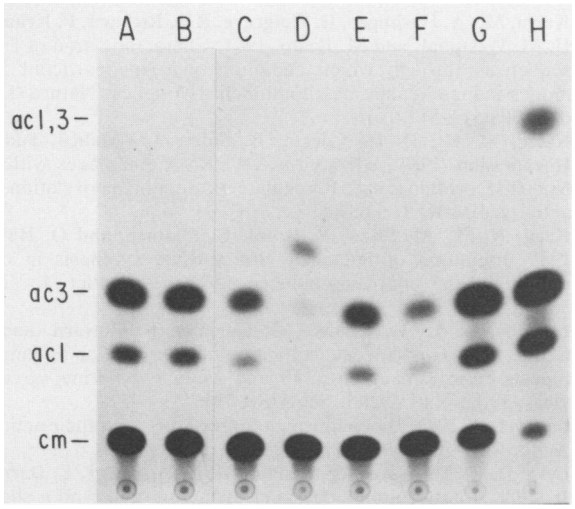

FIG. 2. Effect of IFN-α on CAT activity in PLC/PRF/5 cells transfected with various plasmids. Details concerning transfection protocols, the addition of interferon, and the method used for CAT detection are given in Table 1. Plasmids used for various lanes are as follows: lanes A and B, pSV_2CAT (A, untreated; B, treated with IFN-α); lanes C and D, $pHBE^+C^+CAT$ (C, untreated; D, treated with IFN-α); lanes E and F, $pHBE^+S^+CAT$ (E, untreated; F, treated with IFN-α); lanes G and H, pGEM CAT-E (G, untreated; H, treated with IFN-α). cm, Chloramphenicol.

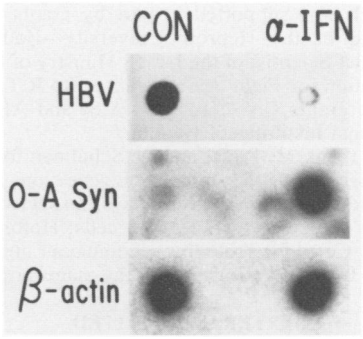

FIG. 3. Dot blots of HBV RNA sequences in extracts from HBV-transfected HepG2 cells (2.2.15) treated with IFN-α. Cells were grown in minimal essential medium supplemented with 10% fetal bovine serum and 200 μg of G418 per ml. Total RNA was extracted from the cells by the guanidinium thiocyanate-CsCl method, and poly(A)$^+$ RNA was prepared by oligo(dT) cellulose chromatography. Poly(A)$^+$ RNA samples were blotted onto replicate nitrocellulose filters by standard procedures and hybridized with an HBV DNA or β-actin probe labeled by random primer extension. The signal was removed from the blot hybridized with HBV DNA by boiling for 10 min in 0.01× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate, and it was rehybridized with a human 2′-5′-oligoadenylate synthetase (O-A Syn) probe (E16), kindly provided by M. Revel. Hybridization was at 65°C for 16 h in a solution containing 5× SSC. Dots using poly(A)$^+$ RNAs from control untreated cells and cells treated with 100 U of IFN-α per ml for 72 h are shown on the left (CON) and right (IFN-α), respectively.

HBV mRNA in cellular poly(A)$^+$ RNA extracts by three- to fourfold, whereas the same extracts showed increased expression of 2′-5′-oligoadenylate synthetase RNA in IFN-α-treated cells but no change in expression of a common cellular gene, β-actin (Fig. 3). Pulse-labeling studies with [^{3}H]uridine also showed a twofold reduction in the labeling of HBV RNA transcripts in HepG2 2.2.15 cells treated with IFN-α (data not shown), suggesting further that the IFN-α-mediated reduction in HBV mRNA results from reduced HBV gene transcription.

Progression of chronic HBV infection to liver cirrhosis and hepatocellular carcinoma is often associated with active HBV replication (26). In clinical trials, IFN has been reported to engender a transient or permanent suppression of viral replication (6, 7, 13, 14). Because IFNs are known to induce negative regulatory effects on the expression of many eucaryotic genes (21), it seemed appropriate to determine whether some of these effects might be exhibited at the transcriptional level on HBV regulatory sequences such as promoters or enhancers, the latter of which can be under either positive (17, 29) or negative (10, 24) control. Although transcriptional inhibition has not been considered a major mechanism of IFN action, there are recent reports of IFN effects on primary transcription of other viral genes, such as influenza virus (19, 28) and vesicular stomatitis virus (2). Changes in mRNA stability or translation are also known to be regulated by IFNs (21). However, the differential suppressive effect of IFN-α on CAT expression driven by pHBV-CAT vectors versus pSV_2CAT or RSV-CAT in the present studies cannot be explained by posttranscriptional mechanisms, since the same mRNA is produced by all these vectors.

IFNS have also been shown to inhibit replication of several DNA viruses, including SV40, herpes simplex virus, and VA RNA mutants of adenovirus, in the former two cases by inhibiting transcription of early viral genes (3, 25, 27) and in the latter case by inhibiting phosphorylation of the α subunit of eIF2 (30) or regulating autophosphorylation of a 68,000-M_r protein kinase (18). Most recently, Hayashi and Koike have reported the inhibition of HBV replication by IFNs in permanently transfected HepGs cells (15); however, the specific mechanism for this inhibition was not elucidated. From the present data, it is reasonable to conclude that IFN-α inhibits transcription of viral RNA. This, in turn, blocks HBV replication because HBV utilizes reverse transcription and a viral RNA plus-strand template for the first step in replication, i.e., synthesis of viral DNA minusstrand (33). Transcriptional inhibition was found to be mediated through an effect of IFN-α on the HBV enhancer, based on our studies of HBV regulatory sequences in CAT plasmid expression vectors and viral RNA levels in HBV-transfected HepG2 cells. In the latter case, it is assumed that the HBV-transfected HepG2 cell line still requires the HBV enhancer for viral gene transcription.

The effect of IFN-α on the HBV enhancer might either be in *trans*, i.e., it induces production or modification of intracellular protein(s) which act as negative transactivators or transrepressors, or in *cis*, i.e., IFN-α itself might be internalized and bind to regulatory sequences in the HBV genome, thereby blocking constitutive transcriptional enhancer activity. Lacking evidence in any system for direct binding of IFNs to DNA sequences, it is our assumption that the effect of IFN-α is indirect, probably occurring via IFN-induced or -modified cellular proteins which bind to the HBV enhancer and partially inhibit its activity. In future studies, we hope to identify and characterize the cellular proteins which mediate IFN-directed transcriptional down regulation of the HBV enhancer. Whatever the precise mechanism, the present study suggests that inhibition of the HBV transcriptional enhancer may contribute to the therapeutic effect of IFN-α in reducing viral gene expression or replication in patients with chronic HBV infection.

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