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Immunosuppressive Function of Hepatitis B Antigens In Vitro: Role of Endoribonuclease V as One Potential *trans* Inactivator for Cytokines in Macrophages and Human Hepatoma Cells

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The mRNAs of transiently expressed cytokine genes contain AUUUA-rich sequences in the 3′ untranslated regions. In order to examine whether the AU-specific endoribonuclease V (EC 3.1.27.8) described previously by us transinactivates those mRNA species, we introduced a 51-nucleotide ATTTA sequence from tumor necrosis factor into the 3′ untranslated region of β-globin gene. Transcripts of that construct, synthesized in vitro, were prone to endoribonuclease V digestion at those AU-rich sequences. Stimulation of human macrophages with lipopolysaccharide resulted in a shift of the association state of the enzyme from the nuclear matrix-associated to the free form. This shift was strongly prevented by the hepatitis B surface antigen (HBsAg) and more weakly by hepatitis B nucleocapsid antigen and hepatitis B antigen of the X region. HBsAg and, to a lesser extent, hepatitis B nucleocapsid antigen and hepatitis B antigen of the X region inhibited the release of alpha interferon, tumor necrosis factor alpha, and granulocyte-macrophage colony stimulating factor, while it had no effect on interleukin-1 production from stimulated macrophages. Using the human hepatoma cell line PLC/PRF/5, we provide further experimental evidence that endoribonuclease V acts in *trans* as a posttranscriptional inactivator for nuclear matrix-associated cytokine transcripts. These results suggest that those cytokine transcripts which contain reiterated (overlapping) AUUUA sequences are degraded by nuclear matrix-associated endoribonuclease V. This degradation was comparably high in cells incubated with HBsAg or cells which produced this antigen.

It is generally agreed that the pathogenesis of hepatitis B virus (HBV)-induced hepatocellular injury is caused by two factors, the viral replication and the immune response of the patient (12). The early replicative phase of HBV infection is characterized by the presence of replicative forms of HBV DNA in liver and the production of hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) (which is derived by proteolytic cleavage from precore protein and the nucleocapsid antigen [HBcAg]), and whole virions. The later nonreplicative phase is marked by the absence of replicative forms of HBV DNA and HBeAg and the presence of integrated forms of viral DNA in the liver and the production of HBsAg (12). The deficiency in cytokine production in chronic HBV infection presented the rationale to treat this disease with alpha interferon (IFN-α) (17, 47). On the basis of the positive results obtained with an IFN therapy (16), the assumption was drawn that factors must be present in HBsAg carriers which down-regulate cytokine production.

It is the aim of the present study to determine the influence of those HBV antigens which are present in chronic hepatitis B on the function of macrophages in in vitro assays to release cytokines. We report that HBsAg, HBcAg, and hepatitis B antigen of the X region (HBxAg) differentially modulate the production of the following macrophage cytokines after incubation of the cells with the suitable inducers: IFN-α (35), tumor necrosis factor alpha (TNF-α) (4), interleukin-1α (IL-1α) and interleukin-1β (IL-1β) (25), and granulocyte-macrophage colony stimulating factor (GM-CSF) (25). The experiments were performed with human macrophages. The results revealed that in vitro incubation of macrophages with HBsAg suppressed the function of the cells to produce the cytokines TNF-α, IFN-α, and GM-CSF, but not the ability to release IL-1. Moreover, experimental evidence is presented which shows that overlapping AUUUA boxes present in the 3′ untranslated region of the cytokine mRNAs contribute crucially to the stability of these mRNAs. To support this conclusion, additional studies were performed with human hepatoma cells (PLC/PRF/5, the Alexander cells) which have been shown to produce the HBsAg (20). These cells were shown to contain high levels of endoribonuclease V (EC 3.1.27.8) (36) activity, which might be the molecular cause for the rapid decay of cytokine mRNAs.

MATERIALS AND METHODS

Materials. The DNA polymerase from *Thermus aquaticus* (*Taq*) was from Cetus, Norwalk, Conn.; lipopolysaccharide (LPS) (L-4130) and concanavalin A (ConA) (C-5275) were from Sigma Chemical Co., St. Louis, Mo.; [^{3}H]poly(A), [^{3}H]poly(U), [^{3}H]poly(C), [^{3}H]poly(G), and [^{14}C]poly(A) · poly(U) were from Miles Laboratories, Inc., Elkhart, Ind.; [^{14}C]poly(dA) was from P-L Biochemicals, Inc., Milwaukee, Wis.; [^{3}H]poly(A · C) and poly(A) · [^{14}C]poly(I) were from Schwarz/Mann, Orangeburg, N.Y.; [α-^{32}P]UTP (3,000 Ci/mmol) was from the Radiochemical Centre, Amersham, England; the T7 RNA polymerase system was from Promega Biotec, Madison, Wis.; the test kit for the determination of the amount of GM-CSF (22) (Insight GM) was from Medical Resources, Surry Hills, New South Wales, Australia.

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PLC/PRF/5 cells. The human hepatoma cell lines (PLC/PRF/5, Alexander cells), which permanently secrete HBsAg (1), were grown in monolayer culture by using Eagle minimum essential medium supplemented with glutamine, nonessential amino acids, 10% heat-inactivated fetal calf serum, penicillin, streptomycin, and amphotericin B (Fungizone). Where indicated, the purified HBV antigen HBsAg (0.4 μg/ml) and ConA or both were added to the assays. The incubation was terminated after 24 or 48 h.

Macrophages. Human macrophages were isolated (43) and seeded at a concentration of 2×10^6 cells per ml in Eagle medium containing 10% fetal calf serum.

Cell culture assays. The cells were adjusted to a concentration of 2×10^6 cells per ml in Eagle medium containing 10% fetal calf serum. The release of the cytokines was stimulated by incubation of the cells (macrophages and PLC/PRF/5 cells) with the following agents: LPS at a concentration of 10 μg/ml to induce TNF-α (32), ConA at 6 μg/ml for IL-1α and IL-1β induction (32), vesicular stomatitis virus (VSV) at 5×10^5 PFU/ml for IFN-α induction (32), TNF-α at 100 ng/ml for GM-CSF induction (22), and ConA at 6 μg/ml for IFN-γ induction (32). Where indicated, purified HBV antigen, HBsAg, HBcAg, or HBxAg, was added at a concentration of 0.4 μg/ml to the assays. The incubation was terminated after 24 or 72 h. Subsequently, 200 μl portions of cell-free samples were taken for the determination of cytokine concentration.

Where indicated, macrophages were incubated for 24 h with the respective cytokine inducers and then exposed to dactinomycin (10 μg/ml) for the time points indicated. Subsequently, the cells were harvested and RNA was extracted.

Assays for cytokine levels. Levels of IFN-α, TNF-α, IL-1α, and IL-1β were determined by an enzyme immunoassay as described previously (11, 32), by using the respective monoclonal antibodies. GM-CSF was quantified as reported previously (22).

The IFN-α levels are given in units per milliliter by using the IFN-α National Institutes of Health (Bethesda, Md.) standard as reference; the TNF-α concentrations are given in nanograms per milliliter (1 ng/ml corresponds to 10 U/ml). The IL-1α and IL-1β levels are given in nanograms per milliliter, and GM-CSF levels are given in picograms per milliliter.

Recombinant DNAs. The following recombinant DNAs were used: human IFN-α cDNA, the 260-base-pair (bp) *Bgl*II-*Bgl*II fragment cloned into pUC19 (15); human TNF-α cDNA, the 620-bp *Xho*I-*Hin*dIII fragment cloned into pTZ19R (26); human IL-1α, the 460-bp *Eco*RI-*Eco*RV fragment and human IL-1β, the 530-bp *Bam*HI-*Nde*I fragment, both cloned into pMG-5 (3, 14); GM-CSF, the exon 2 fragment cloned into pUC19 (41); and IFN-γ, the 450-bp *Hin*dIII fragment cloned into pUC19 (13).

For the Northern (RNA) blot experiments, the β-globin 390-bp restriction fragment D (which covered most of the 3′ exon of the murine major β-globin gene) (7) and the human glyceraldehyde-3-phosphate cDNA (2) were used.

Hepatitis B antigens. The GP25-29 hepatitis B viral envelope antigen HBsAg, the P19 nucleocapsid antigen HBcAg, and the P16-17 HBxAg were purified by following procedures described previously (10, 29, 30, 45).

Amplification technique. DNA was isolated from PLC/PRF/5 cells by sodium dodecyl sulfate-phenol extraction followed by ethanol precipitation (21). Ten microliters of partially purified DNA (1 μg) was added to 90 μl of the standard *Taq* polymerase assay (24) containing the following primers: primer I (5′) GGGCCTCAGTCCGTTTCTCTT (positions 621 to 641 [44]) and primer II (3′) TGGCGACAATGG TTAAAA (positions 766 to 783). An amplified fragment of 162 bp was thus expected. The probe used was a 30-mer (TG GTTCGTAGGGCTTTCCCCCACTGTTTGG; positions 671 to 700). The primers and the probe were synthesized by using a DNA synthesizer (Beckman Instruments, Inc., Fullerton, Calif.). Twenty to 40 cycles of amplification were performed by using an automated temperature-cycling device (Cetus-Perkin Elmer) as described previously (34).

Analysis of amplified DNA. After the last cycle, the sample was heated (10 min; 70°C), precipitated with ethanol, and then analyzed by electrophoresis with 2% electrophoresis grade agarose (Bethesda Research Laboratories, Inc., Gaithersburg, Md.)–40 mM Tris–20 mM NaCl–20 mM sodium acetate–2 mM disodium EDTA (pH 8.0, with acetic acid). Electrophoresis was performed for 1 to 2 h at 5 V/cm. The gels were stained with ethidium bromide or Southern transfer onto nitrocellulose membrane (BA85; Schleicher & Schuell, Inc., Keene, N.H.) was performed as described previously (31). A 30-base oligonucleotide probe specific for the amplified HBV fragment was 5′ end labeled with ^{32}P and hybridized to the filter (33). The autoradiogram was exposed for 3 h with a single intensifying screen.

Endoribonuclease V enzyme and assay. The endoribonuclease V was purified from calf thymus as described previously (36). The specific activity of the purified enzyme was 3,750 U/μg. One unit of enzyme was defined as the amount which forms 1 nmol of soluble oligo(A) in 30 min under standard reaction conditions (36).

The standard reaction mixture (36) contained 50 mM Tris-maleic acid buffer (pH 7.8; 2 mM EDTA, 10 mM *N*-ethylmaleimide), 50 μg of bovine serum albumin, 100 nmol of [^{3}H]poly(A) (specific radioactivity, 10^3 cpm/nmol), and the enzyme sample in a final volume of 60 μl. After incubation at 37°C for 5 to 30 min, the assays were alkalinized with 20 μl of 200 mM Tris hydrochloride (pH 9.0), and 70 μl was placed on DEAE-cellulose DE-81 disks and processed as described previously (23, 36). Where indicated, the [^{3}H]poly(A) substrate was replaced by other synthetic polynucleotides; the specific activities varied between 5×10^2 and 5×10^3 cpm/nmol of phosphate.

Preparation of nuclei, nuclear envelopes, and nuclear matrix. Macrophages were harvested, and nuclei were isolated immediately from the cell pellets (5). The nuclear matrix was prepared from the isolated nuclei as described previously (8). The nuclear envelopes were obtained from the cells by the method of Kaufmann et al. (18).

Plasmid construction and globin RNA synthesis. The plasmids pTZ18UΔG^{AT} and pTZ18UΔG^{GT} were made by insertion of synthetic DNA into a mouse β-globin gene fragment. The mouse β-globin gene fragment (7) (nucleotide 3104 [*Sac*I restriction site] to 6357 [*Ava*III site]) was inserted into the *Sac*I and *Hin*cII sites within the polylinker of the standard plasmid pTZ18U. Two pairs of complementary 51-mers were synthesized with a Beckman DNA synthesizer. Upon annealing, these form the AT sequence of human IFN-α (positions 1053 to 1104; [15]) and the control sequence interspersed with Gs and flanked by 5′-GATC protruding termini. These synthetic oligomers were ligated to pTZ18U, inserted with the mouse β-globin DNA, and cleaved with *Bgl*II.

The G^{AT} and G^{GT} transcripts of modified globin were synthesized in vitro after *Ava*III digestion of the plasmids by using the T7 RNA polymerase (28). Where indicated, the transcripts G^{AT} and G^{GT} were labeled with ^{32}P by using ^{32}P-labeled UTP in the transcription assay; the specific

TABLE 1. In vitro production of IL-1α and IL-1β induced by ConA and viral antigens in supernatants of macrophages[a]

Inducer	IL-1α (ng/ml) at:		IL-1β (ng/ml) at:	
	24 h	72 h	24 h	72 h
None	<0.3	<0.3	<0.3	<0.3
ConA	1.6 ± 0.1	5.0 ± 0.7	23.0 ± 1.1	22.8 ± 4.6
HBsAg	1.7 ± 0.4	2.3 ± 0.4	19.8 ± 2.4	20.2 ± 3.1
HBcAg	0.8 ± 0.7	1.9 ± 0.3	10.5 ± 0.8	14.6 ± 2.9
HBxAg	1.6 ± 0.2	2.3 ± 0.3	21.3 ± 5.2	25.5 ± 4.7
ConA + HBsAg	2.3 ± 0.5	5.0 ± 0.4	25.5 ± 1.4	25.3 ± 3.2
ConA + HBcAg	1.8 ± 0.4	4.7 ± 0.8	23.8 ± 3.8	24.2 ± 2.3
ConA + HBxAg	2.3 ± 0.6	5.6 ± 0.4	25.5 ± 1.6	24.3 ± 0.9

[a] Where indicated HBsAg, HBcAg, or HBxAg was added to the incubation mixture. Results are reported as means ± the standard deviation of five independent experiments.

radioactivities of the transcripts were 3 to 7 cpm per pmol of incorporated nucleotide.

Gel electrophoresis, blot analysis, hybridization, and autoradiography. RNAs (G^{AT} and G^{GT}) were fractionated by electrophoresis in 2 or 1.1% denaturing agarose gels containing 2.2 M formaldehyde and transferred onto nylon filters. Transferred RNA was hybridized with the ^{32}P-labeled probes. After being washed, the filters were exposed to XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.). All procedures were performed by the methods of Perbal (28).

RNA extraction and Northern blot analysis for the determination of the amount of transcripts present in macrophages were performed by following standard procedures (28). The amount of mRNA was determined by scanning densitometry; the values estimated for the cytokine mRNAs were compared with the values measured for the stable glyceraldehyde-3-phosphate dehydrogenase transcripts.

RNA slot blot experiments were performed by following standard procedures (28).

Miscellaneous. Protein was measured as described previously (19), by using bovine serum albumin as the standard.

RESULTS

Cytokine production by macrophages in response to HBV antigens. Macrophages were incubated in vitro and stimulated with the respective inducers for the production of IL-1α and β, TNF-α, IFN-α, and GM-CSF. The results show that macrophages produced IL-1α and IL-1β not only in response to the ConA stimulus but also to HBV antigens HBsAg, HBcAg, or HBxAg (Table 1). A coincubation of the cells with ConA and the antigens resulted in an even higher production of IL-1.

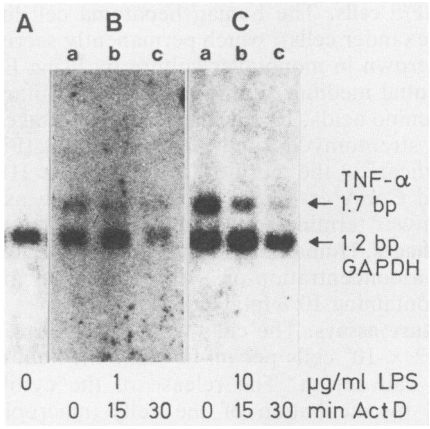

FIG. 1. Degradation of TNF-α mRNA in macrophages. Cells were incubated with 0 (A), 1 (B), or 10 (C) μg of LPS per ml. Then RNA was extracted after an additional treatment with dactinomycin (Act D) for 0 (lane a), 15 (lane b), or 30 (lane c) min, and Northern blot analysis was performed. The blots were assayed with both ^{32}P-labeled IFN-α and ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the probe.

In contrast to the production of IL-1, the release of TNF-α, IFN-α, or GM-CSF by macrophages was not induced by the HBsAg alone (Table 2). HBcAg and HBxAg displayed low induction potencies for the production of TNF-α and GM-CSF, while they failed to induce IFN-α release. A coincubation of HBsAg with the respective inducer resulted in a strong suppression of cytokine production compared with the experiments with the inducer alone.

These data show that HBsAg and, to a lesser extent, HBcAg and HBxAg are potent inhibitors for the in vitro production of TNF-α, IFN-α, or GM-CSF, while they had only a slight effect on IL-1 release.

Stability of mRNA for cytokines. The rate of mRNA degradation in macrophages for the respective cytokines was determined 24 h after stimulation with the cytokine inducers. After incubation, the cells were treated with dactinomycin for 0 to 60 min. In untreated cells, only the reference transcript, glyceraldehyde-3-phosphate dehydrogenase, was detectable (Fig. 1A). In cells incubated with the IFN-α inducer LPS, the IFN-α transcript was detectable at the beginning of dactinomycin treatment (set at 100%) (Fig. 1B and C). After a prolonged incubation period in the presence of the transcription inhibitor, the amount of IFN-α transcripts

TABLE 2. In vitro production of TNF-α, IFN-α, or GM-CSF by macrophages after induction with LPS, VSV, or TNF-α[a]

Inducer[b]	TNF-α (ng/ml)		IFN-α (U/ml)		GM-CSF (pg/ml)	
	24 h	72 h	24 h	72 h	24 h	72 h
None	<1	<1	<1	<1	<1	<1
X	8.3 ± 1.5	4.1 ± 0.7	134.6 ± 19.0	215.9 ± 4.5	70.2 ± 4.3	57.3 ± 3.0
HBsAg	<1	<1	<1	<1	<1	<1
HBcAg	2.3 ± 0.2	<1	<1	<1	68.4 ± 3.3	59.0 ± 1.1
HBxAg	2.1 ± 0.2	<1	<1	<1	48.3 ± 1.9	31.1 ± 0.7
X + HBsAg	4.4 ± 0.6	<1	<1	<1	9.0 ± 2.7	<1
X + HBcAg	7.2 ± 0.9	3.1 ± 0.4	<1	<1	69.3 ± 1.1	57.4 ± 0.9
X + HBxAg	8.1 ± 1.5	4.0 ± 0.8	16.6 ± 6.4	65.2 ± 2.1	53.5 ± 0.9	41.3 ± 0.7

[a] TNF-α was induced with LPS; IFN-α was induced with VSV; and GM-CSF was induced with TNF-α. Results are reported as means ± the standard deviation of five independent experiments.

[b] X, LPS for TNF-α induction, VSV for IFN-α induction, and TNF-α for GM-CSF induction.

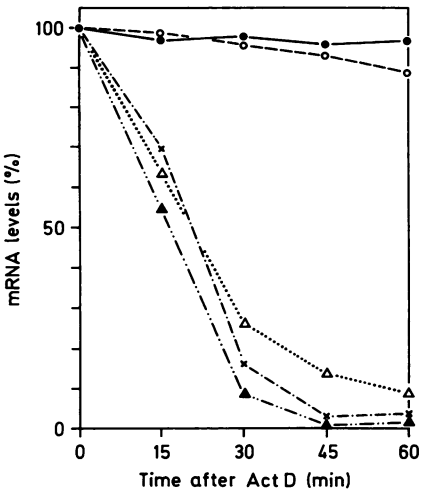

FIG. 2. The rates of mRNA degradation for IL-1α (●), IL-1β (○), TNF-α (x), IFN-α (▲), and GM-CSF (△) in macrophages which had been stimulated for 24 h with LPS (for TNF-α production), VSV (IFN-α), TNF-α (GM-CSF) or ConA (IL-1). After an additional incubation period with dactinomycin (Act D), the RNA was extracted and Northern blot analysis was performed. The amount of mRNA at the various times is expressed as a fraction of the respective mRNA level at time zero, and the data are given in percentages.

decreased and reached a value of 15% after a 30-min incubation period in cells treated with 1 or 10 μg of LPS per ml, whereas the amount of the reference gene remained stable.

Figure 2 summarizes the values determined for mRNA degradation in macrophages after treatment with dactinomycin. It is striking that the levels of IL-1α and β remained almost constant, while the mRNA levels of the other cytokines dropped immediately after dactinomycin addition. The half-lives of IFN-α, TNF-α, and GM-CSF mRNAs were determined to be approximately 15 to 20 min.

These results indicate that the half-lives of mRNAs of GM-CSF, IFN-α, and TNF-α strongly differ from that of IL-1 mRNA. To approach the question of which structure within the mRNA determines the stability of the mRNA, we investigated if the AUUUA-rich boxes present in the 3′ untranslated region of the mRNAs of these cytokines (41) contribute to the rapid degradation. More precisely, we asked whether the A-U-specific endoribonuclease V, which we had described previously (36), degrades AUUUA-rich mRNAs specifically.

Decrease of nuclear matrix-associated transcripts for IFN-α and TNF-α after incubation with HBV antigens. Considering the fact that almost all transcripts are associated with the nuclear matrix (38), the amount of nuclear matrix-associated transcripts for IFN-α and for TNF-α were determined after coincubation of the macrophages with VSV (for IFN-α induction) or with LPS (for TNF-α induction) together with the HBV antigens. The levels of IFN-α transcripts after an incubation period of 12 h decreased in the presence of HBxAg by approximately 60%, in the presence of HBcAg by 80%, and in the presence of HBsAg by 100%; after the prolonged coincubation period to 24 h, no IFN-α transcripts could be detected in the assays supplemented with HBV antigens (Fig. 3A). In contrast to this finding, the decrease of the amount of nuclear matrix-associated TNF-α transcripts was less pronounced (Fig. 3B); even after the 24-h incubation period, the level of TNF-α was reduced in the presence

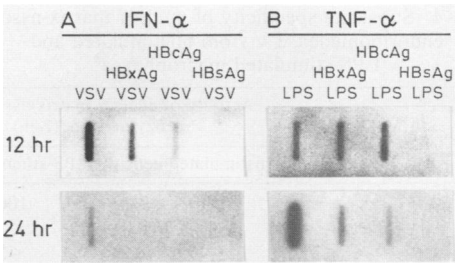

FIG. 3. Slot blot hybridization of RNA from nuclear matrices of macrophages incubated in the presence of VSV (for IFN-α induction) (A) or LPS (for TNF-α induction) (B) in the absence or presence of HBV antigens as indicated; VSV or LPS was added either alone or in combination with HBxAg, HBcAg, or HBsAg. The incubation period was 12 or 24 h. RNA from 50 μg of the respective matrix preparation was analyzed for each slot. Slot blot hybridization was performed with either ^{32}P-labeled IFN-α DNA or ^{32}P-labeled TNF-α DNA probe.

of HBxAg or HBcAg only by 70%, while no transcript could be detected in the nuclear matrix preparation from cells incubated with HBsAg.

Association of endoribonuclease V with the nuclear matrix. Approximately 70% of the total cellular endoribonuclease V activity was found to be present in nuclei from macrophages (data not shown). In unstimulated cells, 67% of the nuclear enzyme was associated with the nuclear matrix and only 2% was associated with the nuclear envelope (Table 3). In contrast, the nuclear matrix fraction from LPS-stimulated cells contained only 11% of the total nuclear enzyme activity (Table 3). These data strongly suggest that during LPS stimulation, most of endoribonuclease V dissociated from the nuclear matrix.

Highly interesting was the finding that the presence of HBsAg in the LPS-stimulated assay prevented the shift of the endoribonuclease V from the nuclear matrix-associated to the free form almost completely, while the two other antigens, HBcAg and HBxAg, had a smaller effect (Table 3). In the absence of the antigens, 13.9 endoribonuclease V units were found to be associated with 1 mg of nuclear

TABLE 3. Nuclear distribution of endoribonuclease V in unstimulated and stimulated macrophages and PLC/PRF/5 cells[a]

Preparation from:	Enzyme activity [U/mg (%)] in:		
	Nucleus	Nuclear matrix	Nuclear envelope
Unstimulated macrophages	122.7 (100)	82.5 (67)	2.3 (2)
LPS-stimulated macrophages			
− HBV antigens	131.3 (100)	13.9 (11)	1.9 (1)
+ HBsAg	129.0 (100)	76.1 (59)	2.1 (2)
+ HBcAg	121.8 (100)	29.5 (24)	1.3 (1)
+ HBxAg	134.2 (100)	24.1 (18)	4.3 (3)
PLC cells			
Unstimulated	159.4 (100)	138.6 (87)	4.9 (3)
ConA-stimulated	153.9 (100)	140.2 (91)	5.7 (4)

[a] Macrophages or PLC/PFC/5 cells were incubated in the absence or presence of 10 μg of LPS or ConA per ml for 24 h. Where indicated, HBsAg, HBcAg, or HBxAg was added to the LPS-stimulated cultures. Then the nuclear fractions were prepared, and the enzyme activity was determined as described in Materials and Methods. The total amount of nuclear enzyme activity was set at 100%. Activity of the enzyme is given in units per microgram of protein. Results are reported as the means of five parallel experiments; the standard deviation was less than 15%.

TABLE 4. Substrate specificity of nuclear matrix-associated endoribonuclease V from unstimulated and LPS-stimulated macrophages[a]

Incubation conditions	Activity of nuclear matrix-associated enzyme from (%):	
	Unstimulated cells	LPS-stimulated cells
Poly(A)	100	100
Poly(U)	47.4	51.1
Poly(C)	<5.0	<5.0
Poly(G)	<1.0	<1.0
Poly(A·C)	<1.0	<1.0
Poly(A·I)	<1.0	<1.0
Poly(A)·poly(U)	<1.0	<1.0
Poly(A) + 10 mM $MgCl_2$	33.4	38.0

[a] The indicated polynucleotide substrates were added to the standard assay at a concentration of 100 nmol. Enzyme from the nuclear matrix of unstimulated macrophages (4.0 U) or enzyme (3.5 U) from the nuclear matrix of LPS-stimulated (10 μg of LPS per ml for 24 h) cells was added to the standard assay. In one experiment, 2 mM EDTA in the standard assay was replaced by 10 mM $MgCl_2$. Calculations of relative degradation were based on the degradation rates in the standard assay with [^{3}H]poly(A) as the substrate. Values are reported as the means of five parallel experiments; the standard deviation was less than 12%.

matrix protein, whereas in the presence of HBsAg, HBcAg, or HBxAg, the nuclear matrix-associated enzyme activity was 5.5-, 2.1-, and 1.7-fold higher, respectively.

The substrate properties of the nuclease from unstimulated and LPS-stimulated nuclear matrices are given in Table 4. Setting the nuclease activity measured in the standard assay containing poly(A) as the substrate at 100%, approximately 50% of degradation was measured with poly(U) as the substrate. None of the other synthetic single- or double-stranded polynucleotides assayed were degraded. These data strongly suggest that the nuclear matrix-associated enzyme is endoribonuclease V, and not endoribonuclease I (pancreas; EC 3.1.4.22), or endoribonuclease IV (EC 3.1.26.6), as discussed previously (23, 36, 42). This nuclease is also distinguished from nuclear matrix-associated poly(A)-specific exoribonuclease (EC 3.1.13.4) (38, 39) by its ability not to be inhibited by *N*-ethylmaleimide and to be inhibited by Mg^{2+} ions (Table 4).

AUUUA specificity of endoribonuclease V. The AT-rich sequence of human IFN-α was synthesized by using complementary oligonucleotides (51-mers) with 5′ GATC overhanging ends in order to ligate it into the generated *Bgl*II cleavage site (Fig. 4). As a control, a second sequence of the same length but with 16 Gs interspersed among the sequence was synthesized. In two separate constructs, the synthetic duplex DNAs were inserted at the *Bgl*II site into the mouse β-globin fragment. The resulting β-globin constructs were termed pTZ18UΔG^{AT} and pTZ18UΔG^{GT} (Fig. 4). By using these expression systems, G^{AT} and G^{GT} RNAs were synthesized in vitro using the T7 polymerase system.

The G^{AT} and G^{GT} RNAs synthesized in vitro were used as substrates in the endoribonuclease V assay. Figure 5 shows that endoribonuclease V degraded the six reiterated AUUUA sequences containing G^{AT} RNA (3,253 bases [lane a]) to fragments of the theoretical lengths of 2,352 and 901 bases (lane b) during an incubation period of 5 min. After an incubation for 20 min, no clear band could be visualized in the Northern blot (lane c). In contrast, the G^{GT} RNA was not degraded during the 5-min incubation period (lanes d and e).

Effect of HBV-antigens on cytokine production in PLC/PRF/5 cells. Two conclusions can be drawn from the experiments hitherto reported: (i) that HBsAg suppresses LPS-, VSV-, or TNF-α-mediated release of macrophage cytokines in vitro, an effect which might (ii) be due to the presence of reiterated AUUUA boxes in the 3′ untranslated region of these mRNA species. These sequences are highly prone to digestion with endoribonuclease V. Hence, we checked whether PLC/PRF/5 cells have the ability to release cytokines in response to a suitable stimulus.

To ensure that the human hepatoma cell line PLC/PRF/5 used contained the HBV viral genome integrated into high-molecular-weight host DNA, a 162-bp fragment of the S gene of the HBV genome was selected for enzymatic amplification analysis. Two opposite-strand complementary oligonucleotides were used as primers for the amplification reaction. After ethanol precipitation and agarose gel electrophoresis, the expected size of the fragment was identified both by

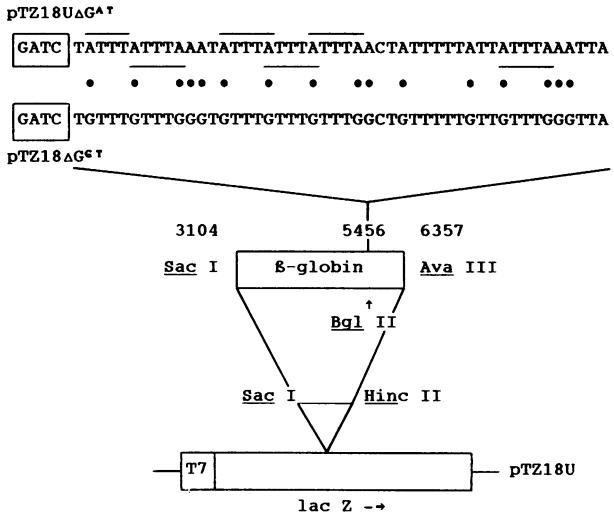

FIG. 4. Construction of plasmids pTZ18UΔG^{AT} and pTZ18UΔG^{GT}. Two complementary 51-mer oligonucleotides with 5′ cohesive ends (boxed) were synthesized. The AT-rich segment of IFN-α (containing the ATTTA motif reiterated six times [underlined]) was inserted into the *Bgl*II site of the plasmid pTZ18U. The double-stranded GT-rich oligonucleotide was synthesized as a control sequence. After *Ava*III digestion of the plasmid, the RNAs G^{AT} and G^{GT} were synthesized in vitro by using the T7 polymerase system. ·, Exchanges of bases.

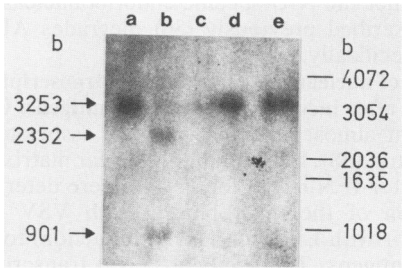

FIG. 5. Cleavage of G^{AT} and G^{GT} RNA in the standard assay system for endoribonuclease V. The reaction mixture was composed of the 50 mM Tris-maleic acid buffer lacking [^{3}H]poly(A), 5 ng of purified endoribonuclease V, and 50 ng of G^{AT} (lanes a to c) or G^{GT} (lanes d and e) RNA. After incubation for 0 (lanes a and d), 5 (lanes b and e) or 20 (lane c) min, the samples were electrophoresed on 2% agarose gels and assayed with ^{32}P-labeled β-globin probe. Molecular weight markers of DNA are indicated at the right. The arrows mark the theoretical values for the degradation products (2,352 and 901 bases) formed from the 3,253-base in vitro-synthesized RNAs.

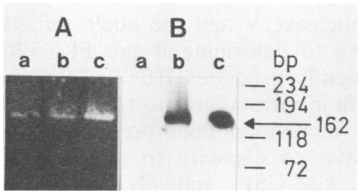

FIG. 6. Amplification of HBV DNA sequences after ethidium bromide staining (A) or hybridization with ^{32}P-labeled oligonucleotide probes (B). DNA (1 μg) from PLC/PRF/5 cells was amplified (20 cycles [lane a], 30 cycles [lane b], and 40 cycles [lane c]) in the standard amplification assay. The amplified products were examined after separation by 2% agarose gel electrophoresis. *Hae*III-digested φX174 replicative-form DNAs (New England BioLabs, Inc., Beverly, Mass.) were used as molecular size markers (base pairs). The arrow marks the size of the DNA product of the amplification reaction.

ethidium bromide staining (Fig. 6A) and by Southern blot analysis by using a probe specific for the internal part of the HBs gene (Fig. 6B).

By using the same incubation and stimulation conditions as those applied to human macrophages, no measurable cytokine (IFN-α, TNF-α, IL-1α, IL-1β, or GM-CSF) production was observed. Also, by applying slot blot hybridization techniques, only a small amount of transcripts of the mentioned cytokines was detected (data not shown).

Impaired IFN mRNA processing in PLC/PRF/5 cells. We postulated that the absence of transcripts for IFN-α, TNF-α, IL-1α, IL-1β, and GM-CSF could be due to the presence of high levels of nuclear matrix-associated endoribonuclease V. The experimental data confirmed this assumption (Table 3). Approximately 90% of the total endoribonuclease V activity present in the nucleus was found to be nuclear matrix-associated. Moreover, no shift of the association state of the enzyme after incubation of the cells in the presence of ConA (Table 3) or LPS and VSV (data not shown) could be detected. This fact is in contrast to the above findings with macrophages and with LPS as the stimulus.

Surprisingly, after incubation of PLC/PRF/5 cells with ConA (10 μg/ml for 48 h), transcripts of IFN-γ were synthesized (Fig. 7). However, no measurable release of IFN-γ (<2 U/ml) could be seen in the in vitro assays (unpublished results). Moreover, it was found that the level of IFN-γ transcripts paralleled the increase of ConA in the cell incubation assay (Fig. 7A). Northern blot experiments revealed that the sizes of the IFN-γ transcripts formed in PLC/PRF/5 cells were 4.6 and 2.4 kilobases (kb) (Fig. 7C, lane b). These species represent the 4.6- and 2.4-kb IFN-γ precursors (13); no mature 1.2-kb IFN-γ transcripts could be detected. In comparison, the only IFN-γ transcript species visualized in RNA extracts from ConA-treated peripheral blood mononuclear cells was the mature 1.2-kb mRNA (Fig. 7C; lanes a and c). These data strongly suggest that processing of IFN-γ transcripts to mature mRNA is impaired in PLC/PRF/5 cells, very likely due to a posttranscriptional degradation.

In the context of the above-mentioned data, which revealed that the amount of transcripts for the respective cytokines in macrophages decreased after incubation with HBV antigens in the presence of the respective cytokine inducers, we asked if the PLC/PRF/5 cells which had been coincubated with ConA and HBsAg also displayed a reduced level of IFN-γ. As summarized in Fig. 7B, the amount of IFN-γ transcript was drastically reduced in the presence of ConA and HBsAg under otherwise identical conditions.

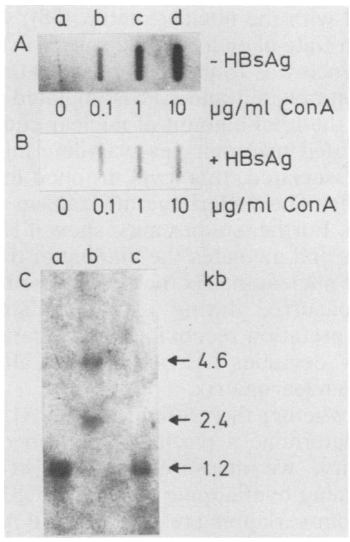

FIG. 7. Synthesis of IFN-γ transcripts in PLC/PRF/5 cells incubated for 48 h in the presence of ConA. (A) Slot blot analysis of IFN-γ mRNA levels in cells incubated in the presence of 0 to 10 μg of ConA per ml and the absence of HBsAg. (B) Cells incubated in the presence of ConA and HBsAg. (C) Northern blot analysis of IFN-γ transcripts isolated from PLC/PRF/5 cells incubated for 48 h in the presence of 10 μg of ConA per ml (lane b). As a comparison, the IFN-γ transcripts from ConA-treated peripheral blood mononuclear cells (48 h, 10 μg of ConA per ml) were analyzed. RNA was extracted from the cells and spotted onto GeneScreen (A and B) or separated according to size by electrophoresis on 1.1% agarose gels containing 2.2 M formaldehyde (C). Hybridization was performed with the ^{32}P-labeled IFN-γ probe. RNA (3 μg per slot [A and B] or 20 μg per lane [C]) was applied.

DISCUSSION

The 3′ noncoding regions of lymphokines, certain oncogenes, and inflammatory mediators are characterized by being rich in AU motifs (6). Evidence has been presented which suggests that AU sequences are a recognition signal for an mRNA degradation pathway (41). Since the mRNA half-life (e.g., for the GM-CSF mRNA) varied from shorter than 30 min (in macrophages) to 2.4 h (2.3 h in the tumor), it has been proposed that a cellular factor(s) must act in *trans* to affect mRNA stability (40).

One aim of this study was the identification of a putative factor regulating mRNA turnover on the posttranscriptional level. In a previous study, we identified and purified a AU-specific endoribonuclease V (36). Now we show that this enzyme hydrolyzes RNAs containing AUUUA motifs at that site. A sequence of 51 nucleotides of the 3′ noncoding region of TNF-α gene containing six partially overlapping ATTTA boxes was inserted into the β-globin gene. This construct was transcribed in vitro and assayed for its susceptibility to endoribonuclease V hydrolysis. The results revealed that, in the initial phase of hydrolysis, the modified β-globin RNA was cleaved in the AUUUA-rich region. Only after a longer period of incubation were other segments of the RNA degraded. These data experimentally confirm earlier theoretic assumptions (40, 46) that it is an endonuclease which acts in *trans* and cleaves AUUUA-rich mRNAs.

On the basis of our earlier findings that (i) heterogeneous nuclear RNA and mRNA molecules are associated with the nuclear matrix (37) and (ii) nucleases are only functionally relevant for heterogeneous nuclear RNA maturation if they

are associated with the nuclear matrix (38), we determined the association state of endoribonuclease V in the nucleus. A distinct difference was found in the association state of the enzyme in unstimulated and LPS-stimulated macrophages. While 67% of the total amount of nuclear endoribonuclease V in unstimulated macrophages was determined to be nuclear matrix-associated, this level dropped to 11% in LPS-stimulated cells. The underlying mechanism of this shift is not yet known. Further studies must show if HBsAg is taken up by the cells and mediates the binding of the endoribonuclease V to the nuclear matrix there. Because no induction of the enzyme occurred during LPS stimulation, the most plausible interpretation reconciling these data is that endoribonuclease V degrades AU-rich mRNAs after its association with the nuclear matrix.

To find out whether the presence of the AUUUA motif is sufficient to determine a particular pattern of cell-specific mRNA stability, we measured the half-lives of mRNA species containing overlapping (e.g., GM-CSF, IFN-α, and TNF-α) and nonoverlapping (e.g., IL-1 [41]) AUUUA boxes in stimulated human macrophages. It was somewhat surprising that the half-life of IL-1 mRNA (both for the α and β forms) was much greater than 60 min, as already described for IL-2 (20 h in human lymphocytes [9]), whereas the half-lives of GM-CSF, TNF-α, and IFN-α mRNA species were less than 30 min. These data strongly suggest that an overlapping AUUUA motif, e.g., AUUUAUUUA, is required for binding of endoribonuclease V to the noncoding region.

In the second part of this study, we determined in in vitro assays whether HBV antigens modulate the release of the cytokines IFN-α, TNF-α, GM-CSF, and IL-1 by macrophages. These cytokines are not exclusively released by macrophages, e.g., TNF-α is released by alloreactive T-cell clones (27). The data showed that HBsAg had a strong inhibitory effect on the production of IFN-α, TNF-α, and GM-CSF, but not on the formation of IL-1, whereas the effects of the other two antigens, HBcAg and HBxAg, were less pronounced. Two mechanisms can be proposed to account for the inhibition of the cytokine production in macrophages by HBsAg. First, there may be an induction of endoribonuclease V, or alternatively, there may be a higher fraction of the enzyme which is present in the nuclear matrix. Our results are most consistent with the notion that in the presence of HBsAg and, to a lesser extent, the two other HB antigens, the shift of endoribonuclease V from the nuclear matrix-associated to the free form is prevented. It must be stressed that HBcAg and HBxAg reduce the LPS-caused TNF-α release to a smaller extent than HBsAg, whereas all three HB antigens suppress VSV-mediated IFN-α production. This finding suggests that the IFN-α mRNA is more susceptible to endoribonuclease V degradation than the mRNAs of the other cytokines. This assumption is supported by sequence data, which revealed that only in the IFN-α mRNA do all AUUUA motifs overlap each other (41); moreover, we could experimentally show that the amount of nuclear matrix-associated IFN-α transcript decreased much faster in the presence of HBV antigens compared with the extent of the decrease of TNF-α transcripts. Hence, we conclude that only twofold-higher nuclear matrix-associated endoribonuclease V activity, caused by HBcAg and HBxAg, is sufficient to degrade IFN-α mRNA, very likely because of the existence of these overlapping AUUUA motifs, which are assumed to be the preferential substrate for this enzyme.

Our experiments with macrophages suggest that HBsAg treatment may function to stabilize the association between the endoribonuclease V and the nuclear matrix. Hence, it was interesting to determine if the PLC/PRF/5 cell line, which permanently produces HBsAg, also displayed a reduced ability in in vitro assays to release cytokines. Applying the same incubation conditions, we determined that these cells have no capacity to produce IFN-α, IFN-β, IL-1α, IL-1β, GM-CSF, and TNF-α in response to the appropriate stimuli. The assumption, which we drew from the new results with macrophages, that a high level of nuclear matrix-associated endoribonuclease V activity (which prevents formation of cytokine mRNAs) might be present in this human hepatoma cell line, was confirmed by the experiments. The only cytokine transcript species we could detect in PLC/PRF/5 cells was the one for IFN-γ. Interestingly, this cytokine was not released after ConA stimulation. Because IFN-γ transcripts also contain AUUUA motifs (although the three sequences are nonoverlapping) (41), we studied their stability. We found that only immature IFN-γ mRNA species are present in ConA-stimulated cells, while mature mRNA species are absent. This means that the processing pathway of INF-γ transcripts is disturbed in these hepatoma cells. One possible interpretation of this result is that mature mRNA for IFN-γ is degraded by endoribonuclease V immediately after formation. A first hint, which might support this assumption, came from the finding reported herein that after incubation of PLC/PRF/5 cells with HBsAg, the amount of the IFN-γ transcripts synthesized in the presence of ConA dropped drastically. However, to prove this assumption, experiments with hepatoma cell lines not expressing the HBsAg have to be performed. By using such a cell line, transfection experiments with an HBsAg expression vector must show whether these cells lose their ability to synthesize mature mRNA for IFN-γ after transfection.

In conclusion, these findings suggest that the reiterated AUUUA sequences in the 3′ untranslated terminus of the cytokine mRNAs are recognition sites for endoribonuclease V. If this enzyme associates with the cytokine RNA-nuclear matrix complex, the RNA is degraded.

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