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Export of Hepatitis B Virus RNA on a Rev-Like Pathway: Inhibition by the Regenerating Liver Inhibitory Factor IκBα

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Nuclear export of hepatitis B virus (HBV) RNA is mediated by a specific RNA element but, in contrast to lentivirus genomic RNA, does not depend on viral proteins. We show that nonetheless, the export of HBV RNA can be blocked by competitive inhibitors of Rev-mediated lentivirus RNA export, suggesting that the export pathways of both viral species share components and might be driven by the same nuclear export machinery. HBV RNA export is also inhibited by overexpression of IκBα, as reported previously for the export of human immunodeficiency virus RNA. Since IκBα is strongly overexpressed during liver regeneration, its inhibition of HBV RNA export might contribute to elimination or silent persistence of HBV.

The nucleocytoplasmic export of hepatitis B virus (HBV) RNA is mediated by an RNA element of ≅600 bases termed the posttranscriptional regulatory element (PRE) (24, 25, 27). The PRE apparently consists of two subelements which function synergistically (12). It enhances the nuclear export of incompletely spliced RNA (24, 25). Similarly, certain lentivirus RNA sequence elements are required for the export of genomic lentivirus RNA. However, lentiviruses such as human immunodeficiency virus (HIV) or human T-cell lymphotropic virus (HTLV) depend on viral gene products of the Rev family to allow efficient export of their RNA (15, 19, 29, 44). These Rev proteins bind to the corresponding viral RNA elements. The complex containing RNA and Rev protein can exit the nucleus by virtue of a short amino acid sequence in Rev termed the nuclear export signal (NES) that contains characteristic hydrophobic residues (14, 18, 34, 38, 49). NES-mediated nuclear export has been shown to compete with the export of 5S rRNA and spliceosomal U small nuclear RNAs (snRNAs) but not with cellular mRNA after injection into *Xenopus* oocytes (14). Several cellular factors that interact with NESs and that might be involved in nuclear export have been identified (5, 7, 17, 45, 46). In contrast to the lentivirus systems, HBV RNA export does not depend on any viral proteins, and its export mechanism is currently unknown.

Adenovirus type 5 modulates mRNA export late during infection: the export of viral mRNA is enhanced, whereas most cellular mRNA species are not allowed to exit the nucleus (2, 4, 39). The 55-kDa E1B protein and the 34-kDa E4 protein are required to influence mRNA export (2, 23, 39, 43). They associate with each other in the nuclei of infected cells (42). We have recently shown that the complex containing the 55-kDa E1B and 34-kDa E4 proteins shuttles between the nucleus and cytoplasm and may thus serve as an RNA transporter. The 34-kDa E4 protein contains a Rev-like NES. The 34-kDa E4 protein but not the 55-kDa E1B protein can effectively compete with the Rev protein of HIV for nuclear export (10).

The cellular protein IκBα (MAD3) is a cellular regulator that inhibits transcriptional activation by proteins of the NF-κB family (3, 32, 48). IκBα binds to the NF-κB complex consisting of the p65 RelA and p50 subunits, thus abolishing these subunits' ability to bind DNA and activate transcription. The trimeric complex of p65, p50, and IκBα localizes mainly to the cytoplasm. Various stimuli induce NF-κB transcriptional activity (22). This can be achieved by proteolytic degradation and/or phosphorylation (28) of IκBα and subsequent entry of the p65-p50 complex into the nucleus. Since NF-κB's transcriptional activity enhances the promoter activity of the HIV 5′ long terminal repeat (LTR), IκBα can be regarded as an antagonist to HIV transcription. However, IκBα has been reported to antagonize HIV gene expression in yet another way: it inhibits Rev-mediated nuclear export of incompletely spliced HIV RNA (51) by an unknown mechanism. Overexpression of IκBα can be observed during the course of liver regeneration: IκBα expression is rapidly and strongly upregulated in rat liver cells after partial hepatectomy, and IκBα has therefore been termed "regenerating liver inhibitory factor" (47). Since IκBα can inhibit HIV RNA export, we asked whether the same might be true for the export of HBV RNA and whether the export mechanisms for both viruses might share components.

To assay for possible interference between lentivirus and HBV RNA export, a construct (pDM138PRE+; generous gift from B. Yen) was used that expresses a transcript containing the HBV PRE sequence together with the coding sequence for chloramphenicol acetyltransferase (CAT) between a splicing donor and a splicing acceptor site. Since the sequence encoding the reporter enzyme is located within an intron, it cannot be expressed after the transcript is spliced. However, the presence of the PRE within the same intron allows the efficient nuclear export of the nonspliced mRNA, and this results in CAT expression (27). Expression plasmids for such transcripts were introduced into HeLa cells by using electroporation. A plasmid (pGL2 control; Promega) expressing firefly luciferase was cotransfected as an internal standard. After 18 h, the cells were harvested and the reporter activities were determined. CAT expression was strongly enhanced by the presence of the PRE when compared to expression of the parental construct pDM138 (Fig. 1A, compare lanes 1 and 2), consistent with previously reported results (24). Next, we cotransfected a plasmid, termed pCFNrex, that expresses a truncated form of the

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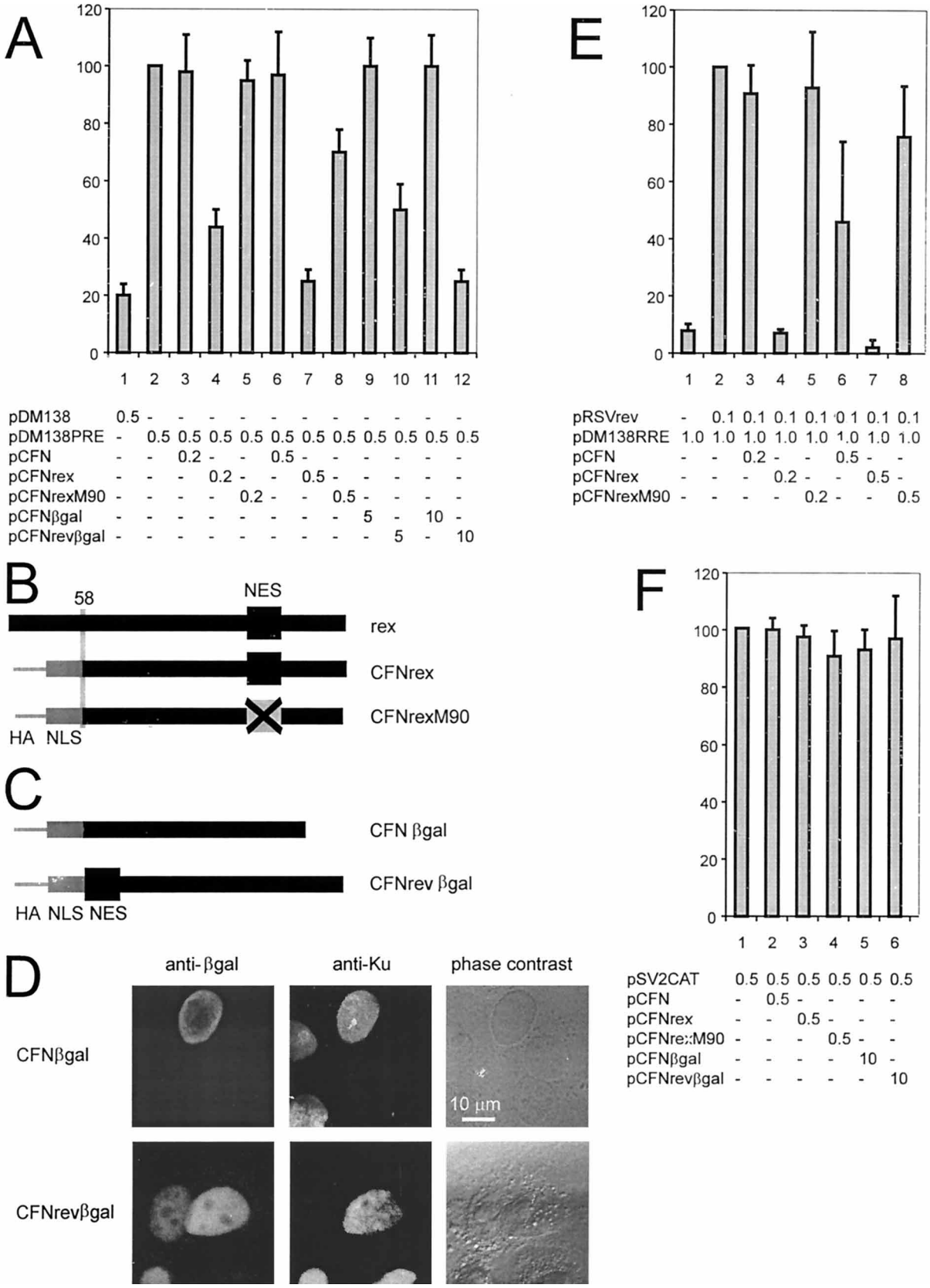

FIG. 1. Impact of known Rev export inhibitors on PRE-dependent reporter expression. (A) An expression construct containing the CAT coding region within an intron (pDM138) was electroporated into HeLa cells (lane 1) and compared to a similar plasmid that additionally contains the HBV PRE in a sense orientation (lanes 2 to 12). Expression plasmids for Rev export inhibitors were cotransfected (lanes 3 to 12) in the amounts indicated below the diagram (micrograms of plasmid). In each

HTLV type 1 Rex protein. In this fragment, the Rex RNA binding domain is replaced by a nuclear localization signal (NLS) from the simian virus 40 (SV40) T antigen (Fig. 1B). The expression of a similar construct has been reported to inhibit the activity of HIV Rev to mediate nuclear export of HIV RNA, presumably by shuttling rapidly between the nucleus and cytoplasm and thus titrating one or several of the cellular factors that are required for Rev export (31). Both expression plasmids pCFNrex and pCFNrexM90 were cloned by PCR, amplifying the 3′ portion of the Rex coding region with the primers TGCTCTAGACCCACTTCCCAGGGTTT GGACAGA and GCGGGATCCTCACGTGGGGCAGGAG GGGCCA from the plasmids pSRαTAgRex and pSRαTAg RexM90 (30), respectively. The PCR products were cloned into the expression vector pCFN (54) by using the enzymes *Xba*I and *Bam*HI. pCFNrex expresses a Rex fusion protein containing both an NLS and an NES. pCFNrexM90 is identical to pCFNrex except that the expressed protein does not contain a functional NES, serving as a negative control. As shown in Fig. 1A, lanes 4 and 7 (compare with lanes 3 and 6), cotransfection of pCFNrex reduced CAT expression from pDM138PRE in a dose-dependent manner. When the NES within the Rex fragment was inactivated by a mutation (pCFNrexM90, Fig. 1B), its ability to reduce CAT activity in this assay was diminished (Fig. 1A, lanes 5 and 8). Rev-dependent RNA export largely parallels these effects (Fig. 1E), confirming previous results (30).

To further elucidate the possibility that NES-mediated export competes with HBV PRE export, a different construct was used as a competitor. The 10-amino-acid NES sequence of the HIV-1 Rev protein was fused to a carrier protein, beta-galactosidase (Fig. 1C), in addition to the NLS from SV40 T Ag by using the following strategy. The *Sal*I Klenow fragment from pMC1871 (Pharmacia) was ligated into the *Sma*I site of pCFN in sense orientation to obtain pCFNβgal (Fig. 1C). The oligonucleotides GCTCTAGACTGCAGCTGCCGCCGCTG and GAACGTCTGACTCTGCGGCCGCGGTACCCC, encoding the Rev NES, were annealed to each other, filled in, and cloned into pCFNβgal by using *Xba*I and *Kpn*I to obtain pCFNrevβgal. Both pCFNβgal and pCFNrevβgal express fusion proteins that mainly localize in the nucleus. The ability of these proteins to shuttle between the nucleus and cytoplasm was determined by a heterokaryon assay as described earlier (10, 40). Transfected human cells were fused to murine cells, followed by a 2-h incubation period and subsequent immunostaining against β-galactosidase and human Ku antigen. The ability of the fusion proteins to shuttle between the nucleus and cytoplasm was judged based on their ability to move from the human to the murine nucleus within a heterokaryon. The fusion protein expressed from pCFNrevβgal rapidly shuttled between the nucleus and cytoplasm (Fig. 1D, lower panel). In contrast, the fusion protein expressed from pCFNβgal apparently did not exit the nucleus after import (Fig. 1D, upper panel). In parallel, pCFNrevβgal, when cotransfected with pDM138PRE+, suppressed PRE export (Fig. 1A, lanes 10 and 12). A similar fusion construct without the Rev NES (pCFNβgal, Fig. 1C) lacked the ability to block PRE export-dependent CAT expression (Fig. 1A, lanes 9 and 11).

None of the export inhibitor constructs had a measurable influence on the expression of CAT from pSV2CAT (20), a control construct with the same promoter (SV40 early promoter) but with the CAT coding region in an exon (Fig. 1F). When compared to each other, the CAT levels after pSV2CAT transfection were three- to fivefold lower than after transfection of pDM138PRE+, suggesting that the observed resistance of CAT expression from pSV2CAT to cotransfection of pCFNrex and pCFNrevβgal was not due to a higher abundance of CAT mRNA that may have titrated out a limiting regulatory factor. Taken together, these data strongly suggest that PRE-mediated nuclear export can be inhibited by NES-containing competitors.

Another protein from a different virus, the adenovirus 34-kDa E4 protein, also competes with HIV Rev for nuclear export (10). We therefore tested whether it blocks the export of HBV RNA, too. As shown in Fig. 2, the expression of the 34-kDa E4 protein from a plasmid (11) reduces PRE-dependent CAT expression in a dose-dependent manner (Fig. 2A, compare lanes 1 and 4 with lanes 2 and 5). In contrast, the adenovirus 55-kDa E1B protein did not show a measurable effect on PRE-mediated export (Fig. 2A, lanes 3 and 6). PRE-independent CAT expression was not affected by the 34-kDa E4 or the 55-kDa E1B protein (Fig. 2B). Parallel results were obtained when Rev-mediated RNA export was analyzed (10). Again, these data suggest that one competitive inhibitor can affect both HBV and HIV RNA export but not the export of completely spliced mRNA.

Next, we tried to identify cellular factors that can interfere with HBV RNA export. Since IκBα is known to inhibit Rev export, we tested whether it might also antagonize the RNA export that is driven by the HBV PRE. A similar reporter construct was used as in the above experiments, except that the SV40 early promoter was replaced by the Rous sarcoma virus (RSV) 3′ LTR. We chose this construct because the promoter activity of the RSV 3′ LTR was shown to be independent from that of the NF-κB transcription factors (51). To generate it, we first ligated both ends of a *Cla*I-*Xba*I Klenow fragment from pRep4 (Invitrogen) to each other to form pRep4Δ (lacking the Epstein-Barr virus-derived sequences present in pRep4). The portion of pDM138PRE (27) encoding mRNA was amplified by PCR, using the primers TTGGCGCGCTAGCCTGGAAG

experiment, the total amount of transfected DNA was brought to 10 μg with pBluescript SK+ (Stratagene) and 0.3 μg of a luciferase expression construct (pGL2 control; Promega) was added for normalization. After 18 h of incubation, the cells were harvested and assayed for CAT and luciferase activities. The relative CAT activities are shown as percentages of the value obtained without export inhibitors (lane 2). Each column represents the average of at least three independent experiments, and the standard error is shown (bars). (B) A competitive inhibitor of Rev-dependent export was created by fusing the C-terminal portion of HTLV Rex (upper panel), containing an NES, to an HA tag and the NLS from SV40-T antigen in the expression plasmid pCFNrex (middle panel). An otherwise identical construct, pCFNrexM90 (lower panel), lacks a functional NES due to a mutation. (C) Another competitive inhibitor of Rev-dependent export contains the HA tag, the NLS from SV40-T antigen and the NES from HIV Rev. These peptide fragments were fused to beta-galactosidase in the expression vector pCFNrevβgal (lower panel). A similar vector, pCFNβgal (upper panel), lacks the Rev NES and served as a negative control. (D) A heterokaryon assay showing the ability of beta-galactosidase fusion proteins to shuttle between the nucleus and cytoplasm. Expression plasmids pCFNβgal and pCFNrevβgal were transfected into HeLa cells by electroporation. Eighteen hours later, the cells were fused to murine BALB/c 3T3 cells and de novo protein synthesis was stopped by using cycloheximide. One hour after fusion, the cells were fixed and double-stained for beta-galactosidase and the human Ku antigen. The shuttling activity of the transfected fusion protein was assayed by its ability to move from the human to the murine nucleus within a heterokaryon. (E) Rev-mediated RNA export was assayed by a reporter carrying the Rev-responsive RNA element (RRE) and the CAT coding region within an intron. This RNA was expressed in the absence (lane 1) or presence (lane 2 to 8) of cotransfected Rev. Transfection was performed by using Superfect reagent (Qiagen). Export inhibitors were cotransfected as indicated, and CAT activity was determined. (F) An expression construct, pSV2CAT, containing the CAT coding region within an exon, was cotransfected (electroporation) with export inhibitors, using the same conditions as described in panel A. CAT activity was determined as described above.

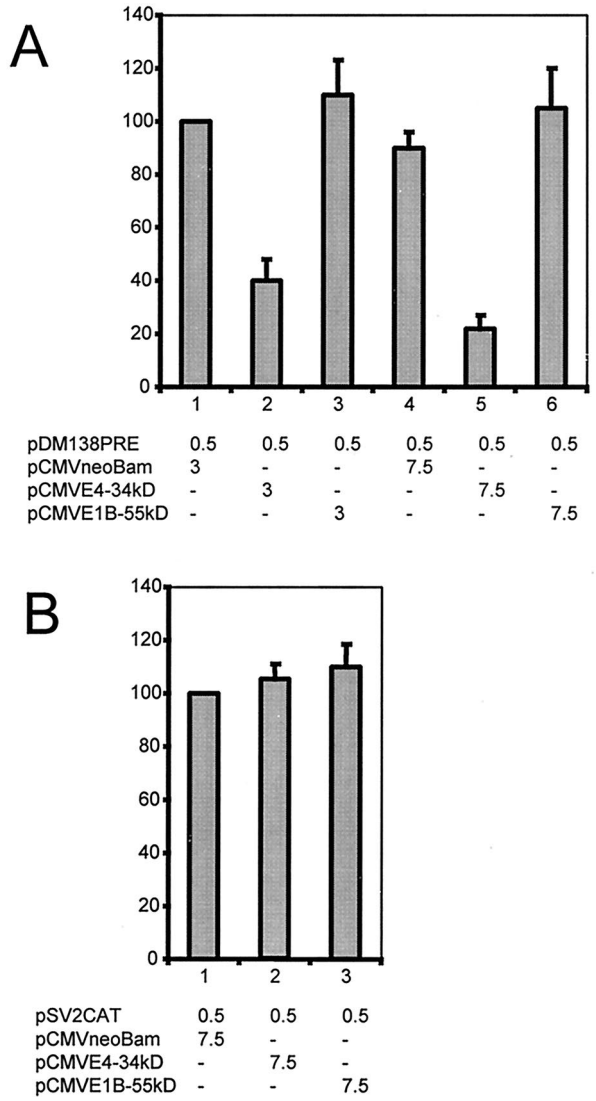

FIG. 2. Effect of adenovirus proteins on PRE-dependent CAT expression. (A) A PRE-dependent construct was assayed for CAT expression as described in the legend to Fig. 1A. Expression plasmids for the adenovirus type 5 34-kDa E4 (lanes 2 and 5) and the adenovirus type 5 55-kDa (lanes 3 and 6) proteins were cotransfected and compared to the expression vector alone (lanes 1 and 4). (B) The same assay was carried out by using pSV2CAT, as described in the legend to Fig. 1F.

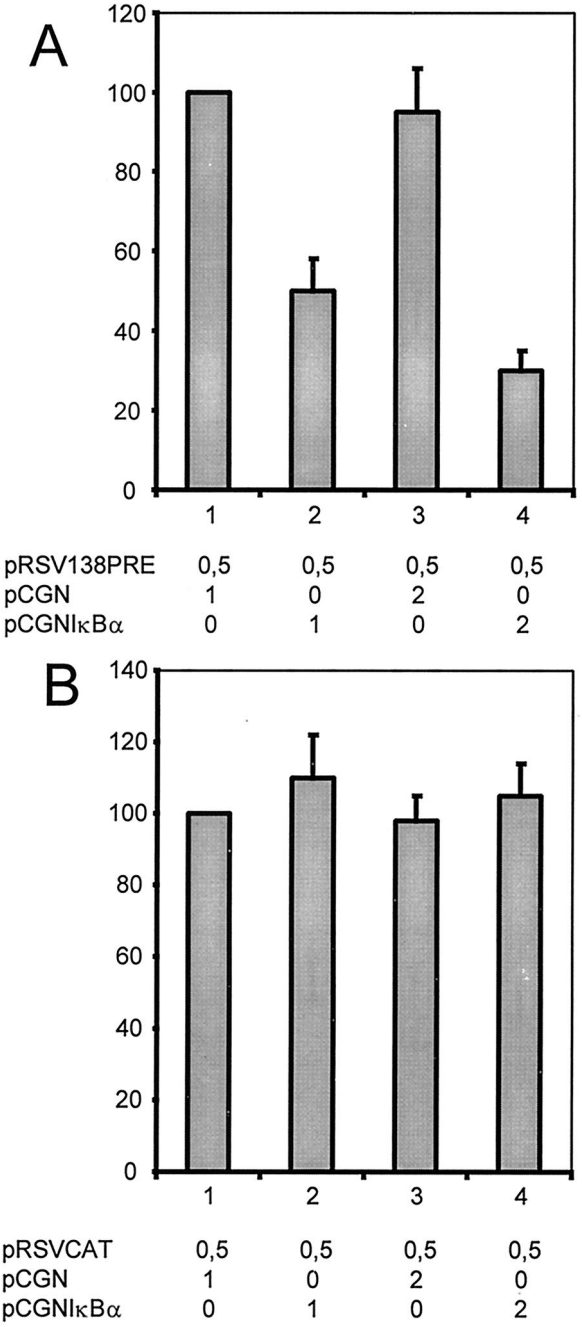

FIG. 3. Influence of IκBα on PRE-dependent CAT expression. (A) A PRE-dependent construct with the RSV 3′ LTR as a promoter was assayed for CAT expression as described in the legend to Fig. 1A. An RSV-driven luciferase expression plasmid (0.5 μg of RSV-luc, a vector containing the RSV 3′ LTR in the background of pGL2basic [Promega]) was cotransfected in each assay for normalization. An expression plasmid for IκBα was cotransfected in different amounts (lanes 2 and 4) and compared to the corresponding empty expression vector (lanes 1 and 3). (B) The same assay was carried out using pRSVCAT, a reporter plasmid driven by the RSV 3′ LTR that contains CAT within an exon.

CATCCAGGAAGTC and CGGGACGTCGACAGATTTTT CCACACTGACTAAAAGGGTCT. The PCR product was treated with *Nhe*I and *Sal*I and ligated into the *Nhe*I-*Xho*I fragment of pRep4Δ to obtain pRSV138PRE. The coding region of human IκBα cDNA was amplified by PCR, using the primers GCTCTAGACGCGCCATGTTCCAGGCGGCCGA GCGC and CGGGATCCGCGGCCGCCCCTTTGCACTCA TAACGTCAGACGCT. The PCR product was cloned into the expression vector pCGN (54), using *Xba*I and *Bam*HI to obtain pCGNIκBα, which expresses the IκBα protein N-terminally fused to an HA epitope. When we cotransfected pRSV138 PRE with pCGNIκBα, PRE-dependent CAT expression decreased, depending on the dose of the IκBα expression plasmid (Fig. 3A, lanes 2 and 4). In contrast, the expression vector pCGN alone did not detectably reduce CAT expression in this assay (Fig. 3A, lanes 1 and 3). IκBα did not change RSV 3′ LTR-driven CAT expression from pRSVCAT (21), a PRE-independent construct (Fig. 3B). Parallel observations to those for PRE were made using Rev-dependent reporter constructs (41a, 51). Thus, IκBα not only interferes with Rev-mediated RNA export but also with the nuclear export of HBV RNA.

Our data reveal common features shared by the nuclear

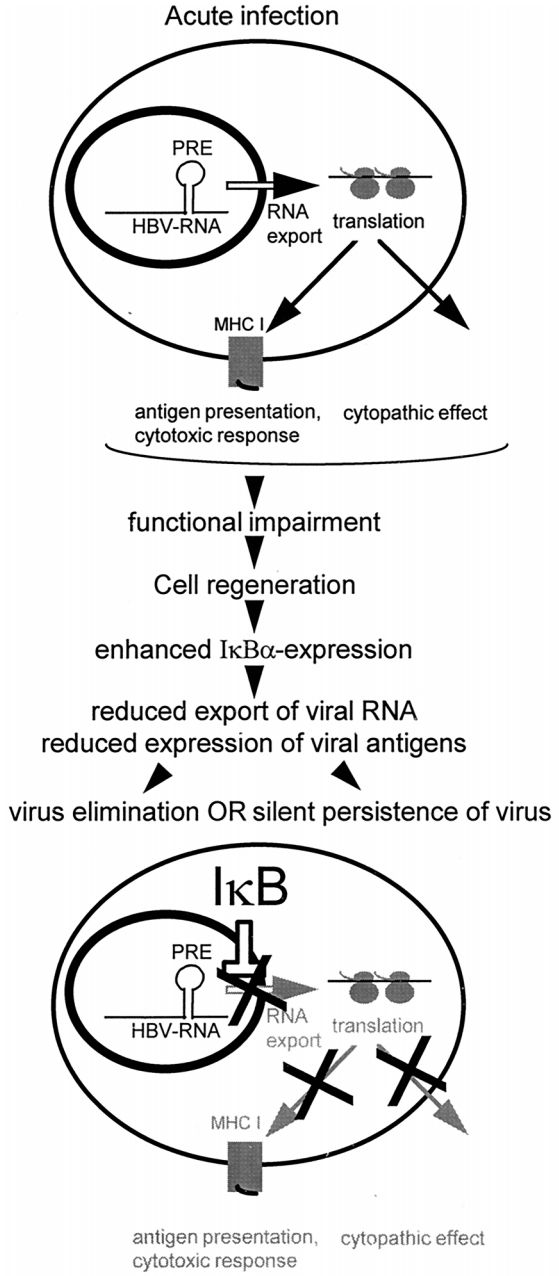

FIG. 4. A possible role in RNA export inhibition of IκBα during HBV infection. Both the expression of viral genes and the formation of new virus particles depend on the export of HBV RNA. The subsequent presentation of viral antigens on the major histocompatibility complex (MHC) and possibly the cytopathic effect itself can lead to functional impairment of the infected cells and to enhanced proliferation of the remaining cell population. This might result in enhanced IκBα expression. Viral RNA export in newly infected cells might then be blocked. This in turn could result in either silent persistence or elimination of the virus.

export pathways for HIV and HBV RNA. Yet there is one fundamental difference between the two export mechanisms. HIV and other lentiviruses use a viral protein to ensure efficient export of their incompletely spliced RNA. In contrast, HBV relies entirely on cellular factors to transport its RNA from the nucleus to the cytoplasm. It is tempting to speculate that a cellular factor specifically binds the PRE portion of HBV RNA and mediates nuclear export. The partial purification of PRE-associated proteins has been reported (26). From our results, such a factor can be predicted to mediate export on a Rev-like pathway, possibly by virtue of an NES. At least one cellular protein, TFIIIA, that binds RNA and contains an NES, has been identified (16). It is therefore conceivable that a cellular PRE-binding factor exists that can be used to export viral RNA. The export of both HIV and HBV RNAs seems to involve common cellular factors, which form a saturable export machinery. This export pathway is also used by a specific class of cellular RNAs. 5S rRNA and U1 snRNA have been identified as members of this class, whereas most cellular mRNA species have been reported to exit the nucleus on a different pathway (14). Therefore, hypothetical factors that export HBV RNA might also mediate the export of 5S rRNA and/or U1 snRNA.

Since three different viruses—lentiviruses, HBV, and adenovirus type 5—seem to employ a common set of cellular export factors, it is tempting to speculate that even more viruses do so. Interestingly, a simple type D retrovirus, Mason-Pfizer monkey virus (MPMV), contains an RNA export element similar to the HBV PRE that also functions in the absence of a viral protein (8, 13). However, it remains to be determined whether MPMV RNA export also uses a cellular export mechanism that overlaps with that of Rev export.

How could IκBα interfere with the export of HIV and HBV RNA? Newly expressed IκBα has been reported to localize in the nucleus (1, 9, 53) and could therefore interact with nuclear export factors. It has been proposed that IκBα not only holds the NF-κB p65-p50 complex in the cytoplasm but might also mediate the export of nuclear p65-p50 (1, 33). If this export occurred on the Rev pathway, the effects of IκBα on Rev and PRE export might arise from competition for export factors. The primary structure of IκBα contains at least one functional NES motif (1, 41a, 52) that meets the NES consensus defined by a randomization selection approach (6), arguing that IκBα might be exported from the nucleus on a Rev-like pathway. On the other hand, this NES motif does not seem to be required to inhibit Rev-mediated RNA export (52), arguing that other mechanisms than simple competition between NESs might be responsible for IκBα's ability to inhibit nuclear export. Further studies are required to elucidate a possible active nuclear export mechanism for IκBα and its impact on viral RNA export and NFκB-mediated transcription.

IκBα is strongly overexpressed in regenerating liver (44). Further, liver cell regeneration is markedly enhanced after clinically manifest HBV infection (35, 50). Our finding that IκBα is an inhibitor of HBV RNA export therefore suggests a model of how export inhibition might limit the progress of HBV infection (Fig. 4). During the initial disease, a substantial proportion of liver cells is functionally impaired. This presumably leads to proliferation of the remaining cells and to enhanced expression of IκBα. Cells that are infected at this stage might be able to suppress HBV gene expression, since overexpressed IκBα specifically inhibits HBV RNA export. As a consequence, these cells might remain silently infected and may escape immunosurveillance, leading to the observable viral persistence (41); alternatively, the mechanism outlined might contribute to the elimination of the virus.

It is presently unknown whether the export of HBV RNA could be modified pharmacologically. The treatment of patients with alpha interferon helps to eliminate chronic HBV in a subset of patients (reference 36 and references therein). Alpha interferon is known to activate the double-stranded RNA-activated kinase PKR, which phosphorylates IκBα to activate NF-κB, which in turn promotes de novo synthesis of IκBα (31). However, it is subject to future studies whether

PKR can modulate IκBα's effect on HBV RNA export. Other agents that are known to specifically induce IκBα degradation, like the fungal metabolite gliotoxin (37), might influence HBV RNA export as well. It remains to be determined whether RNA export represents a suitable target to interfere with HBV infection.

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ADDENDUM IN PROOF

While this paper was under review, it was reported that the constitutive transport element from simian retrovirus type 1 uses an export pathway shared by cellular mRNA but not by lentivirus Rev proteins (C. Saavedra, B. Felber, and E. Izaurralde, Curr. Biol. **7:**619–628, 1997). Thus, the export mechanism for at least one simple retrovirus seems to be fundamentally different from that of lentiviruses, HBV, and adenoviruses.

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