Hepatitis B virus (HBV), a small DNA-containing virus that replicates via reverse transcription, causes acute and chronic B-type hepatitis in humans. The limited success of current therapies for chronic infection has prompted exploration of alternative strategies. Capsid-targeted viral inactivation is a conceptually powerful approach that exploits virion structural proteins to target a degradative enzyme specifically into viral particles. Its principal feasibility has been demonstrated in retroviral model systems but not yet for a medically relevant virus outside the retrovirus family. Recently, we found that C proximal fusion to the HBV capsid protein of the Ca\(^{2+}\)-dependent nuclease (SN) from Staphylococcus aureus yields a chimeric protein, coreSN, that in Escherichia coli coassembles with the wild-type capsid protein into particles with internal SN domains. Here we show that, in HBV co-transfected human hepatoma cells, less than 1 coreSN protein per 10 wild-type core protein subunits reduced titers of enveloped DNA containing virions by more than 95%. The antiviral effect depends on both an enzymatically active SN and on the core domain. CoreSN does not block assembly of RNA containing nucleocapsids but interferes with proper synthesis of viral DNA inside the capsid, or leads to rapid DNA degradation. Our data suggest an intracellular nuclease activation that, owing to the characteristics of HBV morphogenesis, is nonetheless highly virus specific. HBV may therefore be particularly vulnerable to the capsid-targeted viral inactivation approach.

Hepatitis B virus (HBV),\(^1\) an enveloped DNA-containing virus that replicates via reverse transcription (1), is the causative agent of B type hepatitis in humans. Chronic infections affect more than 350 million people worldwide, they have potentially severe consequences such as liver cirrhosis and hepatocellular carcinoma (2), and current treatments are of limited efficacy (3, 4). The sustained response rate of the approved high-dose interferon-α therapy is about 30%. Nucleoside analogues such as lamivudine markedly reduce viral load but suffer, inter alia, from the emergence of drug-resistant virus variants (5). This situation has spurred interest in alternative approaches to interfere with HBV replication (6).

Whereas only primary human, or chimpanzee, hepatocytes are susceptible to HBV infection in cell culture, a few human hepatoma cell lines like HuH7 and HepG2 support virus production upon transfection with cloned viral DNA. The late steps of the infectious cycle (Fig. 1A) are hence understood in some detail (7, 8), and they present novel targets for intervention. After infection, the nucleocapsid transports the partially double-stranded circular 3.2-kb DNA genome to the nucleus (9) for conversion into covalently closed circular DNA; this molecule is the transcriptional template for several subgenomic and genomic RNAs that all act as mRNAs. Of these, the pregenomic RNA (pgRNA) is first used to translate both the capsid, or core, protein and the reverse transcriptase, P protein. Then P protein, together with cellular chaperones (10), binds to a stem-loop structure, ε, close to the 5′-end of the pgRNA (11, 12). Complex formation mediates assembly of immature RNA-containing nucleocapsids (13), and initiation of reverse transcription (14–17). DNA synthesis occurs inside the nucleocapsid and involves several template switches that lead to the characteristic relaxed circular (RC) DNA genome containing a complete (−)-strand and variously extended (+)-strands. Mature DNA-containing nucleocapsids can re-escort the genome to nucleus, or be exported as enveloped virions by budding into a post-endoplasmic reticulum/pre-Golgi compartment. Both events apparently require that at least the (−)-DNA strand be completed (1, 18).

The restriction of HBV genome replication to the nucleocapsid makes this nucleoprotein particle an attractive target for intervention. Apart from nucleic acid-based strategies (19, 20) dominant negative core protein variants have been described that passively interfere with nucleocapsid assembly (21–24). A conceptually more powerful approach is capsid-targeted viral inactivation (CTVI) or, generally, virion-targeted viral inactivation, which exploits a viral capsid protein or other virion-associated protein as carrier to target a degradative enzyme specifically into virus particles (25, 26); alternatively, nucleic acid-based effectors such as ribozymes may be fused to viral packaging signals and thus be used against viruses that, like retroviruses but unlike HBV, encapsidate more than one genome, or genome segment (27, 28). For the protein based approach, the nuclease from Staphylococcus aureus (SN) is considered particularly useful because it requires Ca\(^{2+}\) for activity (29). Intracellular Ca\(^{2+}\) levels are usually below 1 μM, providing a safeguard against attacks on cellular nucleic acids. Serum levels of Ca\(^{2+}\), by contrast, are in the millimolar range; hence...
SN incorporated into a virus is thought to be activated upon release from the cell. The principal feasibility of the approach, pioneered using the yeast retrotransposon Ty1 (25), has been well documented for the model of Moloney murine leukemia virus, a simple C-type retrovirus (30–34). Adapting the approach to human immunodeficiency virus has been hampered by the poor expression and inefficient incorporation into particles of human immunodeficiency virus Gag fusion proteins (35). Effectors fused to the accessory Vpr and Vpx proteins, although efficiently incorporated, can be subject to inactivation by the retroviral protease (36), and the Vpr carrier may induce cell cycle arrest and apoptosis (35). Using expression in E. coli we have recently shown that a chimeric protein consisting of the N-terminal 155 aa of the wild-type core protein followed by the complete SN protein, coreSN, coassembles with wild-type core protein to particles with internal SN domains (37). Here we investigated if, and how, this fusion protein is able to interfere with HBV replication. We show that low levels of coreSN drastically reduce the titers of replication-competent HBV virions in supernatants from transfected HuH7 cells, and we present evidence for an intracellular but virus-specific nuclease activation that may make HBV particularly vulnerable to the CTVI approach.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Effector plasmids pCS1-coreSN and pCS1-coreSNmut were generated by transferring DNA fragments encoding the chimeric proteins from the prokaryotic vectors pPLC-coreSNwt and pPLC-coreSNmut43/87 (37) into plasmid pCS1-C1 (38). This plasmid contains the cytomegalovirus immediate early (CMV-IE) promoter and a polyadenylation signal from SV40. Control constructs encoding an SN fusion to a mutant core protein with aa 80 changed from Ala to Lys (pCS1-coreA80K-SN), and SN without core protein domain were obtained by conventional polymerase chain reaction-mediated mutagenesis and cloning into vectors pCS1-coreSN and pCDNA/Myc-His (Invitrogen), respectively. pCS1-SN and pCS1-SNmut code for an 1–149 of mature SN and its inactivated double mutant, preceded by the dipeptide MD and followed by a C-terminal His-tag; pCDNA6-SN and pCDNA6-SNmut specify active and inactive SN with the same 6-aa propeptide sequence as present in coreSN (see Fig. 1) plus a methionine at the N terminus and a C-terminal His-tag. The HBV core plasmid pCHT-9/3091 carries a slightly overlength HBV genome under control of the CMV-IE promoter (38). Transfection efficiencies were monitored by co-transfection of plasmid pTR-UF5 (39) encoding a fluorescent protein (GFP).

Immunological Techniques—For Western blot detection of wild-type core and coreSN proteins (37, 40), either a polyclonal rabbit antiserum raised against denatured recombinant core protein, or the monoclonal antibody 10E11, recognizing an epitope between amino acids 8 and 20 on denatured core protein (41), served as primary antibodies; for HBsAg monoclonal antibody 4/7B (42) was used. Detection was performed using appropriate peroxidase-coupled secondary antibodies and a chemiluminescent substrate (ECL-Plus, Amersham Pharmacia BioTech). Blots were exposed to x-ray film or, for quantitation, to a Diana charge-coupled device camera; band intensities were evaluated using AIDA software (both Raytest). For immunoprecipitations (38), usually polyclonal rabbit antiserum raised against denatured recombinant core protein aa 1–149 (serum H800) or coreSN particles were employed. In the experiments aimed at directly demonstrating coinfection of coreSN in mixed particles with wild-type core protein, monoclonal antibody mc122 was used. This antibody recognizes a linear epitope within aa 76–84 of core protein (41, 43) which largely coincides with a loop exposed on the spikes of core particles. Therefore, the core variant A80K with a lysine instead of alanine at position 80 reacts much less efficiently with mc122.

Transfections—The human hepatoma cell line HuH7 was used throughout (38). Transfections were performed with FuGENE6 reagent (Roche Molecular Biochemicals) as recommended by the manufacturer. For typical co-transfections 50 μl of FuGENE6 and a total of 21 μg of plasmid DNA/10-cm dish were used. If required, constant amounts of DNA and CMV-IE promoter copies were maintained by adding an appropriate quantity of plasmid pTR-UF5.

Isolation of Secreted and Intracellular HBV Particles—Particles contained in culture supernatants collected from day 3 to 4 post-transfection were enriched by polyethylene glycol precipitation (44) and, loaded on a CsCl step gradient (0.9 ml each of 1.5, 1.4, 1.3, 1.2, and 1.1 g/ml CsCl in 10 ml Tris/Cl−, pH 7.5, 100 mM NaCl). After 17 h at 35,000 rpm at 20°C in a SW 50.1 rotor (Beckman), 25 200-μl fractions were collected from the top. Densities were determined by refractive index. Intracellular particles were obtained 4 days post-transfection by cytoplasmic Nonidet P-40 lysates and subsequent immunoprecipitation with antisera H800 (38). In some experiments, core particles were separated from nonassembled core and coreSN protein by sedimentation in 10 to 60% sucrose gradients as previously described (37).

Characterization of Viral Proteins and Nucleic Acids—For dot blot analyses, the coreSN was transferred to nitrocellulose filters using a Bio-Rad apparatus (Bio-Rad). DNAs were detected with a random primed HBV DNA probe (High Prime DNA Labeling Kit, Roche Molecular Biochemicals) and quantitated using a PhosphorImager (Fuji BAS 1500). Core proteins and HBsAg were detected by Western blotting as described above. For Southern blots, DNAs contained in viral particles from CaCl2 gradients, or in immunoprecipitated intracellular cores, were isolated by proteinase K digestion in the presence of 0.5% SDS, and purified using the QiAamp tissue kit (Qiagen). If desired, aliquots were further treated with avian myoblastosis virus reverse transcriptase (AMV-RT) as previously described (38). DNAs were separated on 1% agarose gels and transferred to nylon membranes using 0.4 M NaOH. Transfection was performed with either a 32P radiolabeled or a digoxigenin-labeled probe (Dig High Prime DNA Labeling Kit; Roche Molecular Biochemicals) as indicated. For Northern blotting of total RNA about one-third of the cells on a 10-cm dish were lysed in RLT buffer and the RNA was purified using the RNeasy Mini kit (both Qiagen). Encapsidated RNA was obtained accordingly from core specific immunoprecipitates. RNAs were separated on 1.2% agarose-formaldehyde gels, transferred to nylon membranes in 10 × SSC buffer, and detected using the 32P radiolabeled HBV probe. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA served as control. Native agarose gel electrophoresis of cytoplasmic cores was performed as described (40), using about 1 to 2% of the cytoplasmic lysate from a 10-cm dish pretreated with DNase I for 30 min at 37°C to remove nonencapsidated DNA. After renaturation (0.5 M NaOH, 1 M NaCl) and neutralization (0.5 M Tris, pH 7.5, 3 mM NaCl), core proteins were detected using H800 serum, and DNA by hybridization with the 32P-labeled probe.

RESULTS

Experimental Design—For lack of a feasible infection system we used co-transfection of HuH7 cells with an HBV target plasmid, pCHT-9/3091, and a coreSN effector plasmid, pCS1-coreSN, as an assay system. The target plasmid produces, under control of the CMV-IE promoter, substantial amounts of pgRNA and the subgenomic RNAs generating all gene products required to form complete virions (38). It should be re-emphasized that, in this setting, there is neither reinfection of the cells nor a significant accumulation of intranuclear HBV covalently closed circular DNA, hence virtually all virions produced derive from the transfected plasmid. The coreSN gene (Fig. 1B) in pCS1-coreSN, although also controlled by the CMV-IE promoter, is only moderately expressed, possibly for lack of introns and/or other elements promoting nuclear export in the mRNA. Here this low expression appeared advantageous since even one fusion protein per particle should be sufficient to exert an antiviral effect (Fig. 1A). To distinguish nucleosome-dependent effects from passive steric hindrance we analyzed in parallel a homologous fusion protein, coreSNmut, with two point mutations in the SN part that drastically reduce enzymatic activity (45).

To test whether coreSN affected expression and/or stability of the wild-type core protein, HuH7 cells were co-transfected with a constant amount of plasmid pCHT-9/3091, and various concentrations of pCS1-coreSN. Core proteins were immunoprecipitated from cytoplasmic lysates using a polyclonal antisera against denatured recombinant core protein, H800, that cross-reacts with the chimeric protein (37). For sensitive Western blot detection a polyclonal antiserum against denatured recombinant coreSN protein was used which reacts much better with coreSN than with wild-type core protein. Indeed, a new
band at the expected 36-kDa position was dose-dependently detected (Fig. 2A). Even at the highest coreSN concentration the intensity of the 21-kDa wild-type core protein band was not significantly reduced; the slightly weakened 21-kDa band in coreSNmut plasmid this assay, after densitometric scanning, was subjected to immunoprecipitation with mc312 and, for control, with the polyclonal antiserum against native coreSN. As shown in Fig. 3, no antigen-specific signal was seen in the single transfection when mc312 was used for immunoprecipitation.

A potential concern was that, at the relatively low concentrations in this eukaryotic setting, the coreSN protein would not be co-integrated into wild-type core particles. Obtaining formal proof for coassembly, e.g. by coimmunoprecipitation, was not trivial for two reasons: (i) using an anti-SN antibody to demonstrate co-precipitation of wild-type core protein would require that the SN domains be exposed on the particle surface, in contrast to our previous Escherichia coli data which strongly suggested an interior location of SN (37); (ii) by necessity of the approach, the core protein part in coreSN must mimic the structure of wild-type core protein as closely as possible, otherwise efficient coassembly would be compromised. To resolve this problem, we resorted to the monoclonal anti-core antibody mc312 which recognizes a linear epitope between aa positions 76 and 84 (41, 43). This sequence overlaps with a surface exposed loop located on the spikes of the core particles (Fig. 3A). Because an entire protein such as GFP can be inserted into this loop without a major impact on particle folding (40), a single aa exchange within the loop sequence would conceivably not affect the assembly capability of a correspondingly altered core protein but inhibit binding of mc312. We therefore generated a mutant core protein, coreA80K, in which the authentic alanine at position 80 is replaced by lysine. This protein formed particles in E. coli, and it reacted very poorly with mc312 on Western blots as well as in immunoprecipitation; a corresponding coreA80K-SN fusion protein (Fig. 3A) was also assembly-competent.² This fusion protein should therefore not be precipitated by mc312 unless it is associated with wild-type core protein. This assumption was first confirmed using mixtures of recombinant wild-type core and coreA80K-SN protein (data not shown). Next, a eukaryotic expression vector for the mutant fusion protein, pCS1-coreA80K-SN, was generated which, except for the single codon exchange, is identical to the coreSN vector. This plasmid was transfected in Huh7 cells, either alone or together with the HBV expression plasmid. Subsequently, equal aliquots of cytoplasmic lysates from both transfections were subjected to immunoprecipitation with mc312 and, for control, with the polyclonal antisemur against native coreSN. The precipitates were then analyzed by Western blotting using the polyclonal antisemur against denatured coreSN. As shown in Fig. 3B, no antigen-specific signal was seen in the single transfection when mc312 was used for immunoprecipitation.
while the protein was well detectable in the precipitate obtained with the polyclonal antiserum. By contrast, coreA80K-SN was precipitated by mc312 when coexpressed with wild-type core protein. This result indicated that wild-type and fusion protein did indeed interact. To further prove that this interaction occurred in the context of complete capsids, aliquots of the same two lysates were subjected to sedimentation in sucrose gradients. Under the conditions chosen, wild-type core proteins are typically found in the center of the gradient (37). The corresponding fractions were again used for immunoprecipitation with mc312. As before, mc312 did not precipitate coreA80K-SN alone but did so when it was coexpressed with wild-type protein (Fig. 3B, right panel). Together these data indicated that the mutant coreSN fusion protein was able to form mixed particles with the wild-type core protein in eukaryotic cells. The suitability of coreA80K-SN as a model compound for coreSN was confirmed by its similar activity against particle-borne HBV DNA (see below).

CoreSN Protein Drastically Reduces HBV DNA in Extracellular Virions—The foremost aim of any antiviral strategy is to reduce the number of infectious virus particles. Therefore we first compared the amounts of genome-containing enveloped virions in supernatants from cells transfected with only HBV, or co-transfected with HBV and equal amounts of the coreSN and core SNmut plasmids. A peculiarity of efficient HBV constructs such as pCHT-9/3091 is the release, by an unknown mechanism, of nonenveloped cores (38). We therefore used CsCl density gradients to separate enveloped and naked particles. Aliquots from the gradient fractions were analyzed by dot blot (Fig. 4A) for HBV-specific nucleic acids, core protein, and HBsAg as indicated. Fraction densities are given at the bottom. The DNA peak at around 1.25 g/ml corresponds to enveloped virions, that around 1.35 g/ml to naked cores. The presence of core protein in the intermediate fractions is probably due to the presence of empty cores. HBsAg appeared in fractions 8 to 13. B, southern blot of DNA in the virion and naked core fractions. DNA present in the indicated fractions was isolated, separated by agarose gel electrophoresis and detected using an HBV-specific 32P-labeled probe. The positions of full-length double-stranded 3.2 kb and single-stranded (ss) HBV DNA, and of marker DNAs of the indicated sizes in kb are shown. The panels marked 10x and 4x are 10- and 4-fold longer exposures to visualize the small amounts of DNA remaining upon co-transfection with coreSN.

FIG. 3. Coassembly of coreSN with wild-type core protein. A, outline of the coprecipitation assay. Monoclonal antibody mc312 recognizes an epitope between aa 76 and 84 of wild-type core protein. This sequence (left) overlaps with a surface exposed loop at the tips of core particle spikes, schematically outlined for core protein dimers on the right. In variant coreA80K-SN the central alanine residue at position 80 was mutated to lysine (symbolized by the gray crosses). The variant should not be precipitated by mc312 unless it is physically associated with wild-type core protein as in mixed particles. The Y-shaped objects symbolize antibody mc312 (not drawn to scale), the zigzag lines on the wild-type core dimer denote the basic C-terminal region not required for assembly but for nucleic acid binding. B, immunoprecipitation. Plasmid pCS1-coreA80K-SN was transfected into HuH7 cells, either without or with HBV plasmid pCHT-9/3091. Equal aliquots from cytoplasmic lysates from the co-transfection lysate was subjected to sucrose gradient sedimentation, and immunoprecipitation was performed from the pooled fractions that typically contain core particles (right panel). Precipitates were resolved by SDS-polyacrylamide gel electrophoresis and detected with a polyclonal rabbit serum (left panel). In addition, an aliquot from the co-transfection lysate was subjected to sucrose gradient sedimentation, and immunoprecipitation was performed from the pooled fractions that typically contain core particles (right panel). Precipitates were resolved by SDS-polyacrylamide gel electrophoresis and detected with a polyclonal rabbit serum against denatured coreSN protein, anti-rabbit antibody-peroxidase conjugate, and a chemiluminescent substrate. The bands marked with an asterisk are derived from the polyclonal rabbit immunoprecipitation antiserum; hc, heavy chain.

FIG. 4. Inhibition of HB virion production by coreSN protein. A, dot blot of density fractionated viral particles from supernatants of transfected HuH7 cells. CsCl gradient fractions were analyzed for HBV-specific DNA, core protein, and HBsAg as indicated. Fraction densities are given at the bottom. The DNA peak at around 1.25 g/ml corresponds to enveloped virions, that around 1.35 g/ml to naked cores. The presence of core protein in the intermediate fractions is probably due to the presence of empty cores. HBsAg appeared in fractions 8 to 13. B, southern blot of DNA in the virion and naked core fractions. DNA present in the indicated fractions was isolated, separated by agarose gel electrophoresis and detected using an HBV-specific 32P-labeled probe. The positions of full-length double-stranded 3.2 kb and single-stranded (ss) HBV DNA, and of marker DNAs of the indicated sizes in kb are shown. The panels marked 10x and 4x are 10- and 4-fold longer exposures to visualize the small amounts of DNA remaining upon co-transfection with coreSN.
tions 8 to 12 around the expected density of 1.20 g/ml.

The presence of active coreSN protein, but not of the inactive variant coreSNmut, dramatically changed the DNA pattern: the virion peak was virtually absent, and the DNA peak from naked cores was substantially reduced. This reduction was not due to global effects on viral gene expression because the signal intensities and distributions of core protein and HBsAg were similar in all three samples.

Next we analyzed the DNAs present in the corresponding fractions by Southern blotting. For better comparison, all samples were run on one gel and transferred to one membrane for hybridization and detection (Fig. 4B). For the HBV-only transfected cells, the virion peak contained mostly mature DNAs at about 3.2 kb, and a weak band at the position of single-stranded DNA (fractions 10 to 13). Naked cores (fractions 16 to 19) contained ssDNA plus additional immature DNA species extending up to the 3.2-kb position. The samples from co-transfection with coreSN, on the same exposure, gave no visible signals in the virion fractions and only weak signals in the naked core fractions; these consisted of ssDNA and a smear of slower, and of faster migrating material that was more pronounced than in the absence of coreSN (panel marked 4x). Further overexposure (panel marked 10x) revealed small amounts of DNA in the virion fractions, broadly distributed with no accumulation at the position of full-length genomes. By contrast, co-transfection with coreSNmut gave, in general, signals similar to those from HBV-only transfected cells. Virion DNA was somewhat reduced and contained a higher proportion of ssDNA. In the naked core fractions, signals were similarly strong but the smear above the ssDNA did not extend as far up.

Quantitation of the overall signal intensities using a PhosphorImager revealed that coreSN diminished the DNA in enveloped particles to 2.5%, or less, of that observed in its absence. Coexpressed coreSNmut reduced the virion signal only to about 70%. For the naked capsids, the signals were lowered to about 10% by coreSN, while coreSNmut had little effect (reduction to 94%). These data confirmed that coreSN efficiently interferes with production of complete virions, and that most of this interference depends on an active nucleosome domain. Whether the number of physical virus particles was reduced could not be definitely answered because the large excess of HBsAg precludes a complete separation from virions; similarly, the core blot signals were not sharply separated between the virion and core particle fractions, possibly due to the presence of empty capsids.

Antiviral Activity of CoreSN Depends on the Presence of the Core Protein Domain—That coreSN dramatically reduced DNA containing virions but had no significant effect on HBsAg production (and on core protein, packaged viral pgRNA, or cellular RNA; see below) strongly suggested a virus-specific action. However, to further prove this specificity we also tested the consequences of expressing active SN, and its enzymatically disabled counterpart SNmut, without the core protein domain. Two possible outcomes were envisaged: either the nuclease would exert a generally toxic effect and nonspecifically inhibit gene expression in, or lead to the death of, the subset of successfully transfected cells (roughly 10% of the cells with our procedure). Alternatively, the low intracellular Ca$^{2+}$ concentration may be insufficient for activation of the enzyme, and no discernable difference between cells expressing active, inactive, or no SN would be expected. Especially the latter case would imply a major difference between free SN, and coreSN incorporated into nucleocapsids.

Two types of expression plasmids for SN and its enzymatically disabled double mutant SNmut were constructed. The first is derived from plasmid pCS1 and encodes the mature nuclease, i.e. aa 1–149, preceded by the dipeptide MD and followed by a His-tag, resulting in a product with a nominal molecular mass of 17.8 kDa. The second is derived from the commercial vector pcDNA6/Myc-His and contains, in addition, the 6-aa propeptide sequence as present in coreSN; its nominal molecular mass is 18.4 kDa. The rationale was that the slightly different products should be discernable by SDS-polyacrylamide gel electrophoresis and thus aid in identifying the corresponding proteins by Western blotting using the anti-coreSN/denat antibodies, as was indeed the case (see Fig. 5).

As an initial test for general toxicity we monitored the effect of SNwt and SNmut on co-transfected GFP. Over 5 days we did not observe significant differences in the number of GFP-positive cells, or the intensity of GFP-fluorescence transfected with active, inactive, or no SN. This was confirmed biochemically
using Western blotting against GFP which also did not reveal significant differences in the amounts of GFP protein (see below). This strongly suggested that even expression of enzymatically active SN was not generally deleterious to the transfected cells.

Next we performed triple transfections, using combinations of plasmids encoding HBV (pCHT-9/3091), GFP (pTR-UF5), and one each of the different SN expression plasmids. Cells transfected with expression plasmids for coreA80K-SN plus either GFP or HBV were analyzed in parallel. Western blots of equal aliquots from the individual transfections, developed using the polyclonal anti-coreSN denat serum, showed the expected results (Fig. 5A). The antiserum detected the coreSN fusions with either wild-type or coreA80K core (Fig. 5A, upper panel, lanes 1, 2, and 8), and the 21-kDa wild-type core protein, in similar amounts, in all transfections containing the HBV expression plasmid (lanes 2–8). In addition, slightly faster migrating bands with small but distinct mobility differences were seen in samples transfected with the nonfused SN expression plasmids coreA80K-SN (Fig. 5A, upper panel, lanes 3 and 4) and pcDNA6-SN (lanes 5 and 6), strongly suggesting that they represented SN and SNmut. Equally sized aliquots of the same lysates were analyzed in parallel (Fig. 5B, lower panel) with monoclonal antibodies to core protein and GFP. The intensities of the wild-type core protein signals paralleled those seen with the polyclonal anti-coreSN serum, and the GFP-specific signals were of comparable intensity in all corresponding samples. The slight signal reduction for wild-type core protein and GFP in the cells transfected with plasmid pcDNA6-SN (lane 5) was due to a slightly lower transfection efficiency because the band corresponding to SN itself as well as a nonspecific background band were also somewhat decreased. Importantly, no difference was seen for SN versus SNmut from the pcS1 vectors although the detectable SN amounts were, if anything, higher than from the pcDNA6 derivatives (Fig. 5A, upper panel, lanes 3 and 5). These data suggested that free SN lacking a core protein domain neither inhibited wild-type core protein nor GFP expression, and they confirmed the absence of a general inhibitory effect on host cell expression by the SN proteins.

Next we compared the DNA signals from enveloped virions, and from naked cores obtained without additive, or with the active and inactive nonfused SN proteins encoded by pcS1-SN and pcS1-SNmut by subjecting the cell culture supernatants from selected transfections to CsCl density gradient centrifugation. As controls, supernatants from cells co-transfected with the original coreSN fusion protein and its variant coreA80K-SN were analyzed in parallel. Fractions covering the density range of enveloped virions were pooled, and the DNA was analyzed by Southern blotting (Fig. 5B, left panel). As before, coreSN led to a drastic signal reduction, and a similarly pronounced decrease was seen with the coreA80K-SN variant (lane cA80K-SN), corroborating its suitability as a model compound in the above described coimmunoprecipitation experiments. By contrast, the nonfused nuclease (lane SN) had no detectable effect when compared with the HBV only (lane 0) or inactivated free SN (lane SNmut) samples. Very similar results were obtained when the DNAs from released naked cores were analyzed (Fig. 5B, right panel). Together, these data further corroborated the specific targeting to viral particles of coreSN, but not free SN, by means of its core protein domain.

coreSN Protein Moderately Reduces the Nucleic Acid Content but Not the Amount of Cytoplasmic Cores—To analyze at what stage of virion morphogenesis coreSN was acting we next compared the intracellular nucleocapsids produced in the absence or presence of coreSN and coreSNmut. Native agarose gel electrophoresis allows to analyze whether the core protein is present as intact particles (46). After transfer to a membrane, both core protein and the nucleic acid contained in the particles can be detected. Cytoplasmic lysates from singly and doubly transfected cells all contained similar amounts of particulate core protein (Fig. 6A). By contrast, the HBV-specific nucleic acid signal from co-transfection with coreSN was selectively decreased. Semiquantitation by densitometric scanning showed a reduction to about 30% of that in the other samples (Fig. 6B) when normalized to the scanned protein signals; similar values (between 15 and 30%) were obtained in several independent experiments. In contrast to encapsidated DNA, RNA may not be stable during the procedure and hence cannot reliably be determined. Cores with coreSN could therefore have contained a higher proportion of pgRNA that was not properly reverse transcribed to DNA, or have a defect in pgRNA encapsidation.

To test for a packaging defect we performed Northern blots comparing total cytoplasmic and encapsidated HBV RNAs (Fig. 6C); for normalization and proof of absence of nonencapsidated RNA in the core-derived samples the blot was also probed for GAPDH. All total RNA preparations gave similar signals, suggesting no significant intracellular activity of coreSN against free viral or cellular RNAs. For the encapsidated RNAs, full-length pgRNA was slightly weakened in the presence of coreSN; however, the smears of smaller products was proportionally stronger. This indicated that coreSN did not influence RNA packaging but possibly led to moderate RNA degradation.

Next we analyzed the DNAs contained in the core particles by Southern blotting. DNA from pure wild-type cores, expectantly, produced a smear of replicative intermediates extending up to the position of 3.2 kb linear HBV DNA, with a prominent single-stranded DNA band (Fig. 7A). A similar pattern was

3 J. Beck and M. Nassal, unpublished data.
FIG. 7. CoreSN reduces the template quality of encapsidated HBV nucleic acids. A and B. DNAs from intracellular cores were separated by agarose gel electrophoresis either without (−AMV), or with prior fill-in reaction (+AMV) with exogenous AMV-RT and detected using a digitonin-labeled HBV probe. M denotes digoxigenin-labeled marker fragments of the indicated sizes; ds refers to a double-stranded 3.2-kb HBV fragment present at 100 pg (lane 4) and 400 pg (lane 7); lane ss contains a heat-denatured, single-stranded aliquot of the same fragment. To visualize the weak bands obtained in the presence of coreSN, overexposures of lanes 1 plus 2, and 8 plus 9 are shown in B. Note the only partial shift toward longer products by AMV-RT.

obtained with coreSNmut. In the samples containing coreSN, however, all signals were reduced, with the strongest remaining band at the position of ssDNA (Fig. 7B). In several experiments, quantitation by PhosphorImaging showed a reduction to 15 to 30% compared with the other samples. This reduction in DNA but not RNA content by coreSN was compatible with either a failure in reverse transcription, or a preferential degradation of the reverse transcribed DNA. Evidence favoring the first possibility was obtained by analyzing the same samples after incubation with AMV-RT and dNTPs (Fig. 5, lanes + AMV). Authentic replicative intermediates isolated from cores can be extended into full-length products using an exogenously added polymerase (47). However, in contrast to the HBV-only, and HBV plus coreSNmut samples, only a small proportion of the material from coexpressed coreSN was extended to the 3.2-kb position (Fig. 7B). A semiquantitative evaluation by densitometric scanning revealed this full-length fraction to account for about 50% of the total signal intensity for the HBV-only and coreSNmut samples but at most 10% for the coreSN sample. This suggests the existence of breaks in the RNA and/or DNA templates that impede elongation.

DISCUSSION

In this report we show the successful application of CTVI to an important nonretroviral human pathogen, HBV. A chimera of the HBV capsid protein with the S. aureus nuclease, coreSN, drastically reduced the titer of DNA-containing enveloped HB virions in supernatants from cells co-transfected with an efficient HBV expression plasmid. The antiviral mechanism depends on nuclease activity because only minor effects were observed with the enzymatically inactive chimera coreSNmut; likewise, the presence of the core protein domain is essential as no significant effects were observed with nonfused SN. CoreSN prevents proper reverse transcription of the encapsidated RNA, or leads to rapid degradation of the genomic DNA. This “destruction from within” (26) is the hallmark of CTVI; unexpectedly, it appears to proceed, to a significant extent, intracellularly.

Antiviral Mechanism of CoreSN—CoreSN protein, when recombinantly expressed in E. coli, fulfilled two fundamental criteria for CTVI; it cointegrates into wild-type capsids, and the nuclease domains are internally localized (37); all data in the present study fully confirm this notion for a eukaryotic setting. However, it was not a priori clear whether this would translate into a detectable antiviral effect against HBV. In particular, the Ca2+ dependence of SN should require the particles to reach a Ca2+-rich milieu. If this was exclusively the extracellular space it would, in addition, require that the HBV envelope be permeable to Ca2+ ions, a factor for which no information exists.

Despite these concerns, coreSN, at 5% to at most 10% the concentration of wild-type core protein, led to an at least 20-fold, and probably higher, reduction in the titers of extracellular DNA containing enveloped particles. By contrast, coreSNmut exerted only minor effects, proving the dependence on nuclease activity of the antiviral mechanism. The apparent lack of full-length DNA forms as well as the failure of nonfused SN to show an antiviral effect corroborated a direct action of coreSN on the packaged genomes. Therefore, the particles had been in contact with sufficient concentrations of Ca2+ for nuclease activation, either after release from the cells, or during export, or inside the cells. Attempts to obtain evidence for extracellular activation by keeping the cells in medium with fetal calf serum as the only Ca2+ source (final concentration about 0.3 mM), or by deliberate addition of Ca2+ to the supernatants gave no conclusive answer because the results were essentially identical in all cases (data not shown). This suggested the nuclease had acted before we analyzed the particles. We therefore also investigated intracellular cores. Whereas their concentration was neither influenced by coreSN nor by coreSNmut protein, selectively the cores produced in the presence of coreSN contained about 70 to 90% less DNA. This reduction was not due to interference with encapsidation of the RNA template, hence coreSN either inhibited reverse transcription, or it led to a preferential degradation of viral DNA. To further minimize degradation after cell lysis, we performed controls with lysis buffers containing 10 mM, or even 100 mM EDTA to chelate the Ca2+ ions released from intracellular Ca2+ stores during work-up (data not shown), again with no significant impact. While post-lysis degradation remains formally possible, the data are fully consistent with an intracellular activation of the nuclease. Direct evidence against a post-lysis artifact is our ability to isolate RNA containing cores in similar amounts in the absence and presence of coreSN although SN degrades both RNA and DNA. Second, even a very low level activation, resulting in a single cut anywhere within the pgRNA, or the (−)-strand DNA, would prevent formation of mature double-stranded DNA. The failure to efficiently extend by an exogenously added polymerase the replicative intermediates produced in the presence of coreSN, but not of coreSNmut, supports this view.

A 70–90% reduction in the DNA content of intracellular cores, and a greater than 95% reduction in extracellular enveloped particles was reproduced in several independent experiments with coreSN, and also with the assembly-competent variant coreA80K-SN. We therefore propose the following stepwise interference model (compare Fig. 1A): in the cytoplasm, coreSN is cointegrated into core particles; the nuclease is activated to a low but sufficient extent to damage the encapsidated viral nucleic acid, resulting in fewer mature nucleocapsids. A second important component is the hepadnavirus-specific coupling of genome maturation and envelopment: of the fewer DNA containing nucleocapsids in the cytoplasm, even fewer 30-rich virions may be subject to further nuclease attack such
that, in sum, genome damage plus secretion inhibition amount to the observed greater than 20-fold overall inhibition.

While an intracellular nuclease activation seemingly contradicts previous reports (25, 34), including safety aspects, the two viewpoints may be reconciled considering the intracellular assembly mechanism of HBV, and the very low level activation required, namely a single hit in any of the strands used as template. Like for Moloney murine leukemia virus Gag-SN fusions (32) we found no evidence that coreSN is cytotonic; similarly, nonfused SN had neither a significant negative influence on cell viability, nor on GFP or HBV expression, suggesting SN itself had no, or only a marginal intracellular activity. A low level activity cannot be excluded because it would not be directed against one specific but against many different targets, and therefore remain undetectable. By contrast, coreSN integrated into the intracellularly assembled HBV core particles is kept in close spatial proximity to its specific nucleic coreSN integrated into the intracellularly assembled HBV core may be inhibition of capsid shell formation (22) and passive interference with RNA packaging and/or reverse transcription (24). Considering the active mechanism of coreSN it should be even more detrimental to HBV infectivity.

Application of CTVI to HBV may be a valuable new tool to combat this important viral infection. However, several important problems, in addition to delivery efficiency, need to be solved before any therapeutic application can be thought of. An essential intermediate step is to prove the concept in an in vivo setting. Toward this end, we are currently generating adenoviruses carrying the coreSN gene for tests in HBV-transgenic mice and other surrogate systems (8). Even more important will be experiments in naturally hepatitis B virus-infected woodchucks. They should also clarify whether induction of cytotoxic T cells against the transduced coreSN protein would soon abolish its antiviral efficacy or, by contrast, would further contribute to virus elimination by concomitantly inducing a response against wild-type core protein in the infected cells.

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