Precore-Mediated Inhibition of Hepatitis B Virus Progeny DNA Synthesis

CLAUDIA LAMBERTS,1 MICHAEL NASSAL,2 IRIS VELHAGEN,1 HANSWALTER ZENTGRAF,1 and CLAUS H. SCHRÖDER1*

Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, 1 and Zentrum für Molekulare Biologie, Universität Heidelberg, Im Neuenheimer Feld 282, 2 D-6900 Heidelberg, Germany

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The capacities to induce the synthesis of hepatitis B virus (HBV) unit-length DNA were compared for two HBV DNAs with an overall sequence diversity of about 10%. They had been cloned from serum (DNA2) and from a hepatocellular carcinoma (DNA4), respectively. As a major difference, DNA4 carries a translational stop signal preventing the synthesis of precore protein. Progeny DNA yields obtained after transfection with respective pregenome transcription units allocated DNA2 to a low-replicator and DNA4 to a high-replicator phenotype. Cotransfection of DNA2 interfered with progeny DNA synthesis induced by DNA4. By mutual exchange of restriction fragments, the region on the viral genome responsible for the differing replicator phenotypes was confined to a sequence comprising the 3'-terminal part of the X gene, core promoter, encapsidation signal e, precore/core gene, and 5'-terminal part of the pol gene. Point mutations in DNA2 abolishing proper expression of the precore gene strongly enhanced the yield of progeny DNA, whereas cotransfection of a precore expression plasmid with DNA4 or with the mutated DNA2 substantially lowered the amount of progeny DNA. Hence, precore expression acts as an inhibitory principle for HBV replication. The same stop mutation as in DNA4 has been found to arise frequently in virus carriers. Loss of precore expression and concomitant conversion to a more severe hepatitis, as observed in the course of a chronic infection, thus can be explained by a relaxation of replication-level control.

For many cytotoxic viruses, there does not appear to be a limit to replication other than the one set by the exhaustion of the host cell. It is likely that for hepatitis B virus (HBV) as a noncytotoxic virus, specific mechanisms exist which avoid the collapse of host cells. An approach to the identification of the viral functions involved can be based on comparative analyses of variants phenotypically differing in replication competence. Natural variants of this type exist (15b); however, determinants have not yet been studied in detail. A defect found to arise during chronic infection which abolishes expression of a secretory 17-kDa core (C) protein, HBV e antigen (HBeAg) (4, 6, 24, 28), so far is not suspected to control replication. Mutants of duck and woodchuck HBVs appeared to cause levels of replication and liver injury in infected animals similar to those observed with wild-type viruses (7, 9, 29).

In contrast to the 21-kDa core protein, HBeAg does not form stable aggregates (20). It derives from a 25-kDa precore protein which is translated from a separate set of mRNAs (pre-C mRNA) carrying on a 5′ extension an AUG codon which adds a precore frame to the core frame. During maturation which ultimately leads to translocation into the endoplasmic reticulum and to secretion, N- and C-terminal domains are removed (5, 12, 26, 34, 37). Antibodies against HBeAg constitute an important marker indicating virus clearance. Forms of HBeAg expressed on the cell surface have been considered as targets for an antibody-mediated elimination of infected cells (30). It is unknown why precore as an element nonessential for replication (38, 43) is conserved between mammalian and avian hepadnaviruses. A role mediating the entry into a chronic infection has been suggested, possibly via the induction of T-cell tolerance (3, 19).

In vitro studies of the replication competence of HBV genotypes are confined to transient expression systems (7, 36). They allow one to monitor on a quantitative basis the key processes in hepadnavirus replication, i.e., the transition from parental DNA via terminally redundant RNA back into progeny DNA (11, 35). In addition to its role as an RNA pregenome, terminally redundant RNA serves as mRNA for polymerase and core protein. Packaging of the pregenome requires in a first step the recognition of the encapsidation signal e by the viral polymerase (2).

In this study, the capacities to induce progeny DNA synthesis of two different HBV DNAs cloned from human serum and from a hepatocellular carcinoma, DNA2 and DNA4, respectively (10, 17), were compared and found to differ significantly. Of the two DNAs, which display an overall sequence diversity of 10.1%, DNA2 is pre-C plus (i.e., it possesses an intact precore frame), while DNA4 is pre-C minus as a result of a nonsense mutation at codon 28, a mutation commonly found on the DNA of precore-minus variants (4, 6, 24, 39). Precore and/or its derivatives, including HBeAg, could be identified as elements limiting replication.

MATERIALS AND METHODS

Plasmids. DNA constructs containing HBV DNA were derived either from DNA 4a1 (17), referred to here as DNA4, or from DNA2 (10). Plasmids pm2 and pm4 contain 3.2-kb unit-length HBV DNA cloned via the unique XhoI site into the pBluescript SK+ vector (Stratagene). Plasmids pd2 and pd4 contain a tandem repeat of XhoI-monomer DNA serving as the pregenome transcription unit. The relative orientation

* Corresponding author.
of viral DNA to vector DNA was identical for the monomer and dimer constructs. Fragment B of viral DNA, defined by restriction sites spanning map positions 1245 (NcoI) to 2306 (AvaI), and subfragment B', spanning positions 1245 to 1597 (DraI), were exchanged between pm2 and pm4 (numbering system starts at the T nucleotide of the unique XhoI site). Resulting plasmids with recombinant monomers were converted into pregenome transcription units (pd4B2, pd2B4, pd4B'2, and pd2B'4). Plasmid pd2fsC was derived from pd2 via cleavage with BglII at position 1857, repair with Klenow polymerase, and subsequent ligation. An insertion of four nucleotides (−1 frameshift) was confirmed (T7 sequencing kit; Pharmacia). The pd2 derivative pd2TAG-28 is pre-C minus as a result of a translational stop at codon 28. It was established via the exchange of a DNA fragment spanning map positions 1755 (Styl) to 2202 (MroI) by the respective fragment excised from plasmid pHIT/TAG-28 (see below).

The pHIT series of plasmids has been previously described (23). In brief, the plasmids contain a slightly overlength HBV2 DNA in which transcription of the pregenome and precore mRNA is driven by the authentic HBV core promoter. The derivatives pHIT/pC-AGG and pHIT/TAG-28 are pre-C minus as a result of mutation of the precore start codon (ATG to AGG) or introduction of a translational stop at codon 28 (TGG to TAG). Plasmids pHM-9/3091 and pHM-3/3097 contain an overlength HBV2 genome with the human metallothionein promoter instead of the HBV core promoter directing pregenome synthesis (15). Plasmid pCS1C1 (22) expresses the core 2 protein from a synthetic C gene (21). Plasmid pCHT-9/3068 is identical to plasmid pCH-9/3091 (22) except that cytomegalovirus immediately promoter-driven transcription starts at HBV position 3065 (numbering system starts at the A nucleotide of the core start codon), corresponding to one of the original precore mRNA sites (15), whereas no detectable amounts of pregenome are transcribed. Hence, predominantly precore protein is produced from this construct.

Cells and transfection. HepG2 cells established from a hepatoblastoma and proven to be negative for HBV markers (1) were transfected by the calcium phosphate method (13), with modifications described previously (16), or the procedure of Chen and Okayama (8) at a dose of 5 μg of plasmid DNA per 10⁶ cells. Results obtained with the two procedures were essentially identical. If not indicated otherwise, cotransfection experiments involved the application of plasmids at a 1:1 mass ratio, with each plasmid at a dose of 5 μg/10⁶ cells.

Preparation and detection of viral unit-length progeny DNA. A cytoplasmic fraction of 10⁶ transfected cells was prepared as described previously (16). To hydrolyze residual transfecting DNA, 90 U of Staphylococcus aureus nuclease (Boehringer Mannheim) was added to an aliquot representing 5 × 10⁵ cells together with 5 mM calcium chloride. Digestion with the restriction endonuclease DprI, which cuts exclusively dam-methylated plasmid DNA, resulted in similar progeny DNA yields and thus confirmed encapsidated viral DNA not to be accessible to the action of S. aureus nuclease. After incubation for 30 min at 37°C, proteinase K (0.2 mg/ml) and sodium dodecyl sulfate (1%) were added to release progeny DNA. After incubation for 2 h at 37°C, the DNA was obtained from the lysate by phenol-

chloroform-isoamyl alcohol (25:24:1) extraction and precipitation in the presence of 10 μg of tRNA. Viral progeny DNA was separated under denaturing conditions on 1% alkaline agarose gels (18).

Southern blot analysis (16, 33) was performed with nitro-

cellulose membranes (Schleicher & Schuell, Dassel, Germany). For detection of progeny DNA, monomer DNA excised from pd2 was ⁳²P labeled via the random priming procedure (labeling kit; Bethesda Research Laboratories) and used at 10⁶ cpm/ml for hybridization. In cotransfection experiments involving core expression plasmids, a subfragment defined by XhoI and AvaI (position 1336) lacking core gene sequences was used as a ⁳²P-labeled probe.

RESULTS

The pregenome transcription units pd2 and pd4 induce different levels of progeny DNA. Monomers of DNA2 and DNA4 cloned via the unique XhoI site into the pBluescript vector were converted into respective dimer constructs. The resulting pregenome transcription units, pd2 and pd4, were tested for replication capacity, defined as the capacity to induce upon transfection of HepG2 cells the synthesis of unit-length progeny DNA. For the identification of this 3.2-kb DNA, particle-associated DNA was isolated from the cytoplasm of transfected cells, separated under alkaline conditions on agarose gels, and subjected to the Southern blot procedure. Plasmid pd4 gave significantly stronger signals for HBV-specific 3.2-kb DNA (Fig. 1). Densitometric measurements indicated that the amount of unit-length progeny DNA exceeded the amount obtained in the pd2 transfection by a factor of 10 (190 pg/10⁶ cells versus 20 pg/10⁶ cells). Comparable yields were obtained in independent experiments with amounts of transfecting DNA ranging from 5 to 20 μg/10⁶ recipient cells (data not shown). A differential release of virus particles from the cells was not observed (data not shown). The difference in progeny DNA yields thus allocated DNA2 to a low-replicator and DNA4 to a high-replicator phenotype.

A region on viral DNA comprising core gene sequences determines levels of progeny DNA. To delineate the region on viral DNA which determines replication capacity, subregions of the transcription units pd2 and pd4 were systematically exchanged. A conversion of the low-replicator phenotype to the high-replicator phenotype and vice versa was obtained upon exchange of a DNA fragment referred to as region B. As depicted in Fig. 2A, this region is defined by restriction sites at positions 1245 (NcoI) and 2306 (AvaI) and comprises core and X-gene sequences (Fig. 2A). Figure 2B documents the replication competence of the respective recombinants, pd4B2 and pd2B4. The exchange of a 5′ portion of B, B' (Fig. 2A), from positions 1245 (NcoI) to 1597 (AvaI), into pd2 and pd4 resulted in substantial increases in replication capacity. The data suggest that a 1-kb segment 3′ of the 3.2-kb progeny DNA is required for full replication capacity.
trans-acting factor unit. (A) Cate relative synthesis. The areas reveal analogous replication. An analogous replication was introduced into the core gene region of pd2 at position 1857 (resulting in pd2fsC). The mutation renders the pregenome transcription unit replication incompetent (Fig. 4A, lanes m2). Progeny DNA synthesis was restored upon cotransfection with both the pm4 and pm2 monomer constructs (Fig. 4A, lanes m4 and m2) but to different levels; pm4 resulted in high, and pm2 resulted in low, yields of progeny DNA, comparable to the yields after transfection with the respective dimer constructs (Fig. 4A, lanes 4 and 2). These results suggest that (i) transcription and encapsidation signals of DNA2 and the polymerase expressed by this DNA are functionally equivalent to those of DNA4 and (ii) precore and core products expressed by DNA2 are inhibitory.

Considering these latter products individually, precore could be conceived as the sole cause for inhibition because it is expressed only by DNA2. However, core 2 cannot be

FIG. 1. Inhibition of transient DNA4 synthesis by cotransfecting pm2. (A) pd4 DNA was cotransfected with pm4 (lanes m4), pm2 (lanes m2), and pBluescript (lanes –). (B) Respective cotransfection experiments with pd2. The profiles in panels A and B were obtained in a single series of experiments. Each cotransfection was carried out in duplicate, applying an independently formed DNA precipitate. Following alkaline gel electrophoresis, the progeny DNA was transferred to one sheet of nitrocellulose, which was exposed for 6 h (A) and for 40 h (B).

To examine whether precore or core mediates inhibition, a frameshift mutation was introduced into the core gene region of pd2 at position 1857 (resulting in pd2fsC). The mutation renders the pregenome transcription unit replication incompetent (Fig. 4A, lanes m2). Progeny DNA synthesis was restored upon cotransfection with both the pm4 and pm2 monomer constructs (Fig. 4A, lanes m4 and m2) but to different levels; pm4 resulted in high, and pm2 resulted in low, yields of progeny DNA, comparable to the yields after transfection with the respective dimer constructs (Fig. 4A, lanes 4 and 2). These results suggest that (i) transcription and encapsidation signals of DNA2 and the polymerase expressed by this DNA are functionally equivalent to those of DNA4 and (ii) precore and core products expressed by DNA2 are inhibitory.

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FIG. 2. An X/core gene region defining the replicator phenotype. (A) Organization of the XhoI dimer as a pregenome transcription unit. Capital letters designate gene products of viral reading frames as follows: polymerase (P), surface proteins (S), X protein (X), and the core proteins (pre-C). The arrow represents pregenomic RNA. The areas labeled B and B’, defined by restriction sites NcoI-AvaI and NcoI-DraI, respectively, represent segments exchanged between pd2 and pd4. For further explanation, see text. (B) Inversion of the replication phenotype after exchange of fragment B. (C) Maintenance of the replication phenotype after exchange of fragment B’. The progeny DNA profiles in panels B and C, each taken from a single autoradiogram, were obtained with the DNAs as indicated: pd2 (lanes 2), pd4 (lanes 4), pd2B4 (lanes 2B4), pd4B2 (lanes 4B2), pd4B’2 (lanes 4B’2), and pd2B’4 (lanes 2B’4). They are based on two series of transfection experiments, each on a set of parallel cultures. Each transfection was carried out in duplicate, applying an independently formed DNA precipitate. Arrows indicate relative positions of the 3.2-kb marker DNA.

(DraI) on constructs pd4B’2 and pd2B’4 did not result in an apparent change in replication capacity (Fig. 2C).

A trans-acting factor specified by DNA2 inhibits progeny DNA synthesis. To examine whether DNA2 expresses a trans-acting factor restricting progeny DNA formation, plasmid pm2 was cotransfected with the high-replicator pregenome transcription unit. The results documented in Fig. 3A reveal strongly reduced progeny DNA yields for the pd4-pm2 cotransfection (lanes m2) compared with a pd4-pm4 (lanes m4) or a pd4-pBluescript cotransfection (lanes –). The results indicate an activity of DNA2 restricting DNA4 replication. Analogous pd2-monomer cotransfection experiments did not reveal an activity of DNA4 which would stimulate DNA2 replication (Fig. 3B).

FIG. 3. Differential transcomplementation of a precore/core defect. (A) Cotransfection at a 1:1 mass ratio of the precore/core defective pregenome transcription unit pd2fsC (2fsC) with pm4 (lanes m4) and pm2 (lanes m2) and transfection with pd4 (lane 4), pd2 (lane 2), and pd2fsC alone (lanes –). The profiles were obtained after a single series of transfection and taken from one autoradiogram. (B) Cotransfection at a 1:1 mass ratio of pd2fsC (2fsC) with pm4 (m4) and pCS1C1 (pCS1aC1). (C) Cotransfection of pd2fsC (2fsC) with pm2 (m2) and pCS1C1 (pCS1aC1) at 1:0.25 and 1:2 mass ratios (pd2fsC constant at 5 μg/10⁷ cells). The profiles in panels B and C were taken from a single autoradiogram and based on a single series of cotransfection experiments. Duplicate lanes are shown for profiles of progeny DNA induced by independently formed DNA precipitates. Arrows indicate relative positions of 3.2-kb marker DNA.
excluded, since its amino acid sequence differs at 11 positions from the sequence of core 4. To distinguish whether the inhibition was caused by precore or core, a construct expressing only core 2 protein (pCS1Cl) was used as helper. Cotransfection of pd2fsC with this construct resulted in progeny DNA levels as high as those observed with the pm4 helper DNA (Fig. 4B). Core 2 protein thus appeared to have the same complementation capacity as does core 4 protein. pd2fsC-pm2 and pd2fsC-pCS1Cl cotransfection at a high dose of the respective helper confirmed the differences in the apparent complementation capacities (Fig. 4C, lanes 1:2); at a low dose of the helper (Fig. 4C, lanes 1:0.25), the same low DNA yields were obtained. These data implicate precore as a mediator of inhibition, active only above a critical concentration.

A precore-expressing plasmid represses progeny DNA synthesis. Inhibition by precore was tested directly by using a DNA2 construct designed to exclusively express precore (pCHT-9/3068). On this construct, a cytomegalovirus promoter directs the synthesis of precore mRNA but not of core mRNA. Predominant precore expression was verified by Western immunoblot analysis (data not shown). Although pCHT-9/3068 closely resembles a pregenome transcription unit, no replication was observed upon transfection (data not shown). Cotransfection of pd4 with pCHT-9/3068 resulted in a strong inhibition which was significant even at low doses of pCHT-9/3068 DNA (Fig. 5A, lanes 1:0.1 and 1:0.05). At a 1:1 mass ratio, no progeny DNA could be detected even after prolonged exposure of the Southern blot (Fig. 5B).

Precore-minus mutations convert DNA2 into high-replicator DNA. Given an inhibitory action of precore, an alteration of DNA2 to the pre-C-minus genotype should increase its apparent replication competence. Mutated DNAs of this type have been established for the pregenome transcription units of the pHT series. These plasmids carry an overlength DNA2 on which transcription of terminally redundant RNA is driven by the authentic core promoter. The wild-type construct pHT-15/190 yields low levels of progeny DNA comparable to those obtained with the respective dimer construct of DNA2, pd2 (Fig. 6A). As predicted, two different point mutations that abolish precore expression by replacing the precore initiation codon (pHT/pC-AGG) or introducing of a translational stop at codon 28 (pHT/TAG-28) led to an increase in progeny DNA to levels observed with pd4 (Fig. 6A; Fig. 6B, lanes +). A similar high replication competence was obtained with pd2TAG-28, a pd2 dimer carrying the pre-C mutation of plasmid pHT/TAG-28 (data not shown).

Cotransfection of pHT/pC-AGG and pHT/TAG-28 with the precore expression plasmid pCHT-9/3068 resulted in an inhibition of progeny DNA synthesis below the limits of detection (Fig. 6B, lanes ±) as observed for the pd4/pCHT-9/3068 cotransfection (Fig. 5). We conclude that precore 2 inhibits, in trans, DNA2 as well as DNA4 progeny DNA synthesis. All of these results were reproducible when small restriction fragments were exchanged between the wild-type constructs and their pre-C-minus derivatives, confirming that the replication phenotypes were due to the pre-C point mutations and not to unintended sequence alterations in other parts of the cloned genomes. The data thus allocate the phenotypic differences of the described pregenome transcription units to precore mutations.

Precore-mediated inhibition of pregenome transcription units driven by a heterologous promoter. In the experiments described above, the expression of pregenome transcription units was governed by the HBV core promoter. Cotransfection of units driven by the human metallothionein promoter
The actual function of precore protein, or its secreted mature product, HBeAg, is still obscure. Though the pre-C region and hence the potential to produce precore protein is common to all hepadnaviruses, it is not essential for viral replication. Artificially introduced pre-C stop codons do not abolish infectivity, as shown for duck HBV (7, 29) and woodchuck HBV (9). Natural pre-C-minus mutants of the human virus which arise in the course of chronic infection support the notion that precore protein is nonessential for replication. The release of inhibition by mutations abolishing precore expression may cause increased replication levels not only in cell culture experiments but also in the infected liver. A more severe hepatitis which has been proposed to be associated with these mutations (25) could be the result of a more active replication of the mutated virus. An additional advantage for precore-minus variants would be the selective elimination of wild-type virus-infected cells expressing HBeAg (30). It is an interesting question why the intact precore frame is a conserved genetic trait despite the apparent advantage for pre-C-minus variants in the infected liver. It is conceivable that HBV as a noncytotoxic virus is capable of maintaining a chronic infection even after the loss of a function essential for infection of a new host. An example would be the surface protein which has been recognized to be dispensable for a virus replicating continuously in tumor tissue that could be passaged in the nude mouse (14). Regarding functions limiting replication, precore likely is not the only but appears to be an important protein, since the pre-C-plus virus is maintained as the epidemiological entity.

As part of a general principle restricting replication, a given precore protein should control not only the genome on which it is encoded. In line with this view, precore 2 inhibited both DNA2 and DNA4 progenome transcription units. If the generation of pre-C-minus variants in the course of a chronic infection is due mainly to an increase in replication competence, other genotypes should be similarly susceptible to precore 2 inhibition, and precore proteins of other strains should be inhibitory as well.

Given the complex nature of the HBV replication process, a loss of precore expression without concomitant alteration of the replication phenotype would not be unexpected and not necessarily in conflict with a prevalent role of precore as an inhibitory element. If observed with a low-replicator phenotype (15b), it may indicate the involvement of other determinants restricting replication; if observed with a high-replicator phenotype (40), it may indicate mutations abrogating inhibition, i.e., mutations affecting either the susceptibility to precore inhibition or the capacity of precore to inhibit.

The mechanism by which precore or its derivatives inhibit could possibly affect secondary rounds of replication, although replicative processes induced by transfecting DNA usually are considered to reflect a single cycle of replication. An infection of transfected cells by newly synthesized progeny virus is generally accepted to be unlikely, but an intracellular recycling of nucleocapsids (42, 45) may occur and direct secondary rounds of progenome synthesis via the accumulation of a nuclear pool of covalently closed circular DNA. In stably transformed cells directing replicative processes from an integrated transcription unit, the accumulation of covalently closed circular DNA is proven and considered to be a consequence of intracellular recycling (31). For the action of precore, however, putative secondary rounds of replication could be excluded as primary targets, since inhibition was also observed with a progenome tran-
progeny

or replicator DNA. This yields not interferon for the interaction RNA. its regulator, the maintenance properties of amine.

Hypothetically, precore could inhibit either transcription or particle maturation. An inhibition of transcription would be consistent with the fact that a precore derivative, P22, becomes translocated into the nucleus (27) and with the relatively low amounts of total viral RNA which were determined for the low-replicator DNA (15a). Although, as shown in this study, core promoter-driven transcription is not required for precore to act, the viral enhancer elements (32, 46) could represent specific targets. Suppression of their activities could be assumed to be the basis for inhibition, as suggested for the downregulation of replication by alpha interferon (41). Inhibition of particle maturation would be consistent with the high doses of precore expression plasmid which were required for inhibition of a cotransfected high-replicator DNA. This finding may indicate that some kind of stoichiometric interaction between core and precore protein prevents encapsidation and reverse transcription of pregenomic RNA.

For defining a role of HBeAg in restricting progeny DNA yields as opposed to a role of precore protein itself or its processing intermediates, it could be of importance to examine mutations known to determine the biophysical properties of HBeAg. Assuming that HBeAg functions as a regulator, its activity may be affected by mutations preventing the maintenance of a nonaggregated state (22a, 44).

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