ORIGINAL ARTICLE

Identification and characterization of a novel bipartite nuclear localization signal in the hepatitis B virus polymerase

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Abstract

AIM: To characterize the nuclear import of hepatitis B virus (HBV) polymerase (P) and its relevance for the viral life cycle.

METHODS: Sequence analysis was performed to predict functional motives within P. Phosphorylation of P was analyzed by in vitro phosphorylation. Phosphorylation site and nuclear localization signal (NLS) were destroyed by site directed mutagenesis. Functionality of the identified NLS was analyzed by confocal fluorescence microscopy and characterizing the karyopherin binding. Relevance of the structural motives for viral life cycle was studied by infection of primary *Tupaia* hepatocytes with HBV.

RESULTS: We identified by sequence alignment and functional experiments a conserved bipartite NLS containing a casein kinase Ⅱ (CKⅡ) phosphorylation site located within the terminal protein domain (TP) of the HBV polymerase. Inhibition of CKⅡ impairs the functionality of this NLS and thereby prevents the nuclear import of the polymerase. Binding of the import factor karyopherin-α2 to the polymerase depends on its CKⅡ-mediated phosphorylation of the bipartite NLS. In HBV-infected primary *Tupaia* hepatocytes CKⅡ inhibition in the early phase (post entry phase) of the infection process prevents the establishment of the infection.

CONCLUSION: Based on these data it is suggested that during HBV infection the final import of the genome complex into the nucleus is mediated by a novel bipartite NLS localized in the TP domain of HBV polymerase.

Core tip: The mechanism mediating import of the hepatitis B virus (HBV) genome into the nucleus is still not fully understood. We describe the identification and characterization of a bipartite nuclear localization signal (NLS) in the HBV polymerase that harbours a phosphorylation site for casein kinase Ⅱ (CKⅡ). Integrity of the phosphorylation site is crucial for the functionality of the NLS. Moreover, inhibition of CKⅡ prevents karyopherin-α2 from binding to the polymerase and thereby the import of the polymerase is impaired. Analysing the viral life cycle we observed that inhibition of CKⅡ blocks the import of the genome into the nucleus resulting in impaired cccDNA formation and so the establishment of the viral infection is prevented.

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INTRODUCTION

Infection with human hepatitis B virus (HBV) can cause acute or chronic inflammation of the liver. At present there are about 400 million chronically infected people worldwide. Moreover, persistently infected individuals have an increased risk of developing primary hepatocellular carcinoma (HCC)[1-6]. HBV is the prototype member of the hepadnaviridae family, which encompasses members isolated from woodchucks, ground squirrels and avian viruses isolated from *e.g.*, pekin duck, grey heron and stork.

The HBV polymerase (P) has four domains (Figure 1A). The terminal protein domain (TP) contains the tyrosine residue that primes DNA synthesis and covalently links P to the viral DNA[7-9]. The spacer domain has no known function other than to connect the terminal protein domain with the rest of P. The spacer domain, however, harbors at aa position 320 a thrombin cleavage site. This generates the possibility that not a full length protein, but a truncated polymerase is linked to the HBV genome due to intracellular proteolytic processing[10]. The reverse transcriptase domain and the RNase H domain contain the two enzymatic active sites catalyzing the reverse transcription of the RNA template to DNA and degradation of the RNA template.

Regarding the life cycle of HBV one open question concerns the fate of the viral nucleocapsid after the virus has entered the cell. There is evidence that the virus enters the cell by receptor mediated endocytosis and at the end of this process the nucleocapsid is released into the cytoplasm[11,12] and transported towards the nuclear pore complex[13,14]. Productive viral infection requires the transport of the HBV genome into the nucleus where the conversion into cccDNA occurs[15].

The phase of nuclear entry is not fully understood. It is discussed that the intact viral capsid shuttles the genome-polymerase complex into the nuclear basket of the nuclear pore complex[16-18] where a partial disassembly of the DNA-loaded nucleocapsid leads to a release of the polymerase-linked genome[13,19-21]. The polymerase-genome complex is too big for free diffusion through the nuclear pore complex.

Due to the facts that that although P is too big for free diffusion through the nuclear pore complex the polymerase-genome complex is imported into the nucleus and that a fraction of P protein is found within the nucleus[22-24] we examined the P protein for conserved motifs that could play a role for nuclear import and aimed to characterize the nuclear import of HBV polymerase-genome complex.

MATERIALS AND METHODS

Cell lines and culture conditions
The human hepatoblastoma cell lines HuH-7[25] and HBV producing cell line HepG2.2.15[26] were cultured in D-MEM medium containing 10% (v/v) fetal calf serum (FCS), 500 U/L penicillin and 100 mg/L streptomycin (PAA, Pasching, Austria). Inducible HBV producing cell line HepAD38[27] were cultivated like HepG2.2.15 but with 400 mg/L G418, 50 μmol/L hydrocortisone and 2.5 mg/L insulin (Sigma-Aldrich, Sleeze, Germany), additionally.

Subcellular fractionation
Subcellular fractionation was performed as described[28,29].

Infection of primary hepatocytes
Primary *Tupaia belangeri* hepatocytes were isolated, cultivated and infected as described[30,31]. Trypsin treatment for removal of attached viral particles was performed as described[12,31-33]. HBeAg and HBsAg synthesis were analysed 120 h after infection.

Generation of expression constructs
Plasmids were sub-cloned in *Escherichia coli* strain DH5α. The relevant mutations in the listed primer sequences are highlighted, restriction sites underlined and the corresponding backward primer sequences of mutation primers are reverse complementary to the forward primer if not citied otherwise.

The 1.2 fold HBV genome pJO19 (subtype ayw, genotype D) was derived from plasmid pSM2[26] by a stepwise truncation of the plasmid with *Bse*A I and *Aat* II. Mutant versions of the wild type genome with alterations in the polymerase coding sequence were generated based on pJO19: The CKII recognition site deficient genome pJO19[T100I] was generated using primer ΔCK II_fw (5'-CAg TTT gTA ggC CCA CTC ATA gTT AAT gAg AAA AgA AgA TTg CAA TTA ATT ATg CCT gCC), the pseudophosphorylated genome pJO19[T100D] was generated with primer *CKII_fw (5'-CAg TTT gTA ggC CCA CTCg ACg TTA ATg AgA AAA gAA gAT TgC AAT TAA TTA TgC CTg CC), the genome with an inactivated NLS pJO19[K105D,K106S] was generated with forward primer ΔNLS_fw (5'-ggC CCA CTC ACA gTT AAT gAg CAg TCT AgA TTg CAA TTg ATT ATg CCT g). GFP wild type expression was obtained by pEGFP-N1 (BD, Heidelberg, Germany). The N-terminal fusions of NLS signals to GFP were generated from a modified version of pEGFP-N1 (pJO21). In pJO21 the first base of the transcriptional START of the GFP reading frame was deleted by site directed mutagenesis to prevent wild type GFP expression using primer GFP Δstart_fw (5'-CCA CCg gTC gCC ACC Tgg TgA gCA
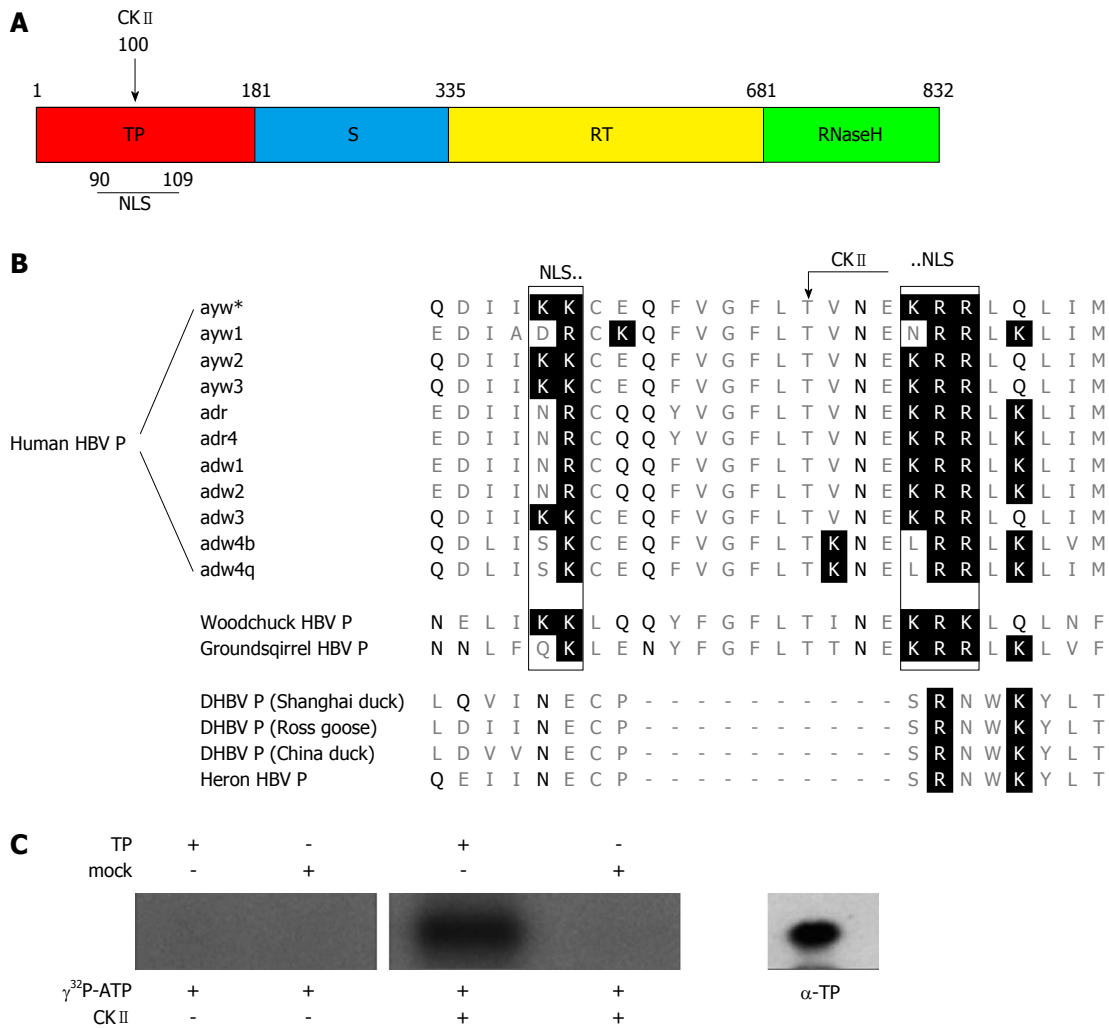
A

B

C

Figure 1 Sequence alignment of the hepatitis B virus polymerase from various virus subtypes and species. A: Scheme of the hepatitis B virus (HBV) polymerase showing the different domains. The terminal protein domain (TP) is shown in red, the spacer domain (S) in blue, the reverse transcriptase domain (RT) in yellow and the RNase H domain in green. The numbers designate the amino acids referred to HBV genotype D. The positions of the casein kinase Ⅱ (CKⅡ) phosphorylation site and of the bipartite nuclear localization signal (NLS) identified in this study (see Figure 1B) are indicated; B: This figure shows amino acid alignment of Q86-M111 referred to the sequence of subtype ayw*, which was used in this study. Basic amino acids are highlighted by a black background and polar (δ+) amino acids are highlighted by black letters. A conserved protein kinase CKⅡ recognition site (arrow) was identified in orthohepadnaviruses at Thr100 (protein kinase CKⅡ: T/S-X-X-E/D; X = any amino acid). A bipartite nuclear localization signal was identified in *orthohepadnaviruses*, which is flanking the CKⅡ recognition site by its two basic amino acid clusters (rectangles). All identified putative motifs were not found in the aligned P proteins of the compared *avihepadnaviruses*; C: Purified TP domain and mock purified proteins (empty vector products) were incubated with [γ-^{32}P] ATP and recombinant protein kinase CKⅡ. To control auto-phosphorylation TP domain was incubated with [γ-^{32}P] ATP in absence of the kinase. The protein specificity was verified by Western blotting using a TP specific antibody (α-TP) on a separate lane. All experiments were performed in triplicate. One representative is shown.

Agg gCg Agg). The plasmid pNLS$_{NP}$-GFP was generated by amplifying human nucleoplasmin from a cDNA library. The polymerase chain reaction (PCR) product was flanked by terminal *Bgl*Ⅱ sites, which were generated by primer N-NLS-3fw (5'-TTT AgA TCT gTT CAg ggC CAg TgC) and primer N-NLS-3bw (5'-TTT AgA TCT TTT TAC TTT TTT CTg Tgg). The PCR product was ligated to the *Bam*HI cut pJO21. An engineered *Bgl*Ⅱ site followed by an optimized Kozak sequence[34] with transcriptional START was inserted immediately upstream of the NLS sequence by site directed mutagenesis using forward primer N-NLS-4fw (5'-ggA TgT gAA ACT CTT AAg TAg ATC TCg CCA CCA Tgg gAA AgC ggT CTg CCC CTg g). The dispensable upstream sequence was removed by a *Bgl*Ⅱ digest. Analogue to pNLS$_{NP}$-GFP, the plasmid pNLS$_{TP}$-GFP was generated by amplifying

the putative NLS of the TP domain from pJO19 using forward primer TP-NLS-3fw (5'-CCC ggA TCC ATg CCC CTA TCC TAT CAA CAC) and backward primer TP-NLS-3bw (5'-TTT AgA TCT TCT TTT CTC ATT AAC Tg). The upstream *Bgl*Ⅱ site, the Kozak signal and the transcriptional START was inserted using primer TP-NLS-4fw (5'-CCT AAT ATA CAT TTA CAC CAA gAC AgA TCT CgC CAC CAT ggT gAA AAA ATg TgA ACA gTT TgT Agg C).

The P-expression constructs were generated based on pJo19 or the corresponding mutants by PCR and subcloned in the pCDNA.3 eukaryotic expression vector.

Site-directed mutagenesis

was performed as described[35] by amplification of the whole plasmid using *Pfu* Turbo Hotstart DNA-Poly-

merase (Invitrogen, Karlsruhe, Germany). All synthetic oligonucleotides are purchased by Tib-Molbiol, Berlin, Germany.

Purification of recombinant proteins

The coding sequence for the TP domain (amino acid 1-181) of HBV polymerase was amplified by PCR and inserted into the eubacterial expression vector pQE60 (Qiagen, Hilden, Germany), which encodes a C-terminal His-tag. Expression was performed at room temperature to reduce the formation of inclusion bodies. The soluble fraction of recombinant TP was purified by affinity chromatography on a Ni-NTA column under native conditions as described recently[36]. TP protein inclusion bodies were solved using 6 mol/L guanidine hydrochloride. Ni-NTA affinity purification under denaturing conditions was performed as described[37]. For further purification the TP containing fractions were pooled, dialyzed to buffer A$_{MS}$ (6 mol/L urea, 20 mmol/L sodium acetate, 2% (v/v) ethanol, pH 5.5) and polished by cationic exchange chromatography using a pre-packed Tricorn MonoS column (GE Healthcare, Freiburg, Germany). The elution was performed by a linear gradient over 20 column volumes (cv) between buffer A$_{MS}$ and A$_{MS}$ containing 1 mol/L sodium chloride.

In vitro phosphorylation

experiments were performed using highly purified *E. coli* produced terminal protein domain dialyzed against kinase buffer (25 mmol/L Tris-HCl, 25 mmol/L beta-glycerophosphate, 10 mmol/L MgCl$_2$, 1 mmol/L DTT, pH 7.5). Phosphorylation was started by addition of 10 µCi [γ-^{32}P] ATP and recombinant human CKⅡ (Merck, Darmstadt, Germany). After 30 min incubation at 30 ℃ the reaction was stopped by addition of SDS sample buffer and heat treatment (5 min, 95 ℃). Proteins were separated by 12% (v/v) SDS-PAGE and detected by autoradiography.

On column phosphorylation of was performed using polished, denatured TP from the cationic exchange chromatography. After addition of 20 mmol/L 2-mercaptoethanol and 100 mmol/L Tris, pH 8 the TP containing fraction was incubated for 1 h at room temperature with 2 cv Ni-NTA agarose, which was pre-washed with buffer A$_D$ (6 mol/L urea, 100 mmol/L Tris, pH 8.0). The coupling efficiency was 90%, which was determined by optical density at 280 nm. Equal amounts of TP-agarose were loaded on two empty chromatography columns. A controlled refolding of TP was initiated by a 30 cv linear gradient of buffer A$_D$ to buffer R (20 mmol/L Tris, 134 mmol/L sodium chloride, 10% (v/v) glycerol, 10% (v/v) sucrose, 20 mmol/L 2-mercaptoethanol, 0.1% (v/v) Tween-20, pH 7.5). The buffer was changed by a 10 cv linear gradient to buffer K (20 mmol/L Tris, 50 mmol/L potassium chloride, 10 mmol/L imidazole, 20 mmol/L 2-mercaptoethanol, 20 mmol/L beta-glycerol phosphate, 0.1 mmol/L sodium ortho-vanadate, 0.1% (v/v) Tween-20, pH 7.5). 2500 U recombinant protein kinase CKⅡ (Merck, Darmstadt, Germany) was injected together with 200 µmol/L GTP in buffer K and the column was incubated for 3 h at 28 ℃. The reaction was stopped by washing the column with 5 cv buffer K.

Binding partner fishing

Six confluent grown 175 cm² culture flasks of HuH-7 cells were lysed by sonication in TBS buffer including protease inhibitor cocktail (1 mmol/L PMSF, 5 mg/L aprotinin, 1 mg/L pepstatin, 4 mmol/L leupeptin, 1 mmol/L EDTA). The crude lysate was cleared by centrifugation at 20000 rpm in a TST41 rotor. The lipid content of the supernatant was reduced by precipitation of the proteins at 75% (w/v) ammonium sulfate. The protein pellet was resolved in TBS buffer and desalted by gel filtration using a HiTrap Desalting column (GE Healthcare, Freiburg, Germany). The desalted 75% (w/v) ammonium sulfate fraction of HuH-7 cell lysate was diluted in buffer B (20 mmol/L Tris, 25 mmol/L beta-glycerol phosphate, 1 mmol/L ortho-vanadate, 20 mmol/L 2-mercaptoethanol, 0.1% (v/v) Tween-20, pH 7.5). Equal amounts of this protein solution were injected to the two terminal protein bound columns and to a blank column only loaded with nickel-agarose. After washing the three columns for 5 cv with buffer B the binding partners were eluted by 1 mol/L sodium chloride and analyzed by western blotting using antibody karyopherin-α2 (C-20) purchased from Santa Cruz, Heidelberg, Germany.

Kinase inhibitor experiments

HepG2.2.15 cells were seeded in 6-well plates with an initial density of 5×10^5 cells/well. Three days after cell seeding CKⅡ inhibitor DMAT (Merck, Darmstadt, Germany) was added to the consumed cell culture medium for 2.5 h. After washing with phosphate buffered saline the cells were incubated for additional 18 h with consumed cell culture medium from the non-HBV producing cell line HuH-7, supplemented with the same concentration of DMAT as already pre-treated. The concentration of the solvent dimethyl sulfoxide (DMSO) was kept at 0.7% (v/v) in all investigated samples.

HBV quantification

Virus genomes were extracted from cell culture supernatant using High Pure Viral Nucleic Acid Kit and determined by LightCyler PCR (Roche, Mannheim, Germany) using a HBx specific probe[38]. cccDNA was extracted from HBV infected *Tupaia* hepatocytes according standard protocols for genomic DNA extraction with phenol/chloroform[39]. Southern blotting of HBV DNA using a HBV specific ^{32}P labeled probe was performed as described[31].

Endogenous polymerase reaction

HuH-7 cells (3×10^5) were seeded in 6-well plates and transfected with 2 µg HBV DNA using Fugene 6 (Roche, Mannheim, Germany). The enveloped viral particles were precipitated 5 d after transfection by sheep anti-HBs polyclonal serum (kindly gift from Klaus-H. Heermann, University Goettingen, Dept. Virology, Germany) and swollen protein-A sepharose beads (Sigma-Aldrich,

Sleeze, Germany) from the cell culture supernatant. The endogenous polymerase reaction (EPR) reaction was performed as described[40] using 10 μCi [α-^{32}P] dCTP (GE Healthcare, Freiburg, Germany) for the labeling.

Confocal laser scanning microscopy

HuH-7 cells (5×10^4) were grown on cover slides in 24-well plates and fixed with 4% formaldehyde/PBS for 30 min at 25 ℃. For visualization of actin filaments, the cells were stained with FITC-labelled phalloidin (Sigma, Munich, Germany). Staining was performed as described[41,42]. Rabbit-derived polyclonal TP-specific or spacer domain-specific sera were used for detection of P. Confocal laser scanning microscopy (CLSM) immunofluorescence was performed using the Zeiss LSM 510 microscope (Zeiss, 20 × and 63 × objectives).

RESULTS

Identification of conserved motifs on HBV polymerase

Previous data of our lab based on cell permeable HBV capsids[13] and studies by M Kann's lab[21] argue against the concept that intact viral capsids[17] or HBcAg shuttles the genome-polymerase complex into the nucleus[16,20]. Based on the data from the TLM-nucleocapsid model system it can be assumed that a partial disassembly of the capsid occurs within the nuclear pore complex or in a perinuclear domain that leads to a release of the genome complex. This raises the question about the final import of the polymerase linked genome into the nucleus.

Sequence analysis of HBV polymerase subtype ayw predicted the existence of a bipartite nuclear localization signal within the terminal domain (TP) (amino acid K90-K91, K104-R106) (Figure 1A). Moreover, a phosphorylation site for CKⅡ (T100) was found within the putative NLS (Figure 1B). The family hepadnaviridae encompasses two genera: orthohepadnavirus and avihepadnavirus. Further analysis revealed that the bipartite NLS and the enclosed CKⅡ phosphorylation site are conserved within the orthohepadna viruses but not within the avihepadna viruses (Figure 1C). The existence of a functional NLS would enable the transfer of the genome-polymerase complex through the nuclear pore complex into the nucleus.

TP domain is phosphorylated by CKⅡ in vitro

To control experimentally whether the predicted phosphorylation site indeed can be phosphorylated by CKⅡ in vitro phosphorylation was performed. Thereto, highly purified recombinant TP domain was incubated with [γ-^{32}P] ATP in the presence of CKⅡ. To exclude any phosphorylation by contaminating kinases the purified TP domain was incubated as described above, but CKⅡ was omitted. As an additional control a mutated TP domain was used in which the predicted CKⅡ phosphorylation site was destroyed by a T to A conversion at aa position 100. Figure 1B shows a significant specific phosphorylation of the TP domain only if CKⅡ is present.

In case of the controls no significant phosphorylation was observed. To confirm the identity of the phosphorylated species with the TP domain Western blotting analysis was performed (Figure 1C, right panel). This indicates that the predicted kinase recognition site on the terminal protein is indeed accessible for phosphorylation.

P protein harbors a functional bipartite NLS, which depends on phosphorylation

To study the functionality of the TP-derived putative bipartite NLS HuH-7 cells were transfected with an expression plasmid encoding for a fusion protein of the putative NLS and GFP (NLS$_P$-GFP). As a positive control served a 17 aa long prototype NLS (K142 to K158) derived from human nucleoplasmin (gi114762) fused to the amino terminus of GFP (NLS$_{NP}$-GFP). The intracellular distribution of the GFP fluorescence was quantified by confocal laser scan microscopy in living cells. Compared to wild type GFP expression, which was found evenly distributed within the cell (Figure 2A), the level of NLS$_P$-GFP was approximately 30% higher in the nucleus then in the cytosol (Figure 2B). In case of the positive control (NLS$_{NP}$-GFP) an about 75% elevated level of GFP specific fluorescence in the nucleus was observed (Figure 2C). This confirms that the predicted sequence indeed acts as a functional NLS. To analyze a putative relevance of CKⅡ-dependent phosphorylation for the functionality of the TP-derived NLS, NLS$_P$-GFP producing cells were incubated for 2 h with CKⅡ inhibitor DMAT prior analysis by confocal microscopy. The quantification of GFP fluorescence revealed that presence of the CKⅡ inhibitor prevented the directed nuclear enrichment of the NLS$_P$-GFP (Figure 2D). Comparable results were obtained for cells expression the NLS$_P$-GFP(T100A) mutant. An equal distribution comparable to GFP was observed (data not shown).

To study the relevance of the identified bipartite NLS for the subcellular distribution of the HBV polymerase cells were transfected with an expression construct encoding for HBV polymerase and analyzed by confocal immunofluorescence microscopy or subjected to cell fractionation. The immunofluorescence microscopy shows that in HBV P overproducing cells a fraction was found within the nucleus. However, in cells overexpressing the T100A mutant that destroys the CKⅡ phosphorylation site the nuclear-localized fraction disappeared and the P was exclusively found in the cytoplasm (Figure 2E). Western blotting analysis of the cytoplasmic and of the nuclear fraction confirmed that in addition to the cytosolic fraction a significant amount of P was detectable in the nucleus. However in cells treated with DMAT or overexpressing the T100A mutant of the putative CKⅡ phosphorylation site no P-specific signal was detectable in the nuclear fraction (Figure 2F).

Taken together these results indicate that the HBV polymerase harbors a bipartite nuclear localization signal which functionality is dependent on CKⅡ-mediated phosphorylation.
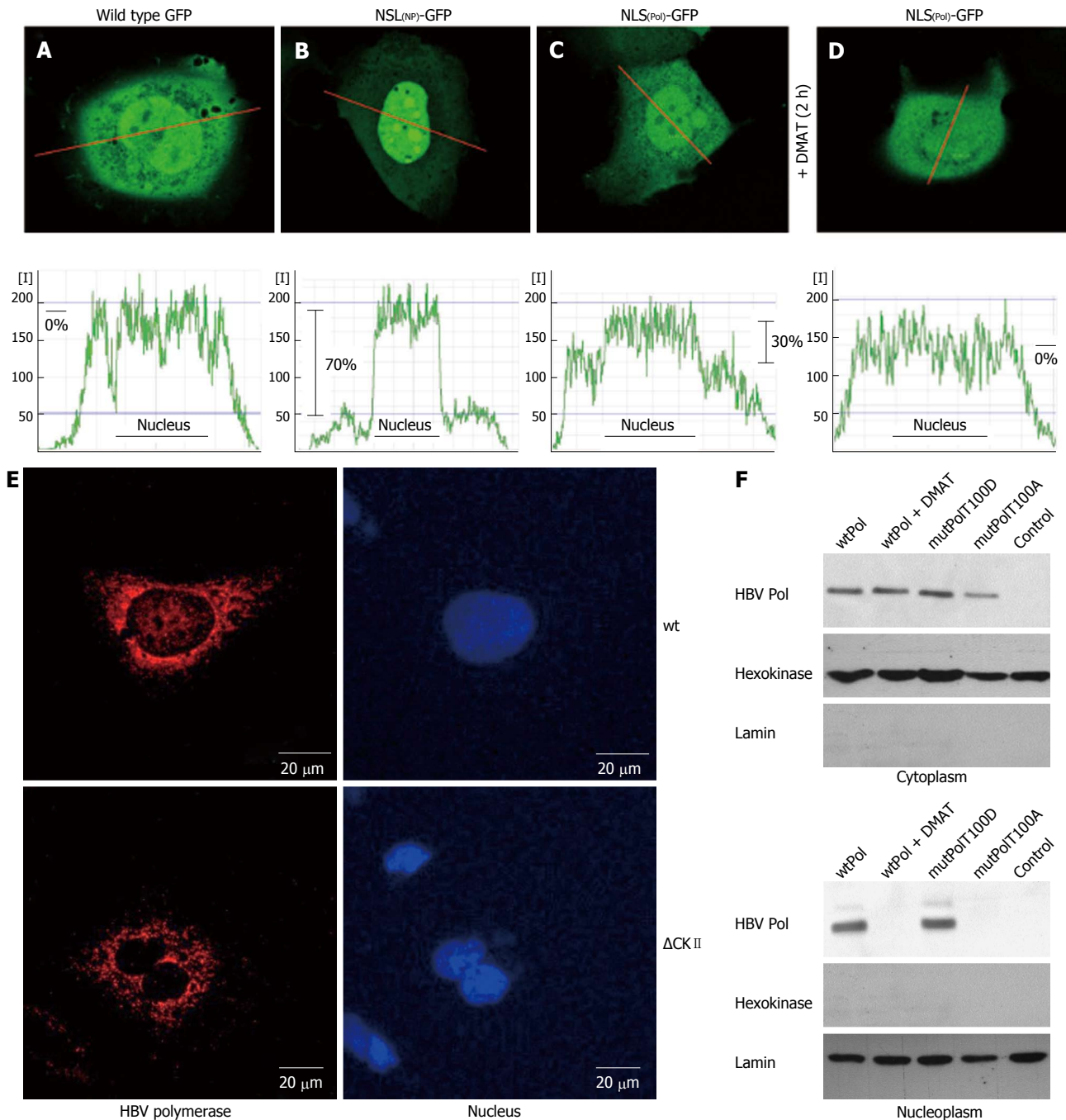
Figure 2 Hepatitis B virus polymerase harbors a functional nuclear localization signal in the terminal protein domain. A-D: HuH-7 cells were transfected with (A) the negative control wild type green fluorescent protein (GFP) (pEGFP-N1), (B) a positive control: GFP fused to a prototype bipartite nuclear localization signal (NLS) of human nucleoplasmin, (C) GFP fused to the putative bipartite NLS of hepatitis B virus (HBV) P protein, (D) the same as (C) but cells were treated with 10 × IC$_{50}$ of casein kinase II (CK II) inhibitor 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) 2 h prior analysis. The fluorescence was measured in living cells by confocal laser scan analysis. The central layer (out of 6) was quantitated along the red indicated line and displayed in the corresponding graph of the lower panel as relative fluorescence intensity [I]. Differences of mean fluorescence intensities in the cytoplasm and within the nucleus (indicated as black line in the graph) were calculated and are indicated in percent. One representative cell for each fusion protein is shown; E: Confocal immunofluorescence microscopy of HuH-7 cells transfected with an expression vector encoding wt P or the mutant ΔCK II (= T100I) that is not phosphorylated by CK II. For detection of P (red) a rabbit-derived spacer domain-specific serum was used. Nuclei were stained with DAPI (blue); F: HuH-7 cells were transfect with the indicated expression vectors and left untreated or treated with 10× IC$_{50}$ of CK II inhibitor DMAT 5 h prior analysis. Transfection with pCDNA.3 served as control. Cells were lysed, the cytosolic and nuclear fraction were isolated by differential centrifugation and analyzed by western blotting. For detection of P a TP-domain specific serum was used. Detection of hexokinase and of lamin served as loading control and as control for the purity of the subcellular fractions. All experiments were performed in triplicate. One representative is shown.

Binding of karyopherin-α2 to TP depends on CK II mediated phosphorylation

Karyopherin-α2 is an essential factor for NLS-mediated nuclear import. Therefore, it was investigated whether the data described above are reflected by an increased binding of karyopherin-α2 to *in vitro* phosphorylated TP as compared to unphosphorylated TP. Equal amounts of terminal protein domain were immobilized on two columns.

Fractions:	2	3	4	2	3	4	2	3	4	FP	F

Karyopherin-α2

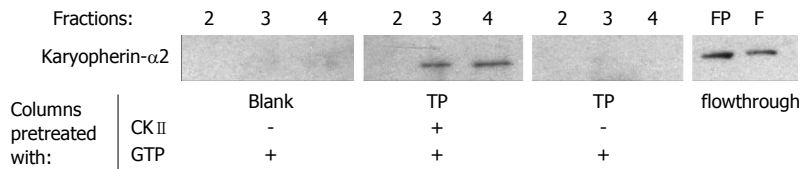| Columns pretreated with:		Blank	TP	TP	flowthrough
	CKⅡ	-	+	-	
	GTP	+	+	+	

Figure 3 Binding of karyopherin-α2 to terminal protein depends on casein kinase Ⅱ mediated phosphorylation. The interaction of soluble protein fraction of HuH-7 cells to immobilized terminal protein (TP) domain was investigated by western blotting of the sodium chloride eluted fractions 2-4. Karyopherin-α2 bound only to the casein kinase Ⅱ (CKⅡ) pre-treated TP column (upper panel). FP: Flow through of the CKⅡ treated TP column; F: Flow trough of the untreated TP column.

One column was *in vitro* phosphorylated by CKⅡ and its substrate GTP the other and a blank control column were treated equally without the addition of the kinase. The pretreated columns were equilibrated and loaded with the desalted 75% (w/v) ammonium sulfate fraction of HuH-7 cell lysate. Finally, binding partners were eluted by sodium chloride and the eluted fractions were analyzed by Western blotting using a karyopherin-α2 specific antibody. Interestingly, karyopherin-α2 was only found in the eluate of the column with CKⅡ pre-treated TP (Figure 3). This indicates clearly that *in vitro* binding of karyopherin-α2, the key enzyme for nuclear import, is dependent on CKⅡ phosphorylation of the terminal protein.

Inhibition of CKⅡ impairs HBV replication in primary Tupaia hepatocytes

To study the relevance of the NLS and of the CKⅡ phosphorylation site for the HBV life cycle the HBV P protein was mutated based on a recombinant 1.2 fold HBV genome (subtype ayw) by site directed mutagenesis. The changes in the DNA sequence did not affect other reading frames or regulatory elements.

In the NLS-deficient mutant (ΔNLS) the NLS is inactivated by manipulating the basicity of the downstream cluster to K105D and K106S. The putative CKⅡ recognition site on the P protein is destroyed by a T100I substitution (ΔCKⅡ), whereas a pseudo-phosphorylated mutant was generated by a T100D conversion (CKⅡ*). However, the attempt to produce mutant virus by transfection of HepG2 or HuH-7 cells with the respective 1.2 fold genomes failed in case of ΔNLS and of the ΔCKⅡ mutant (Figure 4A). In both cases significant less virus was produced (about hundredfold) as compared to the wt genome or the mutant encoding the *CKⅡ** mutant. In a transfection experiment the nuclear import is not the limiting step. It can be concluded that mutations affecting the integrity of the NLS motive or of the CKⅡ phosphorylation site cause secondary effects affecting the functionality of the polymerase. Therefore a direct analysis of the relevance of the NLS and of the CKⅡ substrate domain based on mutated virus was not possible.

The *in vitro* data described above had shown that the functionality of the NLS identified in the TP-domain depends on the CKⅡ-dependent phosphorylation (Figure 2). To study the relevance of CKⅡ-dependent phosphorylation of the TP domain for HBV life cycle primary *Tupaia* hepatocytes were infected with wtHBV particles for 8 h. Adherent HBV particles were removed by trypsin treatment as described above. After infection the cells were grown for 36 h in the presence of the cell permeable small molecular CKⅡ inhibitor DMAT and the virus replication was analyzed by quantification of HBsAg and HBeAg secretion (Figure 4B). Moreover secreted viral particles were quantified by real time PCR. Both approaches revealed that inhibition of CKⅡ by DMAT caused a strong and significant reduction of virus replication. This was further confirmed by analysis of cccDNA formation in infected PTHs. Inhibition of CKⅡ by increasing concentrations of DMAT abolishes cccDNA formation (Figure 4B).

For a more detailed analysis primary Tupaia hepatocytes were infected as described above. One, two, four and seven days after infection the hepatocytes were grown for 36 h in the presence of the cell permeable small molecular CKⅡ inhibitor DMAT and the virus replication was analyzed by quantification of HBsAg and HBeAg secretion (Figure 4C and D). Both approaches revealed that inhibition of CKⅡ by DMAT at 1 and 2 d after infection caused a strong and significant reduction of virus replication, while inhibition after 4 and 7 d *pi* resulted only in a small reduction of virus replication.

To control the specificity of the observed effect the constitutively HBV expressing cell line HepG2.2.15 were instrumental. This cell line harbors a stably integrated HBV genome. Due to the stable integration of the genome the re-import of *de novo* synthesized genomes plays a minor role for maintaining the pool of transcriptional templates. Therefore, inhibition of polymerase import should exert a small effect. HepG2.2.15 cells were treated with DMAT, HBeAg and HBsAg secretion were analyzed by ELISA and virus secretion was quantified by LightCycler PCR (Figure 4B). Under these conditions inhibition of CKⅡ with DMAT did slightly but not significantly reduce virus secretion as compared to the solvent control (Figure 4B). Comparable results were obtained for the cell line HepAD38 (data not shown).

Taken together these data provide indirect evidence that the functionality of the NLS in the TP domain of P is required for the import of the viral genome into the nucleus.

DISCUSSION

It is well as established that HBV replicates its genome inside the nucleus (recent reviews[18,43,44]). This raises the question about the post entry transport of the genome complex to and about its final import into the nucleus.
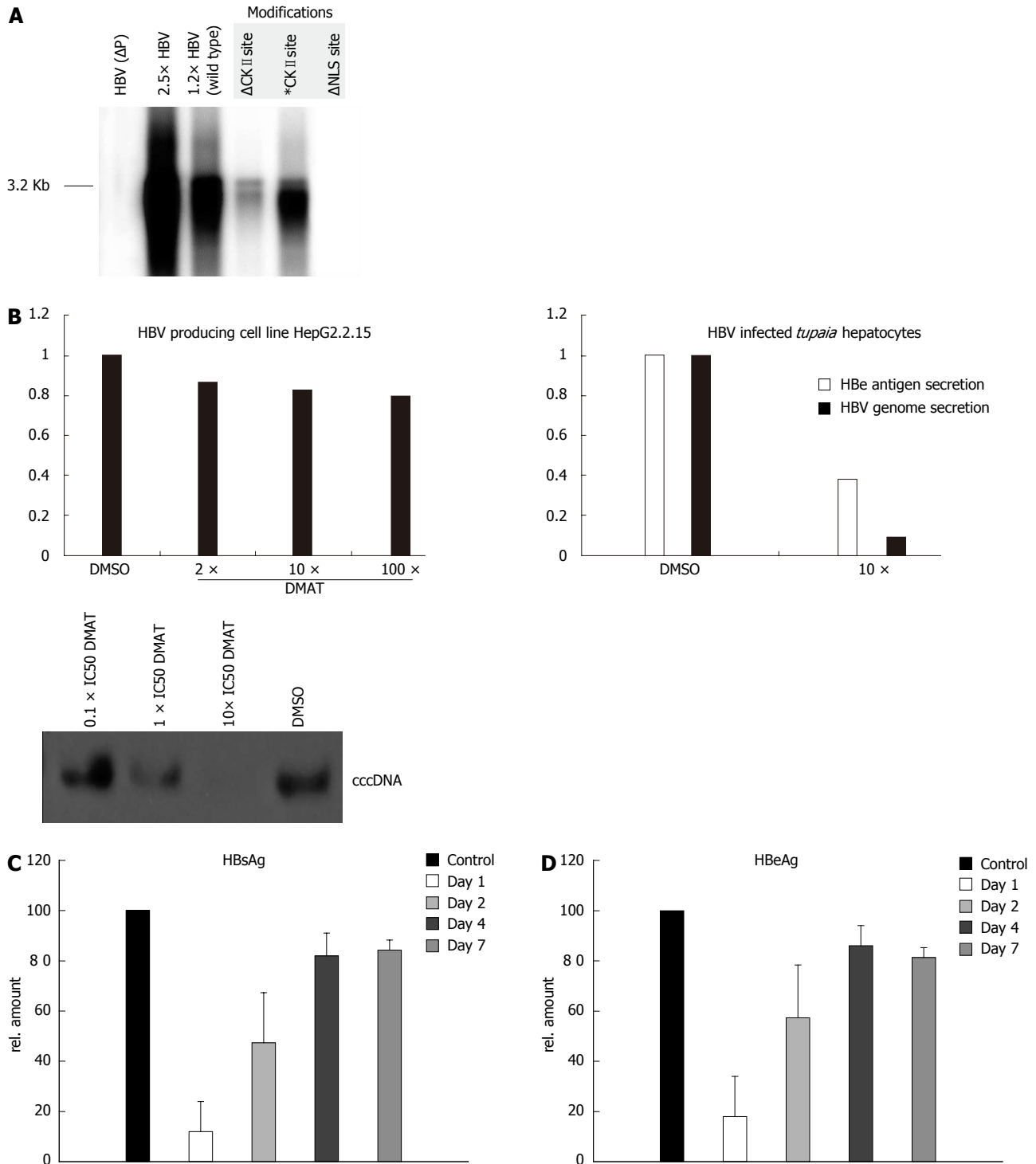
Figure 4 Casein kinase Ⅱ inhibition impairs hepatitis B virus replication in infected primary Tupaia hepatocytes. A: HuH-7 cells were transfected with mutant versions of a 1.2 fold hepatitis B virus (HBV) genome. After 5 d the secreted viral particles were precipitated with a HBs specific antibody and the containing 3.2 kb HBV genomes were visualized by the radioactive tracer [α-^{32}P] dCTP incorporated using the endogenous polymerase activity. The unphosphorylated form of the casein kinase Ⅱ (CKⅡ) recognition site in the P protein was simulated by a T100I substitution (Δ) and the pseudo-phosphorylation was simulated by a T100D substitution (*) in the 1.2 fold HBV wild type genome. The nuclear localization signal (NLS) was inactivated by mutating the downstream basic cluster (K105D and K106S) on the P protein. A 2.5 × HBV genome and a P deficient genome [HBV (P-)] served as controls; B: Primary *Tupaia* hepatocytes were infected with HepAD38 derived HBV and post infection treated for 36 h with solvent DMSO or 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) (10 × IC50). Twelve days after infection the HBV genome secretion was measured by Lightcycler polymerase chain reaction. The cccDNA content of infected *Tupaia* hepatocytes that were incubated for 36 h with the indicated amounts of DMAT was visualized by Southern blot using a HBV specific probe. The specificity of DMAT incubation was analyzed by 2 h inhibitor pre-treatment of the stably HBV transfected cell line HepG2.2.15 followed by treatment of 36 h with 10 × IC50 CKⅡ inhibitor DMAT (IC50 in rat liver = 150 nmol/L) and genome secretion was compared to the solvent control DMSO measured by Lightcycler PCR. All experiments were performed in triplicate. The figure shows one representative experiment; C, D: Primary *Tupaia* hepatocytes were infected with HepAD38-derived HBV and at day 1, day 2, day 4 and day 7 treated for 36 h DMAT (10 × IC50). Twelve days after infection the HBV replication was measured by HBeAg or HBsAg-specific enzyme linked immunosorbent assay. The bars represent the standard deviation.

Previous reports discussed whether the final import of the assembled nucleocapsid in the nucleus harboring the genome complex can occur[17]. More recent reports provide evidence that the mature nucleocapsid disassembles in the nuclear pore complex and the final import of the genome complex could be mediated by an association with HBcAg oligomers that harbor in their C-terminal domain a NLS[20,21,44-47]. For DHBV it was described that completition of plus-strand DNA synthesis triggers genomic DNA deproteinization and conformational changes of the nucleocapsid. This could lead to the exposure of a NLS within the core and thereby could enable the import of the rcDNA[48]. In this context it is interesting to mention that the presence of the identified NLS is not conserved for the genus avihepadnavirus.

Recently we developed cell permeable HBV nucleocapsids as a vehicle for gene transfer (Brandenburg *et al*[13]) Based on this system it was observed that neither HBcAg dimers nor nucleocapsids were visible in the nucleus of TLM-nucleocapsid treated cells although an efficient expression of the packaged, P-linked genome occurred suggesting that neither the nucleocapsid nor HBcAg dimers mediate the final import of the genome complex. Therefore the question arose about an alternative import mechanism: with about 90 kDa the covalent complex of HBV polymerase and genome clearly exceeds the size that freely can pass the nuclear pore complex.

In this context it is interesting that previous in vitro experiments have shown that the HBV genome complex can be efficiently imported into the nucleus. However if the complex is deproteinized, the naked genome fails to enter efficiently the nucleus[23]. These data suggest that the genome-linked polymerase could be relevant for the nuclear entry process.

The bipartite NLS identified in the TP domain of P could mediate the entry of the genome complex into the nucleus. The functional analysis of the genome complex revealed that the predicted NLS indeed has the potential to act as a nuclear localization signal. However, compared to other nuclear localization signals the TP-derived NLS is not a strong signal. This might reflect the different functions of P[18]. On the one hand P recognizes in the cytoplasm the de novo-synthesized 3.5 kb mRNA[43,49] and on the other hand the genome associated Pol is assumed to mediate by its NLS the entry of the genome complex into the nucleus. It is obvious that a too strong NLS signal might counteract the RNA-recognizing function in the cytoplasm. Yet, it was described that in duck HBV replicating cells in addition to the encapsidated polymerase non-encapsidated polymerase exists[24]. The major fraction of the non-encapsidated duck HBV polymerase is found in the cytoplasm a smaller fraction however can be detected within the nucleus[24]. Interestingly, in cells overexpressing HBV polymerase in the absence of other viral proteins a fraction of HBV polymerase is found within the nucleus, co-localized with the p11 protein of PML bodies[22].

A further interesting feature is the CKII phosphorylation site localized in the center of the bipartite NLS.

The functionality of the HBV polymerase-derived NLS depends on the CKII-mediated phosphorylation. CKII is not a very tightly regulated kinase[50]. It can be assumed that CKII exerts a housekeeping phosphorylation function[51]. If subcellular localization and function of HBV polymerase is subjected to a tight control, it is not likely that CKII exerts this function. It is tempting to speculate that phosphatases could play an important role to regulate the subcellular localization and thereby function of HBV polymerase. CKII phosphorylation is reported to influence subcellular localization of various nuclear proteins[52]. For example CKII phosphorylation upstream of the NLS of simian virus 40 T-antigen enhances its nuclear import up to 40 fold[53]. But immediate phosphorylation one or two amino acid upstream of the crucial amino acid of classical monopartite NLS seems to have inhibitory effects on karyopherin binding due to a disturbance of the NLS basicity[54]. In case of bipartite nuclear localization signals this correlation is not evident. For example the spacer of the functional bipartite NLS of the *Agrobacterium tumefaciens* protein nopaline contains four negative charged aspartates, one even immediate located ate the downstream basic cluster[55]. On the other hand an increase of the hydrophobicity of the 10-12 amino acid spacer seems to decrease its functionality[56].

In transfection experiments it was found that destruction of the NLS or of the CKII-site almost completely abolishes HBV replication. Under these experimental conditions the import of the viral genome into the nucleus does not represent the limiting step. The plasmid DNA freely moves into the nucleus. However this observation suggests that perturbation of this domain has further effects on the polymerase function. Since the ΔNLS and ΔCKII-mutants were replication deficient it was not possible to study the replication of the respective mutant viruses. The in vitro data however have shown that the functionality of the TP-derived NLS requires the functionality of CKII. Based on this it could be shown that inhibition of CKII in the early phase of the infection abolished the establishment of HBV infection while inhibition of CKII in a later phase of the infection or in a stable system had no effect on HBV replication.

In conclusion we demonstrate the presence of a bipartite NLS within the TP domain of P and provide evidence for a novel model describing the import of the HBV genome into the nucleus.

ACKNOWLEDGMENTS

COMMENTS

Background

Human hepatitis B virus (HBV) enters the cell by receptor mediated endocytosis and at the end of this process the nucleocapsid that harbours the viral genome is released into the cytoplasm and transported towards the nuclear pore complex. Establishment of a productive viral infection requires the transport of the HBV genome that is covalently linked to the polymerase into the nucleus. In the

nucleus the partial- double stranded DNA genome is converted to covalently closed circular (ccc) double stranded DNA.

Research frontiers

The phase of nuclear entry is not fully understood. It is discussed that the intact viral capsid shuttles the genome-polymerase complex into the nuclear basket of the nuclear pore complex. Here, after a partial disassembly of the nucleocapsid the polymerase-linked genome is released. The polymerase-genome complex however is too big to pass freely through the nuclear pore complex. This raises the question about the import mechanism.

Innovations and breakthroughs

The identification and characterization of a bipartite NLS in the HBV polymerase that harbours a phosphorylation site for casein kinase Ⅱ (CKⅡ) is described in this manuscript. The integrity of the phosphorylation site is crucial for the functionality of the NLS. Moreover, inhibition of CKⅡ prevents karyopherin α2 from binding to the polymerase. Thereby the import of the polymerase is impaired resulting in inhibited cccDNA formation that prevents the establishment of the viral infection. The data identify novel structural and functional prerequisites for the establishment of HBV infection.

Applications

The data describe a potential novel target for antiviral that could block the establishment of a HBV-infection.

Peer review

In this work, they identified a novel NLS located in the terminal protein domain of HBV polymerase and defined a CKⅡ phosphorylation site (threonine) which is adjacent to the NLS. This paper is well written.

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