Hepatitis B Virus Core Antigen Has Two Nuclear Localization Sequences in the Arginine-Rich Carboxyl Terminus†

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Expression of the hepatitis B virus core antigen (HBcAg) in mouse NIH 3T3 fibroblasts has been shown previously (A. McLachlan et al., J. Virol. 61:683–692, 1987) to result in the nuclear localization of this polypeptide. Since the carboxyl terminus of HBcAg contains four clusters of arginine residues which resemble nuclear localization sequences identified in other nuclear proteins, a series of carboxyl-terminus-truncated HBcAg polypeptides were expressed in mouse fibroblasts to examine the role of these sequences in the cellular localization of HBcAg. By immunofluorescence and cell fractionation analysis, it was demonstrated that regions of the HBcAg polypeptide including the most carboxyl-terminal (cluster 1) and amino-terminal (cluster 4) clusters of arginine residues represent distinct and independent nuclear localization sequences for this polypeptide. Substitution of a threonine residue for the second arginine residue in cluster 4 inactivates the nuclear localization signal in this region of the HBcAg polypeptide, demonstrating the importance of this residue to this signal sequence. However, HBcAg fails to accumulate in the nucleus only when both nuclear localization signal sequences are simultaneously deleted or disrupted by mutation. The possible significance of the nuclear localization sequences identified in the HBcAg polypeptide is discussed in the context of the role of the nucleocapsid in the hepatitis B virus life cycle.

Hepatitis B virus (HBV) is a 42-nm particle which possesses a 3.2-kb, partially double-stranded DNA genome (22, 26, 44). The genome is enclosed in a 27-nm nucleocapsid of hepatitis B virus core antigen (HBcAg), which is enveloped by a lipoprotein coat composed of cellular lipid and the hepatitis B virus surface antigen (HBsAg) (49). From the sequence of several cloned HBV DNAs, it was observed that the core antigen open reading frame (ORF) has two in-frame translation codons in several but not all HBV genomes (4, 12, 13, 25, 33–36, 39, 51, 53, 55). By using a variety of eucaryotic expression systems, it has been shown that the polyepitope synthesized from the first initiation codon is hepatitis B virus e antigen (HBsAg) (9, 29, 38, 47). The precore signal sequence directs the product of the precore plus core ORF to the endoplasmic reticulum, where a signal peptidase cotranslationally cleaves the precore signal sequence (9, 15, 29, 54). This 22-kDa precursor polypeptide is subsequently further processed, probably by trypsin- and carboxypeptidase B-like activities in the post-Golgi compartment (11), resulting in the secretion of the mature 18-kDa HBsAg polypeptide (29, 47).

The polypeptide product encoded from the second in-frame initiation codon of the core antigen ORF is the 21-kDa HBcAg polypeptide (29, 45, 47, 54). This polypeptide is not proteolytically processed and has been localized to the nuclear (29, 45) and cytoplasmic (38, 45) cellular compartments depending on the expression system used to produce the HBcAg. Previously, it had been shown that expression of HBcAg in mouse NIH 3T3 fibroblasts, using an amphotropic retroviral expression system, resulted in the nuclear accumulation of this antigen (29).

In the current study, the regions of the HBcAg polypeptide containing the nuclear localization sequences were analyzed by using an amphotropic retroviral expression system and mouse NIH 3T3 fibroblasts. The carboxy-terminal 34 amino acids of the HBcAg polypeptide contain four clusters of arginine residues which define regions of consecutive basic amino acids similar to nuclear localization sequences identified in several nuclear proteins (10, 20, 24, 28, 32, 40, 42, 50, 52). For this reason, this region of the HBcAg polypeptide was analyzed for the presence of nuclear localization sequences. Deletion and point mutational analyses of the HBcAg polypeptide revealed that the carboxyl-terminal (cluster 1) and amino-terminal (cluster 4) clusters of arginine residues reside in regions of the polypeptide constituting nuclear localization sequences. In contrast, the two internal arginine clusters (clusters 2 and 3) do not appear to influence the cellular localization of the HBcAg polypeptide.

MATERIALS AND METHODS

Plasmid constructions. The various steps in the cloning of the plasmid constructs were performed by standard techniques (46). The amphotropic retroviral vector pARV1MT and the expression vector pARV1MTC have been described previously (29). Briefly, the expression vector pARV1MTC, containing the HBV core ORF sequence (HBV DNA sub-type ayw, coordinates 1899 to 2804; GenBank genetic sequence data bank), directs the expression of HBcAg from the mouse metallothionein promoter and the product of the neo gene from the amphotropic retrovirus long terminal repeat. This permits the isolation of antigen-producing cell lines by selection for G418 resistance.

The expression vectors pARV1MTCΔ1 through pARV1MTCΔ3 were derived from the expression vector pARV1MTC by deletion of carboxyl-terminal coding sequences of the HBcAg ORF. This was achieved by digesting the HBV core ORF sequence with StyI (coordinate 2459;
GenBank genetic sequence data bank), digesting the carboxy-terminal coding sequence with BfaI nuclease, and inserting an NheI linker (CTAGCTAGCTAG) to generate a termination codon in all three reading frames. The expression vector pARV1MTCΔ4 was derived from the expression vector pARV1MTC by deletion of the HBcAg coding sequences located carboxy-terminal to the HpaII site (coordinate 2334; GenBank genetic sequence data bank) in the HBcAg ORF and insertion of an NheI linker (CTAGCTAGCTAG). These four expression vectors will direct the expression of HBcAg-related polyptides comprising the 171, 161, 156, and 144 amino-terminal amino acids of the HBcAg ORF, respectively (Fig. 1). In addition, the expression vectors pARV1MTCΔ1 and pARV1MTCΔ4 encode the additional carboxy-terminal sequences LAS and ISLI, respectively, derived from polylinker and NheI linker sequences included in these plasmid constructions.

The expression vectors pARV1MTCΔ1-5, pARV1MTCΔ2-2, and pARV1MTCΔ3-2 were generated from the expression vectors pARV1MTC, pARV1MTCΔ1, pARV1MTCΔ2, and pARV1MTCΔ3, respectively, by modifying the HBcAg ORF codon 151 from CGA (Arg) to ACG (Thr) by site-directed mutagenesis. Site-directed mutagenesis was performed with oligonucleotides containing the nucleotide substitutions at codon 151 and a polymerase chain reaction procedure (21). The sequence of the mutated HBcAg ORFs was confirmed by nucleotide sequence analysis.

Cells and transfections. Mouse NIH 3T3 fibroblasts were grown, transfected, and selected as previously described (29). Clonal cell lines were derived from aminoglycoside antibiotic G418-resistant colonies established from each transfection. Clonal cell lines from each transfection that expressed the highest levels of HBcAg-related poly peptides were used for further analysis.

Antigen assays. The level of HBcAg-related polyptide expression in the transfected cell lines was quantitated as described before (29). HBcAg and HBeAg were measured by enzyme-linked immunosorbent assay (HBEIA Diagnostic Kit; Abbott) with the HBcAg positive control (arbitrarily defined as 1 U/ml, undiluted) used as a standard. Antigen assays were performed on whole-cell lysates and crude nuclear and cytoplasmic cell fractions after suitable dilutions in phosphate-buffered saline (PBS; 10 mM sodium phosphate [pH 7.4], 145 mM NaCl containing 1% [wt/vol] bovine serum albumin, 5% [vol/vol] fetal calf serum, and 0.005% [vol/vol] Tween 20.

Cell fractionation. Whole-cell lysates were prepared by washing 5 × 10⁶ cells in PBS and suspending the cells in 1 ml of PBS containing 1 mM phenylmethylsulfonyl fluoride. The cell suspensions were subjected to three cycles of freezing and thawing, followed by centrifugation for 30 min at 12,800 × g. The supernatant represented the whole-cell lysate.

Crude nuclear and cytoplasmic cell fractions were prepared by washing 5 × 10⁶ cells in PBS and subsequently homogenizing the cells in 0.3 ml of lysis buffer (LB; 10 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 5 mM MgCl₂, 1% [wt/vol] octyl-β-D-glucopyranoside, 1 mM phenylmethylsulfonyl fluoride). After the lysate was incubated on ice for 3 min, nuclei were pelleted by centrifugation at 12,800 × g for 1 min. The supernatant represented the cytoplasmic fraction. The nuclear pellet was resuspended in 0.3 ml of LB without detergent and then subjected to three cycles of freezing and thawing, followed by centrifugation at 12,800 × g for 30 min. The supernatant represented the nuclear fraction. The nuclear and cytoplasmic fractions were dialyzed against 500 volumes of LB without detergent for 2 h at 4°C prior to determination of antigen and protein concentrations. Protein concentrations were determined by the Bradford assay (7).

Immunoblotting. Immunoblotting of cell lysates was performed as described previously (29) with the Amersham RPN.23 blotting detection kit. Membranes were rinsed in TBS (20 mM Tris hydrochloride [pH 7.6], 137 mM NaCl) and then blocked overnight with TBS containing 5% (wt/vol) dried milk and 0.1% (vol/vol) Tween 20 at 4°C. The membrane was probed at 20°C with a polyclonal rabbit anti-HBc (Dako) as the primary antibody and a biotinylated donkey anti-rabbit immunoglobulin as the secondary antibody. The immunoblot was developed by using a streptavidin-alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indolyl phosphate as the substrate according to the manufacturer's (Amersham) instructions.

Immunofluorescence. The cellular localization of the HBcAg-related polyptides was determined by indirect immunofluorescence microscopy (29). The antigens were detected by using a polyclonal rabbit anti-HBc (Dako) as the primary antibody and an affinity-purified fluorescein-conjugated goat anti-rabbit immunoglobulin (Tago) as the secondary antibody.

RESULTS

Production of cell lines synthesizing HBcAg-related polyptides. The expression vectors pARV1MTCΔ1, pARV1
MTCA2, pARV1MTCA3, pARV1MTCA4, pARV1MTC-T, pARV1MTCA1-T, pARV1MTCA2-T, and pARV1MTCA3-T were transfected into mouse NIH 3T3 fibroblasts, and G418-resistant clonal cell lines were established. Several clonal cell lines containing each expression vector were assayed for antigen production, and the cell lines 3T3 pARV1MTCA1, 3T3 pARV1MTCA2, 3T3 pARV1MTCA3, 3T3 pARV1MTC4, 3T3 pARV1MTC-T, 3T3 pARV1MTCA1-T, 3T3 pARV1MTCA2-T and 3T3 pARV1MTCA3-T displaying the highest level of HBeAg-related polypeptide synthesis were characterized further. At least one additional clonal cell line containing each expression vector was also analyzed by indirect immunofluorescence microscopy to confirm the cellular localization of the HBeAg-related antigens (data not shown).

The sizes of the HBeAg-related polypeptides encoded by the various expression vectors were determined by immunoblotting analysis (Fig. 2). The cell lines 3T3 pARV1MTCA1-4 and 3T3 pARV1MTC were shown to synthesize HBeAg-related polypeptides of 19.9, 18.4, 17.7, 16.8, and 21.1 kDa, as was predicted from the sequence of the HBeAg ORFs present in the transfected expression vectors. HBeAg-related polypeptides of the expected sizes were also detected in the 3T3 pARV1MTC-T, 3T3 pARV1MTCA1-T, 3T3
HBcAg-related polypeptides possessing a threonine substitution at codon 151. Analysis of the carboxyl-terminus-truncated HBcAg-related polypeptides located a nuclear localization signal between amino acid residues 145 and 156 (Fig. 3 and 4). With a view to examining the role of the arginine-rich cluster (cluster 4) in this region of HBcAg in the localization of the antigen to the nucleus, the second arginine residue in this cluster (codon 151) was converted to threonine by site-directed mutagenesis (Fig. 1). Since it had been shown for simian virus 40 large T antigen that conversion of the second basic amino acid in the nuclear localization sequence to a threonine residue prevented its accumulation in the nucleus (17, 23, 24), the influence of this modification on the cellular localization of HBcAg was examined (Fig. 5B and 6). From the immunofluorescence analysis of the 3T3 pARV1MTC-T cell line (Fig. 5B), it was apparent that conversion of arginine 151 to threonine did not influence the cellular localization of the HBcAg polypeptide. This suggested that either the threonine 151 substitution had not inactivated the nuclear localization signal located between amino acid residues 145 and 156 or that HBcAg contains a second nuclear localization sequence.

In an attempt to distinguish between these two possibilities, the threonine 151 substitution was introduced into the carboxyl-terminus-truncated HBcAg-related polypeptides, and the cellular localization of the threonine 151-substituted carboxyl-terminus-deleted HBcAg-related polypeptides was examined (Fig. 5C to E). Immunofluorescence analysis of the cell lines 3T3 pARV1MTCΔ1-T, pARV1MTCΔ2-T, and pARV1MTCΔ3-T revealed that the HBcAg-related polypeptides expressed in these cell lines were localized in the cytoplasm. This indicates that HBcAg contains a second nuclear localization sequence located, at least in part, in the carboxyl-terminal 12 amino acid residues. It appears likely that the arginine-rich cluster (cluster 1) located within this region of the HBcAg polypeptide represents part of this localization sequence. In addition, this result demonstrated that the threonine 151 substitution in the arginine-rich cluster 4 inactivated the nuclear localization sequence between amino acid residues 145 and 156. The cellular localization of the threonine 151-substituted carboxyl-terminus-deleted HBcAg-related polypeptide was confirmed by cell fractionation analysis (Fig. 6). Equivalent nuclear and cytoplasmic antigen concentrations were only observed for cell fractions derived from the 3T3 pARV1MTC and 3T3 pARV1MTC-T cell lines. The HBcAg-related polypeptides in the 3T3 pARV1MTCΔ1-T, pARV1MTCΔ2-T, and pARV1MTCΔ3-T cell lines were located almost exclusively in the cytoplasmic fraction (Fig. 6).

**DISCUSSION**

An amphotropic retrovirus vector has been used to express HBcAg-related polypeptides in mouse NIH 3T3 cells. Expression of the 21.1-kDa HBcAg polypeptide in this system has been shown previously to result in the nuclear accumulation of this antigen (29). Since the carboxyl-terminus 34 amino acids of this polypeptide contain four arginine-rich clusters (clusters 1 to 4) (Fig. 1) which resemble the nuclear localization sequences identified in a variety of nuclear proteins (10, 20, 24, 28, 32, 40, 42, 50, 52), the role of these sequences in the nuclear localization of HBcAg was examined by indirect immunofluorescence microscopy and cell fractionation analysis.

Characterization of the cellular localization of a series of carboxyl-terminus-truncated HBcAg-related polypeptides

![Cell fractionation analysis of HBcAg-related antigens expressed in transfected cell lines.](http://jvi.asm.org/)

**FIG. 4.** Cell fractionation analysis of HBcAg-related antigens expressed in transfected cell lines. Cell lines analyzed were 3T3 pARV1MTC (C), 3T3 pARV1MTCΔ1 (CΔ1), 3T3 pARV1MTCΔ2 (CΔ2), 3T3 pARV1MTCΔ3 (CΔ3), and 3T3 pARV1MTCΔ4 (CΔ4). The solid and hatched boxes indicate the specific activities of the HBcAg-related polypeptides in the nuclear and cytoplasmic fractions, respectively.

pARV1MTCΔ2-T, and 3T3 pARV1MTCΔ3-T cell lines by immunoblotting analysis (data not shown). These results confirm that the expression vectors were directing the synthesis of the expected polypeptide products in the transfected cell lines.

**Cellular localization of the carboxyl-terminus-truncated HBcAg-related polypeptides.** The cellular localization of the carboxyl-terminus-truncated HBcAg-related polypeptides was initially analyzed by indirect immunofluorescence microscopy (Fig. 3). As a control, the localization of HBcAg synthesized in the cell line 3T3 pARV2PC (29) was examined and shown to be predominantly cytoplasmic (Fig. 3A), which is consistent with the observation that HBcAg is a secreted antigen (9, 15, 29, 37, 47, 54). In contrast, the 183-amino-acid HBcAg accumulated in the nuclei of the mouse fibroblasts (Fig. 3B). Deletion of 12 (CΔ1), 22 (CΔ2), and 27 (CΔ3) carboxyl-terminal amino acid residues (Fig. 1) from the HBcAg polypeptide did not affect the cellular localization of the HBcAg-related polypeptides (Fig. 3C to E). These deletions consecutively deleted the arginine-rich clusters 1, 2, and 3. However, deletion of 39 (CΔ4) carboxyl-terminal amino acid residues from the HBcAg polypeptide caused this HBcAg-related polypeptide to accumulate in the cytoplasm of the cell (Fig. 3F). This indicates that a nuclear localization sequence is located, at least in part, between residues 145 and 156 of the HBcAg polypeptide.

Within this region of the HBcAg polypeptide is an arginine-rich cluster (cluster 4) which appears similar to regions of consecutive basic amino acids found to serve as nuclear localization sequences in several nuclear proteins (10, 20, 24, 28, 32, 40, 42, 50, 52). The cellular localization of the carboxyl-terminus-truncated HBcAg-related polypeptides was confirmed by cell fractionation analysis of the cell lines 3T3 pARV1MTC, 3T3 pARV1MTCΔ1, 3T3 pARV1MTCΔ2, 3T3 pARV1MTCΔ3, and 3T3 pARV1MTCΔ4 (Fig. 4). All of these cell lines except 3T3 pARV1MTCΔ4 revealed the presence of HBcAg-related polypeptides in the nuclear fraction after cell lysis, indicating that these polypeptides possess a nuclear localization sequence. The presence of cytoplasmic HBcAg-related polypeptides in the fractionation analysis of the 3T3 pARV1MTC, 3T3 pARV1MTCΔ1, 3T3 pARV1MTCΔ2, and 3T3 pARV1MTCΔ3 cell lines is probably due, in part, to the leakage of nuclear antigen into the cytoplasmic fraction during cell lysis (data not shown).

**Cellular localization of the carboxyl-terminus-truncated HBcAg-related polypeptides possessing a threonine substitution at codon 151.** Analysis of the carboxyl-terminus-truncated HBcAg-related polypeptides located a nuclear localization signal between amino acid residues 145 and 156 (Fig. 3 and 4). With a view to examining the role of the arginine-rich cluster (cluster 4) in this region of HBcAg in the localization of the antigen to the nucleus, the second arginine residue in this cluster (codon 151) was converted to threonine by site-directed mutagenesis (Fig. 1). Since it had been shown for simian virus 40 large T antigen that conversion of the second basic amino acid in the nuclear localization sequence to a threonine residue prevented its accumulation in the nucleus (17, 23, 24), the influence of this modification on the cellular localization of HBcAg was examined (Fig. 5B and 6). From the immunofluorescence analysis of the 3T3 pARV1MTC-T cell line (Fig. 5B), it was apparent that conversion of arginine 151 to threonine did not influence the cellular localization of the HBcAg polypeptide. This suggested that either the threonine 151 substitution had not inactivated the nuclear localization signal located between amino acid residues 145 and 156 or that HBcAg contains a second nuclear localization sequence.

In an attempt to distinguish between these two possibilities, the threonine 151 substitution was introduced into the carboxyl-terminus-truncated HBcAg-related polypeptides, and the cellular localization of the threonine 151-substituted carboxyl-terminus-deleted HBcAg-related polypeptides was examined (Fig. 5C to E). Immunofluorescence analysis of the cell lines 3T3 pARV1MTCΔ1-T, pARV1MTCΔ2-T, and pARV1MTCΔ3-T revealed that the HBcAg-related polypeptides expressed in these cell lines were localized in the cytoplasm. This indicates that HBcAg contains a second nuclear localization sequence located, at least in part, in the carboxyl-terminal 12 amino acid residues. It appears likely that the arginine-rich cluster (cluster 1) located within this region of the HBcAg polypeptide represents part of this localization sequence. In addition, this result demonstrated that the threonine 151 substitution in the arginine-rich cluster 4 inactivated the nuclear localization sequence between amino acid residues 145 and 156. The cellular localization of the threonine 151-substituted carboxyl-terminus-deleted HBcAg-related polypeptide was confirmed by cell fractionation analysis (Fig. 6). Equivalent nuclear and cytoplasmic antigen concentrations were only observed for cell fractions derived from the 3T3 pARV1MTC and 3T3 pARV1MTC-T cell lines. The HBcAg-related polypeptides in the 3T3 pARV1MTCΔ1-T, pARV1MTCΔ2-T, and pARV1MTCΔ3-T cell lines were located almost exclusively in the cytoplasmic fraction (Fig. 6).

**DISCUSSION**

An amphotropic retrovirus vector has been used to express HBcAg-related polypeptides in mouse NIH 3T3 cells. Expression of the 21.1-kDa HBcAg polypeptide in this system has been shown previously to result in the nuclear accumulation of this antigen (29). Since the carboxyl-terminus 34 amino acids of this polypeptide contain four arginine-rich clusters (clusters 1 to 4) (Fig. 1) which resemble the nuclear localization sequences identified in a variety of nuclear proteins (10, 20, 24, 28, 32, 40, 42, 50, 52), the role of these sequences in the nuclear localization of HBcAg was examined by indirect immunofluorescence microscopy and cell fractionation analysis.

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lacking one (CΔ1), two (CΔ2), three (CΔ3), or all four (CΔ4) of the arginine-rich clusters (Fig. 1) demonstrated that amino acid residues 145 to 156 constitute at least part of a nuclear localization signal for HBcAg (Fig. 3 and 4). These results indicated that this region, containing a single cluster of arginine residues (cluster 4), is sufficient in the context of the 156 amino-terminal HBcAg residues (CΔ3) to translocate this HBcAg-related polypeptide into the nucleus. Based on the observation that several nuclear localization sequences contain regions of basic amino acids (10, 20, 24, 28, 32, 40, 42, 50, 52), it appeared likely that the nuclear localization sequence located within residues 145 to 156 included the arginine cluster spanning residues 150 to 154 (cluster 4). This possibility was examined by substituting a threonine residue for arginine 151 (Fig. 5 and 6). Within the context of the 183-amino-acid HBcAg polypeptide, this substitution did not affect the cellular localization of the HBcAg polypeptide (Fig. 3B and 5B). However, the deletion of 12 carboxyl-terminal amino acids generated HBcAg-related polypeptides which were dependent on the amino acid encoded by codon 151 for their cellular localization. The polypeptide expressed in the cell line 3T3 pARV1MTCΔ1 was located in the nucleus (Fig. 3C), whereas the polypeptide expressed in the cell line 3T3 pARV1MTCΔ1-T, which differs from that expressed in the cell line 3T3 pARV1MTCΔ1, only by the arginine 151 to threonine substitution, was located in the cytoplasm (Fig.
This observation established the importance of arginine 151 to the nuclear localization sequence located between residues 145 and 156 and suggests that the arginine-rich cluster (cluster 4) represents an essential part of this sequence. In addition, these results establish that the HBcAg polypeptide contains a second nuclear localization sequence located, at least in part, between residues 172 and 183. This sequence includes an arginine-rich cluster (cluster 1) which may represent part of this second nuclear localization sequence. The ability of these two nuclear localization sequences to function independently of other HBcAg sequences will require the analysis of fusion proteins containing these HBcAg nuclear localization sequences in the absence of additional HBcAg polypeptide sequences. Why HBcAg has two nuclear localization sequences is unknown, but two nuclear localization sequences have been observed in other proteins (20, 40, 42, 50).

The identification of two nuclear localization sequences located within residues 145 to 156 and 172 to 183 of the HBcAg polypeptide suggests that at least a portion of each of these regions might be present on the surface of the protein (43). This is presumably required so that HBcAg can be recognized by the nuclear localization sequence receptor(s) (1, 27, 56) and subsequently transported into the nucleus (8). Analysis of protease digestion products of HBcAg (14) and the structure of HBcAg fusion proteins (6) suggests that residues 144 to 149 are at the surface of the HBcAg particle. This would be consistent with the arginine-rich cluster located between residues 150 and 154 (cluster 4) being located on the surface of the HBcAg particle. It should be noted that the quaternary structure of the HBcAg and HBcAg-related polypeptides expressed in mouse fibroblasts has not been established. After lysis of cells expressing HBcAg, it was determined that the majority of the antigen was nonparticulate in nature (29). However, it is not clear whether the nonparticulate nature of the antigen was due to proteolytic degradation or denaturation of HBcAg upon lysis of the cells, since it has been shown that HBcAg-related polypeptides expressed in Escherichia coli and lacking up to 39 carboxyl-terminal amino acids do form particles (5).

The significance of the nuclear localization sequences in HBcAg to the HBV life cycle is currently unclear. However, it would clearly be advantageous to the infecting HBV virion if the nucleocapsid possessed a nuclear localization sequence. This could direct the nucleocapsid and HBV DNA from an infecting virion to the nucleus, where the next steps in the viral life cycle, including the transcription of the viral genome, occur. The observation that immature nucleocapsids containing pregenomic RNA and minus-strand DNA are localized to the cytoplasm (2, 18, 19, 30, 31) suggests that the nuclear localization sequence can be inactivated under certain circumstances. This might occur by a conformational change in the HBcAg polypeptide or as a consequence of the phosphorylation of the serine residues in the carboxy-terminal region of the HBcAg polypeptide (3, 16, 45). Consistent with this second suggestion is the observation that in the duck HBV (DHBV) system, immature nucleocapsid particles are phosphorylated, whereas the nucleocapsids in the mature virions are not phosphorylated (41). In addition, the observation that the pool of covalently closed circular DHBV DNA is amplified in the absence of DHBV envelope antigen synthesis (48) suggests that mature nucleocapsids which do not interact with the envelope antigen to produce virions might be translocated to the nucleus, where their genomic DNA would contribute to the nuclear DNA pool. This suggests that the conversion of the pregenomic RNA in immature nucleocapsids to DNA, producing mature nucleocapsids, might be associated with a change in the phosphorylation status of the nucleocapsid so that the nuclear localization signal is activated. It will be necessary to characterize the effect of mutations which inactivate the HBcAg nuclear localization sequences or modify the nucleocapsid phosphorylation sites on virus production in order to determine their functional roles in the HBV life cycle.

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