Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6459–6463, July 1993
Medical Sciences

Cell cycle regulation of nuclear localization of hepatitis B virus core protein

CHAU-TING YEH*, SUNNY W. WONG*, YUEN-KAI FUNG*†, AND JING-HSIUNG OU*‡

*Department of Microbiology, University of Southern California, Los Angeles, CA 90033; and †Department of Hematology and Oncology, Children's Hospital, Los Angeles, CA 90027

Communicated by William J. Rutter, April 9, 1993

ABSTRACT **The hepatitis B virus (HBV) core protein has been found in the nucleus, the cytoplasm, or both of HBV-infected hepatocytes. However, the mechanism that regulates the subcellular localization of the HBV core protein is still unclear. In this report, we demonstrate that nuclear localization of the HBV core protein is cell cycle-regulated in two different cell lines. The amount of the core protein in the nucleus was increased during the G_1 phase, reduced to an undetectable level during the S phase, and increased again when the cells were confluent and ceased to grow. Thus, the nuclear localization of the core protein during HBV infection can be at least partially attributed to liver injury and regeneration, which cause the hepatocytes to enter cell cycles. Based on the observation that the cytoplasmic core protein was phosphorylated and the nuclear core protein was not, we speculate that nuclear localization of the HBV core protein is negatively regulated by phosphorylation during the cell cycle.**

Hepatitis B virus (HBV) can cause acute and chronic hepatitis in humans and is also the major cause of liver cancer. The HBV core protein, also named core antigen, is the major capsid protein of the virus and is an important serological marker for diagnosis of HBV infection. This antigen has been found in the nucleus, the cytoplasm, or both of hepatocytes infected by HBV (1–3). Although it has been demonstrated that the carboxyl-terminal arginine-rich domain of the core protein contains a signal for nuclear localization (4, 5), the mechanism that regulates the nuclear localization of the core protein is still unclear.

We have studied the subcellular localization of the HBV core protein in two different cell lines and report here our results which indicate that nuclear localization of the core protein is cell cycle-regulated. Further studies indicate that this regulation may be mediated by phosphorylation of the core protein.

MATERIALS AND METHODS

Cell Lines. NIH 3T3 cells and Vero cells are immortalized mouse fibroblast and monkey kidney cell lines, respectively. The 3T3pARV1MTC cell line was a gift of A. McLachlan (The Scripps Research Institute). This cell line was established from a mixture of stable NIH 3T3 transformants that express various amounts of the HBV core protein (6).

For the establishment of the Vero-C3 stable transformant, Vero cells were transfected with the cytomegalovirus (CMV)-derived plasmid pCMV-core and selected with the neomycin analog G418 by our previous procedures (7). Vero-C3 was one of the stable transformants selected. pCMV-core was a plasmid constructed for expressing the HBV core protein. This plasmid was constructed by inserting the 1.7-kilobase (kb) *Hin*dIII–*Xba* I DNA fragment of pECE-core (8) into the *Hin*dIII–*Xba* I polylinker site of the pRC/CMV vector (Invitrogen, San Diego). The 1.7-kb pECE-C DNA fragment used in the construction contained the coding sequence of the HBV core protein (adw subtype). In pCMV-core, the expression of the HBV core protein is under the control of the CMV *IE* promoter.

Cell Cycle Synchronization. Cells (6×10^5) were plated in a 60-mm Petri dish the day before the synchronization experiment. For synchronization with the serum-free medium, cells were incubated in Dulbecco's modified Eagle's medium (DME medium) without serum for 30 h. This resulted in synchronization of cells in the G_0 phase of the cell cycle. The cell cycle was then initiated with DME medium containing 20% (vol/vol) calf serum. For synchronization with aphidicolin, cells were incubated with DME medium containing 10% calf serum and 2 μg of aphidicolin per ml for 48 h. This resulted in synchronization of cells in the late G_1 phase. The cells were either analyzed at this time point or allowed to enter the *S* phase by further incubation in the same medium without aphidicolin for 12 h.

Subcellular Fractionation. For subcellular fractionation, cells were rinsed with phosphate-buffered saline twice and lysed with 0.5 ml of TBS (10 mM Tris·HCl, pH 7.2/150 mM NaCl) containing 0.5% Nonidet P-40 (NP-40). The nuclear fraction (NP-40-insoluble fraction) and the cytoplasmic fraction (NP-40-soluble fraction) of the cell lysates were separated by a brief centrifugation in a microcentrifuge at 1500 × *g*. The nuclear fraction was rinsed once with the lysis buffer and solubilized in 1 ml of radioimmunoprecipitation assay (RIPA) solution (10 mM Tris·HCl, pH 7.0/150 mM NaCl/1% Triton X-100/1% sodium deoxycholate/0.1% SDS). The cytoplasmic fraction was also mixed with an equal volume of RIPA buffer. Two-hundred microliters of each fraction was used for RIA with the Abbott HBe-RIA kit.

[^{3}H]Thymidine Incorporation Assay. For the [^{3}H]thymidine incorporation assay, the cells were labeled with [^{3}H]thymidine at a concentration of 25 μCi/ml (1 μCi = 37 kBq) for 30 min at selected time points after synchronization. After labeling, the cells were rinsed with phosphate-buffered saline twice and lysed with 1 ml of 0.1 M NaOH containing 10 mM EDTA and 0.5% SDS. After being heated at 70°C for 30 min, the cell lysate was mixed with 100 μl of 100% CCl_3COOH and incubated at 4°C overnight. The CCl_3COOH precipitates were then collected on GF/C filter discs (Millipore), rinsed with 5% CCl_3COOH solution and then with 95% ethanol, air-dried, and scintillation-counted.

RESULTS

Cell Cycle Regulation of Nuclear Localization of the Core Protein in NIH 3T3 Cells. 3T3pARV1MTC is a cell line derived from a mixture of NIH 3T3 stable transformants that express various amounts of the HBV core protein (6). To

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HBV, hepatitis B virus; CMV, cytomegalovirus.
‡To whom reprint requests should be addressed.

investigate whether nuclear localization of the HBV core protein is cell cycle-regulated, the growth of 3T3pARV1MTC cells was synchronized at the G_0/G_1 phase with a serum-free medium. The cell cycle was then initiated with a medium containing 20% calf serum. Cells were lysed at various time points after synchronization and fractionated into the nuclear and the cytoplasmic fractions, which were then individually monitored for the amount of the core protein with the RIA. No significant amount of the core protein was detected in the nucleus when the cell cycle was initiated (Fig. 1*A*). However, there was a gradual increase of the amount of the core protein in the nucleus after the initiation of the cell cycle. The ratio reached a peak 9 h after synchronization. At this time point, based on cellular chromosomal DNA content analyzed by flow-cytometry (data not shown), most cells were found to be in the G_1 phase. After this time point, the amount of the core protein in the nucleus was significantly reduced. The core protein was essentially undetectable in the nucleus 15 h after synchronization, when the cells were in the S phase, as determined by the [^{3}H]thymidine-incorporation assay. After the S phase, the amount of the core protein in the nucleus again continued to increase up to 36 h after synchronization.

Similar results were obtained with immunoblotting (Western blotting) experiments. At zero time point, a small amount of the core protein was detected in the cytoplasm, and no detectable amount of the core protein was found in the nucleus (Fig. 1*B*). Eight hours after synchronization when most cells were in the G_1 phase, the amount of the core protein in the cytoplasm was not significantly different from that of the zero time point. However, a significant amount of the core protein was detected in the nucleus at this time point. Sixteen hours after synchronization, when most cells were in the S phase of the cell cycle, despite the increase of the amount of the core protein in the cytoplasm, the amount of the core protein in the nucleus was substantially reduced. Twenty-four hours after synchronization, the amount of the core protein in the nucleus was again significantly increased. Thus, results of both the Western blot (Fig. 1*B*) and the RIA (Fig. 1*A*) demonstrate that the concentration of the core protein in the nucleus is increased during the G_1 phase and reduced during the S phase.

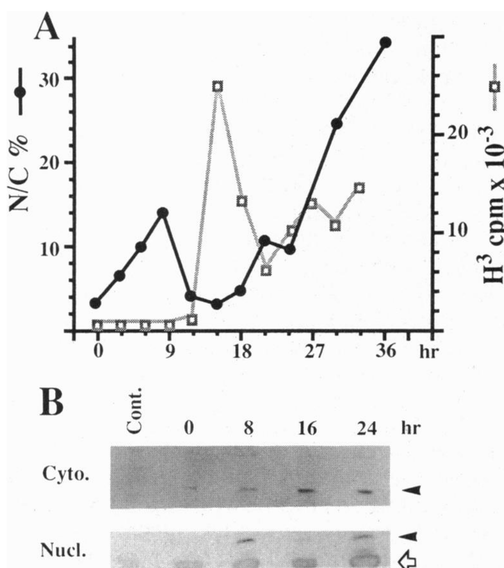

FIG. 1. Cell cycle analysis of nuclear localization of the HBV core protein in 3T3pARV1MTC cells. (*A*) Results of RIA and [^{3}H]thymidine incorporation assay. ●, Relative ratios (N/C ratios) of the amount of the core protein in the nucleus (N) and that in the cytoplasm (C) during the cell cycle as determined by RIA; □, results of the [^{3}H]thymidine incorporation assay. For both the RIA and the [^{3}H]thymidine incorporation assay, the average results of triplicated experiments are shown. (*B*) Western blot analysis of the core protein. Cells were fractionated by the procedures described in text. The samples were mixed with an equal volume of 2× Laemmli sample buffer (21) and electrophoresed on a 12.5% Laemmli gel. The amount of the cytoplasmic (Cyto.) sample loaded on the gel for the Western blot analysis was one-eighth of that of the nuclear (Nucl.) sample. The primary and the secondary antibodies used were rabbit anti-core protein (8) and alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad). Arrowheads mark the core protein bands. The empty arrow indicates the position of a nonspecific protein band that served as an internal control for the quantitation purpose.

To ensure that the observation of nuclear localization of the core protein was not due to subcellular fractionation artifact, we also performed immunofluorescence staining experiments. The staining results of representative cells are shown in Fig. 2 *Upper*, and the relative proportions of cells with different staining patterns are shown in Fig. 2 *Lower*. At the beginning of the cell cycle, the core protein was localized to the cytoplasm of almost all of the positive 3T3pARV1MTC cells (Fig. 2 *Lower*), although the staining signal of the core protein was generally weak, indicating that the amount of the core protein in the cells was low (Fig. 2 *Upper a*). Nine hours after synchronization, the percentage of cells with predominant cytoplasmic staining was reduced to <60%, and the rest of the positive cells were stained either predominantly in the nucleus or in both the nucleus and the cytoplasm (Fig. 2 *Lower*). In Fig. 2 *Upper b–d* are shown representative examples of the cells stained in the cytoplasm, in both the nucleus and the cytoplasm, and in the nucleus, respectively. It is unclear why cells displayed a heterogeneous staining pattern at this time point. It may be due to the heterogeneity of the cell population (6) and its heterogenous growth rate. Eighteen hours after synchronization during the S phase, the core protein was detected in the cytoplasm of almost all of the positive cells (Fig. 2 *Upper e* and *Lower*). Thirty-six hours after synchronization, the cells were confluent and, as revealed by flow-cytometry analysis (data not shown), were mostly arrested in the G_0/G_1 phase possibly because of contact inhibition (9). At this stage, the majority of the positive cells was stained in the nuclei (Fig. 2 *Upper f*), and only ≈15% of the cells was stained in the cytoplasm (Fig. 2 *Lower*). Thus, similar to the results of RIA and Western blotting, results of immunofluorescence staining shown in Fig. 2 also indicate that the concentration of the core protein in the nucleus is transiently increased during the G_1 phase, reduced during the S phase, and increased again when the cells become confluent and cease to grow. Note that although immunofluorescence staining revealed that the nuclear core protein signal was more prominent than the cytoplasmic core protein signal when cells were confluent, the RIA results indicated that the cytoplasmic core protein signal was always predominant. This discrepancy is likely due to the limitation of the immunofluorescence assay, which can only measure qualitatively the relative concentrations of the core protein in the nucleus and the cytoplasm and cannot measure quantitatively the absolute amounts of the core protein in the nucleus and in the cytoplasm.

To ensure that the concentration of the core protein in the nuclei of 3T3pARV1MTC cells was indeed regulated by the cell cycle, a different approach was used to synchronize the cells. If entry of the cell cycle into the S phase was blocked by the chemical aphidicolin (10), the core protein was stained in the nuclei of the great majority of the positive cells (Fig. 2 *Upper h* and *Lower*). Release of the cell cycle from the chemical block resulted in the entry of cells into the S phase (ref. 10; also data not shown) and the localization of the core protein in the cytoplasm (Fig. 2 *Upper g* and *Lower*). Thus, both the serum deprivation and the aphidicolin experiments indicate that the core protein concentration in the nucleus is

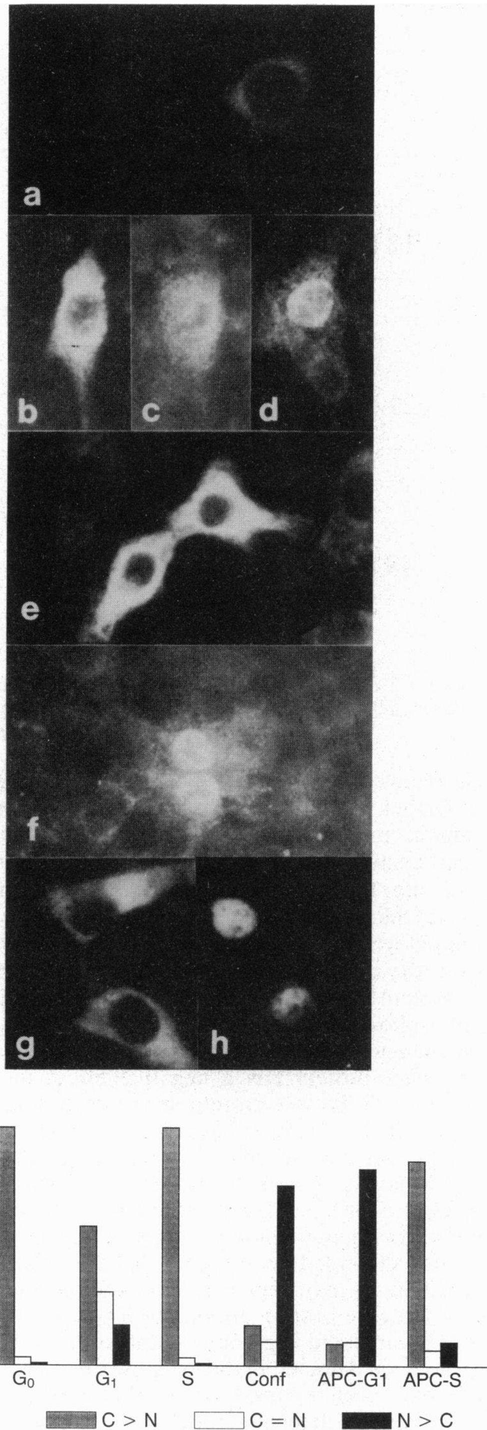

FIG. 2. Subcellular localization of the core protein in 3T3pARV1MTC cells during the cell cycle. Cells synchronized by serum deprivation were stained at various time points as indicated. (*Upper*) Immunofluorescence staining results at various times after synchronization: 0 h (G_0 phase) (*a*); 9 h (G_1 phase) (*b–d*); 16 h (S phase) (*e*); and 36 h (confluent phase) (*f*). Also shown are blockage of the cell cycle at the G_1 phase with aphidicolin at 2 μg/ml for 48 h (APC-G1) (*h*) and the effect 12 h after removal of the chemical aphidicolin (APC-S) (*g*). The procedures for immunofluorescence staining have been described (8). A substantial fraction of the cells expressed little core protein or a nondetectable amount. (*Lower*) The proportions of cells with various staining patterns in different phases of the cell cycle. Roughly 100 positive cells were counted at each time point for calculating the relative proportions of cells with different staining patterns. C > N, predominantly cytoplasmic staining; C = N, staining in both the nucleus and the cytoplasm; N > C, predominantly nuclear staining; Conf, confluent.

increased during the G_1 phase and reduced during the S phase.

Cell Cycle Regulation of Nuclear Localization of the Core Protein in Vero Cells. Because of the heterogeneity of the 3T3pARV1MTC cell population, a similar experiment was conducted with a stable transformant established from Vero cells, an immortalized monkey kidney cell line. This cell line, named Vero-C3, was established by transfecting Vero cells with pCMV-core, a plasmid containing the core protein coding sequence under the expression control of the CMV immediate early gene (*IE*) promoter. Vero-C3 cells were synchronized at the G_0 phase with a serum-free medium for cell cycle studies. The amount of the core protein in the nucleus increased significantly after the initiation of the cell cycle and reached a peak 6 h after synchronization (Fig. 3*A*). At this time point, the amount of the core protein in the nucleus was approximately 60% of that in the cytoplasm (Fig. 3*A*). Considering that the volume of the nucleus is approximately 1/8th to 1/10th of that of the cytoplasm (unpublished observation), the concentration of the core protein in the nucleus was ≈5- to 6-fold of that in the cytoplasm. In fact, the immunofluorescence staining experiment revealed that the core protein was stained mostly in the nuclei of all cells at this time point (Fig. 3*B*). Twelve hours after synchronization when the cells were in the S phase as determined by the [^{3}H]thymidine incorporation assay, the amount of the core protein in the nucleus was greatly reduced and the core protein was found again almost entirely in the cytoplasm (Fig. 3). After the S phase, the amount of the core protein in the nuclei of Vero-C3 cells again increased gradually (Fig. 3*A*). Twenty-one hours after synchronization, as revealed by the immunofluorescence staining experiment, the core protein was found distributed throughout the entire cell, including both the cytoplasm and the nucleus (Fig. 3*B*); and 48 h after synchronization, the core protein was stained primarily in the nucleus (Fig. 3*B*). Note that the amount of the core protein in the nucleus was slightly reduced 24 h after initiation of the cell cycle with the serum-containing medium. A similar reduction was also seen with the 3T3pARV1MTC cells at the same time point after initiation of the cell cycle (Fig. 1*A*). The reason for this reduction is unclear; it may represent the entry of the cells into the G_2 or the M phase of the cell cycle or, alternatively, it may represent entry of a fraction of cells into the S phase of the second cell cycle.

Hypophosphorylation of the Nuclear Core Protein. The carboxyl-terminal arginine-rich region of the core protein contains a nuclear localization signal (4, 5). This signal can be phosphorylated by cellular kinases (11). To investigate the role of phosphorylation on nuclear localization of the core protein, the 3T3pARV1MTC cells were grown to confluence and labeled with [^{32}P]orthophosphate. Although a phospho-core protein signal was detected in the cytoplasmic lysate, no significant phospho-core protein signal was detected in the nuclear lysate (Fig. 4*A*). To ensure that the core protein was present in the nuclear lysate, a Western-blot analysis was performed. Similar to the results shown in Fig. 1*B*, a significant amount of the core protein was detected in both the nuclear and the cytoplasmic lysates (Fig. 4*B*). Thus, these results indicate that, in contrast to the cytoplasmic core protein, the nuclear core protein was hypophosphorylated. This could be due to the presence of a phosphatase activity in the nucleus or, alternatively, to suppression of nuclear import and/or activation of nuclear export of the core protein by phosphorylation.

DISCUSSION

Our results presented in this report indicate that, in two different immortalized cell lines, NIH 3T3 cells and Vero cells, the concentration of the core protein in the nucleus is

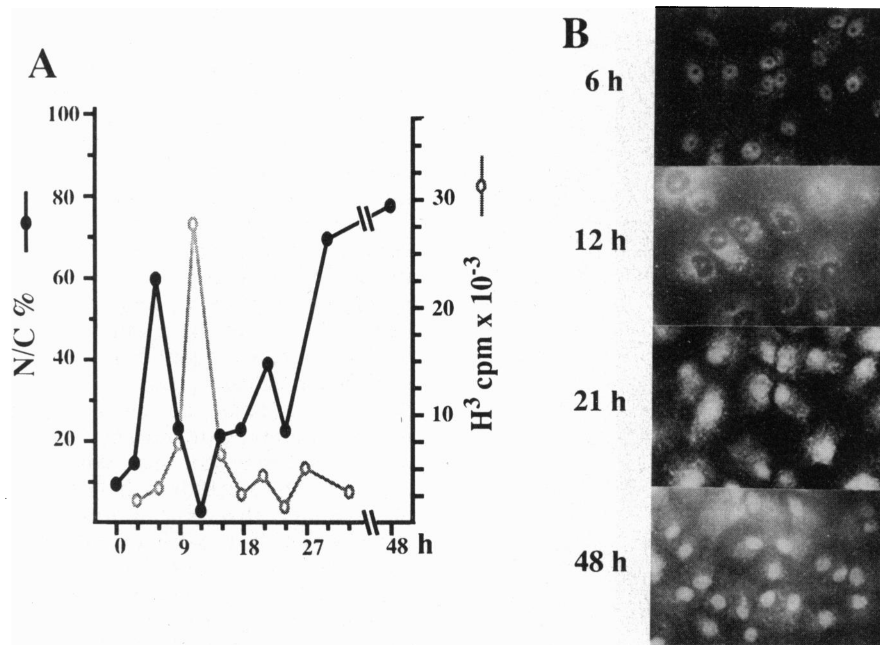

FIG. 3. Cell cycle regulation of nuclear localization of the core protein in Vero-C3 cells. (*A*) RIA and [^{3}H]thymidine incorporation assay results. (*B*) Immunofluorescence staining results. Vero-C3 cells synchronized by serum deprivation were analyzed at various time points by using the procedures described in the legends to Figs. 1 and 2.

regulated by the cell cycle. In our experiments, cells were synchronized in the G_0/G_1 phase with a serum-free medium (12). Initiation of the cell cycle with the serum-containing medium resulted in an increase of the core protein concentration in the nucleus during the G_1 phase. During the S phase, the core protein concentration in the nucleus was reduced to an undetectable level. When the cells were grown to confluence and arrested in the G_0/G_1 phase, the core protein concentration in the nucleus was again significantly increased. It is unclear why the core protein was not detected in the nuclei of serum-starved cells which were also arrested in the G_0/G_1 phase (Figs. 1 and 2). It is possible that serum-starved cells might be in a physiological state that is very different from that of the cells arrested by contact inhibition. This is evidenced by the observation that the core protein concentration is low in serum-starved cells. The cell

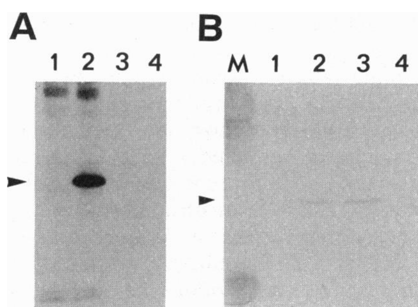

FIG. 4. Phosphorylation of the HBV core protein. (*A*) Phosphorylation of the core protein in 3T3pARV1MTC cells. Cells grown to confluence were labeled with [^{32}P]orthophosphate for 4 h. Similar results were obtained if the cells were labeled with [^{32}P]orthophosphate for only 1 h. (*B*) Western blot analysis of the core protein. Cells grown to confluence were separated into nuclear and cytoplasmic fractions. The protein samples were then blotted using the procedures described in the legend to Fig. 1. The amount of the cytoplasmic sample used for the analysis is approximately one-third of that of the nuclear sample. Lanes in both *A* and *B*: 1 and 4, control NIH 3T3 cells; 2 and 3, 3T3pARV1MTC cells; 1 and 2, cytoplasmic lysates; 3 and 4, nuclear lysates; M, protein molecular weight markers.

cycle regulation of nuclear localization of the core protein was further supported by our observation that the core protein accumulated primarily in the nucleus when entry of the cell cycle into the S phase was blocked by the chemical aphidicolin. Removal of the chemical resulted in the entry of cells into the S phase and the accumulation of the core protein in the cytoplasm.

How the cell cycle regulates nuclear localization of the core protein is unclear. On the basis of the observation that the phospho-core protein was detected in the cytoplasm but not in the nucleus (Fig. 4), we speculate that phosphorylation of the core protein has a negative effect on the nuclear localization of the core protein. In this regard, lack of nuclear core protein during the S phase may be the result of activation of a cellular kinase at this particular stage of the cell cycle. $p34^{cdc2}$ kinase is a kinase that is activated during the transition from G_1 to S phase of the cell cycle (13, 14). We have found that this kinase could phosphorylate the core protein *in vitro* (data not shown). It is possible that this kinase and/or its related kinases may negatively regulate the nuclear localization of the core protein during the S phase.

In HBV-infected hepatocytes, the core protein (core antigen) has been detected in the nucleus, the cytoplasm, or both (1–3). It has been reported that in asymptomatic patients, the core protein is detected largely in the nuclei of infected hepatocytes; however, in patients with more aggressive hepatitis, the core protein is localized in the cytoplasm of a significant fraction of infected hepatocytes (2, 3). On the basis of our observation that the concentration of the nuclear core protein can be regulated by the cell cycle, we speculate that the differential subcellular localization of the core protein during natural infection may be related to cell cycles of hepatocytes. Since during the asymptomatic infection, hepatocytes are largely quiescent and arrested in the G_0/G_1 phase, the core protein is likely localized in the nucleus. However, during aggressive hepatitis, liver injury and regeneration cause many hepatocytes to enter cell cycles, and as a result the core protein will be expected to be detected in the cytoplasm of a significant number of hepatocytes. It has been reported that the core protein was detected primarily in the nuclei of hepatocytes of transgenic mice carrying the entire

HBV genome (15). It should be of interest to examine whether liver injury introduced by partial hepatectomy leads to localization of the core protein to the cytoplasm.

Cell cycle-regulated nuclear localization has been shown to be important for regulating the biological activities of the transcription factors SWI5 (16, 17) and v-jun (18). However, the biological function for the nuclear localization of the core protein remains unclear. It is possible that this process is important for transporting genomic DNA into the nucleus during the early phase of infection. Alternatively, it may be important for amplification of the covalently closed circular DNA for RNA transcription during the course of HBV replication (19). These two possibilities are not mutually exclusive. Recently, it has been demonstrated that the core protein could selectively suppress the expression of the β interferon gene (20). Thus, alteration of the subcellular localization of the core protein during the cell cycle may also have a profound effect on the regulation of cellular gene expression.

We thank Dr. A. McLachlan for providing us with the 3T3pARV1MTC cell line; Dr. D. Chien for providing us with the purified HBV core protein; and Drs. P. Vogt, A. McLachlan, and T. S. B. Yen for critical reading of this manuscript. This work was supported by National Institutes of Health Research Grants AI26244 and CA54533 (to J.-H.O.) and CA44754 and EY07846 (to Y.-K.F.).

1. Mondelli, M., Tedder, R. S., Ferns, B., Pontisso, P., Realdi, G. & Alberti, A. (1986) *Hepatology* **6**, 199–204.
2. Chu, C.-M. & Liaw, Y.-F. (1987) *Gastroenterology* **92**, 220–225.
3. Hsu, H.-C., Su, I.-J., Lai, M.-Y., Chen, D.-S., Chang, M.-H., Chuang, S.-M. & Sung, J.-L. (1987) *J. Hepatol.* **5**, 45–50.
4. Yeh, C.-T., Liaw, Y.-F. & Ou, J.-H. (1990) *J. Virol.* **64**, 6141–6147.
5. Eckhardt, S. G., Milich, D. R. & McLachlan, A. (1991) *J. Virol.* **65**, 575–582.
6. McLachlan, A., Milich, D. R., Raney, A. K., Riggs, M. G., Hughes, J. L., Sorge, J. & Chisari, F. V. (1987) *J. Virol.* **61**, 683–692.
7. Ou, J.-H., Laub, O. & Rutter, W. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1578–1582.
8. Ou, J.-H., Yeh, C.-T. & Yen, T. S. B. (1989) *J. Virol.* **63**, 5238–5243.
9. Freytag, S. O. (1988) *Mol. Cell. Biol.* **8**, 1614–1624.
10. Pedrali-Noy, G., Spadari, S., Miller-Faures, A., Miller, A. O. A., Kruppa, J. & Koch, G. (1980) *Nucleic Acids Res.* **8**, 377–387.
11. Yeh, C.-T. & Ou, J.-H. (1991) *J. Virol.* **65**, 2327–2331.
12. Mudryj, M., Devoto, S. H., Hiebert, S. W., Hunter, T., Pines, J. & Nevins, J. R. (1991) *Cell* **65**, 1243–1253.
13. Nurse, P. (1990) *Nature (London)* **344**, 503–508.
14. Welch, P. J. & Wang, J. Y. T. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 3093–3097.
15. Farza, H., Hadchouel, M., Scotto, J., Tiollais, P., Babinet, C. & Pourcel, C. (1988) *J. Virol.* **62**, 4144–4152.
16. Nasmyth, K., Adolf, G., Lydall, D. & Seddon, A. (1990) *Cell* **62**, 631–647.
17. Moll, T., Tebb, G., Surana, U., Robitsch, H. & Nasmyth, K. (1991) *Cell* **66**, 743–758.
18. Chida, K. & Vogt, P. K. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 4290–4294.
19. Tuttleman, J. S., Pourcel, C. & Summers, J. (1986) *Cell* **47**, 451–460.
20. Whitten, T. M., Quets, A. T. & Schloemer, R. H. (1991) *J. Virol.* **65**, 4699–4704.
21. Laemmli, U. K. (1970) *Nature (London)* **277**, 680–682.