JOURNAL OF VIROLOGY, May 1996, p. 2691–2696 Vol. 70, No. 5
0022-538X/96/$04.00+0

n-Butyrate, a Cell Cycle Blocker, Inhibits Early Amplification of Duck Hepatitis B Virus Covalently Closed Circular DNA after In Vitro Infection of Duck Hepatocytes

FABRICE TURIN,[1] CHRISTELLE BOREL,[1] MEHDI BENCHAIB,[2] ALAN KAY,[1] CATHERINE JAMARD,[1] CHRISTIANE GUGUEN-GUILLOUZO,[3] CHRISTIAN TRÉPO,[1] AND OLIVIER HANTZ[1]*

Unité de Recherche sur les Hépatites, le SIDA et les Rétrovirus Humains, Institut National de la Santé et de la Recherche Médicale 271, 69424 Lyon Cedex 03,[1] Laboratoire de Cytologie Analytique, 69373 Lyon Cedex 08,[2] and Unité de Recherches Hépatologiques, Institut National de la Santé et de la Recherche Médicale 49, Hôpital de Pontchaillou, Rennes Cedex,[3] France

Received 27 November 1995/Accepted 29 January 1996

During chronic hepadnavirus infection, virus persistence depends on the regulation of the pool of covalently closed circular DNA (cccDNA), which is the template for transcription of viral RNA species. The development of in vitro infection of duck hepatocyte primary cultures by duck hepatitis B virus (DHBV) provides a unique opportunity to study the regulation of cccDNA synthesis. After DHBV in vitro infection, cccDNA is detected 1 day later and is amplified to a high copy number after 1 week in culture. We studied whether this amplification occurs during cell cycle progression of duckling hepatocytes. By using [^{3}H]thymidine incorporation, we found that hepatocytes obtained from 3-week-old ducklings spontaneously entered the S phase of the cell cycle when cultured in serum-free medium without added growth factors. Bromodeoxyuridine labeling confirmed that cellular DNA synthesis took place in more than 50% of parenchymal cells. Cytofluorometry analysis revealed the presence of asynchronous populations and polyploidization processes. The addition of a cell cycle blocker, *n*-butyrate, completely inhibited [^{3}H]thymidine incorporation and blocked duckling hepatocytes in the G_1 phase of the cell cycle. Simultaneously, butyrate inhibited cccDNA amplification and allowed the establishment of DHBV infection, as demonstrated by the detection of a basal level of cccDNA in treated hepatocytes. Both effects were reversible since active cell DNA synthesis was restored and cccDNA accumulated after drug withdrawal.

Duck hepatitis B virus (DHBV), an animal model for human hepatitis B virus (21), causes persistent noncytopathic infection of hepatocytes. The hepadnavirus genome is a small, partially double-stranded relaxed circular (RC) DNA molecule that is replicated by reverse transcription of a 3.5-kb terminally redundant mRNA transcript, the pregenome.

A covalently closed circular (ccc) supercoiled viral DNA molecule is detected in the nuclei of hepadnavirus-infected hepatocytes (20). This cccDNA is presumed to be the template for transcription of viral RNA species, including the pregenome RNA, and is generated initially by the direct conversion of infecting RC virion DNA (20).

Virus persistence in infected cells during chronic infections depends on the regulation of the pool of transcriptionally active cccDNA molecules. This control occurs by the recycling of encapsidated viral DNA (core particles) into the nucleus, not by semiconservative replication of cccDNA (35, 37). One critical issue is to identify cell and/or viral signals that determine whether core particles either are matured as virions which exit the cell or are recycled to the nucleus to maintain the pool of cccDNA. It has been demonstrated that the DHBV pre-S envelope protein regulates cccDNA amplification by a negative feedback mechanism (18, 33, 34). It has been speculated that core protein also plays a role in regulating cccDNA levels. Nuclear transport (19, 39) of the hepadnavirus core protein and/or nucleocapsid disassembly (2, 11, 15, 41) may be crucial for the entry of hepadnavirus encapsidated genomic DNA into the nucleus and its conversion to supercoiled DNA.

Cell cycle regulation of the nuclear localization of hepatitis B virus core protein has been demonstrated in transfected cell lines (39). If such a relationship between the cell cycle and regulation of cccDNA synthesis exists, the early amplification of cccDNA observed in the infection of hepatocyte primary cultures (35) may be influenced by duck hepatocyte proliferation. It has been shown that hepatocytes from different species can achieve one or more rounds of DNA synthesis (1, 22). However, until now, no study has described the proliferative activity of duck hepatocytes in vitro. In this paper, we present evidence that duckling hepatocytes can undergo at least two rounds of DNA synthesis without the addition of growth factors. We have used this model system to devise experiments to investigate if the cell cycle can influence cccDNA synthesis. For this purpose, we established DHBV in vitro infection in the presence of sodium butyrate, a cell cycle blocker which has been used to distinguish the dependence on cell cycle control of smaller versus larger DNA virus families (30).

MATERIALS AND METHODS

Infection of primary hepatocyte cultures. Hepatocytes were isolated from 3- to 6-week-old Pekin ducklings by in situ double-step collagenase perfusion of their livers (9). Since we observed lot-to-lot variation in the efficiency of infection of hepatocytes by DHBV, a unique lot of collagenase H (Boehringer Mannhein), selected on the basis of cell viability and susceptibility to DHBV infection, was used in this study. Cell viability was assessed by the trypan blue exclusion test and was usually greater than 80%. When more than 20% of the cells stained with trypan blue, damaged cells were removed by centrifugation through isotonic Percoll solution, as described by Berry et al. (1). Hepatocytes were seeded on six-well plates at a low density of approximately 2.5×10^5 cells per well in L15

* Corresponding author. Mailing address: INSERM U271, 151 Cours Albert Thomas, 69424 Lyon Cedex 03, France. Phone: 72681982. Fax: 72681971. Electronic mail address: u271@lyon151.inserm.fr.

medium supplemented with 5×10^{-5} M hydrocortisone hemisuccinate, 5 μg of bovine insulin per ml, and 10% fetal calf serum. Twelve hours after seeding, the medium was discarded and replaced with the same medium without fetal calf serum. Cultures were incubated at 37°C in a humid atmosphere containing 5% CO_2, and media were renewed daily. The inoculum, containing approximately 4×10^9 DNA genome equivalents per ml, was a pool of DHBV-positive sera obtained from 2-week-old experimentally infected ducks. Cells (on six-well plates) were infected with 0.1 ml of inoculum on day 2 postplating for 2 h at 37°C, as previously described (35).

Cell cycle inhibitor. Sodium butyrate (Sigma) was used to block hepatocytes in the G_1 phase of the cell cycle (4). The stock solution was 0.5 M dissolved in H_2O, with the pH adjusted to 7, and was used at a concentration of 5 mM (31, 32).

[^{3}H]thymidine incorporation. For the measurement of DNA synthesis, cultures on six-well plates were treated with 4 μCi (5 Ci/mmol) of [*methyl*-^{3}H]thymidine (Amersham) for 4 or 24 h before being harvested. Cells were washed twice with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer and were then lysed with 500 μl of 10 mM Tris (pH 7.5)–10 mM EDTA–0.5% sodium dodecyl sulfate (SDS). After overnight incubation at 4°C with 1 volume of 30% trichloroacetic acid, acid-insoluble materials were washed once with cold 10% trichloroacetic acid, twice with cold 5% trichloroacetic acid, and finally with 95% ethanol. They were then dissolved in 0.2 ml of 0.33 N NaOH, and radioactivity was measured in a liquid scintillation counter.

Flow cytometric measurements and data analysis. The preparation and staining of hepatic nuclei with propidium iodide was carried out daily by the procedures described in a Cycle Test Plus DNA Reagent Kit (Becton Dickinson). Ten thousand nuclei per sample were analyzed. A Becton Dickinson FACScan flow cytometer system equipped with a doublet discrimination module and CellFit software was used.

Labeling index by microscopy of BrdU-stained cells. Immunocytochemical staining for bromodeoxyuridine (BrdU) was done by using Labeling and Detection Kit I (Boehringer Mannheim). BrdU (10 mM) was added to the culture medium from 10 h to 96 h, with the medium changed daily. The labeling index was determined by microscope counting of immunostained cells.

Assay of infected hepatocytes for viral DNA. Viral DNA replicative intermediates and cccDNA were assayed as previously described (33). Briefly, cells were lysed with buffer containing SDS and protein-bound replicative forms were precipitated in a protein-detergent complex by the addition of KCl to a final concentration of 0.5 M. After centrifugation, cccDNA was recovered from the soluble fraction by phenol extraction and ethanol precipitation. Replicative forms were dissolved by digestion of the pellet with proteinase K and purified by phenol extraction and ethanol precipitation. Viral DNA was analyzed by 1% agarose gel electrophoresis and blot hybridization with a ^{32}P-labeled full-length genomic DHBV probe, as previously described (9).

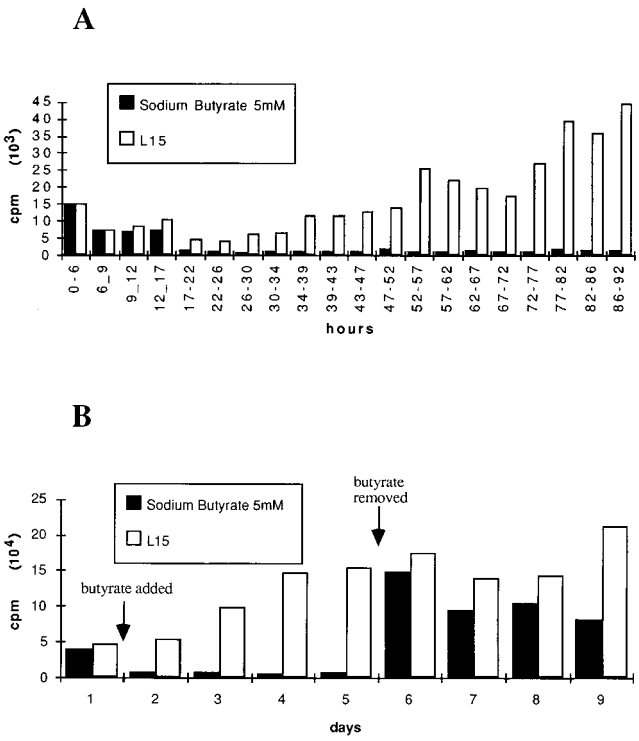


FIG. 1. DNA synthesis in duck hepatocyte cultures treated or untreated with sodium butyrate (5 mM). DNA synthesis was measured as described in Materials and Methods and was expressed as the incorporation of [^{3}H]thymidine into acid-insoluble material per plate during the 4 (A) or 24 (B) h before harvesting. Sodium butyrate was added 10 h after perfusion and was removed from culture medium at 120 h.

RESULTS

Several lines of evidence led us to suspect that duckling hepatocytes in primary cultures are not as quiescent as originally thought (40) (unpublished observations) and that this could affect the accumulation of DHBV cccDNA (19). We therefore decided to investigate to what extent these cells could spontaneously progress through the cell cycle and how this influenced cccDNA levels.

Primary cultures of duckling hepatocytes progress through the cell cycle without a requirement of added growth factor. We investigated whether duck hepatocytes could progress through the cell cycle by measuring cellular DNA synthesis. Hepatocytes attached within 0.5 to 4 hours in L15 medium in the presence of 10% fetal calf serum and were then cultured in the presence of the same medium lacking fetal calf serum, which was renewed daily. Cells displayed the typical polygonal morphology of liver cells for about 3 to 4 days. After 5 days in culture, cells underwent a progressive and extensive flattening process. The kinetics of DNA synthesis were analyzed by measuring daily [^{3}H]thymidine incorporation for either 4- (Fig. 1A) or 24 (Fig. 1B)-h periods. Measurements of [^{3}H]thymidine incorporation during the 4 h before harvesting (Fig. 1A) showed an initial weak round of DNA synthesis during the first 15 h after plating, and after 25 h, incorporation gradually reincreased. [^{3}H]thymidine incorporation measured over 24 h (Fig. 1B) showed that DNA synthesis continued to increase until day 9 of culture, suggesting that more than one round of DNA synthesis occurred. Immunocytochemical staining for BrdU was done to confirm that this DNA synthesis occurred in parenchymal cells. The labeling index obtained by counting immunostained hepatocytes reached 80% after 4 days of culture with continuous labeling with BrdU (Fig. 2A). In three independent experiments, no less than 50% of cells were consistently labeled. Thus, it appeared that a high proportion of duck hepatocytes progressed through the S phase of the cell cycle.

Flow cytometry analysis of nuclei (Fig. 3A) by propidium iodide fluorescence confirmed this progression and allowed us to distinguish several populations with 2, 4, and 8 N DNA contents. The percentage of nuclei with 2 N DNA content declined from around 70% on day 2 to 16% of total nuclei on day 4, indicating that more than 50% of nuclei had progressed through the S phase of the cell cycle during that time. The pool of nuclei with 4 N DNA content included cells in the G_2/M phase of the diploid cycle and cells in the G_1 phase of the tetraploid cycle.

Since the duckling serum used as viral inoculum may contain growth factors, cell cycle progression of uninfected hepatocytes obtained from a 3-week-old duckling was monitored by cytometry analysis. The results showed that in this experiment, there were no major differences in cell cycle transitions between duck serum-treated and untreated hepatocytes (data not shown).

Effects of sodium butyrate on cultured duck hepatocytes. Sodium butyrate was added to the culture medium 24 h after perfusion to a final concentration of 5 mM. Sodium butyrate treatment preserved the differentiated morphological aspect of cells but was associated with toxicity, as evidenced by cell death after 3 to 4 days of treatment. Thus, butyrate was removed from the culture medium after 5 to 6 days of treatment.

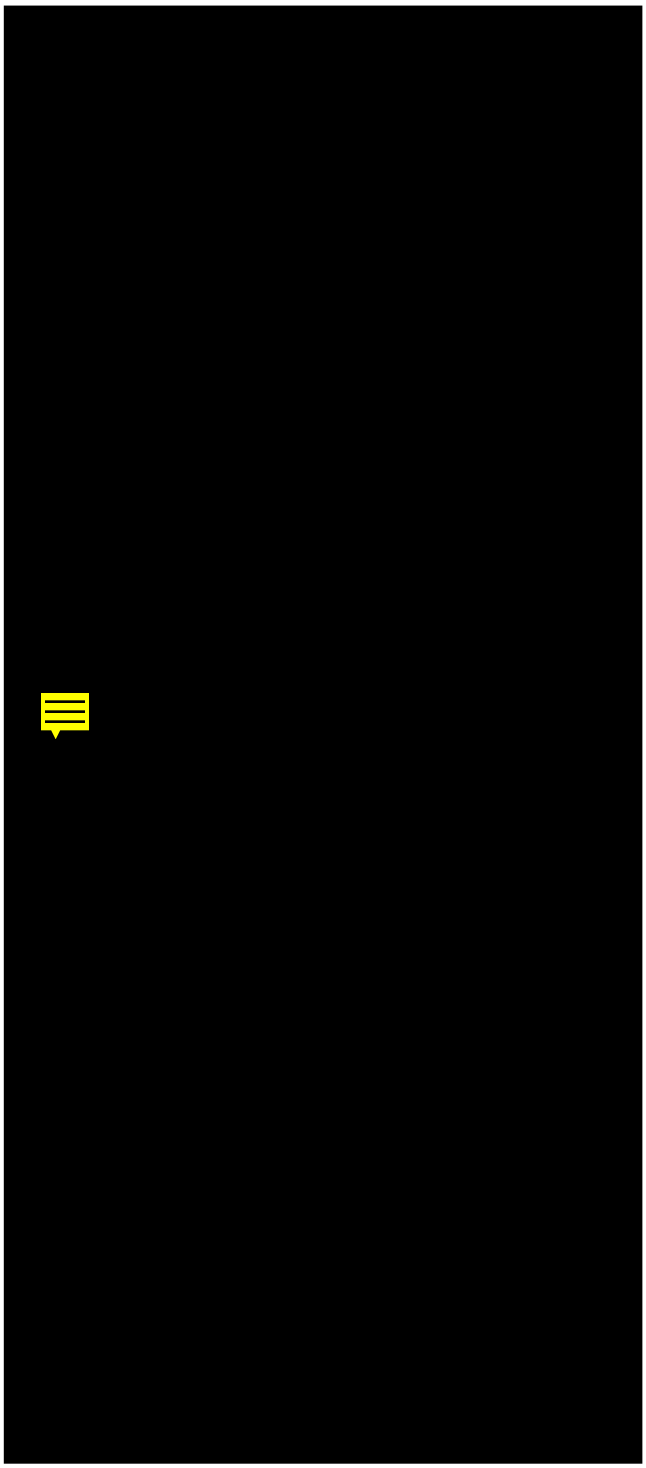


FIG. 2. Immunocytochemistry for BrdU in primary culture of duck hepatocytes in basal medium (A) or in butyrate-supplemented medium (B).

No [^{3}H]thymidine incorporation was detected in butyrate-treated cultures until butyrate was removed (Fig. 1). DNA synthesis inhibition was confirmed with BrdU labeling (Fig. 2B). The withdrawal of butyrate allowed cells to enter the S phase rapidly (Fig. 1B) and was followed by a rapid loss of the hepatocyte-differentiated phenotype, as estimated from the morphological aspect of cells.

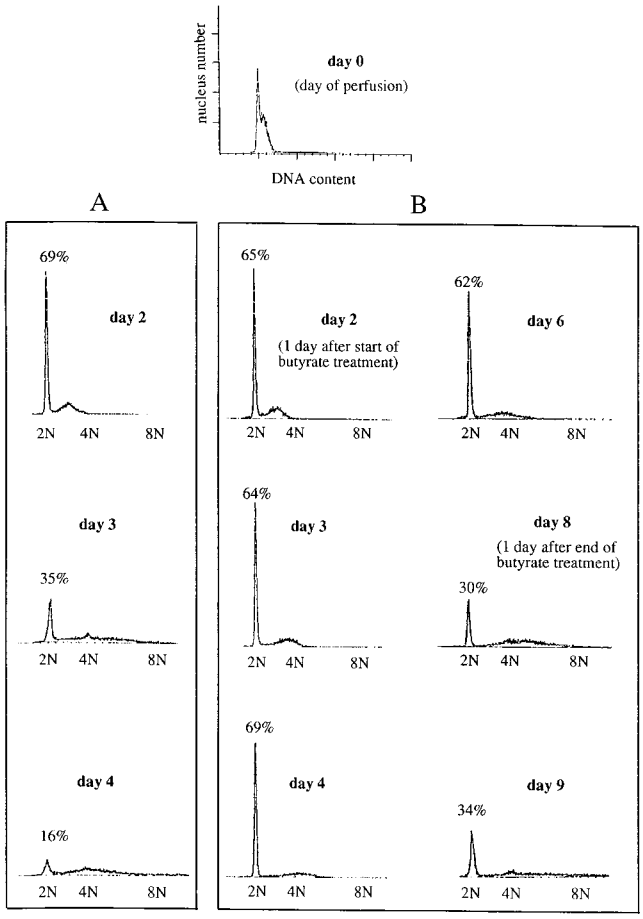


FIG. 3. Flow cytometric analysis of cell transitions in control cultures (A) or in butyrate-treated cultures (B). Butyrate was removed from the culture medium on day 7. Time points indicate intervals subsequent to cell seeding after perfusion (day 0). Data are histograms in which the nuclear DNA content (*x* axis) is plotted against the nucleus number after doublet discrimination. Nuclei with 2 N DNA content represent nuclei in the G_0/G_1 phase of the diploid cycle. Nuclei with 4 N DNA content represent nuclei in the G_2/M phase of the diploid cycle or nuclei in the G_1 phase of the tetraploid cycle. Nuclei with an intermediate DNA content are in the S phase. The percentages of nuclei with 2 N DNA content are indicated.

Flow cytometry analysis confirmed the cell cycle block until the end of treatment. Indeed, on day 4, nuclei with 2 N DNA content represented almost 70% of total nuclei, the same level as on day 2 (Fig. 3B). The marked decrease in the percentage of nuclei with 2 N DNA content on day 8 (only 30% of total nuclei) confirmed that cellular DNA synthesis restarts after drug withdrawal.

cccDNA amplification is reversibly blocked in sodium butyrate-treated cultures. Hepatocytes cultivated in the presence or absence of 5 mM butyrate were infected on day 2 postplating (i.e., 1 day after the start of butyrate treatment). A differential extraction procedure (33) was used to separate protein-free (mostly cccDNA) and protein-bound (other replication intermediates) viral DNAs. An analysis (Fig. 4A) of protein-free viral DNA in control cultures showed a gradual increase in cccDNA from day 1 to day 6 postinfection. In sodium butyrate-treated cultures, cccDNA was detected on day 1 postinfection at the same level as that in control cultures, but no further amplification was observed until the end of treatment on day 5 (Fig. 4A). During treatment, no significant differences in the

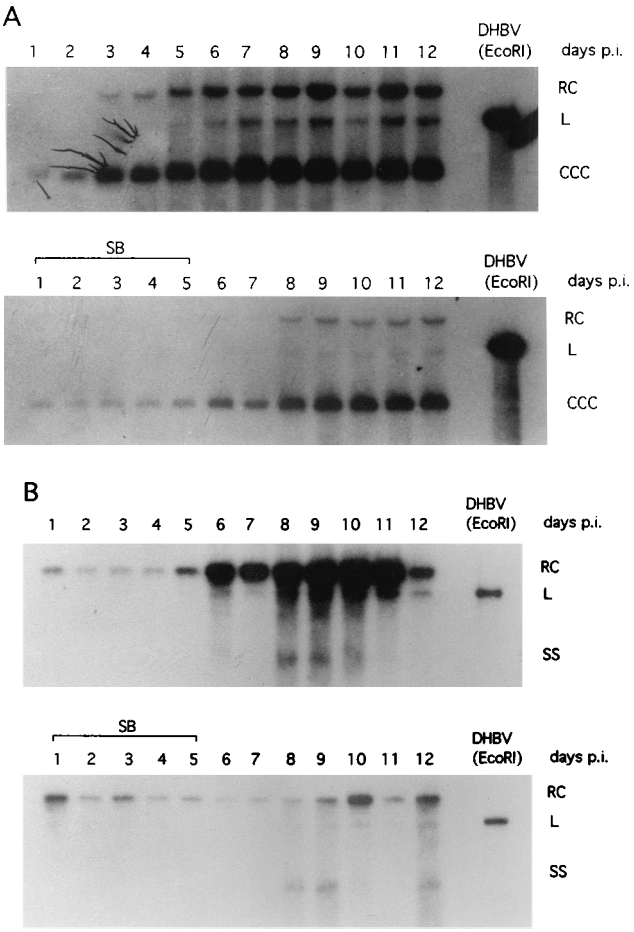


FIG. 4. Southern blot analysis of DHBV DNA extracted as described in Materials and Methods. Nucleic acids were dissolved in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA, and one-fifth of the nucleic acid prepared from each petri dish was subjected to electrophoresis. (A) DNA recovered from the soluble fraction (cccDNA fraction); (B) DHBV protein-bound replicative forms. The upper gel in each panel contained DNAs extracted from control cultures; the lower gel in each panel contained DNAs extracted from butyrate-treated cultures. The number above each lane indicates the day postinfection (p.i.). Infection was established on day 2 postplating. The migrations of RC, linear (L), ccc, and single-stranded (SS) viral DNAs are indicated on the right. Cloned linear DHBV DNA (100 pg) was used as a hybridization control. Sodium butyrate (SB) was added 24 h after perfusion (day 1 postplating) and was removed from the culture medium on day 7 postplating.

levels of RC DNA between treated and untreated cultures were observed (Fig. 4B). The withdrawal of butyrate was followed by a gradual increase in cccDNA for 3 to 4 days. Thus, sodium butyrate did not prevent the initial conversion of the viral genome into cccDNA but reversibly blocked its subsequent amplification.

An analysis of protein-bound replicative viral DNA (Fig. 4B) showed a marked increase in RC DNA and the apparition of single-stranded DNA (at day 6 in control cultures and day 8 in butyrate-treated cultures) when amplification of cccDNA was completed. The levels of cccDNA and single-stranded DNA were clearly diminished in butyrate-treated cultures after the cessation of butyrate treatment.

DISCUSSION

Duck hepatocyte primary cultures have been widely used for DHBV replication studies and antiviral screening. In particular, cccDNA synthesis, which represents a key step in the hepadnavirus life cycle, has been investigated in this culture system (33, 35). In all these studies, the hepatocytes in primary cultures were considered to be quiescent cells (5, 8) and the possible role of cell cycle progression in hepadnavirus replication in this culture system was not taken into account.

The experiments described here demonstrate that, surprisingly, primary cultured hepatocytes of 3- to 4-week-old ducks spontaneously enter the cell cycle. It is known that cell proliferation can be stimulated in liver parenchymal cells maintained in monolayer cultures (1, 22). Rat liver parenchymal cell proliferation in primary cultures is the best documented model (23). Isolated rat hepatocytes can be induced to grow in culture in defined serum-free media. However, growth factors (epidermal growth factor and hepatocyte growth factor) and a variety of auxiliary hormones and metabolites are absolutely required in the culture medium. Nevertheless, spontaneous autonomous growth of hepatocytes has already been described for cells isolated within a few days of birth. Nakamura et al. reported that early neonatal rat hepatocytes grew actively even without serum or hormones (24). Our results show that spontaneous growth also occurs in duckling hepatocyte primary cultures. Moreover, different cell populations with respect to cell cycle kinetics and ploidy distribution were observed. Such a polyploidization phenomenon has already been described for cultured rat hepatocytes (23). The detection of nuclei with 8 N DNA content indicated that these nuclei undergo at least two rounds of DNA synthesis after cell seeding. However, the extent to which cells undergo mitosis or cytokinesis could not be established in this study. In view of these results, one can assume that the cccDNA amplification initially demonstrated by Tuttleman et al. (35) during DHBV infection of duck hepatocyte primary cultures occurred during cell cycle progression.

We attempted to block this cell cycle progression with sodium butyrate. Sodium butyrate is a potent inhibitor of proliferation and an inducer of differentiation in several cell types (16), including hepatocytes in primary cultures (16, 31, 32). Butyrate induces hyperacetylation of histones and hypophosphorylation of histone H1 (6), and it has been reported to cause reversible G_1 block and apoptosis (4, 10, 27). Sodium butyrate clearly blocks duck hepatocytes at 2 or 4 N DNA content. In all probability, butyrate-arrested cells with 2 N DNA content are in the G_0/G_1 phase of the cell cycle; one can postulate that butyrate-arrested cells with 4 N DNA content are blocked in the G_1 phase of the tetraploid cell cycle.

It appears that sodium butyrate also impairs early cccDNA amplification during in vitro infection. Both effects are reversible since cellular DNA synthesis and viral cccDNA amplification occur after drug withdrawal, although fewer cells were able to initiate DNA synthesis after 5 days of butyrate treatment.

How does sodium butyrate affect cccDNA accumulation? Several nonexclusive hypotheses are conceivably involved. Butyrate may act on cccDNA amplification through its action on the cell cycle. Culture conditions which impair hepatocyte cell cycle progression, such as the presence of dimethyl sulfoxide or high cell density (1) (preliminary observations), also result in the maintenance of low steady-state levels of cccDNA (29, 37). Progression through the cell cycle may diminish expression of the viral pre-S protein, which is known to negatively regulate cccDNA synthesis. Pre-S mutants which either are unable to synthesize pre-S protein or produce an altered pre-S protein accumulate high levels of cccDNA (17). It is thought that the pool size of cccDNA molecules is regulated by the ability of the viral large envelope protein to direct newly synthesized viral

DNA into the pathway for virion secretion and thereby to inhibit their utilization for the formation of viral cccDNA. A relationship between cell status and hepatitis B virus envelope protein secretion has been reported for a human hepatoma cell line (28). Thus, cccDNA amplification during the cell cycle progression of duck hepatocytes could correspond to low levels of pre-S expression. A variant of this hypothesis is that while entry into the cell cycle decreases the capacity of hepatocytes to synthesize viral envelope proteins, butyrate and other differentiating agents increase it. Second, the cccDNA amplification process requires the entry of encapsidated cytoplasmic viral DNA into the nucleus. The results of experiments with transgenic mice (11) suggest that because of their size, core particles cannot translocate across the nuclear membrane, despite the existence of a nuclear localization signal on the core protein (7, 38). Thus, the cell cycle could allow cytoplasmic viral DNA to enter the nucleus either through the destabilization of the nuclear membrane during mitosis or by destabilizing the cytoplasmic core particles themselves. Phosphorylation of the core protein by cell cycle-regulated kinases, such as protein kinase C (15) or $p34^{cdc2}$ kinase (19), may alter its nucleic acid binding capacity (12) and lead to destabilization of the nucleocapsid, allowing release of the genome into the nucleus and cccDNA formation. Third, butyrate may exert a more direct role in cccDNA accumulation. DHBV cccDNA exists as minichromosomes associated with histone and nonhistone proteins (3, 25), and the action of butyrate on posttranslational histone modifications may affect cccDNA formation. One argument against such a direct effect is that butyrate does not appear to affect the initial conversion of inoculum virion DNA into cccDNA (Fig. 4). Moreover, the results of preliminary experiments indicate that butyrate does not affect in vitro priming or elongation (36) of DHBV DNA by DHBV polymerase (data not shown). However, butyrate is toxic to primary duck hepatocytes. The reasons for this are not known, and we cannot exclude the possibility that the events leading to this toxicity are also directly responsible for the lack of cccDNA accumulation.

The in vivo biological significance of these results remains to established. In one report (26), it was shown that liver regeneration after a partial hepatectomy stimulated viral production in chronically infected ducks. In contrast, hepatocyte proliferation is associated with the recovery phase during acute infection of woodchucks (14) and ducks (13) or after antiviral therapy of chronically infected ducks (8). In adults, hepatocytes do not normally divide but can still ensure high levels of viral replication. However, immune-mediated liver injury during chronic hepatitis leads to limited but continuous liver cell death and proliferation. A possible increase in the pool of cccDNA in these growing cells may play an important role in the maintenance of viral infection.

ACKNOWLEDGMENTS

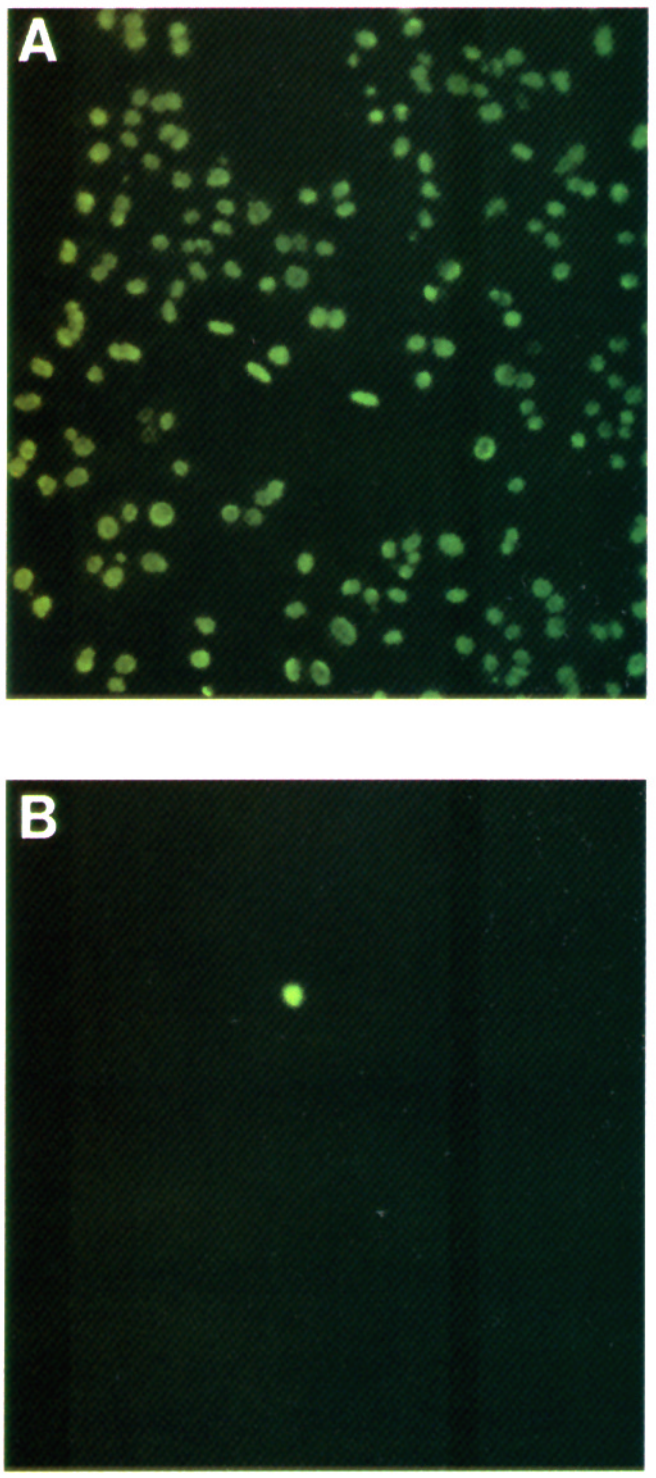
We thank F. Zoulim for critically reading the manuscript.

F. Turin was a recipient of fellowships from Ligue Nationale Contre le Cancer and from Institut Pasteur Lyon. This work was supported by the Institut National de la Santé et de la Recherche Médicale.

REFERENCES

1. **Berry, M. N., A. M. Edwards, and G. J. Barrit.** 1991. Isolated hepatocytes preparation, properties and applications, p. 265–354. *In* R. H. Burdon (ed.), Laboratory techniques in biochemistry and molecular biology—1991. Elsevier Science Publishers, Amsterdam.
2. **Birnbaum, F., and M. Nassal.** 1990. Hepatitis B virus nucleocapsid assembly: primary structure requirements in the core protein. J. Virol. **64:**3319–3330.
3. **Bock, C. T., P. Schranz, C. H. Schroder, and H. Zentgraf.** 1994. Hepatitis B virus genome is organized into nucleosomes in the nucleus of the infected cell. Virus Genes **8:**215–229.
4. **Charollais, R. H., C. Buquet, and J. Mester.** 1990. Butyrate blocks the accumulation of CDC2 mRNA in late G1 phase but inhibits both the early and late G1 progression in chemically transformed mouse fibroblasts BP-A31. J. Cell. Physiol. **145:**46–52.
5. **Civitico, G. M., and S. A. Locarnini.** 1994. The half-life of duck hepatitis B virus supercoiled DNA in congenitally infected primary hepatocyte cultures. Virology **203:**81–89.
6. **D'Anna, J. A., R. A. Tobey, and R. L. Gurley.** 1980. Concentration-dependent effects of sodium butyrate in Chinese hamster cells: cell cycle progression, inner-histone acetylation, histone H1 dephosphorylation and induction of an H1-like protein. Biochemistry **19:**2656–2671.
7. **Eckhardt, S. G., D. R. Milich, and A. McLachlan.** 1991. Hepatitis B virus core antigen has two nuclear localization sequences in the arginine-rich carboxy terminus. J. Virol. **65:**575–582.
8. **Fourel, I., J. M. Cullen, J. Saputelli, C. E. Aldrich, P. Schaffer, D. R. Averett, J. Pugh, and W. S. Mason.** 1994. Evidence that hepatocyte turnover is required for rapid clearance of duck hepatitis B virus during antiviral therapy of chronically infected ducks. J. Virol. **68:**8321–8330.
9. **Fourel, I., P. Gripon, O. Hantz, L. Cova, V. Lambert, C. Jacquet, K. Watanabe, J. Fox, C. Guillouzo, and C. Trepo.** 1989. Prolonged duck hepatitis B virus replication in duck hepatocytes cocultivated with rat epithelial cells: a useful system for antiviral testing. Hepatology **10:**186–191.
10. **Gong, J. P., F. Traganos, and Z. Darzynkiewicz.** 1994. Use of the cyclin E restriction point to map cell arrest in G(1)-induced by n-butyrate, cycloheximide, staurosporine, lovastatin, mimosine and quercetin. Int. J. Oncol. **4:** 803–808.
11. **Guidotti, L. G., V. Martinez, Y.-T. Loh, C. E. Rogler, and F. V. Chisari.** 1994. Hepatitis B virus nucleocapsid particles do not cross the hepatocyte nuclear membrane in transgenic mice. J. Virol. **68:**5469–5475.
12. **Hatton, T., S. Zhou, and D. N. Standring.** 1992. RNA- and DNA-binding activities in hepatitis B virus capsid protein: a model for their roles in viral replication. J. Virol. **66:**5232–5241.
13. **Jilbert, A. R., T.-T. Wu, J. M. England, P. M. Hall, N. Z. Carp, A. P. O'Connell, and W. S. Mason.** 1992. Rapid resolution of duck hepatitis B virus infections occurs after massive hepatocellular involvement. J. Virol. **66:** 1377–1388.
14. **Kajino, K., A. R. Jilbert, J. Saputelli, C. E. Aldrich, J. Cullen, and W. S. Mason.** 1994. Woodchuck hepatitis virus infections: very rapid recovery after a prolonged viremia and infection of virtually every hepatocyte. J. Virol. **68:** 5792–5803.
15. **Kann, M., and W. H. Gerlich.** 1994. Effect of core protein phosphorylation by protein kinase C on encapsidation of RNA within core particles of hepatitis B virus. J. Virol. **68:**7993–8000.
16. **Kruh, J.** 1982. Effects of sodium butyrate, a new pharmacological agent, on cells in cultures. Mol. Cell. Biol. **42:**65–82.
17. **Lenhoff, R. J., and J. Summers.** 1994. Construction of avian hepadnavirus variants with enhanced replication and cytopathicity in primary hepatocytes. J. Virol. **68:**5706–5713.
18. **Lenhoff, R. J., and J. Summers.** 1994. Coordinate regulation of replication and virus assembly by the large envelope protein of an avian hepadnavirus. J. Virol. **68:**4565–4571.
19. **Liao, W., and J.-H. Ou.** 1995. Phosphorylation and nuclear localization of the hepatitis B virus core protein: significance of serine in the three repeated SPRRR motifs. J. Virol. **69:**1025–1029.
20. **Mason, W. S., M. S. Halpern, J. S. England, G. Seal, J. Egan, L. Coates, C. Aldrich, and J. Summers.** 1983. Experimental transmission of duck hepatitis B virus. Virology **131:**375–384.
21. **Mason, W. S., G. Seal, and J. Summers.** 1980. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. J. Virol. **36:** 829–836.
22. **McGowan, J.** 1986. Hepatocyte proliferation in culture, p. 13–38. *In* A. Guillouzo and C. Guguen-Guillouzo (ed.), Isolated and cultured hepatocytes—1986. John Libbey Eurotext, London.
23. **Mossin, L., H. Blankson, H. Huitfeldt, and P. O. Seglen.** 1994. Ploidy-dependent growth and binucleation in cultured rat hepatocytes. Exp. Cell Res. **214:**551–560.
24. **Nakamura, T., M. Nagao, and A. Ichiara.** 1987. *In vitro* induction of neonatal rat hepatocytes by direct contact with adult rat hepatocytes. Exp. Cell Res. **169:**1–14.
25. **Newbold, J., H. Xin, M. Tencza, G. Sherman, J. Dean, S. Bowden, and S. Locarnini.** 1995. The covalently closed duplex form of the hepadnavirus genome exists in situ as a heterogeneous population of viral minichromosomes. J. Virol. **69:**3350–3357.
26. **Qiao, M., E. J. Gowans, and C. J. Burrell.** 1992. Intracellular factors, but not virus receptor levels, influence the age-related outcome of DHBV infection of ducks. Virology **186:**517–523.
27. **Sadaie, M. R., and L. H. Gordon.** 1994. Induction of developmentally programmed cell death and activation of HIV by sodium butyrate. Virology **202:**513–518.

28. **Saito, H., T. Kagawa, S. Tada, S. Tsunematsu, F. M. Guevara, T. Watanabe, T. Morizane, and M. Tsuchiya.** 1992. Effects of dexamethasone, dimethylsulfoxide and sodium butyrate on a human hepatoma cell line PLC/PRF/5. Cancer Biochem. Biophys. **13:**75–84.
29. **Seeger, C., J. Summers, and W. S. Mason.** 1991. Viral DNA synthesis. Curr. Top. Microbiol. Immunol. **168:**41–60.
30. **Shadan, F. F., L. M. Cowsert, and L. P. Villarreal.** 1994. *n*-Butyrate, a cell cycle blocker, inhibits the replication of polyomaviruses and papillomaviruses but not that of adenoviruses and herpesviruses. J. Virol. **68:**4785–4796.
31. **Staecker, J. L., C. A. Sattler, and H. C. Pitot.** 1988. Sodium butyrate preserves aspects of the differentiated phenotype of normal adult rat hepatocytes in culture. J. Cell. Physiol. **135:**367–376.
32. **Staecker, J. L., N. Sawada, and H. Pitot.** 1987. Stimulation of DNA synthesis in primary cultures of adult rat hepatocytes by sodium butyrate. Biochem. Biophys. Res. Commun. **147:**78–85.
33. **Summers, J., P. M. Smith, and A. L. Horwich.** 1990. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. J. Virol. **64:**2819–2824.
34. **Summers, J., P. M. Smith, M. Huang, and M. Yu.** 1991. Morphogenetic and regulatory effects of mutations in the envelope proteins of an avian hepadnavirus. J. Virol. **65:**1310–1317.
35. **Tuttleman, J. S., C. Pourcel, and J. W. Summers.** 1986. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. Cell **47:**451–460.
36. **Wang, G. H., and C. Seeger.** 1992. The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis. Cell **71:**663–670.
37. **Wu, T. T., L. Coates, L. Aldrich, J. Summers, and W. S. Mason.** 1990. In hepatocytes infected with duck hepatitis B virus, the template for viral RNA synthesis is amplified by an intracellular pathway. Virology **175:**255–261.
38. **Yeh, C.-T., Y.-F. Liaw, and J.-H. Ou.** 1990. The arginine-rich domain of hepatitis B virus precore and core proteins contains a signal for nuclear transport. J. Virol. **64:**6141–6147.
39. **Yeh, C. T., S. W. Wong, Y. K. Fung, and J. H. Ou.** 1993. Cell cycle regulation of nuclear localization of hepatitis B virus core protein. Proc. Natl. Acad. Sci. USA **90:**6459–6463.
40. **Yokota, T., K. Konno, E. Chonan, S. Mochizuki, K. Kojima, S. Shigeta, and E. de Clercq.** 1990. Comparative activities of several nucleoside analogs against duck hepatitis B virus in vitro. Antimicrob. Agents Chemother. **34:**1326–1330.
41. **Yu, M., and J. Summers.** 1991. A domain of the hepadnavirus capsid protein is specifically required for DNA maturation and virus assembly. J. Virol. **65:**2511–2517.


A
B