RESEARCH ARTICLE

High-Frequency Transfection and Cytopathology of the Hepatitis B Virus Core Antigen Gene in Human Cells

George H. Yoakum, Brent E. Korba, John F. Lechner
Takayoshi Tokiwa, Adi F. Gazdar, Todd Seeley, Mary Siegel
Lawrence Leeman, Herman Autrup, Curtis C. Harris

Abstract. *A protoplast fusion method was developed to stably transfect human cells with pSV2-derived plasmids at frequencies greater than 10^{-3}. This procedure made it possible to test the biological effect of a hepatitis B virus (HBV) gene independent of the viral structures required for infection. A pSV2gpt$^+$ plasmid constructed to carry a subgenomic fragment of HBV that contained the core antigen gene (*HBc *gene) was transfected into human cells. A human epithelial cell line was stably transfected with the* HBc$^+$ *gene by selecting recipient cells for expression of guanine phosphoribosyl transferase expression. With this* gpt$^+$/HBc$^+$ *cell line it was shown that growth in serum-free medium or treatment with 5′-azacytidine stimulates the production of the HBV core antigen. A hepatocellular carcinoma carrying the entire HBV genome was stimulated to produce the* HBc *gene product in response to the same factors that stimulated HBcAg production in the* gpt$^+$/HBc$^+$ *cell line constructed by transfection. The temporal relation between the cytopathologic response and* HBc *gene expression was similar for both cell types, indicating a primary role for* HBc *gene expression in the cytopathology of HBV-infected human liver.*

Hepatitis B virus (HBV), the major cause of viral hepatitis, has been epidemiologically linked to liver cancer (*1*, *2*). To understand the pathogenesis of HBV infection during acute and chronic disease processes, it is essential to separate various viral genetic elements and to study their biological effects and molecular biology in a model cell system in vitro. The HBV genome has been isolated on multicopy plasmids, and the primary DNA sequence of several subtypes have been described (*3–7*). By adapting the protoplast fusion method of transfection (*8–10*) for transfer of pSV2-derived plasmids (*11*) to a variety of human cells, including cells grown in serum-free media, we can transfect genes into human cells for isolation of genetically stable cell lines at frequencies greater than 10^{-3}. With this procedure, the HBV core antigen (*HBc*) gene was transiently expressed in 70 to 90 percent of recipient cells for 6 to 12 days after transfection, and *gpt*$^+$ (guanine phosphoribosyl transferase positive/*HBc*$^+$) cells were selected at frequencies greater than 10^{-3}. The immediate response of the recipient cells to high-frequency transfection provided a genetic test for cytopathologic effects that can be followed by analysis of the factors that regulate *HBc* gene expression in the stable *gpt*$^+$/*HBc*$^+$ population (*12*).

Total genomic HBV-DNA has been transfected into a variety of mammalian cell recipients by the $CaPO_4$-DNA method (*13–19*). A subgenomic fragment carrying the HBV surface antigen (HBsAg) gene has been transfected and stably expressed in a COS (simian) cell line (*18*). The emphasis of these experiments has been on (i) production of HBsAg, which is immunologically indistinguishable from that produced during HBV infection (*14*, *17–19*), (ii) the production of virus-like particles resulting in transient cytopathologic effects in HeLa cells (*16*), and (iii) the study of viral transcripts (*13*, *15*). However, the effect of *HBc* gene expression was difficult to discern from these studies, since recircularized HBV genomic DNA was transfected without a selectable marker (*16*). These experiments did not result in a stable cell line carrying only the *HBc* gene for studies of *HBc* gene expression. Transfer of tandem duplicates of the HBV genome into rat and mouse cells yielded stable cell lines producing detectable levels of HBsAg and HBeAg, but not HBV core antigen (HBcAg) (*16*, *17*). The HBsAg/HBeAg-positive rodent cells did not demonstrate a cytopathologic response to detectable levels of these HBV gene products (*16*, *17*). Transfection of HeLa cells with recircularized HBV resulted in notable cytopathology when HBsAg, HBcAg, and virus-like particles were observed (*16*). The similarity of these effects to those observed with Dane particle–infected liver hepatocytes (*16*) suggests that human carcinoma cells may provide effective model recipients for transfection experiments with subgenomic fragments of HBV.

High-Frequency Transfection of Human Cells

The frequency of stable transfection into NCI H292 [previously referred to as HuT 292 (*20*, *21*)] cells with pSV2-derived plasmids was 3×10^{-3} after selection for the *gpt*$^+$ (*11*) or *neo*$^+$ (*22*) markers in recipient cells (Table 1). Cells with the *gpt*$^+$ marker can be selected on the basis of their ability to convert xanthine to guanine when grown in medium containing aminopterin and mycophenolic acid (*11*); the *neo*$^+$ (neomycin) marker can be selected by growing the cells in medium containing G418-neomycin (*22*). The adapted protoplast fusion procedure described here yields substantially higher frequencies of stable transfection into NCI H292 cells than the $CaPO_4$-DNA method. The fraction of recipient cell populations that are selectable for stable transfection of the *gpt*$^+$ or *neo*$^+$ genes remains constant when recipient cells are challenged by selection within 72 hours or grown (three to ten divisions) to reach confluence after the transfection procedure. Thus, the biological effects of unstable expression of transferred genes in 70 to 90 percent of the population were observed after transfection, without reducing the *gpt*$^+$-resistant fraction of the original population of recipient cells. NCI H292 cells were tested for their

G. H. Yoakum, J. F. Lechner, and H. Autrup are senior staff fellows, B. E. Korba is a staff fellow, and C. C. Harris is chief in the Laboratory of Human Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205. T. Tokiwa is a visiting scientist, T. Seeley and L. Leeman are summer students, and M. Siegel is a technician, also in the Laboratory of Human Carcinogenesis, National Cancer Institute. A. F. Gazdar is deputy branch chief, National Cancer Institute–Navy Medical Oncology Branch.

ability to be transfected by $CaPO_4$-DNA (*23*) with pSV2 gpt^+ or neo^+ plasmids. Stably resistant cells were unobtainable by the selctions used to obtain transfected cells after protoplast fusion (Table 1). The physical methods used here to introduce exogenous DNA are relatively nontoxic. The development of protoplast fusion for human cells grown in serum-free conditions results in a procedure that is applicable to a variety of human cell types. However, we found that the human cell types we tested were sensitive to this fusion procedure when grown in Dulbecco's minimum essential medium (DMEM). Efficient use of the method required adaptation of these cells to growth in serum-free medium LHC-4 (*20, 21*), RPMI 1640 medium, MCDB 104 (*24*) or MCDB 151 (*25*) nutrient media.

To test the biological effects of the *HBc* gene, we constructed a hybrid plasmid carrying the *HBc* gene on an 1850-base pair (bp) Bam HI fragment ligated to the Bam HI site of pSV2gpt (pKYC200) (Fig. 1). The HBc^+ Bam HI fragment came from plasmid pAM6, which carries the HBV genome on pBR322 (*14*). Although there is significant variation among subtype sequences, the 1850-bp region carrying the *HBc* gene is reasonably conserved, and the only complete structural gene on this fragment encodes the *HBc* gene (*3–7*). The Bam HI site upstream of the *HBc* gene is mapped just within an open reading frame which is designated X since there is no defined transcript or gene product yet assigned to this region. Consistent with mapping data that locate Bam HI within the X-region open reading frame (*3–7*), we have sequenced the Bam HI fragment of pKYC200 and find neither a complete reading frame nor a potential fusion reading frame that would express the X-region of pKYC200. The Bam HI fragment on pKYC200 places the *HBc* gene in opposite orientation to the SV40 promoter of pSV2 that expresses the gpt^+ gene (Fig. 1). This suggests that the *HBc* gene on pKYC200 is probably expressed from a native promoter between the AUG (adenine, uracil, guanine) triplet at bp 451 and the Bam HI site. High-frequency transfection of pKYC200 into a human epithelial cell line with secretory properties such as NCI H292 provides a genetic test for the biological consequences of *HBc* gene expression separate from the rest of the HBV genome.

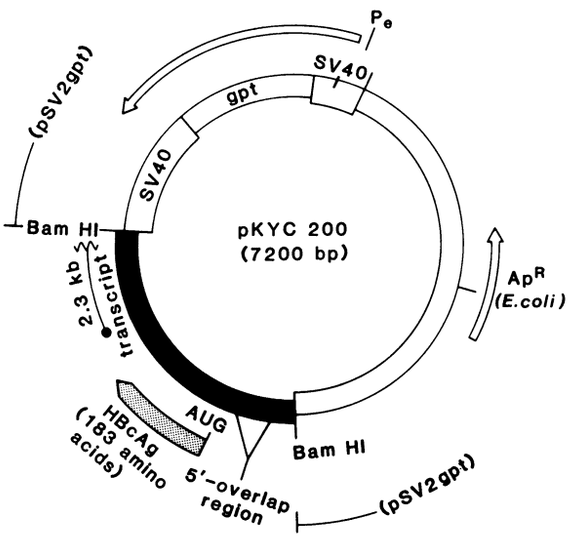

Fig. 1. Construction of plasmid pKYC200. The *HBc* gene was localized on an 1850-bp Bam HI fragment from plasmid pAM6. Plasmid pAM6 contains the HBV genome ligated into pBR322 at the Bam HI site that separates the HBV surface antigen gene (*HBs*) from its native promoter (*19*). The 1850-bp Bam HI fragment, released by the Bam HI/Pst I digest of pAM6 DNA, was isolated by ligation to pSV2gpt DNA at a 100-fold molar excess of fragment DNA to vector DNA. HB101 colonies carrying the pSV2gpt/*HBc* gene fragment were selected for resistance to ampicillin.

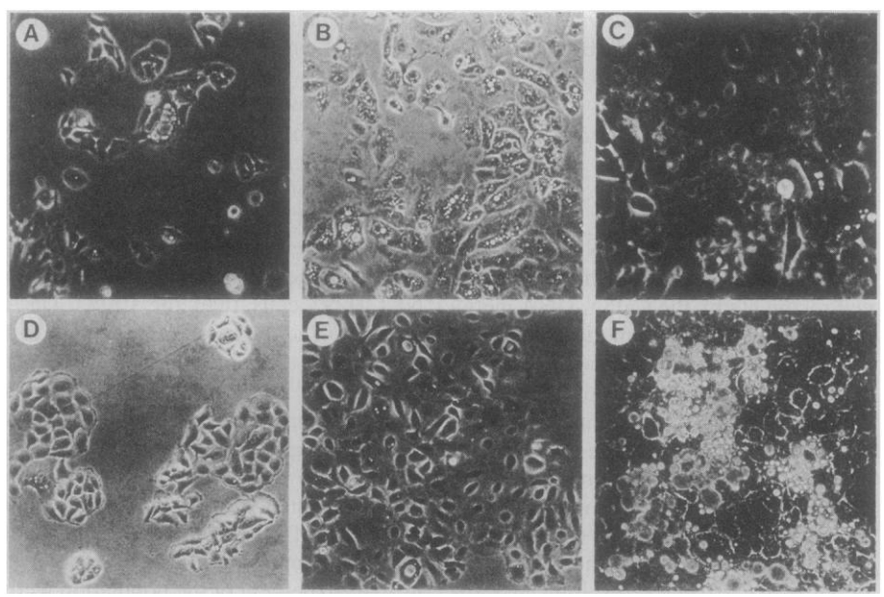

Fig. 2. The morphology of human cells expressing the hepatitis B core antigen gene (*HBc* gene). (A and B) NCI H292 transfected with pKYC200, which carries the 1850-bp Bam HI fragment from the HBV genome (GTC2). (C and D) NCI H292 transfected with the vector pSVgpt (GTC1). These cultures were incubated in LHC-4 medium with 5 percent serum (L4-5S) for 3 days (A and C) and 9 days (B and D) prior to selection of the cultures for stable gpt^+ recipient cells. (E) The biological crisis that occurs when *HBc* gene expression reaches its maximum after 5′-azacytidine treatment of GTC2 and two passages in L4-5S medium. (F) The biological crisis of Alexander cells, which occurs at the second passage of these cells in L4-5S medium or following treatment with 5′-azacytidine treatment and growth in MCDB 104 medium containing 2.5 percent FBS (MC-2.5). The cell detachment in (E) and (F) coincides with the peak production of *HBc* gene product.

After high-frequency transfection of NCI H292 grown in serum-free medium (*20, 21*) with pSV2gpt (GTC1) or pKYC200 (GTC2), transfected cultures were observed during growth to confluence (6 to 12 days). GTC2 cells become vacuolated and granular within 72 hours, and when these cultures approach confluence, cytopathologic changes are apparent in 70 to 90 percent of the population (Fig. 2). However, cells transfected with the vector pSV2gpt remain morphologically indistinguishable from the parent cells (NCI H292) (Fig. 2). The results shown in Fig. 2, A through D, indicate that the transient expression of genes during the period immediately following the procedure provides a useful screening method to determine the cytopathologic potential of subgenomic fragments of viral DNA before obtaining a stable population of cells carrying the viral gene by selection for gpt^+ expression.

High-frequency transfection into human cells required significant modification of the protoplast fusion methods developed for murine, simian, and HeLa cells (*8–10*). Protoplasts were prepared by growth of plasmid-carrying derivatives of HB101 in 250 ml of L-broth to 2×10^8 to 5×10^8 cells per milliliter. Chloramphenicol was added to a final concentration of 200 μg/ml and incuba-

tion at 37°C was continued for 18 to 20 hours to amplify the plasmid copy number. After centrifugation, cell pellets were placed on ice and protoplasts were prepared as follows: (i) the pellets were resuspended in 2.5 ml of HBS-20 buffer (*26*), (ii) 0.8 ml of freshly mixed lysozyme at 10 mg/ml in HBS-20 was added, and (iii) incubation at room temperature for 15 to 45 minutes was followed by microscopic observation of the conversion of *Escherichia coli* cells to spheroplasts to determine when reactions were complete. After the lysozyme had converted 85 to 90 percent of the cells to spheroplasts, the mixture was placed on ice, 0.4 ml of 1.25*M* $CaCl_2$ was added to stop the lysozyme, and 2.5 ml of 0.25*M* EDTA was added to chelate excess Ca^{2+}. This mixture was diluted by slow addition to 12.5 ml of HBS-9 buffer (*26*) resulting in a preparation containing approximately 2×10^9 protoplasts per milliliter.

The fusion procedure was conducted by placing 1.0 ml of 48 percent PEG-1000 preparation in each 60-mm dish containing 5×10^4 cells per dish. The culture dishes were centrifuged at 850*g* for 3 minutes to approximate protoplasts and human cells. The protoplast supernatant was removed and dishes were flooded with 2.5 ml of 48 percent PEG-1000 fusion reagent prepared as follows: (i) PEG-1000 (polyethylene glycol, Baker™ grade) was heated to 42°C; (ii) 300 to 500 ml of this melted reagent was poured into a large beaker and the *p*H was adjusted to 7.4 with concentrated HCl if needed; (iii) 10 g of Bio-Rad mixed-bed resin AG50l-X8(D) was added and the mixture incubated for 4 hours at 40°C; (iv) the PEG-1000 was collected by filtering the mixture through Whatman paper No. 1 covered by 10 g of unexposed resin into a vacuum flask; (v) the fusion grade PEG was weighed while still warm and adjusted to a 48 percent solution by weight by the addition of MCDB 151 nutrient medium stock. This PEG-fusion reagent was passed through a 0.22-μm filter for sterilization and stored at −20°C for as long as 1 year without notable differences in performance. Storage at 4°C was adequate for several weeks. Preparation methods that involve excessive heating (that is, autoclaving) of PEG and exposure to oxygen generate toxic contaminants that obviate most of the advantages obtained by application of fusion methods to human cell culture experiments involving growth in serum-free media.

Cell and protoplast mixtures were treated with PEG-fusion reagent for 45 to 60 seconds, the mixture was removed, and culture dishes were carefully washed three to five times with MCDB 151 medium to remove most of the residual PEG. Washed cells were covered with LHC-4 growth medium and placed in the incubator; the medium was changed at 1-hour intervals for the next 3 hours. The LHC-4 culture medium was subsequently changed each morning for the next 3 days. Within 48 to 72 hours after the procedure, transfected cells could be handled normally, for example, trypsinized for passage. We defined "stable" transfectants for this study as those populations that maintained the selected marker in 80 to 90 percent of the cells after growth for a minimum of ten divisions without selection.

Table 1. The frequency of transfection of human cells (NCI H292) with pSV2-derived plasmids. Transfection was achieved by protoplast fusion with *Escherichia coli* strain HB101 carrying pSV2-derived plasmids and was determined after selection for gpt^+ or neo^+ expression. The growth rates of human cells in culture and frequency of gpt^+ transfectants were measured by using the clonal growth assay (*20, 21*). Those colonies capable of eight divisions in gpt^+ selection or four divisions during neo^+ selection are stably transfected for these marker genes. The fraction of colonies continuing to grow as described above indicates the frequency of transfection for each experiment. Clonal isolation and continued passage indicates that greater than 90 percent of these colonies are genetically stable. The frequencies listed are averages of three experiments, the lower limit for detection is 10^{-5}.

Recipient cell designation	Plasmid	Genotype	Selection		Frequency of transfection	
			gpt^+	neo^+	Protoplast fusion	$CaPO_4$-DNA [3 m*M* to 125 m*M* Ca^{2+}]
GTC1	pSV2gpt	gpt^+	+	−	3.2×10^{-3}	$< 10^{-5}$
GTC2	pKYC200	gpt^+, HBc^+	+	−	3.1×10^{-3}	
GTC10	pSV2neo	neo^+	−	+	3.4×10^{-3}	$< 10^{-5}$

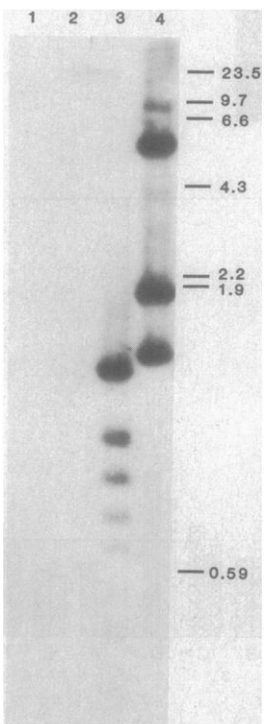

Fig. 3. Southern blot analysis of nuclear DNA from gpt^+ GTC2 cultures. Nuclei were isolated from gpt^+/HBc^+ GTC2 and NCI H292 cells, and high molecular weight DNA was purified for restriction and hybridization with ^{32}P-labeled pAM6 DNA. The HBV-probe revealed the presence of HBV sequences by hybridization to several bands ranging in size from 0.9 to 10.0 kbp. Lane 1 is Msp I/Bam HI and lane 2 is Hpa II/Bam HI digests of parental DNA (NCI H292). Lane 3 is Msp I/Bam HI and lane 4 is Hpa II/Bam HI digests of GTC2 DNA.

Hybridization Analysis of GTC2 Cells

NCI H292 cells stably carrying the pKYC200 plasmid (GTC2) were isolated by selection for the expression of the gpt^+ gene (Table 1). The transfected genes have remained stably integrated after more than 30 passages of GTC2 cells in RPMI 1640 medium containing 10 percent fetal bovine serum (FBS) (HUT medium). The gpt^+ GTC2 cell line was used to test for the physical presence of the *HBc* gene and to ascertain the factors regulating the expression of the *HBc* gene. To detect the physical presence of pKYC200 sequences in GTC2 cells, high molecular weight DNA was isolated from cell nuclei for Southern blot analysis (*27, 28*). GTC2 DNA was probed with pAM6 DNA after Bam HI/Hpa II or Bam HI/Msp I restriction enzyme digests to detect the presence of pKCY200 sequences in nuclear DNA. The pAM6 probe detected sequences between 0.9 and 10 kbp, and no hybridization to NCI H292 DNA was observed (Fig. 3). Hybridization analysis indicates that nuclear DNA from GTC2 cells contains sequences from pKYC200 after transfection and selection for the gpt^+ marker and that such cell lines may be mapped for gene-specific response to 5′-azacytidine treatment.

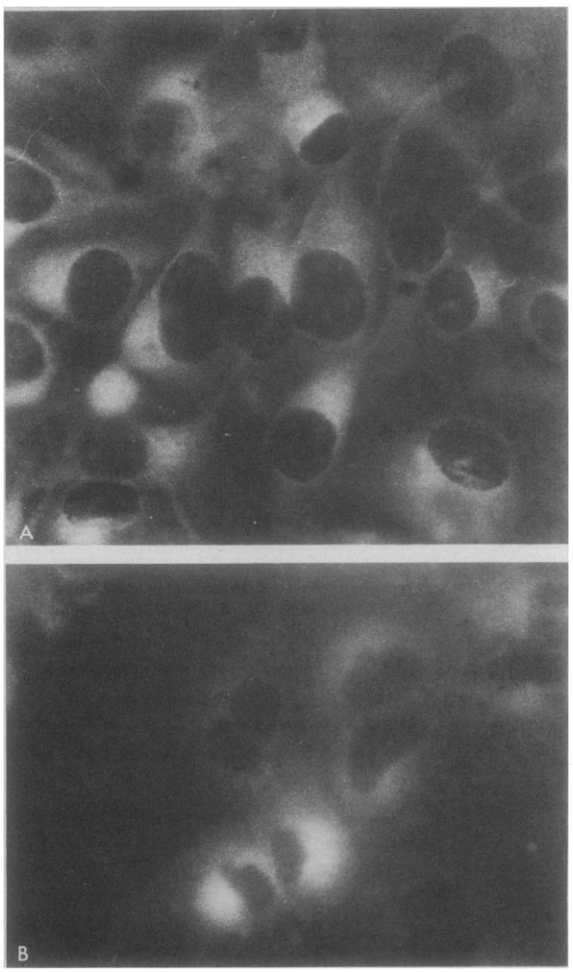

Fig. 4. Indirect immunofluorescence detection of HBcAg after 5′-azacytidine treatment of (A) GTC2 cells and (B) Alexander cells. Cells were treated with 5′-azacytidine as follows: GTC2 cells were grown in HUT medium and Alexander cells were grown in MC-2.5 medium for 3 to 4 days before the addition of 5′-azacytidine (2 to 5 μM for NCI H292 recipient cultures and 5 to 10 μM for Alexander cells). The cells to be tested were grown for 3 to 6 days on Falcon 3006 Optical Film-lined dishes, and the immunofluorescence test for HBcAg was carried out with a 1:50,000 dilution of purified human anti-HBcAg and a 1:5000 dilution of rabbit antibody to human IgG conjugated to fluorescein isothiocyanate.

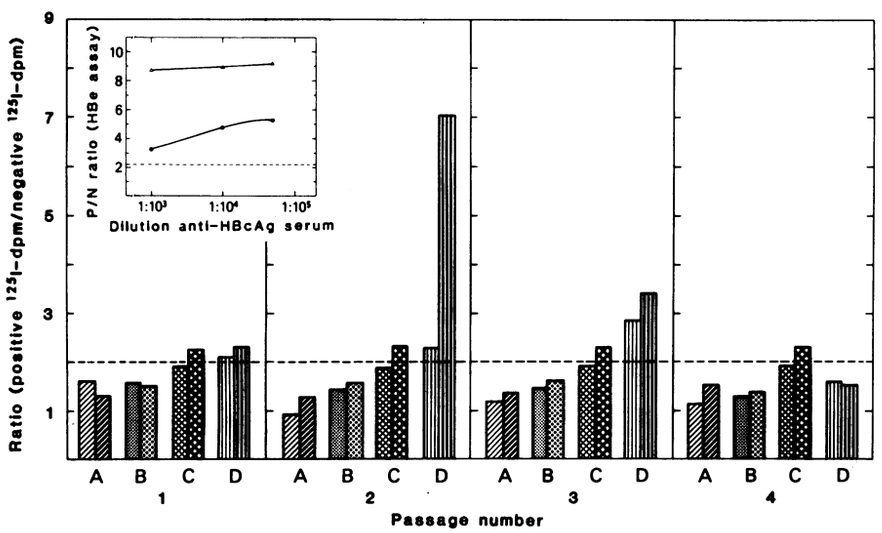

Fig. 5. To quantitatively compare growth conditions that affect the cytopathologic response of GTC2 cells and HBV-carrying Alexander cells for the production of HBcAg, we adapted the commercially available HBe radioimmunoassay diagnostic kit (Abbott) for application to the measurement of HBcAg in extracts from cells grown in culture. The inset shows the response of the HBe assay kit positive control to the human anti-HBcAg between dilutions of 1:1000 and 1:80,000 (top curve). The observation of no blocking activity for human anti-HBcAg indicates a high degree of specificity for the HBcAg. The GTC2 extract (bottom curve) yields a P/N ratio of 5.0; however, reaction of the extract with human anti-HBcAg yielded blocking of the kit response to P/N ratios between 2.8 and 5.0. The P/N ratios measured for cell extracts after growth in HUT medium are shown for each condition tested by less dense marking of the bars. The values reached by cell extracts after growth in L4-5S medium are indicated by heavily marked bars. The bars are labeled as follows: A, measurements of GTC1 cells (pSV2gpt$^+$), grown in HUT or L4-5S medium; B, GTC1 cell extracts after 5′-azacytidine treatment; C, GTC2 cell extracts (pKYC200 *gpt*$^+$/*HBc*$^+$); D, GTC2 cells after 5′-azacytidine treatment.

Measurement of HBcAg in GTC2 and Alexander Cells

GTC2 cells were tested for the presence of a functional *HBc* gene by indirect immunofluorescence assay with an antibody to HBcAg (anti-HBcAg; Abbott), (Fig. 4). Robinson *et al.* have reported that a hepatocellular carcinoma cell line that has carried the HBV genome since its isolation (Alexander cells) expresses the HBcAg after 5′-azacytidine treatment (*29*). Therefore, we treated GTC2 cells with 5′-azacytidine before conducting immunofluorescence assays. Greater than 90 percent of GTC2 cells expressed the HBcAg after treatment with 5′-azacytidine (*30, 31*) and growth in LHC-4 with 5 percent FBS (L4-5S) medium (Fig. 4A). Alexander cells also become positive for expression of HBcAg after 5′-azacytidine treatment (Fig. 4B).

To quantitate the level of HBcAg expressed during various growth conditions, we adapted the HBeAg assay kit (Abbott) for use with cell extracts (Fig. 5). To test the applicability of this kit for HBcAg measurements in cellular extracts, we determined a linear positive response to diluted extracts in the range used for *HBc* gene product (*32, 33*) assay (data not shown). The anti-HBcAg was tested for cross-reactivity to HBeAg using the positive HBeAg control provided with the assay kit (Fig. 5, inset). The anti-HBcAg preparation did not cross-react with HBeAg. The linear blocking response by anti-HBcAg in the 2.8- to 5-fold range of the positive to negative (P/N) ratio indicates that the HBe assay kit can be used to quantitatively compare cell extracts for the production of *HBc* gene product (Fig. 5, inset). The dashed line indicates the P/N ratio at which the measurement reaches 99.4 percent detection confidence (Abbott). We found variations of ±0.05 in the P/N ratio between duplicate measurements of HBcAg when cell extracts were substituted for serum samples and the reaction mixtures formulated as described by the manufacturer (Abbott). The level of *HBc* gene product reached a maximum when GTC2 cells were passaged in L4-5S after treatment with 5′-azacytidine (Fig. 5).

LHC-4 medium is a serum-free nutrient medium, developed for primary human epithelial cell culture, that causes terminal differentiation of epithelial cells when serum is included (*20, 21*). However, when serum is deleted from L4-5S, GTC2 and Alexander cells are stimulated to produce the same level of HBcAg, indicating that one or more of the growth factors and hormones included in LHC-4 medium is positively regulating *HBc*

gene expression. The most notable effect of including 5 percent serum in LHC-4 medium for these experiments is an increase in the growth rate for the GTC2 and Alexander cells. At the second passage of 5′-azacytidine-treated GTC2 cells in L4-5S medium, the maximum level of *HBc* gene product (HBcAg) coincided with a cytopathologic response, and most of the cells detached (Figs. 2E and 5). However, some cells (approximately 10^{-4}) remained attached and grew to confluence (GTC2-1) in L4-5S medium, and the level of HBcAg dropped below detection limits at cell passage 4 (Fig. 5).

Karyotype analysis of GTC1, GTC2, GTC2-1, and NCI H292 showed that these cells carry the same marker chromosomes, indicating a parental relationship to NCI H292 (data not shown). These cell lines are being used to study the methylation patterns and RNA transcripts involved in regulation of the changes in *HBc* gene expression that occur during this sequence. The basal level of expression of HBcAg is maintained by GTC2 cells grown in L4-5S medium without 5′-azacytidine treatment (Fig. 5). Since the anti-HBcAg does not cross-react with HBeAg, and since the antibody preparation blocks 80 to 90 percent of the activity of the GTC2 extract (Fig. 5, inset), most of the *HBc* gene product measured in GTC2 extracts is HBcAg.

The relative response of Alexander cells was determined for the production of HBcAg after growth in L4-5S medium or 5′-azacytidine treatment and growth in MCDB 104 medium with 2.5 percent FBS (MC-2.5). Either of these conditions stimulated the production of HBcAg to P/N ratios of 3.5 to 3.8 and a cytopathologic response for Alexander cell cultures (Fig. 2F). Alexander cells initially increased their growth rate when passaged into L4-5S medium to from 0.8 to 1.0 population doublings per day (PD/day). However, at the second cell passage in this medium, PD/day decreased to 0.65, the cells produced HBcAg at P/N ratios of 3.5 to 3.8, and then they detached in a manner very similar to the response observed for GTC2 cells after 5′-azacytidine treatment and growth in L4-5S medium (Figs. 2 and 5). A similar response was obtained for Alexander cell cultures after treatment with 5′-azacytidine and growth of these cells in MC-2.5. Although Alexander cell extracts did not exceed P/N ratios of 3.5 to 3.8, the morphologic response was similar to that observed for GTC2 cells carrying only the *HBc* gene (Fig. 2). Treatment with 5′-azacytidine deregulates genes controlled by cytosine methylation (*30, 31*), and serum-free medium includes hormones and growth factors (*20, 21*) not present in HUT medium. The cytopathologic response of Alexander cells to conditions that increase the expression of *HBc* gene product and the similarity of this response to that of GTC2 cells indicate that *HBc* gene regulation and the cytopathologic response of GTC2 and Alexander cells are the same.

Discussion

Transfection of subgenomic fragments into appropriately selected human cell lines provides a means to rapidly study the expression, regulation, and pathobiological effects of individual viral genes. We are investigating the role of HBV and its individual genes in the pathogenesis of viral hepatitis and liver cancer. Therefore, we developed a modified protoplast fusion method to transfect human cells grown in serum-free media. The human cell recipient (NCI H292) was selected as a prototype because its growth characteristics in serum-free media are similar in many respects to normal human epithelial cells (*20, 21*). Expression of the transfected *HBc* gene is regulated by similar factors and has similar effects in a hepatocellular carcinoma cell line that carried the HBV genome since its isolation from a patient with chronic hepatitis. This is consistent with a primary role for the *HBc* gene product in the cytopathology found in human liver during HBV infection.

Such information may prove useful for development of therapeutic regimens for chronically infected patients and provide insight about the biological nature of the virus at the genetic and molecular levels. The establishment of HBV-infected lymphoblastoid cell cultures from bone marrow biopsies that were positive for HBsAg (*34*) and the detection of HBV DNA in Kaposi sarcoma tumor tissue (*35*) suggest a broadening pathologic role for HBV infection. The improved fusion method described here for high-frequency transfection of human cells in serum-free growth conditions provides a method for application to general problems in human somatic cell genetics. The ability to stably transfect genes at frequencies greater than 10^{-3} in human cell recipients is sufficient to attempt isolation of single-copy genes from genomic libraries linked to selectable markers.

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