High-Capacity In Vitro Assessment of Anti-Hepatitis B Virus Compound Selectivity by a Virion-Specific Polymerase Chain Reaction Assay

ROBERT W. JANSEN,* LANCE C. JOHNSON, AND DEVRON R. AVERETT

Division of Experimental Therapy, Burroughs Wellcome Co., Research Triangle Park, North Carolina 27709

An integrated assessment system specific for hepatitis B virus (HBV) Dane particle DNA was developed to examine the activity of potential anti-HBV compounds in chronic HBV-producing HepG2-derived 2.2.15 cells. Cell culture, immunoaffinity purification, polymerase chain reaction, and hybrid-capture detection were performed in the microtiter format to facilitate increased throughput by automation. The high sensitivity afforded by the assay provided quantitative detection of less than 0.5 fg of extracellular HBV DNA from 25 µl of cell culture supernatants, and drug-induced reductions in HBV titers greater than 100-fold were easily measured. Fluorometric determination of total cellular DNA from the same 96-well proliferating cell cultures allowed simultaneous evaluation of inhibition of cell growth, thus providing the ability to assess the overall selectivities of candidate compounds in a single experiment. The potent activities of three anti-HBV compounds, the (+) and (−) enantiomers of cis-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxythiolane-5-yl]cytosine (FTC) and D-carbocyclic-2′-deoxyguanosine (CDG), were confirmed by this method. (−)-FTC was more active than its (+) enantiomer (50% inhibitory concentrations, 0.033 ± 0.006 and 0.723 ± 0.160 µM [standard error of the mean; SEM], respectively), while both enantiomers demonstrated a lack of cytotoxicity at 200 µM. CDG was more potent (50% inhibitory concentration, 0.0063 ± 0.0007 µM [SEM]) but was also significantly more toxic, inhibiting cell growth by 50% at 32 ± 6 µM (SEM). These results demonstrate the usefulness of this immunoaffinity-based, quantitative polymerase chain reaction system as a high-capacity in vitro tool for assessment of anti-HBV compound selectivity.

Hepatitis B virus (HBV) infection is one of the most prevalent viral diseases in the world and is known to be a major cause of chronic liver disease, which over time can lead to cirrhosis and/or hepatocellular carcinoma (3, 26, 27). The identification of anti-HBV compounds requires a biological test system of adequate sensitivity and throughput that can distinguish the selective antiviral activities of test compounds from host cell cytotoxicity. Nonproliferating cells of the HepG2-derived HBV-producing 2.2.15 line (21) have been used in this endeavor (1, 11). However, since generalized DNA synthesis inhibitors do not demonstrate toxicity in nonproliferating cells, we wished to investigate the use of growing cells in an effort to obtain more sensitive, simultaneous assessments of the anti-HBV activities and toxicities of various compounds.

The level of HBV produced in proliferating cells is too low to be reliably detected by conventional methods (1, 22). However, the polymerase chain reaction (PCR) can provide sufficient sensitivity to detect such small amounts of DNA (19, 20), but its use often involves tedious sample extraction steps. In order to exploit the high-throughput capability offered by 96-well PCR machines, we explored the use of antibody capture (9, 10) in a microtiter format to isolate HBV from supernatants prior to high-capacity PCR. Furthermore, the emergence of enzyme-linked immunosorbent assay-like techniques for quantitation of DNA (6, 14, 25) makes possible the quantitation of PCR products in the microtiter format.

Here we describe a unique integration of microtiter-based systems for the simultaneous in vitro assessment of both the efficacies and cytotoxicities of potential anti-HBV compounds in growing 2.2.15 cells. We applied this semiautomated procedure in the analysis of three anti-HBV active compounds, the (+) and (−) enantiomers of cis-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxythiolane-5-yl]cytosine (FTC) and D-carbocyclic-2′-deoxyguanosine (CDG), and demonstrated the potential of the procedure for high-capacity in vitro screening of anti-HBV agents.

MATERIALS AND METHODS

Compounds. The syntheses of the (+) and (−) enantiomers of FTC have been described previously (5, 8). The D isomer of CDG was synthesized by S. Daluge at Burroughs Wellcome Research Laboratories.

Automation. Microtiter tissue culture work was performed with a Pro/Pette robotic pipette (Perkin-Elmer, Norwalk, Conn.). For immunoaffinity purification of HBV, PCR, and hybrid-capture techniques, we used an assortment of Pro/Pettes, multichannel pipettes (Costar, Cambridge, Mass.; ICN Flow, Costa Mesa, Calif.), and 12-channel washers (Nunc, Naperville, Ill.).

Cell cultures and determination of growth inhibition. The P5A passage of HBV-producing 2.2.15 cells derived from transfected HepG2 cells (21) was generously provided by B. Korba (Georgetown University). Cells were trypsinized and seeded in 96-well microtiter plates at a density of 2,500 cells per well in 150 µl of medium. The cells were fed every 2 days with various concentrations of potential anti-HBV compounds in antibiotic-free RPMI 1640 medium (GIBCO, Gaithersburg, Md.) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine. The compounds were tested initially with 10-fold serial dilutions beginning at
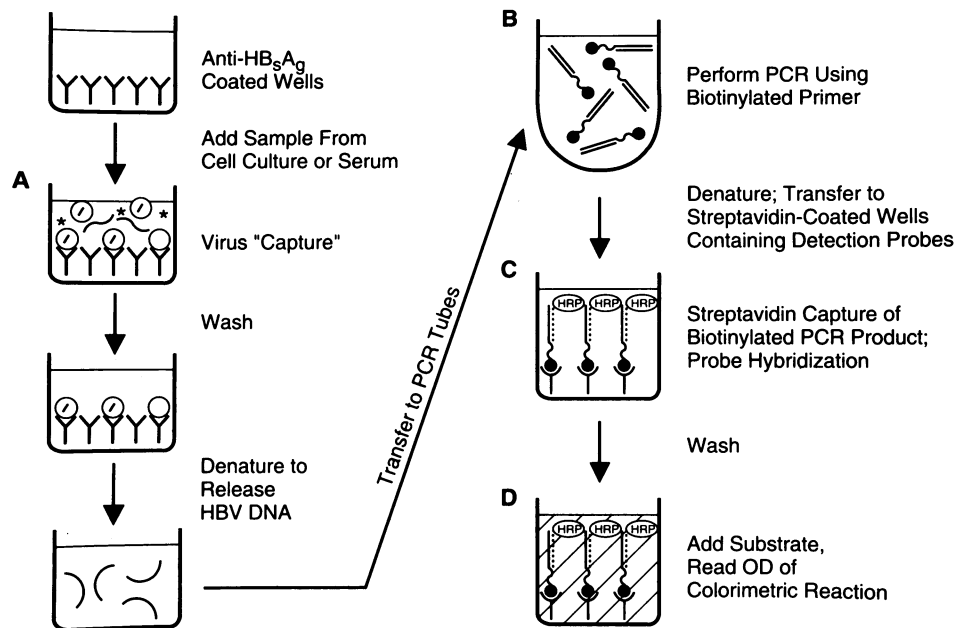
* Corresponding author.

FIG. 1. Flow diagram for microtiter-based detection of HBV. (Step A) Virus-containing samples are applied to antibody-coated wells. The wells are washed after incubation, and bound virions are denatured with NaOH to release the HBV DNA. (Step B) Neutralized samples containing HBV DNA are transferred to PCR tubes, and amplification of DNA proceeds in the presence of a biotinylated primer. (Step C) PCR products which have been denatured to single strands with NaOH are hybridized with detection oligonucleotide probes specific for the biotinylated strand. The incubations are performed in streptavidin-coated wells so that both hybridization and streptavidin binding of the biotinylated DNA strand proceed simultaneously. (Step D) Following stringent washes, the bound probes are detected. In the example shown, the detection probe is labeled with HRP. OD, optical density.

200 μM. Several control wells in each plate were maintained without compound. On the days of harvest, media were transferred to new plates and stored at $-80°C$ for subsequent HBV DNA analysis. The cells were then fixed with 70% ethanol for 30 min, rinsed with unsupplemented RPMI 1640 medium, and incubated with the DNA stain bisbenzimide (H33342 · 3HCl · 4H$_2$O; Calbiochem Corporation, La Jolla, Calif.) at 30 μg/ml for 1 h at 37°C in serum-free RPMI 1640 medium. Fluorescence values (excitation, 355 nm; emission, 460 nm; arbitrary units) were determined with a Titertech Fluoroscan II plate reader (ICN Flow). Evaluations of growth inhibition were made by comparing the values for treated cells with those for the untreated cell controls and were expressed as a percentage of the control values.

Immunoaffinity purification of HBV. For immunoaffinity purification of HBV (Fig. 1, step A), round-bottom microtiter plates (25802; Corning Glass Works, Corning, N.Y.) were coated with 50 μl of a 10-μg/ml solution of anti-HBV surface antigen (anti-HBsAg) murine monoclonal immunoglobulin G (Wellcome Diagnostics) in phosphate-buffered saline (PBS) for 16 h at 4°C. The contents were replaced with 100 μl of 0.1% bovine serum albumin in PBS, and the plates were incubated for 2 h at 37°C and were either used immediately or stored overnight at 4°C. The wells were washed three times with PBS–0.01% Tween 20 immediately before automated additions of 0.035% Tween 20 in PBS (10 μl) and cell culture supernatants (25 μl). The microtiter plates were then incubated at 4°C overnight. These conditions permit the adsorption of the majority (>70%) of the HBV present in the samples. Following incubation, the wells were washed five times with PBS–0.01% Tween 20 and three times with PBS. The residual liquid was removed after a brief, low-speed centrifugation, and the DNA from the

captured virions was released by a 60-min incubation at 37°C in 25 μl of 0.09 N NaOH–0.01% Nonidet P-40. Upon the addition of 1 volume of 0.09 N HCl–100 mM Tris (pH 8.3) to neutralize and buffer the solution, the plates were sealed and stored at 4°C for subsequent PCR analysis. For the purposes of internal control and quantitation, dilutions of a standardized HBV-containing supernatant were included on every plate.

PCR. For PCR (Fig. 1, step B), denatured and neutralized capture samples (5 μl) were added to 20-μl mixtures of PCR components in sterile MicroAmp tubes arranged in 8-by-12 arrays (Perkin-Elmer 9600 PCR system components). The final volume of 25 μl contained 20 mM Tris (pH 8.3), 50 mM KCl, 2 mM MgCl$_2$, 18 mM NaCl, 0.2 mM 2′-deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP), 6.25 pmol of each primer, and 0.625 U of *Taq* polymerase (19). After installation of strip caps, the block of tubes was seated in a Perkin-Elmer 9600 thermal cycler and subjected to the following programs after an initial incubation for 5 min at 94°C: 30 cycles of 94°C for 30 s, 55°C for 15 s, and 72°C for 1 min. The reactions were finally incubated at 72°C for 5 min prior to storage at 4°C.

The primers were selected from a highly conserved region of a consensus of 14 subtype sequences obtained from GenBank (Fig. 2). This region maps at *ayw* positions 372 to 483 in the S gene and is encompassed by the primers EHBV372 (5′-TCG CTG GAT GTG TCT GCG GCG TTT TAT) and EHBV460 ([−]5′-TAG AGG ACA AAC GGG CAA CAT ACC). In addition to mapping to conserved regions, these primers have the advantage of being relatively close in proximity to one another, thus facilitating the efficiency of the PCR. Although this region may randomly exist as single-stranded DNA in the HBV particle (18, 24),

```
            361       370       380       390       400       410       420
             |         |         |         |         |         |         |
Consensus   TGTCCTGG_TATCGCTGGATGTGTCTGCGGCGTTTTATCAT_TTCCTCT_CATCCTGCTG
                                                                           ← (+)372
ayw         TGTCCTGGTTA[TCGCTGGATGTGTCTGCGGCGTTTTATCAT]CTTCCTCTT[CATCCTGCTG
                                                                 [GTAGGACGAC  ← (−)411
aywVar      ------------------------------------------------------------
ayr         --------C------------------------------------A---------------
adw         ---------------------------------------------A---------------
adwl        -------------------------------------------------G-----------
adw2        ---------------------------------------------A---------------
adw2a       -------------------------------------------------G-----------
adw3        -------------------------------------------------G-----------
adr         --------C------------------------------------A---------------
adra        ---------------------------------------------A---------------
adrcg       --------C------------------------------------A---------------
adrm        --------C------------------------------------A---------------
adywcore    ------------------------------------------------------------
chimp       ------------------------------------------------------------


            421       430       440       450       460       470       480
             |         |         |         |         |         |         |
Consensus   CTATGCCTCATCTTCTT_TTGGTTCTTCTGGA_TA_CAAGGTATGTTGCCCGTTTGTCCTCTA
ayw         CTATGCCTCATCTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTGTCCTCTA
            [GATACGGAGTAGAAGAA]                    [CCATACAACGGGCAAACAGGAGAT]  ← (−)460
aywVar      ----------------------------------------------------------------
ayr         --------------------------------------C-------------------------
adw         -----------------A--------------T-------------------------------
adwl        ----------------------------------------------------------------
adw2        -----------------A--------------T-------------------------------
adw2a       ----------------------------------------------------------------
adw3        ----------------------------------------------------------------
adr         --------------------------------------C-------------------------
adra        --------------------------------------C-------------------------
adrcg       --------------------------------------C-------------------------
adrm        --------------------------------------C-------------------------
adywcore    ----------------------------------------------------------------
chimp       ----------------------------------------------------------------
```
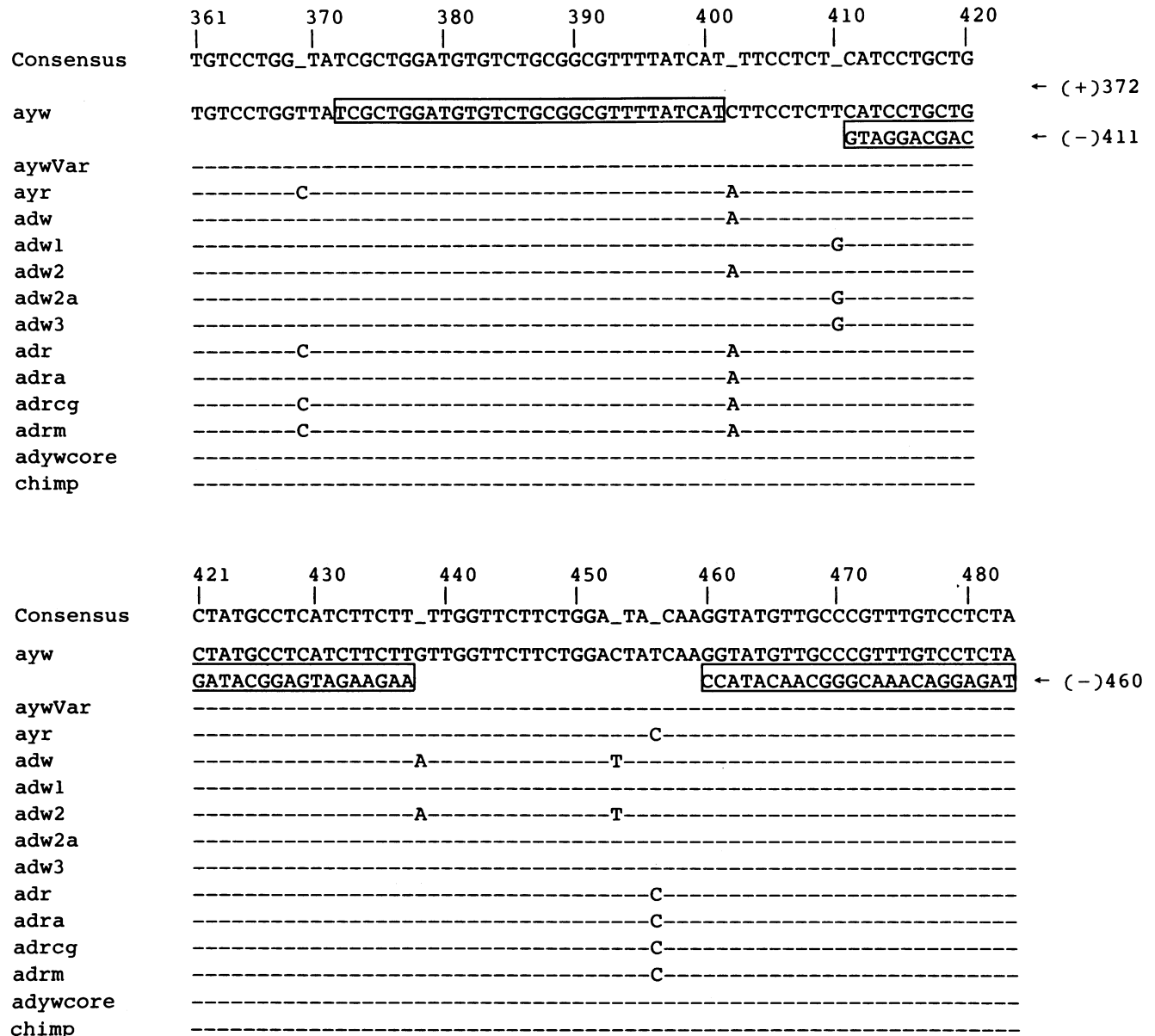
FIG. 2. PCR primer selections. HBV DNA sequences obtained from GenBank (Los Alamos National Laboratories, Los Alamos, N.M.) were aligned by using subtype *ayw* numbering. The pseudo consensus sequence is shown on the top row, with spaces indicating differences in one or more sequences compared with the *ayw* sequence. These differences are shown in capital letters within the indicated sequences, while homology with *ayw* is represented with dashes. Oligonucleotide (−)411 was chosen as a detection probe for hybridization to PCR products generated by primers (+)372 and (−)460. The expected amplimer of 112 bases in length is the only product visible on an agarose gel.

which could potentially lead to a small degree of variation in quantitation, we considered this to be insignificant and offset by the advantages mentioned above. To aid in subsequent PCR product detection, EHBV372 was biotinylated at its 5' end. All primers used in the study, including the horseradish peroxidase (HRP)-labeled oligonucleotide described below, were purchased from Synthecell Corporation (Rockville, Md.).

Hybrid-capture detection. For hybrid-capture detection (Fig. 1, step C), streptavidin-coated microtiter plates or strip wells (Corning 25801 and 24121-12, respectively) were prepared by incubation for 16 h at 4°C with 75 μl of 10 μg of streptavidin (Pierce, Rockford, Ill.) per ml in 50 mM sodium carbonate buffer (pH 9.6). The wells were postcoated with bovine serum albumin as described above for immunoaffinity purification and were stored at 4°C. Immediately before use, the wells were washed twice with 0.1× SSPE (15)–0.05% Tween 20. The PCR products were denatured to single strands directly in the original reaction tubes by the addition of 25 μl of 0.1 N NaOH–0.15 M NaCl and incubation for 5 min at room temperature. Samples (10 μl) were added to 40 μl of a 1.25×-concentrated hybridization solution (containing the detection probes described below) in the streptavidin-coated wells to yield the following final conditions: 4× SSPE, 2.5× Denhardt's solution (15), 100 mM Tris (pH 7.1), 0.25 mg of heat-denatured salmon sperm DNA per ml, 0.5
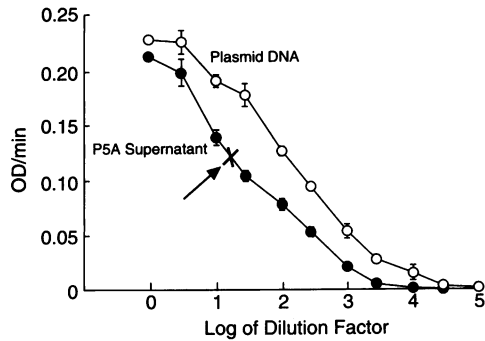
FIG. 3. Generation of PCR standard curves. Plasmid DNA (O) was serially diluted prior to PCR amplification of 5-μl samples. The undiluted concentration was 1.38 pg/5 μl. Supernatant from a confluent P5A cell culture (●) was serially diluted and applied to antibody-coated wells. After washing and denaturation of HBV, the equivalents of 2.5 μl of the original dilutions were amplified by PCR. Results are expressed as rates of color development ± standard deviation after hybridization to an HRP-labeled detection probe. The level of HBV DNA detected in the undiluted supernatant from a growing 96-well P5A culture on day 9 is indicated by the arrow (×). OD, optical density.
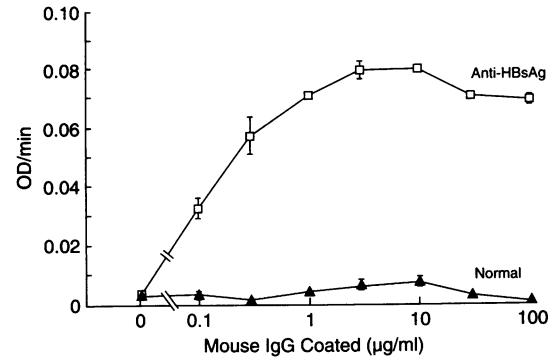

FIG. 4. Specificity of antibody capture. A dilution of the P5A cell culture supernatant containing 12 pg of HBV DNA per ml was applied to wells coated with increasing amounts of normal mouse immunoglobulin G (IgG) (▲) or mouse anti-HBsAg monoclonal antibody (□). Results of PCR amplification of DNA from bound HBV are expressed as rates of color development ± standard deviation after hybridization of the PCR products to the HRP-411 oligonucleotide. OD, optical density.

mg of poly(A) per ml, and 0.05% Tween 20. Thus, streptavidin binding of the PCR-derived biotinylated DNA strand and hybridization of the probe to this strand proceeded simultaneously.

Two different methods were used during hybridization, depending on the type of label bound to the detection oligonucleotide probe (−)411 (Fig. 2). For ^{32}P detection, the hybridization reactions in the streptavidin-coated strip wells contained ~3 × 10⁴ cpm of T4 polynucleotide kinase-labeled probe (1 μCi/pmol). The plates of strip wells were sealed with mylar sheets, and the plates were incubated for 1 h at 52°C. Following five washes with 0.1× SSPE–0.05% Tween 20, the bound radioactivity was counted directly in the absence of scintillation fluid. For HRP-labeled probe detection, 1 pmol of HRP(−)411 was included in each reaction well and was allowed to hybridize for 45 min at 42°C. The wells were washed three times with 0.1× SSPE–0.05% Tween 20 and two times with PBS. Upon the addition of o-phenylenediamine (0.4 mg/ml in phosphate-citrate buffer with sodium perborate; Sigma Chemical Co., St. Louis, Mo.), the rates of the reactions were determined for 2 min at 450 nm in a microplate reader (Thermo Max; Molecular Devices Corporation, Menlo Park, Calif.). When appropriate, final anti-HBV results were expressed as a percentage of the results for untreated controls after determination of the HBV DNA content by comparison with a standard curve.

RESULTS

PCR standardization. A plasmid DNA containing one copy of the ayw subtype genome was used directly in the PCR to generate a standard curve. After 30 cycles of PCR, hybrid capture detected a minimum of 0.045 fg of HBV DNA (2 × 10⁵ amol; ~12 genome equivalents). With amounts of greater than 45 fg, the signal became more erratic, leaving a useful range of detection of between 0.045 and 45 fg of HBV DNA (Fig. 3). The correlation coefficient for this curve from a four-parameter fit was 0.997, with 50% of the maximal signal occurring at 8.6 fg. This titration was used to calibrate the DNA content of a standard HBV-containing cell culture supernatant. The supernatant medium from a confluent P5A

culture was serially diluted prior to capture of the HBV by specific antibody and subsequent PCR. The resulting curve (Fig. 3) paralleled that of the standard plasmid DNA, with an estimated value of 120 pg of HBV DNA per ml of supernatant. A sample of supernatant from the ninth day of culture in untreated, growing P5A cells in a 96-well plate was similarly assayed and also paralleled the two standard curves (data not shown). The level of HBV DNA in this sample was calculated to be 6.7 pg/ml and is indicated in Fig. 3. Of importance is the fact that HBV could still be detected even after a 300-fold dilution from this microtiter plate-derived starting material, indicating that greater than 100-fold reductions in HBV output could be reliably measured in drug-treated growing cultures in high-capacity, 96-well formats.

A series of HBV DNA dilutions were amplified either with the usual primer pair described in Materials and Methods or with another primer pair located farther upstream of, and not overlapping with, the other primers. PCR amplifications with either primer pair produced single DNA bands of equivalent ethidium bromide stain intensity and of the appropriate sizes on an agarose gel. However, when hybridized to the probe specific for the first primer pair amplimers (oligonucleotide 411; Fig. 2), only the DNA amplified with those primers produced a hybridization signal (data not shown). These results confirmed the specificities of both the PCR primers and the hybridization detection probe.

Specificity and capacity of the antibody-capture step. To determine the level of specificity conferred by the immunoaffinity purification of HBV prior to PCR, we varied the amounts of either specific or nonspecific coating antibodies in the wells of a microtiter plate. After incubation with a constant amount of culture supernatant containing HBV, the wells were washed and HBV DNA was assessed by PCR. The resulting PCR signals fluctuated around the background with increasing amounts of normal mouse immunoglobulin G, whereas a large increase in the signal was obtained with increasing amounts of anti-HBsAg (Fig. 4). The maximum signal occurred at 3 μg of anti-HBV coating solution per ml. The use of the mild detergent Tween 20 throughout the immunoaffinity procedure did not reduce the specific binding of HBV to anti-HBsAg, although a significant reduction in
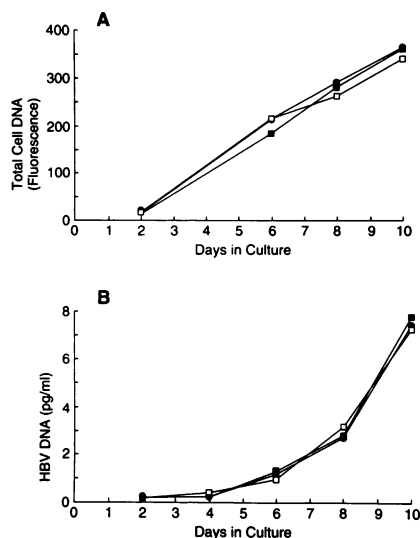
FIG. 5. Characterization of growing P5A cell cultures in replicate microtiter plates. Cells were seeded at 2,500 cells per well in microtiter plates on day 0 and were fed on days 2, 4, 6, and 8. Plates were harvested in sets of three on days 2, 6, 8, and 10. Supernatants were sampled on days 2, 4, 6, 8, and 10. Each point represents the average of 12 samples (wells) from a single microplate. (A) Growth of cells determined by fluorescence of total cellular DNA after staining fixed cells with bisbenzimide. The values are arbitrary fluorescence units. (B) HBV content in supernatants determined by antibody capture and PCR.

FIG. 6. Growth inhibition (toxicity) and anti-HBV activities of (−)-FTC, (+)-FTC, and CDG. P5A cells were stained and supernatants were sampled for antibody capture and PCR analysis 10 days after seeding (8 days of drug treatment). Results are expressed as percentage of the control value ± standard deviation ($n = 4$). (A) Effects of (−)-FTC (□) and (+)-FTC (●). (B) Effects of CDG.

the nonspecific binding of HBV to the wells was observed (data not shown).

The amount of HBV DNA present in the culture supernatant applied in this experiment (12 pg of HBV DNA per ml) was slightly greater than that present in day 9 supernatants of growing 96-well cultures. These results, together with the fact that our routine coating antibody solution is 10 μg/ml, indicate that the anti-HBsAg antibody available in the capture wells is in at least a threefold excess over the surface antigen-containing particles in the supernatants of the growing cells. Also, the conclusion that capture antibody is present in excess is reinforced by the data in Fig. 3, which show that an increase in the amount of HBV by 3- to 10-fold above that in the 96-well culture supernatants produced a significantly higher PCR signal.

Characteristics of growing P5A cells. The HBV-producing P5A cells (untreated controls) in the microtiter wells showed a linear increase in DNA fluorescence over a 10-day span (Fig. 5A). These cell cultures become confluent and fluorescence begins to level off after day 10 (data not shown). HBV DNA levels in the supernatant were steady over 4 days and then rose exponentially through day 10, reaching ~7.5 pg/ml of supernatant (Fig. 5B). The low degree of plate-to-plate variations for both fluorescence and HBV DNA measurements is demonstrated in Fig. 5. The coefficients of variation among the controls (12 each) on a given plate were typically less than 5% for fluorescence and less than 20% for HBV DNA (data not shown).

Simultaneous growth inhibition and anti-HBV analyses. Growing P5A cells were treated with various dilutions of compounds every 2 days, as described in Materials and Methods. At the end of the treatment period (9 to 10 days of culture), the cells were fixed and stained for growth inhibition analysis, while the supernatants were subjected to
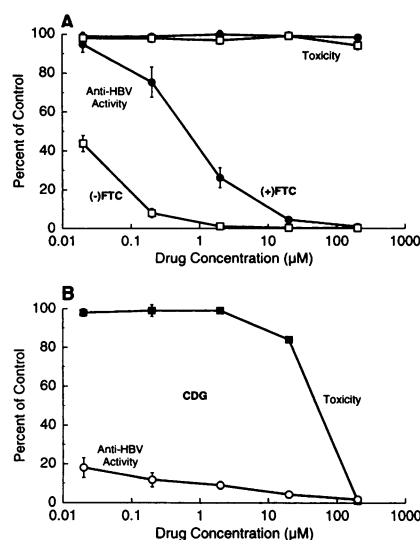
antibody capture and PCR for antiviral assessment. Neither (−)-FTC nor (+)-FTC reduced cell growth at 200 μM, while both exhibited anti-HBV activity (Fig. 6A). In the example shown in Fig. 6A, the potency of (−)-FTC was approximately 40 times that of (+)-FTC, with estimated 50% inhibitory concentrations (IC_{50}s) of 0.015 and 0.63 μM, respectively.

CDG was highly active against HBV but was also cytotoxic (Fig. 6B). This compound inhibited cell growth by as much as 99% at 200 μM, while the level of HBV was reduced to 18% of the control level when CDG was used at 0.02 μM.

Additional experiments were performed with dilutions of compounds that crossed through the IC_{50}s to further characterize their activities (data not shown). Since concentrations greater than 200 μM were not tested, the cytotoxic IC_{50}s for the FTC compounds are represented as >200 μM (growth inhibitions at this concentration were less than 10%). The average growth inhibition IC_{50} of CDG was 32 ± 5.5 μM (standard error of the mean [SEM]; $n = 5$). Average anti-HBV IC_{50}s were 0.033 ± 0.006 μM (SEM; $n = 9$) for (−)-FTC, 0.72 ± 0.16 μM (SEM; $n = 3$) for (+)-FTC, and 0.0063 ± 0.0007 μM (SEM; $n = 3$) for CDG. Selectivities (growth inhibition IC_{50} divided by anti-HBV IC_{50}) based on these averages were >6,100 for (−)-FTC, >280 for (+)-FTC, and 5,100 for CDG.

DISCUSSION

The use of nonproliferating 2.2.15 cells for analysis of the anti-HBV efficacies of compounds has been reported previously (1, 5, 11, 16). In contrast to proliferating 2.2.15 cells, nonproliferating 2.2.15 cells produce HBV at a high level, allowing HBV DNA measurement by conventional detection methods (1, 22). However, these cell conditions may be less suitable for cytotoxicity assessment, since the absence of macromolecular synthesis reduces susceptibility to compounds which may be nonselective inhibitors of, for example, DNA synthesis. The goals we sought were to develop an

assessment methodology based on growing 2.2.15 cells (passage P5A) to eliminate these possible problems and to use a 96-well format throughout the procedure to obtain a high capacity.

Growth inhibition by compounds in 96-well cell cultures can easily be monitored by fluorometric scanning of stained cells in commercially available fluorometers. One drawback in attempting the simultaneous analysis of the HBV secreted by these small, growing cell cultures is the minute amount of the virus present in the supernatants (1). In order to develop a concomitant HBV assessment under these conditions, it was necessary to use PCR, which, until recently, has had limited, single-tube throughput. The availability of commercial 96-well thermal cyclers enables high-capacity PCR. We used an immunoaffinity approach (10, 17) as a prelude to PCR amplification of HBV DNA for several reasons. First, PCR-inhibiting serum components from the culture supernatants can be conveniently removed. Second, HBV DNA species not associated with mature Dane particles (i.e., core particles, spurious DNA from broken cells, etc.) are eliminated from the PCR so that only virus particle DNA is amplified. Third, the procedure can easily be conducted in a microtiter format. The immunoaffinity purification step also provides the ability to quantitatively assay for HBV in a wide variety of samples, including serum (data not shown).

Unlike the case with hepatitis A virus, which, after antibody immobilization, can be efficiently denatured by heating captured virions directly in the PCR tubes (10), we found that immobilized HBV had to be alkali denatured prior to PCR amplification. Through the use of NaOH and Nonidet P-40, a maximal PCR signal occurred after 60 min of lysis incubation at 37°C (data not shown). We assume that the core protein structure surrounding the DNA is responsible for the resistance to heat-driven DNA dissociation from protein.

The high-capacity PCRs were run in low volumes (25 µl) to increase the efficiency of heat transfer and to decrease the cost, which becomes an important consideration when running several hundred reactions on a routine basis. Although the number of cycles during PCR can be adjusted to increase the reaction's sensitivity, we found that 30 cycles were optimal since the maximum signals from the 96-well culture supernatants were near the high end of the standard curve (Fig. 3). This results in a 300-fold range of detection, thus permitting the quantitative detection of greater than 100-fold reductions in HBV content while allowing 50% inhibitions to be measured in the most linear, central section of the standard curve.

The successful use of biotinylated PCR primers for quantitative HRP-based detection of PCR products was first reported by Holodniy et al. (6). Rather than using streptavidin-coated beads, we developed our detection method with streptavidin-coated microtiter wells to allow for a higher capacity and the convenience associated with semiautomation. We also used alkali denaturation rather than heat denaturation of PCR products prior to hybridization since there is no chance for reannealing to occur during transfer of the denatured DNA to the hybridization solution. No decrease in detection efficiency was observed with this approach (data not shown). We also found this technique to be more amenable in multiplate, high-throughput processing. Only a slight loss in sensitivity was observed when HRP instead of ^{32}P was used for detection, and this was overcome by increasing the amount of PCR products incorporated into the hybridization reactions. After we began routine compound screening using these methods, Holodniy et al. (7)

reported a similar adaptation of this methodology to the microtiter format, both at the PCR and the hybrid-capture levels. The success of this approach with both groups further strengthens our view that these technologies will become widely used and will play a vital role in future high-capacity biological assays.

Simultaneous assessment of both growth inhibition and the anti-HBV activities of compounds (Fig. 6) was made possible with the technologies described above and provided information regarding compound selectivity. The significant cytotoxicity observed with CDG (IC_{50}, 32 µM) may reflect the more sensitive nature of the growing cells in this system, since this level of toxicity was not detected in other systems (23). The anti-HBV activity of CDG was substantial and consistent with that observed in other HBV assays (1, 5, 13, 16). The high activity of (−)-FTC (IC_{50}, 0.033 µM) in light of the lack of toxicity at 200 µM, however, makes this compound a more attractive target for development as an anti-HBV drug.

While some error is associated with any multistage quantitative analysis, we found that this was not a major problem in this system. The observation that coefficients of variation were generally less than 20% after the entire four-part procedure (tissue culture, antibody capture, PCR, and hybrid capture) was encouraging. The negative controls that were used throughout the entire procedure demonstrated that cross-contamination and false-positive PCR results were not hindrances in these assays, as long as appropriate procedures were followed (12). These preliminary results, along with those for several other compounds which have been repeated in independent assays (data not shown), indicate that variation from experiment to experiment may not be a major impediment to the use of the system as a primary screen for anti-HBV activity.

Several areas requiring further analyses include a comparison of results between this assay system and more conventional ones (11). Since the HBV-producing cells in the assay described here were growing, the enzymes necessary for activation of a given compound may be present at levels different from those in stationary-phase cells. Thus, there may be differences in results from the two types of assays. Another issue of concern is the distal location of the PCR primers from the origin of plus-strand DNA synthesis in the HBV genome (18, 24). Whether variances in the completeness of the plus-strand DNA in HBV produced in drug-treated cells will have an effect on quantitative PCR analyses remains to be shown. To date, however, results from comparisons of several compounds in the two types of assays indicate that these differences may not be significant (2, 4, 5). We conclude that use of the microtiter-based combination of proliferating P5A cell cultures, antibody capture, PCR, and hybrid capture provides an efficient and reliable means of assessing the selective activities of anti-HBV compounds in vitro.

REFERENCES

1. **Aoki-Sei, S., M. C. O'Brien, H. Ford, H. Fujii, D. A. Gilbert, D. A. Cooney, D. G. Johns, S. Broder, and H. Mitsuya.** 1991. In vitro inhibition of hepatitis B virus replication by 2′,3′-dideoxy-

guanosine,2′,3′-dideoxyinosine, and 3′-azido-2′,3′-dideoxythy-midine in 2.2.15 (PR) cells. J. Infect. Dis. **164**:843–851.

2. **Condreay, L. D. (Burroughs Wellcome Company).** 1992. Personal communication.

3. **Davis, G. L., and J. H. Hoofnagle.** 1986. Interferon in viral hepatitis: role in pathogenesis and treatment. Hepatology **6**:1038–1041.

4. **Davis, M. (Burroughs Wellcome Company).** 1992. Personal communication.

5. **Furman, P. A., M. Davis, D. C. Liotta, M. Paff, L. W. Frick, D. J. Nelson, R. E. Dornsife, J. A. Wurster, L. J. Wilson, J. A. Fyfe, J. V. Tuttle, W. H. Miller, L. Condreay, D. R. Averett, R. F. Schinazi, and G. R. Painter.** 1992. The anti-hepatitis B virus activities, cytotoxicities, and anabolic profiles of the (−) and (+) enantiomers of cis-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxythiolane-5-yl]cytosine. Antimicrob. Agents Chemother. **36**:2686–2692.

6. **Holodniy, M., D. A. Katzenstein, S. Sengupta, A. M. Wang, C. Casipit, D. H. Schwartz, M. Konrad, E. Groves, and T. C. Merigan.** 1991. Detection and quantification of human immunodeficiency virus RNA in patient serum by use of the polymerase chain reaction. J. Infect. Dis. **163**:862–866.

7. **Holodniy, M., M. Winters, and T. C. Merigan.** 1992. Detection and quantification of gene amplification products by a nonisotopic automated system. BioTechniques **12**:36–39.

8. **Hoong, L. K., L. E. Strange, D. C. Liotta, G. W. Koszalka, C. Burns, and R. F. Schinazi.** Enzyme-mediated enantioselective separation of the antiviral agent 2′,3′-dideoxy-5-fluoro-3′-thia-cytidine ((−)-FTC) and related compounds. J. Org. Chem., in press.

9. **Jansen, R. W., J. E. Newbold, and S. M. Lemon.** 1985. Combined immunoaffinity cDNA-RNA hybridization assay for detection of hepatitis A virus in clinical specimens. J. Clin. Microbiol. **22**:984–989.

10. **Jansen, R. W., G. Siegl, and S. M. Lemon.** 1990. Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. Proc. Natl. Acad. Sci. USA **87**:2867–2871.

11. **Korba, B. E., and G. Milman.** 1991. A cell culture assay for compounds which inhibit hepatitis B virus replication. Antiviral Res. **15**:217–228.

12. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature (London) **339**:237–238.

13. **Lampertico, P., J. S. Malter, and M. A. Gerber.** 1991. Development and application of an in vitro model for screening anti-hepatitis B virus therapeutics. Hepatology **13**:422–426.

14. **Landgraf, A., B. Reckmann, and A. Pingoud.** 1991. Direct anal-

ysis of polymerase chain reaction products using enzyme-linked immunosorbent assay techniques. Anal. Biochem. **198**:86–91.

15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

16. **Price, P. M., R. Banerjee, and G. Acs.** 1989. Inhibition of the replication of hepatitis B virus by the carbocyclic analog of 2′-deoxyguanosine. Proc. Natl. Acad. Sci. USA **86**:8541–8544.

17. **Robertson, B. H., B. Khanna, O. V. Nainan, and H. S. Margolis.** 1991. Epidemiologic patterns of wild-type hepatitis A virus determined by genetic variation. J. Infect. Dis. **163**:286–292.

18. **Robinson, W. S., and R. L. Greenman.** 1974. DNA polymerase in the core of a human hepatitis B virus candidate. J. Virol. **13**:1231–1236.

19. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuch, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.

20. **Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230**:1350–1354.

21. **Sells, M. A., M.-L. Chen, and G. Acs.** 1987. Production of hepatitis B virus particles in HepG2 cells transfected with cloned hepatitis B virus DNA. Proc. Natl. Acad. Sci. USA **84**:1005–1009.

22. **Sells, M. A., A. Z. Zelent, M. Shvartsman, and G. Acs.** 1988. Replication intermediates of hepatitis B virus in HepG2 cells that produce infectious virions. J. Virol. **62**:2836–2844.

23. **Shealy, Y. F., C. A. O'Dell, W. M. Shannon, and G. Arnett.** 1984. Synthesis and antiviral activity of carbocyclic analogues of 2′-deoxyribofuranosides of 2-amino-6-substituted-purines and of 2-amino-6-substituted-8-azapurines. J. Med. Chem. **27**:1416–1421.

24. **Summers, J., A. O'Connell, and I. Millman.** 1975. Genome of hepatitis B virus: restriction enzyme cleavage and structure of DNA extracted from Dane particles. Proc. Natl. Acad. Sci. USA **72**:4597–4601.

25. **Syvänen, A.-C., M. Bengtström, J. Tenhunen, and H. Söden-lund.** 1988. Quantification of polymerase chain reaction products by affinity-based hybrid collection. Nucleic Acids Res. **16**:11327–11338.

26. **Tiollais, P., and M. Buendia.** 1991. Hepatitis B virus. Sci. Am. **246**:116–123.

27. **Tiollais, P., C. Pourcel, and A. Dejean.** 1985. The hepatitis B virus. Nature (London) **317**:489–495.