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DNA polymerase activity of hepatitis B virus particles: differential inhibition by L-enantiomers of nucleotide analogs

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Abstract

DNA polymerase activity was assayed in hepatitis B virus (HBV) and core particles isolated from chronic producer lines. The particle-associated DNA polymerase activity, which was found to be limited to incorporation of only a few nucleotides, was inhibited by the 5′-triphosphates of nucleoside analogs. The 1-β-L (1*S*,4*R*) and 1-β-D (1*R*,4*S*) enantiomers of antiviral nucleoside analogs were compared for the ability to inhibit incorporation of natural nucleoside triphosphates into the viral DNA. Previously, both enantiomers of several analogs were found to be substrates for human immunodeficiency virus type 1 reverse transcriptase (HIV RT); the 1-β-D enantiomers of some pairs were preferred as substrates. In contrast, the 1-β-L enantiomers of all pairs tested were the more potent inhibitors of labeled substrate incorporation into hepatitis B virus DNA; the concentration required to inhibit the incorporation of the natural substrate by 50% was 6-fold to several hundred-fold lower than the concentration of the 1-β-D enantiomer required for the same inhibitory effect. This preference for the 1-β-L enantiomers was observed for both RNA-directed synthesis in core particles and DNA-directed synthesis in viral particles. The observed antiviral effect of the nucleoside analogs in cell culture seemed to be limited chiefly by their phosphorylation in cells.

Keywords: Hepatitis B virus; L-Nucleotide; Polymerase

1. Introduction

The DNA polymerase activity associated with hepatitis B virus (HBV) particles has been detected in virus pellets isolated from HBV antigen-positive human serum. Purified particles were disrupted with detergent in the presence of radiolabeled nucleotides and the accumulation of acid-precipitable radioactivity was monitored (Kaplan et al., 1973). The polymerizing reactions were DNA-dependent and were presumed to be incorporation of nucleotides into the second strand of HBV DNA catalyzed by the encapsidated HBV

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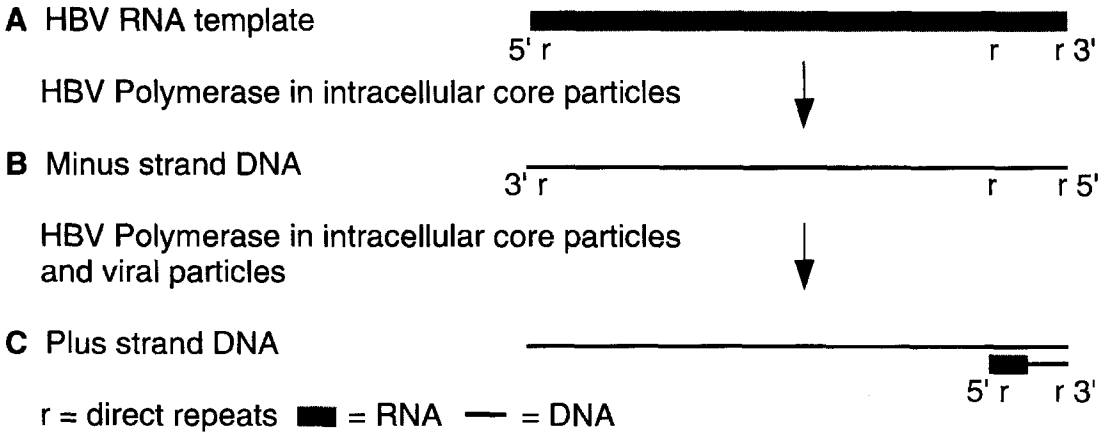

Fig. 1. Nucleic acid templates for DNA polymerase in HBV particles isolated from chronic producer cells. Intracellular core particles from chronic producer cells contain a plus strand RNA template (A) which is reverse transcribed into minus strand DNA (B). Minus strand products appear as a smear of single-stranded DNA. The full length minus strand is copied into a double-stranded molecule containing plus strand DNA (C). Plus strand products appear as a 3.2-kb band (Fig. 2), and both plus and minus DNA is labeled in polymerase reactions containing core particles. Extracellular viral particles, which contain full length minus strand DNA, incorporate radioactivity into plus strand DNA only. This diagram was taken from several contained in McLachlan (1991).

polymerase. A diagram of DNA polymerase templates found in HBV particles is illustrated in Fig. 1. In separate experiments, DNA extracted from HBV particles and incubated with unlabeled nucleotides was examined by electron microscopy (Hruska et al., 1977). The length of the double-stranded region appeared to increase after the polymerization reaction, which would be consistent with a processive reaction in which single-stranded regions of HBV DNA were filled in during the reaction. The polymerization reaction was inhibited by nucleotide analogs; inhibition of the accumulation of acid-precipitable radioactivity into HBV DNA in particles isolated from human serum by triphosphates of nucleoside analogs such as 2′,3′-dideoxynucleoside has been observed (Hantz et al., 1987; Matthes et al., 1991, 1992; Reimer et al., 1991). Particles of human and related animal hepadnaviruses isolated from infected livers (core particles) had reverse transcriptase activity as well as DNA polymerase activity (Miller et al., 1984a,b; Summers and Mason, 1982; Summers et al., 1978). The reverse transcriptase activity in core particles (minus strand DNA synthesis) as well as the DNA–DNA polymerase activity in viral and core particles (plus strand DNA synthesis) was sensitive to inhibition by nucleotide analogs, including the triphosphates of acyclovir (ACV)[1] and azidothymidine (AZT) (Hantz et al., 1987; Lofgren et al., 1989). The unique cycle of HBV replication, in particular, the initiation of DNA synthesis, has been the subject of several recent publications (Seeger et al., 1991; Wang and Seeger, 1992; Wang and Seeger, 1993; Zoulim and Seeger, 1994).

The HBV particles produced by the transfected HepG2 cell line (2.2.15) are infectious (Sells et al., 1987). They also contain DNA polymerase activ-

[1] Abbreviations: 3TC, (1*S*,4*R*) 1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine; (+)3TC, (1*R*,4*S*) 1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine; (−)FTC, (1*S*,4*R*) *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine; (+)FTC, (1*R*,4*S*) *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine; (+)CBV, (1*S*,4*R*) 9-(4-(hydroxymethyl)-2-cyclopenten-1-yl)guanine; (−)CBV, (1*R*,4*S*) 9-(4-(hydroxymethyl)-2-cyclopenten-1-yl)guanine (carbovir); ACV, 9-[(2-hydroxyethoxy)methyl]guanine (acyclovir); PCV, 9-[(4-hydroxy-3-hydroxymethyl-1-butyl]guanine (penciclovir); GCV, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (ganciclovir); d4T, (1*R*,4*S*) 2′,3′-dideoxy-2′,3′-didehydrothymine; L-d4T, (1*S*,4*R*) 2′,3′-dideoxy-2′,3′-didehydrothymine; AZT, 3′-azido-3′deoxythymine; araA, 9-β-D-arabinofuranosyladenine (vidarabine).

ity, which has been measured by following the incorporation of radioactivity into the HBV DNA extracted from the particles after the reaction (Sells et al., 1987; Furman et al., 1992).

In this report, we describe the DNA polymerase activity of particles isolated from the culture supernatants and cytoplasmic fractions of 2.2.15 cells and from other chronic producer cells, HB611 (Tsurimoto et al., 1987). These particles had a consistent genotype and unlimited supply. Both DNA polymerase and reverse transcriptase activity were inhibited by 5′-triphosphates of antiviral nucleoside analogs in assays similar to published studies with serum particles.

2. Materials and methods

2.1. Materials

Unlabeled and α-^{32}P-deoxynucleotides were purchased from DuPont-New England Nuclear. 2′,3′-Dideoxynucleotides were purchased from Boehringer Mannheim GmbH. AraATP and ddC were purchased from Sigma Chemical Co. ddG, PCV, GCV and ACV, as well as the triphosphates of PCV, GCV and ACV, were synthesized according to published procedures at Burroughs Wellcome Co. The 5′-triphosphates of the L derivatives of thymidine were prepared as described by VanDraanen et al. (1992). The 5′-triphosphates of CBV were prepared as described by Miller et al. (1993). 3TC, FTC and the 5′-triphosphates of these compounds were prepared according to procedures described by Liotta (Choi et al., 1991; Hager and Liotta, 1991; Hoong et al., 1992) and Wilson[2]. 1,3-Dimethyl-3,4,5,6-tetrahydro-2(*1*H)-pyrimidone was obtained from Aldrich and redistilled before use. (−)FTC-5′-[^{32}P]MP was kindly provided by Grace Roberts (Burroughs Wellcome Co.). Nucleotide monophosphate kinase, nucleotide diphosphate kinase, pyruvate kinase and lactate dehydrogenase were obtained from Sigma Chemicals.

(−)[α^{32}P]FTC-TP was prepared enzymically using nucleotide monophosphate kinase and nucleotide diphosphate kinase. Pyruvate kinase and lactate dehydrogenase were used during the reaction to regenerate ATP. There was approximately 70% conversion to the 5′-triphosphate as monitored by TLC and FPLC. The final product was purified by anion exchange chromatography on a Pharmacia MonoQ 10/10 column using a linear gradient of ammonium bicarbonate (240 ml, 0–700 mM). (−)[α^{32}P]FTC-TP eluted as a single peak at 0.35 M ammonium bicarbonate. The elution position corresponded to that of authentic, unlabeled (−)FTC-TP.

L-ddCTP was prepared according to the procedure of White et al. (1989), except 1,3-dimethyl-3,4,5,6-tetrahydro-2(*1*H)-pyrimidone was substituted as the solvent. The reaction mixture was extracted with chloroform to remove solvent, and the L-ddCTP (0.01 mmol) was purified by ion-exchange chromatography on a Pharmacia MonoQ 10/10 anion exchange column and eluted with an ammonium bicarbonate gradient (240 ml, 0–700 mM). L-ddCTP eluted as a single peak at 0.21 M ammonium bicarbonate resulting in a final yield of 19%. ^{1}H NMR and ^{31}P NMR obtained after chromatography corresponded with that expected for the dideoxynucleotide triphosphates: L-ddCTP had a $\lambda_{max} = 271$ nm (9000 M/cm, pH 7.0) using ^{31}P NMR (D_2O, pD 7.0) −8.0 (d, γP) −11.0 (m, αP) −22.0 (t, βP); mass spectrometry, $M + H^+$: expected 452.1, observed 452.1.

2.2. Preparation of particles

Five 225-cm^2 flasks of HBV producer cells (2.2.15, gift from B. Korba) were grown to confluence in RPMI medium (Gibco) containing 10% fetal bovine serum (FBS) and 500 μg/ml G418 (Geneticin, Gibco). Five 100-cm dishes coated with collagen (Collaborative Biosystems) of HB611 cells (gift from K. Matsubara) were grown to confluence in Dulbecco's medium (DMEM) (Gibco) containing 10% FBS and 500 μg/ml

[2] Wilson, J.E., Martin, J.L., Aulabaugh, A.E., Wright, L., McPherson, S., Wakefield, J.K., Morrow, C.D., Reardon, J.E. and Furman, P.A. (1995) Human immunodeficiency virus type-1 reverse transcriptase with mutations at methionine 184 in the highly conserved YMDD region: mechanism for reduced viral replication rates and 1-β-L drug resistance. (Manuscript in preparation).

G418. At confluence, medium containing 1% FBS was added, and collected twice a week for 2 weeks. Culture fluids were clarified by centrifugation at $1500 \times g$ for 5 min and the supernatants were mixed with one-fourth volume of solution containing 50% PEG 8000 and 0.6 M NaCl. After overnight refrigeration, particles were pelleted by centrigugation at $10\,000 \times g$. Pellets were resuspended in 20 ml of phosphate-buffered saline containing 50 units (2 μg) of DNase and 0.1 μg of RNase (Boehringer, Mannheim). After incubation for 30 min at 37°C, 25 mM EDTA was added and incubation was continued for 15 min. The cellular debris containing a DNA polymerizing activity was pelleted by centrifugation at $1500 \times g$ for 10 min. The supernatant containing HBV was layered over a step gradient of 7 ml each of 0.3, 0.6 and 0.9 M sucrose in buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl and 5 mM $MgCl_2$ (TKM buffer). The virus was pelleted by centrifugation in an SW27 rotor at 25 000 rpm for 20 h. Virus pellets were resuspended by sonication in 1 ml of TKM buffer and stored in aliquots at −80°C. Minus strand DNA was measured in these samples by slot hybridization; preparations of viral particles from 2.2.15 cells contained 33–75 ng of HBV minus strand DNA per ml. A preparation of particles from HB611 cells contained 6 ng of HBV minus strand DNA per ml.

Cytoplasmic core particles were isolated by shaking the 2.2.15 cell layers with glass beads or by digestion of the HB611 cells with collagenase. Cells were centrifuged at $1500 \times g$ and resuspended in 0.25 M sucrose solution containing 3 mM DTT, 4 ml per 225 cm^2 flask. Cells were homogenized with a Dounce homogenizer and nuclei were pelleted by centrifugation at $1500 \times g$ for 10 min. The cytoplasmic fraction was mixed with 0.7 volumes of 2.5 M sucrose (final concentration 1.35 M sucrose) and layered over a step gradient of 7 ml each of 1.55 and 1.8 M sucrose in TKM buffer. After centrifugation at 25 000 rpm for 20 h in an SW27 rotor, the 1.55 M sucrose layer contained all of the minus strand HBV DNA (determined by slot hybridization of samples). This layer was removed, then mixed with RNase and DNase and pelleted as described for viral particles. Preparations from 2.2.15 cells contained 11–14 ng HBV DNA per ml. A preparation from HB611 cells contained 1 ng HBV DNA per ml.

2.3. Incorporation of labeled nucleotides into HBV DNA

Particles (10 μl containing $0.5–4 \times 10^8$ particles) were mixed with NP-40 and β-mercaptoethanol at a final concentration of 0.1% each. 50 mM $MgCl_2$ and additional 200 mM KCl were added. Aliquots of this mixture (10 μl) were distributed into microcentrifuge tubes containing nucleoside triphosphates and inhibitors. Each ^{32}P-labeled nucleotide was added at 10 μCi, 3000 Ci/mmol, final concentration 0.24 μM or as indicated. Unlabeled nucleotides were added at a final concentration of 25 μM. Inhibitors were added at concentrations from 0.0002 to 24 μM. The final volume was 12.5 μl. Incubations were at 37°C, overnight or as indicated. The reactions were stopped by addition of 2.5 μl each of 0.5 M EDTA, 10 mg/ml proteinase K and 10% SDS. Samples were digested at 37°C overnight, then diluted with 225 μl of a solution containing 10 mM Tris-HCl at pH 8 and 1 mM EDTA. The DNA was purified by extraction with phenol and chloroform and the water layer was mixed with 75 μl of 7 M ammonium acetate and 350 μl of isopropanol. After freezing the samples at −80°C for 1 h, DNA was pelleted by centrifugation for 10 min at 14 000 rpm in a microfuge. Pellets were resuspended in 5X Tris-acetate EDTA gel buffer and subjected to electrophoresis on a 1.5% agarose gel, for separation of plus strand and minus strand products. Gels were fixed by soaking in 2.5% TCA for 20 min, dried and exposed to autoradiography on a phosphorimager screen for several days. The radioactivity associated with HBV DNA was integrated on an image analyzer (PhosphorImager, Molecular Dynamics, Sunnyvale, CA). The incorporation of labeled nucleotide versus concentration was determined for each inhibitor.

The time course of incorporation was determined by incubating viral particles as described with labeled nucleotides between 0.05 and 0.6 μM and removing samples at 15-min intervals for 90

min. The time point samples were mixed with 50 mM EDTA and 1% SDS, and aliquots were spotted onto DE81 paper (Whatman, Clifton, NJ). The unincorporated nucleotides were washed by rinsing the filters with 125 mM Na_2HPO_4. Dried filters were exposed to autoradiography on a phosphorimager screen and scanned by image analyzer. The concentrations of dNTP's at half-maximum rate of incorporation were determined by Lineweaver-Burk analysis of the integrated radioactivity using Enzfitter (R.J. Leatherbarrow, 1987, Biosoft, Cambridge, UK), a nonlinear regression data analysis program.

2.4. Hybridization

Supernatant samples were frozen at −80°C, thawed and 200-μl samples were mixed with 200 μl of 2 M NaOH. After incubation at room temperature for 30 min, 100 μl of 5 M ammonium acetate was added and the samples were filtered through nitrocellulose mounted on a slot apparatus (Minifold II, Schleicher and Schuell, Inc., Keene, NH). Slots were rinsed with 250 μl of 1 M Tris-HCl (pH 7.5) + 2 M NaCl, and finally with 2X SSC. DNA was fixed by baking the nitrocellulose at 80°C for 2 h under vacuum. Filters were hybridized against ^{32}P-labeled riboprobe. The RNA probe was synthesized with T3 RNA polymerase from a template DNA (pGEMEX-1 and Riboprobe Gemini System, Promega) carrying HBV DNA inserted at the *Eco*RI site in the minus orientation.

3. Results

3.1. Labeled nucleotide incorporation into HBV DNA

Incorporation of radiolabeled dATP, dCTP, dGTP and dTTP into HBV DNA in viral as well as core particles was observed; dGTP incorporation is shown in Fig. 2. Radiolabeled UTP was not incorporated in the presence or absence of other nucleotides. Since the viral particles are enveloped, the viral envelope was disrupted by addition of the detergent NP-40 so that nucleotides could penetrate the particles. Incorporation into DNA of viral particles was maximum at 0.1% NP-40. This concentration of detergent was also added to core particle assays to prevent particle aggregation during the incubations, although the presence of detergent did not affect the rate or extent of the reactions. The optimal concentration of unlabeled nucleotides was 10 μM; at 50 μM or above, the total incorporation of radioactive nucleotides was inhibited.

The incorporation of a single labeled nucleotide was enhanced by addition of all three unlabeled nucleoside triphosphates (20-fold over a single labeled nucleotide), but elimination of one of three unlableled nucleoside triphosphates did not completely eliminate incorporation. As an example, the incorporation of dATP in the presence of dTTP and dGTP was 50% of the incorporation in the presence of three unlabeled triphosphates (2-fold reduction). The range of reduction for nine experiments with two instead of three unlabeled nucleotides was 2–10-fold, the average reduction was 5-fold. This would be consistent with a nonprocessive reaction in which only a few nucleotides were added per template. In fact, the number of labeled nucleotides added in the presence of three unlabeled nucleotides was calculated to be one per template (see below).

In the presence of three unlabeled nucleotides, the rate of incorporation of ^{32}P-labeled nucleotides was linear for 45–60 min (Fig. 3) and reached a plateau after several hours. The concentration of dNTP that yielded the half-maximum rate of incorporation was 0.06–0.24 μM labeled nucleotide (dATP = 0.09 ± 0.02 μM, dCTP = 0.15 ± 0.05 μM, dTTP = 0.24 ± 0.02 μM, dGTP = 0.06 ± 0.04 μM). Inhibition assays were carried out at 0.24 μM labeled nucleotide, to allow for comparison between analogs of different nucleotides.

The extent of the inhibition of incorporation into plus strand DNA (DNA-directed DNA synthesis) by nucleotide analogs was the same as inhibition of incorporation into minus strand DNA (RNA-directed DNA synthesis). An illustration of the inhibition of dGTP incorporation into HBV DNA of core particles is shown in Fig. 2. In lanes 10–14, incorporation of [α^{32}P]dGTP

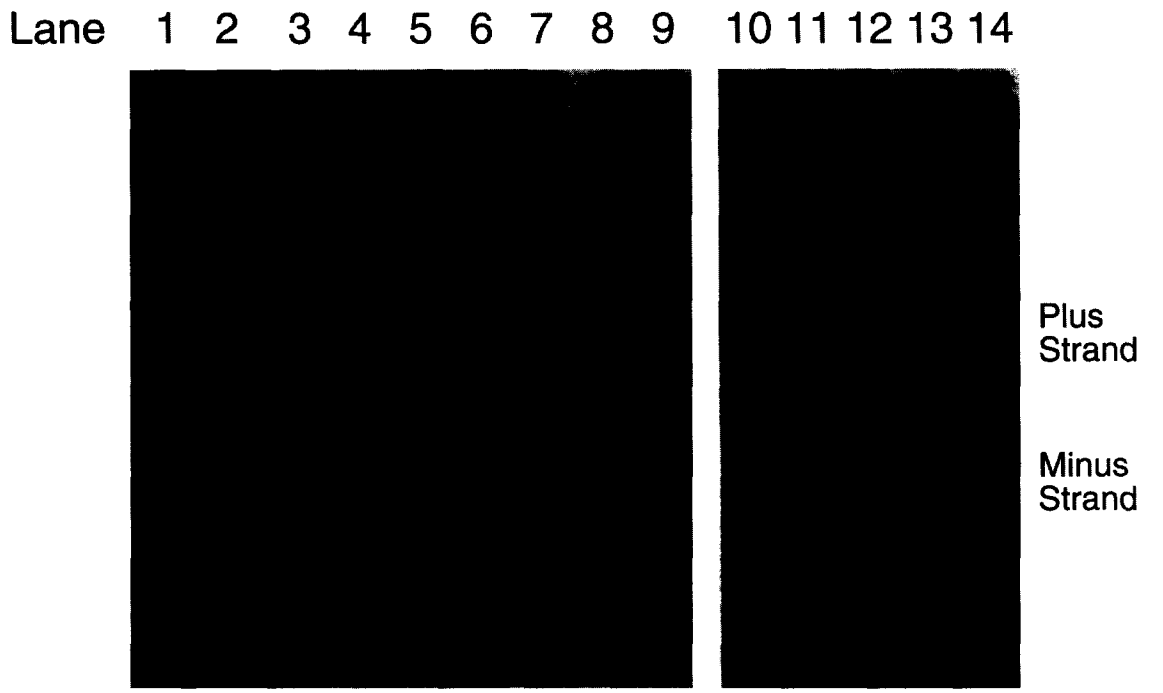

Fig. 2. Inhibition of [α^{32}P]dGTP incorporation into HBV DNA. Viral particles containing 330 pg HBV DNA (lanes 1–9) or intracellular core particles containing 110 pg (lanes 10–14) were incubated with 0.2 μM [α^{32}P]dGTP for 16 h. Inhibitory nucleotides were present at the following concentrations: (−)CBV-TP, 0.2 μM (lane 2), 0.6 μM (lane 3), 2 μM (lane 4); (+)CBV-TP, 0.007 μM (lane 5), 0.02 μM (lane 6), 0.07 μM (lane 7), 0.2 μM (lane 8), 0.7 μM (lane 9); ACV-TP, 0.2 μM (lane 11), 0.6 μM (lane 12), 2 μM (lane 13) and 6 μM (lane 14). In this experiment, the IC_{50} of (−)CBV-TP was 0.3 μM, (+)CBV-TP was less than 0.007 μM and ACV-TP was 0.33 μM for both plus and minus strand incorporation.

into double-stranded DNA is marked as plus strand and the incorporation into single-stranded DNA is marked as minus strand. Ribonucleotides, including L-r-TTP, had no effect on the incorporation of nucleotides into either strand of HBV DNA. The inhibition of incorporation of radiolabel into the DNA of particles isolated from HB611 cell cultures was very similar (data not shown), although the total incorporation was lower; the yield of particles was 10-fold lower than the yield of particles from 2.2.15 cell cultures, leading to lower incorporation of radiolabel.

3.2. Incorporation of one molecule of each deoxynucleotide triphosphate per minus strand DNA template

The total number of radioactive molecules incorporated per minus strand DNA molecule was calculated by spotting onto DE81 paper a radiolabeled HBV DNA sample isolated from viral particles after incubation for 6 h with 0.6 μM ^{32}P-dATP and 10 μM unlabeled nucleotides. After washing away the unincorporated label, the sample was counted in a scintillation counter. The cpm was converted to molecules of nucleotide based on the concentration of nucleotide supplied and the cpm from an unwashed nucleotide sample spotted onto DE81 paper. The concentration of minus strand DNA was determined by slot hybridization. In one experiment, 16×10^6 minus strands of HBV had incorporated 20×10^6 molecules of dATP. In another, 57×10^6 minus strands of HBV had incorporated 28×10^6 molecules of dATP; which is between 0.5 and 1.25 molecules of dATP incorporated per minus strand

template. Similar calculations were made for dTTP incorporation.

In agreement with the incorporation of one radioactive nucleotide per minus strand template, we saw no change in the size of HBV DNA extracted from particles after incubation with nucleotides. After incubation for 1 h up to several days, no change in the size of the plus strand or minus strand DNA products extracted from particles was observed in the presence or absence of detergent and no new sequence was detected (for example, no sequence at the 3′-end of the plus strand could be detected by hybridization) in particles incubated with concentrations of four nucleotides from 0.2 to 250 μM. Although particles isolated from human serum appeared to carry out extended DNA synthesis (Hruska et al., 1977), the particles isolated from chronic producer cells only incorporated a few nucleotides.

The radiolabeled nucleotides were incorporated into HBV DNA of viral particles in a limited region, which we identified by using the radiolabeled HBV DNA as a hybridization probe. After incorporation of dATP overnight, the labeled HBV DNA was extracted from viral particles isolated from 2.2.15 cells, digested with *Hha*I and *Hae*III enzymes to reduce the size of the probe DNA, and hybridized against antisense transcripts of HBV adhered to nitrocellulose (Fig. 4). According to the hybridized fragments, the incorporated radioactivity was concentrated in an area of the plus strand between the origin of the plus strand and 200 bases downstream. The same results were obtained after hybridization of dGTP- or dCTP-labeled DNA. The labeled region corresponds to the sequence between the direct repeats on the primed second strand (see Fig. 1), so that no incorporation was detected in the sequence homologous to the 5′-end of the minus strand downstream from the direct repeat. Thus, only runoff polymerase products were observed, and the particles did not have detectable incorporation into sequences copied after cyclization of the HBV DNA in viral particles (McLachlan, 1991).

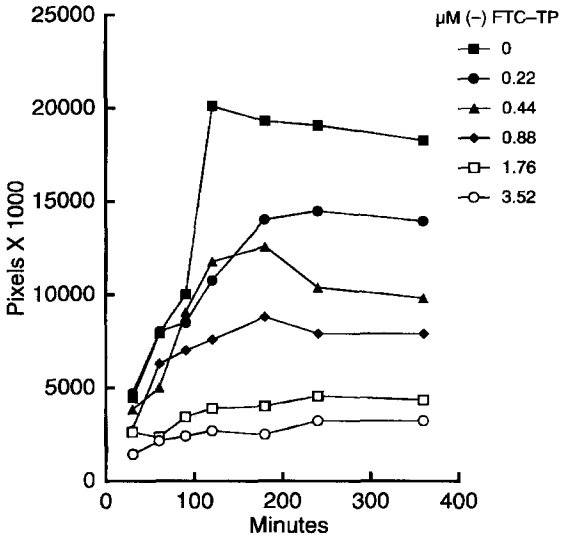

Fig. 3. Time course of incorporation of [α^{32}P]dCTP into HBV DNA in the presence of (−)FTC-TP. Viral particles containing 750 pg HBV DNA were incubated with 0.22 μM [α^{32}P]dCTP and the indicated concentrations of (−)FTC-TP. Samples were withdrawn at 30 min intervals and the extracted DNA was applied to agarose gels which were dried and exposed to a Phosphorimager screen. The radioactivity associated with the 3.2-kb DNA was integrated in units of pixels, shown on the Y axis.

3.3. Incorporation of labeled (−)FTC-TP into HBV DNA

(−)[α^{32}P]FTC-TP was incubated overnight with 1 ml of HBV viral particles containing 33 ng minus strand DNA (about 2×10^{10} particles isolated from 2.2.15 cell culture), unlabeled dATP, dTTP and dGTP, 0.1% NP-40, and buffers in a final volume of 1.5 ml. The DNA was purified by proteinase K digestion, extraction and precipitation, then separated on an agarose gel. The incorporated radioactivity was detected by autoradiography of the dried gel with a Phosphorimager screen. A labeled 3.2-kb DNA was detected (Fig. 5a). Thus, the (−)FTC-TP was incorporated into the DNA of HBV particles. We tested whether this incorporation would result in chain termination by examining the polymerizing activity of particles released from (−)FTC-treated 2.2.15 cells (see below).

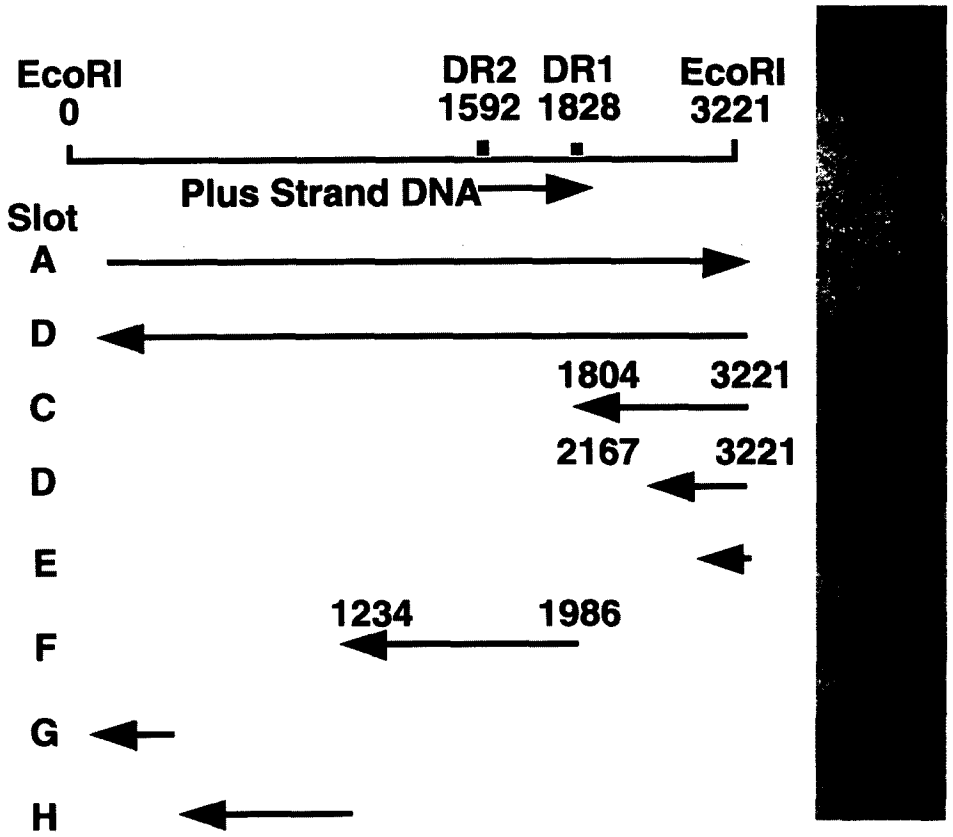

Fig. 4. Hybridization of labeled HBV DNA extracted from viral particles. HBV DNA extracted from viral particles after incorporation of labeled dCTP was hybridized vs. sense (slot A) and antisense (slots B–H) RNA transcripts adhered to nitrocellulose. The RNA transcripts were synthesized from cloned HBV templates corresponding to the arrows shown on the left. Sequence numbers correspond to sequences listed in McLachlan (1991). The radioactivity hybridizing to each template was quantitated by PhosphorImage autoradiography of the hybridized filter. An image of that exposure is shown on the right.

3.4. Lack of polymerase activity in chronic producer cells treated with (−)FTC

The incorporation of radiolabeled dCTP into HBV DNA in particles isolated from cell cultures treated with different concentrations of (−)FTC was compared with the incorporation in particles from control cultures (Fig. 5(b)). The particles produced from cells treated with 10 nM (−)FTC had reduced but detectable polymerase activity; however, particles isolated from cells treated with 100 nM (−)FTC did not have any polymerase activity even though they had detectable HBV minus strand DNA. The particles produced in drug-treated cells did not have any detectable plus strand DNA, which would be consistent with a chain-terminating activity of (−)FTC, preventing the completion of the minus strand DNA and thus preventing initiation of plus strand DNA. Duplicate experiments confirmed that particles PEG-precipitated from cultures treated with 100 nM or higher (−)FTC lacked the ability to incorporate radiolabeled dATP into HBV DNA. The accumulated minus strand DNA forms in (−)FTC-treated cells were primarily less than full length (Davis and Jansen, 1994), in contrast to control cells which contained abundant full-length minus strand DNA.

Similar experiments were carried out with 2.2.15 cultures treated with ddC, ACV, PCV,

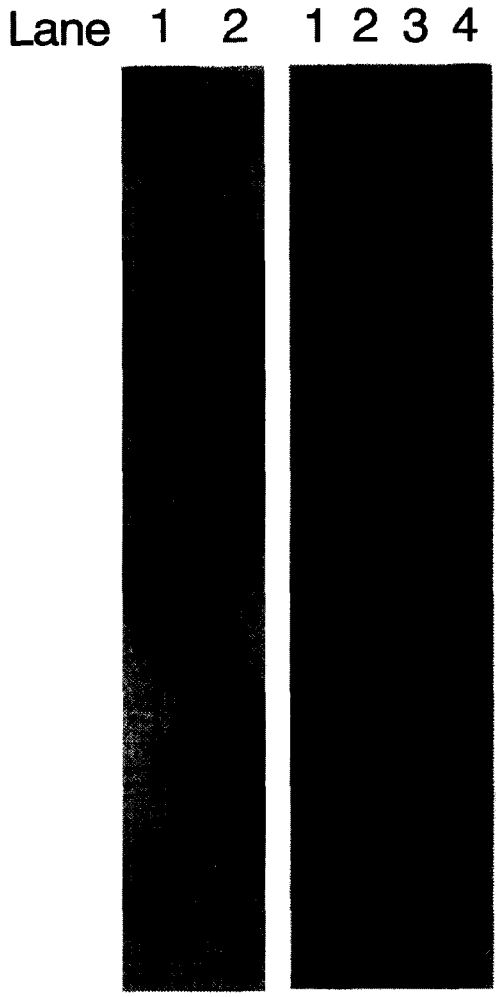

Fig. 5. Incorporation of (−)[α^{32}P]FTC-TP and [α^{32}P]dCTP into HBV DNA. (a) Conditions of the incorporation of (−)[α^{32}P]FTC-TP are described in the text: lane 1, HBV DNA; lane 2, end-labeled fragments of λ DNA. Molecular weights in descending order are 23.1, 9.4, 6.56, 4.36, 2.3 and 2.0 kb. (b) Incorporation of [α^{32}P]dCTP into HBV DNA in particles isolated from (−)FTC-treated cultures. Culture supernatants were collected from 2.2.15 cells on days 8, 9 and 10 of treatment with (−)FTC. The particles were precipitated with PEG as described in Section 2.2. The particles were resuspended in buffer for polymerase assays and for hybridization of HBV DNA. The incorporation of dCTP into HBV DNA was compared with the total minus strand HBV detected in the samples. Lane 1 contained 2 ng HBV DNA, culture was not treated with compound; lane 2 contained 1 ng of HBV DNA and the incorporated label is 37% of lane 1, culture was treated with 10 nM (−)FTC. Lane 3 contained 560 pg of HBV DNA and no detectable dCTP incorporation, culture was treated with 100 nM (−)FTC. Lane 4 contained 240 pg of HBV DNA and no detectable dCTP incorporation, culture was treated with 1000 nM (−)FTC.

GCV and ddG (Table 1). The decline in polymerizing activity of supernatant virus particles produced in drug-treated cells correlated with the decline of plus strand DNA in the supernatant particles. The intracellular HBV DNA detected by hybridization of Southern blots of total cell DNA also decreased in samples treated with these compounds (data not shown).

3.5. Inhibition of labeled nucleotide incorporation by an equal concentration of inhibitor

Since a single labeled nucleotide was added per minus strand DNA template, the chain-terminating nucleotides like (−)FTC-TP competed in the inhibition assays for a single incorporation into the HBV DNA. The time course of this competition was at least 4 h, since the inhibition was measured at the plateau of the reaction (Fig. 3). In the example shown, the concentration of (−)FTC-TP required for 50% inhibition of dCTP incorporation into HBV DNA was about twice the concentration of dCTP. Multiple inhibition studies were carried out with labeled nucleotide at 0.24 μM, close to the concentration of dNTP that yielded the half-maximum rate of incorporation, and the average concentration of (−)FTC-TP required for 50% inhibition of dCTP incorporation was 0.33 μM.

Approximately equal concentrations of chain-terminating nucleotides were required for inhibition of 50% of labeled nucleotide incorporation into HBV DNA in serum particles (Hantz et al., 1987; Matthes et al., 1991, 1992; Reimer et al., 1991; Lofgren et al., 1989). The incorporation reactions described in these published reports were similar to those described here.

3.6. Increased potency of 1-β-L enantiomers of nucleotides as inhibitors of labeled nucleotide incorporation

The L and D enantiomers of 2′,3′-dideoxynucleotides ddCTP and ddTTP, as well as the triphosphates of the L (1*S*,4*R*) and D (1*R*,4*S*) enantiomers of the antiviral compounds d4T,

Table 1
Polymerase activity of virus isolated from 2.2.15 cells during compound treatment

Compound	Concentration (μM)	POL (% control)	HBV DNA (% control)	
			Minus	Plus
(−)FTC	0.01	50	74	27
	0.1	0	31	0
	1	0	26	0
ddC	1	138	71	66
	10	42	38	11
	50	31	32	12
ACV	50	61	96	63
	100	24	67	40
	200	18	58	25
GCV	20	60	67	58
	100	23	54	49
PCV	20	44	62	52
	100	17	25	36
ddG	10	30	67	46
	50	20	26	11

Supernatant virus was pooled from triplicate 24-well cultures of 2.2.15 cells on days 7, 8 and 9 of compound treatment. The HBV minus and plus strand DNA was measured by slot hybridization. The dCTP incorporation activity (POL) was measured in PEG-precipitated samples under conditions described for purified particles. Values are expressed as % control (virus particles collected from untreated cells).

CBV, 3TC and FTC were compared for the ability to inhibit incorporation of natural deoxyribonucleoside triphosphates into the viral DNA (Table 2). For all compounds, the 1-β-L enantiomer was the more potent inhibitor of labeled substrate incorporation into HBV DNA; the concentration required to inhibit the incorporation of the natural β-D-deoxyribosylnucleoside triphosphate by 50% was 6-fold to several-hundred-fold lower than the concentration of the 1-β-D enantiomer required for the same inhibitory effect. This preference for the 1-β-L enantiomers was observed for both RNA-directed synthesis in core particles and DNA-directed synthesis in viral particles, for particles isolated from either 2.2.15 cell cultures or HB611 cultures.

The triphosphates of ACV, GCV and PCV were also tested for the ability to inhibit the incorporation of dGTP into HBV DNA. ACV-TP was recognized as well as ddGTP, but GCV-TP and PCV-TP were at least 40-fold less effective than ddGTP.

4. Discussion

The ability of nucleotide analogs to inhibit the HBV DNA polymerase activity was strongly dependent on the composition and absolute stereochemistry of the sugar component of the analog. For example, the D-dideoxyribonucleotides, as well as AZT-TP, d4T-TP, (−)CBV-TP and ACV-TP competed for incorporation into HBV DNA about the same as the natural deoxyribonucleotides, but ribonucleotides were not recognized as competitors. Also, arabinosides were weak inhibitors (araATP was 4-fold less inhibitory than ddATP), as were D-oxathiolanes ((+)3TC-TP and (+)FTC-TP) (at least 6-fold less inhibitory than ddCTP). ACV-TP was recognized at least 40-fold better than PCV-TP and GCV-TP. The 1-β-L enantiomers of all compounds tested inhibited incorporation of labeled nucleotides by 50% at concentrations about 10-fold less than the concentration of the 1-β-D enantiomer required for the same effect, except for the 1-β-L enantiomers of the compounds containing double bonds in the

Table 2
Incorporation of labeled deoxynucleoside triphosphates into HBV DNA

Nucleotide analogs		Inhibitor IC_{50} (uM)	Fold excess of inhibitor	Preference L/D	#Expt.
Labeled substrates	Inhibitor				
dCTP	ddCTP	0.4	1.7		3
	L-ddCTP	0.05	0.2	8 X	2
	3TC-TP	0.13	0.56	18 X	1
	(+)3TC-TP	2.4	10		1
	(−)FTC-TP	0.33	1.4	14 X	6
	(+)FTC-TP	4.8	20		4
dGTP	ddGTP	0.18	0.75		2
	(−)CBV-TP	0.07	0.3		2
	(+)CBV-TP	0.0007	0.003	100 X	2
	ACV-TP	0.14	0.6		2
	PCV-TP	14	60		2
	GCV-TP	>7	>30		2
dTTP	ddTTP	0.36	1.5		3
	L-ddTTP	0.06	0.25	6 X	3
	d4T-TP	0.19	0.8		3
	L-d4T-TP	0.007	0.003	266 X	2
	AZT-TP	0.24	1		1
dATP	ddATP	0.48	2		1
	araATP	1.9	8		1

Values were averaged for minus and plus strand incorporation for the number of experiments indicated. The range of multiple determinations was 3-fold, and the preference for L nucleotides was consistent through all experiments.

sugar component, L-d4T-TP and (+)CBV-TP, which were preferred at least 100-fold. Similar preference for 1-β-L enantiomers was observed with assays of woodchuck hepatitis virus particles isolated from woodchuck serum (Davis et al., 1994; Schinazi et al., 1994).

In contrast to the 1-β-L enantiomer preference of HBV polymerase, other polymerases including the human immunodeficiency virus type 1 reverse transcriptase (HIV RT) preferred the 1-β-D enantiomers such as ddTTP 50-fold (VanDraanen et al., 1992). In addition, the HIV RT recognized the ribonucleotides as inhibitors. The enantiomers of the oxathiolane compounds 3TC-TP and FTC-TP were recognized equally as inhibitors by wild-type HIV RT (Wilson et al., 1993). Interestingly, when the consensus polymerase amino acid sequence YMDD (amino acids 183–186) was changed to YVDD, the mutant HIV RT preferred the D enantiomers of nucleotide analogs as inhibitors by a factor of at least 80-fold (see footnote 2). The 184V corresponds to the mutation observed in (−)FTC-resistant HIV generated in culture and 3TC-resistant HIV generated in the clinic (Tisdale et al., 1993; Schinazi et al., 1993). Since the areas of homology between HBV and HIV RT include a consensus YMDD in the polymerase amino acid sequence (Larder et al., 1987; Radziwill et al., 1990), and alteration of this sequence in clones of hepatitis virus polymerase eliminated polymerizing activity (Radziwill et al., 1990), we think this 4-amino-acid stretch might be important for the unique substrate specificity of HBV.

The antiviral activity of compounds in culture was a consequence of the concentration of triphosphate accumulated in cells and the activity of the triphosphate as an inhibitor of HBV polymerase. The D enantiomer of FTC ((+) FTC) was phosphorylated in cells but (+)FTC-TP accumulated at a lower concentration than (−)FTC-TP in cells (Furman et al., 1992), and it was a weaker inhibitor of HBV polymerase activity. As a result, the IC_{50} for (+)FTC is almost 100-fold higher than the IC_{50} for (−)FTC.

The dideoxynucleosides and (−)CBV were phosphorylated in cells, and these compounds inhibited HBV production in chronic producer cells. However, HBV production in chronic producer cells was poorly inhibited by AZT and (+)CBV, due to the lack of kinase activation (Miller et al., 1993), although the triphosphates of AZT and (+)CBV were effective HBV polymerase inhibitors. The anti-herpesvirus compounds ACV and PCV had comparable inhibition of HBV production in chronic producer cell cultures (Table 1), although the triphosphate of ACV was a more potent inhibitor of HBV polymerase than the triphosphate of PCV (Table 2). The concentration of PCV-TP in HepG2 cells (2.2.15 cells were derived from HepG2) was more than 100-fold higher than the concentration of ACV-TP (Littler et al., 1994), which accounts for the equivalent inhibition in cell culture. The combination of polymerase inhibition studies and compound screening in chronic producer cells has provided evaluation of compound activity, toxicity and mechanism of action. These studies support the development of L-nucleosides as anti-hepatitis therapies.

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