Efficient pyrophosphorolysis by a hepatitis B virus polymerase may be a primer-unblocking mechanism

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Effective antiviral agents are thought to inhibit hepatitis B virus (HBV) DNA synthesis irreversibly by chain termination because reverse transcriptases (RT) lack an exonucleolytic activity that can remove incorporated nucleotides. However, since the parameters governing this inhibition are poorly defined, fully delineating the catalytic mechanism of the HBV-RT promises to facilitate the development of antiviral drugs for treating chronic HBV infection. To this end, pyrophosphorolysis and pyrophosphate exchange, two nonhydrolytic RT activities that result in the removal of newly incorporated nucleotides, were characterized by using endogenous avian HBV replication complexes assembled *in vivo*. Although these activities are presumed to be physiologically irrelevant for every polymerase examined, the efficiency with which they are catalyzed by the avian HBV-RT strongly suggests that it is the first known polymerase to catalyze these reactions under replicative conditions. The ability to remove newly incorporated nucleotides during replication has important biological and clinical implications: these activities may serve a primer-unblocking function *in vivo*. Analysis of pyrophosphorolysis on chain-terminated DNA revealed that the potent anti-HBV drug β-L-(−)-2′,3′-dideoxy-3′-thiacytidine (3TC) was difficult to remove by pyrophosphorolysis, in contrast to ineffective chain terminators such as ddC. This disparity may account for the strong antiviral efficacy of 3TC versus that of ddC. The HBV-RT pyrophosphorolytic activity may therefore be a novel determinant of antiviral drug efficacy, and could serve as a target for future antiviral drug therapy. The strong inhibitory effect of cytoplasmic pyrophosphate concentrations on viral DNA synthesis may also partly account for the apparent slow rate of HBV genome replication.

antiviral inhibition | core particles | proofreading | lamivudine/3TC

Hepatitis B virus (HBV) is a member of the *Hepadnaviridae* family characterized by small, circular, partially double-stranded DNA genomes that are replicated by reverse transcription (1). Chronic HBV infection causes hepatitis and hepatocellular carcinoma, and remains a significant health problem worldwide (2). Designing antiviral drugs to combat HBV infection is impeded by a poor understanding of the parameters governing viral inhibition. Since currently effective antiviral drugs target the viral reverse transcriptase (RT; reviewed in ref. 3), a detailed characterization of the HBV-RT catalytic mechanism is essential to understanding antiviral drug efficacy.

The HBV-RT is a multifunctional enzyme with RNA- and DNA-dependent DNA polymerase, RNase H, and protein-priming activities (reviewed in ref. 4). Once translated, the RT binds the epsilon stem-loop structure of the pregenomic RNA template and, together with bound cellular factors, this replication holoenzyme complex is encapsidated within a coat composed of the viral core protein (5–7). All viral DNA synthesis catalyzed by the RT subsequently proceeds enclosed within these replicating core particles by the polymerization of deoxynucleoside triphosphates (dNTPs) onto the growing DNA chain (8): $DNA_n + dNTP \rightleftharpoons DNA_{n+1} + PP_i$. Although the DNA synthesis reaction responsible for HBV genome replication has been studied mechanistically (9), other potential reactions catalyzed by the HBV-RT polymerase activity have not been examined.

The necessary byproduct of DNA polymerization is inorganic pyrophosphate (PP_i), and PP_i also serves as a substrate for the reverse reaction, pyrophosphorolysis (10). Typically, DNA replication proceeds in low PP_i concentrations because of the hydrolytic action of cellular pyrophosphatases (PP_iases). Under such replicative conditions, pyrophosphorolysis is inefficient. However, because of its compartmentalized nature, these conditions may not be true of HBV DNA synthesis within replicating cores: viral core particles isolated from infected tissue are impermeable to even small enzymes (11), suggesting that larger multimeric enzymes such as cytoplasmic PP_iases would similarly be excluded from the viral core particle. Furthermore, the positively charged interior surface of cores (12, 13) may also retard PP_i efflux by forming ionic interactions with the negatively charged PP_i produced during DNA synthesis. Therefore, HBV DNA synthesis within cores may proceed in the presence of an elevated local concentration of PP_i. Because of their implied physiological relevance, it is important to examine the effects of PP_i on HBV-RT catalysis.

The general difficulty in obtaining enzymatically active RT from human or animal hepadnaviruses in large quantities and high purity has hampered the study of HBV-RT function (reviewed in ref. 14). Although advances in the expression of the RT in heterologous systems have furthered our understanding of the initiation reaction, these systems are not well suited for enzymological analysis of RT catalysis. Moreover, in examining the effects of PP_i, it is essential to study the HBV-RT in its natural context, encapsidated within replicating cores, to ensure the activities examined reflect their native characteristics. Under physiological conditions, the HBV-RT functions in a multiprotein replication complex with altered properties; RT activity is specifically activated by encapsidated cellular factors such as a multiprotein chaperone complex (7), the viral core protein (15), and the viral RNA (16). Proteolytic processing of the HBV-RT is also thought to be important, although its enzymatic significance is not understood (17, 18). Furthermore, because genome replication occurs compartmentalized within viral core particles, the exact architecture and stoichiometry of the replication complex is likely to influence RT function. For example, it has been proposed that physiological concentrations of PP_i are unlikely to affect DNA polymerase function because the rate of dissociation from the DNA is faster than the rate of PP_i use (19).

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Abbreviations: HBV, hepatitis B virus; RT, reverse transcriptase; DHBV, duck hepatitis B virus; PP_i, pyrophosphate; 3TC, β-L-(−)-2′,3′-dideoxy-3′-thiacytidine; TP, triphosphate; MP, monophosphate; PP_iase, pyrophosphatase.

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Although valid for an isolated polymerase, this parameter may not be true for an encapsidated polymerase that is unable to switch templates. As such, examination of HBV-RT catalysis within replicating cores is most likely to reflect the natural characteristics of this enzyme.

Using the avian HBV model system, a method for producing high activity and purity replicating cores was developed and used to characterize the effects of PP_i on RT catalysis. Contrary to expectation, PP_i-dependent RT activities were able to efficiently remove newly incorporated nucleotides and certain antiviral drugs even under low, cytoplasmic concentrations of PP_i. These activities operating during viral replication could potentially undermine the efficacy of some drugs, and could partly account for the slow rate of genome replication.

Materials and Methods

Replicating Core Purification and Polymerase Activity Analysis. Intracellular replicating cores were purified from the livers of robustly viremic Pekin ducks carrying the wild-type duck hepatitis B virus (DHBV) strain ALTA-16 (GenBank AF047045) by using the extraction and anion exchange chromatography techniques described previously (20). For the analysis of drug-resistant mutants, animal hosts were experimentally infected with DHBV variants corresponding to the clinical L528 M (LM), M555V (MV), and L528 M-M555V (LMMV) mutations and were maintained on a strict drug regimen; replicating cores were purified from the livers of robustly viremic animals.

Endogenous polymerase activity was assayed under optimal conditions in polymerase buffer containing 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 0.5% Nonidet P-40, 50 μM each of dATP, dGTP, and dTTP, and 2 μCi [α-^{32}P]dCTP (3000 Ci/mmol, Dupont/NEN). Reactions had a final volume of 50 μl and were initiated by the addition of 5 μl of the core preparation to the polymerase buffer. After a 60-min incubation at 37°C, the reactions were terminated by dispensing the reaction mixture into 5 ml of ice cold 10% trichloroacetic acid and incubating on ice for 10 min. The resulting trichloroacetic acid suspensions were filtered through Millipore GF/C fiberglass filters, washed three times with 10% trichloroacetic acid and once with 95% ethanol, dried, and counted in a Beckman scintillation counter.

Pyrophosphorolysis. To assay the pyrophosphorolytic removal of dNMPs from primer termini, endogenous viral DNA was first labeled by the incorporation of the appropriate nucleotide catalyzed by the viral RT. This labeling was achieved by the addition of 2 μCi ($\approx$0.012 μM) [α-^{32}P]dNTP (3000 Ci/mmol, Dupont/NEN) to 20 μl of cores, and incubation at 37°C for 10 min. Labeling with chain-terminating nucleotide analogs was performed in the presence of 1 μM of the remaining 3 dNTPs, and either 2 μCi [α-^{33}P]ddNTP (1500 Ci/mmol, Dupont/NEN), [α-^{32}P]ddATP (5000 Ci/mmol, Dupont/NEN), or [α-^{32}P]β-L-(−)-2′,3′-dideoxy-3′-thiacytidine triphosphate (lamivudine triphosphate; 3TC-TP; 5000 Ci/mmol, J. Wilson, University of Alberta) for 60 min. Background radioactivity was determined by blocking [α-^{32}P]/[α-^{33}P]ddNTP incorporation by preincubating with unlabeled dNTP/ddNTP, or by using heat-inactivated core preparations. To assay pyrophosphorolysis, PP_i was then added in polymerase buffer containing 1 μM of the appropriate unlabeled dNTP to ensure that the removed nucleotides could not serve as substrates for a second round of label incorporation. To assay rNTP-dependent pyrophosphorolysis, rNTPs were first pretreated with PP_iase (Amersham Pharmacia) to remove any contaminating PP_i, and then added to labeled cores in polymerase buffer (also with 20 mM $MgCl_2$). The reactions were terminated by dispensing into 10% trichloroacetic acid and processed as described for the polymerase assay. To ensure that the pyrophosphorolysis assay specifically measured DHBV DNA, nucleic acids were also purified with and without protease pretreatment and counted in a scintillation counter (see *Results*). For the quantitative pre-steady-state assay, the purity and integrity of the cores were assessed in detail as variations in core quality significantly affected the derived parameters.

Pyrophosphate Exchange. Because of the high concentration of polymerase activity required to assay PP_i exchange, purified cores were further concentrated 10-fold by ultracentrifugation. PP_i exchange was assayed under optimal conditions at 37°C in polymerase buffer containing 100 μM dNTPs and various concentrations of $^{32}PP_i$ (Dupont/NEN, 5 Ci/mmol). One microliter of the reaction mixture was spotted onto polyethyleneimine-cellulose TLC plates and developed in phosphate buffer as described previously (20). This TLC method resolved all dNTPs, PP_i, and P_i; the identity of the individual dNTPs was verified by UV shadowing co-spotted standards. Labeled products were visualized by using a Fuji phosphorimager, and quantified by using IMAGE GAUGE V. 3.0 software. Although PP_i exchange was linear for at least 6 h, shorter incubation times of 30 min were used in determining the PP_i K_M. The k_{ex} was estimated by quantifying the amount of labeled dNTP product in comparison with known amounts of $^{32}PP_i$ substrate, and by estimating the amount of active polymerase from the single nucleotide extension assay.

Results

Enzymological Analysis of DHBV-RT Catalysis Within Core Particles. The HBV-RT was investigated within the multiprotein replicating core complex assembled during the course of a natural infection. In contrast to other polymerases that are difficult to study within intact holoenzyme complexes, naturally assembled replicating cores can be harvested from the cytoplasm of infected hepatocytes (8). The resulting preparations possess an endogenous polymerase activity corresponding to the activity of the viral RT on the encapsidated viral nucleic acid. It is precisely the activity of these replicating cores that serves as a target for most antiviral therapy. However, core preparations are typically plagued by two limitations that render them unsuitable for enzymological analysis: very low RT activity and purity.

To overcome these limitations, a method for producing high activity and purity replicating cores was developed by using the DHBV model system. The initial development and evaluation of this system has been described elsewhere (20). Ultimately, cores with a 25- to 50-fold higher and sustained polymerase activity were obtained (Fig. 1*a*). To determine whether this polymerase activity was due to the DHBV-RT, polymerase reaction products were examined electrophoretically. Under polymerase assay conditions, the majority of cores completed genome synthesis *in vitro*, in contrast to other core and polymerase preparations, and nucleic acid with an electrophoretic pattern consistent with DHBV replication intermediates was labeled (Fig. 1*b*). This pattern was not observed when protease digestion before the purification of total nucleic acid was omitted. Under these conditions, the DHBV DNA was specifically removed in the organic extraction because of the unique covalent linkage between the viral DNA and the terminal protein used for priming first strand synthesis (21). Because exogenous nucleic acid would remain in the aqueous phase of the organic extraction, the absence of radiolabeled DNA after the organic extraction indicated that no detectable contaminating polymerase activity was present under typical polymerase assay conditions. Consistent with this conclusion, identical preparations from uninfected animals yielded no detectable polymerase activity (Fig. 1*a*). Furthermore, the polymerase activity always coeluted precisely with DHBV DNA, could be immunoprecipitated with anti-core antibodies, and had the expected inhibitor profile (20). As such, the strong polymerase activity of these preparations was due to the DHBV-RT.

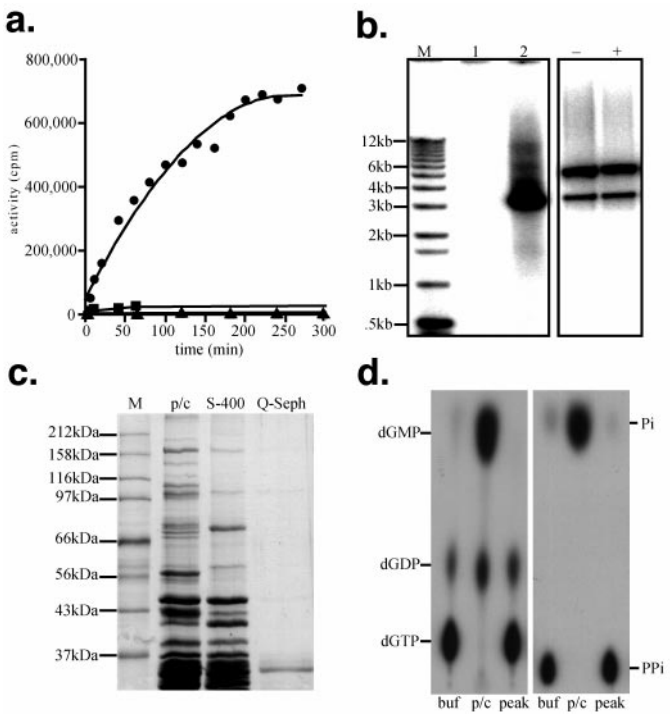

Fig. 1. Characteristics of DHBV core preparations (20). (*a*) Progress curve comparing the polymerase activity of cores purified by the newly developed method (●) versus those isolated by using a previous method (■), and preparations from uninfected animals (▲). (*b*) Agarose gel electrophoretic analysis of DNA polymerase reaction products after extended incubation time. End-labeled 1-kb ladder (GIBCO/BRL) (lane M), polymerase reaction products purified without (lane 1) and with (lane 2) protease pretreatment were subjected to gel electrophoresis and detected by autoradiography. The ability of cores to exclude exogenous enzymes from their interiors was assessed by incubating without (−) or with (+) 10 μg of DNase for 1 h after DNA labeling in a 1 h polymerase assay. (*c*) SDS/PAGE analysis of protein content of precolumn (p/c), Sephacryl S-400 gel filtration purified (S-400), and Q-Sepharose purified core preparations (Q-Seph) standardized by polymerase activity. The predominant band in the Q-Seph lane was verified to be the core protein by immunoreactivity (20). (*d*) Evaluation of dNTPase and PP_iase contamination of purified cores. [α-^{32}P]dGTP, which was most sensitive to degradation, or $^{32}PP_i$ were incubated in buffer (buf), precolumn cores (p/c), and peak Q-Sepharose purified cores at 37°C for 60 min. Breakdown products were separated by TLC, and visualized by autoradiography. Data partly adapted with permission from ref. 20.

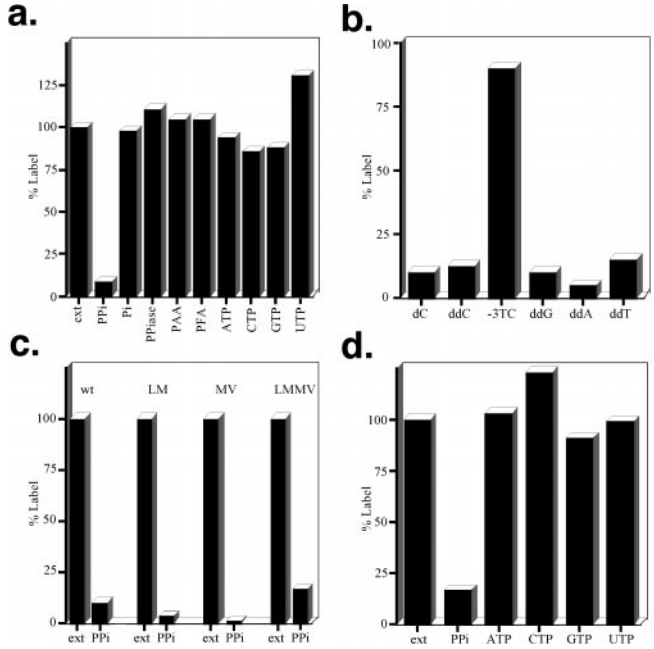

Fig. 2. Pyrophosphorolytic activity of purified DHBV cores. (*a*) The ability of 1 mM PP_i and various PP_i analogs (5 mM rNTPs) to act as substrates for pyrophosphorolysis was assayed by monitoring the removal [α-^{32}P]dCMP from viral DNA. For these and subsequent data, background radioactivity was subtracted, and values are percentages relative to the extension reaction (ext). (*b*) The primer termini were labeled by the incorporation of chain-terminating nucleotide analogs, and their ability to serve as substrates for DHBV-RT pyrophosphorolysis was compared by the addition of 1 mM PP_i. The results are depicted for the end-point of all reactions: ddNTPs were completely removed in less than 1 min, whereas the data of a 1-h incubation with PP_i are shown for 3TC. (*c*) The pyrophosphorolytic activity of various DHBV-RT mutants was determined as described for wild type. The results are for a 1-h incubation with 1 mM PP_i. (*d*) The substrate specificity of the LMMV variant was tested as for wild-type DHBV-RT.

In addition to possessing a robust polymerase activity, these cores were also sufficiently pure for enzymological analysis. Electrophoretic analysis of total protein revealed that these preparations contained predominantly viral protein (Fig. 1*c*). However, because even minor constituents could interfere with enzyme analysis, these preparations were further assessed for contaminating activities directed against the viral DNA and dNTP and PP_i substrates. Most importantly, these preparations exhibited high substrate stability because no dNTP or PP_i degradation could be detected under polymerase assay conditions (Fig. 1*d*). This issue was critical because such contaminants would interfere with studies aimed at characterizing the effects of PP_i on DHBV DNA synthesis. Other cellular activities, such as nuclease activities, were also undetectable in these preparations under polymerase assay conditions. Furthermore, analysis of cores that were impermeable to enzymes by virtue of an intact capsid barrier further ensured that any trace contaminants did not interfere with RT analysis (Fig. 1*b*). Because of their high activity and purity, these core preparations were therefore well suited for investigating the effects of PP_i on DHBV-RT catalysis.

Characteristics of DHBV-RT Pyrophosphorolysis. In the absence of nucleotides, PP_i stimulates pyrophosphorolysis, an activity that proceeds by the nucleophilic attack of PP_i on the phosphodiester backbone of the primer terminus (10, 19, 22, 23). The result is the release of a dNTP and the concomitant degradation of the DNA chain by one: $DNA_n + PP_i \rightleftharpoons DNA_{n-1} + dNTP$. To assay the DHBV-RT pyrophosphorolytic activity, endogenous viral primer termini within core particles were first labeled by the incorporation of [α-^{32}P]dCTP catalyzed by the viral RT. This single nucleotide extension was expected to label every genome where the next required nucleotide was dCTP, approximately one quarter of all active replicating cores. Next, PP_i was added to assess whether the incorporated dCMP could be removed by pyrophosphorolysis. In contrast to other assay systems, this method had the advantage that only active RTs were analyzed and at physiological RT-to-DNA template ratios.

The addition of exogenous PP_i stimulated DHBV-RT catalyzed pyrophosphorolysis (Fig. 2*a*). This activity was specific to PP_i because ortho-phosphate (Pi) and PP_i pretreated with PP_iase could not support this activity. To investigate the substrate specificity of pyrophosphorolysis, PP_i analogs were substituted for PP_i in this assay. The clinically useful PP_i analogs phosphonoacetate (PAA) and phosphonoformate (PFA) were unable to support pyrophosphorolysis. High but physiological concentrations of rNTPs have also been shown to stimulate a pyrophosphorolysis-like activity of the HIV-RT, resulting in the release of a dinucleoside tetraphosphate and the degradation of the DNA chain by one (24). However, substitution of rNTPs in the

DHBV-RT pyrophosphorolysis assay resulted in no [α-^{32}P]-dCMP removal even when 5 mM rNTPs were used under a variety of conditions and extended incubation times. The DHBV-RT thus possessed a pyrophosphorolytic activity with narrow substrate specificity.

To assess the efficiency of DHBV-RT pyrophosphorolysis on chain-terminated DNA, primer termini were labeled by the incorporation of radiolabeled ddNTPs and 3TC-TP, a cytidine analog with a thio substituted sugar in the unnatural enantiomeric form. In contrast to the inefficient removal of chain terminators by some polymerases (10, 19, 25), addition of PP_i resulted in ddNMP removal which was as rapid as dNMP removal (Fig. 2*b*). As such, DHBV-RT pyrophosphorolytic efficiency was not affected by the 3′ hydroxyl moiety, and was not significantly base specific. In notable contrast, no loss of label was detected from 3TC-monophosphate (MP)-terminated viral DNA even under high PP_i concentrations and extended incubation times. Because removal of 3TC-MP was at least 60-fold less efficient than the removal of ddCMP or dCMP, unnatural sugar modification of the terminal primer nucleotide appears to seriously decrease the efficiency of DHBV pyrophosphorolysis (although this could also be a specific characteristic of 3TC).

Examination of pyrophosphorolysis was also extended to clinically relevant HBV variants. HBV-RT mutations conferring resistance to the nucleoside analog 3TC have been mapped to the YMDD active site motif (26). Mutation to YVDD and YIDD are sufficient to confer resistance to 3TC, whereas the second mutation L528 M is frequently associated with the drug resistant variants. Because residues in the homologous YMDD motif of ϕ29 polymerase affect pyrophosphorolytic activity (23), it was first important to determine whether the corresponding drug-resistant variants of DHBV retain this activity. Addition of PP_i resulted in the removal of [α-^{32}P]dCMP, indicating that all variants tested possessed a pyrophosphorolytic activity (Fig. 2*c*). Substitution of rNTPs for PP_i resulted in no decrease in radiolabeled DNA (Fig. 2*d*), indicating that the drug-resistant DHBV variants did not acquire the ability to use rNTPs to catalyze pyrophosphorolysis, in contrast to AZT-resistant HIV-RT (25). Therefore, the 3TC-resistant DHBV-RT variants retain a pyrophosphorolytic activity with unaltered substrate specificity.

Pre-Steady-State Kinetic Analysis of Pyrophosphorolysis. Because understanding the catalytic efficiency of DHBV-RT pyrophosphorolysis would aid in predicting its physiological relevance, a kinetic analysis was undertaken. The rate constant (k_{pyro}) of pyrophosphorolysis on a dNMP substrate was estimated by determining a time course of the pre-steady-state pyrophosphorolysis assay (Fig. 3*a*). Assuming that removal of the labeled dCMP corresponded to the removal of a single dCMP by each polymerase, a minimal k_{pyro} was estimated to be $\approx$0.03 nt/sec under saturating PP_i concentrations. Interestingly, although the k_{pyro} varied between 0.01 and 0.1 nt/sec, it was only about 5-fold slower than that of the synthesis reaction estimated under similar conditions. Also, pyrophosphorolysis was not notably base specific because other labeled dNMPs were removed at similar rates.

In addition to the rate of the reaction, the affinity of the DHBV-RT for PP_i was estimated by varying the concentration of PP_i used in the pre-steady-state pyrophosphorolysis assay (Fig. 3*b*). Surprisingly, even low PP_i concentrations efficiently stimulated pyrophosphorolysis; maximal reaction velocity was achieved at a PP_i concentration of $\approx$150–200 μM, whereas the half maximal rate occurred at $\approx$7.5 μM. These results implied that the DHBV-RT pyrophosphorolytic activity is unexpectedly efficient and may function under physiological conditions.

Steady-State Kinetic Analysis of PP_i Exchange. To define the catalytic efficiency of the reverse reaction more precisely, a robust steady-state system was developed that would emulate DHBV replicative conditions. DNA polymerization occurs in the presence of both dNTPs and PP_i under physiological conditions. In the presence of dNTPs, PP_i stimulates a PP_i exchange activity of DNA polymerases (10, 22, 27), which in simple terms is a polymerization step immediately followed by the removal of the newly incorporated nucleotide by pyrophosphorolysis (28). This system was used to directly measure the K_M of PP_i and the rate of removing newly incorporated nucleotides.

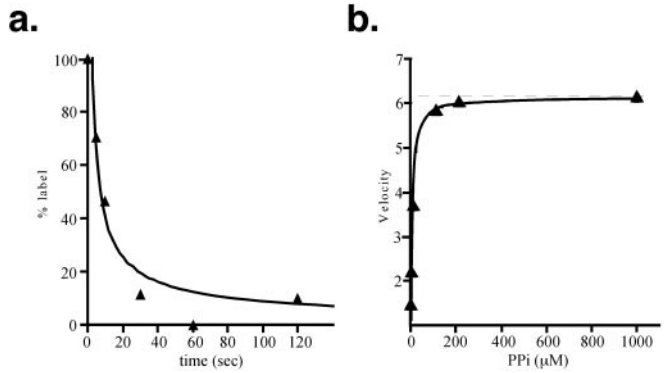

Fig. 3. Pre-steady-state kinetic analysis of DHBV-RT pyrophosphorolysis. (*a*) A time course of [α-^{32}P]dCMP removal from the viral primer terminus was used to estimate the pyrophosphorolysis rate constant. (*b*) The PP_i levels were varied to estimate the PP_i concentration dependence of pyrophosphorolysis. A time course was also determined for individual PP_i concentrations to ensure that the reactions did not plateau before reaction termination.

PP_i exchange was assayed by the addition of radiolabeled $^{32}PP_i$ in the presence of unlabeled dNTPs, with the transfer of the label from PP_i to dNTPs being evidence of PP_i exchange: DNA_n + dNTP + PP_i* $\rightleftharpoons$ DNA_n + dNTP* + PP_i. Under these conditions, the system was pseudofirst order for PP_i, and the DHBV-RT catalyzed PP_i exchange with a K_M of $\approx$19 μM (mean of three experiments), whereas a PP_i concentration of 50–200 μM resulted in maximal reaction velocity (Fig. 4). Although the K_M varied between 3 and 54 μM, it was always 50-fold lower than that of the avian myeloblastosis virus (AMV)-RT determined in parallel, and the lowest known for any polymerase (10, 19, 22, 23). This low K_M for PP_i also agreed with the PP_i binding constant derived for pyrophosphorolysis under pre-steady-state conditions. The PP_i exchange rate (k_{ex}) was $\approx$1 nt/sec. Although

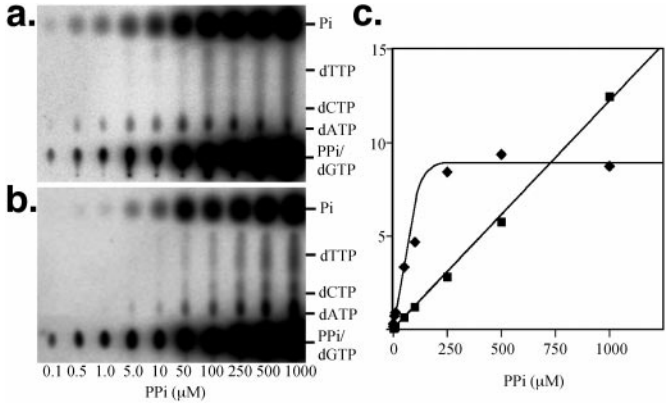

Fig. 4. PP_i exchange activity of DHBV cores (*a*) and avian myeloblastosis virus (AMV)-RT (*b*) determined under identical conditions. After a 30-min incubation with dNTPs and $^{32}PP_i$, reaction mixtures were separated by TLC, the products quantified, and data plotted reaction velocity versus PP_i (*c*) for DHBV (♦) and AMV-RT (■). Although dAMP was removed $\approx$5-fold more efficiently than other dNMPs by both RTs, this disparity was not observed when lower, physiological concentrations of dNTPs were used.

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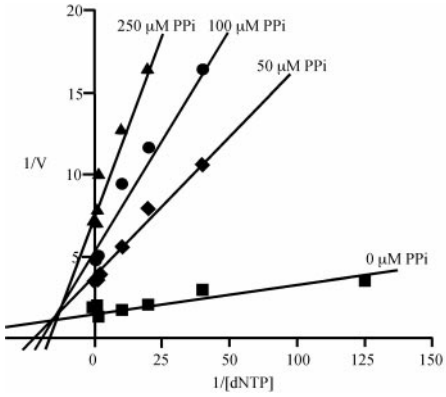

Fig. 5. Steady-state reversible inhibition of DHBV DNA synthesis by PP_i. The effect of 0, 50, 100, and 250 μM PP_i on DHBV-RT DNA synthesis was determined under varying dNTP concentrations. After 60 min at 37°C, the reactions were terminated, and the reciprocal of the reaction velocity was plotted versus the reciprocal of substrate concentration (Lineweaver-Burk plot).

this was about 10-fold faster than the rate derived for pyrophosphorolysis, this disparity is characteristic for polymerases (10). Furthermore, although intracellular cores consist of both RNA- and DNA-templated species, the PP_i K_M and k_{ex} were similar for cores purified from serum, which undergo DNA-templated synthesis, and intracellular cores in the presence of actinomycin D, which undergo RNA-templated synthesis. These results confirmed that DHBV PP_i exchange is unusually efficient, and that the nature of the nucleic acid template does not dramatically influence its efficiency.

Effect of PP_i on DNA Synthesis. Because the DHBV-RT efficiently used low levels of PP_i to remove newly incorporated dNMPs, it was important to assess the effect of PP_i on DNA synthesis. PP_i is both a product of the polymerization reaction and a substrate for the reverse reaction, and therefore acts as an inhibitor of DNA synthesis (10, 22). Accordingly, the addition of PP_i to a polymerase reaction resulted in potent inhibition of DHBV DNA replication (Fig. 5). Analysis under steady-state conditions revealed that PP_i inhibited DHBV-RT DNA synthesis reversibly by a mixed mechanism, with both inhibitor constants (K_i) for PP_i being low, $\approx 12 \pm 6.0$ μM and 59 ± 12 μM (Fig. 5*b*). Since the basis for PP_i inhibition is at least partly because of the reversal of the polymerization reaction (10), this was an independent confirmation of the low PP_i binding constants derived for pyrophosphorolysis and PP_i exchange.

Interestingly, low concentrations of PP_i strongly inhibited DHBV DNA synthesis. To examine more precisely the physiological implications of this inhibition, polymerization was assayed under physiological concentrations of 1 μM dNTPs (29) and 125 μM PP_i (30). Under these conditions, the presence of 125 μM PP_i resulted in 80–90% inhibition of DNA synthesis (Table 1). To determine whether dNTP removal was the basis of this inhibition, labeled PP_i was used to measure dNTP removal, whereas labeled dCTP was used to measure dNTP incorporation. Intriguingly, approximately equal amounts of dNTP removal occurred as stable dNTP incorporation. Therefore, as predicted by the kinetic analyses, dNTP removal may not be only a minor side reaction of the DHBV-RT, but may be as catalytically prevalent under physiological conditions as polymerization.

Table 1. Competition between dNTP incorporation and removal during DHBV replication

	1 μM dNTPs	1 μM dNTPs + 125 μM PPi
dNTP incorporation	480 fmol	75 fmol
dNTP exchange	—	94 fmol
Polymerase activity	100%	15.7%

However, the exact basis of PP_i inhibition may be complex. The mixed mechanism of inhibition suggests that PP_i binds to both the free RT and the RT-dNTP complex. As such, the actual PP_i binding site is likely distinct from the dNTP binding site. The strength of PP_i binding was greater with the free RT than the RT-dNTP complex. However, both pyrophosphorolysis, which is predicted to proceed from the free RT state, and PP_i exchange, which is predicted to proceed from the RT-dNTP state (28), occurred with similar and high affinity PP_i binding constants. Therefore, PP_i may bind to a second, lower affinity site on the RT-dNTP complex to further increase the level of inhibition. Consistent with this model, increasing PP_i resulted in further inhibition, even when PP_i exchange was already maximal. Furthermore, the dramatic inhibition of DNA synthesis by PP_i may be greater than the reciprocal stimulation of PP_i exchange (Table 1).

Discussion

The effects of PP_i on DHBV DNA replication within core particles were examined to characterize further the catalytic properties of the DHBV-RT polymerase activity. In agreement with previous studies of DNA polymerase function (10, 19, 22, 23, 27, 28), PP_i was found to exert three effects on DHBV-RT catalysis: stimulation of pyrophosphorolysis, stimulation of PP_i exchange, and inhibition of DNA synthesis. Because these were activities previously undocumented for any HBV-RT, an enzymological characterization was undertaken.

Of particular significance is the consistently low PP_i binding constant of 7.5–19 μM independently derived from the analyses of all three PP_i effects. This is the lowest PP_i binding constant reported for any polymerase by over an order of magnitude (10, 19, 21, 22). Although pyrophosphorolysis is not thought to be physiologically relevant for any polymerase, estimation of cytoplasmic PP_i concentrations at 150 μM (30) strongly suggests that the DHBV-RT would efficiently catalyze pyrophosphorolysis under physiological concentrations of PP_i. Furthermore, estimation of the rate constants for these activities suggests that they would efficiently compete with polymerization *in vivo*. These activities may therefore have a number of biologically and, if applicable to the human HBV, clinically significant implications.

Both DHBV-RT pyrophosphorolysis and PP_i exchange are able to remove newly incorporated nucleotides under physiological conditions. These activities may allow the DHBV-RT to reverse the polymerization reaction and thus serve a primer-unblocking function during genome replication. The strategy of current antiviral drugs in inhibiting HBV replication relies on their ability to be incorporated into the viral DNA irreversibly as chain terminators. Because RTs lack a $3' \rightarrow 5'$ exonuclease activity that could remove incorporated drugs, it is assumed that an effective inhibitor is one that ultimately achieves incorporation. However, the demonstration of an efficient HBV pyrophosphorolytic activity challenges this dogma: an effective chain terminator is one that is both incorporated and resistant to pyrophosphorolysis. This resistance to removal is thus a novel parameter of anti-HBV drug efficacy that may be important in designing antiviral drugs. Consistent with this hypothesis, ineffective inhibitors such as ddCMP were readily removed by pyrophosphorolysis whereas 3TC-MP, the only effective HBV inhibitor currently licensed for therapeutic use, was found to be resistant to pyrophosphorolytic removal. Because the cellular metabolism and *in vitro* incorporation kinetics of both 3TC and ddC are efficient (A. Severini, unpublished data), these observations suggest that ddC removal by pyrophosphorolysis may be the factor that undermines its potency *in vivo*. It will be of

interest to test whether other effective anti-HBV drugs currently under development are resistant to pyrophosphorolytic removal. Intriguingly, most of these drugs possess unnatural sugar ring modifications, and nucleotides with modified sugars are generally poor substrates for pyrophosphorolysis (31). Conversely, although pyrophosphorolytic removal of ddGTP from DNA was found to be efficient, dGTP analogs are potent anti-HBV drugs that inhibit protein priming (32). Their efficacy may therefore be derived from being less efficiently removed from a protein linkage. In addition to designing pyrophosphorolysis-resistant nucleotide analogs, combination drug therapy with PP_i analogs to inhibit HBV primer unblocking may provide another approach to increasing the activity of previously ineffective drugs.

HBV-RT mutations that enhance pyrophosphorolysis could also result in acquired antiviral drug resistance to chain-terminating nucleotide analogs. A serious problem in treating HBV is the development of resistance to 3TC, the only agent currently licensed for therapeutic use. Although the role of increased primer unblocking by pyrophosphorolysis in conferring AZT resistance has been established for HIV-RT (25, 33), resistance to 3TC likely involves a different mechanism. Structural analysis has revealed that mutations in the HIV-RT active site cause a steric clash with the 3TC-TP and thus occlude its incorporation (34). Furthermore, the recent characterization of 3TC-resistant HIV-RT has also revealed a less efficient pyrophosphorolytic activity on 3TC-terminated DNA (35), suggesting that 3TC resistance does not occur even in part by primer unblocking. It is likely that the same mechanism is responsible for conferring HBV resistance to 3TC because the same amino acids are involved. Therefore, although drug removal by pyrophosphorolysis is unlikely to be involved in HBV resistance to 3TC, it should be noted that drug resistance to other antivirals could develop because of enhanced primer unblocking by the wild-type and 3TC-resistant HBV-RT.

In addition to its potential importance to antiviral therapy, HBV pyrophosphorolysis may also have implications on RT function. Although precise fidelity measurements have not been possible with any HBV-RT for technical reasons, estimation of the viral mutation rate suggests that the HBV genome is replicated with a 10- to 100-fold higher level of accuracy than expected for reverse transcription (36). Although this apparent low mutagenesis may be due to a number of factors, the reversibility of nucleotide incorporation could serve as a fidelity-enhancing mechanism if a mismatched primer terminus is more likely to be a substrate for pyrophosphorolysis than for polymerization. Pyrophosphorolysis has been discounted as a fidelity mechanism for proofreading polymerases: however, less attention has been devoted to the analysis of RTs, which react differently to PP_i (37). Because the HBV-RT may represent the first known RT to catalyze pyrophosphorolysis under physiological conditions, the effect of PP_i on HBV-RT fidelity warrants further investigation.

Although the 3.2-kb HBV genome is the smallest known DNA virus genome, HBV DNA replication is incomplete when the virus is released from the cell. Estimation of the rate of DNA synthesis (38) implies that the HBV-RT is among the slowest polymerases known. Although the mechanistic basis for this slow rate is not yet clear, the strong inhibitory effect of PP_i may partly explain these observations: physiological concentrations of PP_i were found to reduce the rate of DHBV-RT DNA synthesis by 80–90%. However, further analysis is required to determine whether PP_i inhibition is the cause of the slow rate of HBV genome replication, and what other effects PP_i may have on HBV DNA synthesis.

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