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An in vitro system for the enzymological analysis of avian hepatitis B virus replication and inhibition in core particles

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Abstract

A detailed analysis of the hepatitis B virus (HBV) replication reaction is important both in understanding viral biology and in developing effective antiviral drugs. This can best be achieved by studying the viral reverse transcriptase (RT) in its natural context, encapsidated within viral core particles in a multiprotein complex, rather than as an isolated enzyme. In order to facilitate a precise enzymological analysis of the avian HBV-RT reaction and its inhibition within replicating cores, a scheme for the purification and analysis of intracellular core particles derived from infected liver tissue has been devised, optimized and evaluated. The purification scheme itself is simple and rapid, and results in preparations with a 25-fold increase in endogenous polymerase activity that persists for over 5 h under assay conditions. In order to assess the suitability of these preparations for mechanistic studies, a thorough evaluation of purity was undertaken, revealing predominantly pure viral protein and nucleic acid, free of contaminating cellular polymerases and phosphatase activities that potently degrade nucleotides and antiviral drugs. Parameters governing optimal polymerase activity have been determined, and an assay for DHBV-RT activity has been developed which offers the highest purity and specific polymerase activity currently available to study hepadnaviral replication and inhibition. Published by Elsevier Science B.V.

Keywords: Duck hepatitis B virus; Replication; Polymerase; Reverse transcriptase; Antiviral inhibition; Replicating cores

1. Introduction

Hepatitis B virus (HBV) is a small, enveloped, hepatotrophic pathogen which poses a significant health problem world-wide (Mahoney, 1999). Although an effective vaccine is available, it is of no benefit to the large number of infected individuals, and thus antiviral therapy remains a priority. Since all effective antiviral drugs target the viral reverse transcriptase (RT), understanding the HBV-RT catalytic mechanism and the basis for its inhibition at the molecular level promises to facilitate drug design and to further our knowledge of this unique viral replication scheme. However, the general difficulty in obtaining enzymatically active

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RT from human or indeed hepadnaviral animal model systems in large quantities and in sufficient purity has seriously hampered the study of hepadnaviral RT function.

Recent advances have succeeded in the expression of enzymatically active hepadnaviral RT in numerous heterologous systems, each with their own advantages and limitations (as reviewed in Hu and Seeger, 1996). These successes have, in turn, led to the elucidation of numerous characteristics of the viral replication mechanism, most notably initiation of viral DNA synthesis (as reviewed in Scaglioni et al., 1996). However, these expression systems remain hindered by a number of shortcomings which prevent a precise enzymological analysis of hepadnaviral RT catalytic function. The expressed polymerase activity is generally weak and transient, and the preparations are also impure, with the viral polymerase constituting only a minor proportion of the total protein. More importantly, these preparations suffer from the inherent limitation of isolating one component of the multiprotein hepadnaviral replication complex that operates under physiological conditions. Upon translation of the viral polymerase, the pregenomic RNA template, viral polymerase, and potentially certain cellular factors are encapsidated within a proteinaceous shell composed of the viral core protein (Hirsch et al., 1990; Hu et al., 1997). Since all genome replication occurs within these replicating cores in the cytoplasm of an infected hepatocyte, it is of particular importance to study the hepadnaviral RT in the context of the replicating core. Indeed, the activity of the hepadnaviral RT is significantly affected by the viral core protein (Seifer et al., 1998) and encapsidated cellular factors (Hu et al., 1997). Furthermore, the hepadnaviral RT responsible for genome replication could be a post-translationally modified form which may behave unlike the form produced in heterologous systems (Bavand and Laub, 1988; Oberhaus and Newbold, 1993). These observations underscore the importance of studying the entire replication complex produced during the course of a natural viral infection.

The avian hepadnavirus duck hepatitis B virus (DHBV) infects Pekin ducks and has been developed as a tractable model system for studying various aspects of HBV biology. Intracellular replicating cores can be isolated from the liver of infected animals and the resulting preparations possess an endogenous polymerase activity resulting from the action of the encapsidated hepadnaviral RT on the viral template (Summers and Mason, 1982). However, the preparations typically require lengthy purification schemes and ultimately achieve only a weak polymerase activity of low to moderate purity which is not suitable for enzymological analysis. In order to study the DHBV replication mechanism within replicating cores more precisely, a biochemical purification scheme that overcomes most inherent limitations of conventional core preparations was first devised and optimized. A thorough evaluation of core preparation activity and purity was then undertaken in order to determine their suitability for enzymological analysis. Compared to conventional core preparations, the improved strategy yielded replicating cores with a 25-fold increase in the recovered viral polymerase activity which was sustained for over 5 h under standard assay conditions. Electrophoretic analysis revealed essentially pure viral protein and nucleic acid. Furthermore, the replicating core preparations were found to be free of contaminating cellular polymerase and phosphatase activities, which interfere with enzymological studies of DHBV-RT function. These highly pure core preparations were used to establish a steady-state system for the enzymological analysis of the DHBV-RT catalytic mechanism. As well as facilitating a more precise and consistent examination of the viral replication reaction, these improvements in activity and purity have permitted the characterization of novel activities associated with DHBV core particles which may serve as future targets for antiviral therapy (S. Urban, manuscript in preparation).

2. Materials and methods

2.1. Isolation of DHBV core particles

Replicating cores were typically purified from fresh liver tissue derived from 2–4 week old Pekin ducks (usually 18–36 g of liver tissue). The ani-

mals were either congenitally or experimentally infected with various DHBV genotypes, serum viremia was screened by dot blot hybridization using standard protocols (Suzuki et al., 1988), and robustly viremic animals were chosen for subsequent core purification.

In order to preserve the polymerase activity, all buffers and instruments were prechilled, and samples were maintained on ice or at 4°C during the course of the purification. Liver tissue resected from freshly euthanized animals was minced and homogenized in NEB extraction buffer (20 mM Tris–HCl pH 7.5, 50 mM NaCl, 7 mM $MgSO_4$, 0.25 M sucrose, 0.1% β-mercaptoethanol, 0.5% NP40 — BDH Laboratory Supplies) with 10 strokes in a Potter-Elvehjem homogenizer (2 ml NEB per g liver tissue). The homogenate was clarified by centrifugation at 10 000 rev./min in a Beckman JA-20 rotor for 30 min, and layered 7 ml per tube onto a discontinuous sucrose gradient (composed of 11-ml steps of 15, 22.5 and 30% sucrose prepared in NEB and layered into Beckman Ultra-Clear SW28 ultracentrifuge tubes). Following ultracentrifugation at 23 000 rev./min for 7 h in a SW28 Beckman rotor, the 22.5 and 30% sucrose steps excluding pelleted material were collected by side puncture with a syringe. Under these ultracentrifugation conditions, the majority of cellular proteins and lipid vesicles generally did not penetrate past the top sucrose step, while very large structures such as organelles were pelleted. Viral core particles and large cellular complexes such as ribosomes were distributed in the lower two sucrose gradient steps. Although the 22.5 and 30% sucrose layers are typically discolored, hazy, and possess two flocculent bands, inclusion of NP40 in the extraction buffer resulted in entirely clear steps due to the increase in purity. The harvested cores contained within the sucrose layers were pelleted at 28 000 rev./min in a SW28 rotor for 12 h, and resuspended in 1 ml of chromatography buffer (CB-150 — 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% NP40) supplemented with 20 mM $MgCl_2$. Unencapsidated nucleic acids were fragmented by the addition of 10 μg of DNase I and incubation at 37°C for 15 min, followed by the addition of 10 μg of RNase A and EDTA to a concentration of 20 mM, and incubation at 37°C for a further 15 min. This enzymatic treatment served to partially remove contaminating ribosomal proteins prior to column chromatography since ribosomal proteins aggregate and precipitate under these conditions (Summers and Mason, 1982). The resulting ribosomal protein precipitate was removed by microcentrifugation at 14 000 rev./min for 5 min prior to column chromatography.

2.2. Column chromatography of DHBV replicating cores

A 16 mm × 180 mm Q-Sepharose High Performance anion exchange column was poured and packed according to manufacturer's instructions (Pharmacia Biotech, 1991). However, the Q Sepharose resin required pretreatment with 0.5 M NaOH in order to promote strong binding of the viral core particles (see Section 3). The exact duration of the pretreatment that proved to be effective varied significantly and had to be determined empirically (for example, see Fig. 2A), but always resulted in core elution at a salt concentration equal to or greater than 500 mM. It should be noted that the salt concentrations used for the washing and elution stages were adjusted according to the strength of binding achieved with the column pretreatment (i.e. for weaker binding, a wash with CB-300 and an elution with CB-750 were used). Once the desired level of pretreatment was achieved, the column was reused for subsequent core purifications and yielded consistent results.

The column was equilibrated with 5–10 column volumes of CB-150, and was then loaded with the enriched core particle sample. A low flow rate of 0.25 ml/min (approximately 7.5 cm/h) was used throughout the column chromatography steps due to the large size of the viral particles. The column containing bound cores was washed with approximately two column volumes of CB-500 (CB + 500 mM NaCl), and the cores were eluted with CB-1000 (CB + 1 M NaCl). The majority of contaminating proteins and lipids were removed in these binding and washing steps, while essentially pure viral core particles were eluted with CB-1000. One

hundred 0.5-ml fractions were collected, and the presence of the core particles was detected using both the endogenous polymerase assay (see below) and dot blot hybridization with a DHBV specific probe (Suzuki et al., 1988). The core particle peak was rapidly frozen in 20-μl aliquots in a dry ice ethanol bath, and stored at −80°C until use. In order to preserve the viral polymerase activity, it was important to freeze the peak fractions as soon as possible (generally no later than 48 h after commencement of the purification scheme). The enzymatic activity of the purified core particles was stable for at least 1 year and likely indefinitely under these conditions.

2.3. Endogenous polymerase assay

Endogenous polymerase activity of the core preparations was assayed under optimized conditions in polymerase buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% NP40, 50 μM each of dATP, dGTP, dTTP, and 2 μCi (approximately 0.012 μM) [α^{32}P]dCTP (NEN Dupont, 3000 Ci/mmol). Although the limiting quantity of the dCTP tracer resulted in sub-optimal polymerase activity, it was sufficient for all diagnostic polymerase assays and was used throughout the core purification scheme. 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 which also facilitated achieving the optimal 150 mM salt concentration by diluting the high salt eluted core preparation substantially. Following 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 (TCA) and incubating on ice for 10 min to allow complete nucleic acid precipitation. The resulting TCA suspensions were filtered through Millipore GF/C fiberglass filters, washed three times with 10% TCA and once with 95% ethanol, dried, and counted in a Beckman scintillation counter. Alternatively, in order to examine the reaction products electrophoretically, the reactions were terminated by boiling, and protein was digested with 400 μg/ml proteinase K at 55°C for 3–16 h. The labeled nucleic acids were extracted with phenol, ethanol precipitated, and detected by autoradiography following agarose gel electrophoresis under standard conditions (Sambrook et al., 1989).

2.4. Analysis of substrate and inhibitor stability

The peak polymerase fractions of each purification run were routinely assessed for phosphatase contamination. 1 μl of each fraction was incubated in polymerase buffer lacking unlabeled dNTPs with 2 μCi [α^{32}P]dGTP (NEN Dupont, 3000 Ci/mmol), [α^{32}P]3TC-TP (synthesized by J. Wilson), or ^{32}PPi (NEN Dupont, 5 Ci/mmol) in a final volume of 10 μl for 60 min at 37°C. Although all dNTPs were used as substrates for the phosphatase assay, dGTP was routinely used to assess phosphatase contamination of core preparations since dGTP was found to be most susceptible to dephosphorylation. 1 μl of each reaction was spotted onto a polyethyleneimine (PEI) cellulose thin layer chromatography (TLC) plate that had been prerun in 0.15 M KH_2PO_4 + 15% ethanol, thoroughly rinsed in water, and dried (Reha-Krantz and Nonay, 1993). The TLC plates were developed in 0.15 M K_2HPO_4 + 15% ethanol, dried, and exposed to a Fuji phosphorimager plate for detection and quantification.

2.5. Protein analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western analysis were performed using standard protocols (Sambrook et al., 1989). Core protein was detected with rabbit polyclonal serum raised against the recombinant DHBV core antigen. Polymerase activity gel analysis was performed using a method described previously (Oberhaus and Newbold, 1993).

3. Results

3.1. DHBV core purification scheme

In developing a strategy for the enzymological analysis of DHBV-RT function and its inhibition, it was important to ensure that the system would

ultimately reflect the in vivo characteristics of the viral polymerase. In this respect, it was essential to study the DHBV-RT in the multiprotein replicating core complex assembled during the course of a natural Pekin duck infection as opposed to recombinant enzyme produced in heterologous systems. However, conventional core preparations suffer from two main limitations which render them unsuitable for precise enzymological analysis; low polymerase activity and low purity. In an attempt to overcome these shortcomings, an optimized core purification scheme was first developed. Since the viral polymerase activity was found to be unstable at 4°C, it was important to reduce the overall manipulation time by minimizing the number of steps used in the purification strategy. The final procedure required only 2 days to perform and was comprised of three main stages; homogenization, fractionation, and column chromatography. The homogenization and fractionation methods were modified from a DHBV isolation protocol described previously (Summers and Mason, 1982). Infected liver tissue was homogenized in extraction buffer containing NP40, and the tissue and cellular debris were removed by centrifugation to yield a clarified lysate. The lysate was then fractionated according to size using sucrose gradient ultracentrifugation. Following concentration of the isolated core particles, unencapsidated nucleic acids were enzymatically fragmented, and ribosomal protein was precipitated in the presence of EDTA. The enriched core particles were subjected to Q Sepharose anion exchange chromatography, rapidly frozen, and stored at −80°C. The enzymatic activity of the purified core particles was stable for at least 1 year and likely indefinitely under these conditions.

Typical purification runs consistently yielded replicating cores possessing a 25-fold higher polymerase activity than achieved by other methods (for example, see Fig. 1A and Fig. 6), and in sufficient quantities for 400 endogenous polymerase reactions. While core particle yields correlated precisely with the starting animal viremia, the standardized polymerase activity of replicating cores purified with the new method always resulted in the incorporation of approximately 25 000 cpm in the endogenous polymerase assay per ng of core associated viral DNA. Overall, yields of approximately 2 μg of viral nucleic acid and 250 μg of viral core protein were achieved, as assessed by dot blot hybridization and SDS-PAGE, respectively. Based on a typical elution peak of 2 ml, the average particle density was 10^{13}–10^{14} replicating cores per ml.

Although the purification scheme is relatively quick and simple, its success is largely derived from the optimization of the extraction buffer components and the implementation of high resolution ion exchange chromatography. Due to their importance, the optimization of these two parameters will be first described in greater detail, followed by a thorough evaluation of the suitability of these core preparations for enzymological analysis.

3.2. Role of the extraction buffer

In addition to optimizing the purification strategy itself, individual components of the extraction buffer were also tested for their effect on core particle activity and purity in order to optimize the isolation of core particles. Since DHBV core particles are exceptionally stable (Radziwill et al., 1988), various detergents were also included in the test. Although most components, including other detergents, were found to have only minor effects on core particle purity and activity, inclusion of the nonionic detergent NP40 in the extraction buffer during the course of the purification consistently resulted in a 25-fold increase in the recovered endogenous polymerase activity (Fig. 1A). Subsequent analyses revealed that NP40 increased purity by greater than 10-fold (Fig. 1B), while only increasing the yield of core particles by two-fold (data not shown). As such, it appears that NP40 may also be providing an overall stabilizing effect on the core particles during the course of the purification as it is not clear whether the increased purity and yield conferred by NP40 can fully account for its dramatic effect on the recovered polymerase activity. In any case, since inclusion of NP40 in the extraction buffer resulted in a

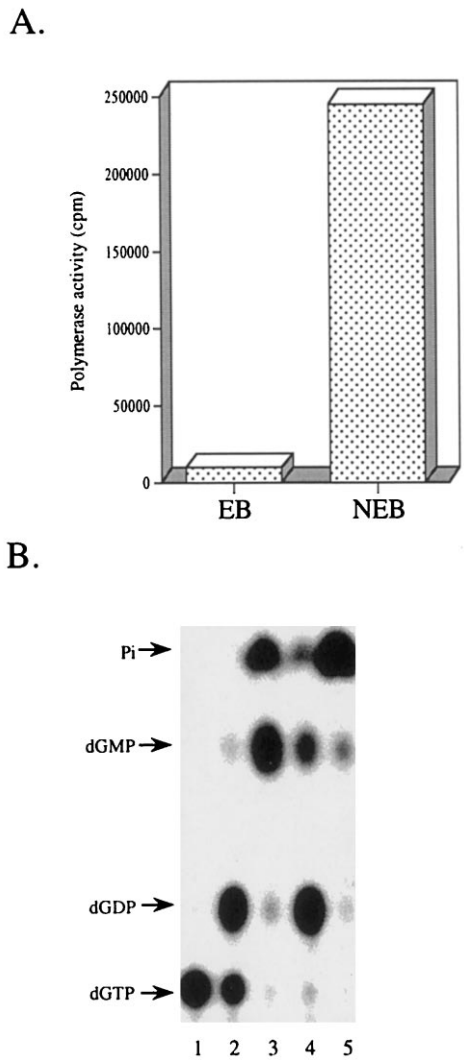

Fig. 1. Effect of NP40 on the purification of DHBV core particles. (A) Replicating cores were isolated from DHBV-infected liver tissue in parallel using extraction buffer lacking (EB) and containing 0.5% NP40 (NEB). Note that the protocol of Summers and Mason was used without the addition of column chromatography. Inclusion of NP40 in the extraction buffer during the course of the purification increased the final endogenous polymerase activity 25-fold. (B) Thin layer chromatogram of [α^{32}P]dGTP breakdown products. [α^{32}P]dGTP was incubated in the presence of buffer for 30 min (1), NEB purified cores for 5 min (2), EB purified cores for 5 min (3), NEB purified cores for 30 min (4), and EB purified cores for 30 min (5). Over 10-fold less phosphatase activity was present when NP40 was included in the extraction buffer during the course of the purification. However, even when NP40 was used, essentially complete dGTP hydrolysis occurred after 30 min.

25-fold increase in the recovered polymerase activity, the contents of the extraction buffer were the most important parameter of homogenization and fractionation efficiency.

3.3. Anion exchange chromatography of replicating cores

Although optimized homogenization and fractionation conditions dramatically increased core particle activity, the overall purity of the preparations remained unacceptably low (see Fig. 1B and Fig. 3, precolumn data). Further sucrose gradient ultracentrifugation and gel filtration chromatography did not increase the purity of the preparations adequately. Since differences in molecule size, shape and density were the basis of separation used in the fractionation stage of the purification scheme, it was clear that addition of further techniques that exploit similar principles could only yield moderate improvements in purity.

In contrast to partition techniques such as gel filtration chromatography, adsorption chromatography techniques possess the potential of yielding substantially higher resolution by exploiting other physical attributes of molecules. To this end, anion exchange chromatography was applied to and optimized for DHBV core particle purification. However, application of anion exchange chromatography was initially difficult as the core particles differed from conventional proteins in several ways with respect to their chromatographic behavior. First, although the binding capacity of the Q Sepharose resin is very high, core particles were completely excluded from the interior of the resin beads. As such, the amount of resin required for a purification run had to be determined empirically and was unexpectedly high. Second, under standard conditions the Q Sepharose resin bound only a minor fraction of the applied core particles and these coeluted with a large fraction of cellular proteins at low salt concentrations (Fig. 2A). The affinity of the resin for core particles was markedly increased by a pretreatment with NaOH. Under these conditions, the Q Sepharose column bound cores very strongly, and a high salt concentration was required for their elution. Since the majority of

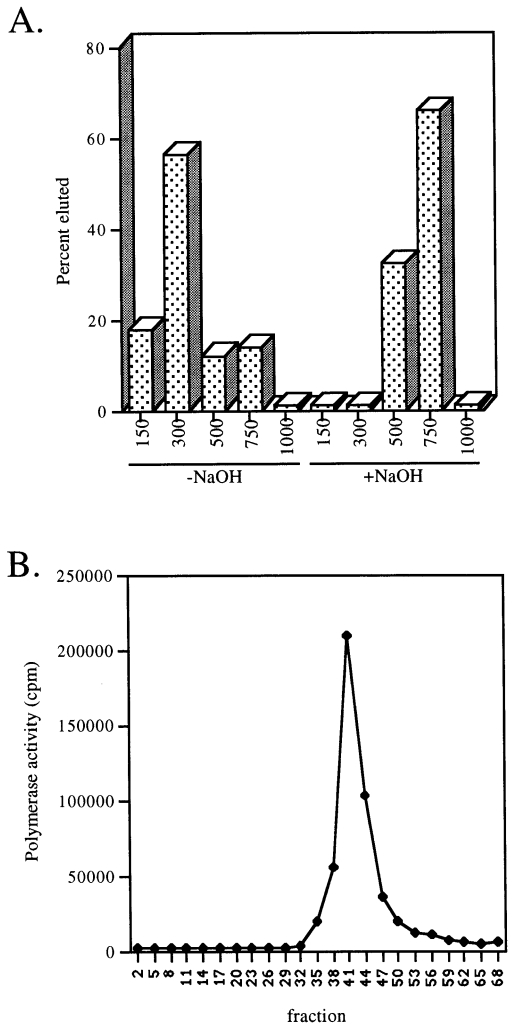

Fig. 2. Anion exchange chromatography of DHBV replicating cores. (A) Core particle retention by the Q Sepharose resin was dependent on a pretreatment with NaOH (data from a 9-h 0.5 N NaOH pretreatment is shown). The majority of cores either did not bind to the resin or eluted at low (300 mM) salt concentrations from an untreated column. However, pretreatment resulted in the majority of cores requiring a high salt concentration (500–750 mM) for elution, yielding higher selectivity and purity. Core elution was assessed by dot blot hybridization and quantification by comparison to known viral DNA standards. (B) A typical elution profile of DHBV polymerase activity from a Q Sepharose column eluted with CB-1000 under optimized chromatography conditions (see text). Slow flow rates resulted in elution peaks that were sharp and symmetric with a small amount of 'tailing' present. One hundred 0.5-ml fractions were collected and every third fraction was tested for endogenous polymerase activity.

cellular proteins were removed using low and moderate salt concentrations, essentially pure core particles eluted at the high salt concentration. Therefore, NaOH pretreatment resulted in a great differential increase in core particle binding, which allowed very high selectivity and very efficient purification. Flow rates and elution conditions also required modification for core particle purification. The ion exchange column required very slow flow rates as higher flow rates resulted in incomplete binding to the resin and extremely broad elution peaks. Typically, binding, washing, and elution were performed at a flow rate of 7.5 cm/h, which is approximately 12-fold slower than suggested for conventional protein chromatography (Pharmacia Biotech, 1991). Finally, due to the inherent heterogeneity of the replicating cores, the core particles required broad salt steps for elution; the use of a linear salt gradient resulted in a very broad peak while smaller salt steps resulted in incomplete elution at various salt concentrations. Ultimately, conditions were established that yielded efficient chromatography results (Fig. 2B).

3.4. Evaluation of core particle purity

A serious limitation of conventional core purification schemes is the inability to remove contaminants which interfere with enzymological analyses of viral replication and inhibition. Having established an efficient method for the purification of core particles that possessed very high polymerase activity, a thorough evaluation of core preparation purity was undertaken in order to assess whether these preparations were amenable to enzymological analysis.

Cellular phosphatases typically contaminate core preparations and potently degrade nucleotides, antiviral drugs, and pyrophosphate with differing efficiencies, thus compromising the stability of substrates and inhibitors used to study viral replication (see Fig. 3A precolumn data and Severini et al., 1995). Such differential nucleotide instability seriously impeded precise mechanistic studies of the replication reaction and its inhibition. In developing different purification strategies, it was clear that although both sucrose gradient ultracentrifugation and gel filtration

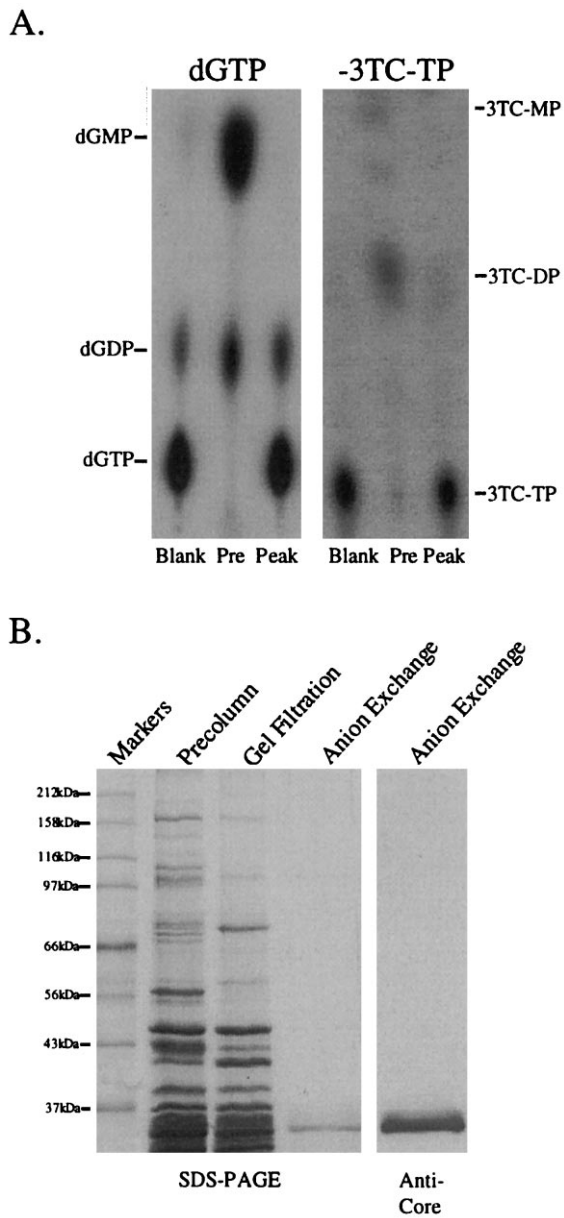

Fig. 3. Purity analysis of DHBV replicating cores following anion exchange chromatography. (A) Incubation of the DHBV precolumn and peak fractions eluting from the Q Sepharose column with [α^{32}P]dGTP and [α^{32}P](−)3TC-TP for 60 min resulted in no detectable dephosphorylation above background hydrolysis. (B) SDS-PAGE analysis of DHBV cores prior to and following gel filtration and anion exchange chromatography. Anion exchange chromatography resulted in essentially pure viral protein. Subsequent analysis with anti-core antisera verified the identity of the prominent band as the viral core protein. Under more sensitive staining conditions bands corresponding to the expected sizes of the DHBV-RT were observed (data not shown).

chromatography marginally improved purity, only anion exchange chromatography eliminated phosphatase contamination (Fig. 3A). The resulting core preparations were found to have only spontaneous levels of hydrolysis with respect to all substrates and inhibitors tested. Therefore, these core preparations possessed the very high substrate and inhibitor stability required for precise enzymological studies.

In addition to contaminating phosphatase activities, electrophoretic analysis of previous core preparations and preparations purified by gel filtration chromatography revealed that viral proteins constituted only a minor proportion of the total protein present (Fig. 3B). These contaminating proteins would be expected to affect replication dynamics as they were subsequently found to sequester nucleotides and pyrophosphate by ligand blot analysis (S. Urban, unpublished observations). In contrast, implementation of anion exchange chromatography resulted in essentially pure viral protein; the predominant band present is the viral core protein as determined by both estimated size and reactivity with anti-core antisera (Fig. 3B). Since electrophoretic analysis of nucleic acid also revealed essentially pure viral nucleic acid in the core preparation (Fig. 4A), the resulting core particles were judged to be pure.

3.5. Evaluation of polymerase purity

Before a mechanistic analysis of the viral polymerase reaction could be undertaken using these high activity and purity preparations, it was first necessary to evaluate whether the detected polymerase activity was entirely attributable to the DHBV-RT. Even a trace contaminating cellular polymerase activity would be expected to produce misleading results with respect to replication and inhibition analysis. Several initial observations suggested that the detected polymerase activity was predominantly due to the action of DHBV-RT (data not shown). First, it was clear that the

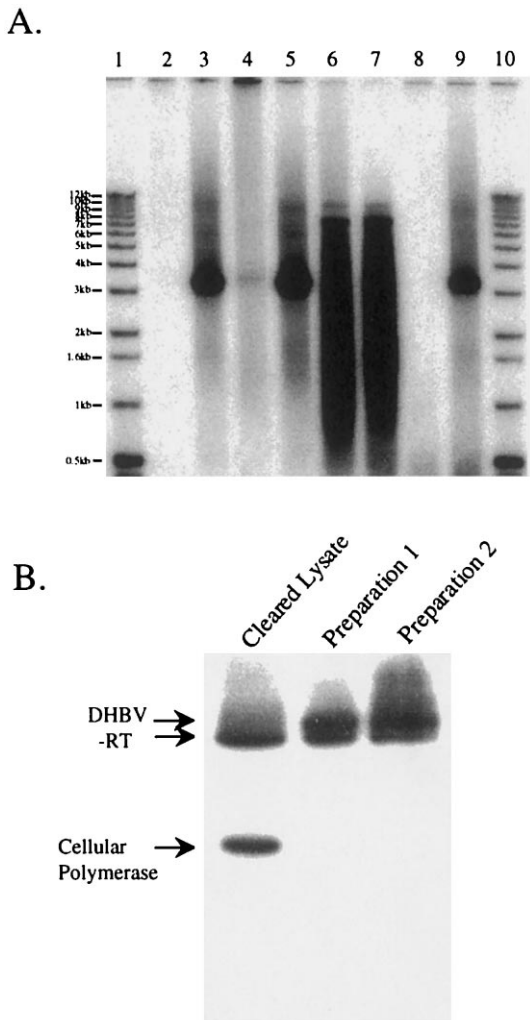

Fig. 4. Polymerase activity analysis of purified replicating core preparations. (A) Products of the endogenous polymerase assay were purified and examined electrophoretically. Lanes 1, 10, end-labelled 1-kb ladder (GibcoBRL); lanes 2, 3, core preparation; lanes 4, 5, core preparation + primed M13mp18; lanes 6, 7, core preparation + primed M13mp18 + 5 U Klenow; lanes 8, 9, core preparation + 5 U Klenow. DNA samples corresponding to lanes 2, 4, 6, and 8 were not subjected to proteinase K digestion in order to allow the specific removal of protein-linked viral DNA by phenol extraction. (B) Activity gel analysis of core preparations. Core preparations were subjected to SDS-PAGE through gels impregnated with 1 mg/ml activated DNA. Following renaturation, polymerase activity was detected by the incorporation of radiolabeled dNTPs into the DNA present in the gel matrix. The only detectable polymerase activity in the purified core preparations corresponded to the expected two bands of DHBV-RT (Oberhaus and Newbold, 1993).

activity represented a polymerase activity and was not due to background radionuclide binding or utilization since incubation with only radiolabeled dCTP lowered TCA precipitable radioactivity to essentially background levels. In contrast, a reaction containing the radiolabeled dCTP and excess unlabeled dATP, dGTP, and dTTP resulted in a 100-fold increase in precipitable radioactivity. Second, the polymerase activity eluting from the column precisely correlated with the viral genome peak as assessed by nucleic acid dot blot hybridization. Third, the final polymerase activity and yield correlated with the serum viremia of the infected animals, with uninfected animals yielding no detectable polymerase activity. Finally, the polymerase activity was partially resistant to actinomycin D, an inhibitor of DNA templated synthesis (Reich and Goldberg, 1964), and stimulated by nonionic detergents (Fig. 5A) (Wu and Cetta, 1975), both characteristics of viral reverse transcriptase.

In order to investigate whether a trace cellular polymerase activity could be contaminating the core preparations, the nature of the polymerase reaction products was examined electrophoretically. Incubation of the core preparation with radiolabeled dCTP and the remaining three unlabeled nucleotides resulted in the labeling of nucleic acid with an electrophoretic pattern consistent with DHBV replication intermediates (Fig. 4A). This pattern was not observed when protease digestion prior to the purification of total nucleic acid was omitted. Under these conditions, the DHBV DNA was specifically removed in the organic extraction due to the unique covalent linkage between the viral DNA and the terminal protein used for priming first strand synthesis (Gerlich and Robinson, 1980; Wang and Seeger, 1992). Since any exogenous nucleic acid would be expected to remain in the aqueous phase of the organic extraction, the absence of radiolabeled DNA following the organic extraction indicated that no detectable exogenous polymerase activity was present under typical polymerase assay conditions. In order to test whether the absence of an exogenous polymerase activity was due to the absence of a contaminating polymerase, template DNA, or both, exogenous primed single-stranded

M13 DNA or recombinant Klenow fragment polymerase were added to the core preparation polymerase assay. Electrophoretic analysis showed that both contaminating polymerase and templates were absent in the core preparations as only DHBV DNA was labeled. Furthermore, an activity gel analysis of the core preparation which facilitates direct visualization of the active polymerase species showed the presence of only the expected DHBV-RT bands (Fig. 4B) (Oberhaus and Newbold, 1993). Therefore, the robust polymerase activity of the core preparations was entirely attributable to the DHBV-RT, and no contaminating polymerase activity or template DNA could be detected in these preparations.

3.6. Analysis of DHBV-RT activity

Parameters affecting DHBV-RT activity were examined as a final step towards developing a system for the enzymological analysis of the DHBV replication reaction. Interestingly, the optima of these high activity and purity core preparations differed significantly from those previously reported (Fourel et al., 1987). A 4- to 10-fold lower and physiological magnesium concentration was optimal for the viral polymerase activity (Fig. 5). Although the polymerase activity was not markedly affected by different salt concentrations and pH, optimal activity was observed under physiological conditions of 150 mM salt and a pH of 7.5. NP40 was found to be a potent stimulator

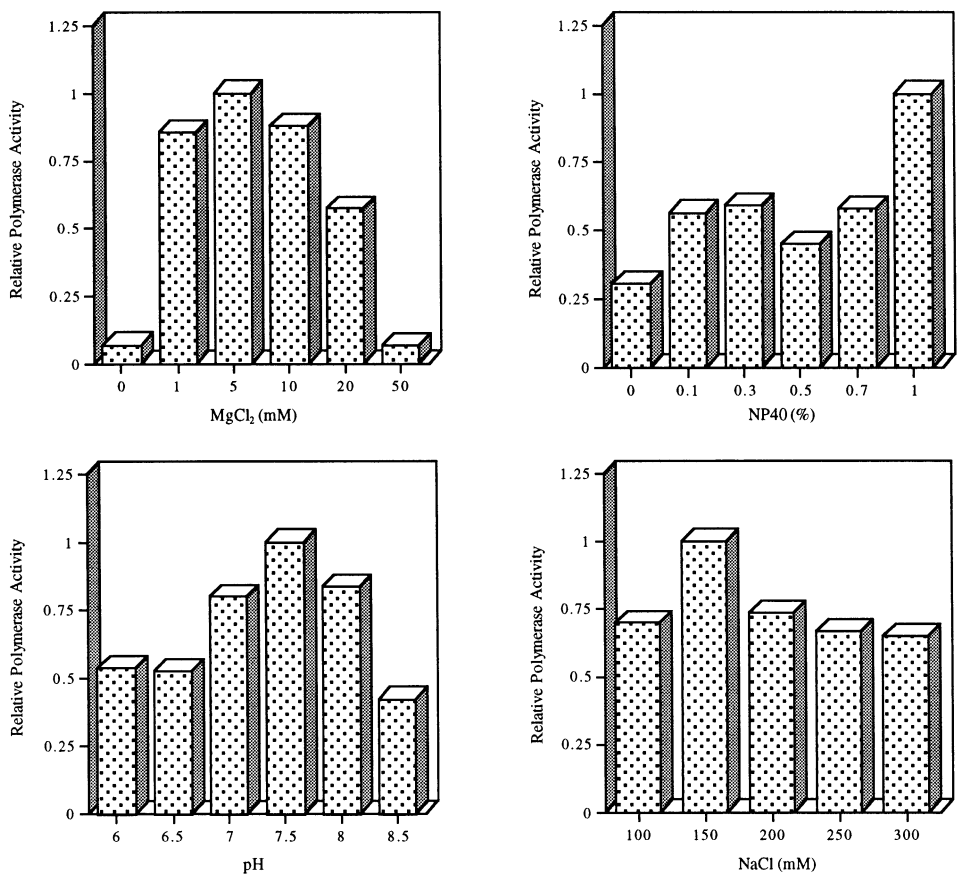

Fig. 5. Parameters governing optimal polymerase activity of purified DHBV core particles. Polymerase assay conditions were systematically tested to optimize the polymerase activity of core particles isolated using the new method.

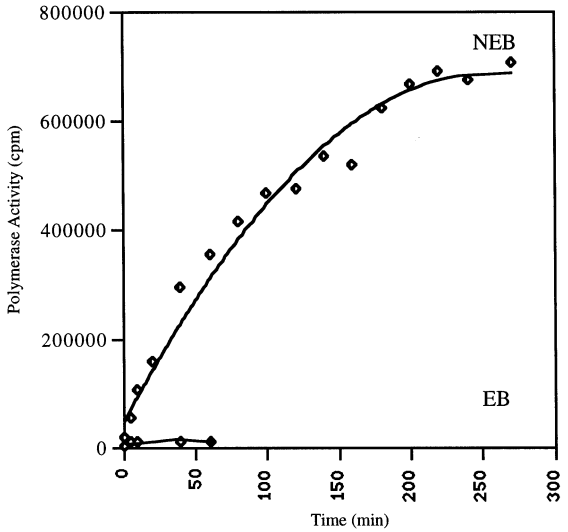

Fig. 6. Polymerase reaction progress curve of core preparations purified using the new protocol (NEB) versus those used previously (EB) (Severini et al., 1995). While the polymerase activity of conventional core preparations reached a plateau after 20–30 min, the polymerase activity of the new core preparations was approximately linear for 5 h.

of the DHBV-RT activity, which is consistent with its effect on other viral reverse transcriptases (Wu and Cetta, 1975). Ultimately, the DHBV-RT polymerase activity was assayed under optimal conditions in buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, and 0.5% NP40.

A reaction progress curve was also determined since a sustained and linear polymerase activity is also a prerequisite for establishing an assay for the steady-state analysis of DHBV-RT function and its inhibition within replicating cores. In contrast to the polymerase activity of conventional core preparations which reached a plateau after 20–30 min, the polymerase activity of the new core preparations was sustained in excess of 5 h (Fig. 6). As expected, the progress curve was initially approximately linear, with the small deviation likely being due to the time-dependent completion of genome synthesis by a heterogeneous mixture of replicating cores. Therefore, due to its high and approximately linear activity, the DHBV-RT within core particles is now amenable to substrate and inhibitor analysis using steady-state kinetics.

4. Discussion

An efficient scheme for the purification and analysis of replicating cores has been developed, optimized, and evaluated in order to facilitate a detailed enzymological study of DHBV-RT function and its inhibition by antiviral drugs. Studying DHBV-RT catalysis using this system offers significant advantages over other available methods. First, these core preparations possess a dramatically increased and stable polymerase activity compared to that of other replicating core preparations. Furthermore, the specific activity of the polymerase appears considerably higher than that of hepadnaviral polymerases expressed in heterologous systems (for example, see Fig 2 in Xiong et al., 1998), and may be the highest currently available for examining hepadnaviral replication. Such a robust polymerase activity is crucial for precise measurement of polymerase reaction parameters such as catalytic and substrate binding constants, and for the analysis of other activities catalyzed by the DHBV-RT. Also, the high polymerase activity should advance the study of drug-resistant mutants of DHBV-RT which appear to be much less active than the wildtype enzyme (Melegari et al., 1998). A robust polymerase activity is also important in quantifying antiviral inhibition as the difference between full polymerase activity and assay background, which is greater than 100-fold in these preparations. In addition to the strong polymerase activity, it has been established that no trace contaminating polymerase activity is present within these preparations to contribute to a background level of activity that may obscure polymerase analysis. Thus, a much more accurate and quantitative assessment of RT function and inhibition can be performed using this system.

Great care has also been taken to develop preparations which are essentially free of phosphatase contamination and thus provide high substrate and inhibitor stability. Other preparations have been found to be contaminated with phos-

phatase activities that potently degrade nucleotides, antiviral drugs, and pyrophosphate under standard polymerase assay conditions. The presence of these phosphatases occludes quantitative analysis of RT function and its inhibition. Furthermore, since these phosphatases degrade different nucleotides and antiviral drugs differentially, comparative studies using contaminated preparations can produce misleading disparities in substrate and inhibitor efficiencies resulting from susceptibility to degradation rather than inefficiency of RT interaction. Indeed, in studying the differential inhibitory efficacy of the plus versus the minus enantiomer of 3TC-TP, it was noticed that the plus enantiomer was much less stable than the minus enantiomer under polymerase assay conditions due to the phosphatase contamination of previous core preparations (Fig. 3A and S. Urban, unpublished observations). As such, the great disparity in DHBV-RT inhibition in vitro by the two enantiomers was partly due to the degradation of (+)3TC-TP by the phosphatases, and partly due to a weak interaction with the DHBV-RT. Since indirect effects may have plagued previous analyses of other antiviral agents, these core preparations should facilitate a more accurate analysis of antiviral drug action as they are pure of contaminating phosphatase activities.

Using this replicating core system for studying DHBV-RT function also has the important advantage of analyzing DHBV-RT function in its natural context. Since the DHBV-RT is contained within the macromolecular replicating core complex that has been assembled during the course of natural DHBV replication, the viral polymerase present in these preparations is likely to reflect the physiological characteristics of the enzyme. The polymerase has been folded and post-translationally modified under natural conditions, and is associated with both viral and cellular proteins in the replicating core which have recently been shown to significantly modulate the efficiency of the RT reaction (Hu et al., 1997; Seifer et al., 1998). Furthermore, the encapsidated RT may behave unlike the free polymerase due to the compartmentalization of the replication components within viral core particles. Indeed, RT dissociation from the viral nucleic acid may not be as relevant in replicating cores as in free solution. This could be of particular importance for inhibition analysis, as the DHBV-RT may be more likely to overcome inhibitor action when strongly associated with the primer-template DNA. As such, it may be essential to study the compartmentalized DHBV-RT when assessing DHBV-RT function and inhibition.

In addition to an enzymological analysis, elucidating the contents and structural architecture of the replicating cores is important in understanding hepadnaviral replication at the molecular level. This purification method, although originally aimed at preserving enzyme activity, results in pure core preparations under mild conditions that do not seem to denature the target molecules. With a typical yield of approximately 250 μg of viral core protein (Fig. 3B) and a resulting particle density of approximately 10^{13}–10^{14} particles per ml, these preparations may be well suited for structural analysis of native replicating core particle architecture by cryoelectron microscopy as was very successful for recombinant particles (Crowther et al., 1994).

Finally, the purification and analysis scheme may be applicable to the general study of hepadnaviral particles. Although the purification scheme was originally designed for the purification of intracellular core particles, it has also been successfully adopted for the purification of high activity core particles from virions present in serum (S. Urban, unpublished observations). Also, given the similarities between the various members of the hepadnaviral family, it seems possible that the purification and analysis scheme could be extended to the study of replicating cores from other systems with the same level of success. These advances should, in turn, provide a more detailed view of the enzymological similarities and differences of the various hepadnaviral RTs.

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