Expression of Hepatitis B Virus Polymerase in Ty1-his3AI Retroelement of Saccharomyces cerevisiae*

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Hepatitis B virus (HBV), although a DNA virus, replicates using reverse transcriptase encoded by the HBV polymerase (pol) gene. The biochemical dissection of HBV pol has been hampered by failure to liberate enzymatically active protein from nucleocapsids. Here, we have employed a yeast-based genetic approach to express the HBV reverse transcriptase. In this strategy, the reverse transcriptase of yeast retrotransposon Ty1 element is replaced with the HBV pol gene to produce the hybrid Ty1/HBV element. Additionally, the indicator gene his3AI is combined in an antisense orientation to the transcripts of the hybrid Ty1/HBVRT element. The splicing of his3AI, cDNA synthesis of the Ty1/HBVRT RNA and subsequent integration relies on the reverse transcriptase activity. The production of histidine prototrophs results from the successful reverse transcription of Ty1/HBVRT/ his3AI transcripts followed by either homologous recombination or integrase-mediated insertion and subsequent expression of HIS3 gene. Using this approach we successfully detected the reverse transcriptase activity of HBV in yeast strains defective in endogenous Ty1 expression. Consistent with the unique priming activity associated with HBV pol, the minus strand DNA synthesis was protein-primed. Deletion of HBV reverse transcriptase (RT) or RNase H domains resulted in a dramatic drop in histidine prototrophs. The addition of HBV encoded HBx protein in virus-like particles during in vitro RT reaction stimulated the RT reaction by severalfold. Furthermore, in the presence of 3TC, a known inhibitor of HBV reverse transcriptase, yeast His+ growth of His prototrophs was not observed. Thus, this approach, which is based on genetic selection in yeast, is safe, economic, and a reliable strategy with a potential for large scale screening of cofactors and inhibitors of HBV polymerase functions.

Hepatitis B virus is one of the causative agents of acute/chronic hepatitis in humans, which is a major health problem worldwide. It is estimated that over 500 million individuals are hepatitis B carriers worldwide and about 1 million deaths are attributed annually to the effects of HBV1 infection (1). There is a strong correlation between chronic hepatitis B infection and the incidence of hepatocellular carcinoma. Immunization against HBV has been effective in reducing the number of new infections. Additionally interferon and nucleoside analog treatments have been employed for HBV-infected individuals with limited success. Approaches other than the vaccine have not been successful in eradicating the disease entirely. Therefore, there is an urgent need to develop alternative therapeutic methods and effective drug treatments to combat and or reduce the onset of chronic liver disease.

Human hepatitis B virus contains a small 3.2-kilobase DNA genome and is a member of the hepadnaviridae family, a group of viruses with a strong tropism for the liver (2). Other members are found in woodchucks, ground squirrels, and several avian species. At least four proteins (surface protein, core protein, polymerase, and HBx) are translated from the mammalian HBV genome. HBV utilizes the strategy of replication via reverse transcription. The polymerase (pol) protein encoded by HBV consists of three domains: terminal protein (TP), pol/reverse transcriptase (RT), and RNase H (RH). TP domain is separated from the other two domains by a spacer or tether sequence. The pol activity is utilized during HBV replication to convert a greater than genome-length RNA intermediate, known as progenome RNA, into an asymmetric or partially duplex DNA that is maintained in a circular conformation. The details of HBV replication have emerged from the work of several laboratories (3–6). According to these reports, the first step of HBV replication appears to be the recognition of progenomic RNA by polymerase at a stem loop structure termed ϵ located within its own mRNA (7, 8). Studies of in vitro translated (4) and yeast transposon-derived duck HBV-like virus pol (3) provided the first clues by revealing that a novel protein priming reaction initiates the minus strand DNA synthesis. This priming step yielded a discrete 3–4-deoxyribonucleotide oligomer that is covalently linked to the TP domain of HBV pol protein. The dNTPs are polymerized onto a tyrosine 63 acceptor residue located in the N-terminal priming TP domain. Priming is templated by a bulge within the ϵ stem-loop structure located near the 5’ end of the pregenomic RNA. The ϵ motif and an additional 10–11-nucleotide sequence termed DR are also found in the 3’ end of the terminally redundant pregenome RNA. This redundancy facilitates the translocation of initiated DNA-protein complex to the 3’ end of pregenomic RNA and ensures the continuation of minus strand synthesis. The pol covalently linked to initial minus strand DNA upon translocation to a complementary sequence in the 3’ end of pregenome RNA resumes minus strand DNA synthesis. RNaseH activity of pol protein hydrolyzes the pregenome RNA following its reverse transcription. A ribonucleotide primer of 15 nucleotides including the CAP structure is recruited to initiate plus strand DNA synthesis at the DR2 sequence near the 3’ end. The series

drophoresia; VLP, virus-like particle; 3TC, (−)-β-L-2’,3’-dideoxy-3’-thiacytidine.

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of reactions result into a DNA product that has a circular conformation. Core particles in which these reactions occur are either recycled through nucleus or enveloped with surface antigen to bud off as mature virion particles from cell surface (2).

Reverse transcription has been observed in a wide variety of genetic elements including all retroviruses, transposons, bacteria, animals, and plants (9). Yeast has several classes of endogenous retrotransposons (Ty) elements that transspese through RNA intermediate, and their replication is dependent upon Ty element-encoded reverse transcriptase (23). Hybrid Ty and other retroelements have been used to demonstrate the RT activity of duck HBV-like virus (3), human long interspersed nuclear elements (10), and long interspersed nuclear element-like elements from trypanosomatids (11). Although the retroviruses reverse transcription is understood in great detail, the biochemical dissection of HBV pol has been hampered by failure to obtain enzymatically active protein from nucleocapsids. In this report, we have employed a genetic selection scheme recently described for HIV RT that utilizes a Ty1 element-based expression system in Saccharomyces cerevisiae (18, 19). In the construct pTy1/HBVVRT, the Ty1 RT (TyB) coding sequences were substituted with HBV pol open reading frame. For genetic selection HIS marker was added in the construct that exists in an antisense orientation. Reverse transcription yields a cDNA copy of the hybrid element carrying a functional HIS gene. The conversion of Ty1 RNA into DNA occurs within the virus-like particles known as VLPs. Upon transposition in yeast chromosome either by homologous recombination or via Ty integrase-mediated insertion, expression of HIS gene leads to histidine prototrophy. Thus, the expression of his marker in the present system totally relies on the HBV pol activity. Although our biochemical analyses and genetic selection support the functional activities of HBV pol, the system was also capable of detecting the failure of HBV RT activity in the presence of a nucleoside triphosphate 3TC on solid medium. Thus this approach suggests the feasibility of the system for large scale screening of inhibitors of HBV RT/RH activities. 3TC is a known inhibitor of HBV pol activities (12).

**MATERIALS AND METHODS**

**Yeast Strains—** The yeast strain 1052 (Mata ura-167 trpl-1 GB spt3-101 his200 Rad52) was used to monitor reverse transcription of hybrid Ty1/HBVRT retroelement. The spt3 mutation blocks the expression of endogenous Ty element. The His200 mutation deletes the entire HIS3 gene. Rad52 mutation blocks the integration of plasmid borne Ty element into chromosome by homologous recombination. The genotype of JSSS611-B (spt' yeast strain) is Mata gal' CanL' cys' his 33I trp-7 leu-112 ura-32.

**Plasmids—** A 2.4-kilobase fragment flanked by the Clal and Smal restriction sites encompassing the entire HBV open reading frame including TP, RT, and RNS H domains was polymerase chain reaction-amplified from plasmid pNET. This fragment was cloned into the CiaI and SmalI sites of plasmid pHART to generate a hybrid construct Ty1/HBVRT this3AI (pTy1/HBVRT). Galactose-regulated Ty-his3AI plasmid pHuveo08 was used as a control (19). Both pHART and pHuveo08 were kindly gifted by Drs. Dwight Nisal, David Garfinkel, and Jeffrey Strathern of ABL of the National Cancer Institute (Frederick, MD). pHART contains the Ty1 protease, an integrase, and HIV reverse transcriptase (RT) domain (18, 19). The plasmid Ty1/HBVRT was constructed by replacing the HIV RT sequences in the pHART plasmid. The junctions of Ty integrate with HBV pol open reading frame was confirmed by DNA sequencing. Two deletion mutants of HBV pol open reading frame were generated. pTy1/HBVART contains a deletion of an 1.4-kilobase BanHI fragment that includes a major part of RT domain. The second deletion mutant pTy1/HBVARR contains a 70-base pair Notl-SstI deletion fragment encompassing the RNase H domain of the pol protein.

**In Vivo Reverse Transcription Assay—** Yeast cultures containing the appropriate constructs were grown in YM1 medium with 2% glucose, lacking uracil. Saturated yeast culture were then harvested and pellet washed in sterile water. Cells were grown for 1-2 h 2% raffinose.

For induction of reverse transcription, 1 ml of yeast culture was then grown in YM1 medium with 2% galactose, 2% glycerol, and 2% ethanol at either 30 or 20 °C for 12-16 h. After induction the yeast cells were diluted in water and simultaneously plated on plates with histidine for cell counts and without histidine for scoring His' prototrophs. The ratio of colonies/total number of yeast colonies was shown in Table I. Purification of VLPs—Ty virus-like particles were isolated by a modification of the method of Mathias et al. (11). Yeast strain 1052 containing Ty-derived plasmids or their derivatives were grown in 2000 ml of YM1 synthetic medium with 2% glucose to an A590 of 2. Cells were collected and diluted 2.5-fold in 5C-ura medium (0.16% yeast nitrogen base without amino acids) with 2% raffinose and grown with 2-3 h. Cultures were induced by the addition of 2% galactose, 2% glycerol, and 2% ethanol and grown for 16-24 h. Cells were collected and suspended in 6 ml of B/EDTA buffer (15 mM KCl, 10 mM HEPES, pH 8.5, 5 mM EDTA, 3 mM 1,4-dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and 0.1 unit/ml aprotinin). Cells were lysed by vortex mixing with glass beads (12 gm) for 3-5 min. The lysates were clarified by centrifugation at 5,000 rpm in Sorval SW54 rotor for 10 min. The supernatant was loaded onto a step gradient of 20%/30%/70% sucrose in 60 mM Tris, pH 8.0, 12.5 mM MgCl2, and 2.5% v/v 2-mercaptoethanol. The second deletion mutant pTy1/HBV containing [32P]dCTP and 10 μg of protein was incubated for 75 min at 24 °C in 30 μl containing [α-32P]dCTP and 10 μg of DTG, dATP, and dITP each, in 60 mM Tris, pH 8.0, 12.5 mM MgCl2, and 2.5% v/v 2-mercaptoethanol. Where indicated VLPs were incubated with 10 units of micrococcal nuclease (Roche Molecular Biochemicals) in 7 mM CaCl2 for 30 min to remove the endogenous DNA within VLPs prior to labeling reaction. Micrococcal nuclease activity was terminated by adding 15 μM EGTA. Actinomycin D (100 μg/ml Roche Molecular Biochemicals) was added to the reaction mixture. Where indicated the labeled products of the polymerase reaction were treated with mung bean nuclease at 10 units of protein was incubated for 75 min at 30 °C in 30 μl containing [α-32P]dCTP and [32P]dCTP and 10 μg of protein was incubated for 75 min at 24 °C in 30 μl containing [α-32P]dCTP and 10 μg of DTG, dATP, and dITP each, in 60 mM Tris, pH 8.0, 12.5 mM MgCl2, and 2.5% v/v 2-mercaptoethanol and protease inhibitors).

**RESULTS**

**Expression Strategy—** In this study, we used yeast Ty1 retrotransposon-based chimeric construct to express the functional HBV pol protein. Ty1 retrotransposition is a replicative process involving reverse transcription of Ty1 mRNA and integration of Ty1 cDNA into the yeast genome. Yeast Ty1 elements carry two overlapping genes, TyA and TyB, expressed in vivo. TyB encodes a structural protein that directs the formation of virus like particles. TyB encodes a multiprotein product with protease, reverse transcriptase, and integrase activities. TyB is initially expressed as TyA-TyB polyprotein by ribosome frameshifting (15, 23). This protein along with the mRNA is incorporated into VLPs where the protease activity encoded by the N terminus of TyB liberates TyB reverse transcriptase/RNase H protein. An in vivo assay was developed by Carreau and Garfinkel (17). The selection of RNA intermediate transcription of the Ty1 element, in which a HIS3 gene interrupted by an artificial intron in the antisense orientation was tagged to Ty genome at the 3' end. The intron is located in the sense orientation to Ty1 RNA. Splicing and retrotransposition of marked Ty1 transcripts leads to expression of histidine marker, which is scored as his' colonies on solid medium lacking histidine amino acid. Re-
cently, this strategy was employed to express HIV RT activity (18, 19). In the present study, we have utilized a similar strategy to express HBV reverse transcriptase activity. The coding region of HBV pol was cloned in frame with the TyA protein immediately 3′ to the protease and integrase domain to produce the plasmid pTy1/HBVRT (Fig. 1A). Two additional constructs, Ty1/HBVΔRT and Ty1/HBVΔRH, were developed to serve as negative controls, in which RT or RH domains were deleted, respectively (Fig. 1B). All constructs were regulated by Gal1 promoter. To reduce RT activity attributable to expression of endogenous Ty1 and Ty2 elements, a spt3 mutant host strain was used. Upon galactose induction, yeast carrying these Ty1 plasmids produce and accumulate large amounts of VLPs (15). By using this assay, we estimated the Ty1/HBVRT transposition rate between 1 × 10⁻⁴ and 1.5 × 10⁻⁴ transpositions per Ty1/HBVRT hybrid element per generation (Table I). Variations in the transposition rate of individual hybrid Ty1/HBVRT elements may be due to the relative abundance of their transcripts. Table I summarizes the production of His prototroph colonies by different constructs at 20 and 30 °C, respectively. It is important to note that the His3 reporter gene was active only if marked Ty1/HBVRT RNA was spliced prior to reverse transcription carried out by HBV pol protein.

To demonstrate the presence of HBV pol, VLPs purified from both spt⁻ and spt⁺ yeast strains were examined by immunoblotting. The presence of TyA protein was readily detectable by Coomassie Blue staining and by immunoblotting using TyA antibodies (data not shown). The separated proteins were then transferred onto nitrocellulose and probed with a polyclonal antiserum directed against human HBV pol. An expected ~96-kDa (also includes amino acid residues derived from TyB protein and HIV integrase domain) mature HBV pol protein was detected only after induction of spt⁻ (Fig. 2A, lane 4) and spt⁺ (Fig. 2B, lane 6) yeast strains with wild type Ty1/HBVRT particles. This band was absent in uninduced spt⁻ and spt⁺ yeast strains (Fig. 2, A, lane 5, and B, lane 1, respectively). The RT and RH deletion mutant VLPs showed no band (Fig. 2, A, lanes 2 and 3, and B, lanes 4 and 5, respectively). Uninduced yeast containing wild type hybrid Ty1/HBVRT element and induced yeast containing Ty1/HBVΔRT and Ty1/HBVΔRH (deletion mutants of HBV RT and RH, respectively) produced no VLPs as tested by immunoblotting using the TyA antibodies (data not shown). Therefore, for immunoblotting and subse-

Table I

<table>
<thead>
<tr>
<th>Frequency of His⁺</th>
<th>30 °C</th>
<th>20 °C</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>spt3RAD52</td>
<td>spt3rad52</td>
</tr>
<tr>
<td>Ty1/HBVRT</td>
<td>1.8 × 10⁻³</td>
<td>2.2 × 10⁻⁴</td>
</tr>
<tr>
<td>Ty1/HBVΔRT</td>
<td>1.0 × 10⁻⁷</td>
<td>2.8 × 10⁻⁷</td>
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<td>5.5 × 10⁻⁷</td>
<td>6.6 × 10⁻⁷</td>
</tr>
<tr>
<td>Ty1/pGEM74</td>
<td>3.2 × 10⁻⁷</td>
<td>2.0 × 10⁻⁷</td>
</tr>
<tr>
<td>pYES-2</td>
<td>2.7 × 10⁻⁷</td>
<td>1.7 × 10⁻⁸</td>
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Activity of Ty1/HBVRT hybrid retroelement in yeast

The frequency of reverse transcription mediated by HBVRT/RH was monitored by selection of histidine prototrophy following the induction of yeast containing pTy1/HBVRT. Galactose-induced expression leads to an RNA molecule carrying an antisense copy of his3A1 at the 3′ end. The his3 coding sequences are interrupted by an artificial intron in the backward orientation. Splicing and reverse transcription results in the synthesis of a cDNA carrying a functional HIS3 gene. Ty PR and HIV IN stands for Ty protease and HIV integrase functions, respectively. Once integrated either by homologous recombination or through transposition mediated by Ty integrase, the expression of cDNA leads to histidine prototrophy. B, schematic representation of the Ty1/HBVRT mutants. All constructs are under the control of Gal1 promoter. Histidine prototrophy following the induction of spt3 reporter gene was readily detected only after induction of spt⁺ (Fig. 2A, lane 4) and spt⁻ (Fig. 2B, lane 6) yeast strains with wild type Ty1/HBVRT particles. This band was absent in uninduced spt⁻ and spt⁺ yeast strains (Fig. 2, A, lane 5, and B, lane 1, respectively). The RT and RH deletion mutant VLPs showed no band (Fig. 2, A, lanes 2 and 3, and B, lanes 4 and 5, respectively). Uninduced yeast containing wild type hybrid Ty1/HBVRT element and induced yeast containing Ty1/HBVΔRT and Ty1/HBVΔRH (deletion mutants of HBV RT and RH, respectively) produced no VLPs as tested by immunoblotting using the TyA antibodies (data not shown). Therefore, for immunoblotting and subse-

Expression of HBV Polymerase in Ty1-his3AI Retroelement
quent analysis, we used an equal amount of precipitated protein fraction in 5% sucrose/B/EDTA, which was previously collected from the interphase of 30%/70% stepwise sucrose gradient. HBV pol antibodies also showed weak cross-reactivity to HIV RT (Fig. 2B, lane 3). Although the same amounts (10 μg) of VLPs were analyzed for immnoblotting, the expression of HBV pol in spt1 strain appears to be much higher (Fig. 2B, compare lane 4 of A with lane 6 of B). This suggests that encapsidation of HBV pol in the VLPs within spt1 yeast strain may be facilitated by endogenous Ty reverse transcriptases. Collectively these results suggest that HBV pol (reverse transcriptase/RNaseH) is contained within the VLPs isolated from histidine prototrophs. In this system, as mentioned earlier, the expression of HBV polymerase in Ty1-his3AI Retroelement

production of His+ colonies entirely relies on the reverse transcription function provided by HBV pol protein.

Hybrid Ty1/HBVRT Retroelements Express the Bona Fide HBV RT Activity—A highly unusual behavior of HBV pol is its ability to prime the DNA synthesis. In doing so the first nucleotide (dGTP) is covalently linked to the tyrosine 63 residue. This causes the attached (dGTP)DNA to partition into the phenol phase. In the present analysis, the HBV pol protein becomes radiolabeled by the transfer of the first [32P]dGTP. VLPs were
Expression of HBV Polymerase in Ty1-his3AI Retroelement

Incubated in the RT buffer containing only the α-32P-labeled dGTP in the reaction. The HBV pol trans-labeled product of this reaction was separated by 8% SDS-PAGE (Fig. 3A). A radiolabeled HBV pol protein band of expected molecular mass was observed (Fig. 3A, lane 1). No α-32P-dGTP-labeled proteins were observed in uninduced (Fig. 3A, lane 2) or induced yeast cells expressing Ty1/HIVRT (lane 3). HIV RT like other retroviruses utilizes a tRNA molecule for priming minus strand synthesis (9). A weak priming activity could be seen when [α-32P]dCTP was included in the HBV pol reaction (data not shown). These results clearly support the conclusion that the HBV pol protein in the VLPs is functional and is able to initiate priming reaction with dGTP.

Next, we examined an RNA-dependent DNA synthesis initiated by the HBV pol protein within the VLPs. This was accomplished by incubating the VLPs in RT buffer in the presence of dNTPs including α-32P-labeled dCTP. Reactions were terminated by incubating with proteinase K for 90 min. Proteins were removed by phenol-chloroform extraction, and labeled DNA products were resolved by either 4% urea-polyacrylamide or 1.5% alkaline-agarose gel electrophoresis. Where indicated RNase (10 units), actinomycin D (100 μM), and micrococcal nuclease (0.1 μg/ul) were used prior to DNA synthesis. A series of controls were included in this analysis to establish the bona fide HBV pol activity. The RT products analyzed by 4% urea-PAGE gel electrophoresis are shown in Fig. 4A. A prominent DNA band was observed in an induced yeast culture (lane 2) and was absent in uninduced cells (lane 1). Micrococcal nuclease treatment of the VLPs prior to RT reaction yielded an enhanced DNA synthesis (lanes 5 and 6). This increase may have resulted due to the digestion of the synthesized labeled DNA is covalently linked to HBV pol, a unique characteristic of hepadaviruses polymerases.

To monitor the extent of DNA synthesis, partially purified VLPs were incubated with dNTPs containing α-32P-labeled dCTP. Reactions were terminated by incubating with proteinase K for 90 min. Proteins were removed by phenol-chloroform extraction, and labeled DNA products were resolved by either 4% urea-polyacrylamide or 1.5% alkaline-agarose gel electrophoresis. Where indicated RNase (10 units), actinomycin D (100 μM), and micrococcal nuclease (0.1 μg/ul) were used prior to DNA synthesis. A series of controls were included in this analysis to establish the bona fide HBV pol activity. The RT products analyzed by 4% urea-PAGE gel electrophoresis are shown in Fig. 4A. A prominent DNA band was observed in an induced yeast culture (lane 2) and was absent in uninduced cells (lane 1). Micrococcal nuclease treatment of the VLPs prior to RT reaction yielded an enhanced DNA synthesis (lanes 5 and 6). This increase may have resulted due to the digestion of the synthesized DNA by micrococcal nuclease within VLPs (compare lanes 3 and 6). Although RNA-dependent DNA synthesis is sensitive to actinomycin D, at concentrations used here a unique characteristic of hepadaviruses polymerases.

Enter to RT reaction. Lane 10, no proteinase K added. Lane 11, mung bean nuclease added after the completion of RT reaction. Lane 12, reaction performed in the presence of dideoxy NTPs. Lane 13, labeled RT product recovered from phenol phase by acetone precipitation. The standard RT reaction performed in the presence of 10 μM 3TC. B, RT products analyzed by 1.5% alkaline-agarose gel electrophoresis. Several DNA species ranging from 200 to 800 nucleotides are also visible. Lane 1, uninduced spt yeast. Lanes 2–4, induced yeast expressing Ty1/HBV/ΔRT (lane 2), Ty1/HBV/ΔRH (lane 3), and Ty1/Gem74 (lane 4). Lane 5, standard RT reaction of Ty1/HBVRT lanes 6–8, RNase treatment prior to the RT reaction (lane 6), after the RT reaction (lane 8), and in the presence of 100 μM actinomycin D (lane 7). MW, molecular weight standard. C, RT products from spt yeast strain analyzed by 1.5% alkaline-agarose gel electrophoresis. Lane 1, normal reaction. Lanes 2 and 3, RNase was added either prior to the reaction (lane 2) or after completion of the reaction (lane 3). Lanes 4 and 5, no proteinase K or Mg2+ added respectively. Lanes 6 and 7, reaction performed in the presence of actinomycin D and ddNTPs, respectively. Lane 8, products recovered from the phenol interphase, treated with proteinase K, and separated.

Fig. 4A, DNA synthesis in VLPs. Purified VLPs (10 μg) were subjected to RT reaction as described in the legend to Fig. 3B and subjected to 4% urea-PAGE. Lane 1, uninduced yeast containing Ty1/HBVRT. Lanes 2–14, induced yeast containing Ty1/HBVRT. Lane 3, standard reverse transcription reaction followed by RNase treatment. Lane 4, actinomycin D added during the RT reaction. Lanes 4–7, micrococcal nuclease was added prior to the RT reaction. Lane 6, RNase digestion after the RT reaction within the micrococcal nuclease treated VLPs. Lane 7, actinomycin D added during the RT reaction, after micrococcal nuclease treatment of VLPs. Lane 8, no Mg2+. Lane 9, RNase treatment prior to RT reaction. Lane 10, no proteinase K added. Lane 11, mung bean nuclease added after the completion of RT reaction. Lane 12, reaction performed in the presence of dideoxy NTPs. Lane 13, labeled RT product recovered from phenol phase by acetone precipitation. The standard RT reaction performed in the presence of 10 μM 3TC. B, RT products analyzed by 1.5% alkaline-agarose gel electrophoresis. Several DNA species ranging from 200 to 800 nucleotides are also visible. Lane 1, uninduced spt yeast. Lanes 2–4, induced yeast expressing Ty1/HBV/ΔRT (lane 2), Ty1/HBV/ΔRH (lane 3), and Ty1/Gem74 (lane 4). Lane 5, standard RT reaction of Ty1/HBVRT. Lanes 6–8, RNase treatment prior to the RT reaction (lane 6), after the RT reaction (lane 8), and in the presence of 100 μM actinomycin D (lane 7). MW, molecular weight standard. C, RT products from spt yeast strain analyzed by 1.5% alkaline-agarose gel electrophoresis. Lane 1, normal reaction. Lanes 2 and 3, RNase was added either prior to the reaction (lane 2) or after completion of the reaction (lane 3). Lanes 4 and 5, no proteinase K or Mg2+ added respectively. Lanes 6 and 7, reaction performed in the presence of actinomycin D and ddNTPs, respectively. Lane 8, products recovered from the phenol interphase, treated with proteinase K, and separated.
dramatic reduction was seen (lane 4), which is consistent with its previously observed inhibitory activities against viral polymerases and reverse transcriptases (6, 13). Most importantly, these DNA products disappeared from the aqueous phase during phenol extraction if protease K digestion was omitted (compare lanes 2–7 and 10). Acetone precipitation was performed to recover the DNA from phenol phase (lane 13). No RT products were observed in the absence of Mg\(^{2+}\) or when VLPs were treated with RNase prior to RT reaction (Fig. 4A, lanes 8 and 9). In the reaction with RNase treatment, Mg\(^{2+}\) was included. These data suggest that an authentic reverse transcription reaction has occurred within the purified VLPs. Absence of a band in the mung bean nuclease treated reaction indicates that the RT product is a single-stranded DNA (lane 11). The addition of dideoxy-NTPs did not completely obliterate the RT activity (lane 12). This is consistent with the observation of others that a pool of sufficient amount of nucleotides is present in the VLPs to initiate the RT reaction (6). 3TC is a known inhibitor of reverse transcriptase activities of HIV and HBV. Here, we have used 3TC triphosphate (10 \(\mu\)M) to examine its influence on HBV pol activity. As shown in lane 14, 3TC inhibited this reaction. Collectively, these results show that the RT activity is authentically initiated by the HBV polymerase in accordance with the known characteristics of this protein. Further, this also rules out the concerns that endogenous yeast RT activity may have co-purified with the VLPs.

To precisely determine the size of the DNA fragments in the RT reactions within the VLPs purified from pTy1/HBVRT-expressing HIS\(^+\) yeast cells, the reaction mixtures were analyzed by alkaline-agarose gel electrophoresis (Fig. 4B). Shown in Fig. 4B (lanes 5 and 8) are the various sizes of DNA bands. There are two discrete DNA fragments of approximately of ~5000 and 2000 nucleotides and a diffuse pattern of DNA products ranging from 200 to 800 nucleotides. These DNA bands were absent in uninduced yeast cells and those transformed with HBV RT and RNase H deletion mutants or with Ty1/Gem74 vector (lanes 1–4). No DNA synthesis was observed when VLPs were treated with RNase prior to RT reaction (lane 6). However, when RNase was added after the RT reaction, RT product was not degraded, suggesting that the DNA synthesis has occurred (lane 8). As was seen before (Fig. 4A, lane 4), the DNA synthesis was sensitive to actinomycin D at a concentration of 100 \(\mu\)g (lane 7). For comparison, we also carried out the RT analysis within the purified VLPs from an spt\(^+\) yeast strain JSS 56 11-B (Fig. 4C). These results are consistent with those shown in Fig. 4B. In all lanes, bona fide RT products of ~5500 and ~600 nucleotides were seen; however, the 2000-nucleotide band was not as pronounced in spt\(^-\) strain (Fig. 4B). In this analysis several minor species ranging from 0.8 to 3.5 kilobases were also seen (lanes 1, 3, and 6–8). Similarly, when no proteinase K was added to the mixture, no RT products were seen (lane 4). The RT products covalently bound with the HBVRT were separated in the interphase. These products were recovered from the interphase by chilled acetone precipitation, treated with proteinase K, and separated (lane 8). These result firmly establish that the VLPs purified under the his selection, from both spt\(^+\) and spt\(^-\) yeast strains contain the functional HBV RT activity.

**HBV Reverse Transcription Is Inhibited by 3TC**—One of the promising features of the genetic selection of Ty1/HBVRT expression strategy is the ability to screen for inhibitors of RT activity. Recently, Nissley et al. (19) were able to show the inhibitory activity of several compounds by the absence of Ty/HIVRT-expressing yeast growth on solid medium. We have used this method to show HBV RT inhibitory activity of 3TC triphosphate on solid medium. First, the toxicity of 3TC in yeast was determined by growing yeast in the presence or absence of 100 \(\mu\)M 3TC, which appeared to be the same. Yeast cells were grown in YMIN with 2% galactose and plated onto solid medium lacking histidine but containing 100 \(\mu\)M 3TC-triphosphate (left) or lacking histidine but containing 100 \(\mu\)M 3TC-triphosphate for 16 h. No growth was observed. This result establishes the feasibility of selection mixture, respectively. Lanes 5 and 6, 100 ng each of bacterially purified His-Xaa and Thio-His-Xaa added. Lane 7, anti-HBx serum added along with His-Xaa protein during RT reaction. The conditions for RT reaction are same as described in the legend to Fig. 3B. The RT products were separated by 4% urea-PAGE.

**FIG. 5.** HBV RT activity is inhibited by 3TC. Inhibition of RT activity in yeast was tested by plating yeast expressing Ty1/HBVRT on plates lacking histidine and RT inhibitor 3TC-triphosphate (right) or lacking histidine but containing 100 \(\mu\)M 3TC-triphosphate (left). Prior to the plating on both plates, cells were induced for reverse transcription in liquid culture with 2% galactose and 100 \(\mu\)M 3TC-triphosphate for 16 h.

**FIG. 6.** HBV RT activity is stimulated by HBx protein. Lane 1, HBV/RT reaction of VLPs from uninduced yeast. Lanes 2–7, HBV/RT reaction of VLPs from induced yeast. Lanes 3 and 4, 100 ng each of bovine serum albumin and bacterial lysates were added to the reaction mixture, respectively. Lanes 5 and 6, 100 ng each of bacterially purified His-Xaa and Thio-His-Xaa added. Lane 7, anti-HBx serum added along with His-Xaa protein during RT reaction. The conditions for RT reaction are same as described in the legend to Fig. 3B. The RT products were separated by 4% urea-PAGE.
HBx protein interacts with single-stranded nucleic acids in a manner that is not sequence-specific (25). In light of these properties of HBx, we reasoned that HBx protein may influence the HBV RT reaction. To test this hypothesis, bacterially purified histidine-tagged HBx protein was added into the VLPs, during in vitro RT reaction (Fig. 6, lanes 5 and 6). As a control bovine serum albumin and bacterial lysates were included in the RT reaction mixture (lanes 3 and 4, respectively). The results of this experiment show that HBx displays stimulatory activity in the RT reaction by at least 3–5-fold. This stimulatory activity was inhibited when anti-HBx serum was included along with HBx in the reaction mixture (lane 7). VLPs are believed to constitute a loose structural conformation that is permeable to a large variety of reagents including small proteins.

DISCUSSION

Although the HBV genome is a DNA molecule, the viral replication occurs via an RNA intermediate (13). A pregenomic RNA is packaged in immature core particles, wherein it is reverse transcribed by the HBV-encoded polymerase. Reverse transcription occurs within the core particles (13–16). Despite several attempts to express the pol gene, the biochemical dissection of the protein has lagged behind because of the unavailability of soluble enzyme in amounts required for such an analysis. In this communication we have employed a novel hybrid yeast-based Ty retrotransposon to express the HBV pol protein. The strategy was first developed by Curcio and Garfinkel (17) and subsequently utilized to express HIV reverse transcriptase activity (18, 19) that is genetically marked particularly useful for large scale screening of novel inhibitors of HBV reverse transcriptase activity.

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Expression of Hepatitis B Virus Polymerase in Ty1-his3Al Retroelement of *Saccharomyces cerevisiae*
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