



# Reverse Transcriptase Activity of Hepatitis B Virus Polymerase in Eukaryotic Cell Extracts *In Vitro*

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## Summary

*In hepadnaviruses, reverse transcription is primed by the viral reverse transcriptase (RT) and requires the specific interaction between the RT and the viral RNA encapsidation signal termed  $\epsilon$ . To study the activity of the RT in vitro, the current procedure uses in vitro translated duck hepatitis B virus polymerase, but not the hepatitis B virus polymerase itself, in the rabbit reticulocyte lysate expression system.*

*Here, the hepatitis B virus (HBV) polymerase has been successfully expressed in a translational extract that was obtained from monolayer human hepatocyte cells HuH-7. The translated polypeptide retained the RNA-directed polymerase (reverse transcriptase) activity on the viral RNA template containing the  $\epsilon$  signal. We suggest that the reverse transcription event of the viral RNA coding for the polymerase and containing an  $\epsilon$  structure is concomitant to the translation of the viral polymerase to the messenger RNA. In contrast to the duck polymerase, only a fraction of the reverse transcribed complementary DNA (cDNA) was covalently bound to the HBV polymerase in this system. When the  $\epsilon$  signal was missing on the mRNA, the translated full-length HBV polymerase could not reverse transcribe the viral RNA template. A truncated HBV polymerase that was lacking the YMDD catalytic active site for the initiation of reverse transcription was unable to reverse transcribe the viral mRNA template containing the  $\epsilon$  signal. The reverse transcription activity could also be partially inhibited by employing nucleoside analogues, such as 2'-3'-dideoxy-3'-thiacytidine (3TC; lamivudine) in the expression system.*

*The procedure described here provides a method for the in vitro screening of new anti-HBV compounds directed against wild-type and mutants of this crucial viral protein, the HBV polymerase, without the use of animals (ducks) or animal extracts (rabbit reticulocyte lysate).*

**Zusammenfassung:** *In vitro* reverse Transkriptase Aktivität der Hepatitis B Virus Polymerase in eukariotischen Zellextrakten

*In Hepadnaviren wird die reverse Transkription von der viralen reversen Transkriptase (RT) ausgeführt. Hier findet eine spezifische Interaktion zwischen der RT und dem viralen Verpackungssignal  $\epsilon$  statt. Um die Aktivität der RT in vitro zu untersuchen, wird im aktuellen Protokoll in vitro translatierte Entenhepatitis B Virus Polymerase, aber nicht die Hepatitis B Virus Polymerase selbst, in einem Kaninchen Retikulozytenlysats Expressionssystem eingesetzt.*

*In dieser Arbeit wurde die Hepatitis B Virus (HBV) Polymerase erfolgreich in einem translationalen Extrakt aus einer einlagigen Kultur der humanen Hepatozytenzellen HuH-7 exprimiert. Das translatierte Polypeptid behielt die RNA-gerichtete Polymerase (reverse Transkriptase) Aktivität auf dem viralen RNA Template mit einem  $\epsilon$  Signal. Wir schlagen vor, dass die reverse Transkription der viralen RNA, welche die Polymerase kodiert und eine  $\epsilon$  Struktur besitzt, gleichzeitig mit der Translation der viralen Polymerase in die Boten-RNA (mRNA) abläuft. Im Gegensatz zur Entenpolymerase war nur ein geringer Anteil der revers transkribierten komplementär DNA (cDNA) in diesem System kovalent an die HBV Polymerase gebunden. Wenn die mRNA kein  $\epsilon$  Signal enthielt, konnte die translatierte HBV Polymerase das virale RNA Template nicht revers transkribieren. Eine verkürzte HBV Polymerase, welcher die katalytische Aktivdomäne YMDD zur Initiation der reversen Transkription fehlte, konnte das virale mRNA Template trotz  $\epsilon$  Signal nicht revers transkribieren. Die reverse Transkriptionsaktivität konnte auch partiell durch den Einsatz von Nukleosidanaloga, wie 2'-3'-Dideoxi-3'-thiacytidin (3TC; Lamivudin) im Expressionssystem inhibiert werden.*

*Diese Arbeit beschreibt eine Methodik mit der in vitro neue anti-HBV Wirkstoffen gegen Wildtyp und Mutanten dieses zentralen viralen Proteins, der HBV Polymerase, ohne den Einsatz von Tieren (Enten) oder tierischen Extrakten (Kaninchen Retikulozytenlysate) gesucht werden können.*

**Keywords:** hepatitis B virus, HBV, viral polymerase, reverse transcriptase, hepatocyte, cell extract, HuH-7, translation, RNA, reverse transcription, inhibitors

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## 1 Introduction

Hepatitis B virus (HBV) is a major public health problem with over 350 million chronically infected people worldwide. HBV, as a member of the hepadnavirus family, is an enveloped virus with partially double-stranded DNA. The viral infection is associated with the development of hepatocellular carcinoma and liver cirrhosis. Although HBV is a DNA virus, the mode of replication involves reverse transcription of an RNA pregenome, a process that occurs in intracellular viral cores.

The unique virus-encoded reverse transcriptase (RT) is able to initiate DNA synthesis *de novo*, using the RT itself as a protein primer (Hu and Seeger, 1996). The protein priming requires the specific interaction between the RT and a short RNA signal, termed  $\epsilon$ , located at the 5' end of the pregenomic RNA (pgRNA) that serves as a template for the reverse transcription (Nassal and Rieger, 1996). For the polymerase protein of DHBV, but not that of HBV, the priming reaction for reverse transcription could be efficiently reconstituted by *in vitro* translation of the protein in the rabbit reticulocyte lysate (Wang and Seeger, 1992). This event required the presence of a functional  $\epsilon$  signal on the mRNA (Beck and Nassal, 1998). In the case of HBV, some reports show that the purified polymerase protein alone efficiently reverse transcribes the mRNA containing the  $\epsilon$  signal (Jeong et al., 1996; Lanford et al., 1997). The catalytic site is located in the YMDD nucleus of the polymerase (Jeong et al., 1996) and is susceptible to mutation during antiviral therapy. In infected patients, the HBV polymerase is expressed by internal initiation and acts preferentially in *cis* on the pgRNA (Chang et al., 1989). The viral polymerase exhibits RNA dependent-DNA polymerase, DNA dependent-DNA polymerase, and RNase H activity (Roychoudhury et al., 1991). Because of problems in obtaining sufficient amounts of purified RT proteins *in vitro*, it has been difficult to study the hepadnavirus functions by biochemical and structural analyses. The most useful system to date has been a cell-free translation system, the rabbit reticulocyte lysate, developed for the study of the cognate duck hepatitis B virus (DHBV) reverse transcription activity (Wang, 1992). In this system, the viral

polymerase is translated *in vitro* in a first step and thereafter the reverse transcription activity is analysed in a subsequent reaction involving radiolabelled ribonucleotides. To date, all attempts at producing the biologically active HBV polymerase itself in the reticulocyte lysate system have not been fully convincing (Jeong et al., 1996; Kim and Jung, 1999). It was hypothesised that this might be due to the lack of additional protein factors or divalent cations (Jeong et al., 1996; Li and Tyrrell, 1999). Finally, several attempts have also been made to produce the HBV polymerase in insect cells by employing the baculovirus expression system (in which only a very minor percentage of the polymerase is biologically active) (Lanford et al., 1997), in *E. coli* (Jeong et al., 1996) or in yeast (Tavis and Ganem, 1993) in order to produce large amounts for further biochemical and structural studies.

A novel and simple *in vitro* system for the rapid analysis of the HBV polymerase is thus needed. Recently, we developed an efficient cell-free translational system using eukaryotic cells grown as monolayers (Favre and Trépo, 2001). This expression system is particularly suited for the translation of exogenous viral mRNAs originating from various sources. By employing an *in vitro* transcribed viral RNA coding for the full length HBV polymerase, which also comprised an  $\epsilon$  structure at the 5' end, the HBV polypeptides with the expected sizes of 94 and 81 kDa were successfully translated in a cytoplasmic extract that was obtained from the hepatocyte cell line HuH-7. When the translation of the HBV polymerase was concomitantly performed with the reverse transcription reaction, the incorporation of the radiolabelled nucleotide into the nascent DNA molecule was shown to be a fast and efficient process. Surprisingly, only a small percentage of this DNA was covalently bound to the newly synthesised HBV polymerase. This newly synthesised reverse transcript was indeed HBV DNA, as revealed by Southern blotting analysis. The activity was dependent on the presence of an  $\epsilon$  signal on the viral RNA, since the absence of  $\epsilon$  did not allow full length HBV polymerase to reverse transcribe the viral RNA. Moreover, a truncated HBV polymerase lacking the catalytic site YMDD, which is required for the onset

of the RT reaction, was unable to reverse transcribe the template RNA despite the  $\epsilon$  signal. The reverse transcriptase activity was tributary to the new translation of the HBV polymerase, since no RT activity was detected in the presence of both the mRNA and an inhibitor of the initiation of the protein synthesis. As expected, the use of various dideoxynucleotides for the inhibition of the reverse transcriptase activity revealed that the incorporation of the first nucleotides was a T followed by G and A, respectively. The additional use of the nucleoside analogue 2'-3'-dideoxy-3'-thiacytidine triphosphate (3TC; lamivudine) inhibited the RT reaction by about 40% in this system. The RT activity of the HBV polymerase was also obtained in the rabbit reticulocyte lysate in which the translation reaction was concomitantly performed with the RT reaction; however the efficiency was lower than that obtained in the translational extracts prepared from eukaryotic cells grown as monolayers.

The above results support the conclusion that, in addition to the presence of the very conserved YMDD catalytic motif in the viral polypeptide and the presence of an  $\epsilon$  signal on the template RNA, the reverse transcriptase activity of the HBV polymerase is only detected during a reaction in which the translation and the reverse transcriptase reactions are concomitantly performed. The generation of an *in vitro* assay for the study of the reverse transcriptase activity of the HBV polymerase will undoubtedly allow broad *in vitro* screening for new antiviral molecules directed against this important protein. Moreover, it might be employed for the *ad hoc in vitro* screening of antiviral molecules directed against mutants of the HBV polymerase that do not or poorly respond to the treatment with 3TC or other inhibitory molecules. This represents another novel approach to the putative prevention of the development of liver cancer that can thus reduce or replace the use of ducks, ducklings and rabbit reticulocyte lysate.

## 2 Materials and Methods

### Plasmids

Plasmid HH3, used for *in vitro* expression of full-length HBV reverse transcriptase, was constructed by cloning the

HBV polymerase coding sequence from the ayw strain into plasmid pSP64 that was digested with restriction endonuclease *SacI* and filled with Klenow enzyme. Plasmid HH3 contains the SP6 promoter cassette upstream of the HBV polymerase gene (Fig. 1).

#### *In vitro transcription*

To synthesise the RNA message used for *in vitro* translation of the HBV polymerase gene, the plasmid HH3 was linearised with the restriction endonuclease *PvuII*, which cuts outside of the viral sequences and leaves the  $\epsilon$  signal downstream of the intact polymerase open reading frame (ORF), or with *FspI*, which cuts right at the stop codon of the polymerase ORF and thus no  $\epsilon$  is present on the transcript. The *in vitro* transcription reaction of capped mRNAs was performed as described elsewhere (Svitkin et al., 1994). Following the transcription reaction, plasmid DNA was removed with DNase I and RNA was purified by phenol and chloroform extraction followed by passage into a Sephadex G-50 column and precipitation, as described elsewhere (Svitkin et al., 1994). For controls, the mRNA coding for the DHBV polymerase was transcribed from linearised plasmid pHP containing the DHBV polymerase gene under the control of the SP6 promoter, as described elsewhere (Wang and Seeger, 1992). This plasmid was linearised with restriction enzyme *SalI*. The resulting transcribed mRNA codes for full-length DHBV polymerase protein and contains an  $\epsilon$  signal at the 3'-end.

#### *Cell culture and generation of translational extracts*

The HuH-7 hepatocyte cell line (Nakabayashi et al., 1982) was grown in Eagle's Modified Essential Medium supplemented with 10% foetal calf serum, 1% sodium pyruvate, 2 mM L-glutamine and antibiotics (100 U/ml penicillin, 100 micrograms/ml streptomycin). The chicken hepatoma (LMH) and the baby hamster kidney (BHK) cell lines were grown as described elsewhere; the translational extract was prepared essentially as described (Favre and Trépo, 2001). In order to allow efficient incorporation of radiolabelled amino acids such as methionine in the *in vitro* translated polypeptides,

the cells were preincubated for 30 to 45 min at 37°C under 5% CO<sub>2</sub> with DMEM lacking methionine (Sigma #21013 supplemented with cysteine). The cells were then washed with washing buffer (20 mM Hepes [pH 7.4], 33 mM NH<sub>4</sub>Cl, 7 mM KCl, 150 mM sucrose) and thereafter the cytoplasmic membranes of the cells were lysed for 90 s by using 100  $\mu$ g lysolecithin, palmitoyl (Avanti Polar Lipids; stock at 10 mg/ml in chloroform/methanol (1:1) at -20°C) per ml in washing buffer, on ice. Following the complete removal of lysolecithin from the Petri dish, the cells were scraped into extraction buffer containing 100 mM Hepes-KOH [pH 7.4], 120 mM potassium acetate [pH 7.4], 2.5 mM magnesium acetate, 1 mM dithiothreitol, 2.5 mM ATP, 1 mM GTP, 100  $\mu$ M S-adenosyl-methionine, 1 mM spermidine, 20 mM creatine phosphate, 100 mM sucrose, 40  $\mu$ M hemin and 40  $\mu$ M of each essential amino acid except methionine (Promega). The cells were then passed ten times through a 25-gauge needle, and the lysate was centrifuged 5 min later at 4°C and 800g for 2 min. A Petri dish of 10 cm-in-diameter provides 0.2 ml of translational extract obtained from about 10<sup>7</sup> cells. The extracts were then frozen at -70°C until further use. Where indicated, the translation extracts were treated with micrococcal nuclease to hydrolyse the endogenous mRNAs prior to translation, as described elsewhere (Favre and Trépo, 2001).

#### *In vitro translation*

[<sup>35</sup>S]-labelled HBV polymerase was translated *in vitro* by employing eukaryotic cytoplasmic extracts that were obtained from cells grown as monolayers, as described elsewhere (Favre et al., 2001) with a minor modification: before the translational reaction, one  $\mu$ l of 40 mg/ml reactivated, biologically active creatine kinase in 50% (vol/vol) glycerol stored at -20°C (Favre and Muellhaupt, 2005) was added to 200  $\mu$ l of freshly thawed translational extract. This also allowed the optimal *in vitro* regeneration of the energy regenerating system based on ATP and creatine phosphate (Favre and Trépo, 2001). *In vitro* translation in 20  $\mu$ l was carried out by mixing 15  $\mu$ l of the extract in which [<sup>35</sup>S]methionine (translation grade; >1,000 Ci/mmol) and

exogenous mRNA (final concentration: 5 to 10  $\mu$ g per ml) were added. Translation reactions were carried out at 30°C for 60 min. As controls, translation reactions using rabbit reticulocyte lysate were performed according to the manufacturer's instructions (Promega).

#### *In vitro reverse transcription assay*

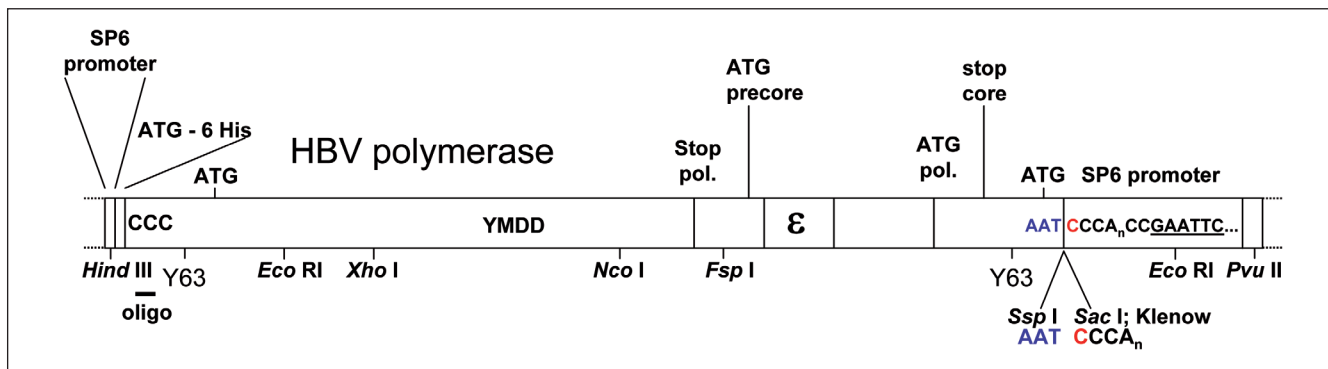
Reverse transcription assays were performed as described above for the *in vitro* translation in the presence or absence of exogenous mRNA with the addition of [ $\alpha$ -<sup>32</sup>P]radiolabelled deoxyribonucleotide triphosphate (3,000 Ci/mM, 0.2  $\mu$ M), and with 40  $\mu$ M non-radiolabelled methionine instead of [<sup>35</sup>S]methionine, referred to here as a coupled translation/reverse transcription reaction. Coupled translation/reverse transcription reactions were carried out at 30°C for 60 min. Where indicated, the reactions were performed in the presence of TMN buffer (100 mM Tris [pH 7.4], 20 mM MgCl<sub>2</sub>, 30 mM NaCl), as described elsewhere (Seigner et al., 2001).

#### *DNA extraction, electrophoresis and Southern blot analysis*

Where indicated, the DNA products from *in vitro* polymerase reactions were digested with proteinase K (1 mg/ml) in TES buffer (10 mM Tris [pH 7.8], 5 mM EDTA, 0.5% SDS) for 2 h at 56°C. DNA was extracted first with phenol and then with chloroform. Then it was ethanol precipitated in the presence of glycogen (2  $\mu$ g) and resuspended in water. DNA products were separated on 1% agarose gels and transferred to a nylon membrane (Hybond N+, Amersham). The membrane was hybridised with a genomic HBV DNA radiolabelled probe (Favre et al., 2003). Acrylamide gel electrophoresis was then performed.

#### *SDS-PAGE*

Reactions were disrupted in electrophoresis sample buffer containing 2% sodium dodecyl sulphate (SDS) and 2% 2-mercaptoethanol and were heated to 100°C for 5 min. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels containing both the stacking and the resolving portions of the gel were fixed with 30% methanol plus 10% acetic acid,



**Fig. 1: HBV polymerase expression plasmid HH3**

The phage SP6 promoter employed for the *in vitro* transcription of the HBV polymerase gene is located on a plasmid linearised with restriction enzyme *Pvu* II. Restriction enzyme *Fsp* I generates a linearised plasmid allowing transcription of mRNA lacking the  $\epsilon$  signal required for the reverse transcriptase activity of the polymerase polypeptide. Due to the cloning strategy, the HBV polymerase coding sequence begins with ATG followed by 6 codons for histidine and the CCC codon coding for Pro 2 in the HBV polymerase. The position of Tyr 63 and the YMDD catalytic site are indicated.

washed in water, dried, and autoradiographed (15 min to 2 h exposure on Kodak X-ray film).

### 3 Results

#### 3.1 Hepatitis B virus polymerase is translated *in vitro* in eukaryotic cell extracts

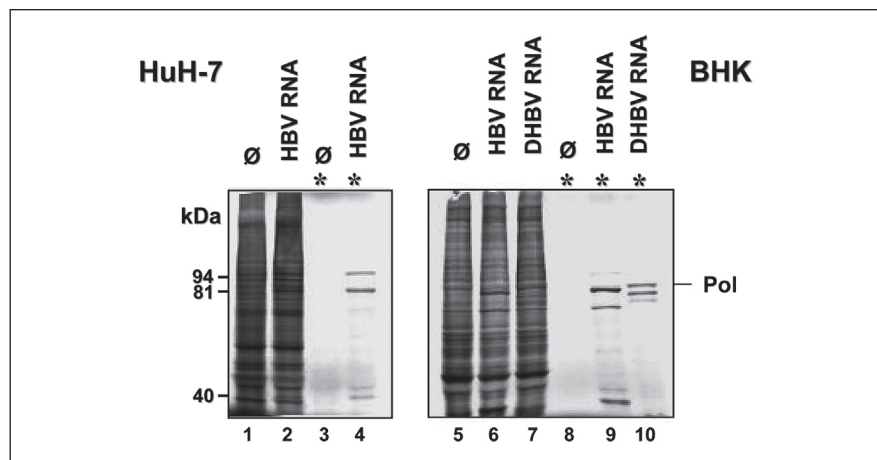
In an attempt to establish an *in vitro* assay for HBV polymerase, the enzyme was expressed in translational extracts obtained from eukaryotic cells grown as monolayers. For this, cytoplasmic extracts were prepared from the hepatocyte cell line HuH-7 and from the baby hamster kidney cell line BHK, treated or not with micrococcal nuclease in order to hydrolyse the endogenous mRNAs, and used for the translation of mRNA coding for the HBV polymerase. As illustrated in Figure 2, the HBV polymerase is translated in both cell extracts at the expected molecular masses of 94 and 81 kDa (Fig. 2, lanes 2, 4, 6 and 9). The 94-kDa component corresponds to the full-length HBV polymerase protein. This is consistent with a predicted MW for 845 amino acids. The appearance of the second, more rapidly migrating protein product can be attributed to the initiation at a second AUG codon, which is located 113 amino acids downstream of the first AUG codon of the polymerase open reading frame. A minor polypeptide of 40 kDa was also consistently expressed. The latter polypeptide might arise from the amino-terminal portion of the protein,

as suggested elsewhere (Li and Tyrrell, 1999). No polypeptide was translated without the addition of the exogenous HBV polymerase mRNA in the extracts that were previously treated with micrococcal nuclease (Fig. 2, lanes 3 and 8). As a control, the mRNA coding for the duck HBV polymerase was also efficiently translated, with a major apparent molecular weight of 86 kDa (Fig. 2, lanes 7 and 10), which is in good agreement with the expected size of 90.5 kDa calculated from the predicted amino acid product of 836 amino acids. The appearance of a more

rapidly migrating codon is also attributed to initiation at a second AUG codon located 50 amino acids downstream of the first AUG codon, as suggested elsewhere (Wang and Seeger, 1992).

#### 3.2 Expression and characterization of HBV polymerase expressed in an *in vitro* coupled translation-reverse transcription system

The initial aim was to develop an *in vitro* system originating from eukaryotic cells, but not from rabbit reticulocytes, for the



**Fig. 2: *In vitro* translation in cytoplasmic extracts obtained from HuH-7 and BHK cells grown as monolayers**

Extracts were previously treated with micrococcal nuclease to hydrolyze endogenous mRNAs (lanes 3, 4, 8-10), or were not treated (lanes 1, 2, 5-7). Translation of exogenous HBV polymerase mRNA (lanes 2, 4, 6 and 9). Translation of exogenous DHBV polymerase mRNA (lanes 7, 10). Control reactions without exogenous mRNAs (lanes 1, 3, 5, 8). [ $^{35}$ S]methionine-labelled polypeptides were resolved in 10% SDS-PAGE followed by autoradiography.  $M_r$ , relative molecular mass (kDa).



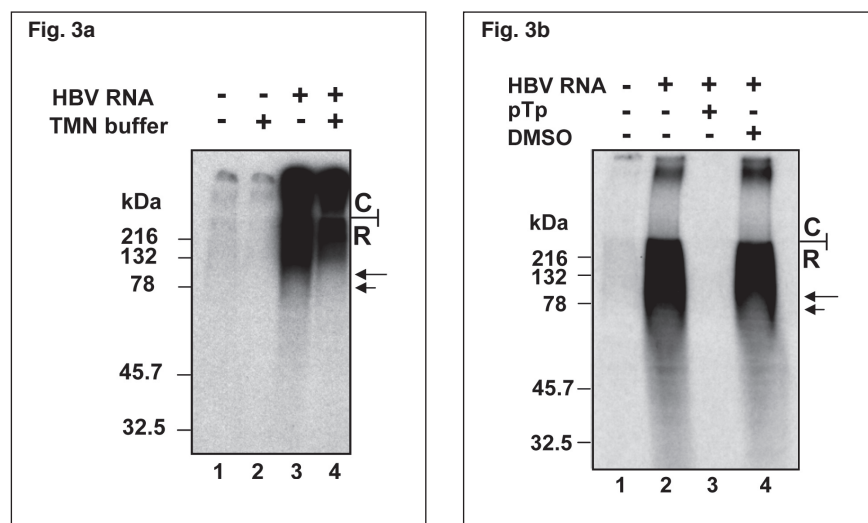
analysis of the reverse transcriptase activity of the HBV polymerase. The HBV polymerase was expressed in translational extracts in the presence of all 20 non-radioactive amino acids, and also with the concomitant presence of a [ $^{32}$ P]-radiolabelled deoxynucleotide triphosphate for the incorporation in the newly synthesised DNA that is generated by the reverse transcription on the HBV polymerase mRNA. Since the translational extracts still contain endogenous deoxynucleotides, no exogenous dNTPs were added in the coupled translation-reverse transcription reaction. Moreover, it is known that divalent cations are required for the HBV polymerase activity. Thus, the extracts were employed without prior treatment with micrococcal nuclease, in order to avoid the presence of the calcium chelating agent EGTA in the reactions. As shown in Figure 3a, a strong signal is obtained with the concomitant translation/reverse transcription of the HBV polymerase, with a [ $^{32}$ P]-radiolabelled signal present in both the stacking and the resolving portions of the gel (Fig. 3a, lane 3). This signal was totally absent when no exogenous HBV polymerase mRNA was added to the translational reaction (Fig. 3a, lane 1). The two 81 and 94 kDa bands corresponding to the HBV polypeptides might be masked by the radioactive signal. The presence of additional ions such as  $Mn^{++}$  and NaCl has been shown to increase the biological activity of the DHBV polymerase (Seigneres et al., 2001). In contrast, the presence of these two ions has a minor inhibitory effect on the biological activity of the HBV polymerase in this assay (Fig. 3a, lane 4).

Separate control reactions were also performed with the *in vitro* translation of the HBV polymerase for 1 h at 30°C performed in the presence of all 20 non-radiolabelled amino acids, followed in a subsequent reaction by the reverse transcription reaction itself for 1 h at 30°C, with the separate addition of each of the four [ $^{32}$ P]-labelled deoxynucleotides. When the translation and the reverse transcription were performed in two separate steps, no radiolabelled signal was obtained after SDS-PAGE analysis (not shown). Thus, this experiment revealed that both the translation of the

HBV polymerase mRNA and the reverse transcription on the HBV mRNA have to be performed concomitantly in order to generate a strong radiolabelled signal during reverse transcription.

It was then determined, whether the [ $^{32}$ P]-radiolabelled signal, which was obtained after the coupled *in vitro* translation/reverse transcription reaction, was indeed due to the *de novo* translation of the HBV polymerase protein and was not due to the presence of a putative contaminating endogenous reverse transcriptase activity in the HuH-7 cell extracts. To test this hypothesis, the HBV polymerase mRNA was translated in the presence of the specific inhibitor of the initiation

of translation, 2'-deoxythimidine, 3'-5-diphosphate (pTp) (Skup and Millward, 1977) during the coupled translation/reverse transcription reaction. The addition of this translation inhibitor did not allow the generation of a [ $^{32}$ P]-radiolabelled signal during the concomitant translation/reverse transcription reaction (Fig. 3b, lane 3). However, in the absence of the translational inhibitor, the generation of the [ $^{32}$ P]-radiolabelled signal was obtained (Fig. 3b, lane 2). Dimethylsulphoxide, in which pTp was resuspended, had no effect on the generation of the signal when the HBV polymerase mRNA was present (Fig. 3b, lane 4). This result thus suggested that the *de novo* transla-



**Fig. 3: [ $\alpha$ - $^{32}$ P]dNTP labelling of expressed HBV polymerase in a coupled translation/reverse transcription assay**

a) Coupled translation/reverse transcription of the HBV polymerase mRNA. HBV polymerase mRNA was transcribed *in vitro* from linearised plasmid HH3 and thereafter translated in a coupled translation/reverse transcription reaction in the absence of [ $^{35}$ S] methionine, but in the presence of all 20 unlabelled amino acids. During translation, additional [ $\alpha$ - $^{32}$ P]dGTP or [ $\alpha$ - $^{32}$ P]dTTP was added for the reverse transcription of the newly synthesised minus-stranded HBV DNA, thus generating a coupled translation/reverse transcription reaction. The products of the coupled reaction are shown in lanes 3 and 4, in the absence (lane 3) or presence (lane 4) of additional TMN buffer. The products of the control reactions without polymerase expression mRNA (lanes 1 and 2), and with the absence (lane 1) or presence (lane 2) of TMN buffer, are also presented. Similar results were obtained with the use of the three other radiolabelled dNTPs.

b) *De novo* translation of HBV polymerase protein is necessary for biological activity. Coupled translation/reverse transcription reactions were performed with HBV polymerase mRNA (lanes 2 to 4) and in the absence (lanes 2 and 4) or the presence (lane 3) of additional 2'-deoxythimidine 3',5'-diphosphate (pTp; 1 mM) in order to inhibit the initiation of translation. During translation, additional [ $\alpha$ - $^{32}$ P]dTTP was employed for the reverse transcription of the newly synthesized minus-stranded HBV DNA. Control reaction with DMSO that has served to suspend pTp (lane 4). Polypeptides were resolved in 10% SDS-PAGE followed by autoradiography for 15 to 30 min. Both the concentrating (C) and resolving (R) portions of the gel were analysed. Mr, relative molecular mass (kDa).

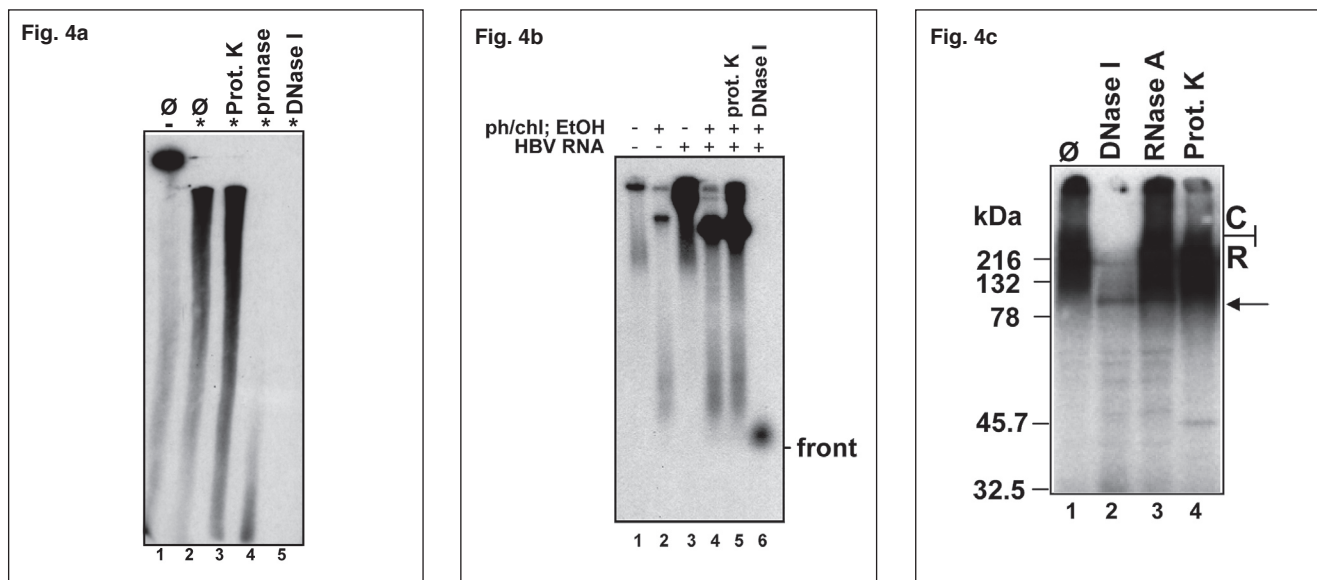
tion of the HBV polymerase mRNA is a prerequisite for obtaining a specific [ $^{32}\text{P}$ ]-radiolabelled signal, and that no contaminating endogenous reverse transcriptase activity was responsible for the radioactive signal. Finally, micrococcal nuclease treatment of the cell extracts had no deleterious effect on the HBV polymerase activity (not shown).

The purified, reverse transcribed DNA was then shown to be solely composed of a [ $^{32}\text{P}$ ]-radiolabelled DNA. To show this, the radiolabelled HBV nucleic acid was extracted with organic solvents after generation in a coupled translation/reverse transcription reaction, precipitated in the presence of glycogen, resuspended in water, and subjected to various enzymatic treatments. This revealed

that the radiolabelled nucleic acid was composed of a major nucleotide at least 1,000 bases long, as revealed by migration in urea-acrylamide gel (Fig. 4a, lane 2; purified probe without further enzymatic treatments). The hydrolysis of the radiolabelled DNA with DNase I for 2 h completely abolished the radioactive signal (Fig. 4a, lane 5). Treatment with proteinase K had no effect on the appearance of the radioactive signal (Fig. 4a, lane 3). Treatment with pronase abolished the signal, thus probably reflecting the contamination of this enzyme with some contaminating DNase activities (Fig. 4a, lane 4). Finally, the analysis of a crude preparation of the translation/reverse transcription reaction revealed a radioactive signal that did not enter into

the gel, thus probably reflecting the fact that the [ $^{32}\text{P}$ ]-radiolabelled HBV DNA was trapped in a large molecular weight complex (Fig. 4a, lane 1).

The reverse transcribed, [ $^{32}\text{P}$ ]-radiolabelled HBV polymerase DNA was further analysed by agarose gel electrophoresis. For this, the [ $^{32}\text{P}$ ]-radiolabelled HBV DNA was extracted or not with organic solvents after the coupled translation/reverse transcription reaction, precipitated and analysed by autoradiography after migration in an agarose gel and transfer on a nitrocellulose membrane. A major, high molecular weight, [ $^{32}\text{P}$ ]-radiolabelled band was obtained with the analysis of the crude reaction that was not extracted with organic solvents (Fig. 4b, lane 3). After extraction of the



**Fig. 4: Analysis of HBV reverse transcribed DNA**

The reverse transcribed HBV polymerase DNA was synthesized in the *in vitro* coupled translation/reverse transcription assay as described in the Fig. 3a, by using [ $\alpha$ - $^{32}\text{P}$ ]dTTP as a radiolabel.

a) The nucleic acids were extracted with organic solvents (phenol/chloroform; ph/chl) and precipitated. Purified, radiolabelled HBV polymerase DNA was then incubated with various enzymes and finally analysed by urea-acrylamide (4%) gel electrophoresis and autoradiography. Extraction with organic solvents alone (lane 2). Extraction with organic solvents followed by treatment with proteinase K (lane 3), pronase (lane 4), DNase I (lane 5). Crude, coupled translation/reverse transcription reaction without further extraction of the radiolabelled nucleic acids (lane 1), revealing that the majority of the radioactive signal did not enter into the gel.

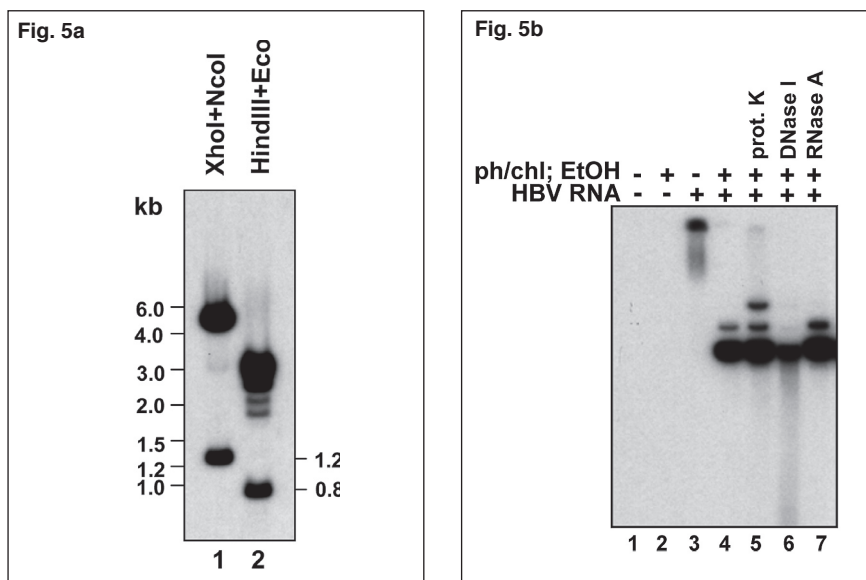
b) After the coupled translation/reverse transcription assay using HBV polymerase mRNA, the reactions were incubated or not with proteinase K (lane 5) or DNase I (lane 6), as indicated. The nucleic acids were thereafter extracted (ph/chl +) or not (ph/chl -) with organic solvents, precipitated, migrated in 1% agarose gel, transferred to a nitrocellulose membrane, and finally autoradiographed. Control reactions were performed in the absence of exogenous HBV polymerase mRNA (lanes 1 and 2). In the absence of extraction with organic solvents, a substantial portion of the radioactive signal could barely enter into the agarose gel. The position of the front of migration is indicated.

c) The coupled translation/reverse transcription reactions were thereafter incubated with DNase I (lane 2), RNase A (lane 3) or proteinase K (lane 4), and the proteins were analysed by 10% SDS-PAGE followed by autoradiography. Both the concentrating (C) and resolving (R) portions of the gel were analysed. Mr, relative molecular mass (kDa).

samples with organic solvents followed by ethanol precipitation, a major radiolabelled DNA band was obtained (Fig. 4b, lane 4). Interestingly, an additional signal of higher molecular weight appeared with treatment of the coupled translation/reverse transcription reaction with proteinase K prior to the extraction of the nucleic acids with organic solvents (Fig. 4b, lane 5). The majority of the radioactive bands disappeared after a 2-hour DNase I treatment (Fig. 4b, lane 6). As controls, translation/reverse transcription reactions were performed in extracts that were not supplemented with exogenous HBV polymerase mRNA. Only unspecific bands were obtained (Fig. 4b, lanes 1 and 2).

In parallel, the coupled translation/reverse transcription reactions were also incubated with DNase I, RNase A or proteinase K after the completion of the coupled translation/reverse transcription reaction. The proteins were thereafter analysed by 10% SDS-PAGE followed by autoradiography. This revealed that the radioactive signal was nearly completely eliminated after hydrolysis of the nucleic acids with DNase I, with the major exception of the presence of a radiolabelled polypeptide of around 90 kDa and various minor polypeptide bands ranging between 48 and 65 kDa (Fig. 4c, lane 2). The hydrolysis of the RNAs using RNase A did not reduce the intensity of the radioactive signal (Fig. 4c, lane 3) when compared to the control reaction performed with HBV polymerase and without further enzymatic treatments (Fig. 4c, lane 1). However, treatment with RNase A also generated minor radioactive bands ranging between 48 and 65 kDa. Finally, treatment with proteinase K did not interfere with the presence of a strong radioactive signal (Fig. 4c, lane 4) when compared to the reaction without further treatment (Fig. 4c, lane 1).

We further tested whether the [ $^{32}$ P]-radiolabelled signal obtained in the coupled translation/reverse transcription reaction was indeed specifically the reverse transcribed HBV polymerase DNA. To test this, the [ $^{32}$ P]-radiolabelled, reverse transcribed DNA was treated with proteinase K, extracted with organic solvents, precipitated in the presence of glycogen and finally employed as a molecular probe



**Fig. 5: Analysis of the HBV polymerase DNA by Southern blot**

a) The reverse transcribed HBV polymerase DNA that is synthesized in the *in vitro* coupled translation/reverse transcription reaction can be employed as a radiolabelled molecular probe for the detection of the HBV polymerase DNA. Reactions were performed as described in Fig. 3a, followed by treatment with proteinase K. The [ $^{32}$ P]-radiolabelled, reverse transcribed DNA was thereafter extracted with organic solvents, purified, and employed as a molecular probe for detection in a Southern blot analysis of the *Xho* I/ *Nco* I and the *Hin* dIII/ *Eco* RI DNA fragments of the HBV polymerase digested in plasmid HH3, as indicated. These 1,250 and 870 bp corresponding fragments, excised from the N-terminal and C-terminal coding sequence of the HBV polymerase, respectively, are both hybridised by the radiolabelled reverse transcribed, HBV polymerase DNA probe.

b) The newly reverse transcribed HBV polymerase DNA can be hybridized by a radiolabelled HBV DNA probe. The coupled translation/reverse transcription reactions were performed with HBV polymerase mRNA using all 20 unlabelled amino acids, and were then treated with proteinase K (lane 5), DNase I (lane 6) or RNase A (lane 7), or not treated (lanes 3 and 4). Control reactions were performed in the absence of exogenous HBV polymerase mRNA (lanes 1 and 2). Where indicated, the nucleic acids were then extracted with organic solvents, precipitated, migrated in 1% agarose gel, transferred to a nitrocellulose membrane, and finally analyzed by Southern blotting using a radiolabelled HBV DNA probe followed by autoradiography. Sizes are indicated in kilobases (kb).

for the detection of various HBV DNA fragments in a Southern blot analysis. As shown in Figure 5a, both the *Xho* I/ *Nco* I and the *Hin* dIII/ *Eco* RI fragments digested from plasmid HH3 were recognised by the reverse transcribed, [ $^{32}$ P]-radiolabelled probe. This revealed that the probe was specifically HBV polymerase DNA. The reverse transcription performed by the HBV polymerase generated a large DNA fragment, since the 870 bp-long *Hind*III/ *Eco*RI fragment from plasmid HH3 was hybridized.

In order to confirm more precisely the fate of the reverse transcribed DNA after synthesis in the coupled translation/reverse transcription reaction, a Southern

blot analysis using a radiolabelled probe composed of the genomic HBV DNA was performed. In a first step, the reverse transcript was generated by coupling the translation/reverse transcription reaction in the presence of the HBV polymerase mRNA, but in the absence of any radiolabelled deoxynucleotide. The reactions were further treated or not with various enzymes followed by extraction of the nucleic acids with organic solvents and precipitation. The nucleic acids were then separated by agarose gel electrophoresis, transferred to a nitrocellular membrane, and finally hybridized with a radiolabelled HBV DNA probe. This revealed that the HBV DNA probe specifically hy-

bridized to the HBV DNA sequences that were reverse transcribed in the coupled translation/reverse transcription reaction. Indeed, a specific major band was recognized (Fig. 5b, lanes 3 to 7). Larger additional bands were also obtained (Fig. 5b, lanes 4, 5 and 7). Importantly, proteinase K treatment prior to the extraction of the nucleic acids revealed the presence of an additional higher band, thus revealing the reverse transcript covalently bound to the HBV polymerase (Fig. 5b, lane 5). Moreover, the treatment (15 min) of the nucleic acids with DNase I led to the extinction of the larger nucleotide bands and to the diminution of the intensity of the lower signals (Fig. 5b, lane 6), thus probably reflecting the fact that a longer incubation period in the presence of DNase I might be required for the total extinction of this signal. The hydrolysis of the nucleic acids with RNase A did not affect the intensity of the radioactive signal (Fig. 5b, lane 7). Analysis of the crude reverse transcribed HBV polymerase DNA revealed a signal that did not enter into the agarose gel, but remained in the well (Fig. 5b, lane 3). This can be explained by the fact that the newly reverse transcribed DNA might be surrounded by proteins. As an internal control, the endogenous nucleic acids present in extracts obtained from HuH-7 cells were analysed after extraction or not with organic solvents followed by precipitation. This revealed that the HBV DNA probe did not hybridise to contaminating HBV DNA sequences in this cellular extract (Fig. 5b, lanes 1 and 2).

### 3.3 The HBV polymerase requires an $\epsilon$ structure on the mRNA and a catalytic YMDD amino acid sequence for reverse transcription activity

It was crucial to determine whether the  $\epsilon$  signal was necessary for the reverse transcription activity of the HBV polymerase in the coupled translation/reverse transcription assay. To test this hypothesis, plasmid HH3 was linearised with restriction enzyme *Fsp* I. The *in vitro* transcription of the linearised plasmid generated an mRNA without the  $\epsilon$  signal at the 3' end. The *in vitro* translation of this transcript generated a full length HBV polymerase (not shown). However, the translation/

reverse transcription of this mRNA resulted in a polypeptide incompetent of reverse transcription activity on the HBV polymerase mRNA (Fig. 6, lane 3). As a positive control, the mRNA coding for the HBV polymerase and containing an intact  $\epsilon$  signal was translated into a polypeptide providing reverse transcription activity on the HBV polymerase mRNA (Fig. 6, lane 2). Thus, the absolute dependence on an  $\epsilon$  signal on the mRNA was proven for the reverse transcription activity. Moreover, this result confirmed that the biologically active, full-length HBV polymerase was not able to reverse transcribe putative contaminating HH3 plasmid DNA in the RT reaction. Indeed, HBV polymerase mRNA lacking an  $\epsilon$  signal was unable to synthesise a radiolabelled molecular DNA probe that could be employed for

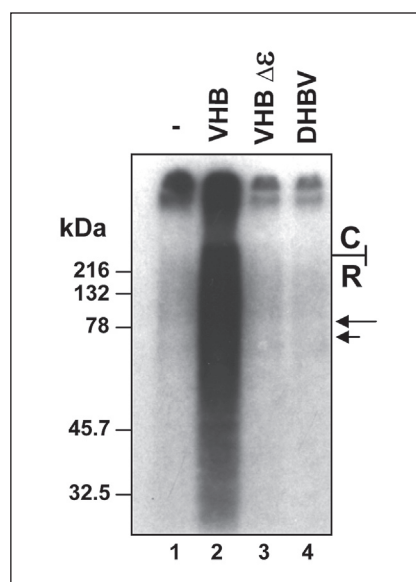
the detection of HBV polymerase DNA fragments in a Southern blot analysis in a coupled translation/reverse transcription reaction (not shown), thus suggesting that the HBV polymerase per se was unable to reverse transcribe either an RNA lacking an  $\epsilon$  signal or a putatively contaminating plasmid DNA in the coupled translation/reverse transcription reaction.

On an other hand, the presence of the catalytic YMDD motif in the viral RNA-dependent DNA polymerase is a prerequisite for its activity (Poch et al., 1989). We therefore tested, whether an YMDD motif was necessary for the reverse transcription activity in the HBV polymerase. To test this hypothesis, an HBV coding sequence containing a point mutation at amino acid position 152 (new stop codon TAG instead of TGG) was generated. After linearisation of the plasmid with restriction endonuclease *Pvu* II (thus maintaining the  $\epsilon$  signal on the mRNA), *in vitro* transcription followed by *in vitro* translation, a truncated HBV polymerase protein of about 18 kDa was synthesised (not shown). When the mRNA containing the point mutation was employed in the coupled translation/reverse transcription reaction, no [ $^{32}$ P]-radiolabelled polypeptide was obtained (not shown), even though the mRNA contained an  $\epsilon$  structure at the 3' end. This suggested that the  $\epsilon$  structure alone did not confer reverse transcription activity to the HBV polymerase in the absence of the catalytic YMDD motif.

### 3.4 HBV polymerase activity can be obtained in cell extracts other than hepatocytes

Translational extracts were also prepared from other eukaryotic cell lines growing as monolayers, such as LMH and baby hamster kidney (BHK). The extracts were then employed in the coupled translation/reverse transcription assay by using the mRNA coding for the HBV polymerase. This revealed that in addition to the HuH-7 cell extracts (Fig. 2a), the use of both LMH and BHK cell extracts for the coupled translation/reverse transcription reaction of the HBV polymerase could generate a specific radioactive signal (data not shown).

We then tested whether the reticulocyte lysate expression system could be employed for the study of the biological



**Fig. 6: Absence of reverse transcription activity of the HBV polymerase on HBV polymerase mRNA lacking an  $\epsilon$  signal**  
Coupled translation/reverse transcription reactions were performed as described in Fig. 3a, in the presence of an mRNA encoding full length HBV polymerase but lacking the  $\epsilon$  signal at the 3'-end (lane 3). Control reaction with mRNA containing the  $\epsilon$  signal at the 3'-end (lane 2). Control reaction without exogenous HBV polymerase mRNA (lane 1). Proteins were analyzed by 10% SDS-PAGE followed by autoradiography. Both the concentrating (C) and resolving (R) portions of the gel were analyzed. Mr, relative molecular mass (kDa).



activity of the human HBV polymerase. For this, the coupled translation/reverse transcription assay was performed in lysate supplemented with all 20 amino acids and with mRNAs coding for either the DHBV or HBV polymerases. Both polymerases were efficiently translated with their expected molecular masses in this system, as seen with the [ $^{35}\text{S}$ ]methionine incorporation into the newly synthesised polypeptides (data not shown). The synthesised HBV polymerase generated a smeary radioactive signal during the coupled translation/reverse transcription on the HBV polymerase mRNA (Fig. 7, lane 5), however with a 90% lower efficiency when compared to the activity obtained in the HuH-7 cell extract (compare with Fig. 3a). Interestingly, the coupled translation/reverse transcription of the DHBV

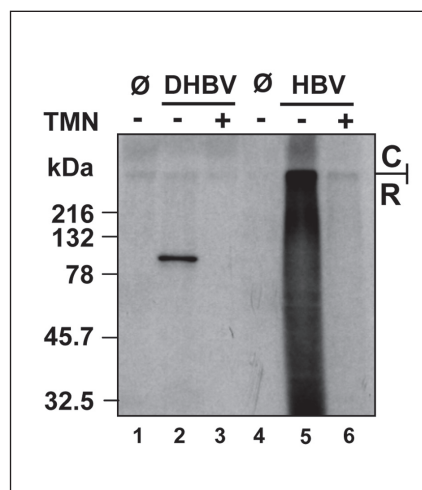
polymerase mRNA generated a single radiolabelled polypeptide band of about 90 kDa (Fig. 7, lane 2). This suggested that only the priming reaction was obtained with DHBV mRNA in a coupled translation/reverse transcription reaction. The addition of TMN buffer in the coupled translation/reverse transcription reaction abolished the generation of radioactive signals with both mRNAs (Fig. 7, lanes 3 and 6).

### 3.5 HBV polymerase activity can be inhibited *in vitro* with dideoxynucleotides triphosphate and nucleoside triphosphate analogues

Using the HuH-7 cell extract, we analysed the inhibitory effect of dideoxynucleotides and nucleoside triphosphate

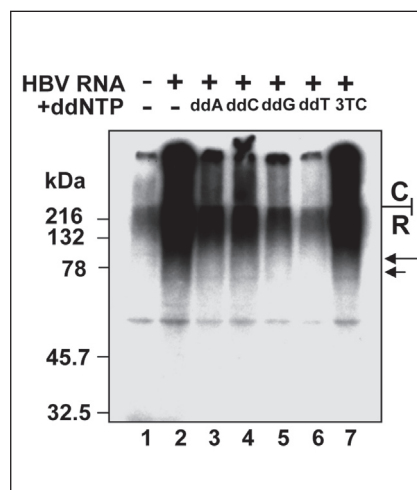
analogues on the HBV polymerase activity *in vitro*. For this, the extracts were employed in coupled translation/reverse transcription reactions using the HBV polymerase mRNA in the presence or absence of the various inhibitors. The level of inhibition of [ $^{32}\text{P}$ ]-labelled DNA synthesis was assessed by quantifying the incorporation of [ $\alpha\text{-}^{32}\text{P}$ ]dCTP into the nascent viral DNA. Although no exogenous dNTPs were added in the reactions and since there is a pool of endogenous dNTP-TPs in the HuH-7 extracts (as was shown elsewhere with the use of the DHBV RNA in the reticulocyte lysate; Le Guerhier et al., 2001), the results showed that the incorporation into the nascent viral DNA was significantly inhibited with the presence of ddNTPs triphosphate (Fig. 8). The most potent inhibition was obtained with the use of ddTTP-TP (Fig. 8, lane 6) with nearly 100% inhibition, when compared to the control reaction performed in the presence of the exogenous HBV polymerase mRNA but without additional ddNTP-TP (Fig. 8, lane 2) (mean of three experiments). This was followed by ddGTP-TP (Fig. 8, lane 5; 90% inhibition), ddATP-TP (Fig. 8, lane 3; 80% inhibition) and ddCTP-TP (Fig. 8, lane 4; 70% inhibition), respectively. This result was consistent with an order of nucleotide addition 5'-dT-dG-dA during the reverse transcription of the HBV polymerase.

The use of 2'-3'-dideoxy-3'-thiacytidine triphosphate (3TC-TP; lamivudine triphosphate) could act as an inhibitor of the elongation activity of the HBV polymerase in the coupled translation/reverse transcription reaction. To test this hypothesis, increasing concentrations of 3TC-TP were employed. The results showed that 50% inhibition of dCTP-TP incorporation into the nascent DNA was obtained with the use of 2.5  $\mu\text{M}$  of 3TC-TP (mean of three experiments; not shown). Thus, the results showed that a chain terminator of the reverse transcription activity of the HBV polymerase could be analysed *in vitro* in eukaryotic cell extracts. We thereafter tested whether other nucleotide analogues or inhibitory molecules could inhibit the activity of the HBV polymerase *in vitro*. This revealed that candidate inhibitors such as PFA (foscarnet), DXG-TP or DAPD-



**Fig. 7: Analysis of the HBV polymerase activity in the rabbit reticulocyte lysate system**

The mRNAs coding for either DHBV polymerase (lanes 2, 3) or HBV polymerase (lanes 5, 6) were employed with the use of [ $\alpha\text{-}^{32}\text{P}$ ]dGTP or [ $\alpha\text{-}^{32}\text{P}$ ]dTTP, respectively, as a radiolabel. Coupled translation/reverse transcription reactions were also performed with additional TMN buffer (lanes 3 and 6). Proteins were analysed by 10% SDS-PAGE followed by autoradiography. Both the concentrating (C) and resolving (R) portions of the gel were analysed.  $M_r$ , relative molecular mass (kDa).



**Fig. 8: Inhibition of HBV polymerase activity by dideoxynucleotides triphosphate and nucleoside triphosphate analogue *in vitro***

Coupled translation/reverse transcription reactions were performed as described in Fig. 3a. Coupled translation/reverse transcription reactions were performed in the presence of [ $\alpha\text{-}^{32}\text{P}$ ]dCTP plus various combinations of unlabelled ddNTPs, as indicated in the Figure. Weak labelling was obtained with the use of ddTTP followed by ddGTP, ddATP and finally ddCTP, in agreement with the synthesis of a primer with an authentic sequence. Proteins were analysed by 10% SDS-PAGE followed by autoradiography. Both the concentrating (C) and resolving (R) portions of the gel were analysed.  $M_r$ , relative molecular mass (kDa).



TP possessed 50% inhibitory concentrations ( $IC_{50}$ ) of 0.5 mM, 0.8-2  $\mu$ M and 0.15-0.30  $\mu$ M, respectively. When the conventional inhibitor 3TC-TP (lamivudine triphosphate) was employed, 50% inhibition of reverse transcriptase activity was obtained at a concentration of 2.5  $\mu$ M (data not shown). These inhibitory concentrations are relevant for reduction of hepadnaviral reverse transcription *in vitro*, as compared to other similar inhibitory studies such as the rabbit reticulocyte lysate system.

## 4 Discussion

### 4.1 The HBV polymerase and the role of an *in vitro* expression system

Several animal studies have been performed in the past for the screening of new antiviral molecules that inhibit the hepadnavirus replication, either *in vivo* with studies involving the sacrifice of the experimental animals (Addison et al., 2002; Cao and Tavis, 2006; Cavanaugh et al., 1997; Colledge et al., 1997; Cullen et al., 1997; Fourel et al., 1994; Genovesi et al., 2000; Hafkemeyer et al., 1996; Howe et al., 1996; Lin et al., 1998; Lofgren et al., 1996; Luscombe et al., 1996; Nicoll et al., 2000; Nicoll et al., 1998; Offensperger et al., 1993a; Rahn et al., 1997; Rajagopalan et al., 1996; Seigner et al., 2000; Severini et al., 1995; Tomita et al., 2000; Urban et al., 2001; Witcher et al., 1997) or *in vivo* with additional *in vitro* studies using the rabbit reticulocyte lysate system (Aguesse-Germon et al., 1998; Doong et al., 1991; Guo et al., 2007; Jacquard et al., 2006; Le Guerhier et al., 2001; Offensperger et al., 1993b; Robaczewska et al., 2005; Seifer et al., 1998; Seigner et al., 2001; Seigner et al., 2003; Shaw et al., 1996; Zoulim et al., 1996) (Tab. 1).

Reliable ways and means can be employed in order to investigate human biology and health issues *in vitro*, for example in the field of viral infections. The assessment method builds up from molecules and cells to the individual via tissues and organs. It is in strong contrast to the top-down approach of the animal model, which faces at the outset the full complexity of the animal. By producing

**Table 1: RRL (rabbit reticulocyte lysate)**

Scientific article	Number of animals
Offensperger et al., 1993	18 ducklings
Severini et al., 1995	One duck
Rajagopalan et al., 1996	3 woodchucks
Howe et al., 1996	24 ducklings
Luscombe et al., 1996	18 ducklings
Zoulim et al., 1996	13 ducklings
Hafkemeyer et al., 1996	11 ducklings
Lofgren et al., 1996	20 ducks
Colledge et al., 1997	Primary duck hepatocytes
Rahn et al., 1997	Primary duck hepatocytes
Witcher et al., 1997	3 woodchucks
Cullen et al., 1997	25 woodchucks
Cavanaugh et al., 1997	56 transgenic mice
Aguesse-Germon et al., 1998	23 ducklings, RRL, primary duck hepatocytes
Nicoll et al., 1998	18 ducklings
Genovesi et al., 1998	23 woodchucks
Lin et al., 1998	18 ducks
Seifer et al., 1998	1 woodchuck, RRL
Seigner et al., 2000	29 ducklings
Tomita et al., 2000	30 ducks
Nicoll et al., 2000	16 ducklings
Seigner et al., 2001	Primary fetal duck hepatocytes, RRL
Le Guerhier et al., 2001	67 ducklings
Urban et al., 2001	4 ducks
Addison et al., 2002	14 ducks
Seigner et al., 2003	141 ducks, primary fetal duck hepatocytes, RRL
Zhang et al., 2004	48 ducklings
Hu et al., 2004	
Robaczewska et al., 2005	RRL, primary duck hepatocytes
Wang et al., 2005	16 ducklings
Jacquard et al., 2006	RRL
Guo et al., 2007	< 36 ducks ; recombinant mice (n= ?)

a biologically active HBV polymerase *in vitro*, it might be envisioned that the molecular responses of the polymerase to antiviral compounds can be studied on a broad scale, by means of reactions on biochips, for example. Hundreds or thousands of putative inhibitory molecules can be selected for their specific activity in inhibiting the initiation and the elongation of the HBV polymerase. This will undoubtedly lead to a better comprehension of the processes involved in the inhibition of this medically important enzyme by reducing, refining and replacing (3Rs) the use of animals and animal extracts.

### 4.2 General considerations on the synthesis of hepatitis virus polymerases

Analysis of the HBV polymerase has been hampered for many years due the inability to efficiently express this functional enzyme in a recombinant system *in vitro*. For this reason, the investigations have mostly relied on studies of the duck hepatitis B virus (DHBV) system. For the polymerase protein of DHBV, but not that of HBV, one of the systems described has shown that the priming reaction and primer elongation can be artificially reconstituted by *in vitro* translation of the protein in the rabbit reticulocyte lysate (Wang and Seeger, 1992). The other system packages an active fusion protein of DHBV polymerase in a virus-like particle

from the yeast retrotransposon Ty1 (Tavis and Ganem, 1993). These two systems yield a polymerase that possesses accurate protein-primed reverse transcriptase activity that synthesises minus-strand DNA originating at  $\epsilon$  and DR1 (Tavis et al., 1994). The polymerase mRNA employed contained a 3' copy of  $\epsilon$ , but no 5' copy of this sequence. Recently, a third polymerase expression system that utilises purified human HBV polymerase that was expressed *via* the baculovirus-insect cell expression system has been described. The purified HBV polymerase was active for *in vitro* priming and reverse transcriptase reactions. Some other reports claimed that the human HBV polymerase was active in the rabbit reticulocyte lysate expression system (Kim and Jung, 1999) or in *E. coli* (Jeong et al., 1996). In this respect, a major breakthrough was performed with the coupling of the transcription and translation of the HBV polymerase mRNA in the rabbit reticulocyte lysate expression system (Li and Tyrrell, 1999). Importantly, both the priming and the elongation reactions were performed in this system by the concomitant transcription and translation of the mRNA in the presence of either a protein priming buffer or a polymerisation buffer, respectively, containing an additional radiolabelled deoxynucleotide. In addition to the generation of radiolabelled polypeptide bands at 94, 81 and 40 kDa, radioactivity appeared at the top of the gel in both the stacking and resolving portions. The polymerase generated DNA fragments of around 200 and 400 bp, the latter seemingly representing a DNA product with a length twice that of the original RNA template (Li and Tyrrell, 1999).

#### 4.3 Coupling translation with reverse transcription *in vitro*

Recently, we generated an *in vitro* translation system from eukaryotic cells that were grown as monolayers (Favre and Trépo, 2001). Since this expression system is particularly suited for the translation of viral mRNAs originating from various sources, it was thus employed for the generation of a biologically active HBV polymerase *in vitro*. It has been shown that translational extracts from HepG2 and HuH-7 cell lines still con-

tain free deoxynucleotides (Gaillard et al., 2002). Since the reverse transcription reaction of the HBV polymerase occurs in the cytoplasm of infected cells, we considered that the generation of translational extracts without further equilibration and complementation, and also without the hydrolysis of the endogenous mRNAs, was providing an expression system that would approach the natural cellular conditions present in eukaryotic cells very closely.

We have shown that both the HBV and DHBV polymerases were efficiently translated in the cytoplasmic extracts that were obtained from eukaryotic cells, as revealed with the [ $^{35}$ S]methionine labelling of the polypeptides (Fig. 2). For the HBV polymerase, three major polypeptides of 94, 81 and 40 kDa were synthesised. These results were identical to those obtained with the translation of the HBV polymerase in the rabbit reticulocyte lysate (Li and Tyrrell, 1999). Thus, the expression system described here was a strong candidate for the *in vitro* generation of an HBV polymerase that would possess inherent and efficient reverse transcriptase activity. In initial trials, the HBV polymerase was translated in a first reaction involving the mRNA and all unlabelled amino acids, and thereafter the reverse transcriptase assay was performed in a second reaction in which additional radiolabelled dNTPs were added. It turned out that this procedure did not allow the generation of radiolabelled reverse transcripts. It finally turned out that the stumbling block was the stepwise procedure initially employed. Circumstantial evidence confirms that the reverse transcribed DNA is indeed the expected HBV polymerase DNA.

First, a strong radioactive signal was solely obtained under conditions in which the translation reaction was tightly coupled to the reverse transcription reaction *per se*, as revealed by the incorporation of radiolabelled dNTPs into the newly synthesised DNA (Fig. 3a). It has been shown that the removal of the endogenous template from purified HBV polymerase by nuclease treatments resulted in the loss of the nucleotide priming activity. Because of the inherent procedure employed for the expression of the HBV polymerase in extracts obtained from eukaryotic

cells, it was not feasible to hydrolyse the RNA template, because this would have led to the absence of HBV polymerase expression. Anyway, the presence of the HBV polymerase mRNA in a coupled translation/reverse transcription reaction performed in the presence of the specific inhibitor of the initiation of protein synthesis pTp, resulted in the total lack of a radiolabelled signal, thus confirming that no putative unexpected endogenous reverse transcriptase activity was present in the reactions. Thus, the translation of the HBV polymerase mRNA was a prerequisite for the generation of a radioactive signal (Fig. 3b).

Second, the reverse transcriptase activity generated a large DNA transcript that was sensitive to DNase I treatment (Fig. 4a). The reverse transcript was covalently bound to protein, as revealed by Southern blot analysis of the nucleic acids after the proteinase K treatment (Fig. 4b). Surprisingly, it appeared that a substantial portion of the reverse transcript was not covalently bound to protein. Indeed, there are multiple potential reasons why full-length HBV polymerase is not fully covalently bound to the minus strand of the reverse transcript. This might be due to the inherent differences in the assay systems employed for the generation of a biologically active HBV polymerase, as well as for the DHBV polymerase.

Third, a single radioactive polypeptide of 94 kDa was obtained after the digestion of the nucleic acids with DNase I, thus suggesting that this polypeptide was HBV polymerase covalently bound to primed dTTP (Fig. 4c). A substantial amount of radioactive signal was obtained after the digestion of the proteins with proteinase K. These striking features might be explained by the fact that the DNA could somehow enter into the protein gel due to the net negative charge of the unbound, radiolabelled reverse transcribed DNA. In this respect, we can ask ourselves, whether the claims of the presence of extension products of the HBV polymerase in previously published articles (Tavis and Ganem, 1993) were in fact not due to the presence of DNA molecules covalently bound to the polymerase, but instead of free radiolabelled, reverse transcribed HBV DNA molecules. Fourth, we have shown that the newly



reverse transcribed DNA was specifically the HBV polymerase DNA, since it could be specifically employed as a molecular probe for the detection of HBV DNA in a Southern blot analysis (Fig. 5).

For the polymerase of DHBV, but not that of HBV, the reverse transcription reaction could be initially artificially reconstituted by *in vitro* translation, and this activity relies on the specific  $\epsilon$  structure either in cis or in trans on the mRNA. By contrast, the HBV polymerase activity of the HBV polymerase expressed from recombinant baculoviruses could be obtained in the absence of the  $\epsilon$  signal, however to a lesser extent than with an  $\epsilon$  signal on the mRNA (Lanford et al., 1997). The same phenomenon has been demonstrated in *Xenopus* oocytes in the absence of  $\epsilon$  as well, however without characterisation of the priming reaction in this system (Seifer and Standring, 1993). The requirements for *in vitro* priming are less stringent than *in vivo*, since the 5' copy of  $\epsilon$  in HBV genomic replication is absolutely required, as shown by mutation studies (Nassal and Rieger, 1996). In contrast to the results obtained in these two systems, we have shown that the deletion of the  $\epsilon$  signal on the mRNA coding for the full-length HBV polymerase did result in a complete lack of reverse transcription activity (Fig. 6). Thus, it appeared that the interaction of the HBV polymerase with the viral  $\epsilon$  stem-loop structure on the mRNA during the coupled translation/reverse transcription reaction was necessary to induce or activate the reverse transcription activity of the HBV polymerase, at least in translational extracts obtained from human hepatocytes.

Finally, significant levels of polymerase mRNA have been shown to co-purify with the polymerase, albeit in a highly degraded state. Interestingly, only 1% of the purified HBV polymerase protein expressed with the baculovirus system displayed reverse transcription activity. Thus, we can ask, whether this residual reverse transcription activity was indeed not due to the potential priming and the extension reactions of the HBV polymerase on these remaining RNA molecules that potentially contain an  $\epsilon$  signal.

#### 4.4 Inhibition of the HBV polymerase *in vitro*

We have shown that the use of about 2.5  $\mu$ M 3TC-TP in the assay inhibited 50% of the reverse transcription activity of the HBV polymerase. This inhibitory activity closely resembles that obtained using the DHBV polymerase in the rabbit reticulocyte lysate (Seigneres, 2003). It is clear that the exact determination of the IC<sub>50</sub> measured in our work requires further investigation, because the assay was based on the presence of the endogenous deoxynucleotides for the reverse transcription activity. For a more precise measure of the inhibitory activity of the antiviral compounds, the translational extracts should first be passed through a sepharose column in order to remove low molecular weight molecules (such ions and nucleotides), and thereafter supplemented for standardisation with various molecular compounds, as described elsewhere (Ochoa and de Haro, 1979; Pelham and Jackson, 1976). Our initial attempts to standardise the translational extracts by passage through a column and supplementation failed to generate extracts in which efficient *in vitro* translation on exogenous mRNAs occurred. Further work is thus needed to circumvent this technical problem. This peculiar minor bias kept in mind, we have analysed the fate of various putative candidate molecules to inhibit the activity of HBV polymerases containing mutations in the YMDD catalytic site. The hepadnavirus polymerases are sensitive to mutations in respect to the viral replication cycle. It has been shown that the YVDD and YIDD mutants fail to respond to lamivudine therapy. Plasmids HH3 containing these point mutations in the YMDD catalytic sites were constructed. In preliminary experiments, we were able to show that the promising antiviral molecules DAPD-TP and DXG-TP were effective as inhibitory compounds for the inhibition of the HBV polymerase containing the mutations in the YMDD catalytic site (D. Favre, unpublished observations). Further work is needed to study these inhibitory activities in more detail.

We have observed that the expression of the HBV and DHBV polymerases in

the rabbit reticulocyte lysate showed striking differences (Fig. 7). A single polypeptide band was observed with the DHBV polymerase, thus suggesting that only the priming reaction took place. In contrast, the expression of the HBV polymerase resulted in a smear, without the appearance of a defined polypeptide. Moreover, it was also intriguing to observe that the DHBV polymerase was solely active in the initiation step but not in the elongation step in this coupled translation/reverse transcription assay. These observations might be explained by the fact that several differences might exist between the HBV and the DHBV polymerases. These are for example the inherent differences in the assay systems employed, and also the intrinsic behaviour of these two polymerases.

#### 5 Concluding remarks

In conclusion, this study provides the first evidence that HBV polymerase produced in translational extracts obtained from eukaryotic cells grown as monolayers could initiate reverse transcription *in vitro* in the absence of other viral proteins. We favour a model in which a mechanism is active for the regulation of the ribosome and the HBV polymerase on the same mRNA template. Once translation is completed, the polymerase immediately initiates (-) strand synthesis. A similar mechanism has been proposed for poliovirus (Herold and Andino, 2001) and barley yellow dwarf virus (Barry and Miller, 2002).

The expression system described in this work might represent an important initial step towards the detailed understanding of hepadnaviral genomic replication. The availability of a biologically active HBV polymerase in a coupled translation/reverse transcription assay provides a means for the *in vitro* screening of inhibitory molecules against the reverse transcription activity of the HBV polymerase. Moreover, it may be employed for the screening of antiviral molecules directed against mutants of the polymerase, such as the YIDD or the YVDD mutants (instead of YMDD in the catalytic site of the wild-type polymerase). Such inhibi-



tors could provide novel approaches to antiviral therapy without the use of large amounts of laboratory animals in the screening process.

This scientific article is a contribution to the 3Rs, for the following reasons:

- With the use of this *in vitro* procedure, it can reduce the number of animals and animal extracts that are employed for the study of the HBV polymerase.
- It is refining the procedure employed in the rabbit reticulocyte lysate with duck HBV polymerase: an ameliorated procedure can be performed in eukaryotic extracts by doing the concomitant transcription/translation of the HBV polymerase. This was not possible in the rabbit reticulocyte lysate with duck or human HBV polymerase.
- It can replace the use of the latter animals and animal extracts; there is no further need for ducks, ducklings and rabbit reticulocyte lysate.

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