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DUCK HEPATITIS B VIRUS POLYMERASE - CLONING AND EXPRESSION, ANTIVIRAL THERAPY AND TRANSLATION REGULATION

by

ANITA YEE MEI HOWE 

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled DUCK HEPATITIS B VIRUS POLYMERASE - CLONING AND EXPRESSION, ANTIVIRAL THERAPY AND TRANSLATION REGULATION in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Virology.

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Abstract

Our laboratory has used duck hepatitis B virus (DHBV) infected primary duck hepatocytes to screen compounds for activity against hepadnaviral replication. Results from these studies indicated that 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR) and 2',3'-dideoxyguanosine (ddG) were potent inhibitors of DHBV replication, whereas pyrimidine 2',3' dideoxynucleosides were not. We have hypothesized that ddGTP (metabolized from ddDAPR) might block DHBV DNA replication by binding to the protein primer domain of the viral polymerase. The work presented in this study was undertaken to prove the above hypothesis.

DHBV polymerase was expressed *in vitro* using bacteriophage T7 polymerase transcription and rabbit reticulocyte lysate translation systems. The *in vitro* translated polymerase exhibited DNA dependent DNA polymerase and reverse transcriptase activities when tested on DNA or RNA templates, respectively, primed with DNA-hexamers. In the presence of actinomycin D, there was about 70% inhibition of the DNA dependent DNA polymerase activity, whereas the reverse transcriptase acitvity remained unchanged. The self-priming property of this *in vitro* translated polymerase was demonstrated by its reverse transcriptase activity on endogenous templates and its binding to dGTP, the first nucleotide which covalently links the polymerase to the rest of the DHBV genome.

The mechanism of inhibition of DHBV replication by ddDAPR or ddG was studied using both *in vitro* and *in vivo* experimental conditions. In DHBV infected primary duck hepatocyte cultures, ddDAPR or ddG was found to compete with dG for hepadnaviral DNA replication. A selective and irreversible inhibition of DHBV DNA replication was observed in ducklings treated with high doses of ddDAPR (20 - 50 mg/kg), but not with similar doses of ddC. The inhibition mediated by ddDAPR occured at a very early stage of the reverse transcription. However, the DNA polymerase and reverse transcriptase activities of the polymerase were found to remain active when tested on exogenous templates in activity gels. I have demonstrated direct binding of [α-^{32}P] ddGTP to the *in*

vitro expressed DHBV polymerase. These results suggested that the binding of ddGTP to the polymerase blocked the initial DNA synthesis, plausibly at the priming stage of the hepadnaviral replication.

Formation of the nucleocapsid requires selective encapsidation of the pregenomic RNA template and the viral polymerase by the core proteins. It has been shown that an encapsidation signal located at the 5' end of the pregenomic RNA is responsible for its interaction with the polymerase. Using the *in vitro* translated polymerase, I have shown that a second region located at nt 401 - 820 in the pregenomic RNA may interact with the viral polymerase in duck hepatitis B virus (DHBV). Interaction of the polymerase with this region resulted in selective suppression of core mRNA translation. Insertion of this putative binding sequence into the CD4 gene also led to a selective inhibition of CD4 mRNA translation in the presence of polymerase. Using a UV cross-linked label transfer technique, I demonstrated that DHBV polymerase bound directly to RNA. These results suggested that DHBV nucleotide sequence 401- 820 of the pregenomic RNA might be a target for polymerase binding. Interaction of the polymerase with this RNA sequence might occlude ribosomal entrance to the 5' end of the pregenomic RNA, leading to translational arrest of core mRNA and permitting exposure of the ε sequence to signal encapsidation.

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Chapter 1

Introduction

I. HISTORY

Viral hepatitis is a general term describing liver infections caused by at least five markedly different viruses. Some of these, such as Hepatitis A virus (HAV) and Hepatitis B virus (HBV), have been identified and well characterized while others, such as viruses of Hepatitis C, D and E, still remain less well understood.

The earliest descriptions of hepatitis were recorded by the Babylonian Talmud and Hippocrates in the fifth century B.C. (Lyons, 1978). Detailed observations of parenterally transmitted forms of hepatitis were reported in the eighteenth and nineteenth centuries when outbreaks of hepatitis occurred in Europe. In all of these cases, inoculations of human serum as vaccines for immunization against smallpox, measles and yellow fever were believed to be the primary cause of the infection (McNalby, 1937; Lurman, 1885; Findlay and MacCallum, 1938). In 1942, a widespread epidemic of hepatitis in United States military troops resulted in 28,000 cases with 62 deaths (Sawyer *et al.*, 1944). These series of outbreaks stimulated hepatitis research.

Repeated attempts to isolate and propagate the etiological agents from susceptible laboratory animals or tissue culture were not successful. Based on the seroepidemiological characteristics of the diseases, Krugman *et al.* (1967) defined two distinct types of hepatitis - infectious (Type A) and serum hepatitis (Type B). Infection by one hepatitis agent conferred no immunity against the other virus.

In 1963, Blumberg examined thousands of blood samples from patients with hemophilia for isoprecipitins and subsequently discovered that a serum sample from an

Australian aborigine contained an antigen that specifically reacted with serum from an North American hemophiliac patient (Blumberg, 1967). This antigen was called Australian (Au) antigen and was subsequently found to be relatively rare in European or North American patients but prevalent in some African and Asian populations. The discovery of this antigen provided an essential key to the understanding of the clinical and epidemiological behavior of HBV. However, the infectious virion for HBV remained unclear until 1970 when Dane *et al.* detected the complete 42 nm spherical virion using electron microscopy. Subsequent research confirmed the existence of the virus and its association with the previously described Australian-antigen-associated hepatitis (Magnius and Espmark, 1972).

In 1975, the first hepatitis vaccine using purified plasma-derived Au antigen, now designated as hepatitis B surface antigen (HBsAg), was developed. With the advance of the biotechnology and molecular cloning techniques, safe and effective vaccines have been prepared in different non-human organisms (Hammond *et al.*, 1991; Penna *et al.*, 1992). However, no satisfactory antiviral chemotherapeutic agents have been developed for the treatment of chronic carrier of HBV.

II. EPIDEMIOLOGY

1. Mode of Spread

Parenteral or percutaneous transmission, in particular the exchange of blood or body fluids, is the major route of HBV infection. Although HBV levels in blood are 100 to 1000 times higher than those in other body secretions (saliva, semen, and vaginal secretions of an infected person), these secretions have also been shown to be infectious (Heathcote *et al.*, 1974; Dusheiko and Hoofnagle, 1991). As a result of higher probability of exposure to contaminated blood, hemophiliacs, hemodialysis patients and staff, and intravenous drug abusers are considered to be high risk groups.

Sexual contact with partners infected with HBV increase the risk of acquiring HBV infection. This mode of transmission is predominantly found with homosexuals and sexually promiscuous individuals. Studies have shown that 16 - 40% of sexual partners of individuals with clinical or subclinical hepatitis B infection will contract the disease (Wright, 1975; Inaba *et al.*, 1975).

Maternal-neonatal transmission is the principle mechanism for maintenance of HBV carrier populations in highly endemic areas. The virus does not cross the placenta easily, therefore perinatal transmission is believed to occur during labor or delivery in most cases of maternal-neonatal transmission (Schweitzer *et al.*, 1973). Theoretically, the virus can be passed post-partum through breast feeding, however this practice has not been shown to pose an added risk to infants (Beasley *et al.*, 1975).

2. Geographic Distribution

There are approximately 350 million HBV carriers worldwide. The prevalence of chronic HBV infection varies from less than 2% to more than 8% of the population. Developing countries such as Southeast Asia, China, Philippines, Indonesia, Africa and Pacific Islands constitute the highly endemic areas. Maternal-neonatal transmission is the major mode of spread in these countries (Perrillo, 1993b). In North America and Western Europe, the prevalence of HBV infection is relatively low. In contrast to the pattern of transmission in highly endemic areas, parenteral and sexual transmission are the principle sources of infection in North America and Western Europe. Although the rate of HBV infection has declined in hemophiliacs and homosexual populations due to blood screening and increased awareness of safer sexual practices, hepatitis B seems to be increasing among heterosexuals with multiple partners (Hadziyannis and Schiff, 1992).

III. HEPATITIS B INFECTION

The outcomes of hepatitis B infection are heterogeneous, ranging from subclinical infection to acute or fulminant hepatitis, cirrhosis and/or hepatocellular carcinoma (Nisini and Rizzetto, 1993). Approximately 90% of the patients contracting hepatitis B experience inapparent or anicteric infections (Figure 1-1). The majority of these patients recover completely, while in some the disease progresses to chronic hepatitis. Persistent infection with hepatitis B is associated with higher risk of developing cirrhosis or hepatocellular carcinoma. In patients who experience acute hepatitis, the mortality rate is estimated to be about 0.5 - 1% (Hollinger, 1990).

Viral hepatitis B infection has an incubation period of about 45 - 120 days (Barker and Murray, 1972; Havens, 1946). The duration of the incubation period and the disease progression is related to the virus-host interaction (Barker and Murray, 1972). The pathological sequelae to infection are believed to be mediated by the host humoral and cellular immune responses rather than by a direct cytopathic effect caused by the virus (Feitelson, 1989; Guilhot *et al.*, 1992; Kamogawa *et al.*, 1992).

1. Clinical Markers

Hepatitis B virus is a common human pathogen which causes extensive mortality and morbidity. Although the virus exists in extrahepatic sites such as pancreas, spleen, lymph nodes and kidneys (Omata, 1990), the primary site of infection is the liver. The liver is essential for the metabolism of carbohydrates, proteins and lipids, and plays an important role in drug, toxin, bilirubin, vitamin and metal metabolism. Degeneration of liver function is often associated with abnormal biochemical markers. These include: (1) elevated levels of hepatic enzymes involved in protein metabolism, specifically serum alanine (ALT) and aspartate (AST) aminotransferase; (2) an increase in serum bilirubin concentrations which result from impaired uptake and transport of serum bilirubin in hepatocytes; (3) an elevated level of serum alkaline phosphatase, possibly the result of biliary obstruction; (4) a decrease in blood clotting factors and (5) a reduction in serum

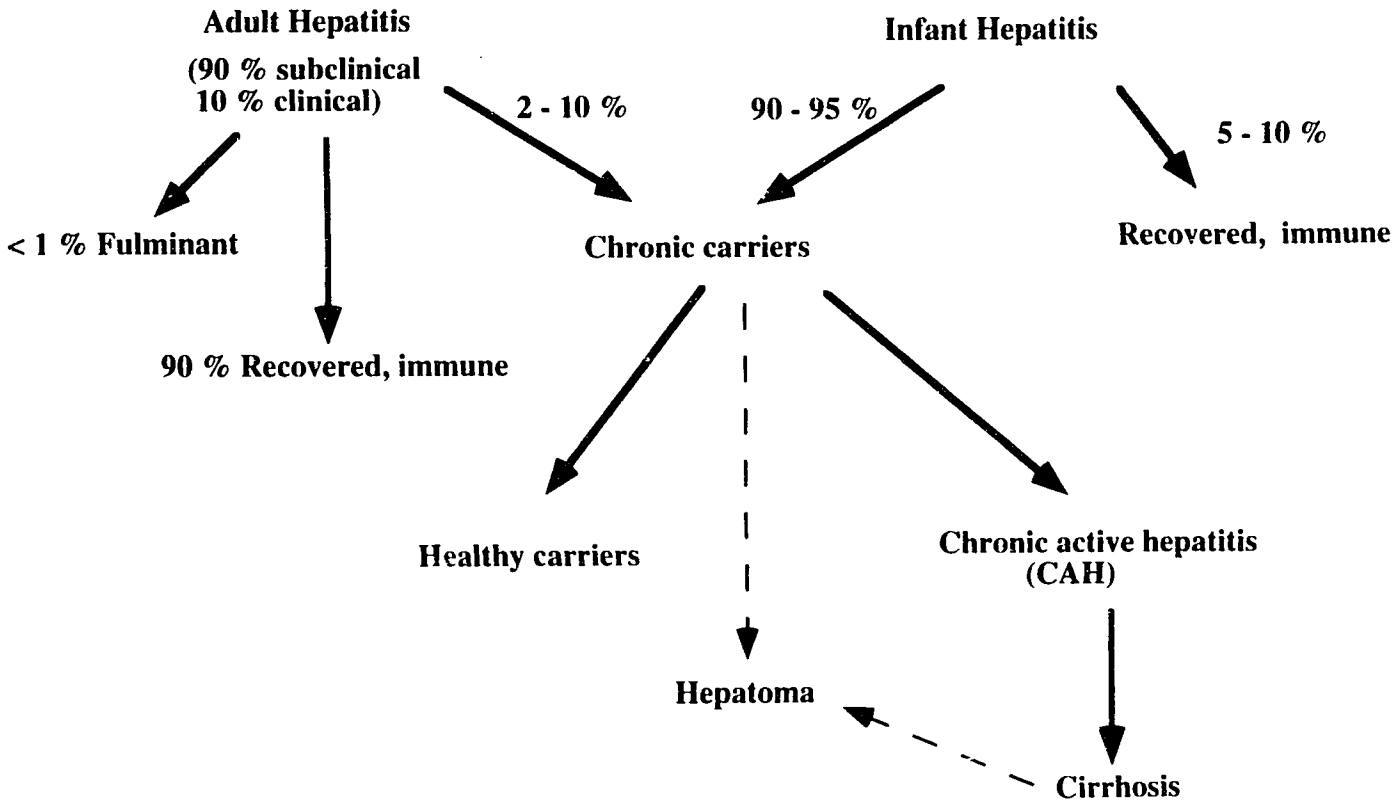


Figure 1-1 Outcome of hepatitis B infection

(Information contained in this figure was obtained from Nisini, R. and Rizzetto, M., 1993)

albumin level with an inverse rise in serum globulin concentration (Hollinger, 1990; Chen and Chen, 1977; Israel and London, 1991).

2. Acute Hepatitis

The severity of hepatitis is related to the location and the extent of the hepatocellular necrosis. Four morphological patterns are commonly observed: classical hepatitis with spotty necrosis; hepatitis with bridging necrosis, hepatitis with panlobular necrosis, or hepatitis with periportal necrosis.

In classical hepatitis with spotty necrosis, the hepatic injury is focal with balloon degeneration, eosinophilic degeneration, and infiltration of lymphocytes, polymorphonuclear granulocytes and monocytes in sinusoids and portal tracts (Scheuer, 1985).

In hepatitis with bridging necrosis, diffuse inflammation is observed between centrilobular areas and portal tracts (Boyer and Klatskin, 1970). Balloon degeneration with rapid lysis which may result in 'dropping out' of hepatocytes from hepatic plates is the major cause of the hepatic damage. Cellular infiltration in sinusoids is predominantly composed of phagocytic cells (Kupper cells). A poorer disease prognosis is anticipated in patients experiencing bridging necrosis than in those having spotty necrosis of the liver.

Acute hepatitis with panlobular necrosis is characterized by massive and severe lesions. Patchy loss of hepatocytes is found throughout the entire lobules with gross disarray of the parenchymal structure. Death appears to be inevitable if necrosis involves more than 65 - 80% of the hepatocytes (Hollinger, 1990).

Hepatitis with periportal necrosis is marked by an accumulation of inflammatory cells around the biliary epithelium, portal and periportal areas. Degenerative changes include paranuclear vacuolization, pyknosis and cytoplasmic eosinophilia (van Thiel *et al.*, 1990). Piecemeal necrosis is seen when the inflammation occurs at the interface of the parenchymal and the connective tissue of the hepatic lobule. Patients with periportal necrosis are at high risk of developing chronic hepatitis and cirrhosis (Bianchi *et al.*, 1979).

3. Chronic Hepatitis

Chronic carriers are patients that have HBsAg detected in their sera for more than 6 months, some of whom will have chronic hepatitis. It occurs in about 2 - 10% of patients following acute infection (Figure 1-1). Histologically, chronic hepatitis can vary from a mild inflammation with minimal cellular infiltration (chronic persistent hepatitis and chronic lobular hepatitis) to more significant parenchymal damage (chronic active hepatitis, CAH).

Chronic persistent hepatitis is usually asymptomatic, although mild hepatomegaly with normal to slightly elevated ALT levels are found in some patients. The prognosis for patients with chronic persistent hepatitis is normally excellent.

Chronic lobular hepatitis is distinguished by series of remissions and relapses. During relapses, ALT level may be increased. Jaundice, acidophilic degeneration, and balloon degeneration localized to the centrilobular region are seen (Peter, 1975; Popper and Schaffner, 1971).

Chronic active hepatitis may occur with or without bridging necrosis and with or without cirrhosis. In the mildest form of CAH, fatigability, anxiety, anorexia and malaise are the most common clinical symptoms. As the disease progresses, elevated levels of ALT, bilirubin and autoimmune antibodies are detected. When present without bridging necrosis, the lobular architecture is preserved, although piecemeal necrosis is seen in periportal and adjacent parenchymal areas (Hollinger, 1990).

Chronic active hepatitis with bridging necrosis is characterized by confluent necrosis across the portal to centrilobular areas. Piecemeal necrosis with lymphocytic and macrophage infiltration are found in the interstitium and parenchyma. The lobular structure is extensively eroded. Regenerating cells often form tube-like clusters (rosettes). Over-activity of the regeneration process as a result of the stimulation from hepatic necrosis may lead to the formation of hyperplastic nodules, a characteristic feature of cirrhosis (Isreal and London, 1991).

IV. HEPADNAVIRIDAE FAMILY

Hepatitis B virus is the prototype member of this family of viruses. All members of the family share similar morphological structures and replication schemes. Other members including woodchuck hepatitis virus (WHV) (Summers *et al.*, 1978), ground squirrel hepatitis virus (GSHV) (Marion *et al.*, 1980), duck hepatitis B virus (DHBV) (Mason *et al.*, 1980) and heron hepatitis B virus (HHBV) (Sprengel *et al.*, 1988) have been identified and characterized. Infection with mammalian hepatitis B viruses causes diverse clinical manifestations, whereas no pathogenic sequelae have been associated with avian hepadnaviral infection. The name Hepadnavirdae denotes the hepatotropic character of these DNA viruses.

1. Morphological Structure

Electron microscopic analysis of sera from HBV infected patients reveals three distinct forms of viral particles: spherical particles of 22 nm in diameter, filamentous particles of the same diameter and various lengths (50 - 1000 nm) (Bayer *et al.*, 1968; Bond and Hall, 1972), and more complex structures known as Dane particles. The Dane particle, a sphere with a diameter of approximately 42 nm, is the mature virion and consists of a 28-nm diameter electron dense inner capsid surrounded by a 7-nm outer envelope (Dane *et al.*, 1970; Jokelainen *et al.*, 1970). The partially double-stranded viral DNA is associated with the inner capsid (Robinson *et al.*, 1974). The 22-nm spherical and filamentous particles are devoid of genetic material and viral polymerase activity (Gerin *et al.*, 1971; Robinson *et al.*, 1973), hence they are non-infectious empty sub-viral particles.

The ultrastructures of DHBV are similar but not identical to those of HBV. The mature DHBV virion is a 40-nm sphere. The inner capsid is characterized by spike-like projections (Mason *et al.*, 1980). In addition to the mature virions, pleomorphic spherical sub-viral particles of 35 - 60 nm in diameter are also found in DHBV-infected liver

(McCaul *et al.*, 1985) . However, no filamentous forms of the empty viral particles have been detected.

2. Genome Organization and Viral Proteins

2.1. Genome Organization

Hepatitis B is a circular partially double-stranded DNA virus with a genome length of approximately 3.2 kbp (Ganem and Varmus, 1987). The circular structure of the genome is maintained by a 225-base-pair cohesive overlap of the plus- and minus-strand DNA molecules (Figure 1-2A). The complete or minus-strand DNA is covalently linked to a protein at the 5' end and has a terminal redundancy of 5 - 10 nucleotides at the 3' end (Summers and Mason, 1982; Will *et al.*, 1987). The incomplete or plus-strand DNA, which encompasses about 15 - 50% of the genome, has a fixed 5' end, but heterogeneous 3' ends. A 19-nucleotide capped oligoribonucleotide has been shown to be linked to the 5' end of the plus-strand DNA (Lien *et al.*, 1987). Unlike the mammalian hepadnaviruses which have incomplete plus-strand DNAs, the plus-strand DNAs of the avian hepadnaviruses are almost full length (> 90% genome length) (Schödel *et al.*, 1990).

Several other functionally important elements in the genome have been identified (Figure 1-2A). Firstly, the 11-base-pair direct repeat and inverted repeat sequences located near the cohesive overlap region have been shown to be important for viral replication (Molnar-Kimber *et al.*, 1984; Seeger *et al.*, 1986). Secondly, there are two transcriptional enhancer elements (only one enhancer element has been identified in DHBV) which are responsible for tissue specificity and regulation of viral gene expression (Schaller and Fischer, 1991). Thirdly, an 18-base-pair glucocorticoid responsive element (GRE) which enhances the viral surface antigen expression in HBV-infected patients *in vivo* has been identified. The presence of this GRE is believed to contribute to a more aggressive replication profile of HBV in males than females (Robinson, 1990). Fourthly, a polyadenylation signal which is used by all major transcripts is located downstream of the core promoter. Fifthly, a conserved 60 - 70 bp sequence with high degree of homology

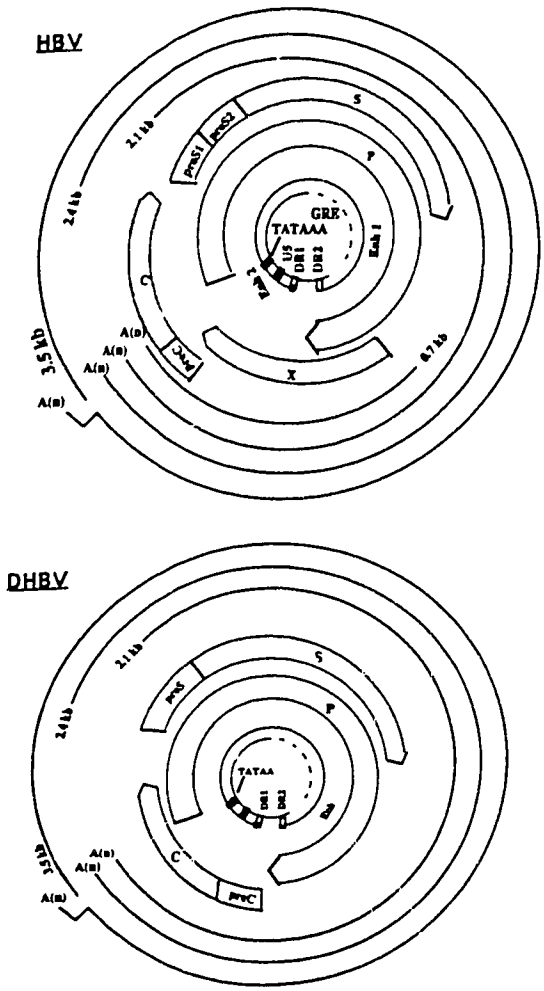


Figure 1-2. Structure and genome organization of HBV and DHBV.

Circular maps depict the genome organization of HBV (upper panel) and DHBV (lower panel). The inner circle with dashed line represents the incomplete plus-strand DNA with heterogenous 3' end. The outer circle represents the complete minus-strand DNA initiated with a protein primer (black dot) at the 5' end. Open arrows represent the functional open reading frames (ORFs) of genes in HBV and DHBV. PreS1, preS2 and S are the three in-frame regions of the envelope gene. Only preS and S regions are found in DHBV envelope gene. PreC and C are the two in-frame regions of the core gene. P and X denote the polymerase and X genes, respectively. The four circles around the outside of the ORFs in HBV represent four major classes of transcripts of 3.4, 2.4, 2.1 and 0.7 kb as indicated. The 0.7 kb transcript is not present in DHBV. Functionally important elements: A(n), poly(A) sequence; TATAAA, polyadenylation signal; GRE, glucocorticoid response element; U5, retrovirus U5-like element; Enh, enhancer elements; DR1 and DR2, direct repeat sequences are indicated.

with the U5 sequence of the LTR of retroviruses has been identified. The function of this sequence is presently unclear (Miller and Robinson, 1986; Simonsen and Levinson, 1983). Table 1-1 compares the characteristic elements present in the DNA genomes of HBV and DHBV.

2.2. Viral Proteins

HBV genome contains four major genes: preS1/preS2/S (collectively known as the envelope gene), preC/C, P and X (Figure 1-2). The envelope gene contains the preS1, preS2 and S regions delineated by three in-frame initiation codons. The preC/C gene contains the preC and C regions, also delineated by two in-frame initiation codons. The P gene contains a long open reading frame (ORF) which overlaps the C-terminus of the C gene, the entire S gene and the N-terminus of the X gene. The X gene carries the smallest ORF in the mammalian hepadnaviral genome and is not present in avian hepadnaviruses.

Transcription of the HBV viral genome is believed to be mediated by the cellular RNA polymerase II. There are four major classes of transcripts generated from four distinct promoters in HBV (Figure 1-2) (Chisari *et al.*, 1989). The 3.5 kb genomic RNA transcripts contain all four major ORFs. The subgenomic RNA transcripts are 2.4, 2.1 and 0.65 kb, respectively. The 2.4 kb transcript contains the PreS1, PreS2 and S regions of the envelope gene, whereas the 2.1 kb transcript contains only the preS2 and S regions of the envelope gene. All these transcripts are unspliced, of plus-strand polarity, capped at the 5' ends and polyadenylated at a common 3' terminus (Chisari *et al.*, 1989; Ganem and Varmus, 1987). Similar features are found in DHBV RNA transcripts except that the smallest 0.65 kb RNA transcript is absent (Büscher *et al.*, 1985). Proteins essential for viral replication are generated from these transcripts. The molecular masses of these viral proteins and their corresponding mRNAs in HBV and DHBV are listed in Table 1-2.

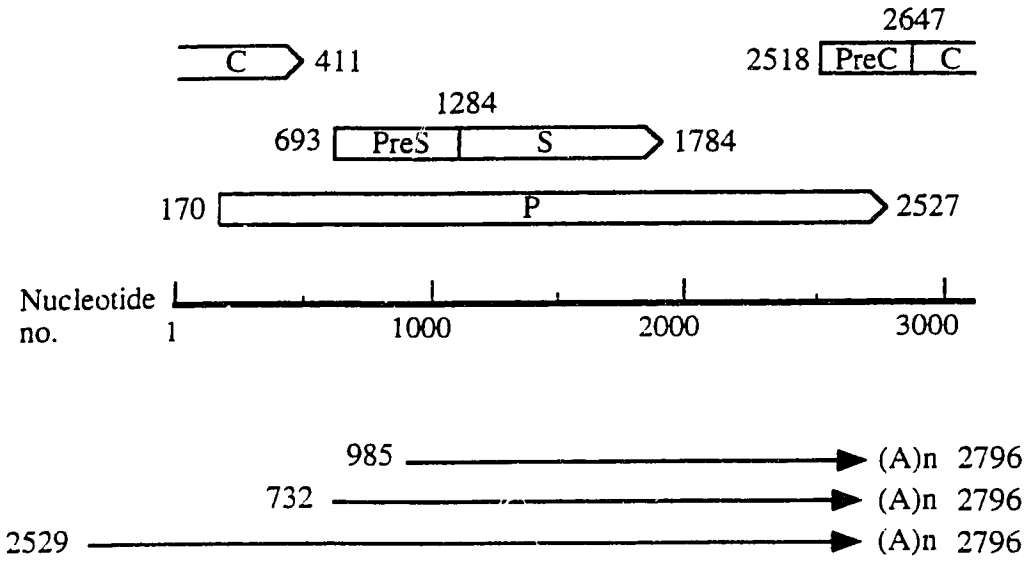


Figure 1-2B. Linear genome map of DHBV.

The boxes represent open reading frames (ORF) of core (PreC and C), envelope (PreS1, PreS2 and S) and polymerase genes. The scale in the center symbolizes the coordinates of the genome. The nucleotide numbering of the genome is given according to Mandart et al. (1984). The three major transcripts are depicted by the arrows. The positions of the 5' and 3' ends of the ORFs and the transcripts are indicated. (A)n represents the polyadenylation sequence at the 3' end of the transcripts.

Table 1-1 HBV and DHBV genome organization[1]

	HBV	DHBV
Genome length	3182 bp	3021 bp
Minus-strand DNA 5' end[2] terminal redundancy	 nt 1826 8 b	 nt 2539 10 b
Plus-strand DNA 5' end[2]	 nt 1600	 nt 2486
Cohesive overlap	200 - 300 bp	69 ± 4 bp
Direct Repeats (DR1/DR2)	11 bp	12 bp
Inverted Repeats	10 - 12 bp	10 bp

[1] Information contained in this table was obtained from Will,H. *et al.* (1987), Raney and McLachan (1990),and Molnar-Kimber,K.L. *et al.* (1984)

[2] Nucleotide numbering of HBV genome and DHBV genome is given according to the nomenclature of Pasek *et al* (1979) and Mandart *et. al.* (1984), respectively.

Table 1-2 HBV and DHBV gene products[a]

	Gene Products	Molecular Mass (kDa)[b]		Apparent size of mRNA (kb)[c]
		HBV	DHBV	
Envelope	Large (PreS1 protein)	45	36	2.4 (preS- mRNNA)
	Middle (PreS2 protein)	33	17	2.1 (S-mRNA)
	Major (HBsAg)	25		2.1 (S-mRNA)
Core	HBcAg	21	32	3.5 (C-mRNA)
	HBeAg	18	27	3.5 (preC- mRNA)
Polymerase		94	90	
X protein		17		0.65 (X mRNA)

[a] Data were obtained from Robinson, 1990; Schodel *et al.*, 1990; and Schaller and Fischer, 1991.

[b] Molecular mass indicated were estimated from unglycosylated proteins by SDS-PAGE.

[c] Approximate length as judged from Northern Blot analysis.

Surface proteins

The envelope gene in HBV encodes three species of surface proteins; large (preS1 protein), middle (preS2 protein) and major (HBsAg protein). The large surface protein contains polypeptides encoded from the preS1, preS2 and S regions; whereas the middle surface protein contains polypeptides encoded from the preS2 and S regions. The smallest major surface protein is encoded from the S region only and lacks of the amino acid sequences encoded from the preS1 and preS2 regions. In contrast to the gene in HBV, the envelope gene in DHBV encodes only two proteins, the large (preS) and major (S) proteins (Table 1-2 and Figure 1-2). In HBV, the preS1 protein is translated from the 2.4 kb mRNA, whereas both the preS2 protein and the HBsAg are generated from two in-frame translation initiation codons of the same 2.1 kb transcript. These surface proteins which exist in both glycosylated and non-glycosylated forms (Ganem and Varmus, 1987; Peterson, 1981) are the integral components of the viral envelope (Chisari *et al.*, 1989; Michel and Tiollais, 1987). Functionally, both the preS1- and preS2- proteins are believed to be involved in recognition of the hepatocyte receptors (Neurath *et al.*, 1986). The HBsAg contains a group-specific determinant "a" and either the "d" or "y" and either the "w" or "r" subtype determinants (McCaul *et al.*, 1985). Thus there are four major serotypes of HBsAg: "adw", "adr" "ayw", "ayr" (Bancroft *et al.*, 1972; Le Bouvier, 1976).

Precore and core proteins

There are several species of RNA transcripts which are longer than genome length and with heterologous 5' ends. These transcripts are generated from the same core promoter which produces two closely related but functionally diverse subsets of RNA species. In general, the one which contains the precore translation initiation site is designated as preC-mRNA, whereas the one which does not contain the precore translation initiation site is known as pregenomic or C-mRNA. The preC-mRNA functions exclusively for the production of precore protein which is post translationally modified into

a secretory protein, HBeAg. The pregenomic RNA serves dual functions : as a pregenomic RNA template for viral DNA replication (Nassal and Junker-Niepmann, 1990), and as a messenger RNA for the synthesis of both core (HBcAg) and polymerase (Pol) proteins (Blum *et al.*, 1992).

Translation of the precore protein is initiated at the first AUG codon of the preC region of the C gene, whereas translation of the core protein begins at the second AUG codon of the C region of the C-gene (Figure 1-2). Therefore, precore protein contains a short amino-terminal sequence which is lacking in the HBcAg. This amino-terminal portion, also known as the preC sequence, serves as a signal peptide for mediating the translocation of the precore protein to the endoplasmic reticulum. After translocation, the precore protein is processed at both the amino- and carboxy-termini, generating a secretory HBeAg in serum (Garcia *et al.*, 1987; Roossinck *et al.*, 1986). In contrast to HBcAg which plays a significant role in nucleocapsid formation, HBeAg appears to be non-essential for viral replication and its role in the biology of HBV remains unclear.

Core protein is the principle component of the virus replicating core which also contains a viral polymerase and a pregenomic RNA template (Crowther *et al.*, 1994; Chen *et al.*, 1992; Lavine *et al.*, 1989). The amino-terminal portion of the HBcAg is essential for nucleocapsid assembly (Brinbaum and Nassal, 1990; Beamies and Lanford, 1993), whereas the carboxy-terminal protamine like region of the HBcAg is required for encapsidation of the pregenomic RNA template (Gallina *et al.*, 1989; Hatton *et al.*, 1992). Although there are several cysteine residues present in HBcAg, formation of disulfide bridges among these cysteine residues are not required for nucleocapsid assembly (Zhou and Standring, 1992; Nassal, 1992). Recently, it has been shown that capsid assembly can be initiated autonomously provided that HBcAg has accumulated to a threshold concentration of 0.7 - 0.8 μM (Seifer *et al.*, 1993). Besides mediating nucleocapsid assembly, the phosphorylated form of HBcAg stimulates viral DNA replication in nucleocapsids and facilitates establishment of infection by mature virions (Schlict *et al.*,

1989; Yu and Summers, 1994). On the other hand, the unphosphorylated form of HBcAg is associated with covalently closed circular DNA amplification and virus production (Gallina *et al.*, 1989; Yu and Summers, 1994).

Polymerase

The polymerase (P) ORF encompasses about 80% of the entire viral genome. Although both the preC- and C-mRNAs contain the P-ORF, the P gene product is preferentially translated from the C-mRNA (Blum *et al.*, 1992). Unlike the mechanism in retroviruses in which P gene product is translated through ribosomal frameshifting, biosynthesis of polymerase in HBV is believed to be translated from either ribosomal leaky scanning or internal entry (Chang *et al.*, 1989; Jean-Jean *et al.*, 1989; Lin and Lo, 1992; Fouillot *et al.*, 1993). HBV polymerase has three main functional domains; terminal protein at the amino-terminus, DNA polymerase/reverse transcriptase in the central region and RNase H at the carboxy-terminus (Bartenschlager and Schaller, 1988; Radziwill *et al.*, 1990). The terminal protein serves as a protein primer for the first (minus) strand DNA synthesis (Molnar-Kimber *et al.*, 1983; Gerlich and Robinson, 1980). The DNA polymerase/reverse transcriptase domain functions as the principle enzyme mediating viral DNA synthesis (see 3 below). The carboxy-terminal RNase H domain acts to degrade the pregenomic RNA template from the DNA-RNA hybrid during the first strand DNA synthesis. Cleavage of the RNA template also produces an oligonucleotide which serves as a primer for the second (plus-) strand DNA synthesis (Lien *et al.*, 1987).

X-protein

Hepatitis B virus X gene encodes a transcription activator which stimulates the HBV promoters as well as a wide variety of other viral and cellular promoters (Nakatake *et al.*, 1993; Kwee *et al.*, 1992; Aufiero and Schneider, 1990; Seto *et al.*, 1990). Although results from transfected cells indicated that viral replication is not affected in the presence of mutant X gene products (Blum *et al.*, 1992), recent findings suggested that the X gene product is required for establishment of viral infection *in vivo* (Zoulim *et al.*, 1994; Chen *et*

al., 1993). X protein has also been shown to be associated with the transforming phenotype in transgenic mice expressing high levels of HBV X gene product (Kim *et al.*, 1991), and in cell lines previously immortalized with simian virus 40 T antigen (Hohne *et al.*, 1990). These observations are intriguing in that patients with chronic persistent hepatitis are predisposed to have an 100-fold higher frequency of hepatocellular carcinoma (HCC) (Beasley *et al.*, 1981; Loncarevic *et al.*, 1990). It may be significant that HCC is frequently found in woodchucks and ground squirrels infected with WHV and GSHV, respectively; whereas no HCC is observed in ducks infected with DHBV which does not have an X gene.

3. Hepadnavirus Replication

Species and tissue specificity, conversion of an asymmetric genome to a covalently closed circular DNA (ccc DNA) and reverse transcription initiated via a protein primer followed by a second round of DNA-DNA replication are characteristic of hepadnaviral replication. Hepatitis B infection begins with the attachment of the virus to a cell, presumably through a cellular receptor. Upon entry into the cell, the nucleocapsid which contains the partially double stranded DNA genome and the viral polymerase is released from the envelope and translocated to the cell nucleus. At this stage of the infection, the viral genome may remain in a latent state, undergo replication or, rarely, integrate into the host genome. Unlike retroviruses, in which integration of the viral DNA into the host genome is a pre-requisite for subsequent replication (Coffin, 1990), hepadnaviral replication does not require viral-host DNA integration. Although integrated HBV DNA has been identified in biopsies isolated from HBV infected patients (Tokino and Matsubara, 1991; Matsubara and Tokino, 1990; Dejean and de Thé, 1990), this integration invariably results in interrupted ORF of HBV proteins such that replicative competent virions will not be produced from viral-host DNA integration.

Inside the nucleus, conversion of the partially double stranded viral DNA into ccc DNA occurs. Transcription of the ccc DNA results in a pool of viral transcripts which will

subsequently be translated into viral proteins. One species of longer than genome length RNA transcript, the pregenomic RNA, also serves as the RNA template for subsequent DNA replication. Encapsidation of this pregenomic RNA takes place in the cytoplasm leading to the formation of virus replicating core wherein the DNA replication takes place.

Replication of the viral DNA involves two stages: (i) the synthesis of the minus-strand DNA from the RNA template and (ii) the synthesis of the plus-strand DNA from the *de novo* synthesized minus-strand DNA. These two stages of the replication are believed to be mediated by the viral polymerase, which also possesses an RNase H domain responsible for degradation of the pregenomic RNA template from the DNA-RNA hybrid during the minus-strand DNA synthesis.

The final step of replication involves the association of the mature nucleocapsid with the appropriately arranged assembly of envelope antigen molecules present within the endoplasmic reticulum membrane. The complete virion is secreted from the cell through the endoplasmic reticulum and Golgi apparatus pathway.

Figure 1-3 depicts an overall scheme of the life cycle and DNA replication of Hepadnaviridae. A more detailed discussion of each step of the DNA replication is presented in the following sections.

3.1. Virus attachment and internalization

Of the three surface antigens present in the viral envelope, preS1 protein has been reported to play the major role in recognizing the viral receptors. In particular, amino acids 21 - 47 of preS1 protein have been shown to contain the epitope responsible for binding the putative receptors in hepatocytes (Neurath *et al.*, 1986; Yuasa *et al.*, 1991; Sureau *et al.*, 1992). Monoclonal antibodies targeted to this region have been shown to block the attachment of HBV virion to HepG2 cells (Petit *et al.*, 1991a; Petit *et al.*, 1991b). Circumstantial evidence, however, has indicated that HBsAg may play a significant role in viral attachment and internalization into human hepatocytes. Radiolabeled HBsAg (lacking polypeptides of preS1 and preS2 regions) has been shown to bind to intact human

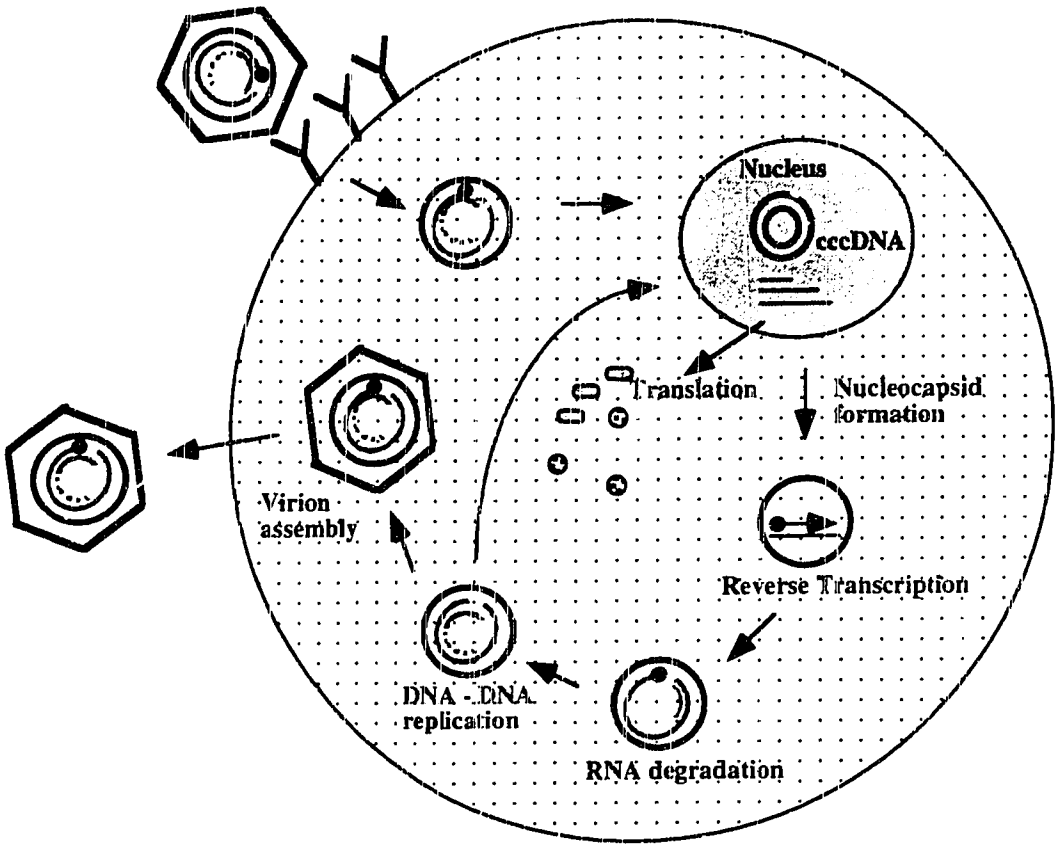


Figure 1-3. Replication cycle of HBV in an infected hepatocyte

hepatocytes and hepatoblastoma cells (HepG2) (Petit *et al.*, 1991; Kaiser *et al.*, 1986; Leenders *et al.*, 1992). Monoclonal antibodies (MAb) raised against HBsAg were found to inhibit viral particles from binding to hepatocytes more efficiently than those raised against preS1 protein (Petit *et al.*, 1991). Furthermore, vaccination with recombinant HBsAg can elicit neutralizing antibodies against viral infection (McAleer *et al.*, 1984).

In spite of the massive amount of information available regarding viral antigens required for viral-host interaction, the cell surface receptor responsible for ligand recognition has not been identified conclusively. Studies aimed at identifying viral receptors have been hampered due to the lack of a tissue culture system which would support an HBV infection. Failure to infect cell lines with HBV could be explained by the absence of viral receptors on cell lines being studied, or by the absence of transcription factors required for viral gene expression. The latter can be exemplified by successful production of virus particles in non-permissive cell lines using transfected HBV DNA cloned under an exogenous promoter (Junker *et al.*, 1987; Seeger *et al.*, 1989). This suggests that the block of virus production occurs at the level of transcription. On the other hand, cell lines such as HepG2 and HuH7 which are believed to be permissive for HBV replication are still shown to be refractory to infection (Qiao *et al.*, 1994).

Recently, several putative receptors for HBV on liver cells have been reported. Using synthetic preS1 peptides (a.a. 21 - 47) as ligands for binding to the putative receptors, several laboratories have identified glycoproteins of molecular masses of 35 and 50 kDa capable of binding specifically to recombinant HBV preS1 proteins. These peptides also block the attachment of preS1-positive HBV particles to HepG2 cells (Petit *et al.*, 1992; Dash *et al.*, 1992). Using the DHBV model, Kuroki *et al.* (1994) have shown that a cell surface glycoprotein (180 kDa) binds with high affinity to the DHBV preS region of the large surface protein. Receptors for major surface antigens have also been reported. Endonexin II, a 34 kDa protein present on human liver plasma membranes, has been shown to bind specifically to HBsAg (Hertogs *et al.*, 1993). Immunization of rabbits with

human liver endonexin II or the F(ab')2 fragment of anti-human liver enodnexin II IgG resulted in spontaneous development of anti-HBsAg antibodies (Hertogs *et al.*, 1994).

The concept of 'intermediate carrier' for facilitating entry of HBV virus particles into hepatocytes has drawn increasing attention. Polymerized human serum albumin has long been demonstrated to facilitate attachment of both preS1 and preS2 proteins to liver plasma membranes (Machida *et al.*, 1984; Michel *et al.*, 1984). This binding presumably occurs through the recognition of the preS2 sequence of both the preS1 and preS2 proteins (Pontisso *et al.*, 1989). In 1993, Budkowska *et al.* demonstrated that a binding factor (50 kDa) in normal human serum was able to compete with the specific binding of preS1- and preS2- specific MAbs to their corresponding epitopes in an inhibition ELISA assay. This observation suggested that the 50 kDa binding factor may serve as an intermediate carrier during viral attachment (Budkowsha *et al.*, 1993). Human apolipoprotein H was found to recognize HBV HBsAg and is believed to mediate viral entry to the hepatocytes through chylomicron and high density lipoprotein complexes (Mehdi *et al.*, 1994). This belief is further supported by the prior observation that myristoylation of the preS protein in DHBV was essential for infectivity in DHBV (Macrae *et al.*, 1991).

In the aforementioned studies, binding of the putative receptors to HBV envelope proteins have been demonstrated, however internalization of the corresponding envelope proteins or the complete virus particles has not been observed. Furthermore, the thought that HBV virus particles gain their entry to the intected cells through receptor mediated endocytosis has recently been challenged by Rigg and Schaller (1992). The results in their study suggested that infection of hepatocytes is not dependent on low pH and possibly takes place through fusion of the virus and the host cells (Rigg and Schaller, 1992).

In summary, HBV attachment and internalization into hepatocytes are thought to be mediated through one of the three processes: receptor mediated endocytosis, facilitated entry through an intermediate carrier or virus-host fusion.

3.2. Formation of ccc DNA and its regulation

After entry into the liver cells, the hepatitis B virion is believed to uncoat and release the nucleocapsid, which is thought to deliver the viral genome into the nucleus. The actual process of the nucleocapsid translocation is not well understood. However, experiments have indicated that nuclear membrane of the infected cell presents a barrier to nucleocapsid transport (Qiao *et al.*, 1994; Guidotti *et al.*, 1995). It is not known whether the nucleocapsid dissembles at the nuclear membrane or after entry into the nucleus. It is clear, however, that the partially double-stranded viral DNA is released from the nucleocapsid into the nucleus. Conversion of the partially double-stranded DNA to the covalently closed circular form (ccc DNA) involves several steps: (1) removal of the terminal protein which is attached to the 5' end of the minus-strand DNA; (2) completion of the plus-strand DNA; (3) removal of the oligonucleotide primer linked to the 5' end of the plus-strand DNA; (4) elimination of the terminal redundancy of the minus strand DNA; and (5) ligation of the 5' and 3' ends of the plus- and minus- strand DNA, respectively. The mechanism of conversion of relaxed circular (rc) DNA to cccDNA is not clear, however Köck and Schlicht (Köck and Schlicht, 1993) have demonstrated that this repair mechanism is not dependent on the viral polymerase activity.

In primary hepatocytes isolated from ducklings congenitally infected with DHBV, the steady state concentration of ccc DNA was found to be a 50-fold excess of that derived from the infecting virus (Tuttleman *et al.*, 1986b). In addition, *de novo* amplification of ccc DNA has been observed in hepatocytes infected with DHBV *in vitro* (Tuttleman *et al.*, 1986a). In 1986, Tuttleman *et al.* demonstrated elegantly that the synthesis of ccc DNA occurs intracelluarly in a non-conservative manner (Tuttleman *et al.*, 1986b). This involves *de novo* synthesis of viral DNA in the cytoplasm by reverse transcription of the RNA transcribed from the infecting viral DNA (Tuttleman *et al.*, 1986b). This finding was further supported by Wu *et al* (1990) who demonstrated that amplification of ccc DNA can

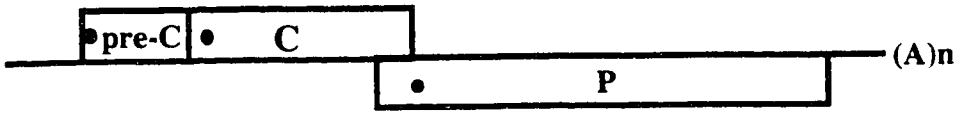
occur efficiently in the presence of suramin, a compound which blocks DHBV superinfection.

In ducklings persistently infected with DHBV, the amount of ccc DNA per cell in steady-state varies from 3 - 50 copies (Miller and Robinson, 1984). The maintenance of the ccc DNA pool during the course of infection is under stringent control. Using DHBV as a model, Summers *et al.* have shown that the presence of the large surface protein suppresses *de novo* synthesis of the ccc DNA (Summers *et al.*, 1990). Infection with DHBV harboring a mutant preS gene resulted in defective enveloped virus production with a concomitant increase in ccc DNA level as compared to that of the wild type (Lenhoff and Summers, 1994). It is not clear how the large surface protein regulates ccc DNA amplification. However, it is believed that efficient packaging of the nucleocapsid by the preS and S envelope proteins may prevent the intracellular recycling of the nucleocapsid for further amplification of ccc DNA.

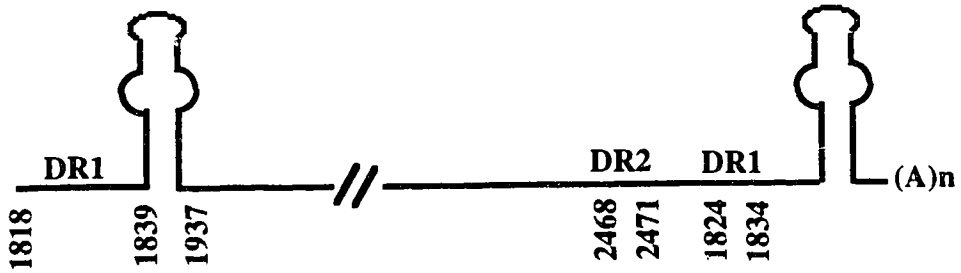
3.3. Encapsidation of the pregenomic RNA

Once the ccc DNA is formed in the nucleus, synthesis of the RNA transcripts occurs. As indicated in Section 2.2., the C-mRNA or pregenomic RNA is utilized as the template for subsequent DNA replication (Nassal and Junker-Niepmann, 1990; Enders *et al.*, 1987). Hepadnaviral DNA replication occurs in the virus replicating core, which consists of the core protein, the viral polymerase and the pregenomic RNA template.

Packaging of the pregenomic RNA into the nucleocapsid requires interaction of the *cis*-acting sequence on the pregenomic RNA with the polymerase, core or polymerase-core complex. In HBV, the *cis*-acting sequence (known as the ε sequence) was shown to reside at the 5' end of the pregenomic RNA (Chiang *et al.*, 1990; Chiang *et al.*, 1992; Junker-Niepmann *et al.*, 1990). This element encompasses a 99- nucleotide sequence near the DR1 region (Figure 1-4). In DHBV, however, a larger segment of about 1200 nucleotides located approximately 30 nucleotide downstream from the initiation site of the pregenomic RNA is required for encapsidation (Hirsch *et al.*, 1991). A more detailed analysis by



HBV pregenomic RNA:



DHBV pregenomic RNA:

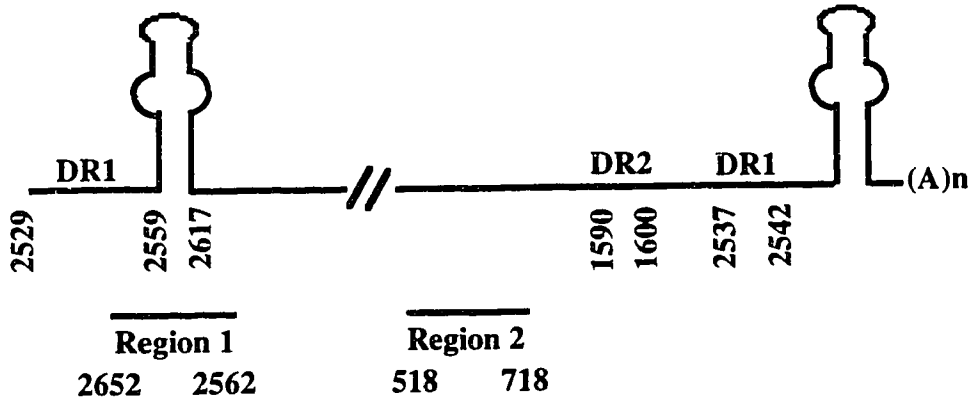


Figure 1-4. Schematic diagram of encapsidation signal in HBV and DHBV pregenomic RNAs

The diagram on the top represents the pregenomic RNA with preC-, C- and P- open reading frames (ORF). Translation initiation of each ORF is represented by a black dot. Initiation codons of PreC, C and P genes are at nt 1816, 1903 and 2299, respectively in HBV (adw2); whereas initiation codons of preC, C and P are at nt 2519, 2647 and 170 , respectively in DHBV 16. The middle and the lower panels illustrate the pregenomic RNA with stem-loop encapasidation signals in HBV and DHBV, respectively. Region 1 and 2 are the sequences required for encapsidation according to Calvert and Summers, 1994. Nucleotide numbering are that according to Pasek *et al.* (1979) in HBV and Mandart *et al.* (1984) in DHBV.

Clavert and Summers revealed that two discrete regions within this segment are crucial to encapsidation. Region 1 is located at nucleotides 36 - 126 and region 2 is located at nucleotides 1046 - 1214, respectively, downstream from the 5' end of the pregenomic RNA. Both region 1 and region 2 are required for encapsidation in DHBV (Calvert and Summers, 1994). Since the ε sequence of HBV and the region 1 of DHBV are located at the terminal redundant region of their pregenomic RNAs, two copies of these sequences will be expected. Hirsch and coworkers have deleted the 3' copy of the ε sequence in HBV without decreasing encapsidation suggesting that only the 5' copy of the ε sequence is recognized during the encapsidation process (Hirsch *et al.*, 1991).

Computational prediction of the ε region in all hepadnaviruses revealed a stable stem-loop structure containing a lower stem, a 6-nucleotide bulge, an upper stem with a single unpaired U residue, and a 6-nucleotide loop (Pollack and Ganem, 1993) (Figure 1-5). Mutational analyses of the requirement for encapsidation showed that conformation and base-pairing, but not primary sequence, of the lower stem, bulge and the lower portion of the upper stem are important for efficient packaging; whereas the primary sequence of the loop and the upper portion of the upper stem region is critical for RNA encapsidation (Pollack and Ganem, 1993; Tong *et al.*, 1993). Interestingly, naturally occurring HBV variants with mutations within this stem-loop region have been found to have either a more stable secondary structure or complementary mutations which will maintain the stability of this stem-loop structure (Laskus *et al.*, 1994; Hasegawa *et al.*, 1994).

Binding of the polymerase to the ε sequence of the nascent pregenomic RNA has been demonstrated (Köchel *et al.*, 1991; Pollack and Ganem, 1994; Bartenschlager *et al.*, 1990; Hirsch *et al.*, 1990). Complementation experiments using constructs containing mutated polymerase indicated that structural domains, but not functional activities, of the polymerase are necessary for encapsidation (Bartenschlager *et al.*, 1990; Radziwill *et al.*, 1990; Blum *et al.*, 1991; Roychoudhury *et al.*, 1991; Chen *et al.*, 1992; Chang *et al.*, 1990). However, package-defective polymerase such as one with C-terminal truncation

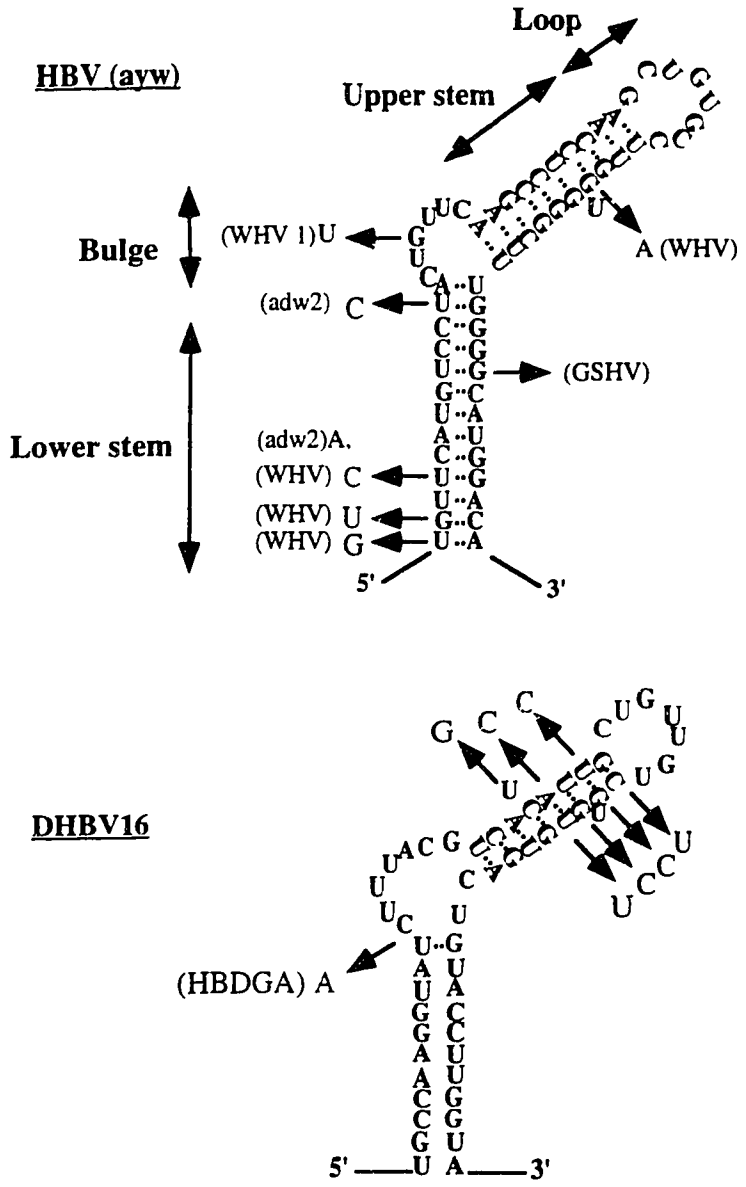


Figure 1-5. Computer prediction of RNA secondary structure of ε region in mammalian and avian hepatitis B viruses.

Computer-based RNA secondary structure of ε region in HBV (ayw) and DHBV16 are shown. Nucleotide changes in other hepadnavirus isolates are indicated by arrows followed by the corresponding isolates in parentheses. Information illustrated in HBV, human hepatitis B virus; DHBVand HBDGA, duck hepatitis B virus; WHV, woodchuck hepatitis virus and GSHV, ground squirrel hepatitis virus was obtained from Pollack and Ganem (1993).

was shown to retain its binding capacity to the ε sequence (Pollack and Ganem, 1994; Chen *et al.*, 1994). This suggests that efficient nucleocapsid assembly is quite complex and may require other proteins such as core or host factors in addition to polymerase.

3.4. Synthesis of minus strand DNA

Minus-strand DNA synthesis is initiated by the primer domain of the polymerase utilizing the pregenomic RNA as the template (Molnar-Kimber *et al.*, 1983; Gerlich and Robinson, 1980; Summers and Mason, 1982; Bartenschlager and Schaller, 1988; Wang and Seeger, 1992). Recently, it has been shown that tyrosine 92 or tyrosine 96 of the polymerase in HBV or DHBV, respectively, is covalently attached to the deoxyguanosine residue : the 5' end of the minus-strand DNA (Nassal and Schaller, 1993; Wang and Seeger, 1992; Zoulim and Seeger, 1994; Weber *et al.*, 1994).

The discovery of the ε sequence in the pregenomic RNA has radically altered our view of the earlier model of hepadnaviral replication, in which the priming site was assigned to the 3' DR1 of the pregenomic RNA (Bartenschlager and Schaller, 1988; Ganem and Varmus, 1987; Seeger *et al.*, 1991). Recent studies suggest that interaction of the polymerase and the ε sequence appears to be crucial for packaging the RNA template and initiating the reverse transcription (Pollack and Ganem, 1994; Bartenschlager and Schaller, 1992). Unequivocal evidence obtained by Pollack and Ganem, and Tavis *et al.* indicate that hepadnaviral polymerase initiates DNA synthesis by first binding to the bulge region of the stem-loop structure in the 5' ε sequence (Pollack *et al.*, 1994; Tavis *et al.*, 1994). Synthesis of 4 - 5 nucleotides occurs *in situ*, followed by translocation of the polymerase-nucleotide complex from the 5' ε to the 3' DR1 (Figure 1-6). Efficient transfer of the polymerase-nucleotide complex occurs when the primary recognition sequence in the 5' bulge region is homologous to that within the 3' DR1, although a minor amount of mismatch between the 5' donor and the 3' acceptor is tolerated (Nassal and Rieger, 1994).

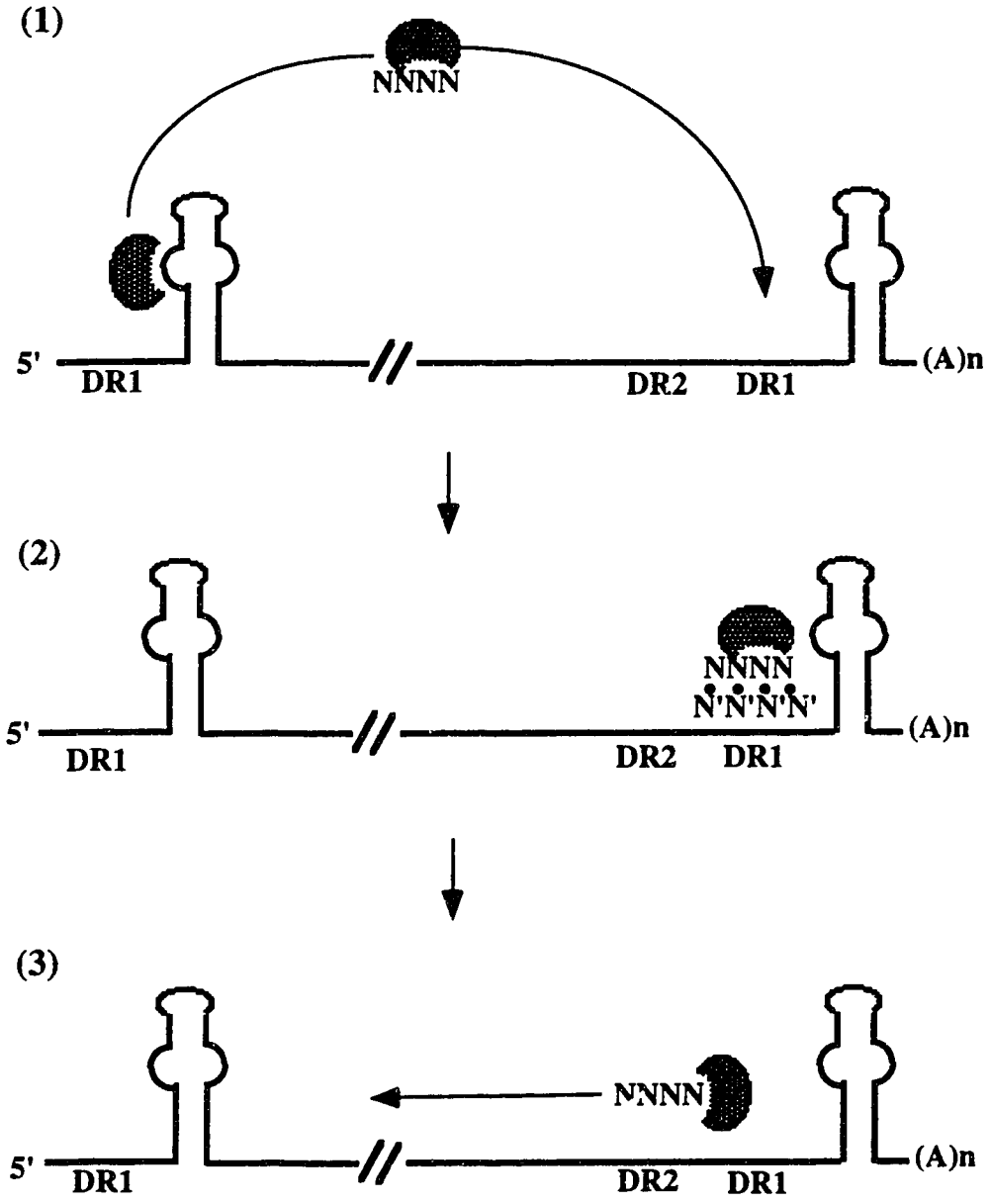


Figure 1-6. Synthesis of minus-strand DNA.

Schematic diagram illustrates the initiation of minus-strand DNA by the polymerase. (1) Binding of the polymerase to the bulge region of the stem-loop structure present in the 5' ε sequence. Synthesis of about 4 nucleotides occurs *in situ*. (2) Translocation of the polymerase - nucleotide complex from the 5' ε to the 3' DR1. The four nucleotides are complementary to those present in the DR1. (3) Synthesis of the minus-strand DNA continues.

In hepadnaviral replication, binding of polymerase to the bulge region of the stem-loop structure and translocation of the polymerase-nucleotide complex to the 3' DR1 demonstrate high specificity. Although ε is present twice in the pregenomic RNA, only the 5' copy is functional in encapsidation and reverse transcription (Hirsch *et al.*, 1991; Junker-Niepmann *et al.*, 1990). Similarly, although there are two copies of DR1 on the RNA template, the 5' end copy of DR1 is not used as the initiation site for the reverse transcription (Will *et al.*, 1987; Wang and Seeger, 1993; Seeger and Maragos, 1990). In fact, the aforementioned 4 nucleotides (UUAC in DHBV and UUCN in HBV, WHV and GSHV) responsible for DNA initiation are also found in 5' DR1, DR2 as well as other parts of the pregenomic RNA besides 3' DR1. However, for reason not presently understood, specific transfer of the polymerase-nucleotide complex to the 3' DR1 is highly preferred (Seeger and Maragos, 1990; Wang and Seeger, 1993; Loeb and Tian, 1994; Tavis *et al.*, 1994). Although the mechanism of minus-strand DNA initiation is different from what was previously thought, the specific transfer of the polymerase-nucleotide complex to 3' DR1 explains the results of the earlier analyses that 5' terminus of the minus-strand DNA was mapped to the 3' DR1 region (Will *et al.*, 1987; Seeger and Maragos, 1990; Saldanha *et al.*, 1992). Physical constraint within the nucleocapsid and/or flanking sequences around the 5' ε and the 3' DR1 may play a role in determining the specificity of the 5' ε - 3' DR1 transfer.

Translocation of the hepadnaviral polymerase-nucleotide complex from the 5' end to the 3' end of the pregenomic RNA template is reminiscent of the process in retroviral replication, in which synthesis of the first strand of DNA also requires a transfer of the oligonucleotide primer from the 5' end to the 3' end of the RNA template. Similar to retroviral replication, hepadnaviral reverse transcription of the minus-strand DNA proceeds after priming and with a concomitant degradation of the RNA template in the DNA-RNA hybrid (Loeb *et al.*, 1991b; Coffin, 1990).

3.5. Synthesis of plus strand DNA

The clue to understanding the initiation of plus-strand DNA synthesis came from mapping the 5' terminus of the plus-strand DNA, which was found to be located at the 3' end of the DR2 (Figure 1-2) (Molnar-Kimber *et al.*, 1984; Will *et al.*, 1987). Lien *et al.* have shown that the length of the plus-strand DNA decreased by 18 - 19 nucleotides after RNase or alkaline treatment suggesting that the primer for plus-strand DNA synthesis is RNA. (Lien *et al.*, 1987). On further analysis of this oligonucleotide primer, it was identified as the 5' end of the pregenomic RNA (Büscher *et al.*, 1985). This oligonucleotide primer was shown to be capped at the 5' terminus (Lien *et al.*, 1987). Subsequent mutational analyses of the polymerase as well as the DR1 and DR2 regions of the pregenomic RNA template led to the deduction of the following model (Figure 1-7): during the synthesis of the minus-strand DNA, the RNase H domain of the polymerase degrades the RNA template from the DNA-RNA hybrid (Radziwill *et al.*, 1990). If the RNase H domain is located at a distance of 40 Å from the polymerase domain (a space equivalent to about 15 - 18 nucleotides), completion of the minus-strand DNA synthesis will position the polymerase at the 3' DR1 of the minus-strand DNA (Luo and Taylor, 1990; Oyama *et al.*, 1989). Further run-off of the polymerase generates a capped 15 - 18 ribonucleotide primer which is equivalent to the 5' DR1 of the pregenomic RNA (Loeb *et al.*, 1991). Translocation of this RNA primer from 3' DR1 to DR2 of the minus-strand DNA initiates the subsequent DNA replication (Seeger and Maragos, 1989). However, extension of the plus-strand DNA is soon stymied as the polymerase reaches the 5' end of the minus-strand DNA. Presence of the terminal redundancy in the minus-strand DNA facilitates template switching through annealing of the 3' end of the newly synthesized plus-strand DNA to the 3' end of the minus-strand DNA. After this template switch, plus-strand DNA synthesis continues using the minus-strand DNA as the template.

In this model, cleavage and translocation of the RNA primer are two distinct events. RNA cleavage is sequence independent, whereas efficient primer translocation requires the

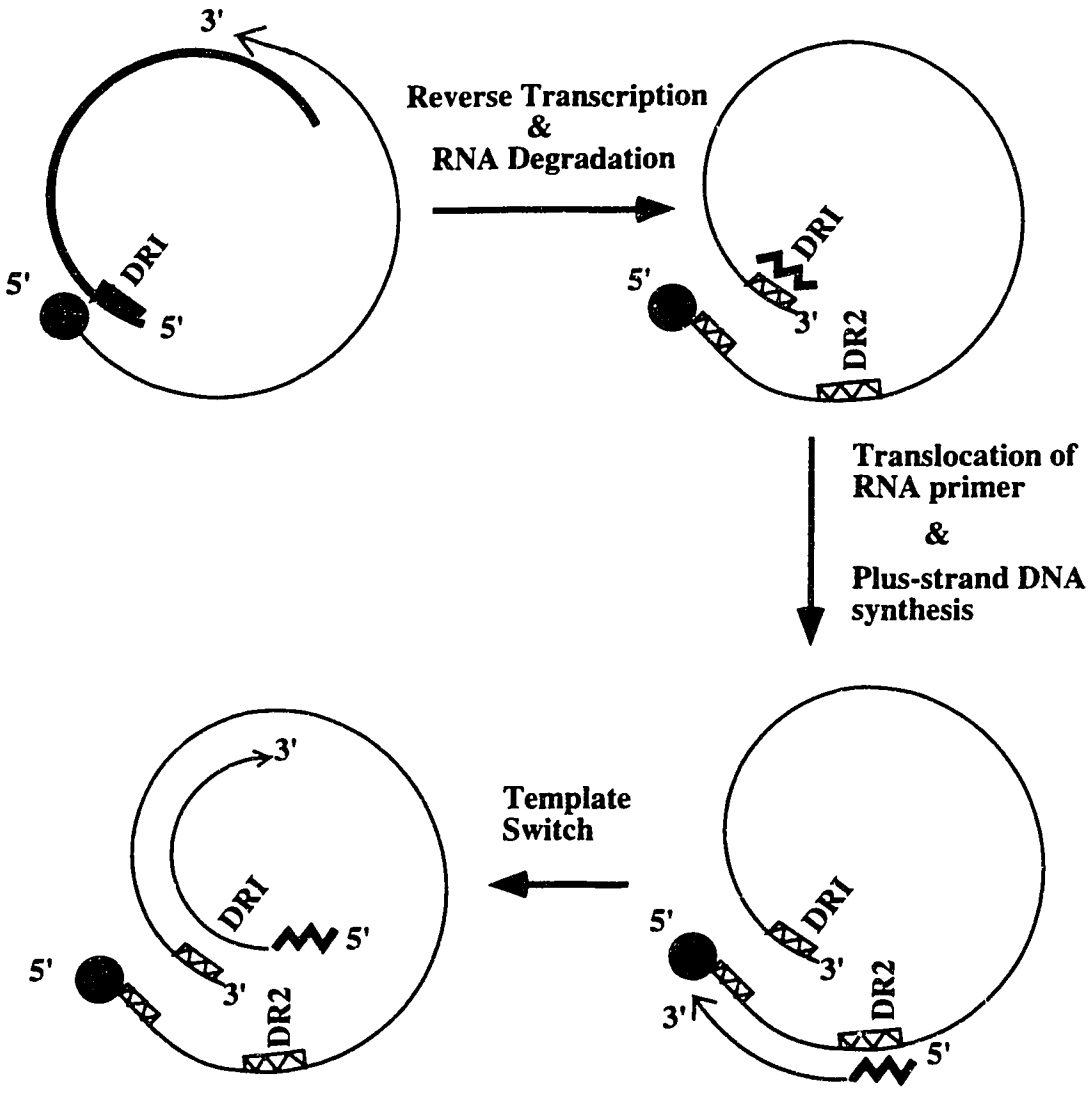


Figure 1-7. Synthesis of plus-strand DNA.

Schematic diagram depicting plus-strand DNA synthesis in hepadnaviruses. Synthesis of minus-strand DNA proceeds with a concomitant degradation of RNA template. RNA primer generated from the degradation translocates from DR1 to DR2 of the minus-strand DNA. Synthesis of plus-strand DNA begins until it reaches the 5' end of the minus-strand DNA. Template-switching from the 5' end to the 3' end of the minus-strand DNA occurs. Plus-strand DNA synthesis continues using the minus-strand DNA as template.

wild type primary sequence of DR1 and its flanking region as well as sequence homology between DR1 and DR2 (Seeger and Maragos, 1989; Staprans *et al.*, 1991; Seeger *et al.*, 1991). Strikingly, mutation of DR1 or its flanking sequences results in aberrant *in situ* priming of the plus-strand DNA synthesis at 3' DR1 of the minus-strand DNA without translocation. This *in situ* synthesis of plus-strand DNA leads to the formation of a linear double-stranded DNA in the progeny nucleocapsids (Condreay *et al.*, 1992; Staprans *et al.*, 1991).

3.6. Assembly of mature virion

As noted previously in section 2.2, the hepadnaviral envelope is composed of three surface proteins; preS1, preS2 and HBsAg. The envelope of the mature virion in serum contains preS1 protein, preS2 protein and HBsAg in a 1:1:4 ratio, whereas the empty subviral particles are made up of mainly preS2 protein and HBsAg in a 1:4 ratio (Robinson, 1990). Like most of the eukaryotic secretory proteins, these surface proteins are cotranslationally inserted into the endoplasmic reticulum where the envelope proteins will undergo postranslational processing such as glycosylation and myristoylation (Eble *et al.*, 1990; Eble *et al.*, 1987). Since budding of the nucleocapsid into the cisternae of the endoplasmic reticulum which contained the pre-arranged envelope proteins has been observed microscopically, this suggests that formation of the Dane particle occurs intracellularly in membrane-limited vesicles. Transport of the mature virion to the exterior is believed to be mediated through a constitutive secretory pathway involving the Golgi apparatus (Kelly, 1985).

V. ANTIVIRAL THERAPY

As treatmments for HBV infection, immunomodulators as well as antiviral agents targeted at different stages of the viral replication, have evolved to the stage of clinical trials. To date, however, results have been very disappointing with few antiviral agents

showing significant clinical benefits. Even agents which have recognized activity do not produce permanent eradication of the virus. Failure to eliminate hepadnaviral ccc DNA is a likely reason for reinitiation of viral replication following completion of therapy.

1. Interferon

Clearance of HBV infection requires efficient antibody and cell-mediated immune responses; in particular, cytotoxic T lymphocyte- (CTL-) and natural killer cell- (NK-) mediated lysis of the infected hepatocytes. Progression to chronic infection is thought to be associated with poor immune response or deficient cytokine production in infected patients (Thomas *et al.*, 1986; Fourel *et al.*, 1994a). This observation forms the rationale for interferon therapy for hepatitis B infection. Interferons (IFNs) are known to suppress viral replication. In RNA viruses, induction of the 2',5'-oligoadenylate synthetase and the 68 kDa-eIF-2 kinase are thought to block viral protein expression (Lengyel, 1982). Inhibition of replication in DNA viruses has also been observed, although the mechanism remains to be elucidated. In HBV, alpha-interferon has been reported to suppress virus enhancer activity and gene transcription (Janssen *et al.*, 1991). Besides their antiviral effects, IFNs also enhance the expression of HLA class I antigens, hence allowing recognition of the infected cells by CTL (Hadziyannis and Schiff, 1992). Other immunoregulatory mechanisms influenced by IFNs include activation of CTL and NK responses (independent of HLA class I expression) and increased release of cytokines such as TNFα and IL-1β (Dienstag *et al.*, 1982).

There are three main subclasses of interferons: alpha (IFNα), beta (IFNβ) and gamma (IFNγ). Of these, IFNα is most effective in inhibiting HBV replication (Di Bisceglie *et al.*, 1990). Clinical trials using 5 - 10 MU of recombinant human IFNα three times a week for 3 - 6 months resulted in clearance of HBeAg, serum HBV DNA and polymerase activity in over 30% of the treated patients. There are general reductions in symptoms and improvement in liver histology as a result of the treatment (Lai *et al.*, 1987;

Coppens *et al.*, 1989; Perrillo, 1993a). However, a significant number of "responders" relapse after interferon therapy has been discontinued (Perrillo, 1993a).

The disadvantages of interferon therapy include the expense, need for parenteral administration and side-effects, which include flu-like symptoms, fever and chills, myalgia, headaches, anxiety, depression and thrombocytopenia (Tyrrell, 1993). In spite of these drawbacks, interferon is one of the most promising antiviral agents available today.

2. Nucleoside analogues

Keys to designing antiviral therapy for HBV infection come from the unique mechanism in the viral replication. Since reverse transcription is one of the required steps during HBV replication, most of the antiviral agents that have been studied were designed to target the viral polymerase.

Nucleoside analogues which have not proven effective in cell cultures include Zidovudine (AZT) (Gilson *et al.*, 1991), adenine arabinoside (Garcia *et al.*, 1987), 2',3'-dideoxycytidine (ddC) (Civitico *et al.*, 1990), 2',3'-dideoxythymidine (ddT) (Löfgren *et al.*, 1989), 2',3'-dideoxyinosine (Lee *et al.*, 1989 and Fried *et al.*, 1992) and in particular fialuridine (FIAU) (Brahams, 1994) which was also found to be extremely cytotoxic. However, there is considerable interest in some newer nucleoside analogues. Initial screening and pilot studies has shown that some of these compounds confer significant effects against HBV. From these studies, the most effective agents appear to be purine analogues. The carbocyclic analogue of 2'-deoxyguanosine (2'CDG) has been shown to produce long-lasting antiviral effects against both HBV and DHBV (Price *et al.*, 1989; Fourel *et al.*, 1994b; Price *et al.*, 1992; Fourel *et al.*, 1994a). 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR) has been reported to selectively inhibit DHBV replication, presumably through blocking the terminal protein priming of the minus-strand DNA synthesis (Lee *et al.*, 1989; Suzuki *et al.* 1988). Oxetanocin-G was found to incorporate into HBV DNA strands and inhibit the HBV endogenous polymerase reaction (Nagahata *et al.*, 1994). Although pyrimidine dideoxynucleosides (ddC or ddT) are not effective

inhibitors of hepadnaviral replication (Lee *et al.*, 1989 and Löfgren *et al.*, 1989), some modified pyrimidine analogues have been shown to be potent anti-HBV *in vitro* . In particular, the fluorinated pyrimidine analogues such as 2',3'-dideoxy-3'fluorothymidine (FddThd) (Matthes *et al.*, 1991), 2',3'-dideoxy-3'fluoro-5-mehtylcytidine triphosphate (FMetdCTP) (Matthes *et al.*, 1990) and 2',3'-deoxy-3'thia-5-fluorocytidine (FTC) (Meisel *et al.*, 1990 and Doong *et al.*, 1991) are potent inhibitors of HBV DNA polymerase. Recently, clinical trials of 2',3'-dideoxy-3'thiacytidine (3TC) have yielded promising results. The use of 3TC in prevention of hepatitis B recurrence after orthotopic liver transplantation has shown to be very promising (Ma *et al.* 1995, in preparation).

Several compounds thought to disrupt HBV supercoiled (sc) or ccc DNA have been evaluated (Civitico *et al.*, 1990; Locarnini *et al.*, 1991). In spite of the reported efficacy in sc DNA clearance, there is a growing concern regarding the cytotoxicity of these compounds. More studies are warranted to ensure the safety of these compounds.

VI. OBJECTIVES OF THE PROJECT

The aim of the work described in this thesis is to use DHBV as a model to study the mechanism of action of ddDAPR, a purine 2',3'-dideoxynucleoside analogue, on HBV replication. This work is an extension of our previous finding that purine 2',3'dideoxynucleosides are effective inhibitors of DHBV replication, whereas pyrimidine 2',3'dideoxynucleosides are not (Lee *et al.*, 1989; Suzuki *et al.*, 1988). In particular, 2',3'-dideoxyguanosine (ddG) and ddDAPR were found to be a thousand times more effective than ddC in inhibiting hepadnaviral replication. We have hypothesized that ddGTP, a metabolite of ddDAPR, might inhibit the viral replication through binding to the primer protein domain of the polymerase.

The majority of the work undertaken in this study was focused on seeking evidence to support the above hypothesis. Specific objectives are outlined as follows:

(1) To determine whether or not ddDAPR or ddG compete with the natural compound, dG, in DHBV replication in primary duck hepatocyte cultures.

(2) To use replicating cores isolated from ddDAPR treated ducklings to deduce the mechanism of action and the site of inhibition of DHBV replication by ddGTP (ddDAPR)

(3) To clone and express an enzymatically active DHBV polymerase in order to demonstrate direct binding of the drug to the polymerase.

During the course of this work, it became apparent that DHBV polymerase might have another role in hepadnaviral replication besides DNA synthesis. This led to another objective: to investigate the regulatory role of DHBV polymerase on core mRNA translation.

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Chapter 2

Duck Hepatitis B Virus Polymerase Produced by *in vitro* Transcription and Translation Possesses DNA Polymerase and Reverse Transcriptase Activities

I. INTRODUCTION

Hepatitis B virus (HBV) DNA synthesis begins with the replication of a minus strand DNA from a pregenomic RNA template. This process is mediated by the virus encoded polymerase which is a multifunctional protein. Studies on the functions of the polymerase have indicated that the amino-terminal portion of the protein is associated with priming properties (Bartenschlager and Schaller, 1988; Weber *et al.*, 1994; Zoulim and Seeger, 1994; Wang and Seeger, 1993) whereas the central region of the protein confers DNA polymerase and reverse transcriptase activities (Wu *et al.*, 1990; Bavand *et al.*, 1989; Faruqi *et al.*, 1991; Chang *et al.*, 1990). As with the retroviral polymerases, HBV polymerase also possesses RNase H activity which is encoded by the carboxy-terminal region of the protein (Radziwill *et al.*, 1990; Faruqi *et al.*, 1991). Recently it has also been shown that the polymerase is directly involved in the process of encapsidation (Bartenschlager *et al.*, 1990; Bartenschlager and Schaller, 1992; Chen *et al.*, 1994) and has RNA and DNA binding properties (Köchel *et al.*, 1991; Pollack and Ganem, 1994).

Although it is well known that hepatitis B virus polymerase plays a pivotal role in the process of the viral replication, characterization of the polymerase at biochemical level has been hampered by the lack of well-characterized antibodies to this protein and an inabilility to produce an active polymerase. Several laboratories have attempted to express the polymerase, however, the protein produc[illegible] have only been identified by im[illegible]ogical

methods, and neither DNA polymerase nor reverse transcriptase activities could be detected when I began this study (Mack *et al.*, 1988; Chang *et al.*, 1989; Noonan *et al.*, 1991; Stemler *et al.*, 1988). In the following section, I will review the different expression strategies employed by several laboratories.

Although syntheses of retroviral polymerases using prokaryotic expression vectors have been successfully achieved (Kotewicz *et al.*, 1985; Hostomsky *et al.*, 1992), expression of a full length HBV polymerase in *E.coli* has not been reported. Most of the HBV polymerase gene products were expressed as fusion proteins with bacteriophage MS2 polymerase, or *E. coli* Trp E or β-galactosidase linked to a truncated polymerase protein at the amino-terminus (Stemler *et al.*, 1988; Chang *et al.*, 1989). A tribrid fusion protein containing 8 amino acids of β-galactosidase at the amino-terminus, 262 amino acids of aminoglycoside phosphotransferase at the carboxy-terminus and 143 amino acids from the central region of the polymerase was produced in *E. coli* (Mack *et al.*, 1988). Although substantial amounts of the fusion proteins have been obtained, none of these products have polymerase activity. Using these fusion proteins as immunogens, generation of anti-polymerase antisera from rabbits or detection of anti-polymerase antibodies in HBV infected patients have been reported (Stemler *et al.*, 1988; Chang *et al.*, 1989; Will *et al.*, 1986).

Eukaryotic expression of HBV polymerase has recently been described. Full length polymerase proteins of 85 - 93 kDa were expressed using the insect cell/baculovirus system (Ayola *et al.*, 1993; Noonan *et al.*, 1991; McGlynn *et al.*, 1992). Recombinant baculoviruses containing the entire HBV polymerase open reading frame (pol ORF) or the nucleotide sequences derived from the preC-mRNA were used for the expression. It is worth noting that two proteins of molecular masses of 72 and 93 kDa were expressed from the pre-C-like mRNA produced by the recombinant baculovirus (McGlynn *et al.*, 1992). Protein species of similar molecular masses were also detected in virus particles isolated from an HBV integrated cell line or HBV infected liver tissue (Oberhaus and Newbold,

1993; Bavand *et al.*, 1989; Bavand and Laub, 1988). Whether these 72 and 93 kDa protein species correspond to the presence of two polymerase gene products during natural viral infection warrants further investigation. Expression of HBV polymerase in a recombinant baculovirus in *Spodoptera frugiperda* (Sf9) cells derived from ovarian tissue of fall army worms resulted in only a product that was detected by ^{35}S-methionine labeling followed by immunoprecipitation using antisera raised against synthetic peptides deduced from the pol ORF (McGlynn *et al.*, 1992; Noonan *et al.*, 1991). A higher level of expression of HBV polymerase was obtained using a baculovirus expression system in a *Trichoplusia ni* (Tn) cell line derived from the eggs of the cabbage looper. In this case, expressed products were visualized with Coomassie blue staining (Ayola *et al.*, 1993). However, no enzymatic activities of the expressed polymerase was reported in any of these studies.

In 1992, Bartenschlager *et al.* reported the use of recombinant vaccinia viruses to express both the complete protein and the terminal domain of the HBV polymerase (Bartenschlager *et al*, 1992). Immunoprecipitation with antiserum directed against the carboxy-terminal sequence of the polymerase protein indicated the presence of a full length product of about 90 kDa, whereas a series of shorter polypeptides were detected when antiserum specific for the amino-terminal region of the polymerase protein was used. These results suggested that the carboxy-terminal region might be more susceptible to proteolytic cleavage or degradation. Introduction of a protein kinase A site at either the amino-terminal or the spacer region of the polymerase allows high specific labeling of the protein after expression. Using this technique, the half-life of the expressed polymerase protein was estimated to be about 40 minutes.

There were two reports describing the expression of enzymatically active hepadnavirus polymerases in eukaryotic hosts (Seifer and Standring, 1993; Tavis and Ganem, 1993). Recombinant plasmids containing pol ORF with or without the *cis*-sequences for DNA initiation were constructed for expression in yeast or Xenopus oocyte, respectively. In yeast expression, duck hepatitis B virus (DHBV) polymerase was expressed as a fusion

protein which contained a portion of the yeast retrotransposon Ty1 fused at the amino-terminus of the polymerase. Gene products of 138 and 131 kDa, presumably these larger molecular masses were accounted for by the presence of the Ty determinants at the amino-termini, were detected by immunoblot using monoclonal antibodies specific for the hemagglutinin epitope inserted at the spacer region of the polymerase protein (Tavis and Ganem, 1993). Microinjection of polymerase mRNAs into Xenopus oocytes resulted in an HBV polymerase protein of 96 kDa, which was detected by immunoprecipitation of a ^{35}S-methionine labeled cell lysate with monoclonal antibodies directed against the L-epitope introduced at the spacer region of the polymerase protein (Seifer and Standring, 1993). Hepadnavirus polymerases expressed by both systems were shown to prime DNA replication. In particular, since the mRNAs used for protein expression in oocytes did not contain any *cis*-sequences essential for hepadnaviral DNA replication, the polymerase activity seen in this study suggested that HBV polymerase can uncouple from both the nucleocapsid and the replication origin.

In the present study, we have expressed the duck hepatitis B virus (DHBV) polymerase by using an *in vitro* rabbit reticulocyte translation system. The product demonstrates both DNA polymerase and reverse transcriptase activities when tested on exogenous templates of DNA or RNA to which DNA random primers had been hybridized (DNA primed DNA templates or DNA primed RNA templates, respectively). In addition, the *in vitro* expressed polymerase is found to prime DNA synthesis using the nascent mRNA as template. During the course of this work, two other laboratories expressed the hepadnaviral polymerases using a similar approach (Lin and Lo, 1992; Wang and Seeger, 1992). Lin and Lo (1992) expressed an HBV polymerase gene product of approximately 94 kDa. However, these authors did not report whether or not their expressed protein was enzymatically active. In 1992, Wang and Seeger described *in vitro* translation of a functional DHBV polymerase. The results reported in their study agreed with the characterization of our *in vitro* translated polymerase, which was in press at the time of their report (Howe *et al*, 1992).

II. MATERIAL AND METHODS

1. Construction of pTZ19RPol expression vector. A plasmid pD2Eco11 containing a full length DHBV-16 sequence in pUC19 was first cut at unique sites (nucleotides 141 and 2843 respectively) with HincII and EcoRI. To separate the vector from the gene of interest, the remaining pUC19 vector was further digested with PvuI. The HincII-EcoRI DHBV fragment was gel purified and ligated into pTZ19R (United States Biochemical, Cleveland, Ohio) previously cut with the corresponding enzymes. pTZ19R contains a T7 promoter upstream of the multiple cloning sites. The resulting recombinant plasmid was transformed into *E.coli* DH5α, and the desired plasmid was verified by restriction enzyme mapping and hybridization to a DHBV DNA probe.

2. *In vitro* transcription and translation. Recombinant plasmids were first purified by polyethylene glycol precipitation as described by Sambrook *et al.* (1989). The purified plasmid DNA was linearized by digesting with EcoRI before transcription. All RNase present in the preparation was removed by treating the plasmid DNA with Proteinase K (200 μg/ml) and SDS (0.5%) at 37 °C for 30 min, followed by a phenol-chloroform extraction. *In vitro* transcription by bacteriophage T7 polymerase was carried out using Ambion Megascript kits (Ambion Inc. Austin, Texas). Briefly, approximately 1 μg of linearized DNA template was incubated with 6 mM m^7Gppp(5')G analogues and 1.5 mM dGTP, 7.5 mM each of dATP, dCTP and dTTP in the transcription buffer and enzyme mix provided by the kit. The final volume of the reaction is 20 μl. After 4 h of incubation at 37 °C, DNA templates were removed by treating the reaction mixture with 1μl of RNase-free DNase at 37 °C for 15 min. The reaction was terminated by adding 115μl of RNase-free water and 15μl of ammonium acetate stop solution provided in the kit. RNA templates were recovered by extracting once with an equal volume of phenol-chloroform (1:1 v/v) and twice with chloroform, followed by an ethanol precipitation in the presence of one-fifth volume of 3M ammonium acetate, [pH4.0].

In vitro translation was performed using a rabbit reticulocyte lysate system (Promega, Madison, WI) with incubation at 30 °C for 1 h. Approximately 0.5 - 1 µg of the *in vitro* synthesized RNA templates were mixed with 40 U RNasin ribonuclease inhibitor (Ambion Inc., Austin, Texas), 20 µM amino acid mixture (minus methionine), 4 µl of ^{35}S-methionine (1,200 Ci/mmol) and 35 µl of rabbit reticulocyte lysate. The reaction mix was brought up to a final volume of 50 µl with RNase free water. To synthesize unlabeled polymerase, 1 µl of 1 mM complete amino acid mixture was added into the translation reaction. Efficiency of translation was determined by measuring ^{35}S-methionine incorporation into proteins that were TCA precipitable. The *in vitro* translated proteins were further analyzed by 10% SDS-PAGE, stained with Coomassie blue dye and then immersed in 1 M sodium salicylate before autoradiography.

3. DNA Polymerase and Reverse Transcriptase assays.

Preparation of DNA primed DNA templates - Five pmoles of random hexamers (custom synthesis on an Applied Biosystems 392 DNA/RNA synthesizer, Department of Biochemistry, University of Alberta) were incubated with approximately 3 pmoles of linearized plasmid DNA (pMJ601 cut with Afl II) in an annealing buffer (50 mM Tris.HCl [pH 8.0], 50 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol) in a final volume of 30 µl. The reaction mixture was boiled at 94 °C for 2 min, centrifuged five seconds at 12,000 rpm at room temperature and incubated at 37 °C for 2 h. Conditions for successful annealing of the DNA-random primers was verified by first 5' end-labeling the random primers with [γ-^{32}P]ATP and bacteriophage T4 polynucleotide kinase, separating the annealed DNA primed DNA templates by electrophoresis in a 0.8% agarose gel and then subjecting the gel to autoradiography.

Preparation of DNA primed RNA templates - RNA templates were first generated by the *in vitro* transcription of the pTZ19RPol construct as described above. The DNA templates used for the *in vitro* transcription were removed by treating the mixture with 1µl of RNase-free DNase (Promega, Madison, WI). The *in vitro* synthesized RNA was

further purified by phenol-chloroform extraction and an ethanol precipitation. Five pmoles of the random primers were hybridized to about 3 pmoles of the RNA templates as described for the preparation of the DNA primed DNA templates. The conditions for annealing the DNA primers to the RNA templates was again verified by using 5' end-labeled random primers and cold RNA templates as described above.

Assay of enzymatic activity with exogenous templates . Ten microliters of the DNA primed DNA or RNA templates (approximately 0.3 pmoles) were mixed with 15µl of 3X polymerase buffer (3X buffer contains 150 mM Tris. HCl [pH 7.5], 150 mM NaCl, 60 mM MgCl, 0.3% NP-40, 150 µM each of dATP, dGTP and dTTP), 10µl of diluted [α–^{32}P]dCTP (2µl of 3000Ci/mmol [α –^{32}P]dCTP diluted in 50µl of water) and either 5µl of the *in vitro* translated reticulocyte lysate products, 1µl (6 units) of Klenow DNA polymerase (BRL, Burlington, Ont.) or 1µl (10 units) of avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI). The total volume of the mixture was brought to 45 µl with water. For the reverse transcriptase activity assay, 1 µl of human placental RNase inhibitor (20 U) (BRL, Burlington, Ont.) and RNase-free water were used in the reaction. After incubation at 37 ℃ for 45 min, the reaction was stopped by transferring the samples directly into tubes containing 4 ml of 10% ice cold TCA and leaving at 4 ℃ for 10 min for precipitates to form. The precipitates were then collected through GF/C filters (Millipore, Mississauga, Ont.) and counted on the open channel in a Beckman LS 6000TA counter. DNA polymerase or reverse transcriptase activity was estimated by measuring [α–^{32}P]dCTP incorporation into TCA precipitable polynucleotides.

Priming and endogenous reverse transcriptase assay. Five microliters of *in vitro* translated products or rabbit reticulocyte lysate were incubated with 50 mM Tris.HCl [pH 7.5], 50 mM NaCl, 20 mM $MgCl_2$, 0.1% NP-40, 500 µg/ml actinomycin D, 3 mM ATP, 5 pmoles of capped DHBV polymerase mRNA generated from *in vitro* transcription of pTZ19RPol, 1 µl of human placental RNase inhibitor (20 U) (BRL, Burlington, Ont.), 50 µM each of dTTP, dGTP, dATP and 33 nM of [α–^{32}P]dCTP. The reaction mix was

brought up to a final volume of 50 µl with diethyl pyrocarbonate (DEPC)-treated water. After incubation at 37 °C for 45 min, the reaction was stopped by transferring the samples directly into tubes containing 4ml of 10% ice cold TCA. Reverse transcriptase activity was estimated by measuring [α–^{32}P]dCTP incorporation into TCA precipitable polynucleotides as described above.

For reaction products intended for analysis by SDS-PAGE, 15 µl of *in vitro* translated DHBV polymerase lysate or reticulocyte lysate were incubated with 15 µl of 2X reaction buffer (100 mM Tris.HCl [pH7.5], 30 mM NaCl, 20 mM $MgCl_2$, 26 µM each of dATP, dCTP and dTTP, and 20 µCi of [α–^{32}P]dGTP (3000 Ci/mmol). The reaction was carried out at 30 °C for 40 min and stopped by the addition of 270 µl of protein loading buffer (Sambrook *et al.*, 1989). The samples were analyzed by 10% SDS-PAGE. After electrophoresis, the gel was stained with Coomassie blue G-250, dried and autoradiographed.

III. RESULTS

1. Expression in prokaryotic or eukaryotic hosts fails to yield full length DHBV polymerase.

I have attempted to express DHBV polymerase in both prokaryotic and eukaryotic systems. Neither system yielded a full length, enzymatically active product. The recombinant constructs used for the expression are shown in Appendix III. For prokaryotic expression, I employed the glutathione S-transferase (GST) expression system in which full length or the amino-terminal portion of the pol ORF was cloned in frame at the 3' terminus of the Schistosoma japonicum GST ORF. A fusion protein of about 37 kDa was obtained from the recombinant construct containing the GST-amino-terminus of the polymerase after IPTG induction. No expression product was obtained from the recombinant construct containing the GST-full length polymerase. Attempts to grow cells to high density before induction resulted in a very faint band of a protein species of about 80 - 90 kDa as revealed in SDS-PAGE. Expression using another cloning vector, pTrcHis, did not yield full length polymerase either.

Attempts have also been made to construct recombinant vaccinia viruses which contain either a full length or the carboxy-terminal half of the DHBV polymerase. Recombinant vaccinia viruses were identified by selection for thymidine kinase negative clones grown in the presence of bromodeoxyuridine, and the presence of β-galactosidase. Although "blue plaques" were obtained in cells containing either construct, only the recombinant viruses containing half of the DHBV polymerase were able to be enriched in cell culture.

I have attempted to inject the *in vitro* synthesized polymerase mRNAs into *Xenopus* oocytes. I found that 62 out of 70 oocytes injected with the DHBV polymerase RNA died after 2 days of incubation, whereas all placebo-injected oocytes survived the injection and incubation period. Further experiments will be required to determine the cause of toxicity.

The difficulty encountered in expressing the active polymerase gene in biological systems led me to believe that the presence of hepadnavirus polymerase may be toxic in both prokaryotic or eukaryotic cells. To overcome this problem, I attempted to express the polymerase gene of DHBV *in vitro* using a rabbit reticulocyte lysate system.

2. *In vitro* transcription /translation of DHBV polymerase.

There are two potential translation initiation sites present (at nucleotides number 20 and 170 respectively) in the DHBV polymerase gene. The codon at nt 170 is highly conserved among different DHBV isolates and is likely to be used as the translation initiation in DHBV. The initiation codon at nucleotide 20 is located in the sequence UGA AAG CUU AUG C, which is an unfavorable context according to Kozak (1989). The sequence surrounding the initiation codon at 170 is GAG GTA GAG AUG C. The purines at positions -3, -6 and -9 in this flanking sequence are believed to accommodate more stable ribosomal binding for translation initiation (Kozak, 1987; Kozak, 1991). The recombinant plasmid construct used in the present study for generating the DHBV polymerase transcriptis shown in Figure 2-1. In this construct, the AUG codon at nucleotide 170 was chosen as the first initiation codon for *in vitro* translation.

The *in vitro* transcribed capped mRNA was translated in the rabbit reticulocyte lysate and the ^{35}S-methionine labeled protein products are shown in Figure 2-2. The addition of transcription products from firefly luciferase was used as the positive control which gave a protein band at 61 kDa (Figure 2-2, lane 1). The RNA product transcribed from pTZ19RPol supported the synthesis of a new protein at 90 kDa as predicted from the nucleotide sequence of the pol ORF of DHBV-16 (Figure 2-2, lane 2). The construct inserted in the plasmid in the opposite direction failed to support the synthesis of this protein (data not shown). No protein synthesis was seen in the lysate incubated without the addition of RNA template, which shows that there was no endogenous RNA in the

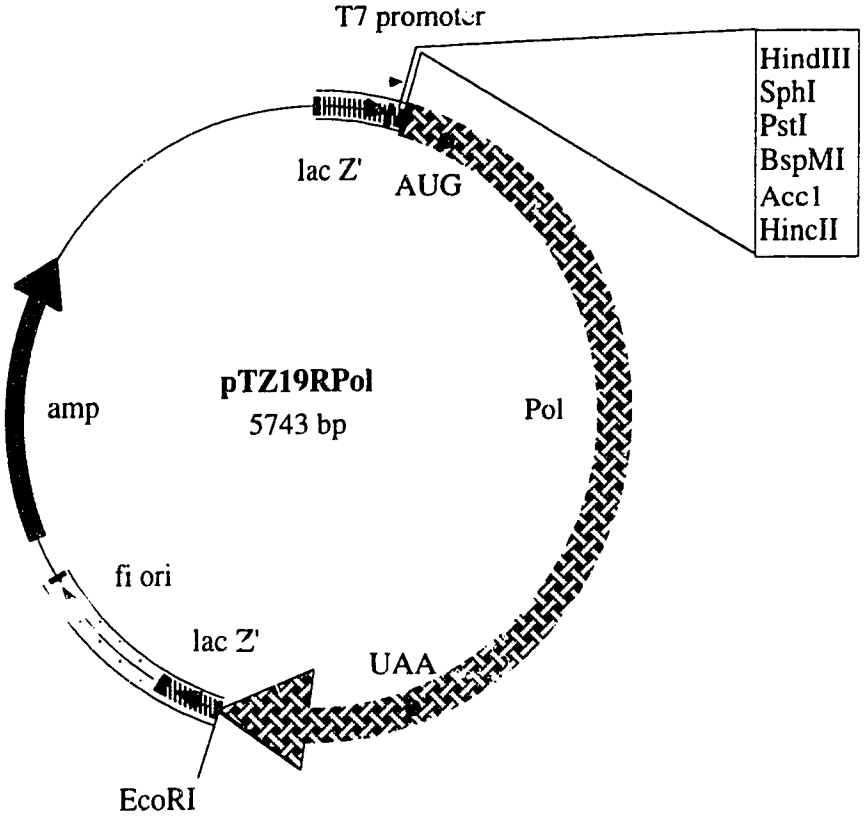

Figure 2-1. Recombinant construct pTZ19RPol for the expression of DHBV polymerase gene.

A fragment of the DHBV polymerase gene was inserted into the multiple cloning sites of an expression vector pTZ19RPol at HincII and EcoRI, respectively. Translation was initiated at nucleotide 170 and terminated at nucleotide 2526 as indicated by AUG and UAA in the diagram. The arrow indicates the direction of transcription from the T7 promoter. A run-off transcript was generated by cutting the construct at the EcoRI site.

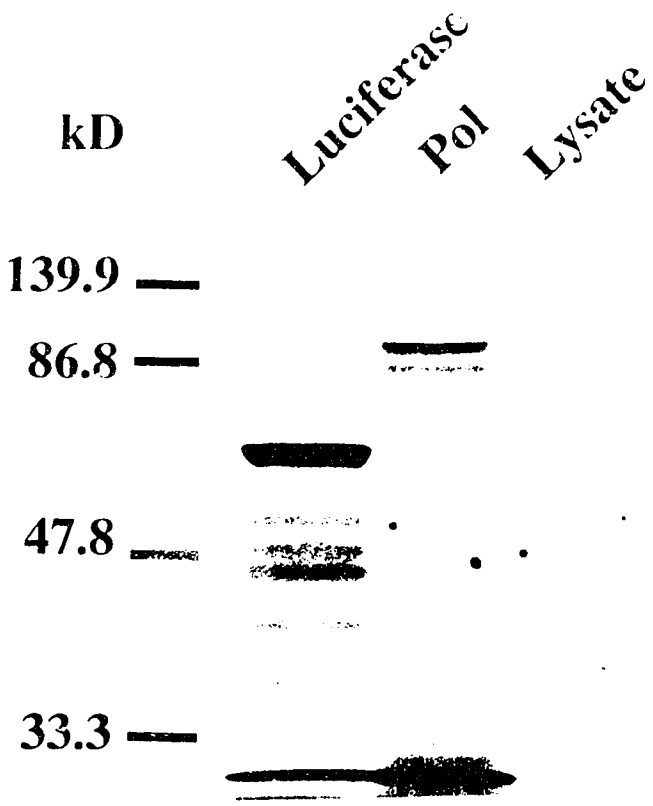

Figure 2-2. SDS-PAGE and autoradiography of ^{35}S-methionine labeled *in vitro* translation products.

Transcripts generated by *in vitro* transcription were translated using a rabbit reticulocyte *in vitro* translation system in the presence of 40 µCi of ^{35}S-methionine. Radioactively labeled proteins were separated on a 10% SDS-polyacrylamide gel and subjected to autoradiography. Luciferase, *in vitro* translated products from mRNA transcripts of firefly luciferase. Pol, *in vitro* translated products from mRNA transcripts generated from pTZ19RPol recombinant construct. Lysate. Rabbit reticulocyte lysate without the addition of mRNA during translation.

nuclease treated reticulocyte lysate system used in these studies (Figure 2-2, lane 3). The typical efficiency of expression of the DHBV polymerase transcripts in this translation system was about 15 fold above background as judged by the ^{35}S-methionine incorporated into the TCA precipitable material. From the specific activity of the incorporated ^{35}S-methionine, I estimated that approximately 75 fmoles of polymerase were produced in each reaction (total volume 50 µl).

I attempted to immunoprecipitate the expressed products using rabbit polyclonal antibodies raised against synthetic peptides deduced from the nucleotide sequences of pol ORF. Unfortunately, none of the antisera produced by immunization with these peptides were immunoreactive with the *in vitro* translated polymerase. A summary of the synthetic peptides used for raising polyclonal antibodies is given in Appendix IV.

3. *In vitro* translated polymerase possesses enzymatic activities.

The protein product from the rabbit reticulocyte translation was used in assays for DNA polymerase and reverse transcriptase activities. Each of these assays were repeated two to three times. Representative data are shown in Figures 2-3, 2-4, 2-5 and 2-6. The DNA polymerase activity is shown in Figure 2-3. In this experiment, purified *E. coli* Klenow DNA polymerase was used as a positive control. There was a potent DNA polymerase activity associated with the product expressed from the recombinant DHBV polymerase construct (pTZ19RPol). This activity was not seen in the reticulocyte lysate alone and was abolished by heating.

Evidence for the reverse transcriptase activity is shown in Figure 2-4. Incorporation of [α –^{32}P]dCTP in the reaction containing AMV reverse transcriptase is shown and indicated that the DNA primed RNA templates serve as an adequate substrate for this assay. The expressed DHBV polymerase also demonstrated a significant amount of reverse transcriptase activity. This activity was not found in the reticulocyte lysate alone, nor in

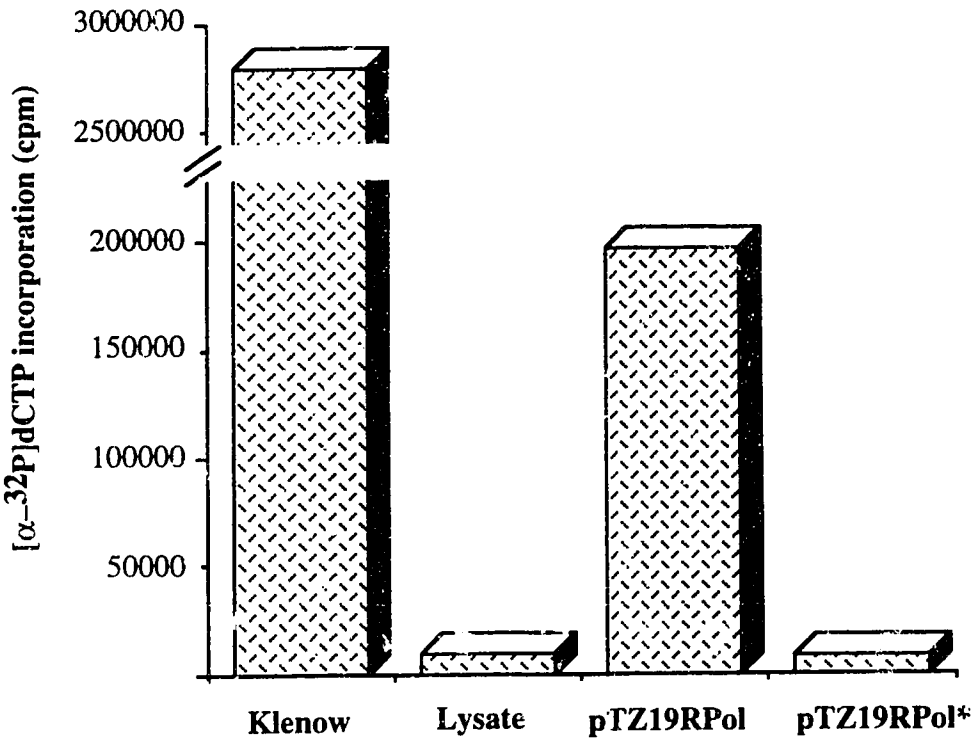

Figure 2-3. DNA polymerase activity assay.

In vitro translated products or positive control (Klenow) were incubated with linearized exogenous DNA primed DNA templates at 37 ℃ for 45 minutes in the presence of [α-^{32}P]dCTP. Klenow represents 1µl of a modified *E.coli* DNA polymerase available commercially (BRL, Burlington, Ont.). Lysate and pTZ19RPol are the *in-vitro* translated products of rabbit reticulocyte lysate without the addition of mRNA or in the presence of mRNA transcribed from pTZ19RPol. pTZ19RPol* represents the *in vitro* translated products of pTZ19RPol after heat inactivation at 94 ℃ for 5 minutes. DNA polymerase activities were estimated by measuring the radioactive counts (CPM) of [α-^{32}P]dCTP incorporation into TCA precipitable polynucleotides.

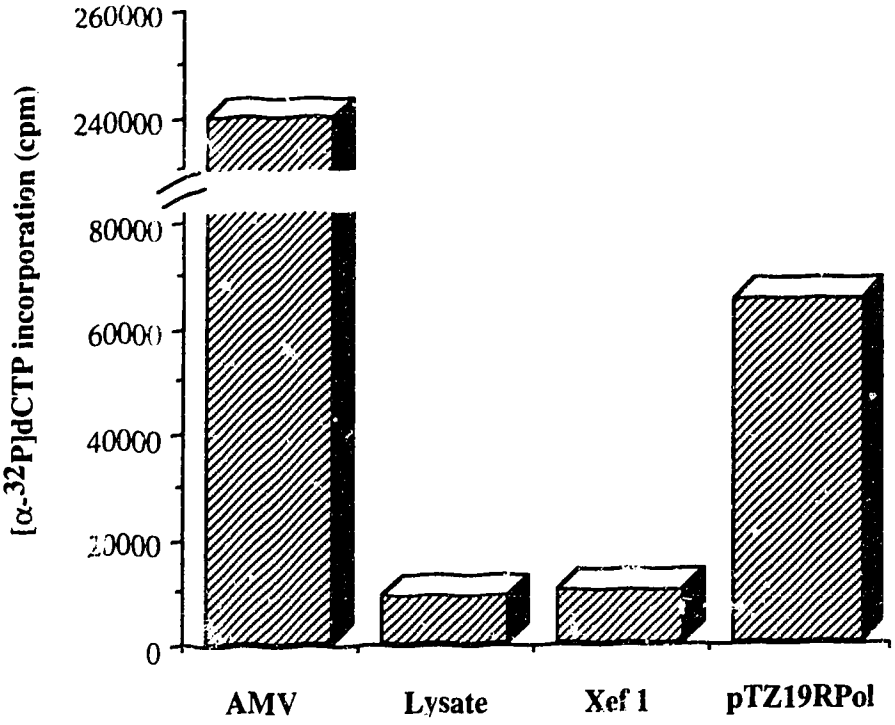

Figure 2-4. Reverse transcriptase activity assay.

In vitro translated products or positive control (AMV reverse transcriptase) were incubated with DNA primed D RNA templates at 37 °C for 45 minutes in the presence of [α-^{32}P]dCTP. AM represents a reaction containing 1µl of commercial AMV reverse transcriptase (BRL, Burlington, Ont). XEF 1 are translation products of *Xenopus* Elogation Factor 1 mRNAs. Lysate and pTZ19RPol are *in vitro* translated products of rabbit reticulocyte without the addition of mRNA or in the presence of mRNAs transcribed from pTZ19RPol. Reverse transcriptase activities were estimated by measuring the radioactive counts (CPM) of [α-^{32}P]dCTP incorporation into TCA precipitable polynucleotides.

another *in vitro* expressed product (*Xenopus* Elongation Factor 1) which is known not to have reverse transcriptase activity. Similar results were obtained in assays using poly rC-$dG_{(12-18)}$ as substrates (results not shown).

The DNA polymerase and reverse transcriptase activities of the *in vitro* expressed DHBV polymerase were determined by the addition of actinomycin D (100 µg/ml) to the reaction mixture. Actinomycin D is a compound which is known to intercalate into DNA-DNA templates, hence blocking the DNA polymerization process. However actinomycin D will not intercalate into DNA-RNA templates and should not affect reverse transcriptase activity. As shown in Figure 2-5, there was about 70% inhibition of the DNA polymerase activity in the presence of actinomycin D, whereas the reverse transcriptase activity remained unchanged by the addition of actinomycin D.

One of the distinctive features displayed by all hepadnavirus polymerases is their ability to prime DNA replication using the nascent pregenomic RNA as templates (Bartenschlager *et al.*, 1990). To be sure that the above assays measured activities from a *bona fide* DHBV polymerase, I tested the *in vitro* translated product for DNA-priming. Since the linearized mRNA used for *in vitro* translation of the polymerase contained both the ε and the DR1 regions which are essential for initiation of DNA replication in hepadnaviruses, it served as a suitable template for reverse transcription. In this assay, the *in vitro* translated products were incubated with [α-^{32}P]dGTP and the three other unlabeled deoxyribonucleotide triphosphates in the absence of exogenous templates. The reaction products were analyzed either by SDS-PAGE or TCA precipitation. The results of these experiments are shown in Figure 2-6A and 2-6B. No radioactive band is seen with products translated from a construct with pol ORF inserted in reverse orientation (Figure 2-6A, lane 1). In Figure 2-6A, lane 2, a radioactive band of 90 kDa and a radioactive smear of molecules with slower mobilities were observed when the reaction contained *in vitro* translated polymerase. The radioactive band at 90 kDa is believed to represent the initiation complex formed by the *in vitro* translated polymerase and the [α-^{32}P]dGTP, whereas the radioactive smear is believed

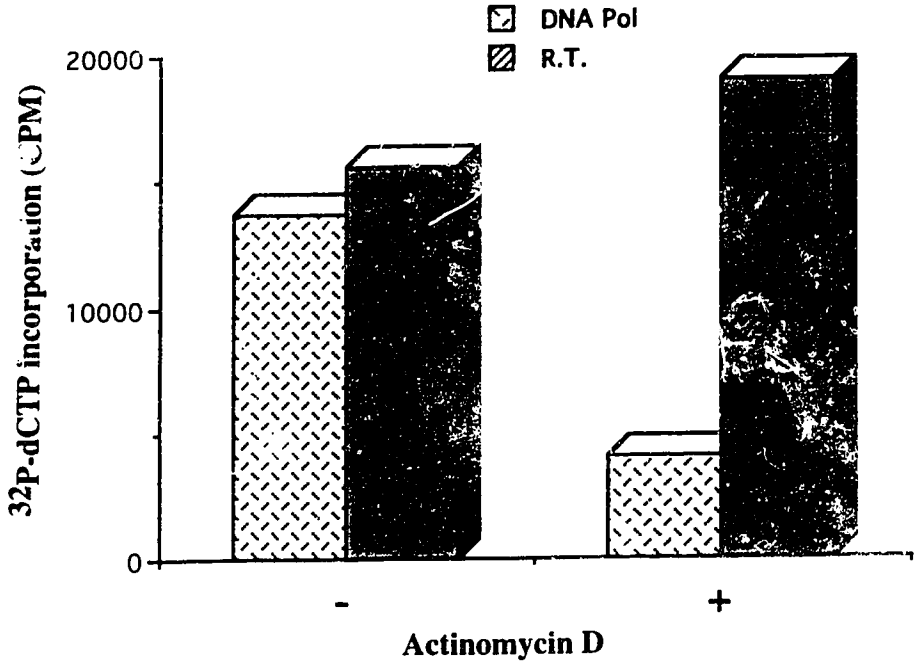

Figure 2-5. DNA polymerase and reverse transcriptase activities of the *in vitro* translated DHBV polymerase in the absence or presence of actinomycin D.

DHBV polymerase from *in vitro* translation was incubated with exogenous DNA primed DNA templates or DNA primed DHBV RNA templates in the presence of [α-^{32}P]dCTP plus or minus 100 µg/ml actinomycin D. The reaction mixture was assayed for DNA polymerase and reverse transcriptase activities, respectively. DNA polymerase or reverse transcriptase activities were estimated by measuring the radioactive counts (CPM) of [α-^{32}P]dCTP incorporation into TCA precipitable polynucleotides.

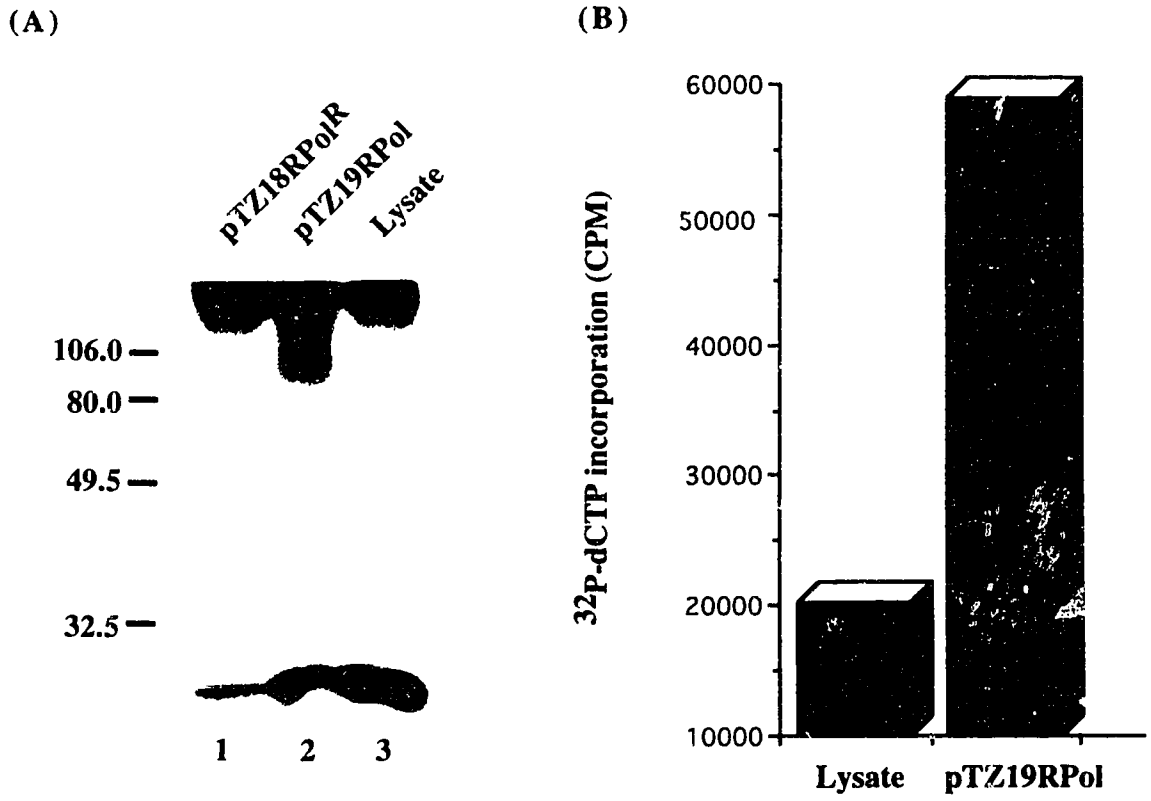

Figure 2-6. DNA priming activity of the *in vitro* translated polymerase.

(A) Analysis of reaction product in SDS-PAGE. *In vitro* translated products were incubated in the presence of dTTP, dCTP, dATP and 20 μCi of [α-^{32}P]dGTP at 30 °C for 40 min. The reaction products were analyzed by 10% SDS-PAGE. Lane 1 and 2 represent *in vitro* translated products from constructs with pol ORF inserted in reverse (pTZ18R^{R}) or same (pTZ19R) orientation as T7 promoter. Lane 3 represents sample of rabbit reticulocyte lysate (Lysate) without addition of mRNA for the polymerase.
(B) Endogenous reverse transcriptase activity. Reverse transcriptase assay was carried in the absence of exogenous templates as described in MATERIAL AND METHODS. Lysate represents rabbit reticulocyte lysate without addition of mRNA. pTZ19RPol contains translation products of reticulocyte lysate plus polymerase mRNA. Endogenous reverse transcriptase activities were estimated by measuring the radioactive counts (CPM) of [α-^{32}P]dCTP incorporation into TCA precipitable polynucleotides.

to indicate the *de novo* synthesized DNA linked to the polymerase. No band was observed on incubation with the reticulocyte lysate alone (Figure 2-6A, lane 3). The high molecular weight radioactive bands found in all samples are likely due to non-specific binding of [α-^{32}P]dGTP to the reticulocyte lysate proteins. In Figure 2-6B, endogenous reverse transcriptase activity is present in sample containing *in vitro* translated polymerase, whereas no activity is detected in reticulocyte lysate alone.

IV. DISCUSSION

An enzymatically active DHBV polymerase was expressed using an *in vitro* transcription/translation system. The *in vitro* synthesized mRNA was translated in a rabbit reticulocyte lysate and produced an ^{35}S-methionine labeled 90 kDa band on SDS-PAGE. The expressed product shows DNA polymerase and reverse transcriptase activities on exogenous templates of DNA or RNA (respectively) with random DNA hexamer primers. The reverse transcriptase activity of this translated product is not inhibited in the presence of actinomycin D, whereas the DNA polymerase activity is found to be significantly inhibited by actinomycin D. Furthermore, the priming activity of this *in vitro* translated protein is evident when tested on endogenous RNA templates. The characteristics found in the protein expressed in this translation system are compatible with those of the DHBV polymerase.

We and investigators in other laboratories have encountered difficulties in cloning and expressing an enzymatically active polymerase. Various expression systems have been explored. These include prokaryotic expression in *E.coli*, a recombinant vaccinia virus expression system and microinjection of polymerase mRNAs into Xenopus oocytes. In spite of these efforts, no full length active polymerase was obtained. This could be due to the putative cytotoxicity of hepadnavirus polymerases as previously suggested (Chang *et al.*, 1989, [illegible] and [illegible]n, 1993). Expression of the amino-terminal portion of the HBV polymerase in [illegible] cells was found to down-regulate interferon α and γ mediated cellular responses (Foster *et al.*, 1991). Results from the same study also indicated that the reverse transcriptase plus the RNase H domains of the polymerase might be responsible for the toxicity of the polymerase in host cells. Alternatively, hepadnavirus polymerases might be extremely short-lived. Instability of the polymerases can be inferred from its short half-life and extensive proteolytic degradation observed in different expression systems (Bartenschlager *et al.*, 1992; Chang *et al.*, 1989; Blum *et al.*, 1992).

To date, functional hepadnaviral polymerases have been reported in three different expression systems: the yeast retrotransposon Ty1 system (Tavis and Ganem, 1993), microinjection of mRNAs into Xenopus oocytes (Seifer and Standring, 1993) and *in vitro* transcription/translation using rabbit reticulocyte lysate (Wang and Seeger, 1992; Howe *et al.*, 1992). The presence of DR1 and ε regions were found in the recombinant constructs used in both yeast expression and *in vitro* transcription/translation. In both cases, specific polymerase activities accompanied by precise DNA initiation from the pregenomic RNA template were described (Tavis and Ganem, 1993; Wang and Seeger, 1992). A recent report by Lanford *et al.* (1994) indicated that expression of a functional HBV polymerase in insect cell/ baculovirus was possible when the DR1 and ε regions were included in the recombinant construct. In the light of these studies, it can be speculated that the presence of these *cis*-sequences might be essential for stabilizing and facilitating the enzymatic activities of the polymerase during expression.

In conclusion, results from my experiments indicated that it is possible to express an enzymatically active DHBV polymerase by using an *in vitro* transcription/translation system. I believe this approach will be generally applicable to other hepadnavirus polymerases, including human HBV. This technique may have broad applications for studying the structure/function of hepadnavirus polymerases and the interaction of antiviral drugs with the polymerases.

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Chapter 3

Selective ›bition of the Reverse Transcription of Duck Hepatitis B ›irus by Covalent Binding of ddGTP to the Viral Polymerase

I. INTRODUCTION

Hepatitis B infection can result in diverse clinical manifestations, ranging from subclinical infections to fulminant hepatitis, or chronic infection which may lead to cirrhosis or hepatocellular carcinoma. Although effective vaccines against hepatitis B virus (HBV) using surface antigens have been developed, there are still approximately 300 million carriers worldwide (Sherlock, 1990). Since the vaccine is of no benefit to the carriers, an attempt to find antiviral agents to treat chronic carriers is justified. Unfortunately, antiviral therapy against HBV infection has met with very limited success (Davis and Hoofnagle, 1986; Lai *et al.*, 1991; Di Bisceglie *et al.*, 1990; Garcia *et al.*, 1987). Recently, several nucleoside analogues have been shown to be effective inhibitors of hepadnaviral replication (Lee *et al.*, 1989; Matthes *et al.*, 1990; Kassianides *et al.*, 1989; Löfgren *et al.*, 1989), but the mechanism of action of these agents remain unclear.

HBV is a partially double-stranded DNA virus with a genome length of about 3200 nucleotides. Viral DNA synthesis, which is mediated by the viral polymerase, involves two stages: the synthesis of minus strand DNA by reverse transcription using the pregenomic RNA as template, and the synthesis of plus strand DNA using the newly synthesized minus strand DNA as template (reviewed in Robinson, 1990; Blum *et al.*, 1989; Schödel *et al.*, 1990). Initiation of minus strand DNA synthesis has been shown to

be mediated by a primer domain of the viral polymerase which is also required for packaging the pregenomic RNA through interaction with the RNA packaging signal, ε (Bartenschlager and Schaller, 1988; Wang and Seeger, 1992; Will *et al.*, 1987; Hirsch *et al.*, 1991; Bartenschlager and Schaller, 1992). After addition of four nucleotides to the polymerase at the bulge sequence of the 5' copy of ε, the polymerase-deoxynucleotide complex is then transferred to the 3' copy of DR1 where minus strand DNA synthesis continues (Tavis *et al.*, 1994; Wang and Seeger, 1993). In the case of human HBV and duck hepatitis B virus (DHBV), the first nucleotide of the minus strand DNA is found to be a deoxyguanosine residue (primer domain-GAAAAA... in HBV; primer domain-GTAATT... in DHBV) (Molnar-Kimber *et al.*, 1984; Saldanha *et al.*, 1992). As well as serving as the key enzyme for DNA replication, the polymerase is also involved in capsid formation (Bartenschlager *et al.*, 1990; Blum *et al.*, 1991). Given that the polymerase plays a pivotal role in viral replication, it is a promising target for antiviral agents.

Previous studies in our laboratory using primary duck hepatocytes to screen dideoxynucleoside analogues for activity against DHBV indicated that purine 2',3'-dideoxynucleosides were potent inhibitors whereas pyrimidine 2',3'-dideoxynucleosides were not (Suzuki *et al.*, 1988). In particular, 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR), a presumptive prodrug of 2',3'- dideoxyguanosine (ddG), was found to be a thousand-times more active than 2',3'-dideoxycytidine (ddC) (Lee *et al.*, 1989). We have also found that ddDAPR was oxidatively deaminated to ddG in cultured cells or *in vivo* and that both ddG and ddC were phosphorylated efficiently in duck hepatocytes (Kitos and Tyrrell, 1995). Therefore, I have considered ddDAPR equivalent to ddG for the purpose of the following discussion. Since the synthesis of the minus strand DNA is initiated with a deoxyguanosine residue at the 5' terminus, we have hypothesized that the introduction of ddGTP (metabolized from ddDAPR) may compete with dGTP for a binding site on the primer domain (Suzuki *et al.*, 1988). The bond between the primer domain and ddGMP

(or ddGTP) might be resistant to repair mechanisms whereas dideoxynucleotides (ddNtds) incorporated downstream might be removed by proofreading.

In the present study, I investigate the mechanism by which these compounds inhibit viral replication. First of all, I ask whether or not these purine 2',3'-dideoxynucleoside analogues compete with the natural nucleoside for utilization in hepadnaviral DNA replication in primary duck hepatocytes isolated from ducklings congenitally infected with DHBV. Results from these studies suggest that ddG or ddDAPR compete with dG for the same target(s) during DHBV replication. To pursue the mechanism of inhibition mediated by ddDAPR observed *in vitro*, I have treated ducklings congenitally infected with DHBV with various dosages of ddDAPR or ddC. By monitoring the reverse transcriptase activity and the amount of DNA and RNA present in the virus replicating cores, I show that synthesis of viral DNA is selectively inhibited by ddDAPR but not ddC. I also demonstrate that the inhibition occurs at a very early stage during the minus strand DNA synthesis. Such inhibition is not due to the loss of enzymatic activity of the polymerase. Finally, by expressing an active DHBV polymerase using an *in vitro* rabbit reticulocyte translation system, I show that there is direct binding of ddGTP to the *in vitro* translated polymerase. These results suggest that ddDAPR (pro-drug of ddG) inhibits DHBV replication through a novel mechanism by the direct binding of ddGTP to the polymerase, hence irreversibly blocking subsequent viral DNA synthesis.

II. MATERIALS AND METHODS

1. Nucleoside analogues and animals. 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR) and 2',3'-dideoxycytidine (ddC) were synthesized by Raylo Chemicals (Edmonton, Alberta, Canada). Fertilized Pekin duck eggs were raised from breeder flocks of Pekin ducks at the University of Alberta. Newly hatched congenitally DHBV-infected ducklings were identified by detection of the presence of DHBV DNA in sera by dot blot hybridization .

2. Experimental animals and cell culture. Primary cultures of duck hepatocytes were prepared from a 10 - 14 d old DHBV infected duckling using procedures described by Tuttleman *et al.* (1986) with some modifications. The duckling was anesthetized by an intravenous injection of approximately 0.5 ml of pentobarbital sodium [Nembutal], (Abbott Laboratories Ltd., Montreal). The liver was perfused aseptically with perfusion buffer which contains 0.5 mM ethylene glyco-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 50 IU/ml penicillin G, 10 µg/ml streptomycin sulfate in Minimum Essential Medium Eagle for suspension cultures (Flow Laboratories, Mississauga, Ont.). All solutions used for perfusion were maintained at 37 °C throughout the procedures. Perfusion of the liver was performed by inserting a number 18 I.V. catheter into the right ventricle of the heart. Perfusion buffer was pumped from the heart into the liver and exited via the portal vein which was incised. After flushing the liver with 200 ml of the perfusion buffer, an additional 200 ml of buffer supplemented with 0.5 mg/ml collagenase, type IV (Sigma Chemical Co., St. Louis) and 2.5 mM $CaCl_2$ was perfused through the liver over approximately 15 minutes. The liver was gently massaged to assist the dissociation of hepatocytes from the connective tissue. After perfusion, the liver was removed and cells were dispersed in L-15 medium (Gibco BRL, Burlington, Ont.) containing 250 U/ml Nystatin (GibcoBRL, Burlington, Ont.), 5% fetal bovine serum, 50 IU/ml penicillin G, 10 µg/ml streptomycin sulfate, 15 mM HEPES, 1.2 µg/ml

insulin, 1.7 μg/ml glucose and 11 μM hydrocortisone 21-hemisuccinate (Sigma Chemical Co., St. Louis, Mo.). Cells were filtered through a sterile nylon gauze and centrifuged at 50 x g for 5 min. The cell pellet was washed three times with L-15 medium and resuspended in the same medium to a final volume of about 50 ml. Viable cells were evaluated by the trypan blue exclusion method and quantified using a hemocytometer. Approximately 2 x 10^6 cells were seeded in a 60-mm tissue culture dish. Cultures were incubated in a 37 °C humidified incubator and the medium was changed every other day.

3. *In vitro* drug treatment. To evaluate the effect of ddG vs dG and ddDAPR vs dG on DHBV replication, 1 μM of ddG or ddDAPR was added to the medium with 0, 0.1, 1, 5 or 10 μM of dG. Media containing nucleoside analogues were added to the hepatocyte cultures on day 2 and changed every second day. Cells were harvested at day 20 and tested for the presence of DHBV DNA by dot blot hybridization. All drug treatments in each experiment were performed in triplicate, and each experiment was repeated twice.

4. Preparation of cell lysate and DNA extraction. Cells in each 60 mm tissue culture dish were lysed with 1 ml of STEEN buffer (0.2% SDS, 150 mM Tris.HCl [pH 8.0], 5 mM EGTA, 10 mM EDTA and 150 mM NaCl). Cell lysates were digested with 0.5 mg/ml Pronase E (Sigma Chemical Co., St. Louis, Mo.) at 37 °C for at least 2 h. Nucleic acids were extracted once with an equal volume of phenol saturated with 20 mM Tris.HCl [pH 8.0] - 0.5 mM EDTA and 0.1% 8-hydroxyquinoline, once with an equal volume of phenol-chloroform (1:1 v/v) and once with an equal volume of chloroform. Nucleic acids were precipitated in the presence of 0.2 M ammonium acetate and 3 volume of 95% ice cold ethanol. After centrifugation, the nucleic acid pellet was washed with ice cold 75% ethanol, dried and dissolved in 500 μl of TE buffer (Tris.HCl [pH 7.5] and 1 mM EDTA).

5. Radioactive probes. Plasmid pD2Eco11 contained the complete genome of DHBV DNA fused to a pUC19 vector at the EcoR1 site. The radioactive probe was

prepared by nick-translation using a Boehringer Mannheim nick translation kit (Boehringer Mannheim, Quebec, Canada). Oligonucleotides AH129 (5' CCC GAG CAA ATA TAA TCC 3') and AH2369 (5' ATG CCC CGA CCA TTG AAG 3') are located at nt 2410 and 170 of DHBV-16 respectively (Mandart *et al.*, 1984). These oligonucleotides are 129 and 2369 nucleotides, respectively, downstream from the expected initiation site for the minus strand DNA. When used as radioactive probes, 20 pmoles of these oligonucleotides were 5' end-labeled with [γ-^{32}P]ATP (7000 Ci/mmol)(Du Pont, Canada) and bacteriophage T4 kinase (BRL, Ont.) according to standard procedures (Sambrook *et al.*, 1989).

6. Dot blot hybridization. Ten microliters of the DNA sample were applied onto a nylon filter (Hybond-N, Amersham Corp., Arlington Heights, Ill.) with a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Richmond, Calif.). DNA on the filter was denatured with 0.5 M NaOH -1.5 M NaCl for 20 min and neutralized with 0.5 M Tris.HCl [pH 7.5] -1.5 M NaCl for another 20 min. The filter was briefly dried, exposed to UV light (250 - 280 nm) for about 5 min and prehybridized for 3 - 10 h at 42 °C in 50% formamide, 5X SSPE (20X SSPE is 3.6 M NaCl, 200 mM NaH_2PO_4 [pH 7.4], 20 mM EDTA), 5X Denhardt solution (50X Denhardt solution is 10 g Ficoll, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin dissolved in water to a final volume of 1 liter), 0.1% SDS and 100 µg/ml of denatured salmon sperm DNA. Hybridization was carried out by the addition of the radioactive DHBV probe at 1 x 10^6 cpm/ml under the same conditions used for prehybridization at 42 °C for 10 - 16 h. The filter was washed once with 0.1% SDS - 2X SSC (20X SSC is 3 M NaCl - 0.3 M sodium citrate, [pH7.0]) at 65 °C for 20 min, once with 2X SSC for 20 min at 65 °C for 20 min and once with 2X SSC at room temperature for 20 min. The filter was dried and autoradiographed at -70 °C with Kodak XAR film. Effects of nucleoside analogues on DHBV replication were evaluated by quantifying signals on the X-ray film with a scanning densitometer (Chromoscan 3, Joyce-Loebl Ltd., England).

7. *In vivo* drug treatment. The drugs (ddDAPR or ddC) were dissolved in 20 mM Tris-HCl [pH 7.5], 0.9% NaCl and stored at -20 °C. Five week-old congenitally DHBV-infected male ducklings were treated with ddDAPR (10-50 mg/kg) or ddC (20 or 40 mg/kg) or 2 ml of placebo (20 mM Tris-HCl [pH 7.5], 0.9% NaCl) by intramuscular injection every 6 or 12 h daily for 2 days. Animals were sacrificed 1.5 h after the final injection. Livers were removed from anesthetized animals, immediately frozen in liquid nitrogen and stored at -70 °C.

8. Isolation of virus replicating cores from liver. Intact replicating cores were prepared according to the procedures described by Summers and Mason (1982). Six grams of liver tissue were homogenized with 18 ml extraction buffer (EB) (20 mM Tris-HCl [pH 7.4], 7 mM $MgSO_4$, 50 mM NaCl, 0.1% ß-mercaptoethanol, 100 µg/ml bovine serum albumin, fraction V (BSA) and 0.25 M sucrose) using a Dounce homogenizer. Cell debris and nuclei were removed by centrifugation at 10,000 rpm for 20 min at 4 °C in a JA20 rotor. Supernatant was immediately layered on a 33 ml discontinuous 15 - 30% sucrose gradient in EB. Gradients were centrifuged at 23,000 rpm for 7 h at 4 °C in a Beckman SW27 rotor. Fractions (1.5 ml) were collected from the gradients and assayed for endogenous reverse transcriptase activity. Fractions containing activity were pooled (approximately 35 ml) and centrifuged at 23,000 rpm for 15 h at 4 °C in a Beckman SW27 rotor. The replicating core pellet was resuspended in 1 ml EB buffer. Exogenous DNA and RNA were removed by digestion with 1 µg/ml bovine pancreatic DNase I (BRL, Burlington, Ont.) at 37 °C for 10 min followed by an addition of 0.1 µg/ml RNase A (Sigma Chemical Co., St Louis) and 20 mM EDTA at 37 °C for 5 min. Cloudy precipitates containing ribosomal proteins were removed by centrifugation at 2500 rpm for 10 min in a Sorvall refrigerated centrifuge (Sorvall Instruments, DuPont, Mississauga, Ont.) Replicating cores were concentrated by layering the opalescent supernatant (about 1 ml) on a discontinuous 15 - 30% sucrose gradient made up in EB. The purified replicating cores were resuspended in 1 ml EB, briefly sonicated and stored in aliquots at -70 °C.

9. Estimation of the amount of replicating cores. The quantity of replicating cores in liver was estimated by direct enzyme linked immuno-substrate assay (ELISA). A 96-well plate was first coated with 100 µl of replicating core preparations diluted 1:1000 in coating buffer (15 mM $Na_2CO_3.10H_2O$, 35 mM $NaHCO_3$ and 3 mM NaN_3, [pH 9.6]). The plate was placed in a moist chamber at 4 °C overnight and then washed three times with phosphate buffered saline - Tween 20 (PBST) which contains 137 mM NaCl, 1.46 mM KH_2PO_4, 3.8 mM Na_2HPO_4, 2.68 mM KCl and 0.05% Tween 20. One hundred microliters of a solution of anti-DHBcAg antibodies (kindly provided by Dr. J. Summers) diluted in 1:100 in PBST-BSA (1:50 BSA in PBST, w/v) were added into each core-coated well. After 2 h of incubation at 4°C in a moist chamber, the plate was washed three times with PBST, followed by an addition of a 1:10.000 dilution (in PBST-BSA) of goat anti-rabbit IgG antibodies conjugated with peroxidase (Boehringer Mannheim, Quebec, Canada). The plate was further incubated in a moist chamber at 4 °C for 3 h. The reaction mixture was developed with p-nitrophenyl phosphate according to the manufacturer's instruction (Boehringer Mannheim, Quebec, Canada). The plate was read in a Titerek Multiskan ELISA plate reader at 410 nm. The amount of replicating core in each sample was estimated from a standard curve constructed using replicating cores prepared from a placebo treated duck liver (1/10 to $1/10(1/2)^{15}$ dilutions).

10. Endogenous reverse transcriptase activity assay. Fifteen microliters of each intact replicating core samples were used for endogenous reverse transcriptase activity assay. The replicating core sample was incubated with 100 µg/ml actinomycin D, 50 mM Tris-HCl [pH 7.5] , 50 mM NaCl, 20 mM $MgCl_2$, 0.1% Nonidet-40, 50 µM each of dATP, dGTP, dTTP (Pharmacia Inc. Quebec, Canada) and 0.1 µM [α–^{32}P]dCTP (DuPont, Canada). The final reaction volume was 45 µl. After 40 min of incubation at 37 °C, the reaction was stopped by an addition of 5 µl of 10% SDS-0.5 M EDTA. The reaction mixture was further incubated at 37 °C for 10 min. Endogenously radioactively labeled DNA was precipitated by ice cold 10% trichloroacetic acid (TCA) and collected on a

Whatman GF/C glass filter. The filters were washed thoroughly with 10% TCA and dried. Radioactivity was measured by scintillation counting. Reverse transcriptase activities present in the replicating cores were expressed as a percentage of [α–^{32}P]dCTP incorporation relative to the mean value of two placebo treated controls.

11. DNA extraction and Southern blot. One hundred microliters of each core preparation were treated with 0.1% SDS, 20 mM EDTA, 0.1 M NaCl, 0.5 mg/ml proteinase K and 10 µg yeast tRNA. After 1 h of incubation at 37 °C, DNA was extracted with phenol-chloroform and precipitated with ethanol. The DNA pellet was washed with 70% ethanol, dried and redissolved in 50 µl TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) at 68 °C for 5 min. Twenty microliters of the DNA samples were electrophoresed in a 0.8% agarose gel and transferred to a nylon filter with standard procedures (Sambrook *et al.*, 1989). DNA on the filter was denatured with 0.5 M NaOH -1.5 M NaCl for 20 min and neutralized with 0.5 M Tris.HCl [pH 7.5] -1.5 M NaCl for another 20 min. The filter was briefly dried, exposed to UV light (250 - 280 nm) for about 3 min and prehybridized for 3 - 10 h at 42 °C in 50% formamide, 5X SSPE, 5X Denhardt solution, 0.1% SDS and 100 µg/ml of denatured salmon sperm DNA. Hybridization was carried out by addition of the denatured radioactive DHBV probe at 1 x 10^6 cpm/ml under the same prehybridization condition at 42 °C for 10 - 16 h. The filter was washed once with 0.1% SDS - 2X SSC (20X SSC is 3 M NaCl - 0.3 M sodium citrate, pH7.0]) at 65 °C for 20 min, once with 2X SSC for 20 min at 65 °C for 20 min and once with 2X SSC at room temperature for 20 min. The filter was dried and autoradiographed at -70 °C with a Kodak XAR film.

12. RNA extraction and Northern blot. RNA was extracted according to the protocol described by MacDonald *et al.*(1987). Intact replicating cores prepared from 2 g of liver samples were homogenized in 2 ml of 7.5 M guanidine-HCl [pH 7.0] and 10 mM dithiothrietol (DTT), followed by an addition of 0.1 ml of 10% sodium lauryl sarcosinate. Insoluble debris was removed by centrifugation at 2000 rpm for 5 min at room temperature in a bench-top Sorvall refrigerated centrifuge (Sorvall Instruments, DuPont, Canada). The

supernatant was mixed with 100 µl of potassium acetate [pH 5.5] and 160 µl of 1 M acetic acid, and slowly added with 1 ml absolute ethanol. Nucleic acid-protein complexes were precipitated at -20 °C overnight and collected by centrifugation at 10,000 rpm for 10 min at 4 °C in a SW 27 rotor. The pellet was dissolved in 1 ml unbuffered 7.5 M guanidine-HCl (previously neutralized with NaOH to [pH 7.0] and supplemented with 10 mM DTT) to remove proteins associated with the RNA, and RNA was precipitated with 50 µl of 2 M potassium acetate [pH 5.5] and 0.5 ml ethanol at -20 °C for 2 h. Precipitates were collected by centrifugation at 10,000 rpm for 10 min at 4 °C in a SW 27 rotor, washed with ethanol and re-centrifuged under same conditions. The pellet was dissolved in 1 ml of 20 mM EDTA [pH 7.0], extracted with 2 ml of chloroform-n-butanol (4:1 v/v), and briefly centrifuged to separate the organic and aqueous phases. The interphase and organic phase were further extracted with 1 ml of 20 mM EDTA until the amount of material at the interphase no longer decreasing. Aqueous phases from these extractions were combined. RNA was precipitated with 2 volumes of 4.5 M sodium acetate [pH 7.0] at -20 °C overnight and collected by centrifugation at 10,000 rpm for 10 min at 4 °C in a SW 27 rotor. This salt precipitation removes residual DNA and glycogen. The purified RNA pellet was dissolved in 200 µl of 0.1 M MOPS [pH 7.0], 40 mM sodium acetate and 5 mM EDTA [pH 8.0]. Twenty microliters of the RNA sample were first mixed with 3.5 µl formaldehyde and 10 µl formamide and electrophoresed in 0.8% formaldehyde gel. Northern blotting was performed as described by Sambrook *et al.* (1989). RNA on the filter was prehybridized and hybridized under conditions similar to those used for DNA in Southern blotting.

13. Oligonucleotide dot-blot hybridization. DNA was first extracted from replicating cores which had been previously isolated from 1 g samples of liver. The DNA pellet resuspended in 50 µl of TE buffer was loaded directly onto a nylon filter (Hybond-N; Amersham Corp., Arlington Heights, Ill.); denatured with 0.5 M NaOH - 1.5 M NaCl at room temperature for 15 min and then neutralized with 0.5 M Tris-HCl [pH 7.5] - 1.5 M

NaCl. The filter was exposed to UV for 3 min and prehybridized under the conditions described for Southern blots. Radioactive 5' end-labeled oligonucleotide probe, AH129 or AH2369, was added to the filter in the presence of the prehybridization fluid. Hybridization proceeded at 42 °C overnight. After hybridization, the filter was washed with 0.5X SSC for 1 h at 37 °C and subjected to autoradiography.

14. Primer Extension. Replicating core DNA samples extracted from 2 g of liver tissue were used as the templates for primer extension. The samples were adjusted to contain approximately equal amounts of replicating cores. The DNA template added into a tube containing 20 mM Tris-HCl [pH 8.0], 25 mM KCl, 0.05% Tween 20, 0.1 mg/ml gelatin, 15 mM $MgCl_2$ and 20 pmoles of either AH129 or AH2369 oligonucleotide primer. The reaction mixture was made up to 90 µl with water and boiled for 5 min, followed by an addition of 10 µl of 0.5 mM dNtds mix, 1 µl of [α–^{32}P]dCTP and 1 unit of Taq polymerase (BRL, Burlington, Ont.). The reaction mixture was overlaid with 100 µl of mineral oil. The extension reaction (94 °C, 1 min; 55 °C, 1 min; and 72 °C, 1 min) was recycled 35 times in a thermal cycler (Ericomp Inc., San Diego, CA.). Ten microliters of the reaction product were electrophoresed in a 12% urea polyacrylamide denaturing gel. With the extension reaction using AH129 as primer, the reaction product was digested with Bgl II, phenol-chloroform extracted and ethanol precipitated before electrophoresis. After electrophoresis, the gel was sealed in a plastic bag and subjected directly to autoradiograhy.

15. Activity gels.

DNA polymerase activity gel - Replicating cores prepared from 4 g of liver were resuspended in 1 ml EB buffer without BSA. The mixture was centrifuged through a 20% sucrose-EB (without BSA) cushion at 160,000 x g for 6 h at 4 °C in a Beckman SW40 rotor. DNA polymerase activity gel was performed using procedures suggested by Bavand and Laub (1988). The pellet was resuspended in 25 µl of Buffer E (50 mM Tris-HCl, [pH7.0], 140 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethysulfonyl fluoride, 0.1%

aprotonin) and 5 µl of Buffer C (65 mM Tris-HCl [pH 6.8], 2 mM EDTA, 20% glycerol, 0.6 µl β-mercaptoethanol, 4% SDS). The samples were electrophoresed at 4 °C in an 8% SDS-polyacrylamide gel which contained 0.02% SDS and 100 µg/ml of nicked salmon sperm DNA. After electrophoresis, the polymerase was renatured *in situ* by washing with 6 x 1 liter of ice-cold 50 mM Tris-HCl [pH 7.5] over a 20 h period. The gel was placed in a sealed bag which contained 70 mM KCl, 10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol, 20 µM aphidicolin, 13.5 each of dATP, dTTP and dGTP and 4 µCi/ml of [α-^{32}P]dCTP (3000 Ci/mmol). After 24 h of incubation at 37 °C, the reaction was stopped by washing the gel with 6 x 500 ml chilled 5% TCA-1% sodium pyrophosphate on a shaker at 4 °C for 24 h. The gel was sealed in a plastic bag and subjected to autoradiography.

Reverse transcriptase activity gel - Reverse transcriptase activity gels were performed with procedures according to Oberhaus and Newbold (1993). Ten grams liver tissue were homogenized in 25 ml H-buffer (0.02 M Tris.HCl [pH 7.4], 0.05 M NaCl, 0.007 M $MgCl_2$, 0.1% β-mercaptoethanol and 8% sucrose). The homogenate was centrifuged at 10,000 rpm for 20 min at 4 °C in a JA20 rotor to remove cell debris. Polysomes present in the supernatant were pelleted by a second centrifugation at 27,000 rpm for 90 min at 5 °C. The supernatant was collected, EDTA was added to a final concentration of 1 mM to dissociate the remaining polysomes to monosomes and 0.1% β-mercaptoethanol was added to prevent oxidation of the core particles. Core particles were concentrated by pelleting through a sucrose cushion which contains 4 ml 15% and 4 ml 30% sucrose in core buffer, CB (0.02M Tris.HCl [pH7.4], 50 mM NaCl, 1 mM EDTA, 0.1% β-mercaptoethanol and 0.01% triton X-100). After centrifugation at 20,000 rpm for 15 h at 5 °C in a SW27 rotor, the pellet was resuspended in 500 µl CB for 4 h and then brought to a final volume of 5 ml. Large proteins were moved by precipitation with 5% polyethylene glycol 8000 (total volume 25 ml), followed by an immediate centrifugation at 10,000 rpm for 20 min at 5 °C in a JA 20 rotor. Core particles in the supernatant were

precipitated with 12% polyethylene glycol 8000 - 0.5 M NaCl in ice bath for at least 4 h, and centrifuged at 10,000 rpm for 20 min at 5 °C in a JA 20 rotor. The core pellet was resuspended in 1 ml CB and sedimented in 30-ml continuous 15 - 30% sucrose gradients at 27,000 rpm for 4 h at 5 °C in a SW 27 rotor. One milliliter fractions were collected from the gradients and endogenous polymerase activity was assayed using 15 µl from each fraction. Fractions showing activity were pooled, diluted with CB and centrifuged at 27,000 rpm at 5 °C overnight in a SW27 rotor. The purified core pellet was resuspended in 50 µl of sample buffer (50 mM Tris [pH 6.8], 5% glycerol, 0.67% SDS, 0.1 µM ß-mercaptoethanol, 0.33 mM EDTA, 0.002% bromophenol blue) and 15 µg of nuclease-free BSA (BSA which had previously been heated at 94 °C for 1 h). The samples were electrophoresed at 4 °C in an 8% SDS-polyacrylamide gel containing 0.02% SDS and 118 µg/ml of polyrC.dG$_{18}$ at a molar ratio of 1:10. The conditions and procedures for renaturation and reaction were essentially the same as those with the DNA polymerase activity gel except that only 4 µCi/ml of [α-^{32}P]dGTP (3000 Ci/mmol) with no other dNtds was added to the reaction mixture.

16. Radioactive labeling of DHBV polymerase. DHBV polymerase was expressed *in vitro* using a rabbit reticulocyte lysate translation system as described in Chapter 2 (Howe *et al.*, 1992). [α–^{32}P]ddGTP was synthesized by Dr. J. Wilson with procedures according to Hoard and Ott (1965) and Symons (1970). *In vitro* binding of [α–^{32}P]ddGTP to DHBV polymerase was done by incubating 15 µl of *in vitro* translated DHBV polymerase lysate with 15 µl of 2X reaction buffer (100 mM Tris.HCl [pH7.5], 30 mM NaCl, 20 mM MgCl$_2$, 26 µM each of ddATP, ddCTP and ddTTP, and 20 µCi of [α–^{32}P]ddGTP). *In vitro* binding of [α–^{32}P]dGTP to DHBV polymerase was performed under a similar condition except that 26 µM each of dATP, dCTP and dTTP and 20 µCi of [α–^{32}P]dGTP were used. The reaction was carried out at 30 °C for 40 min and stopped by the addition of 270 µl of protein loading buffer (Sambrook *et al.*, 1989). The samples

were analyzed in a 10% SDS-PAGE. After electrophoresis, the gel was stained with Coomassie blue G-250, dried and autoradiographed.

III. RESULTS

1. ddDAPR, ddG competes with dG for hepadnaviral DNA replication *in vitro*.

Hepadnaviral replication is selectively inhibited by purine 2',3'-dideoxynucleoside analogues. At concentrations of 4 μM, ddDAPR or ddG reduced intracellular viral DNA to non-detectable levels (Lee *et al.*, 1989). In the present study, we attempted to determine if increasing concentrations of dG could prevent the inhibition of viral replication by ddDAPR or ddG. We cultured primary hepatocytes prepared from congenitally infected ducklings in the presence of ddG or ddDAPR plus increasing concentrations of dG. If ddDAPR (a prodrug of ddG) and ddG inhibit viral replication by competing with dG, we would expect to see an increase in intracellular viral DNA in cultures containing increasing concentrations of dG. Primary duck hepatocyte cultures were maintained under the specified conditions for 22 days. The amounts of intracellular viral DNA were quantified by densitometry reading of the dot blot hybridization. As shown in Figure 3-1 and 3-2, there was a gradual increase in viral DNA levels in cultures containing increasing concentrations of dG, consistent with the hypothesis that ddG or ddDAPR competes with dG during viral DNA replication. When the concentration of dG exceeded that of its competitor (1 μM of ddG or ddDAPR), there was no further increase in viral DNA levels. This suggested that viral DNA replication reached its normal steady-state, since cultures of infected primary duck hepatocytes which were maintained without drug present produced similar levels of intracelluar viral DNA (data not shown).

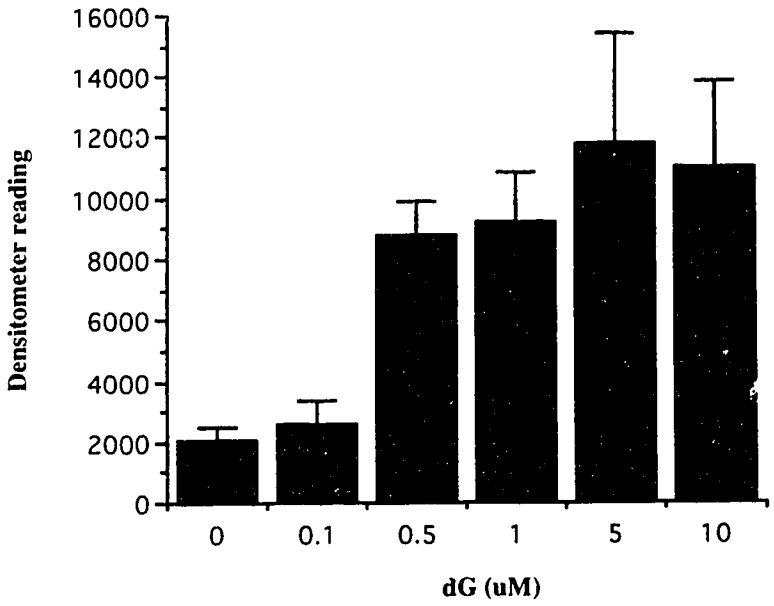

Figure 3-1. Effect of ddDAPR and dG on DHBV replication.

Primary duck hepatocyte cultures were prepared from ducklings congenitally infected with DHBV. Cell cultures were maintained in L-15 medium containing 1 μM of ddDAPR in the presence of increasing concentrations of dG as indicated. DNA was extracted from cell lysates at the end of 22 days and tested for the presence of DHBV DNA by dot blot hybridization. The amounts of DNA were quantified by densitometry scanning of the dots on the autoradiogram.

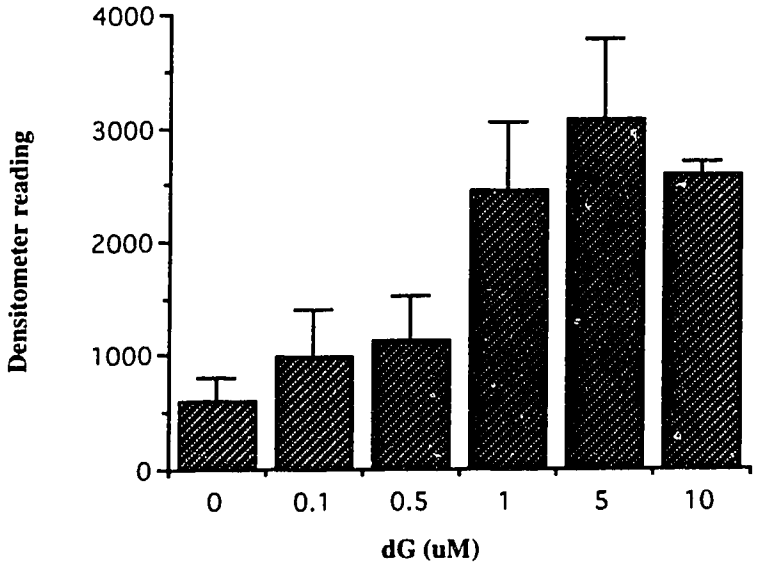

Figure 3-2. Effect of ddG and dG on DHBV replication.

Primary duck hepatocyte cultures were prepared from ducklings congenitally infected with DHBV. Cell cultures were maintained in L-15 medium containing 1 μM of ddG in the presence of increasing concentrations of dG as indicated. DNA was extracted from cell lysates at the end of 22 days and tested for the presence of DHBV DNA by dot blot hybridization. The amounts of DNA were quantified by densitometry scanning of the dots on the autoradiogram.

2. Selective inhibition of DHBV DNA replication by ddDAPR but not by ddC *in vivo*.

Our previous studies using ddDAPR to treat DHBV infected ducklings resulted in rapid clearance of the virus DNA from the sera of treated animals (Lee *et al.*, 1989). In the present study, congenitally DHBV-infected ducklings were treated with increasing doses of ddDAPR (10-50 mg/kg), ddC (20 or 40 mg/kg) or placebo (normal saline) every 6 or 12 hours. After 48 hours of treatment, reverse transcriptase activity in the isolated replicating cores of the treated animals was measured. Potent dosage-dependent inhibition was observed in the replicating cores from animals treated with ddDAPR (Table 3-1). Dosages of 80 mg/day or higher consistently produced a 95-98% inhibition of reverse transcriptase activity. In contrast, ddC had no inhibitory effect on the reverse transcriptase in replicating cores even at high doses.

Both ddGTP and ddCTP are known to be effective chain terminators in the replication of DNA in many species (Doering *et al.*, 1966; Molnar-Kimber *et al.*, 1983; Sanger *et al.*, 1977). However the results of our work suggest there is selective inhibition of DHBV replication by ddGTP when compared to ddCTP. Our results in a study on the enzyme kinetics of reverse transcriptase in replicating cores indicate that ddGTP and ddCTP have similar Ki values (in preparation). This has led us to explore the possibility that ddGTP inhibits DHBV replication by a unique mechanism.

To verify that a decrease in the amount of replicating cores was not responsible for the dramatic decrease in the residual polymerase activity after 48 hours of ddDAPR treatment, the intact replicating cores were isolated by means of sucrose gradient centrifugation; and the amount of viral core proteins in the hepatocytes was quantified by ELISA using polyclonal anticore antibodies. The amount of core protein present in ddDAPR-treated samples was compared with that of the placebo-treated control. Variations in the amount of replicating cores could be due to differences in the initial virus load in different ducklings or a decrease could be an effect of drug treatment (Table 3-2). Overall, there was some

Table 3-1. Reverse transcriptase activity in replicating cores after 48 hours of ddDAPR or ddC treatment.

	Percentage [a-^{32}P]dCTP incorporation[a]	
	ddDAPR	ddC
Placebo	100	100
ddDAPR or ddC		
10 mg/kg, q12h	55	n.d.[b]
10 mg/kg, q6h	38	n.d.
20 mg/kg, q12h	11	129
20 mg/kg, q6h	3	100
40 mg/kg, q12h	4	111
40 mg/kg, q6h	2	110
50 mg/kg, q12h	5	n.d.
50 mg/kg, q6h	4	n.d.

[a] The quantity, in cpm, of [α-^{32}P]dCTP incorporated into DNA is given relative to that of the sample from the placebo treated animals (taken as 100%). Each data entry represents a mean of the results obtained from two animals.

[b] n.d. - not determined.

Table 3-2. Estimation of the quantities of replicating cores by ELISA

Samples	Relative quantities of replicating cores[a]
Placebo	1.00
ddDAPR	
10 mg/kg, q12h	0.84
10 mg/kg, q6h	1.08
20 mg/kg, q12h	0.84
20 mg/kg, q6h	0.48
40 mg/kg, q12h	0.33
40 mg/kg, q6h	0.43
50 mg/kg, q12h	0.82
50 mg/kg, q6h	0.55

[a] The numbers represent the quantity of replicating cores present in the ddDAPR- treated samples relative to that of the placebo-treated control. Each value represents the mean of the results from two similarly treated animals after 48 hours of treatment with ddDAPR.

decrease in the amount of replicating cores in the ddDAPR-treated samples, but this decrease was not large enough to account for the marked decrease in the reverse transcriptase activity in ddDAPR-treated ducklings.

3. Inhibition of DHBV DNA replication by ddDAPR occurs at a very early stage of the reverse transcription.

Viral DNA and RNA were isolated from replicating cores purified after 48 hours of drug treatment. The amount of viral DNA decreased with increasing doses of ddDAPR (Figure 3-3). In particular, there was a substantial reduction of replicating DNA (partially double-stranded and single-stranded DNA) prepared from samples treated with ddDAPR at dosages of $\geq$40 mg/kg/day. In contrast, the amounts of viral RNA present in replicating cores from drug-treated animals were comparable to those in the replicating cores of the placebo-treated animals (Figure 3-4). An accumulation of full length viral RNA in samples isolated from the ddDAPR-treated animals was not observed. Partially degraded viral RNA was consistently observed on the Northern blots. The degradation of the viral RNA could be due to the prolonged procedure required to isolate the replicating cores. Nevertheless, results in Figure 3-4 represent viral RNA present inside the replicating cores since the exogenous nucleic acids would have been removed by the DNase and RNase treatment steps during the isolation procedure.

The process of reverse transcription consists of two stages: priming and polymerization (Ganem and Varmus, 1987). Blocking either process could lead to inhibition of DNA synthesis. In order to determine which stage was inhibited by ddDAPR treatment, the extent of the inhibition of minus strand DNA in the replicating cores was examined. Blocking the priming step would result in the complete absence of minus strand DNA, whereas inhibition of the polymerization stage would likely result in a population of DNA molecules of various lengths.

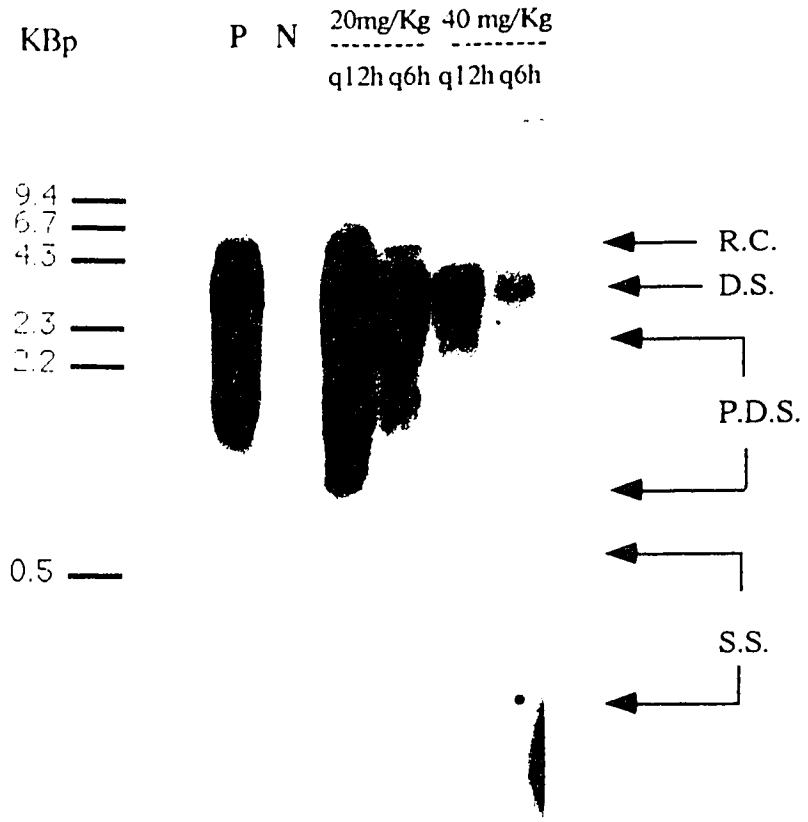

Figure 3-3. Effect of ddDAPR on DHBV DNA in replicating cores.

Five week-old ducklings congenitally infected with DHBV were treated with increasing doses of ddDAPR or placebo (P) for 48 h. DNA was extracted from the replicating cores in the livers, electrophoresed in a 0.8% agarose gel and analyzed by Southern blot hybridization. N represents a sample isolated from a non-infected duckling. RC, relaxed circular DNA; D.S., double-stranded DNA; P.D.S., partially double-stranded DNA and S.S., single-stranded DNA.

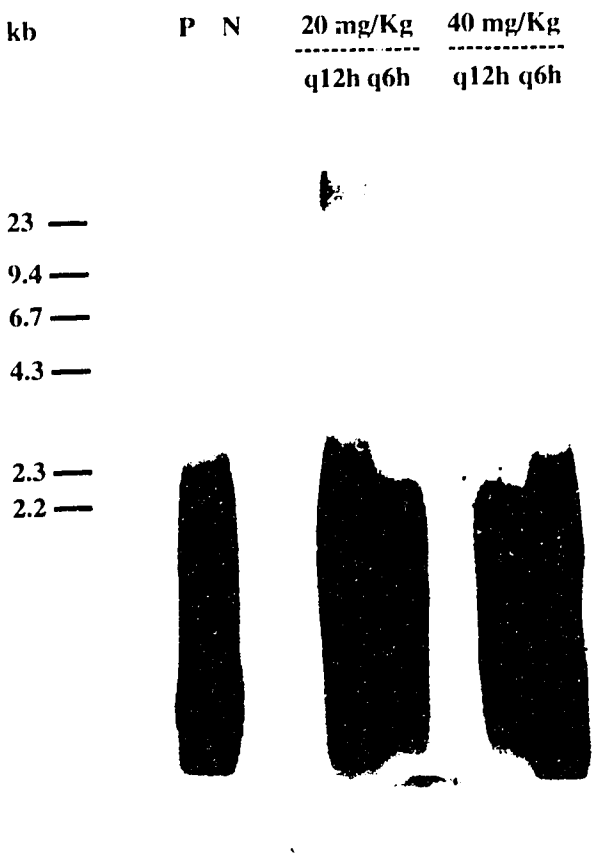

Figure 3-4. Effect of ddDAPR on DHBV RNA in replicating cores.

RNA was extracted from replicating cores isolated from the livers of ducklings which had previously been treated with ddDAPR or placebo (P). The RNA samples were electrophoresed in a 0.8% formaldehyde gel and analyzed by Northern blot hybridization. N represents a sample prepared from a non-infected duckling.

Minus strand specific oligonucleotide probes were targeted to 129 bp (AH129) or 2369 bp (AH2369) downstream from the putative initiation site of the minus strand DNA to detect the presence of the minus strand DNA (Figure 3-5A). Replicating cores isolated from ducklings which had previously been treated with high doses of ddDAPR (50 mg/kg, q12h) or ddC (40 mg/kg, q6h) were used for the analyses. To enhance the sensitivity of detection, I used dot blots with 5' end-labeled oligonucleotide probes rather than Southern blots. As shown in Figure 3-5B, the presence of minus strand DNA was detected in samples from the placebo and ddC treated animals but not in the samples from ddDAPR-treated ducklings. The conditions used for the hybridization were specific since no signal was detected in samples prepared from a non-infected duckling or a plasmid containing a HSV specific DNA sequence. The absence of minus strand DNA in the replicating cores from ddDAPR-treated animals was confirmed by an amplified primer extension. DNA samples isolated from replicating cores were annealed with primer AH129. The extension reaction was repeated 35 times using Taq polymerase in a thermal cycler. A band of approximately 129 nucleotides was produced in the samples prepared from placebo or ddC treated ducklings, however only a faint band was detected in the samples from ddDAPR-treated ducklings (Figure 3-5C). A similar pattern was observed when the downstream AH2369 primer was used (not shown). Larger amounts of DNA products were detected in replicating cores isolated from ddC-treated animals than placebo-treated controls in this experiment which may reflect differences in the "viral load" in different animals.

Treatment of ducklings with ddDAPR results in inhibition of DHBV DNA replication at a very early stage of minus strand DNA synthesis. This conclusion was supported by the results of the oligonucleotide dot blot hybridization and the primer extension studies. These results also suggest that the priming stage was the likely target for inhibition rather than the polymerization stage of reverse transcription. However, I cannot exclude the possibility that polymerization might be terminated at a position earlier than can be detected by the AH129 probe used in these experiments.

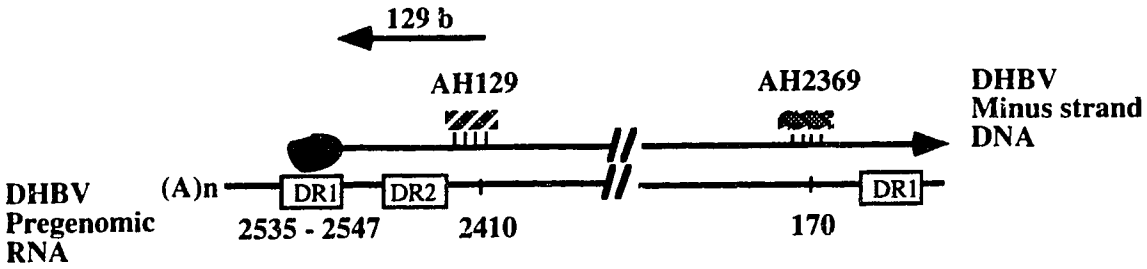

Figure 3-5A. Detection of the presence of minus strand DNA: Schematic diagram of the oligonucleotide dot-blot hybridization and primer extension.

Synthesis of genomic minus-strand DNA with primer domain linked to the 5' end is initiated at DR1 as shown. Oligonucleotide probes AH129 and AH2369 are located at 129 and 2369 nucleotides, respectively, downstream from the expected initiation site for the minus strand DNA. Extension from primer AH129 produces a DNA fragment of approximately 129 b.

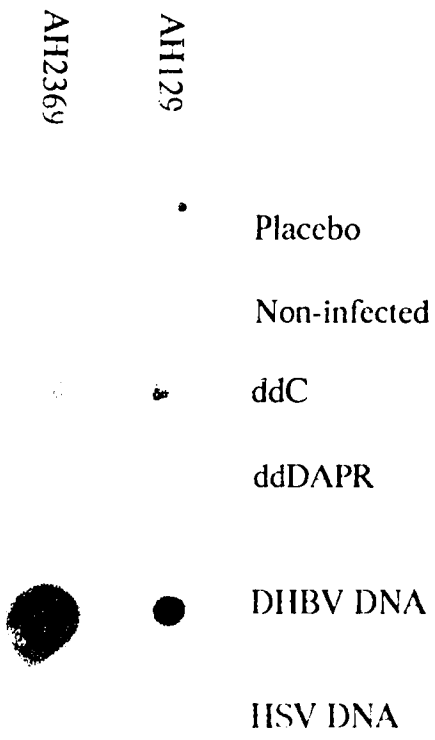

Figure 3-5B. Detection of the presence of minus strand DNA: Oligonucleotide dot-blot hybridization.

DNA was extracted from replicating cores which were isolated from infected ducklings treated with placebo, ddC (40 mg/kg, q12h) or ddDAPR (50 mg/kg, q6h) for 48 h. and from a non-infected duckling. DHBV DNA and HSV DNA are samples from plasmids containing DHBV and HSV DNA sequences respectively. The DNA samples were dot-blotted onto a nylon membrane and analyzed with minus strand specific oligonucleotide probes (AH129 or AH2369).

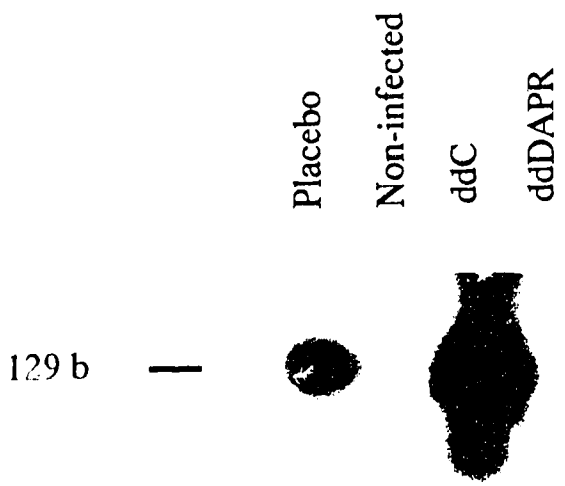

Figure 3-5C. Detection of the presence of minus strand DNA: Primer extension.

DNA isolated from placebo, ddC- or ddDAPR-treated, or non-infected ducklings was analyzed by an amplified primer extension assay. Oligonucleotide AH129 was used as the primer for the extension. The reaction products were analyzed in 12% urea-PAGE. The location of the extended DNA fragment of 129 bases is indicated.

4. DHBV retains its DNA polymerase and reverse transcriptase activities after ddDAPR treatment.

In this section, I describe attempts to prove that DHBV polymerase retains its DNA polymerase and reverse transcriptase activities after treatment with ddDAPR. I employed the technique developed by Bavand and Laub (1988) to detect the DNA polymerase activity in replicating cores. In the DNA polymerase activity gel shown in Figure 3-6A, a positive control, which contained commercially available purified Klenow fragment and *E.coli* polymerase I (BRL, Burlington, Ont.), revealed the expected radioactive bands at 76- and 109-kDa respectively. Radioactive bands of approximately 80 kDa were detected in the activity gel using replicating cores prepared from the placebo, ddDAPR or ddC treated animals. This indicated that DHBV polymerase activity was not inhibited by ddDAPR treatment even though *in vivo* DNA synthesis was inhibited. No activity was detected in the sample isolated from a non-infected duckling.

To demonstrate the reverse transcriptase activity, I employed the protocol suggested by Oberhaus and Newbold (1993). In the reverse transcriptase activity gel shown in Figure 3-6B, a commercially available murine Moloney leukemia virus reverse transcriptase (BRL, Burlington, Ont.) in which the RNase H domain had been deleted was used as the positive control. A 55-kDa radioactive band was detected in this positive control indicating that the conditions were adequate for the detection of the reverse transcriptase activity. In the samples of replicating cores prepared from the ddC, ddDAPR or placebo treated animals, the major radioactive band was detected at approximately 80-kDa in the activity gel. This result indicated that despite the inhibition of the DNA synthesis during the reverse transcription phase of DHBV replication, the enzymatic function of the DHBV reverse transcriptase was not inhibited by ddDAPR treatment. No radioactive band at 80-kDa was detected in the sample isolated from a non-infected duckling.

Detection of DNA polymerase and reverse transcriptase activities by activity gels requires precise experimental techniques. Many laboratories have failed to detect

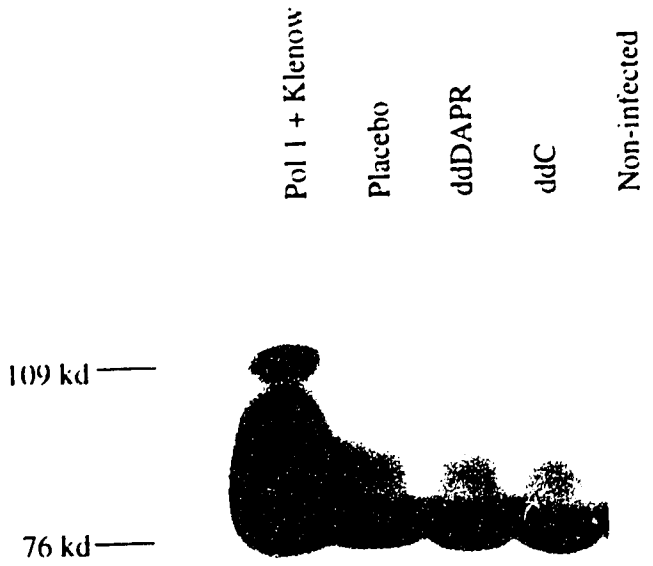

Figure 3-6A. Activity gel assays for DNA polymerase and reverse transcriptase activities associated with replicating cores - DNA polymerase activity.

Replicating cores were isolated from livers of placebo, ddDAPR- (50 mg/kg, q6h for 48 h), and ddC- (40 mg/kg, q12h for 48h) treated ducklings. A cytoplasmic lysate from a liver of non-infected duckling was prepared in a similar manner. DNA polymerase activities associated with these samples were analyzed in an 8% SDS-polyacrylamide gel containing nicked salmon sperm DNA as primer-template substrates. Commercially available purified *E.coli* polymerase I (109-kDa) and Klenow fragment (76-kDa) were included as a positive control and molecular markers for the activity gel.

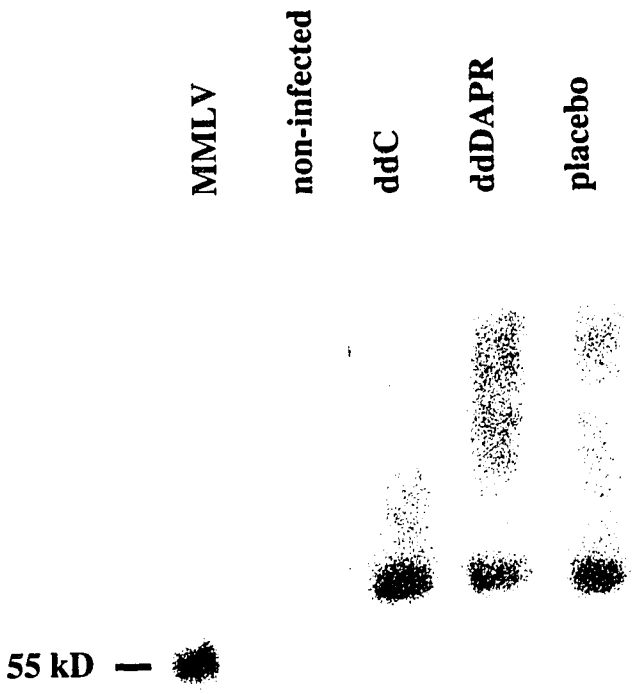

Figure 3-6B. Activity gel assays for DNA polymerase and reverse transcriptase activities associated with replicating cores - Reverse transcriptase activity.

Samples from placebo, ddDAPR- or ddC-treated DHBV infected ducklings and from non-infected ducklings were analyzed in an 8% SDS-polyacrylamide gel containing polyrC.dG18 as primer-template substrates. MMLV superscript reverse transcriptase (55-kDa) was used as a positive control.

polymerase activity using this assay. Low abundance of the polymerase gene products in virus particles may account for the failed attempts. In the present studies, replicating cores prepared from at least 10 g liver tissue were required for detection of reverse transcriptase activities. Furthermore, I observed decreased polymerase activities using replicating cores prepared from CsCl density gradients. This observation has been reported by others (Oberhaus and Newbold, 1993). In order to exclude the possibility that the replicating core preparation might trap cellular polymerases, aphidicolin, a specific inhibitor for cellular DNA polymerase α, δ and ε, was added to the reaction. Eukaryotic polymerase α, δ, ε and γ all have molecular masses over 125 kDa, while polymerase β has molecular mass of about 36 - 38 kDa (Kornberg and Baker, 1992). The radioactive bands detected in the activity gels lying in the range of 75 - 80 kDa (Figure 3-6A and B) and do not coincide with the molecular masses of any of the known eukaryotic polymerases, Therefore, I believe that the radioactive bands detected in the activity gel assays are indicative of DHBV polymerase.

In summary, I have shown that treatment of ducklings with ddDAPR resulted in abrogation of the viral DNA replication. However, the drug treatment did not affect DNA polymerase or reverse transcriptase activities when assayed on activity gels using exogenous templates.

5. Direct binding of ddGTP with DHBV polymerase.

Since ddDAPR was found to be oxidatively deaminated to ddG by host adenine deaminase in serum, and subsequently entered the cell and was phosphorylated to ddGTP (Kitos and Tyrrell, 1995), I believe that ddGTP is capable of covalently binding to the polymerase and blocking hepadnaviral DNA synthesis. The following study was designed to demonstrate ddGTP binding to DHBV polymerase. [Alpha-^{32}P]ddGTP was chemically synthesized using the methods of Hoard and Ott (1965), and Symons (1970). Active DHBV polymerase was expressed using the *in vitro* rabbit reticulocyte lysate translation

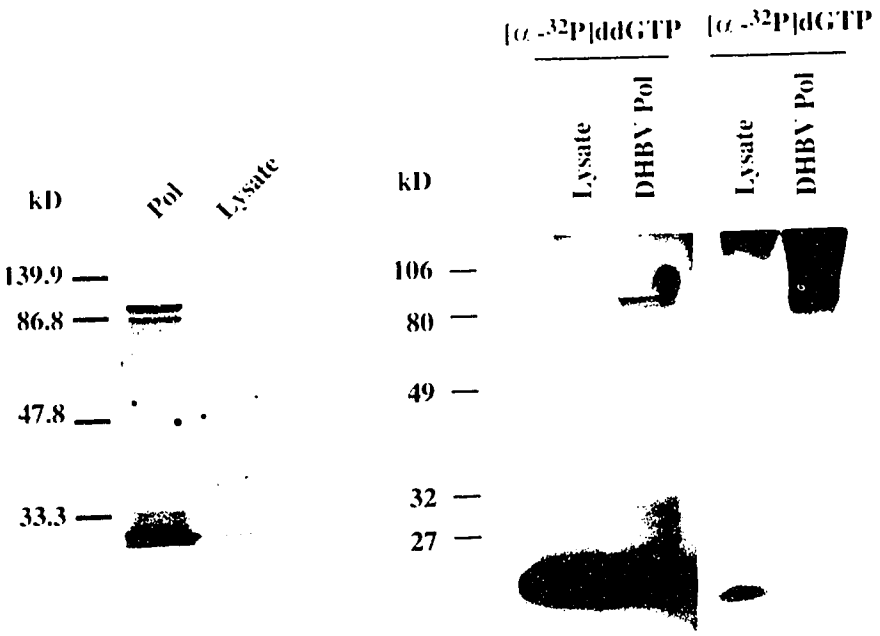

Figure 3-7. Binding of ddGTP to an *in vitro* translated DHBV polymerase.

Full length [^{35}S]methionine-labeled DHBV polymerase (90 kDa) was expressed *in vitro* using a rabbit reticulocyte lysate translation system (left panel). *In vitro* binding of the expressed polymerase with [α–^{32}P]ddGTP or [α–^{32}P]dGTP was carried out as described in Materials and Methods. The reaction products were analyzed in a 10% SDS-PAGE (right panel). The lanes designated Lysate represent *in vitro* translated reticulocyte lysate in the absence of mRNA

system as previously reported (Figure 3-7) (Howe *et al.*, 1992; Wang and Seeger, 1992). The active DHBV polymerase was incubated with [α-^{32}P]ddGTP in the presence of other ddNtds. As a positive control, the *in vitro* translated polymerase was incubated with [α-^{32}P]dGTP and three other deoxynucleotides (dNtds). After 40 min of incubation, the lysate was boiled in SDS-PAGE loading buffer and analyzed by 10% SDS-PAGE. The labeling with [α-^{32}P]dGTP in the presence of dNtds resulted in radioactive bands of 90 kDa and higher, an indication of self-primed DNA synthesis. (Figure 3-7). This result is consistent with the previous observation reported by Wang and Seeger (1992). However, a radioactive band (~ 90 kDa) was also detected in the sample incubated with [α-^{32}P]ddGTP (Figure 3-7). The intensity of this band is weaker than that labeled with [α-^{32}P]dGTP as would be expected, since elongation of DNA is not possible when the polymerase is bound to ddGTP.

In the following experiments, I attempted to show that the binding of ddGTP to the *in vitro* translated polymerase resulted in inhibition of DNA replication. *In vitro* translated polymerase was first incubated with ddGTP before exposure to dNtds and increasing concentrations of dGTP. No activity was detected after the polymerase was preincubated with ddGTP (Figure 3-8A). This result is compatible with the observations in our *in vivo* study in which treatment of ducklings with ddDAPR (metabolized to ddGTP) resulted in inhibition of the viral DNA replication. In the experiment illustrated in Figure 3-8B, I attempted to reverse the inhibition of the endogenous polymerase activity in replicating cores obtained from ddDAPR treated ducklings by increasing concentrations of dGTP. The ddDAPR induced inhibition of reverse transcriptase in replicating cores was not reversed by dGTP.

In summary, I have demonstrated that there is a direct covalent linkage between ddGTP and the polymerase. The binding of ddGTP to the polymerase results in inhibition of DNA replication. I have also shown that the inhibition of DNA replication by ddGTP is irreversible.

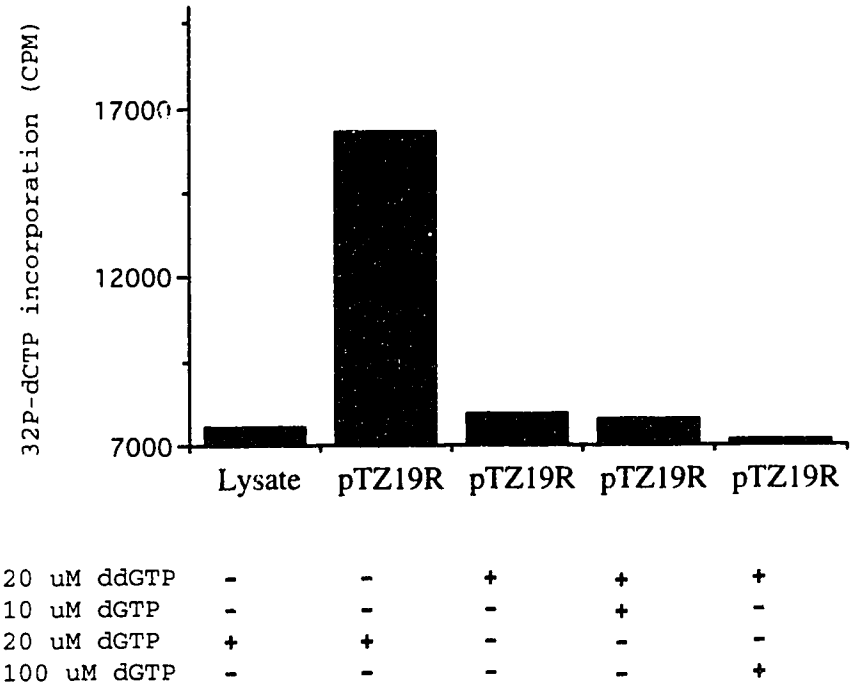

Figure 3-8A. Irreversible inhibition of in vitro transcribed/translated DHBV polymerase by ddGTP

Lysate represents reaction products from rabbit reticulocyte lysate which was translated without an *in vitro* transcribed DHBV polymerase RNA. pTZ19R, an *in vitro* translated DHBV polymerase, was incubated in the absence or presence of 20 μM of ddGTP, respectively, for 10 min at 37 °C. Reverse transcriptase activity was assayed in the presence of 20 μM each of dATP and dTTP, 0.1 μM of [α–^{32}P]dCTP and various concentrations of dGTP (as indicated). Reverse transcriptase activity was measured as cpm of [α-^{32}P]dCTP incorporated into TCA precipitable DNA.

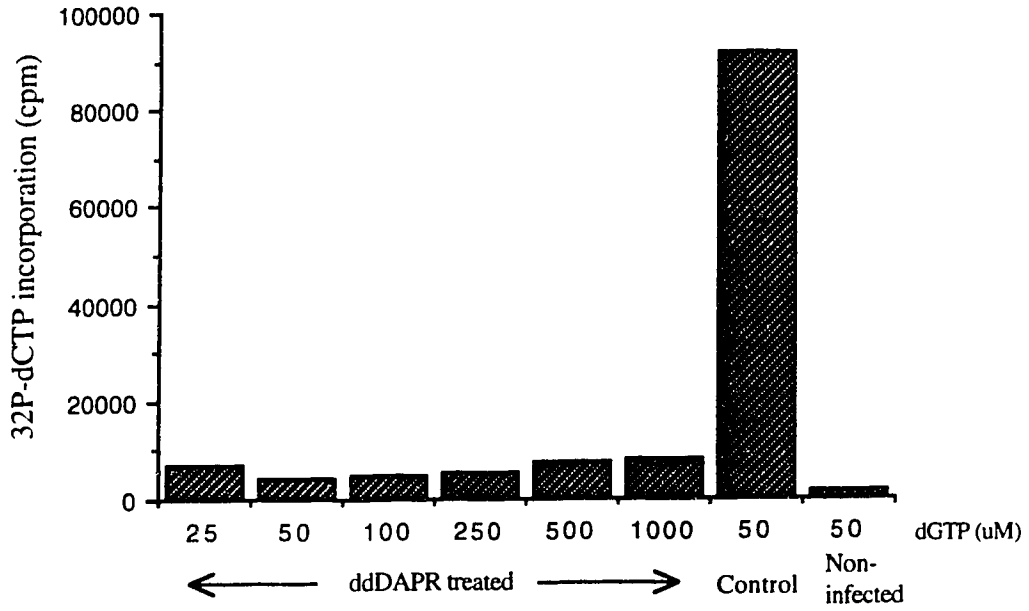

Figure 3-8B. Irreversible inhibition of DHBV polymerase by ddDAPR (ddGTP) - Endogenous reverse transcriptase activity in replicating cores.

Intact replicating cores were isolated from livers of ducklings which had been treated with ddDAPR (50 mg/kg, q6h for 48 h). The endogenous reverse transcriptase assay was performed in the presence of 50 μM each of dATP and dTTP, 0.1 μM of [α-^{32}P]dCTP and increasing concentrations of dGTP (25 - 1000 μM). Control, replicating cores isolated from a placebo treated duckling; non-infected, cytoplasmic extract prepared from a non-infected duckling. Both control and non-infected preparations were assayed with 50 μM dGTP. Reverse transcriptase activity was estimated by measuring the cpm of [α-^{32}P]dCTP incorporated into TCA precipitable DNA.

IV. DISCUSSION

1. ddGTP inhibits DHBV DNA replication through binding with the polymerase.

Kornberg originally envisioned that *E.coli* polymerase I has an active center which is able to accommodate a DNA template, the primer terminus and incoming nucleotides during DNA synthesis (Kornberg, 1969). Eukaryotic polymerases α, δ and ε, and the DNA polymerases of adenovirus, vaccinia virus, herpes simplex virus and bacteriophage T4 may also contain a similar active site (Delarue *et al.*, 1990; Earl *et al.*, 1986; Spicer *et al.*, 1988; Wang *et al.*, 1989). Although an active center in HBV polymerase has not been delineated, there are three functional domains present in the polymerase: the primer domain, the DNA polymerase/reverse transcriptase domain and the RNase H domain located at the amino-terminus, the central region and the carboxy-terminus, respectively (Radziwill *et al.*, 1990; Chang *et al.*, 1990). By inference, polymerase antagonists which target any of these domains are thought to inhibit HBV DNA replication.

It is intriguing that there is such a marked difference in the ability of ddC and ddDAPR (prodrug of ddG) to inhibit DHBV replication, as reported previously and confirmed in this study (Suzuki *et al.*, 1988). The discrepancy could result from one or more of several different mechanisms. There could be differences in cellular transport and metabolism of these nucleoside analogues. However, careful analysis of the uptake and metabolism of ddC and ddG in duck hepatocytes has failed to demonstrate differences sufficient to explain the marked difference in the ability of ddC and ddG to inhibit DHBV replication (Kitos and Tyrrell, 1995). Another possible mechanism which we have previously suggested is selective irreversible blockage of the protein priming of DHBV DNA synthesis by ddGTP (Suzuki *et al.*, 1988). In the present study, the demonstration that ddG competes with dG for hepadnaviral replication in DHBV infected primary duck hepatocytes (Figure 3-1 and 3-2), and ddGTP binds to DHBV polymerase (Figure 3-7) supports this hypothesis. In

addition, I have shown that binding of ddGTP to the polymerase irreversibly blocks the endogenous synthesis of viral DNA *in vivo* and *in vitro* (Table 3-1 and Figure 3-8 A and B). Our results do not agree with those reported by Staschke *et al.* (1994) who indicated that ddGTP failed to bind to the primer domain of the polymerase. However, our results are consistent with the findings of Zoulim and Seeger (1994) showing that dGTP binds to tyrosine 96 of the primer domain of DHBV polymerase.

2. ddC is an ineffective inhibitor of DHBV DNA replication.

Dideoxynucleoside triphosphates are commonly used as chain terminators during DNA synthesis (Sanger *et al.*, 1977). It has been shown that all ddNtds are incorporated into DNA albeit with lower efficiency than the dNtds (Atkinson *et al.*, 1969). However, my results show no decrease of the endogenous polymerase activity in replicating cores isolated from ddC treated ducklings. This suggests that either the DHBV polymerase is refractory to binding and incorporation of ddCTP into DHBV-DNA, or the polymerase might possess a 3' to 5' exonuclease activity, yet to be described. According to the nucleotide sequence of DHBV DNA, dCTP will be the seventh nucleotide incorporated after the initiation of DNA synthesis (primer domain - GTAATTC...). If DHBV polymerase has a 3' to 5' exonuclease activity, dideoxynucleotides incorporated into the DNA chain downstream from the primer protein could be removed by proof-reading. In contrast, the covalent binding of ddGTP to the primer domain by a nucleotide-protein bond would likely be resistant to proofreading by the 3' to 5' exonuclease. The ineffectiveness of ddC for inhibiting DHBV DNA replication could be explained by either its lack of incorporation onto the growing DNA chain or its incorporation and deletion by a 3' to 5' exonuclease proofreading mechanism.

3. Implications for DHBV replication.

It is not known if DHBV polymerase (approximately 90 kDa) undergoes proteolytic processing during encapsidation and DNA synthesis. Several lines of evidence have suggested that the primer domain seperates from the polymerase domain. Polymerase gene products (70 kDa in HBV and 80 kDa in DHBV) were detected in activity gel assays (Oberhaus and Newbold, 1993; Bavand and Laub, 1988; Bavand *et al.*, 1989). The 70 kDa protein species of HBV reacted with antisera directed against the carboxy terminus of the polymerase gene product, but not with an antiserum against the amino terminus of the protein (Bavand *et al.*, 1989). I will refer to the DNA polymerase without primer protein as the "processed" polymerase. Less than full length pol gene products were also found to be capable of producing replicative DNA in cells transfected with frameshift polymerase mutants (Wu *et al.*, 1990).

It is interesting that ddGTP blocks DNA replication without inhibiting the viral polymerase activities. The replicating cores purified from the livers of ducks treated with ddDAPR showed virtually complete inhibition of endogenous polymerase activity and DHBV DNA synthesis (Table 3-1 and Figure 3-3). However, the same viral polymerase preparation was shown to have DNA polymerase and reverse transcriptase activities when tested on exogenous templates in activity gels (Figure 3-6A and B). The demonstration of viral polymerase activities in activity gels requires that the enzyme utilizes exogenously primed templates. In contrast, the endogenous polymerase activity utilizes an endogenous template which requires protein priming (Bartenschlager and Schaller, 1988; Wang and Seeger, 1992). The discrepancy between results seen in these two different experiments can be explained by inhibition of the protein priming step of the endogenous reverse transcriptase activity, a step which is not required in the activity gel assay. These results suggest that priming and elongation are two distinct processes, each of which might be independently regulated, and selectively inhibited by different compounds. Therefore, the 70-80 kDa band detected in my activity gels (Figure 3-6 A and B) likely represents an

active "processed" DHBV polymerase, which does not serve as the target for ddDAPR. On the other hand, the 90 kDa band as seen in the labeling with [α-^{32}P]ddGTP represented the unprocessed polymerase which was bound to ddGTP (Figure 3-7). I am currently investigating which domain in the polymerase is responsible for binding to ddGTP.

Selective inhibition of viral replication requires quantitatively or (preferably) qualitatively unique steps in the virus replicative cycle that can serve as targets for antiviral therapy. I have shown that ddDAPR, a prodrug of ddG, is a potent and selective inhibitor of DHBV replication. Inhibition of the unique priming stage of viral DNA synthesis suggests that this is an important target for development of selective antiviral therapy for the treatment of hepadnaviral infections.

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Chapter 4

In vitro Translated Duck Hepatitis B Virus Polymerase Acts as a Suppressor of Core mRNA Translation

I. INTRODUCTION

The hepadnaviruses are a family of small enveloped hepatotropic DNA viruses. To date, five members of this virus family have been identified: the hepatitis B viruses of humans (HBV) (Gust *et al.*, 1986), woodchucks (WHBV) (Summers *et al.*, 1978), ducks (DHBV) (Marion *et al.*, 1980), ground squirrels (GSHBV) (Mason *et al.*, 1980) and herons (HHBV) (Sprengel *et al.*, 1988). All hepadnaviruses have similar genome sizes (approximately 3.0 - 3.2 kb), morphological structures and replication strategies (Robinson, 1990; Schödel *et al.*, 1990; Will *et al.*, 1987). Viral DNA replication takes place in a replicating core which consists of a RNA template, a viral polymerase and core protein molecules (Bartenschlager and Schaller, 1992; Lavine *et al.*, 1989; Chen *et al.*, 1992; Köchel *et al.*, 1991). Encapsidation of the RNA template requires stringent regulatory control. Several species of the genome length RNA transcripts have been identified in HBV or DHBV infected cells (Yaginuma *et al.*, 1987; Büscher *et al.*, 1985). All these transcripts have colinear 3' ends but heterogeneous 5' ends (Ganem and Varmus, 1987; Blum *et al.*, 1989). The long genome length transcripts, which are referred to as preC RNAs, encompass both the preC- and the C-open reading frames (preC-ORF and C-ORF, respectively) at the 5' end, whereas the shortest genome length transcript, generally known as pregenomic RNA, contains only the C-ORF at the 5' end. The pregenomic RNA transcript is selectively encapsidated.

Recently, several laboratories have identified the region (termed ε) in the pregenomic RNA transcript which is essential for packaging and DNA replication (Chiang *et al.*, 1992; Köchel *et al.*, 1991; Calvert and Summers, 1994; Hirsch *et al.*, 1991). This encapsidation region is characterized by the presence of a stem-loop structure which is believed to serve as a docking site for the binding of the polymerase (Pollack and Ganem, 1993; Tong *et al.*, 1993; Tavis *et al.*, 1994). In HBV, the encapsidation region contains a 99-b sequence downstream from the 5' DR1 of the pregenomic RNA (Chiang *et al.*, 1992; Köchel *et al.*, 1991); whereas in DHBV, a large region of approximately 1200 b starting from 35 nucleotides downstream from the cap site of the pregenomic RNA is required for encapsidation (Hirsch *et al.*, 1991). Since mutant DHBV genomes bearing insertions within this region are packaged efficiently, it is believed that the actual recognition elements for encapsidation contain discontinuous sequences located within this region (Hirsch *et al.*, 1991). Further detailed analysis of DHBV by Calvert and Summers (Calvert and Summers, 1994) has shown that two regions, both of which are within the 1200-b stretch, are required in *cis* for encapsidation.

It is interesting that both the preC and the pregenomic RNAs contain this stem-loop structure (see Figure 4-1), yet only the pregenomic RNA transcript is encapsidated. The discrimination against the preC RNAs has been attributed to translational inactivation (Nassal and Junker-Niepmann, 1990). In the case of preC RNAs, translating ribosomes which advance into the encapsidation sequence will prevent polymerase or polymerase-core complex from binding to the stem-loop structure, hence excluding the preC RNAs from being encapsidated. On the other hand, scanning ribosomes advancing on the pregenomic RNA are believed to be inhibited by the stable secondary structure present in the ε region (Nassal and Junker-Niepmann, 1990; Kozak, 1986; Kozak, 1989a), thus leaving the encapsidation site available for binding to the polymerase. Although this model explains the selective advantage of the pregenomic RNA over the preC RNAs during encapsidation, it does not explain how the encapsidation of the pregenomic RNA takes place in the midst

of translation where the translation initiation site and C-ORF (located within the ε region) are presumably loaded with translating ribosomes (Kozak, 1989b; Wolin and Walter, 1988). This model predicts that presence of translating ribosomes on the pregenomic RNA would interfere with the encapsidation process.

In this chapter, I present evidence that DHBV polymerase interacts with a sequence located at the 3' periphery of the C-ORF. Binding of the polymerase to this region suppresses core protein synthesis. This observation suggests that translating ribosomes on the C-ORF might be displaced by the polymerase-RNA interaction, hence exposing the encapsidation signal in the pregenomic RNA for interaction with the polymerase or polymerase-core protein complexes during the process of nucleocapsid assembly.

II. MATERIALS AND METHODS

1. Materials. Restriction enzymes were purchased from Gibco BRL (Burlington, Ont. Canada) or Boehringer Mannheim (Quebec, Canada) and were used according to the manufacturer's protocols. ^{35}S-methionine and [α-^{32}P]UTP were purchased from Du Pont, Canada. The MEGAscript *in vitro* transcription kit was purchased from Ambion Inc. (Austin, Texas). The rabbit reticulocyte lysate *in vitro* translation kit was purchased from Promega Co. (Madison, WI.).

2. Plasmids. Plasmid pD2Eco11 was constructed from an infectious DHBV-16 virion DNA isolated from the serum of a DHBV infected ducking. It contains a complete genome of DHBV DNA fused to a pUC19 vector at the EcoRI site. pTZ19RPol was derived from pD2Eco11 in which a fragment between the HincII and EcoRI sites was deleted and ligated with a vector pTZ19R (Pharmacia Inc., Quebec, Canada) which had been previously cut with the corresponding enzymes.

Since the C-ORF is disrupted at the EcoRI site in pD2Eco11, a plasmid pD2HT which contains an 1.5 mer head-to-tail DHBV genome encompassing a continuous C-ORF was first constructed. pD2Eco11 was cut with BamHI to generate a 4.3 kbp fragment which was gel purified and self ligated to form pD2BamHI. To construct pD2HT, a unit length DHBV fragment restricted at EcoRI sites was isolated from pD2Eco11 and inserted into pD2BamH1 which was previously cut with EcoRI and dephosphorylated with calf intestine alkaline phosphatase according to Sambrook *et al.* (1989). Head-to-tail orientation of the genome in pD2HT was verified by restriction enzyme mapping as well as PCR using primers located at nt 2632 and 960 respectively. All nucleotide numbering is in the convention established by Mandart *et al.* (1984), beginning at the unique EcoRI site of DHBV. pTZ19RCore, which was used as the parental construct for *in vitro* transcription/translation of core protein, was derived from pD2HT A fragment of 1.3 kbp DNA containing the entire C-ORF was amplified by PCR using primers AH2632 (5'-GAT

CAG CTG CAG GAT TCT TGC TTA TAT ATG GAT-3') and AH960 (5'-ACT GAA TTC CCA TTG TTT GCA CAT GGT CTA-3') at nt 2632 (AUG codon of C-ORF at nt 2647) and 960 respectively. The amplified DNA fragment was phosphorylated with T4 kinase and digested with PstI which had been incorporated in primer AH2632. The PstI-blunt DHBV DNA fragment was subsequently cloned into pTZ19R previously cut with PstI and SmaI.

pSP72Tat which contains the HIV tat open reading frame cloned downstream from a T7 promoter and pSP72-T4 containing the entire human CD4 sequence under a T7 promoter were kindly provided by Dr. L. -J. Chang. pDCD4BstEII and pDCD4BX are two DHBV-CD4 recombinant constructs in which a 471 bp fragment of the DHBV sequence was cloned in frame at the BstEII site within the CD4 open reading frame (CD4-ORF) or at the BamHI site downstream from the CD4-ORF, respectively. pDCD4BstEII was constructed by amplifying a DHBV fragment (nt 401 - 872) with primers DHBV401BstEII (5'-GTA AAA TGG GTT ACC CGC CTA GGA AAT AAA TTA CCT-3') and DHBV872BstEII (5'-AGG GTC CTG GGT AAC CCA AGT TGG TTT AAC AGT ATT TCT-3'). The amplified fragment was digested with BstEII, gel purified and ligated with pSP72-T4 which had been linearized with BstEII and dephosphorylated with calf intestine alkaline phosphatase using standard procedures (Sambrook *et al.*, 1989). For pDCD4BX, the DHBV fragment was amplified by PCR using primers DHBV402BamHI (5'-ACA CTT GGA TCC GCC TAG GAA ATA AAT TAC CTG-3') and DHBV872XhoI (5'-GAC CTC CTC GAG AGT TGG TTT ACC AGT ATT TCT-3'). The amplified fragment was digested with BamHI and XhoI and ligated with pSP72-T4 previously cut with the corresponding enzymes.

3. *In vitro* transcription and translation. Plasmid pTZ19RPol was linearized with EcoRI, purified and transcribed *in vitro* using an Ambion MEGAscript kit. Translation of DHBV polymerase was carried out as described previously in Chapter 3.

DNA templates which were used to generate the parental (P) and the truncated (D1-D5) core mRNAs were linearized at SstI, HincII, SmaI, BglII, SspI, or AatII sites, respectively, prior to *in vitro* transcription. In the case of pSP72tat, pSP72-T4, pDCD4BstEII and pDCD4BX, the plasmids were linearized with SphI, BamHI, BamHI or XhoI, respectively. *In vitro* transcription and translation were carried out using procedures recommended by the suppliers (Ambion Inc and Promega Co.) and are described in Chapter 3.

4. Label Transfer. Radioactive DHBV RNA probes (nt 315 - 872) used for label transfer were transcribed directly from PCR products amplified from a DHBV sequence. The PCR products were generated using a 5' primer (T7DHBV315, 5'-AAC TCG GAA TTC TAA TAC GAC TCA CTA TAG AAA GTC CCG GGA AAG GAG-3') which contains a T7 promoter at nt 161. The underlined sequence represents the T7 promoter. Oligonucleotide DHBV872BstEII was used as the 3' primer for the PCR reactions. Radioactive CD4 RNA and truncated core RNA (D1, Figure 4-5A) probes were generated from the linearized DNA templates of pSP72-T4 and pTZ19RCore restricted at the BamH1 and HincII site, respectively. To synthesize ^{32}P-labeled RNA probes, the DNA templates were transcribed in the presence of 1 µl of [α-^{32}P]UTP (3000 Ci/mmol) using the protocol suggested in the transcription kit (Ambion Inc., Austin, Texas).

Radioactively labeled RNAs (about 200,000 cpm) were incubated with 10 µl of the *in vitro* translated DHBV polymerase in the presence of 10 mM HEPES [pH 7.6], 3 mM $MgCl_2$, 40 mM KCl, 2 mM DTT, 5% glycerol, 0.5% NP-40, 10 µg/ml heparin sulfate and 0.2 µg/ml poly rC. The final reaction volume was 20 µl. After incubation at 30 ℃ for 30 min, the reaction was put on ice and irradiated for 15 min with a germicidal lamp (Spectroline model EF-160C, Spectronics Co. Westbury, N. Y.) located approximately 5 cm above the samples. The UV cross-linked sample was digested with 1 mg/ml RNase A at 37 °C for 15 min. The reaction was terminated by the addition of 40 µl of protein sample

treatment buffer and boiled for 5 min before being analyzed by 10% SDS-PAGE. Radioactively labeled polymerase was detected by means of autoradiography.

5. Immunoprecipitation. Fifty microliters of the *in vitro* translated products were incubated on ice with 150 µl of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM of Tris.HCl [pH 8.0]) and 2 µl of polyclonal rabbit anti-DHBcAg antibodies (kindly provided by Dr. J. Summers) or anti-tat antibodies (a gift from Dr. L. -J. Chang). After 1 h of incubation, 100 µl protein A - Sepharose (10% v/v in RIPA) (Pharmacia Inc., Quebec, Canada) were added. The suspension mixture was rocked at 4 °C for 1 h. and transferred to a spin-X tube (Fisher Scientific, Edmonton, AB, Canada). The immunoprecipitated complex was washed 3 times with RIPA, boiled in 50 µl of SDS-sample treatment buffer and analyzed by 10% SDS-PAGE (Sambrook *et al.*, 1989). After fluorography, the gel was dried and exposed to Kodak XAR film at room temperature. Typical exposure times were 12-16 h.

III. RESULTS

1. Inhibition of core RNA translation in the presence of increasing amounts of polymerase protein.

I have previously expressed DHBV polymerase *in vitro* using a rabbit reticulocyte lysate. The *in vitro* translated polymerase possesses both DNA polymerase and reverse transcriptase activities when tested on exogenous or endogenous templates (Howe *et al.*, 1992). Since only a small amount of the polymerase with unstable enzyme activities was produced, I attempted to stabilize the polymerase by coexpression with core protein. I constructed an expression plasmid pTZ19RCore which contains the DHBV C-ORF cloned under a T7 promoter (Figure 4-1). Transcription and translation using this construct generated a protein of approximately 31 kDa (Figure 4-3, lane 1). This protein was identified as the DHBV core antigen by immunoprecipitation using anti-DHBcAg antibodies. A smaller species (~ 27 kDa) which might have resulted from an internal initiation of the C-ORF during translation was also detected. In the cotranslation studies using both core and polymerase RNAs, the efficiency of translation as measured by ^{35}S-methionine incorporation was markedly decreased when compared to that of the translation of core mRNA in the absence of polymerase RNA (Figure 4-2).

The following experiments were designed to demonstrate the effect of polymerase protein on the translation efficiency of core mRNA. Polymerase protein was first synthesized *in vitro* using rabbit reticulocyte lysate. The production of the polymerase protein was verified by measuring the ^{35}S-methionine incorporation and analyzing the lysate by SDS-PAGE. Previous studies had shown the polymerase to be active (see Chapter 2 ; Howe *et al.*, 1992). The translation of the core mRNA in the presence of increasing amounts of the lysate containing the polymerase protein was examined. The final reaction products were immunoprecipitated using rabbit anti-DHBcAg antibodies and analyzed in 10% SDS-PAGE. The core protein produced by *in vitro* translation is shown

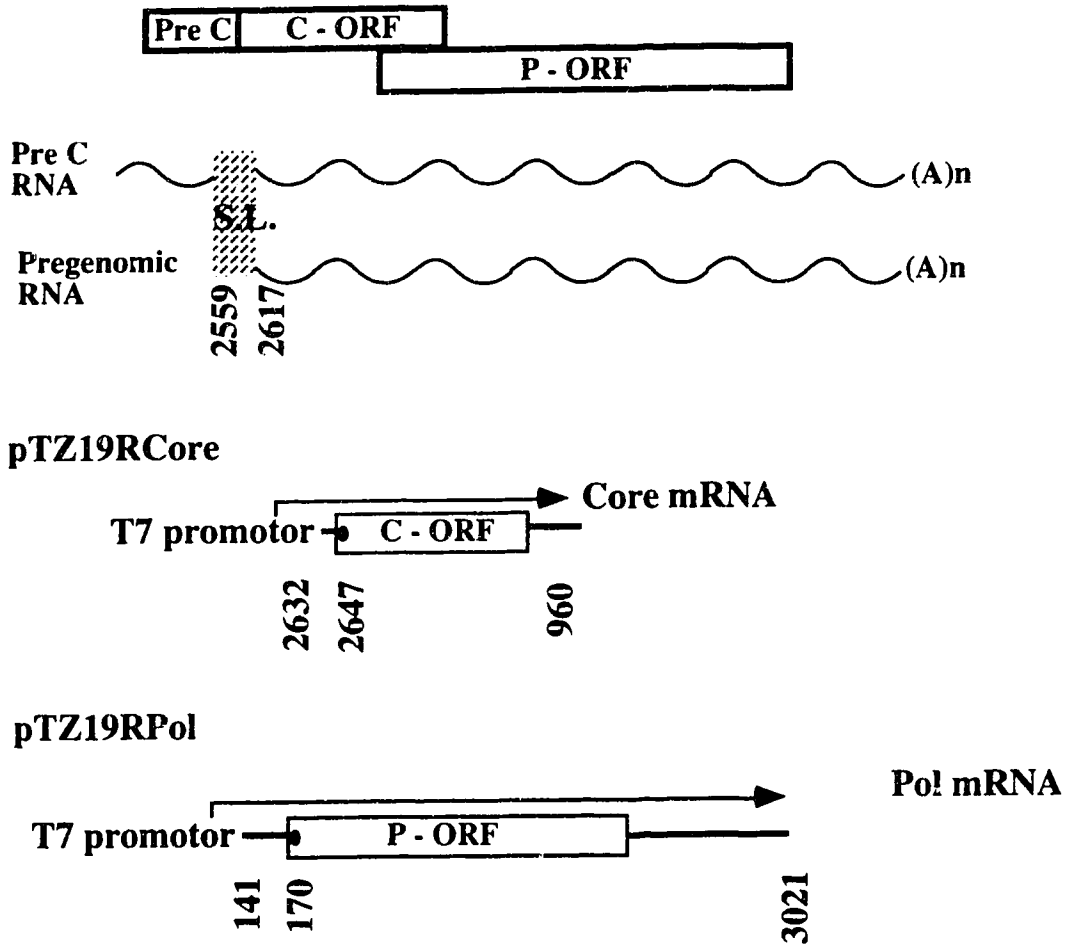

Figure 4-1. Schematic diagram of DHBV open reading frames, the RNAs, and the expression constructs used in this study.

Open reading frames for precore (preC), core (C-ORF) and polymerase (P-ORF) are shown as open boxes. Wavy lines beneath the diagram indicate the preC RNA and pregenomic RNA. The shaded area represents the putative stem-loop structure suggested by Hirsch *et al.* (Hirsch *et al.*, 1991). Expression constructs pTZ19RCore and pTZ19RPol show that the transcription of the core and polymerase RNAs are under the control of the T7 promoter. Transcripts produced from pTZ19RCore will not contain the putative stem-loop structure described by Hirsch *et al.* (1991). Translation initiation sites of core and polymerase open reading frames are indicated as black dots within the open boxes.

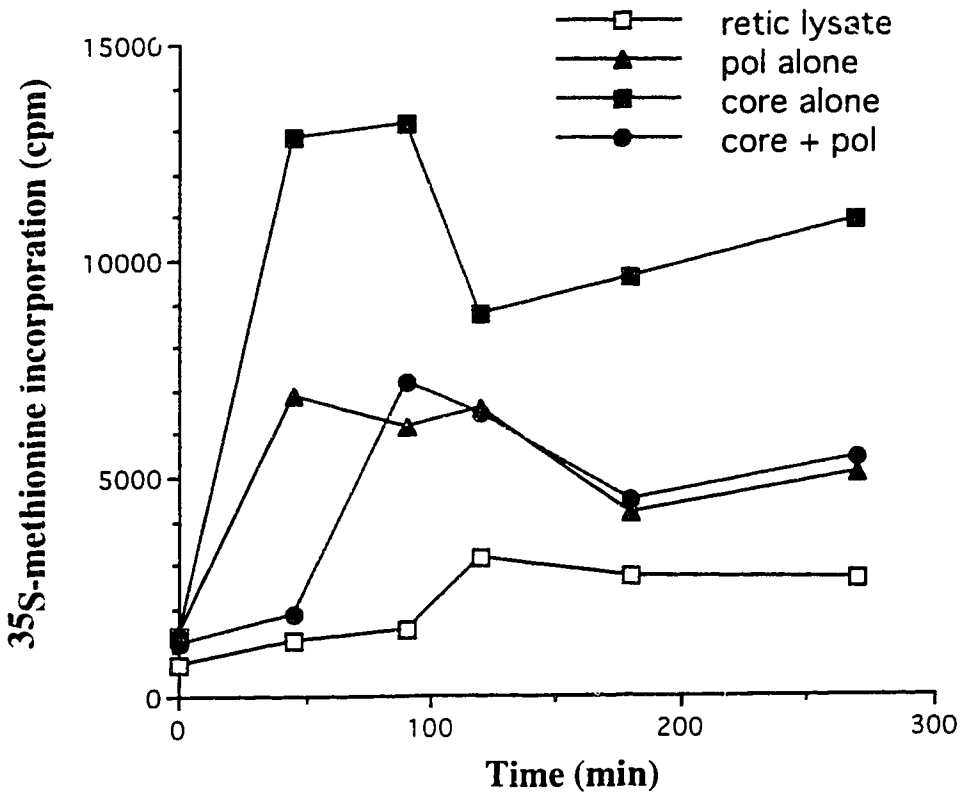

Figure 4-2. Effect of cotranslating core and polymerase mRNAs on translation efficiency.

Two pmoles each of core (—■—), polymerase (—▲—), or both core and polymerase (—●—) RNAs were mixed with a rabbit reticulocyte lysate. (—□—) represents a sample containing the reticulocyte lysate alone. Translation was carried out in the presence of ^{35}S-methionine. Efficiency of the translation was estimated by means of TCA precipitable ^{35}S-methionine incorporation. Each point represents the mean of 3 replicative samples.

in Figure 4-3 lane 1. With increasing amounts of the lysate containing polymerase protein, a decrease in the amount of core protein synthesized was observed (Figure 4-3, lane 2-5).

The lysate used in this study contained both the polymerase protein and its corresponding mRNA. Previous studies have shown that polymerase protein binds to pregenomic RNA during encapsidation (Bartenschlager *et al.*, 1990; Blum *et al.*, 1991; Chen *et al.*, 1992; Bartenschlager and Schaller, 1992; Roychoudhury *et al.*, 1991). In addition, a polymerase - β-galactosidase fusion protein expressed in *E.coli* was found to bind HBV RNAs (Köchel *et al.*, 1991). Therefore, I postulated that the inhibition of core translation by polymerase may be due to binding of the polymerase protein to the core mRNA.

2. Suppression of translation mediated by DHBV polymerase protein is specific and exclusive to DHBV core RNA.

In order to show that the inhibition of core mRNA translation in the presence of the polymerase protein was not a general inhibitory effect seen with coexpression of proteins in this system, I performed a similar experiment with increasing amounts of HIV tat protein added to the reticulocyte lysate. The results shown in Figure 4-4A demonstrate that core protein synthesis was not inhibited in the presence of increasing amounts of lysate containing tat protein (lane 2-4). A similar result was seen using an *in vitro* expressed lysate containing proteins produced from five different species of Brome Mosaic virus mRNAs (result not shown). These results suggest that the inhibition of core mRNA translation was not non-specific inhibition by mRNAs or proteins. I attributed the observed translational arrest of core mRNA to specific inhibition mediated by the polymerase protein.

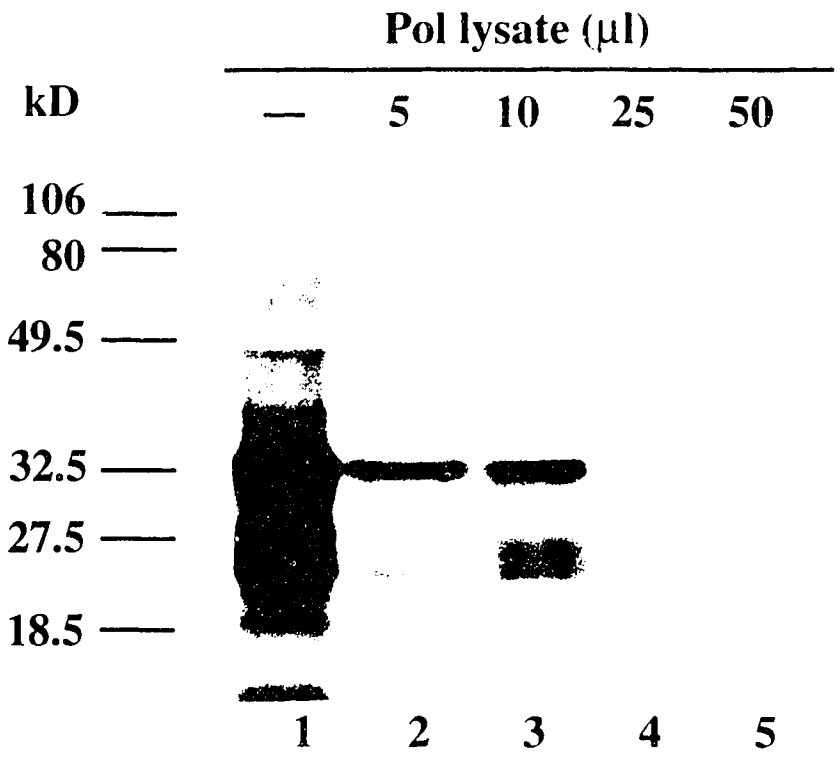

Figure 4-3. Inhibition of core mRNA translation in the presence of increasing amounts of lysate containing DHBV polymerase.

One microgram of polymerase RNA was translated *in vitro* using rabbit reticulocyte lysate at 30 °C for 1 h (polymerase lysate). Two micrograms of core mRNA were mixed with rabbit reticulocyte lysate and translated in the presence of increasing amounts of polymerase lysate. The final volume of each reaction was 100 µl. In lane 1, no polymerase lysate was added. In lanes 2 through 5, 5, 10, 25 and 50 µl of polymerase lysate were added, respectively. *In vitro* translated core proteins (^{35}S-methionine labeled) were immunoprecipated, electrophoresed on 10% SDS-PAGE and detected by autoradiography.

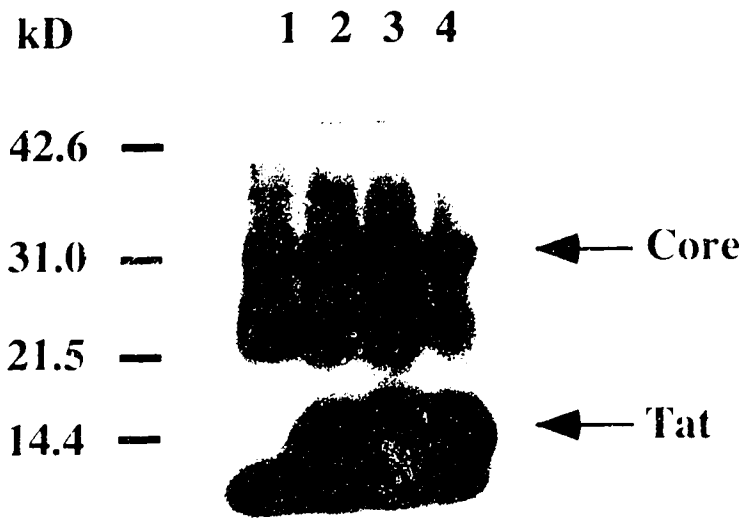

Figure 4-4A. Specificity of inhibition of core mRNA translation by polymerase: Translation of core mRNA in the presence of HIV tat protein lysate.

One microgram of tat mRNA was translated *in vitro* using rabbit reticulocyte lysate. One microgram of core mRNA was mixed with no exogenous protein lysate (lane 1); 5 (lane 2), 7.5 (lane 3) or 12.5 µl (lane 4) of *in vitro* translated HIV tat protein lysate. Each reaction mixture was brought up to a final volume of 25 µl with rabbit reticulocyte lysate translation mix. Translated products were ^{35}S-methionine labeled and immunoprecipitated by anti-DHBcAg and anti-tat specific antibodies and analyzed by 12% SDS-PAGE.

I also considered the possibility that the specific inhibition of core mRNA translation mediated by the polymerase protein could be due to the binding of the polymerase to translational factors rather than interacting directly with the core mRNA. To distinguish these two possibilities, I translated the human CD4 mRNA in the presence or absence of the polymerase protein. If polymerase protein interacts with translational factors, suppression of CD4 mRNA translation would be expected. No inhibition of CD4 translation in the presence or absence of polymerase was observed (Figure 4-4B, lane 1 and 2). In contrast, in samples which contained both core and CD4 mRNAs, only the core mRNA translation was inhibited in the presence of the polymerase protein (Figure 4-4B lane 3 and 4). I have also examined the effect of the polymerase protein on the translation of Xenopus elongation factor 1 mRNA and no inhibition was observed (result not shown).

3. DHBV Polymerase interacts with the 3' periphery of the core open reading frame.

I attempted to define the region in core mRNA which is responsible for binding the polymerase protein. The expression plasmid pTZ19RCore, which was used in the previous experiments, was constructed by inserting a PCR fragment of DHBV sequence starting at nt 2632 (17 nucleotides upstream from the AUG codon of the C-ORF) to nt 960. The transcript generated from this construct will not contain the putative stem-loop structure described by Hirsch *et al.* (Figure 4-1) (Hirsch *et al.*, 1991). Since the encapsidation sequence in DHBV was found to consist of an extensive region (Hirsch *et al.*, 1991), it is probable that the polymerase may interact with the regions downstream from the cap site. I constructed a series of 3' truncated core mRNAs (... D5) (Figure 4-5A), and compared their translation efficiency in the presence or absence of the polymerase protein. The translation products were immunoprecipitated and analyzed by 10% SDS-PAGE. As shown in Figure 4-5B, translation of the core mRNA transcribed from the parental

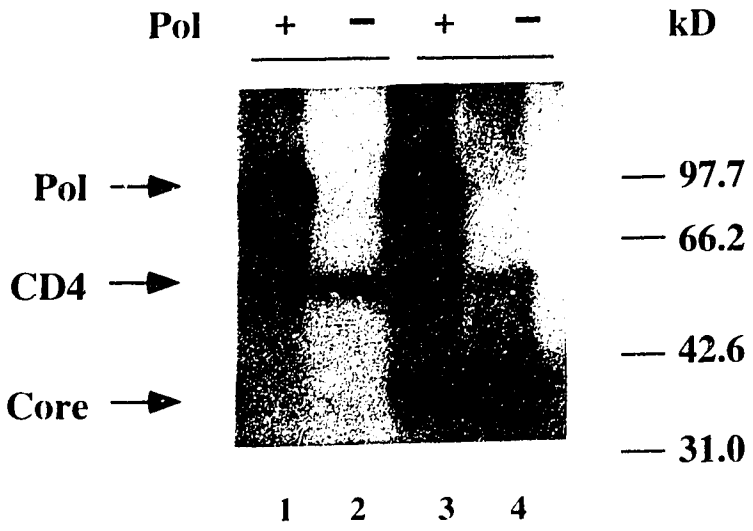

Figure 4-4B. Specificity of inhibition of core mRNA translation by polymerase: Effect of polymerase on the translation of core and CD4 mRNAs.

Translation reactions containing rabbit reticulocyte lysate (total volume, 50 µl) were incubated with 1.5 µg of CD4 mRNA (lanes 1 and 2) or a mix of 1.5 µg of core mRNA and 0.8 µg of CD4 mRNA (lanes 3 and 4). Translation was carried out in the presence (lanes 1, and 3) or absence (lanes 2 and 4) of polymerase lysate (10 µl). The ^{35}S-methionine reaction products were electrophoresed on 12% SDS-PAGE and detected by autoradiography.

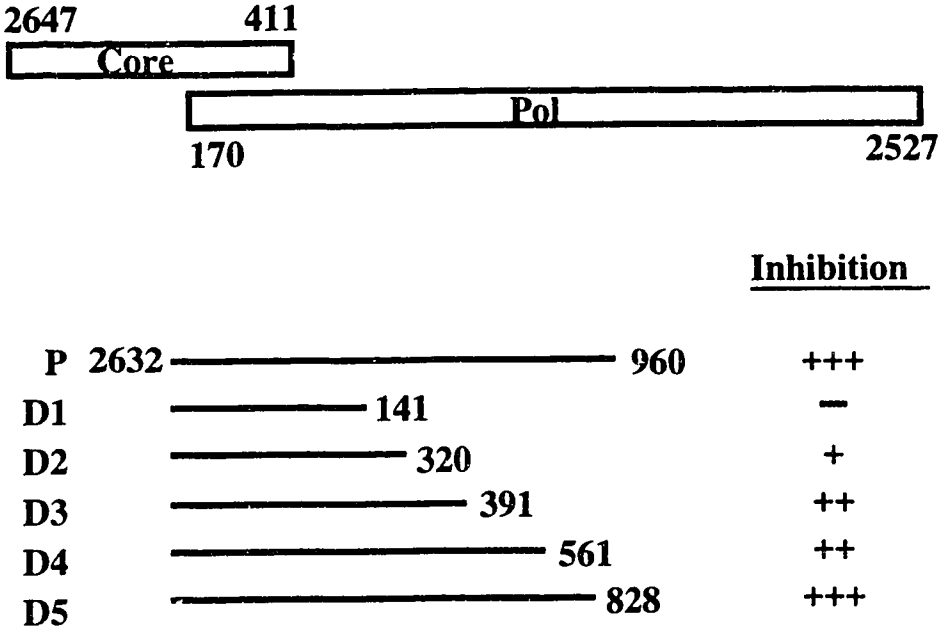

Figure 4-5A. Inhibition of translation of truncated core RNAs by polymerase lysate: Schematic diagram of the truncated core mRNAs.

Locations of the open reading frames of core (Core) and polymerase (Pol) proteins are shown as open boxes. P represents core mRNA generated from pTZ19RCore. D1 - D5 are the 3' truncated core mRNAs transcribed from pTZ19RCore which has been linearized at the positions indicated. Core proteins translated from truncated core mRNAs were quantified using a PhosphorImager. Mean results obtained from four experiments are summarized on the far right. <5% inhibition (-); <25% inhibition (+); approx. 50% inhibition (++); and > 95% inhibition (+++).

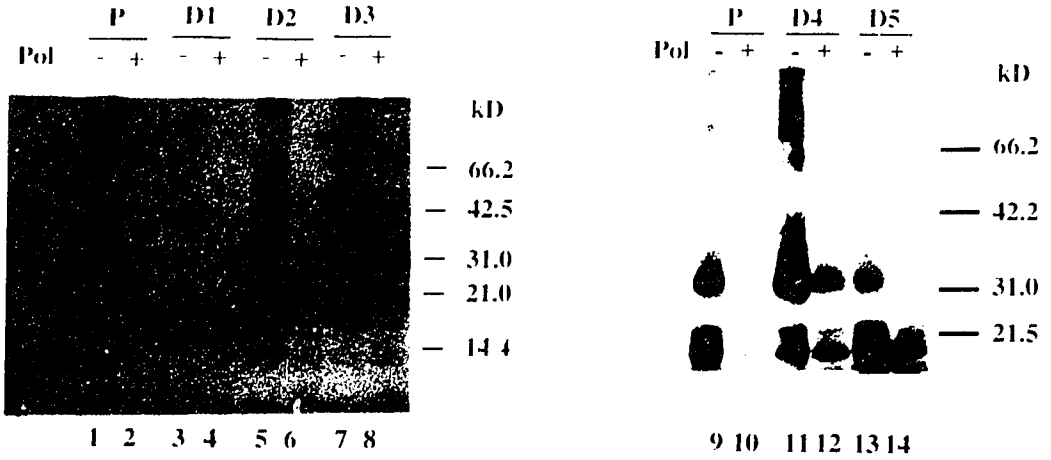

Figure 4-5B. Inhibition of translation of truncated core RNAs by polymerase lysate: Translation of truncated core mRNAs in the absence or presence of polymerase.

The translation products (final volume, 25 µl) containing 1 µg of parental (P) (lanes 1, 2, 9 and 10) or 3' truncated core RNAs (D1 - D5) (lanes 3 - 8, 11 - 14) were incubated in the absence (lanes 1, 3, 5, 7, 9, 11, 13) or presence (lanes 2, 4, 6, 8, 10, 12, 14) of 12.5 µl of polymerase lysate. The ^{35}S-methionine labeled translation products were immunoprecipitated using polyclonal rabbit anti-DHBcAg antibodies, analyzed by 12% SDS-PAGE and detected by autoradiography.

construct was inhibited in the presence of the polymerase protein (Figure 4-5B, lane 1 and 2). No inhibition was found in D1 (Figure 4-5B, lane 3 and 4) indicating that sequences upstream of nt 140 may not contain the region responsible for binding with the polymerase pro[illegible]. However, inhibition of translation in the presence of polymerase began to appear with truncated mRNAs (D2 - D4) which contained increasing lengths of the 3' downstream sequence (Figure 4-5B, lane 5 - 12). With the D5 truncated mRNA, translation was inhibited to the same extent as that of the parental core mRNA (Figure 4-5B, lane 13 and 14 vs lane 9 and 10). The reduced amount of translation product obtained with D5 truncated mRNA (Figure 4-5B, lane 13) relative to that with the other mRNAs (P - D4) (Figure 4-5B, lane 1,3,5,7,9,11) was not seen in repeat experiments (data not shown).

In summary, results from the translation of the 3' truncated core mRNAs in the presence of the polymerase protein identified a region of about 700 nucleotide (nt 140 - nt 828) at the 3' periphery of the C-ORF which may be responsible for binding the polymerase protein. With increasing lengths of the sequence within this region, progressive inhibition of core mRNA translation was observed.

4. Insertion of the DHBV sequence into a heterologous RNA resulted in translation inhibition.

To confirm that this region is involved in the downregulation of the core mRNA translation, presumably by binding with the polymerase, I examined the possibility that addition of this sequence to a heterologous mRNA would render it susceptible to inhibition by polymerase. Translation of CD4 was found not to be affected by the presence of the polymerase protein (Figure 4-4B). We inserted the DHBV sequence (nt 401- nt 870) into a plasmid pSP72-T4 which contains a human CD4 coding sequence cloned downstream from a T7 promoter. Two recombinant constructs were made (Figure 4-6A). One construct (pDCD4BstEII) contains the DHBV sequence inserted in frame at 877 nucleotides

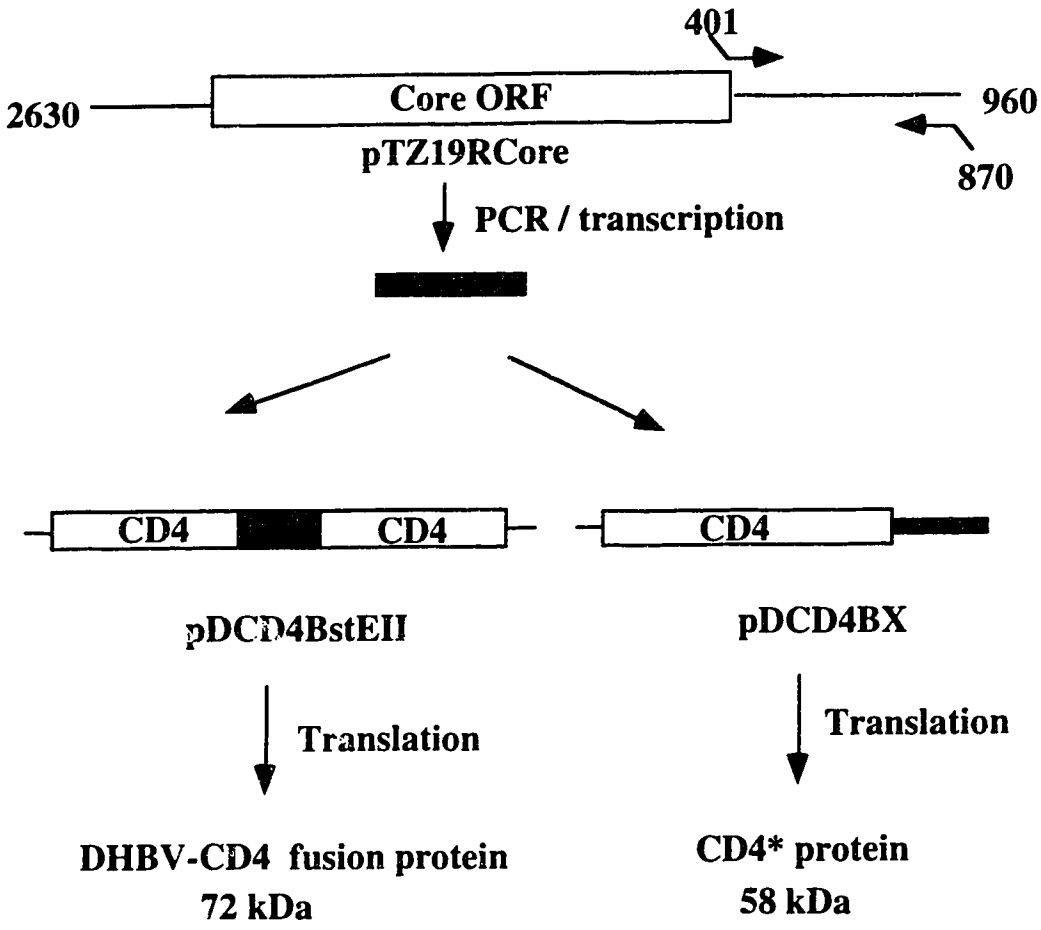

Figure 4-6A. Inhibition of translation of DHBV-CD4 recombinant mRNAs by polymerase lysate: Schematic representation of generation of DHBV-CD4 recombinant constructs.

Linearized pTZ19RCore was amplified by PCR using primers located at nt 401 and 870 as shown. The shaded box represents the amplified DHBV DNA fragment. pDCD4BstEII is a recombinant construct with a DHBV sequence cloned in frame within the CD4 coding sequence. pDCD4BX is another recombinant construct having the DHBV sequence cloned downstream from the CD4 open reading frame. CD* represent a CD4 protein translated from mRNAs generated from the pDCD4BX construct.

downstream from the translation initiation site. Transcription and translation using this construct will produce a fusion protein of approximately 72 kDa. Another construct (pDCD4BX) contains the DHBV sequence cloned immediately downstream from the CD4-ORF. Transcription and translation of this construct will produce a CD4 protein (~ 58 kDa) similar to that from the parental plasmid pSP72-T4.

Figure 4-6B shows the translation products obtained from these DHBV-CD4 recombinant constructs. In the left panel, a DHBV-CD4 fusion protein (~ 72 kDa) and a CD4 protein (~ 58 kDa) were produced from pDCD4BstEII and pDCD4BX, respectively. The constructs produced proteins of the predicted molecular weights. Cotranslation of mRNAs generated from pSP72-T4 and pDCD4BstEII yielded the corresponding proteins (Figure 4-6B, right panel, lane 1), indicating that these two mRNAs together can be translated efficiently. However, cotranslation of these two mRNAs in the presence of the polymerase resulted in the production of the 58 kDa species only (Figure 4-6B, right panel, lane 2), indicating that translation of the pDCD4BstEII RNA was selectively inhibited. Likewise, the presence of the DHBV sequence in pDCD4BX also resulted in suppression of CD4 protein synthesis in the presence of the DHBV polymerase protein (Figure 4-6B, right panel, lane 3 and 4). Since the DHBV sequence was inserted downstream from the CD4-ORF in pDCD4BX, this result suggests that the interaction of polymerase with RNA sequences outside the open reading frame can mediate inhibition of translation of that mRNA. These results strongly suggest that the presence of the DHBV sequence (nt 401 - 870) in a heterologous system is sufficient to cause translational arrest of that recombinant mRNA in the presence of the DHBV polymerase protein.

5. Direct binding of DHBV polymerase to the core RNA.

To demonstrate that there is direct binding of the polymerase protein to the core mRNA, I generated radioactive RNA probes from the putative binding region (nt 315 - 872) and

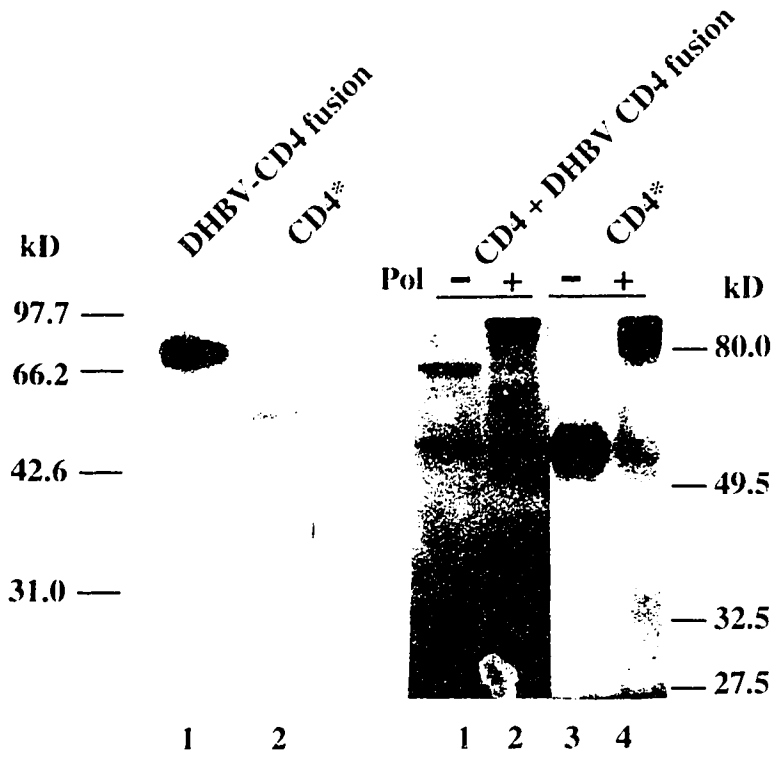

Figure 4-6B. Inhibition of translation of DHBV-CD4 recombinant mRNAs by polymerase lysate: Translation inhibition of DHBV-CD4 recombinant RNAs by polymerase.

The panel on the left indicates the translation products of pDCD4BstEII and pDCD4BX, respectively. The panel on the right shows the products of *in vitro* translation of mRNAs from pSP72-T4 (2 μg) and pDCD4BstEII (2 μg) in lanes 1 and 2 or pDCD4BX in lanes 3 and 4. The translation products are shown in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of polymerase lysate (5 μl). The final volume of each reaction was 25 μl. The ^{35}S-methionine labeled translation products were analyzed by 10% SDS-PAGE and detected by autoradiography.

performed a label-transfer experiment. Radioactive RNA probes, CD4 and D1, the translation of which were shown previously not to be affected by the presence of the polymerase protein (Figure 4-4B and Figure 4-5B, lane 3 and 4), were also included as negative controls. *In vitro* translated polymerase was incubated with the ^{32}P-labeled RNAs in the presence of poly rC and heparin sulfate as non-specific competitors. The reaction products were UV cross-linked, digested with RNase A and subjected to SDS-PAGE. Radiolabeled DHBV RNA which contained the putative binding region was found to bind to the polymerase protein as shown in Figure 4-7, lane 1. There is a band in the 90 kDa region and a smear of radioactivity below this band. Radiolabeled RNAs of CD4 or the region upstream from the putative polymerase binding site failed to show any binding to the *in vitro* translated polymerase (Figure 4-7, lane 2 and 3). No radioactive band was found in the sample containing the reticulocyte lysate (without polymerase protein) and the radioactive RNA probes (Figure 4-7, lane 4, 5 and 6). I have attempted to eliminate the smear below the 90 kDa band by digesting the reaction products with a cocktail of RNases (T1, T2 and C), however, this digestion failed to reduce the smear. No such smear was found in the sample containing the lysate and the radiolabeled RNA (Figure 4-7, lane 4). Therefore, although I have no explanation for the presence of this smear, I believe that it is not due to the non-specific binding of the radiolabeled RNA probes to the proteins present in the reticulocyte lysate.

I have attempted to perform a gel shift experiment using the radioactive RNAs and the *in vitro* translated polymerase. My failure, thus far, to obtained a retarded band could be due to insufficient amount of the *in vitro* expressed polymerase as well as high background of the reticulocyte protein. Nonetheless, I believe that the results obtained in the labeled RNA transfer experiment provide evidence for direct binding of the polymerase and other low molecular weight proteins to the pregenomic RNA containing the suppressor site downstream of the C-ORF.

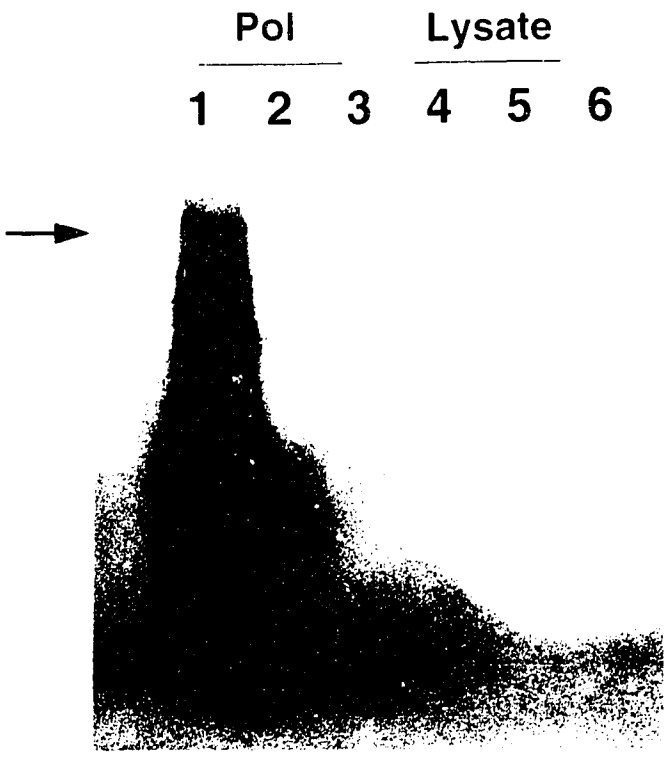

Figure 4-7 Binding of the polymerase protein to DHBV RNA.

Radioactive RNAs, DHBV nt 315 - 872), CD4 and D1, were transcribed from the corresponding DNA templates in the presence of [α-^{32}P]UTP. Radioactive RNA probes were incubated with the lysate containing *in vitro* translated DHBV polymerase (Pol, lanes 1, 2, and 3) or the rabbit reticulocyte lysate alone (Lysate, lanes 4, 5 and 6) in the presence of poly rC and heparin sulfate. Each reaction mix was UV-crosslinked, digested with RNase A and analyzed by 10% SDS-PAGE. Lanes 1 and 3 contained radiolabeled DHBV RNA from nt 315 - 872, lanes 2 and 5 contained radiolabeled CD4 mRNA and lanes 3 and 6 contained truncated core mRNA, D1.

IV. DISCUSSION

Translational regulation is a common mechanism for controlling gene expression in both prokaryotes and eukaryotes (Kozak, 1991; Kozak, 1987). Translation auto-suppression was first characterized in bacteriophage R17 in which the coat protein binds to the RNA synthetase mRNA and suppresses its translation (Klausner and Harford, 1989). Since then, similar mechanisms have been described in eukaryotic systems such as mRNAs of yeast ferritin, human thymidylate synthase and human dihydrofolate reductase (Chu *et al.*, 1991; Chu *et al.*, 1993; Klausner and Harford, 1989). In this study, I have demonstrated that DHBV may also use translational suppression as a mean of regulating core protein synthesis.

In this chapter, I have shown that DHBV polymerase serves as a translational suppressor for core mRNA translation. Suppression of the translation is not due to a systematic effect such as competition of the messenger RNAs for the translating ribosomes, interference with translation by an exogenous protein, or interaction of the polymerase protein with the translational factors or ribosomes (Figure 4-4A and B). I have attributed the translation suppression to the direct binding of the polymerase to the core mRNA. This hypothesis is supported by the observation that insertion of the putative binding sequence of DHBV into or downstream of the CD4 coding sequence leads to an inhibition of the recombinant CD4 mRNA translation in the presence of the DHBV polymerase protein (Figure 4-6B). Furthermore, I have identified the binding region of the core mRNA to the 3' end and downstream of the C-ORF (Figure 4-5B). Binding of the polymerase to this region of the core mRNA was further confirmed by the results of the UV-crosslinked label-transfer experiment.(Figure 4-7).

It is interesting that the putative binding region resides at the 3' end and downstream of the C-ORF in DHBV. Translational arrest caused by binding of the polymerase at this region may necessitate bending of the pregenomic RNA by the polymerase in order for it to

interfere with the translation initiation or elongation. Changes in RNA conformation have been seen in other RNA-protein interactions such as HIV TAR-tat and RRE-Rev, and alfalfa mosaic virus RNA4-CP (Tan and Frankel, 1992; Baer *et al.*, 1994; Daly *et al.*, 1990).

From the results of the present study, I have gained important insights into the mechanism of hepadnaviral nucleocapsid assembly. During the process of nucleocapsid formation, there is a *cis* preference for encapsidation of the nascent messenger RNA (Hirsch *et al.*, 1990). A stem-loop structure present at the 5' end of the HBV pregenomic RNA has been identified as the recognition site for the polymerase (Pollack and Ganem, 1993; Tong *et al.*, 1993). The results of my study, however, suggest that there is a second region in DHBV which may also serve as a docking site for the polymerase. With the data presently available, I favor the concept of coordinate control of translation and encapsidation of the pregenomic RNA. In this model, binding of the polymerase downstream of the core ORF may displace the ribosomes from the entrance at the 5' end of the pregenomic RNA, hence exposing the 5' stem-loop structure and facilitating the selective uptake of the pregenomic RNA into the nucleocapsid. The region identified in my study for suppression of translation coincides with the 'region II' suggested by Calvert and Summers (1994). They reported that deletion of this region abrogates nucleocapsid assembly in transfected cells (Calvert and Summers, 1994).

In keeping with the above concept, the binding of the polymerase to this region may also interfere with its own translation. The scarcity of the polymerase protein in HBV infected cells has often been attributed to the infrequent events of ribosomal leaky scanning or internal ribosomal entry to the polymerase open reading frame during translation (Jean-Jean *et al.*, 1989; Fouilliot *et al.*, 1993; Lin and Lo, 1992). However, *in vitro* translation of the polymerase transcripts consistently resulted in a substantially lower yield as compared with that from an equimolar amount of the core mRNAs (Figure 4-2). In this system, both the mRNAs were generated from constructs in which the translation initiation codon of the

corresponding open reading frames was the first AUG encountered by the ribosomes. I speculate that the polymerase protein may act via translation autoregulatory control through binding to one or more mRNA sequences. Translational auto-regulatory control of the polymerase protein may account for the difficulties in cloning and expression of the polymerase protein which has been encountered in our and other laboratories.

In summary, there are three observations in this chapter that I believe help to explain several important concepts about the unique molecular biology of hepadnaviruses:

a) The presence of the DHBV sequence (nt 401 - 820) in a heterologous system can cause translational arrest of the heterologous mRNA in the presence of DHBV polymerase.

b) The DHBV nucleotide sequence 401 to 820 in the mRNA of core protein appears to be the target of DHBV polymerase binding and translation arrest of core mRNA.

c) The presence of the same sequence in pregenomic RNA may be the "region II" discovered by Calvert and Summers (1994). This region is required for pregenomic RNA encapsidation. Binding of polymerase protein to this region could i) inhibit translation of polymerase (a problem encountered in low expression of polymerase in exogenous systems) and ii) target pregenomic RNA for encapsidation by preventing ribosomal entrance of the 5' end of pregenomic RNA and permitting exposure of the stem-loop structure to signal encapsidation.

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Chapter 5

General Discussion and Conclusions

I. DISCUSSION AND OVERALL SUMMARY

The objective of this project was to investigate the mechanism of action of 2,6 diaminopurine 2',3'-dideoxyriboside (ddDAPR) on hepatitis B virus (HBV) replication, using duck hepatitis B virus (DHBV) as a model system.

This project was a continuation of previous work by Lee *et al* (1989) and Suzuki *et al* (1988), who screened dideoxynucleoside analogues for activity against hepadnaviral replication in infected primary duck hepatocyte cultures. Results from their studies in the avian system indicated that purine but not pyrimidine dideoxynucleoside compounds were potent inhibitors of DHBV replication. In particular, ddDAPR, a prodrug of ddG, was found to be an extremely effective inhibitor of hepadnaviral replication. There are several possible explanations for the selective inhibition by purine but not pyrimidine dideoxynucleoside analogues. Firstly, there could be differences between the transport and rates of metabolism of purine and pyrimidine analogues in hepatocytes. Secondly, purine dideoxynucleoside analogues might be more potent inhibitors of the hepadnavirus polymerase than the pyrimidine dideoxynucleoside compounds. Thirdly, there might be a specific target for the purine dideoxynucleoside in the hepadnaviral replication cycle. Extensive studies carried out by T. Kitos on the metabolism of ddG and ddC in liver cells indicated that the total intracellular ddG nucleotide pool was comparable to ddC nucleotide pool on exposure to these nucleoside analogues (Kitos, 1994). In studies on the enzyme kinetics of the DHBV polymerase, the inhibitory effects of ddGTP and ddCTP were not significantly different. This work has been done by X. Liu and A. Severini (in

preparation). Therefore, results from concurrent studies in our laboratory suggest that the first and second possibilities are unlikely to account for the selective inhibition of DHBV replication by ddDAPR or ddG. From the published results on the sequence of hepadnaviruses, we noted that the first nucleotide attached to the primer domain of the polymerase is a deoxyguanosine. On this basis, we proposed a hypothesis as follows: ddGTP (metabolized from ddDAPR) inhibits viral DNA replication through binding to the polymerase (presumably the primer domain of the polymerase). The covalent binding of the dideoxyguanosine to the polymerase might resist proof-reading.

The series of projects undertaken in this study evolved around the above hypothesis. Both indirect and direct evidence regarding the mechanism of inhibition of hepadnaviral replication by ddDAPR (ddG) was sought in several different experimental settings. I used ddC, an ineffective anti-DHBV agent, as a control throughout the study.

Chapter 2 describes a system used to express an enzymatically active DHBV polymerase using an *in vitro* transcription and translation system. Attempts to use other expression systems such as *E.coli*, recombinant vaccinia viruses and Xenopus oocytes failed to yield a full length polymerase. Attempts to clone the polymerase gene have also been reported by other laboratories. These studies report the low expression of the polymerase protein, but no enzymatic activity of the cloned polymerase has been reported (Noonan *et al*, 1991; McGlynn *et al*, 1992; Chang *et al*, 1989; Stemler *et al*, 1988). The putative toxicity associated with the hepadnavirus polymerase may account for the difficulty of achieving successful expression of hepadnaviruse polymerse in biological hosts. Data presented in Chapter 2, however, show that the *in vitro* translated DHBV polymerase (~ 90 kDa) exhibited DNA-DNA polymerase and reverse transcriptase activities when tested on DNA or RNA templates primed with DNA-hexamers, respectively. These two enzymatic activities were studied in the presence of actinomycin D; the presence of which inhibits the DNA-DNA polymerase activity but has no effect on the activities displayed by the reverse transcriptase. The self-priming property of this *in vitro* translated polymerase is illustrated

by its reverse transcriptase activity on endogenous templates; and its binding to dGTP, the first nucleotide which links the polymerase protein and the DHBV genome. My work on the functional characterization of the *in vitro* translated polymerase yielded results, reported in Chapter 2, that are compatible with the results reported by Wang and Seeger (1992) who also expressed the DHBV polymerase using a similar approach.

In chapter 3, I studied the selective inhibitory effect of ddDAPR on hepadnaviral replication observed *in vitro* using an *in vivo* system. There are two reasons for using an *in vivo* system. First, the effect of the drug can be examined in an integrated system which closely mimics a natural infection and drug treatment. Second, larger amounts of virus replicating cores can be prepared in order to study the mechanism of inhibition. Using this *in vivo* system, I obtained important indirect evidence to support the hypothesis. The strongest evidence for the hypothesis on the mechanism of inhibition by ddDAPR (ddG) on DHBV replication came from the demonstration of direct binding of ddGTP to the *in vitro* expressed polymerase.

I used a stepwise approach to deduce the mechanism of action mediated by ddDAPR. Initially, I identified the site of inhibition by examining the quantities of the nucleic acids inside replicating cores. A drastic reduction of viral DNA without substantial loss of viral RNA indicated that inhibition occurred at the stage of reverse transcription. Consistent with our working hypothesis that ddGTP (metabolized from ddDAPR) inhibits viral replication through blocking the priming step of the DNA synthesis, the results from amplified primer extension and oligonucleotide dot-blot hybridization showed virtually an absence of even the very earliest stages of DNA in samples from ddDAPR treated animals. The inhibition of DNA synthesis was not due to the loss of enzymatic function since the presence of reverse transcriptase and DNA-DNA polymerase activities from the same core preparations was demonstrated in activity gel assays. The binding of ddGTP to the *in vitro* translated polymerase provided direct evidence in support of the hypothesis. Binding of ddGTP to the *in vitro* translated polymerase was also shown to lead to inhibition of DNA

replication in endogenous nascent templates under *in vitro* experimental conditions. The potent inhibitory effect of ddGTP observed with *in vitro* translated polymerase parallels the antiviral effect of ddDAPR seen with *in vivo* treatment of DHBV infected ducklings. At present, we have not identified the domain in the polymerase which is responsible for binding to ddGTP, however tyrosine 96 in the primer domain of the polymerase is the most likely candidate. Binding of [α-^{32}P]dGTP to an *in vitro* translated DHBV polymerase has been shown by Zoulim and Seeger (1994).

The distinct event in hepadnaviral replication in which the polymerase protein primes DNA replication serves as an unique and selective target for antiviral therapy. The data shown in Chapter 3 corroborate the potentiality of using ddG and its prodrug ddDAPR for chemotherapeutic intervention in the hepadnaviral replicative cycle. Metabolic studies conducted by T. Kitos have indicated that hepatocytes are able to maintain relatively high ddGTP nucleotide pools further support the pharmacokinetic feasibility of this compound being used for antiviral therapy (Kitos and Tyrrell, 1995). While the mechanism of action of ddG or ddDPAR has been studied thoroughly in an avian system, further research in mammalian system will be necessary to ensure the efficacy and safety of using this agent as an anti-HBV therapeutic.

As discussed above, Chapters 2 and 3 have focused on the mechanism of action of ddDAPR in DHBV replication using both *in vitro* and *in vivo* systems. Chapter 4 may be seen as a digression from the subject, although it does concentrate on the role of the polymerase in hepadnaviral replication. Results presented in Chapter 4 illustrate translational suppression of core mRNA in the presence of polymerase protein. The suppression mediated by polymerase protein was specific for mRNAs carrying the nucleotide sequence 401 to 820 of the DHBV genome. Further analyses reveal that nucleotide sequences at the 3' end and downstream of the core ORF were responsible for interacting with the polymerase protein leading to the translational arrest. Binding of the polymerase protein to this region has been demonstrated.

The translational regulation observed with polymerase protein and core mRNA leads to important insights to the mechanism of nucleocapsid assembly. In hepadnaviruses, a stem-loop structure (also known as ε) present at the 5' end of the pregenomic RNA has been identified as a recognition site for interaction with the polymerase (Pollack and Ganem, 1994; Köchel *et al*, 1991). The interaction between polymerase and ε facilitates nucleocapsid assembly and initiates minus-strand DNA synthesis (Pollack and Ganem, 1993; Hirsch *et al*, 1991; Pollack and Ganem, 1994; Tavis *et al*, 1994; Wang and Seeger, 1993). In Chapter 4, I have speculated that a second region located at the 3' end and downstream of core ORF might assist in DHBV nucleocapsid assembly through coordinate translation auto-regulation. It can be envisaged that interaction of the polymerase protein with the region of the pregenomic RNA might displace translating ribosomes and expose the stem-loop sequence (ε). This would allow polymerase to bind to ε to signal encapsidation. All of the work described in Chapter 4 was done using the *in vitro* rabbit reticulocyte lysate system. To confirm the biological relevance of these observations, it will be necessary to demonstrate the interaction of polymerase with viral mRNA in DHBV infected or transfected cells.

II. SUGGESTIONS FOR FUTURE RESEARCH

There are essentially three main projects described in this thesis - (i) cloning and expression of DHBV polymerase; (ii) studies of on the mechanism of action of ddDAPR on DHBV replication and (iii) translation suppression of core mRNA by polymerase protein. Each project yielded interesting insights towards understanding hepadnaviral replication. However, each project also uncovered new ground for further research. In the following section, I will discuss areas related to the above projects which I believe require further investigation.

(i) on cloning and expression of hepadnavirus polymerases

As described in Chapter 2, it has been difficult to clone and express an enzymatically active hepadnavirus polymerase. In systems which successfully expressed the active polymerases, only minute amounts of the products were obtained (Seifer and Standring, 1993; Wang and Seeger, 1992; Tavis and Ganem, 1993; Howe *et al*, 1992). Circumstantial evidence, however, suggests that the presence of the ε region may stabilize the polymerase and facilitate enzymatic activities of the polymerase during expression (Lanford *et al*, 1994; Bartos *et al*, 1994). Use of recombinant constructs containing pol ORF plus the ε sequence should be considered as a means of enhancing the expression of hepadnavirus polymerases.

Failure to express hepadnavirus polymerases has been attributed to the putative toxicity associated with the protein (Chang *et al*, 1989; Foster *et al*, 1991). To circumvent this problem, use of a secretory expression system would allow the newly synthesized protein to be secreted, which would prevent accumulation of the toxic protein in the host cell. Expression vectors with signal peptide sequence upstream of the multiple cloning sites could be used to clone and express the hepadnavirus polymerases in eukaryotic cells.

As speculated in Chapter 4, binding of polymerase protein to RNA sequences at the 3' end and downstream of the core ORF may suppress its own translation. Mutations in the nucleotide sequences without alteration of the amino acid sequences may help to evade the translation inhibition and allow for higher expression of hepadnavirus polymerase.

(ii) on the mechanism of action of ddDAPR

Substantial evidence regarding the mechanism of action of ddDAPR on DHBV replication has been sought in this project. Results obtained so far are compatible with our hypothesis that ddGTP terminates DNA replication at the priming stage. Binding of ddGTP to DHBV polymerase has been demonstrated. However, which domain in the polymerase is responsible for binding to ddGTP has not yet been elucidated. More efficient synthesis of proteins should be attempted using the newly described TNT reticulocyte lysate *in vitro* translation system (Promega, Madison, WI). With this system, it should be able to obtain larger amounts of DHBV polymerase protein which would assist with the labeling with [α-^{32}P]ddGTP. The region responsible for binding to ddGTP may be identified by partial tryptic digestion of the radioactive labeled protein, followed by separation of the proteolytic fragments and amino acid mapping of the labeled polypeptides. Alternatively, the amino acid responsible for formation of the phospodiester linkage between ddGTP and the polymerase protein could also be evaluated by acid hydrolysis of the [α-^{32}P]ddGTP labeled polymerase, followed by two dimensional thin layer chromatography. Results obtained from these experiments should provide information on the region in the polymerase protein responsible for binding to ddGTP. If the binding site is the primer domain of the polymerase, the data will provide further support for our hypothesis.

At present, it is not clear whether the phosphodiester bond between the primer domain of the polymerase and the guanosine nucleotide is established at the α, β or γ position. So far, all viruses with genome-linked proteins have been found to have phosphodiester bonds

established at the α position (Salas, 1991; Kornberg and Baker, 1992). Using radioactive dGTP labeled at the α, β or γ position and the experimental strategies suggested above, it should possible to clarify the nature of this protein-nucleotide bond in hepadnaviruses.

From the point of view of those interested in chemotherapy of HBV, it is essential to determine whether or not ddDAPR or ddG is effective in inhibiting mammalian hepadnaviral replication. An anti-HBV effect of ddG has been reported in tranformed cell lines (Aoki-Sei *et al*, 1991; Korba and Milman, 1990; Korba and Gerin, 1992) as well as in treatment of HBV infected chimpanzees (unpublished results, Dr. D .L. J. Tyrrell). However, it is not clear that the mechanism of action of ddG in DHBV is the same in mammalian hepadnaviruses. Since woodchuck hepatitis virus (WHV) has extensive nucleotide sequence homology (over 70%) with the HBV genome (Mandart *et al*, 1984), WHV may serve as a useful model in studying the mechanism of action of ddDAPR or ddG in mammalian hepadnaviral replication.

(iii) on translation suppression of core mRNA by polymerase protein

Evidence to support the translational control of core mRNA by polymerase protein has been presented in Chapter 4. I have identified a region of 700 nucleotides which may be responsible for interacting with the polymerase protein and facilitating nucleocapsid assembly. I also believe that polymerase may mediate a feed-back inhibition of its own translation through binding to this region. In order to make this information useful for subsequent molecular genetic studies, it will be necessary to identify more precisely the contact sites between the polymerase protein and the RNA sequences. Translation of mRNAs containing deletions or mutations within this region in the presence of polymerase protein may help to define the sequence of interest. In addition, the secondary structure of this region should also be examined to see if it contains a specific structure which interacts with the polymerase. Comparison of the secondary structure found between within this 700 nucleotide sequences and the ε region may also give some clues in understanding the

polymerase-RNA binding. Once a more defined "contact" sequence is identified, mutagenesis can be carried out to locate the nucleotides responsible for the interaction with the polymerase.

To study the significance of the translational suppression of core mRNA by polymerase protein observed in the *vitro* experimental conditions to hepadnaviral replication, experiments can be conducted by cotransfection of cell line with core producing plasmids containing mutagenized sequences within the "inhibitory binding region" as identified in Chapter 4, and plasmids expressing polymerase protein. The amounts of core proteins or intact replicating cores could be quantified by immunoprecipitation of the cell lysate using anti-DHBcAg antibodies. Results from these studies may provide insights into the coordinated translational control in hepadnaviral nucleocapsid assembly.

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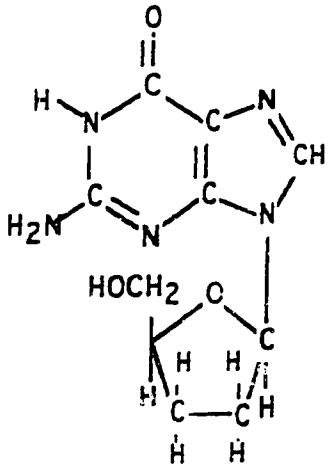
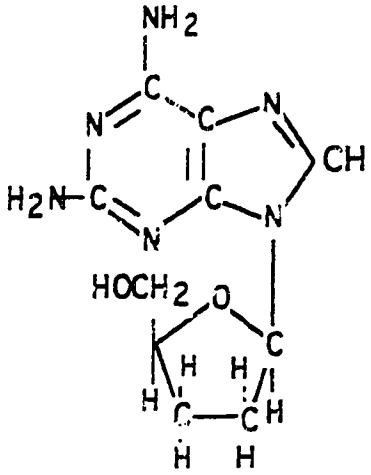
Appendix I

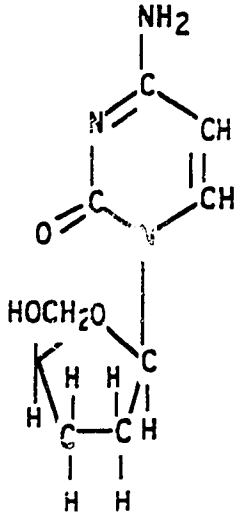
Chemical structures of ddDAPR, ddG and ddC



2', 3' dideoxyguanosine
(ddG)



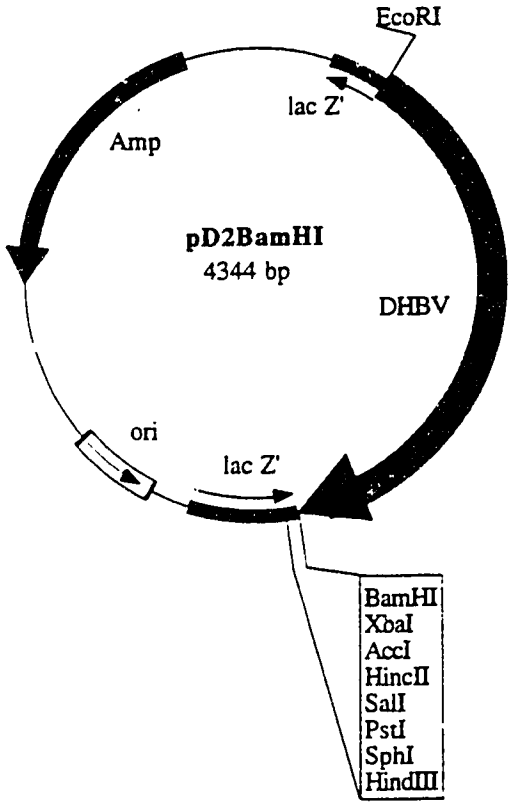
2, 6 diaminopurine
2',3' dideoxyriboside
(ddDAPR)



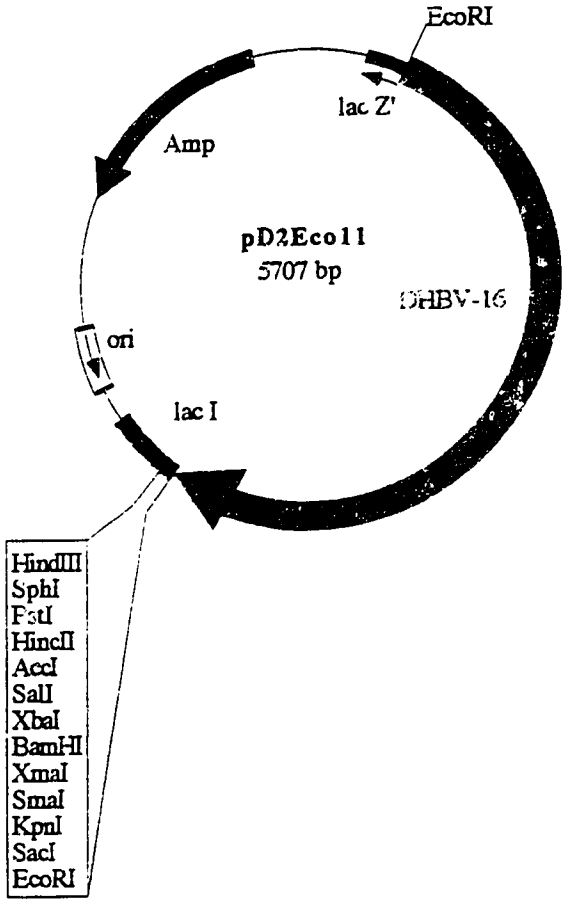
2', 3' dideoxycytidine
(ddC)

Appendix II

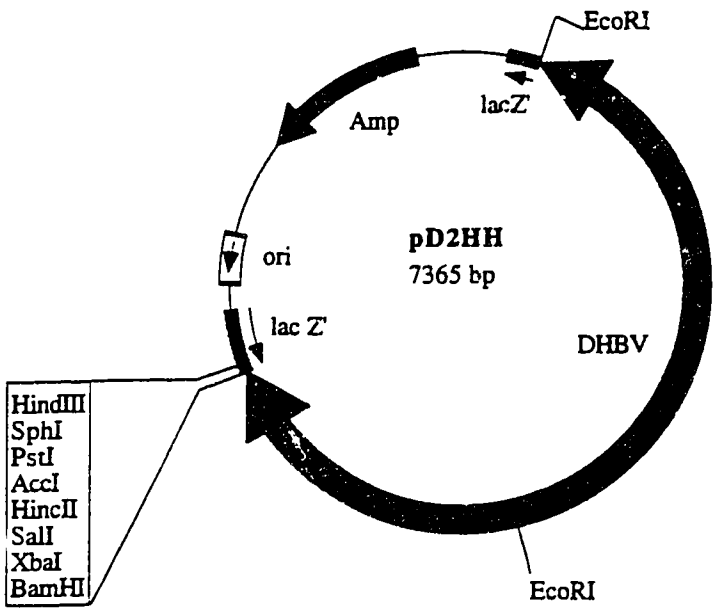
Recombinant plasmid constructs used for the study



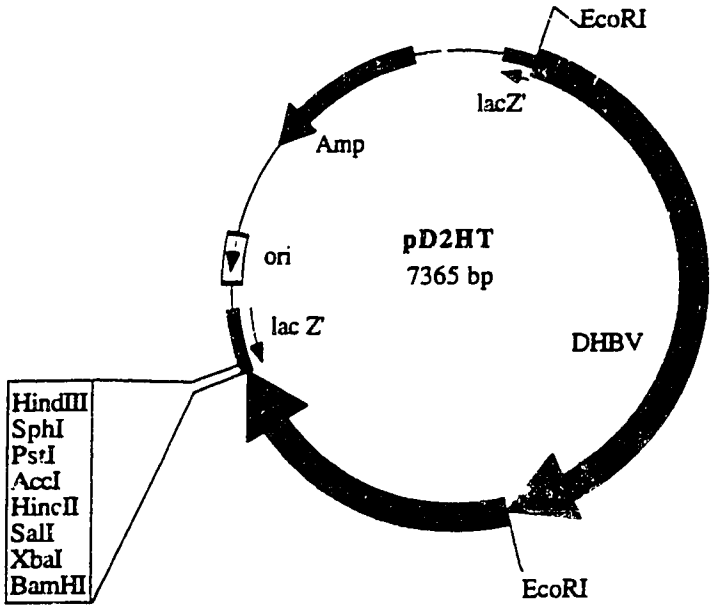
Plasmid name: pD2BamHI
Plasmid size: 4344 bp
Constructed by: A. Y. M. Howe
Construction date: 1993
Comments/References: pD2BamHI is a truncated derivative of pD2Eco11 in which the 3' fragment of the parental plasmid (from BamHI site) is removed. Tha plasmid contains DHBV-16 sequence from nt 1 to nt 1658 in a pUC19 vector. The plasmid is transformed into E.coli SURE.



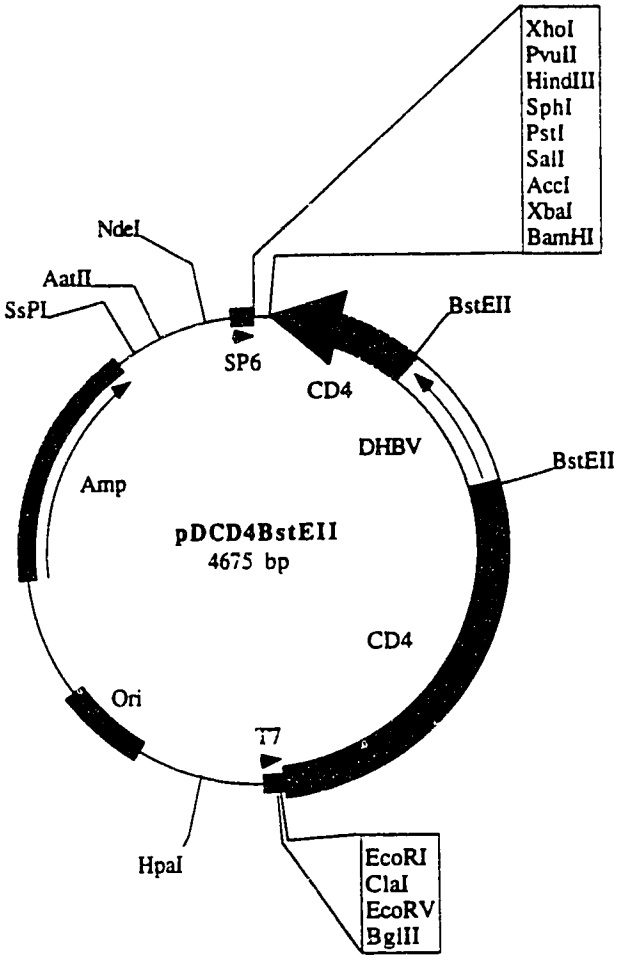
Plasmid name: pD2Eco11
Plasmid size: 5707 bp
Constructed by: K. Fischer
Construction date: 1985
Comments/References: pD2Eco11 contains the entire DHBV-16 genome in a pUC19 vector. The DHBV sequence is inserted at EcoRI site. The plasmid is transformed into E.coli JM109



Plasmid name: pD2HH
Plasmid size: 7365 bp
Constructed by: A. Y. M. Howe
Construction date: 1993
Comments/References: pD2HH is a derivative of pD2BamHI in which the DHBV sequence is inserted in a head to head manner . The plasmid contains DHBV-16 sequence from nt 3021 to nt 1/3021 to nt 1658 in a pUC19 vector and is transformed into E.coli SURE.



Plasmid name: pD2HT
Plasmid size: 7365 bp
Constructed by: A. Y. M. Howe
Construction date: 1993
Comments/References: pD2HT is a derivative of pD2BamHI in which the DHBV sequence is inserted in a tail to head manner. The plasmid encompasses DHBV-16 sequence from nt 1 to nt 3021/1 to nt 1658 in a pUC19 vector and is transformed into E.coli SURE.



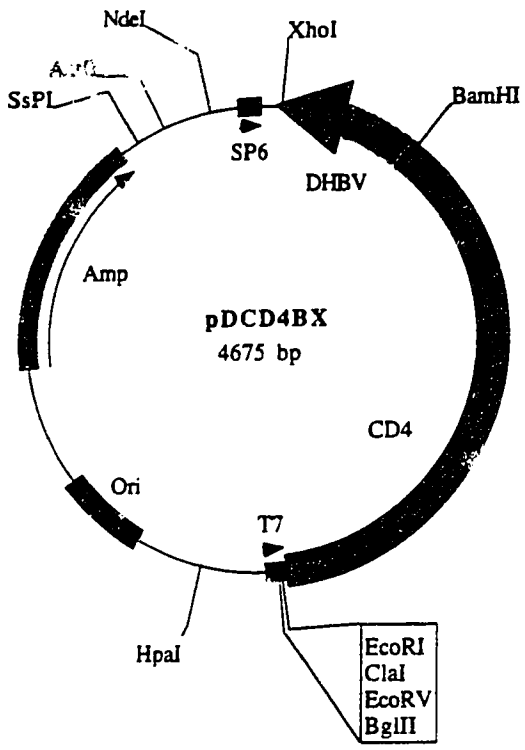
Plasmid name: pDCD4BstEII

Plasmid size: [illegible]

Constructed by: [illegible]

Construction date: [illegible]

Comments/References: pDCD4BstEII is a derivative of pSP72-T4 in which a DHBV sequence (nt 401 - nt 872) is inserted in-frame into the CD4 ORF at BstEII site. The cloning vector is pSP72. The plasmid is transformed into E.coli DH5-alpha.



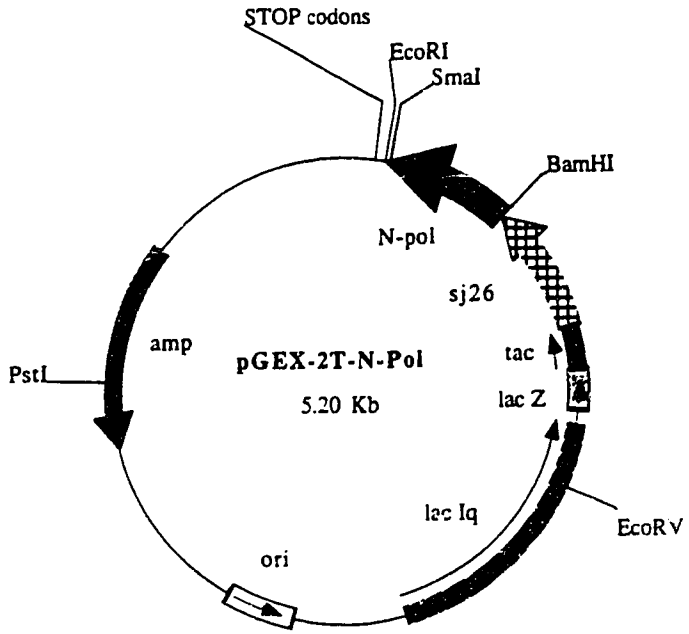
Plasmid name: pDCD4BX

Plasmid size: 4675 bp

Constructed by: A. Y. M. Howe

Construction date: 1994

Comments/References: pDCD4BX is a derivative of pSP72-T4 'is which a DHBV sequence (nt 401 - nt 872) is inserted at BamHI site, downstream from CD4 ORF. The cloning vector is pSP72. The plasmid is transformed into E.coli DH5-alpha.



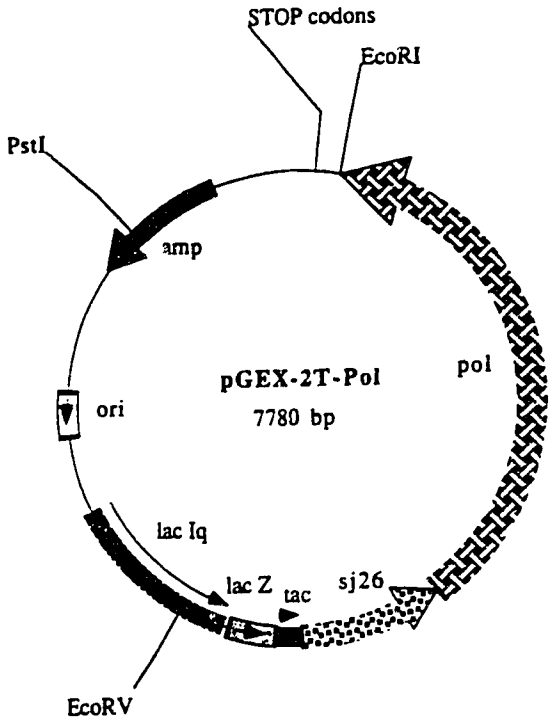
Plasmid name: pGEX-2T-N-Pol

Plasmid size: 5.20 kb

Constructed by: A. Y. M. Howe

Construction date: 1991

Comments/References: pGEX-2T-N-Pol contains N-terminal portion of pol ORF (nt 14 - nt 320) in a pGEX-2T vector. The pol ORF is fused in-frame to the GST ORF at the 3' terminus. The plasmid is transformed into E. coli DH5-alpha.



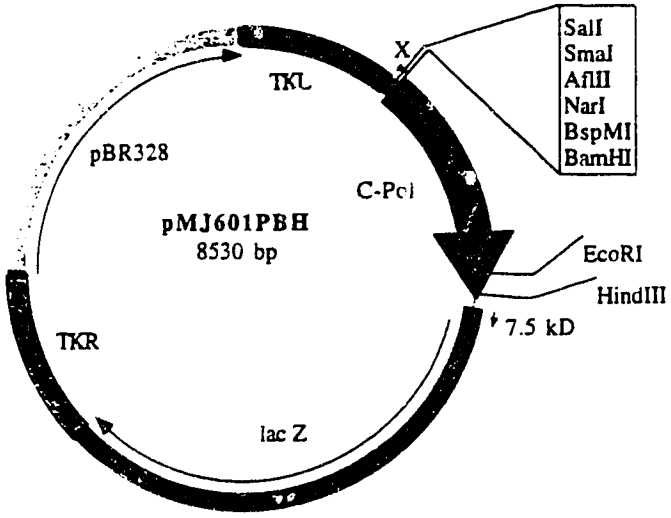
Plasmid name: pGEX-

Plasmid size: 7780 bp

Constructed by: A. Y. M. Howe

Construction date: 1991

Comments/References: pGEX-2T-Pol contains full length pol ORF (nt 14 - nt 3021) in a pGEX-2T vector. The pol ORF is fused in-frame downstream of the GST ORF. The SmaI and HincII sites of pGEX2T and DHBV, respectively, are destroyed. The plasmid is transformed into E.coli



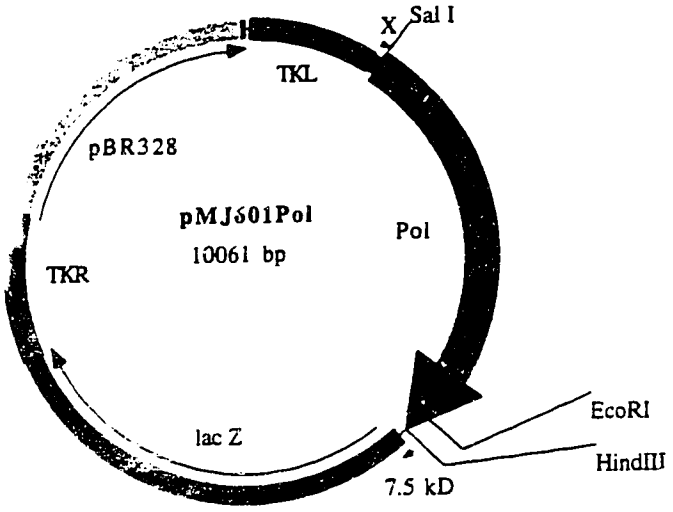
Plasmid name: pMJ601PBH

Plasmid size: 8530 bp

Constructed by: A. Y. M. Howe

Construction date: 1993

Comments/References: pMJ601PBH contians DHBV sequence nt 1658 to nt 14 (C-termianl half of pol ORF) in a pMJ601 vector. The DHBV sequence is flanked by the vaccinia thymidine kinase gene. The plasmid is transformed into E.coli DH5-alpha.



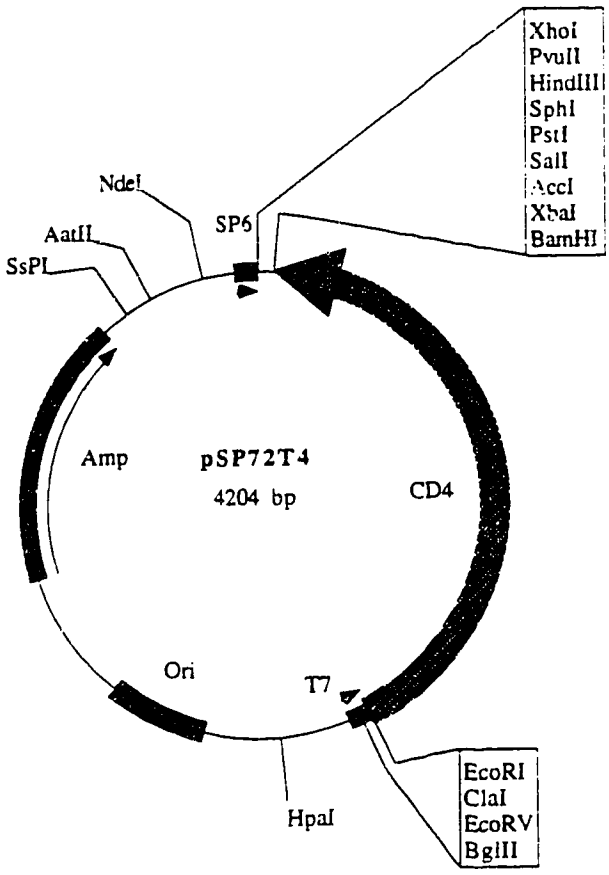
Plasmid name: pMJ601Pol

Plasmid size: 10061 bp

Constructed by: A. Y. M. Howe

Construction date: 1993

Comments/References: pMJ601Pol encompasses DHBV sequence nt 141 to nt 3021/1 to nt 14 (full length pol ORF) in a pMJ601 vector. The SmaI and HincII sites in pMJ601 and DHBV, respectively, are destroyed. The plasmid is transformed into E.coli DH5-alpha.



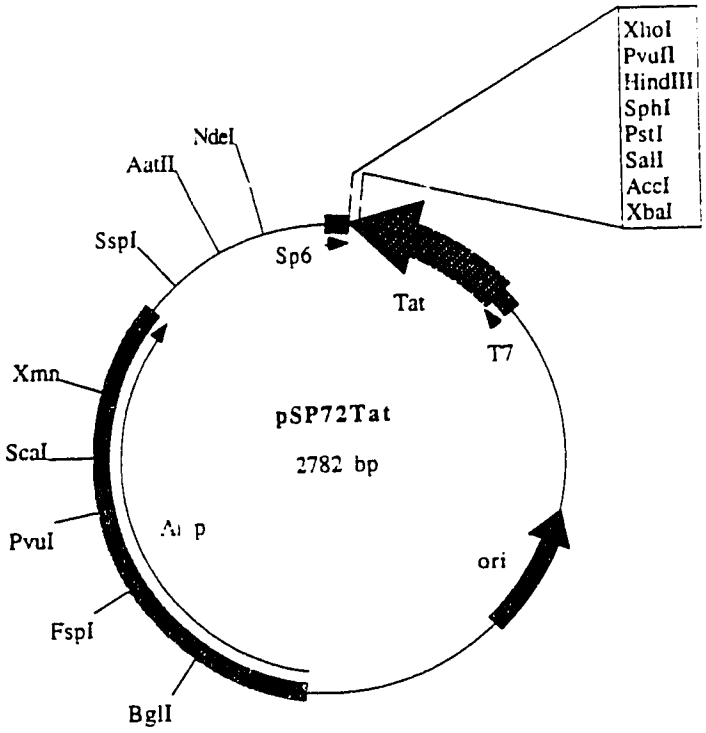
Plasmid name: pSP72T4

Plasmid size: 4204 bp

Constructed by: a gift from L.-J. Chang

Construction date: 1993

Comments/References: Human CD4 gene is cloned under the T7 promoter in a Sp72 vector. The cloning sites are 5' EcoRI and 3' BamHI. The plasmid is transformed into E.coli DH5-alpha.



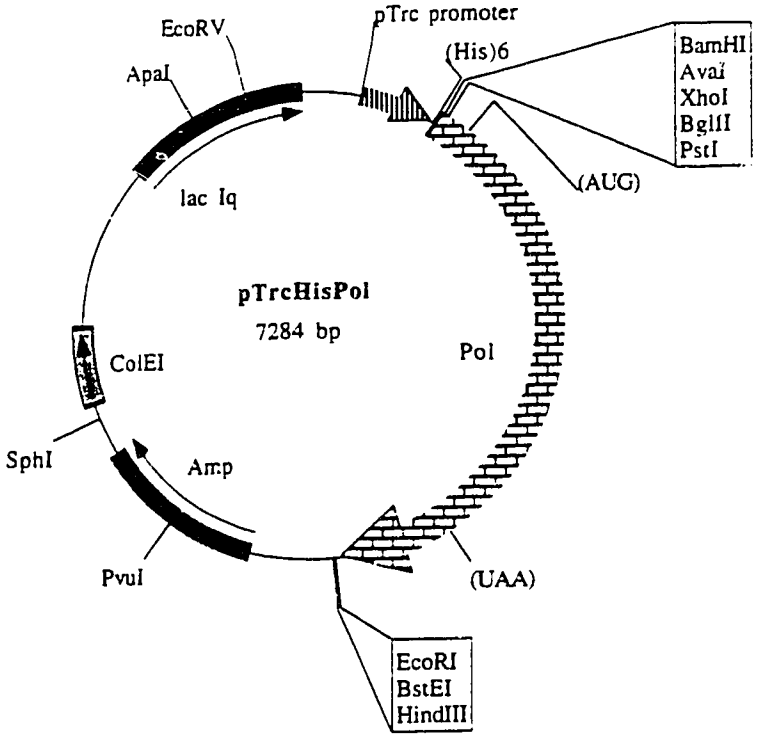
Plasmid name: pSP72Tat

Plasmid size: 2782 bp

Constructed by: L. -J. Chang

Construction date: 1992

Comments/References: pSP72Tat contains the HIV Tat ORF in a pSP72 vector. The Tat gene is obtained by PCR with primers incorporated with BamHI and BglII sites at the 5' and 3' ends, respectively. The PCR fragment is cloned into BglII and BamHI sites in a pSP72 vector.



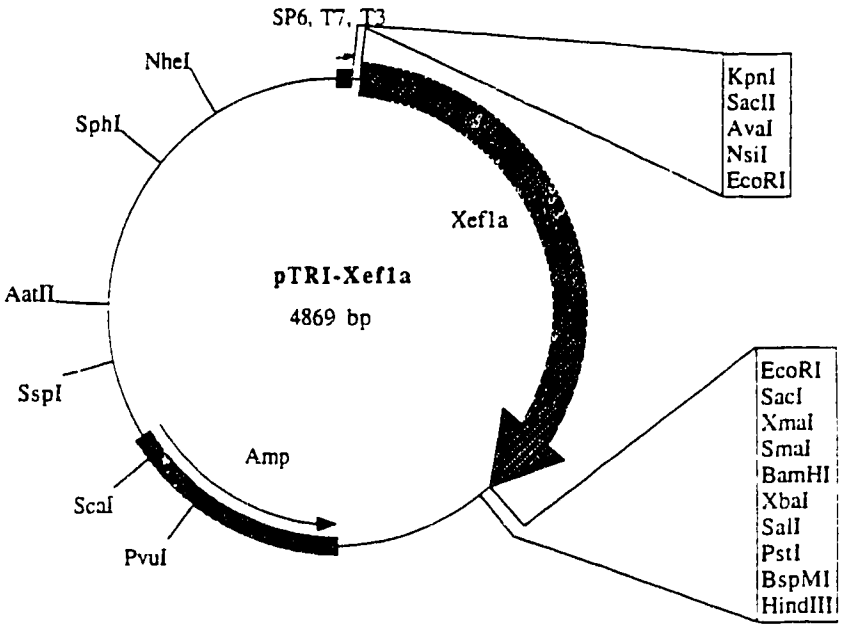
Plasmid name: pTrcHisPol

Plasmid size: 7284 bp

Constructed by: A. Y. M. Howe

Construction date: 1993

Comments/References: pTrcHisPol contains DHBV pol ORF in-frame downstream of sequence encoding 6 (His). The DHBV sequence is obtained by cutting pTZ19RPol at PstI and EcoRI sites and cloned into a pTrcHisC vector. The palsmid is tranformed into DH5-alpha.



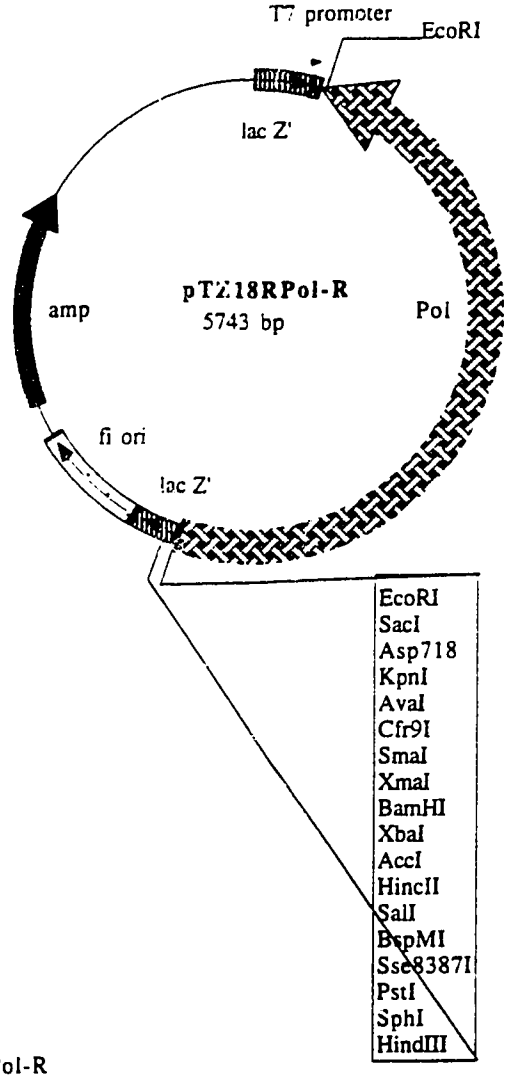
Plasmid name: pTRI-Xef1a

Plasmid size: 4869 bp

Constructed by: Ambion

Construction date:

Comments/References: pTRI-Xef 1a contains 1705 bp fragment of Xenopus elongation factor-1a chain with a poly(A) region at the 3' end. The Xef1 fragment is cloned into a pTRIPLEscript vector. The plasmid can be linearized by digesting with HindIII or XbaI.



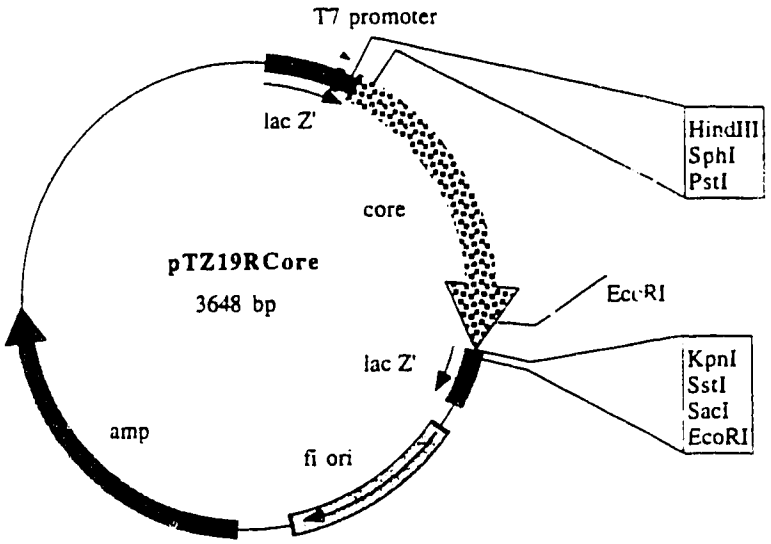
Plasmid name: pTZ18RPol-R

Plasmid size: 5743 bp

Constructed by: A. Y. M. Howe

Construction date: 1992

Comments/References: pTZ18RPol-R contains DHBV pol ORF (nt 1 - nt 3021) inserted at EcoRI site of pTZ19R vector. The pol ORF is in reverse orientation with respect to the T7 promoter. The plasmid is transformed into E.coli DH5-alpha



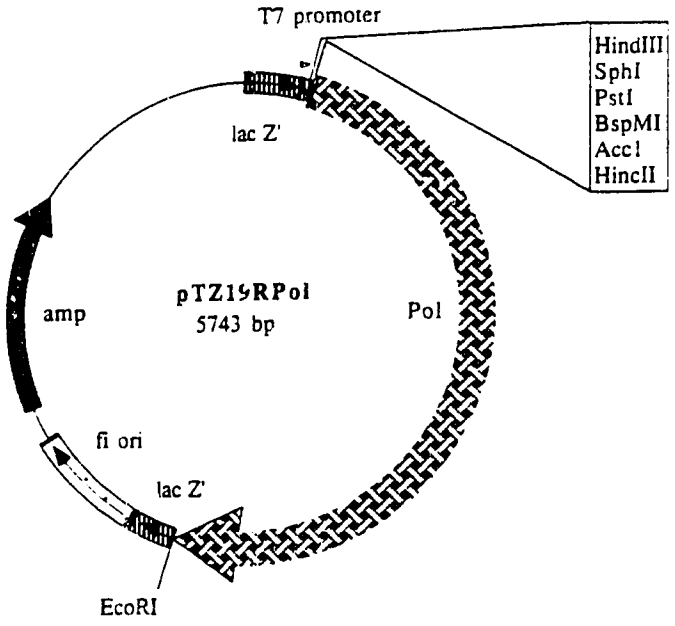
Plasmid name: pTZ19RCore

Plasmid size: 3648 bp

Constructed by: A. Y. M. Howe

Construction date: 1993

Comments/References: pTZ19Core contains the entire core ORF from nt 2630 to nt 960 in a pTZ19R vector. The core sequence is obatined by PCR and inserted at PstI and SmaI sites of pTZ19R. The SmaI site is destroyed. The plasmid is transformed into E. coli SURE.



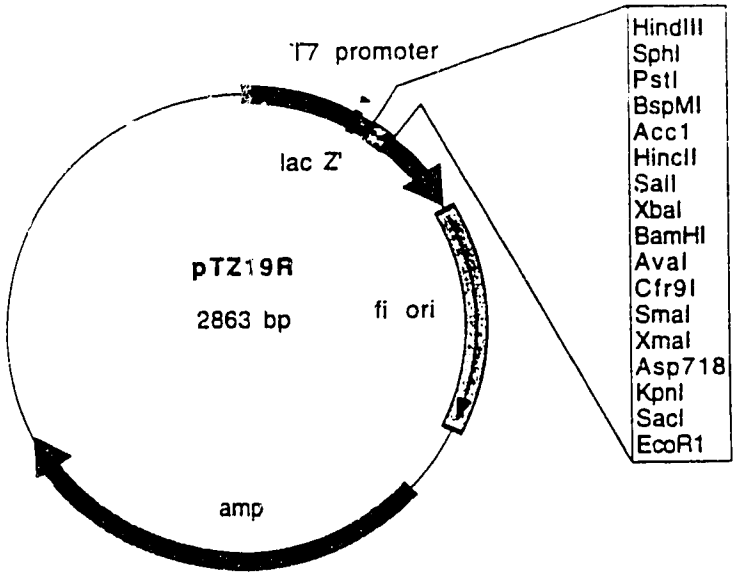
Plasmid name: pTZ19RPol

Plasmid size: 5743 bp

Constructed by: A. Y. M. Howe

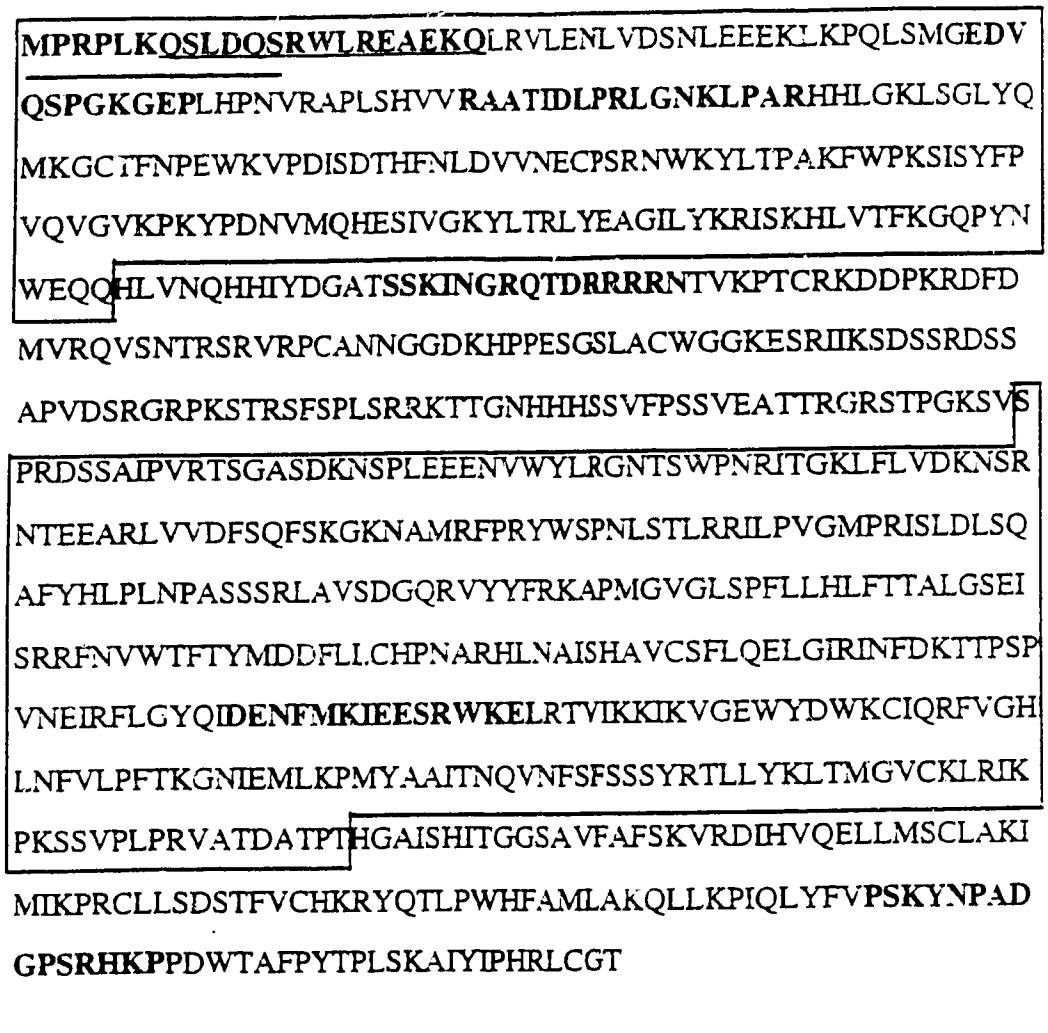
Construction date: 1992

Comments/References: pTZ19RPol contains DHBV pol ORF (nt 141 - nt 3021) inserted at HincII and EcoRI sites of pTZ19R vector. The plasmid is transformed into E.coli DH5-alpha


T7 promoter
lac Z'
pTZ19R
2863 bp
fi ori
amp
HindIII
SphI
PstI
BspMI
Acc1
HincII
SalI
XbaI
BamHI
AvaI
Cfr9I
SmaI
XmaI
Asp718
KpnI
SacI
EcoR1

Appendix IV

Synthetic peptides[1] used to raise polyclonal antibodies in rabbits.



[1] Synthetic peptides deduced from the nucleotide sequence of pol ORF are shown in bold. MPRPLKQSLDQS and QSLDQSRWLREAEKQ are two synthetic peptides as indicated by underlines.