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UNIVERSITY OF ALBERTA

CLONING, EXPRESSION AND FUNCTIONAL STUDY OF

HUMAN HEPADNAVIRAL POLYMERASE

by

ZHI LI

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A thesis submitted to the Faculty of Graduated Studies and Research in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

VIROLOGY

DEPARTMENT OF MEDICAL MICROBIOLOGY AND IMMUNOLOGY

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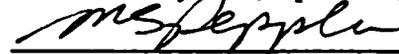
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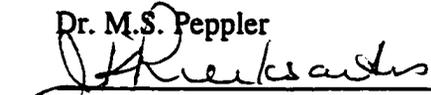
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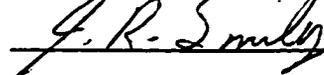
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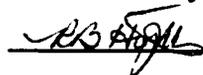
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ABSTRACT

Human hepatitis B virus (HBV), the prototype of the hepadnavirus family, is the most common cause in humans of acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma. Although an effective vaccine has been developed against HBV, more than 300 million chronic carriers worldwide cannot benefit from vaccination. Our laboratory is, therefore, engaged in the development of antiviral therapy. Since the pivotal role of viral polymerase in the viral replication cycle makes it an attractive target for antiviral agents, the work presented in this thesis was undertaken to investigate the molecular biology, synthesis and function of the viral polymerase.

HBV polymerase has been cloned and expressed in a rabbit reticulocyte lysate transcription-translation-coupled system. *In vitro* site-directed mutagenesis confirmed that the recombinant polymerase cDNA produces three translation products: a full-length (~94 kDa), a truncated N-terminal protein (~40 kDa) and an internally initiated protein (~80 kDa). The recombinant polymerase possessed protein priming activity, as demonstrated by ^{32}P-dGTP-labeling of the full size polymerase and the truncated N-terminal polymerase in an *in vitro* priming assay. The recombinant polymerase exhibited polymerization activity, as detected in an *in vitro* polymerase assay by incorporation of radionucleotides into acid-precipitable polynucleotides and by synthesis of HBV specific DNA in the range of 100–500 nucleotides in length. Both protein priming and polymerization activities of the recombinant polymerase were dependent on the RNA template bearing the HBV DR1 and epsilon stem-loop sequences.

Further studies showed that the recombinant HBV polymerase specifically inhibited the translation of HBV core mRNA in a rabbit reticulocyte lysate translation

system. In addition, we demonstrated a cross-species translational regulation in the hepadnaviridae family. Previous studies by Anita Howe in our laboratory showed that DHBV core mRNA translation was inhibited by DHBV polymerase. I have further shown that DHBV polymerase inhibited the translation of HBV and WHV core mRNAs. Similarly, HBV polymerase inhibited DHBV and WHV core mRNA translation. Such a cross-species suppression of hepadnaviral core mRNA translation by the viral polymerase strongly suggests that this function is conserved. This translational regulation may provide a mechanism by which pregenomic RNA (pgRNA) is designated to function as either mRNA or a genomic element.

Previous deletion studies by Anita Howe showed that the 3' periphery of DHBV core mRNA may be involved in the inhibitory effect of the polymerase. I have extended these studies utilizing an RNA folding computer program to analyze the 3' periphery of HBV, DHBV or WHV core mRNAs, referred to as the *trans* repression element (TR), and identified a conserved epsilon stem-loop-like structure within this region. It is worthwhile to note that hepadnaviral polymerase interacts with a 5' epsilon stem-loop structure (encapsidation signal) of pgRNA to initiate viral encapsidation. Other studies have indicated that an additional noncontiguous *cis*-element downstream from the encapsidation signal, which completely overlaps our proposed TR sequence, is also required for genomic encapsidation (Calvert et al. 1994; Hirsch et al. 1991). We hypothesized that hepadnaviral polymerase may interact with this conserved structure in the TR region for its translational inhibitory effect and for its involvement in viral encapsidation. The proposed role of the TR region was further examined by cloning the DHBV-TR sequence into a chloramphenicol acetyl transferase (CAT) expression plasmid downstream from CAT ORF. The *in vitro* translation as well as the CAT activity of the translational product of the chimeric mRNA was 90–95% reduced in the presence of the DHBV polymerase-containing lysate. The translational inhibition of the chimeric mRNA by the DHBV polymerase was dependent on the DHBV-TR sequence

since DHBV polymerase has no inhibitory effect on the translation of CAT mRNA containing no DHBV-TR sequence or CAT mRNA containing a backbone vector sequence. Silent mutation at the 3' end of the TR only slightly released the translational inhibitory effect of the polymerase while the mutation at the 5' end of the TR, which changed the predicted epsilon stem-loop like structure in this region, relieved about 80% of the translational inhibition of the polymerase. We conclude that the 5' region of the TR, containing the conserved epsilon stem-loop-like structure, is the primary element required for the translational inhibitory effect of the polymerase. By inhibiting pgRNA translation, the polymerase liberates the pgRNA from the translational machinery for encapsidation.

To my family and my teachers

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ABBREVIATIONS AND DEFINITIONS

ALT	alanine aminotransferase
ATP	adenosine-5'-triphosphate
bp	base pair
CAT	chloramphenicol acetyltransferase
cccDNA	covalently closed circular DNA
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
C-terminus	carboxyl-terminus
CTL	cytotoxic T lymphocyte
dCTP	2'-deoxycytidine-5'-triphosphate
ddDAPR	2,6-diaminopurine 2',3'-dideoxyriboside
ddH$_2$O	distilled deionized water
DDT	dithiothreitol
DEPC	diethyl pyrocarbonate
dGMP	2'-deoxyguanosine-5'-monophosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
DHBV	duck hepatitis B virus
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxyribonucleoside-5'-triphosphate
DR	direct repeat
dsDNA	double-stranded DNA

E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra acetic acid
EM	electron microscopy
ER	endoplasmic reticulum
Enh	enhancer
gp	glycoprotein
GSHV	ground squirrel hepatitis virus
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCl	hydrochloric acid
HHBV	heron hepatitis B virus
HNF	hepatocyte nuclear factor
hr	hours
hsp90	heat shock protein 90
IFN	interferon
IL	interleukin
kb	kilobase
kDa	kilodalton
kV	kilovolt
LacZ	β-galactosidase gene
LMP	low melting point

M	molar; number of moles per liter of solution
mAb	monoclonal antibody
MgCl$_2$	magnesium chloride
MHC	major histocompatibility complex
min	minute
ml	milliliter
µl	microliter
mol	mole
mRNA	messenger RNA
N-terminus	amino-terminus
NaCl	sodium chloride
ng	nanogram
nm	nanometer
nt	nucleotide
NTP	ribonucleoside-5'-triphosphate
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Pfu	Pyrococcus furiosis DNA polymerase
pgRNA	pregenomic RNA
Pol/RT	DNA polymerase/reverse transcriptase
poly A	polyadenylation signal
PRE	post-transcriptional regulatory element
RNA	ribonucleic acid

RNase	ribonuclease
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
sec	second
Sf9	Spodoptera frugiperfa cell line used in baculovirus expression system
Taq	Thermus aquaticus DNA polymerase
TBE	Tris/borate/EDTA buffer
3TC	(-) 2',3'-dideoxy-3'-thiacytidine
3TC-TP	(-) 2',3'-dideoxy-3'-thiacytidine-5'-triphosphate
TCA	trichloroacetic acid
TE	Tris/EDTA buffer
TES	Tris/EDTA/SDS buffer
Th	T helper lymphocyte
TLC	thin-layer chromatography
Tn	Trichiplusia ni cell line used in baculovirus expression system
TNF	tumor necrosis factor
TP	terminal protein domain of hepadnaviral polymerase
TR	*trans*-repression
Ty	yeast retrotransposon
UTP	uridine-5'-triphosphate
UTR	untranslated region
vol	volume
WBC	white blood cell

WHV   woodchuck hepatitis virus

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

I.   Molecular Biology of Hepatitis B Virus

A.   Introduction

Human hepatitis B virus (HBV) is the prototype member of a family of DNA viruses that primarily infect the liver and share a similar viral morphology and cellular life cycle (Gust et al. 1986; Robinson et al. 1982). HBV is the most common cause in humans of acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma. Throughout the last three decades, we have made tremendous gains in understanding this virus, answering questions ranging from molecular biology to public health issues (Ganem 1996; Nassal and Schaller 1993; Raney and McLachlan 1991). These advances have been possible because of the advent of molecular biological techniques and discovery of animal models of the viral infection (Fujiyama et al. 1983; Galibert et al. 1979; Marion 1988; Marion et al. 1980; Mason et al. 1980; Pasek et al. 1978; Summers et al. 1978; Valenzuela et al. 1980). In 1967, Blumberg discovered the Australia antigen (now termed HBsAg) and Prince revealed its association with hepatitis B (Blumberg et al. 1967; Prince et al. 1968). HBsAg was prepared in quantity and constitutes the immunogen in highly effective vaccines used for the prevention of HBV infection (Andre 1989; Hilleman 1987; Hollinger 1987). Although an effective vaccine has been developed, antiviral drug therapy is still required for the more than 300 million chronic carriers worldwide who cannot benefit from vaccination.

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Further advances in our understanding of HBV molecular biology are essential not only for efficient screening of antiviral compounds but also for rational design of antiviral inhibitors.

B. Organization of hepadnaviridae

1. Hepadnavirus family

HBV belongs to a distinctive family of animal viruses known as hepadnaviruses (hepatotropic DNA viruses). The other members of this group are 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 less well characterized heron hepatitis B virus (HHBV) (Sprengel et al. 1988). All these viruses share a common viral morphology and a characteristic replication mechanism (Ganem 1996). The major hepadnaviruses with their biological properties are summarized in Table 1-1. The mammalian viruses are more closely related to one another than to the avian viruses (Mandart et al. 1984). For instance, mammalian hepadnaviruses encode three envelope proteins (large, middle and small) and contain the X gene whereas avian viruses encode only two envelope proteins (large and small) and do not have the X gene (Pugh et al. 1987; Schlicht et al. 1987). Only mammalian viruses cause chronic active hepatitis and their hosts may develop hepatocellular carcinoma (HCC) (Marion et al. 1986; Popper et al. 1987; Szmuness 1978).

2. Morphological structure

During HBV infection, three distinct morphological forms of virus particles

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Table 1-1 A Comparison of the biological properties of the hepadnaviral family[a]

	HBV[b]	WHV[c]	GSHV[d]	DHBV[e]
Genome (kb)	3.2	3.3	3.3	3.0
ORFs[f]	S,C,P,X	S,C,P,X	S,C,P,X	S,C,P
Hosts	Humans	Wood-chucks	Ground-squirrels	Ducks
	Chimps			Geese
Replication sites	Liver	Liver	Liver	Liver
	Kidney	Kidney	Kidney	Kidney
	Pancreas	Pancreas	Pancreas	Pancreas
	WBC[g]	WBC		Spleen
Diseases	ACS[h]	ACS	ACS	ACS
	Hepatitis	Hepatitis	Hepatitis	Hepatitis
	Cirrhosis			
	HCC[i]	HCC	HCC	

[a] Data are obtained from Ganem 1996.

[b] Hepatitis B virus.

[c] Woodchuck hepatitis virus.

[d] Ground squirrel hepatitis virus.

[e] Duck hepatitis B virus.

[f] Open reading frames.

[g] White blood cell.

[h] Asymptomatic carrier state.

[i] Hepatocellular carcinoma.

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can be identified in the blood by electron microscopy analysis: (1) 42-nm diameter double-shelled particles, termed "Dane particles" representing the intact virion (Figure 1-1, Dane et al. 1970); (2) 22-nm diameter spherical empty envelope particles usually occurring in 10^3 to 10^6-fold excess over virions and (3) 22-nm diameter filamentous empty envelope particles of variable lengths occurring in smaller quantities (Bayer et al. 1968). All three particles have an envelope composed of the large (L), middle (M) and small (S) hepatitis B surface glycoproteins embedded in host-derived lipids (Aggerbeck and Peterson 1985; Gavilanes et al. 1982). However, the distribution of the three envelope glycoproteins differs among the viral particles (Heermann et al. 1984). The subviral particles are composed predominantly of S protein, whereas the Dane particles are substantially enriched for L protein. As L proteins are thought to carry the receptor recognition domain (Klingmüller and Schaller 1993; Pontisso et al. 1989), this distribution may enhance the infectivity, assembly and export of the virion (Bruns et al. 1998).

The Dane particle consists of an outer lipoprotein envelope and an inner electron-dense icosahedral nucleocapsid or core (Dane et al. 1970; Crowther et al. 1994, Kenny et al. 1995). The capsid is composed of the capsid structural phosphoproteins of hepatitis B core proteins (HBcAg) (Petit and Pillot 1985), a 3.2-kb partially double-stranded circular viral DNA molecule (Robinson et al. 1974) and a virus-encoded DNA polymerase/reverse transcriptase (P or Pol) covalently attached to the 5' end of the minus-strand DNA (Figure 1-1) (Gerlich and Robinson 1980; Landers et al. 1977; Robinson and Greenman 1974).

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Figure 1-1 Schematic cross section of a complete HBV virion (Dane particle). The outer envelope of the virion contains the L (large), M (middle) and S (small) surface glycoproteins that are embedded in the host-derived lipid bilayer. The inner nucleocapsid or core contains 240 identical subunits of structural core protein assembled in an icosahedral symmetry and the viral genome, a circular and partially double-stranded DNA. The viral DNA polymerase (shaded oval) is covalently attached to the 5' end of the minus (–) strand DNA via the terminal protein (shaded small circle) of the polymerase and a short RNA primer (dashed line) is covalently linked to the 5' end of the plus (+) strand DNA. (Modified from Nassal 1997).

Core protein

DNA genome

DNA polymerase

Nucleocapsid

Lipid bilayer

Envelope

L

S

M

5'

5'

−

+

6
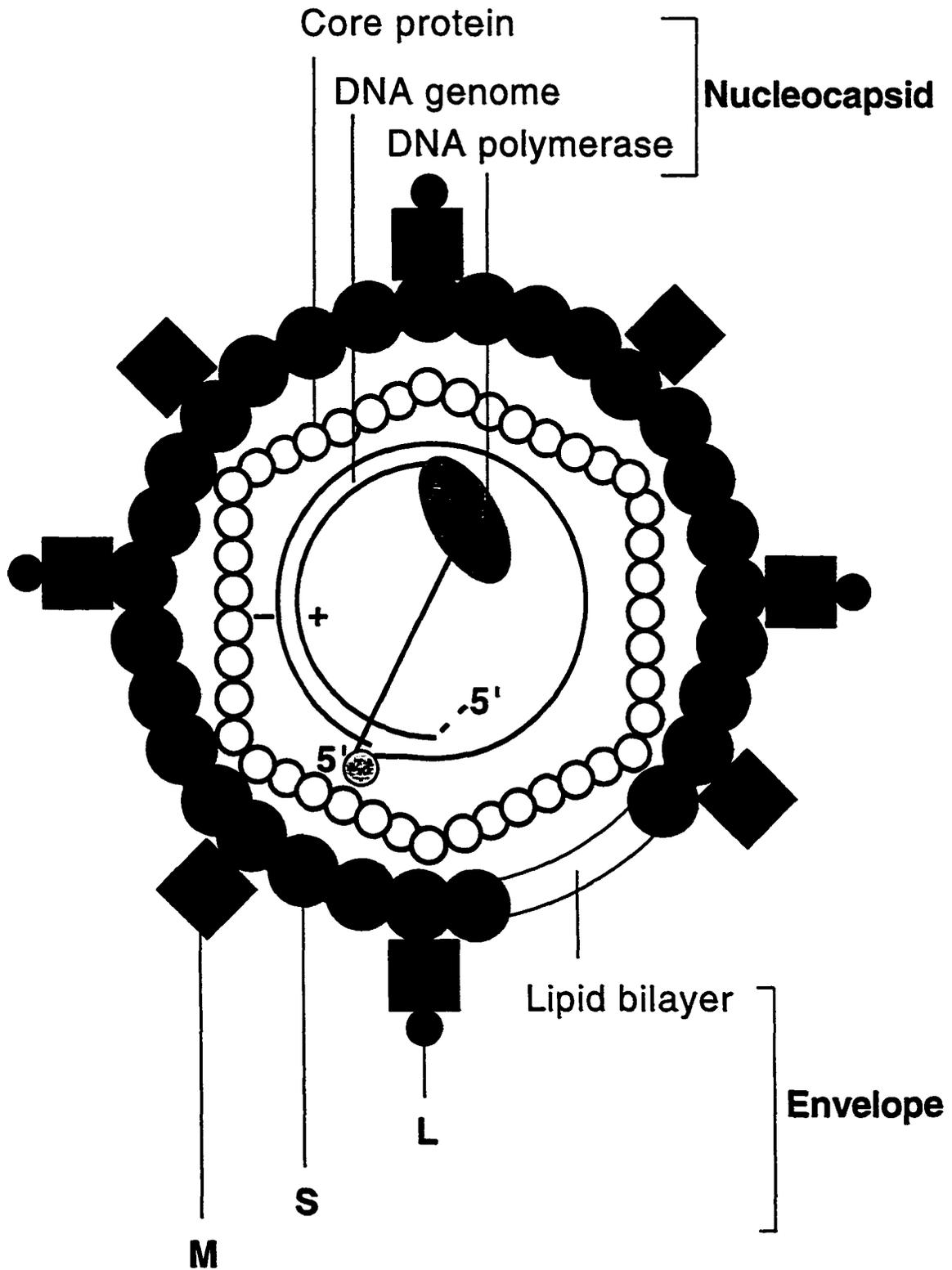
The 22-nm spheres and filaments are subviral envelope particles, composed mainly of small envelope proteins, some middle envelope proteins but with few or no large envelope proteins (Bayer et al. 1968; Ganem 1996). These particles lack viral nucleic acid and viral polymerase, therefore are noninfectious. However, these particles are highly immunogenic and efficiently induce a neutralizing anti-HBs-antibody response (Szmuness et al. 1980; Valenzuela et al. 1982). During a natural infection, these particles are always present in larger abundance than infectious virions. The reasons for the excess production of these particles are still uncertain, but it has been speculated that the excess HBsAg particles may function as decoys to trap neutralizing antibodies and so enhance viral infection (Bruns et al. 1998).

3.   Genome organization

Hepadnaviruses have among the smallest genomes of all known animal DNA viruses (Ganem and Varmus 1987). Human hepatitis B virion DNA is a relaxed circular, partial duplex of approximately 3.2 kb in length with neither strand covalently linked. The circularity is maintained by the overlapping cohesive 5' ends of the minus and plus strands (Figure 1-2) (Sattler and Robinson 1979; Seeger et al. 1986; Siddiqui et al. 1979). This molecule has an unusual structure in that its two DNA strands are not perfectly symmetric. The minus strand is complete and has the terminal protein of the viral polymerase, as a primer, linked to its 5' end (Gerlich and Robinson 1980; Molnar-Kimber et al. 1983). The plus strand within the circulating virion is an incomplete strand (Seeger et al. 1991), having a variable 3' end and a fixed position at the 5' end bearing a short (approximately 18 nucleotides), capped oligoribonucleotide as a primer (Lien et al. 1986). The 5' ends of minus- and plus-strand DNAs map to the region of

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short (12 nucleotides) direct repeats, DR1 and DR2, respectively (Figure 1-2) (Molnar-Kimber et al. 1984; Lien et al. 1986; Seeger et al. 1986; Will et al. 1987).

The hepadnavirus genome is extremely compact and efficiently organized (Miller et al. 1989). All nucleotides have coding functions and much of the genome has multiple functions (Figure 1-2) (Galibert et al. 1979; Valenzuela et al. 1980). The genomes of the mammalian viruses have four overlapping open-reading frames (ORFs) in the minus-strand DNA (Figure 1-2). By utilizing three in-frame initiation codons (Figure 1-2), the envelope gene (S ORFs) of preS1/preS2/S regions encodes the L, M and S surface glycoproteins, respectively (Heermann et al. 1984; Valenzuela et al. 1979). These proteins have a common S domain at their C-termini. Similarly, by utilizing two in-frame initiation codons (Figure 1-2), the preC/C gene (C ORFs) expresses the core-related serum protein known as HBeAg and the nucleocapsid structural protein (core protein) (Ou et al. 1986; Pasek et al. 1979). The P gene (P ORF), which covers three-fourths of the genome and overlaps with the three of the other genes, codes the viral DNA polymerase which is the enzyme responsible for genome replication (Bavand et al. 1989; Bosch et al. 1988; Mack et al. 1988; Toh et al. 1983). The X gene (X ORF), present exclusively in the mammalian viruses, encodes a regulatory product that transactivates the expression of numerous viral and cellular genes (Aufiero and Schneider 1990; Spandau and Lee 1988; Twu et al. 1989; Twu and Robinson 1989; Twu and Schloemer 1987; Sprengel et al. 1985).

Several functionally important elements have been identified in hepadnaviral genomes (Figure 1-2). Four promoters have been defined. The core/genomic

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Figure 1-2 Structure and organization of HBV genome. The narrow lines in the center of the diagram represent the partially double-stranded circular virion DNA genome. (+) and (–) denote the plus- and minus-strand DNA. The 3' end of the plus-strand DNA is variable as shown by the dotted line. The closed small circle and the dashed line, at 5' ends of the (–) and (+) DNA, indicate the terminal protein of the polymerase and the short capped oligoribonucleotide primer, respectively. The wide lines with arrows surrounding the genome represent the four genes (C, P, S, X). The preS1, preS2 and S are the three in frame coding regions of the viral surface protein gene. The preC and C are the two in frame coding regions of the C gene. Other functionally important elements: the direct repeat sequences (DR1 and DR2), the enhancer 1 and 2 (Enh1 and Enh2), the four promoters (Cp, preS1p, preS2p, Xp), the polyadenylation signal (poly A), and the packaging signal (U5-like domain) are indicated. (Modified from Lau and Wright 1993 and Nassal and Schaller 1993).

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preS1p

preS2p

preS1

preS2

preS1p

P

S

(-)

(+)

HBV
3.2 kb

5'

C

Poly A

U5

DR1

DR2

5'

preC

Cp Enh2

X

Xp Enh1

1 0
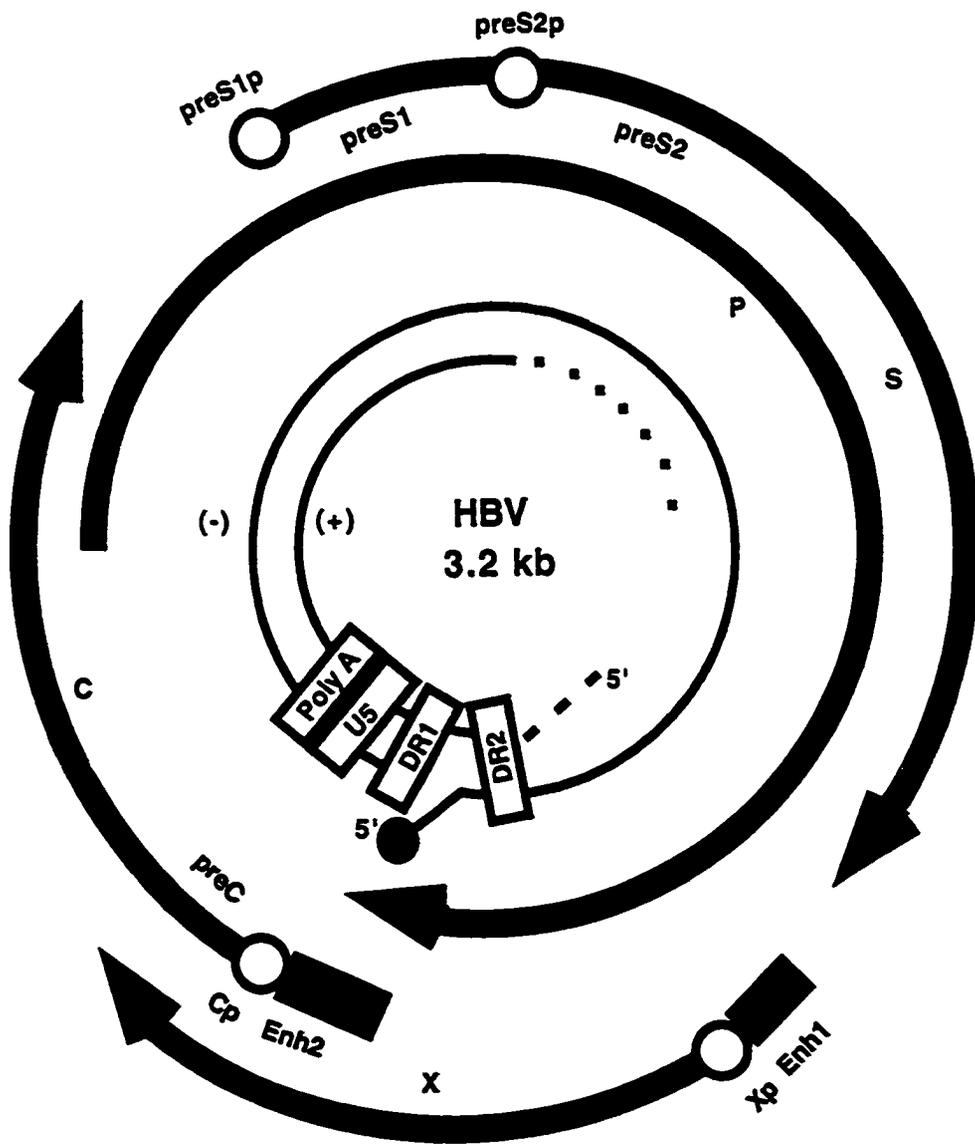
promoter drives the expression of preC and C genomic RNAs; the preS1 promoter drives the expression of L surface protein mRNA; the preS2 promoter drives the expression of M and S surface protein mRNAs and the X promoter drives the expression of X protein mRNA (Schaller and Fischer 1991; Shaul 1991; Yen 1993). Two enhancer elements (Enh1 and Enh2), located upstream of or overlapping with the X and C promoters, respectively, are tissue specific and function efficiently in hepatocytes (Shaul et al. 1985; Wang et al. 1990; Yee 1989; Yuh and Ting 1990). Although Enh1 exhibits liver-specific properties, it has been observed that it is also active in nonhepatocytes (Shaul 1991; Yen 1993). Overall, the regulation of HBV gene expression is governed by both Enh1 and Enh2 acting cooperatively (Su and Yee 1992). However, there is considerable evidence suggesting that Enh2 plays a greater role in regulating the activities of C promoter and preS2 promoter (Zhou and Yen 1990). A polyadenylation signal (TATAAA) for all the major viral transcripts is located within the beginning of the core coding region (Russnak and Ganem 1990). Other elements are DR1 and DR2, involved in the initiation of minus- and plus-strand DNA synthesis, located within the overlapping cohesive region of the two DNA strands (Molnar-Kimber et al. 1984; Seeger et al. 1986), and the U5-like domain of the viral encapsidation signal located in the preC and C coding regions.

C. Hepatitis B virus replication cycle

Much more is known about the later stages of the hepadnavirus replication cycle than about the early events. The overall virus replication cycle is presented schematically in Figure 1-3. The production of the asymmetric genome by reverse

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Figure 1-3 HBV replication cycle. The parental virus attaches to a specific receptor on the surface of a susceptible cell (step 1) which leads to fusion and entry of the capsid (step 2). The capsid is transported to the nuclear membrane (step 3) and the viral genome is released into the nucleus (step 4). The partially double-stranded genome is converted into cccDNA which serves as a template for transcription (step 5). The cccDNA is transcribed by cellular RNA polymerase II (step 6). The viral transcripts are transported to the cytoplasm where the viral messengers are translated (step 7). The shortest genomic RNA, pregenomic RNA (pgRNA), is selectively encapsidated into the immature nucleocapsid through the interaction of the pgRNA with its translation products: core protein and polymerase (step 8). Within the nucleocapsid, pgRNA is reverse transcribed to minus strand DNA (step 9) which serves as template for plus strand DNA synthesis (step 10). The mature nucleocapsid buds into the endoplasmic reticulum, acquires viral envelope proteins, and finally the progeny virus is released (step 11). The progeny virion initiates another round of the replication cycle in a another uninfected cell (step 12). The mature nucleocapsid can also be transported to the nucleus for amplification of cccDNA (step 13). (Modified from Nassal 1997).

13
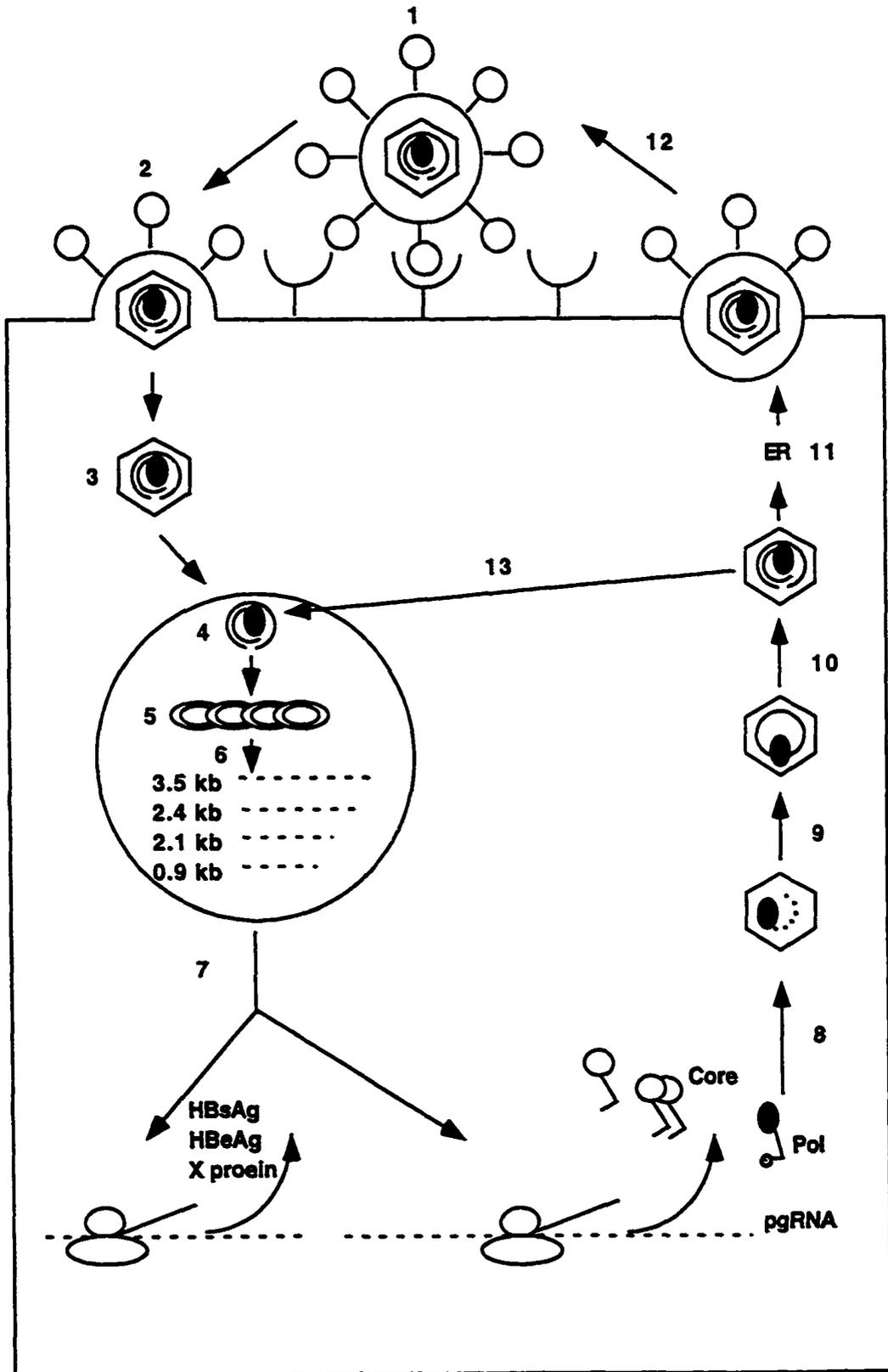
transcription of an RNA intermediate is fascinating and complicated (Summers and Mason 1982). It is therefore helpful to begin with a basic view of the viral life cycle before discussing each step of the cycle in detail. HBV replication is initiated by attachment of the virion to a susceptible host cell, possibly via cellular receptor(s) (Klingmüller and Schaller 1993; Pugh et al. 1995; Tong et al. 1999; Urban et al. 1998), followed by the release of the nucleocapsid into the cytoplasm by membrane fusion and endocytosis (Köck et al. 1996; Offensperger et al. 1991). The capsid is then transported to the nuclear membrane to deliver the encapsidated viral genome into the nucleus, where the viral DNA is converted into covalently closed circular molecules (cccDNA) in an extrachromosomal form (Köck and Schilicht 1993). The cccDNA is transcribed by host RNA polymerase II to give rise to genomic RNA transcripts with terminally redundant sequences and subgenomic RNA transcripts that function as messenger RNAs. Shorter genomic RNA or pregenomic RNA (pgRNA) is selectively encapsidated with viral DNA polymerase into viral core particles in the cytoplasm (Enders et al. 1987; Summers and Robinson 1982). Within these immature viral core particles, viral DNA synthesis is initiated by reverse transcription and concomitant degradation of the pgRNA to form the minus strand DNA, followed by plus strand DNA synthesis on the *de novo* synthesized minus strand DNA template. Progeny cores bud into the endoplasmic reticulum (ER) to acquire their envelopes (Kamimuna et al. 1981; Roingeard et al. 1990) or may be reimported to the nucleus for amplification of cccDNA. The enveloped virions are finally exported from the cell via the constitutive pathway of vesicular transport (Kelly 1982), ready for a new round of infection.

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1. *Virus attachment and entry*

The earliest molecular events that lead to viral entry into hepatocytes are poorly understood. It is generally believed that hepadnavirus interacts with species specific cell-surface protein(s) followed by receptor-mediated endocytosis of virions into hepatocytes. Recently, several lines of evidence have indicated that preS proteins participate in cellular receptor binding (Ishikawa and Ganem 1995; Klingmüller and Schaller 1993; Neurath et al. 1986; Petit et al. 1992; Pontisso et al. 1989). In particular, amino acid residues 80–104 of the DHBV preS domain and residues 21–47 of the HBV preS1 domain are critical for the host interaction (Dash et al. 1992; Li et al. 1996; Neurath et al. 1986; Sunyach et al. 1999). A mutation in the DHBV virion within the preS domain inhibited the DHBV virus binding to the primary hepatocyte and abrogated its infectivity. The binding of HBV virions to human liver cell and human HepG2 cell plasma membranes is blocked by monoclonal antibodies (mAbs) to the HBV preS1 domain (Sureau et al. 1992; Petit et al. 1991). Other evidence that preS protein plays a pivotal role in viral entry into the hepatocyte comes from genetic studies of viral host range determinants. Replacement of the preS1 domain of HHBV with the DHBV counterpart allows this chimeric HHBV to infect duck hepatocytes (Ishikawa and Ganem 1995). Other investigators suggest that the S surface protein contains the putative binding sites for the hepatocyte receptor(s). Binding of the radiolabeled S surface protein to hepatocytes and HepG2 cells has been demonstrated (Hertogs et al. 1993; Leenders et al. 1990). Upon binding, fusion between the viral envelope and cellular membrane followed by endocytosis is required for entry of the virus (Köck et al. 1996; Offensperger et al. 1991) and the proteolysis-dependent exposure of a fusion domain within the envelope protein of HBV has been reported (Lu et al. 1996).

The cellular receptor molecule(s) have not yet been identified. A handful of proteins have been reported to bind with high affinity to hepadnaviral envelope

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glycoproteins or to peptides arising from them. However, for none of them is there convincing evidence for a role in the infection. Two groups of investigators have identified a hepatocyte surface glycoprotein, gp170/180, that binds specifically to the preS domain of the DHBV envelope protein (Ishikawa et al. 1994; Kuroki et al. 1994; Tong et al. 1995). Subsequent cDNA cloning revealed that the gp170/180 is a carboxypeptidase (Kuroki et al. 1995), however, deletion studies indicated that the carboxypeptidase activity is not required for the preS binding (Eng et al. 1998). The interaction of the gp170/180 with the preS region of the large surface protein is blocked by DHBV virions or by neutralizing mAbs to the preS domain. Non-DHBV infectible cell lines, by the expression of gp170/180 in the cell, were able to bind and internalize DHBV virus. However, no viral replication was demonstrated (Breiner et al. 1998; Tong et al. 1999). Moreover, the direct role of the gp170/180 as the primary DHBV receptor was tested by the ability of truncation mutants of preS proteins to compete with virions for interaction with gp170/180 (Breiner et al. 1998; Urban et al. 1999). Another preS binding protein with a size of 120 kDa (p120) was also identified in DHBV infectible tissues such as liver, kidney and pancreas (Li et al. 1996). Other investigators have reported that proteins with molecular masses of 35 and 50 kDa, extracted from HepG2 cells, can specifically bind with recombinant preS1 peptides (a.a. 21–47) (Petit et al. 1992). Other cellular proteins have also been reported to interact with the small envelope proteins. Human hepatocyte membranes, but not HepG2 membranes, appear to bind S surface protein via membrane bound endonexin II which may be involved in intravesicle transport (Hertogs et al. 1993). Other nonhepatocyte membrane proteins, for instance, polymerized human serum albumin (Machida et al. 1984; Michel et al. 1984), 50 kDa human serum factor (Budkowsha et al. 1993) and serum apolipoprotein H (Mehdi et al. 1994) also have been shown to bind viral envelope proteins, which has introduced a concept of "intermediate carrier"

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for facilitating delivery of virion from the periphery of circulation system to the hepatocyte or for facilitating attachment and entry of virion. In general, it seems that the viral entry may be mediated via a multiple step process involving various cellular factors.

2. *Generation of cccDNA*

The cellular events following viral entry are even less well understood (Qiao et al. 1994). However, the viral genome has to be delivered to the nucleus, where the viral DNA is converted to cccDNA to serve as the template for viral transcription. It has been shown that the nucleocapsid cannot be transported across the intact nuclear membranes, since the size of the nucleocapsid exceeds the size of the nuclear pores (Crowther et al. 1994; Feldherr et al. 1984; Guidotti et al. 1994). This suggests that the nucleocapsid needs to disassemble in the cytosol, perhaps near the nuclear pore (Bock et al. 1996; Kann et al. 1997), similar to the disassociation of capsids in adenovirus infection (Phillipson et al. 1968). Despite the size of the nucleocapsids relative to that of the nuclear pores, it has been reported that transportation of the nucleocapsid into the nucleus may be mediated by the core protein which is known to contain nuclear localization signals in its C-terminus (Eckhardt et al. 1991; Ou et al. 1989; Yeh et al. 1990). The nuclear localization of C- protein seems to be ATP-dependent and regulated by a phosphorylation (Hild et al. 1998; Kann et al. 1999; Liao and Ou 1995; Machida et al. 1991).

In the nucleus, the partially double-stranded viral genome is converted into cccDNA. The production of the cccDNA involves a series of steps which include: (1)

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Figure 1-4 Schematic diagram of the genomic and subgenomic transcripts of hepatitis B virus. The viral functional transcripts of 3.5, 2.4, 2.1 and 0.9 kb in length are indicated by wavy lines and labeled accordingly. The poly A tail (AAA) is located at the 3' end of each. The arrows depict the HBV genes labeled as P, C, S and X for polymerase, core/precore proteins, surface proteins and X protein, respectively. The open circles represent the promoter elements (Cp, preS1p, preS2p, Xp). The enhancer elements Enh1 and Enh2 are shown as solid rectangles and labeled accordingly. The open rectangles, labeled accordingly, represent the polyadenylation site, the U5-like domain and the direct repeats DR1 and DR2. (Modified from Ganem 1996; Raney and McLachlan 1991; Will et al. 1987).

2.1 kb preS2 and S mRNA

2.4 kb preS1 mRNA

3.5 kb genomic RNA

0.9 kb X mRNA
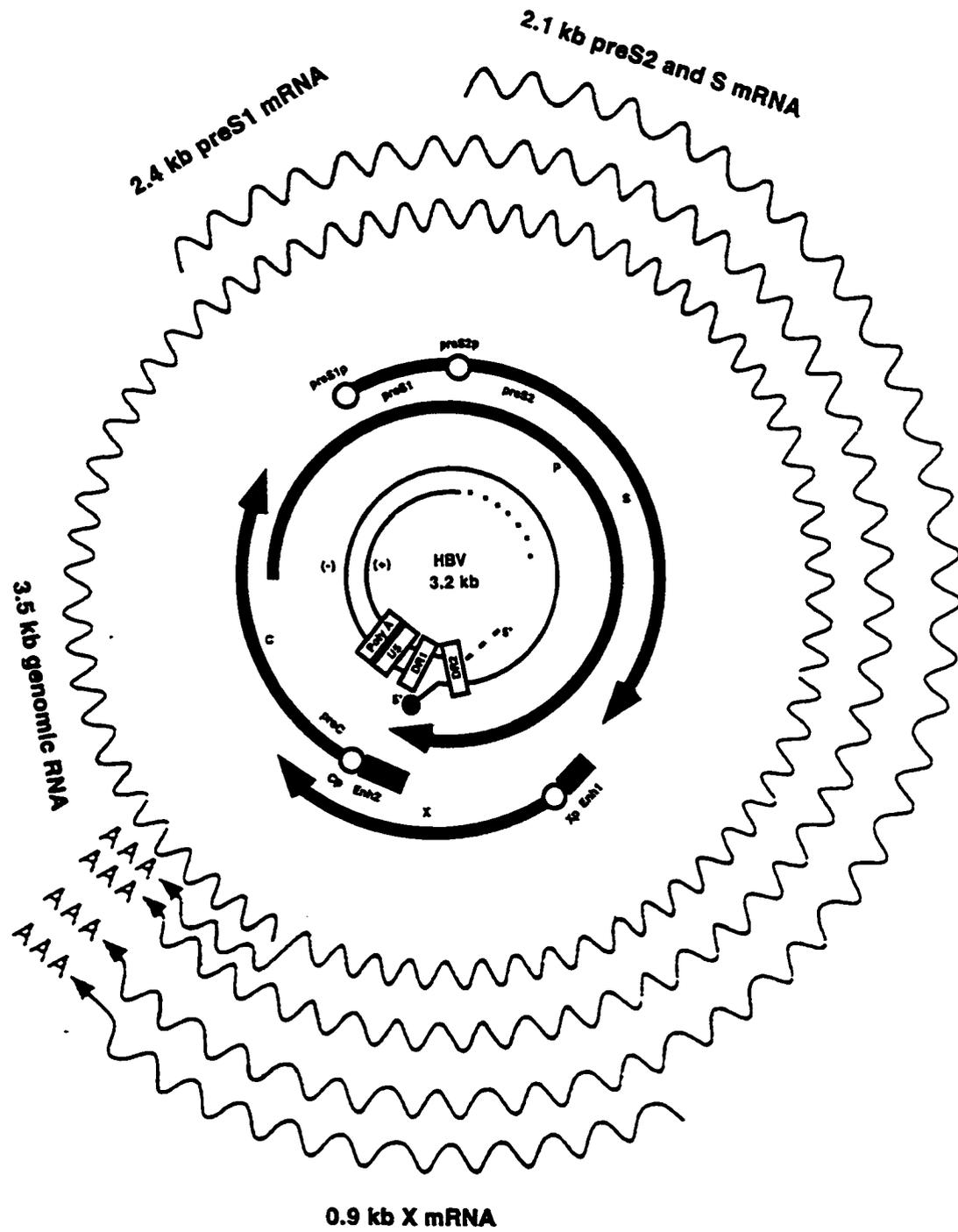
repair of the single-stranded gap; (2) removal of the 5' covalently linked terminal structures, specifically the terminal protein domain of the polymerase on the minus strand and the short capped oligoribonucleotides on the plus-strand DNA; (3) deletion of the 5' terminally redundant sequences of the minus strand DNA; and (4) ligation of the 5' and 3' ends of both DNA strands. The formation of the cccDNA appears mediated largely by the host machinery since a known viral polymerase inhibitor does not block the cccDNA formation (Köck and Schlicht 1993).

The cccDNA exists in the nucleus in an episomal form and is present in a steady state level of about 10–20 or more copies per nucleus of the infected hepatocytes. *De novo* synthesis of the cccDNA has also been reported in the infected hepatocyte (Tuttleman et al. 1986a, b; Wu et al. 1990). The copy number of the viral cccDNA has been reported to be highly regulated. Summers have reported that the *de novo* synthesis of the cccDNA is inhibited by the viral large envelope protein (Summers et al. 1990). Moreover, a mutant DHBV that is defective in the production of the viral large envelope protein resulted in the increased production of cccDNA (Lenhoff and Summers 1994; Summers et al. 1991).

3.   Transcription of viral RNA

Viral transcription is governed by the host RNA polymerase II using viral cccDNA as a template. The regulation of viral transcription, however, is mediated by four viral promoters (Cp, preS1p, preS2p and Xp) and two viral enhancers (Enh1 and Enh2) in a complex way (Figure 1-4) (Antonucci and Rutter 1989; Schaller and Fischer 1991; Shaul 1991; Yen 1993). The C promoter is highly liver specific, therefore playing an important role in determining viral hepatotropism (Seeger et al. 1989). The tissue specificity of the C promoter is dependent on both Enhs via interaction of liver-

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enriched cellular *trans*-acting factors, such as CCAAT/enhancer binding protein (C/EBP) and hepatocyte-nuclear factors (HNF) (Courtois et al. 1988; Honigwachs et al. 1989; Lopez-Cabrera et al. 1990, 1991; Su and Yee 1992). A negative regulation mechanism may be also involved in the C promoter activity (Guo et al. 1993; Lo and Ting 1994; Chen and Ou 1995). The C promoter controls the production of multiple RNAs, collectively called genomic RNAs, with 5' heterogeneous ends (Honigwachs et al. 1989; Karpen et al. 1988; Lopez-Cabrera et al. 1990; Roossinck et al. 1986; Yaninuma and Koike 1989; Yuh et al. 1992). The preS1 promoter is also liver specific and directs the production of the large (2.4 kb) envelope mRNA (Chang and Ting 1989; Chang et al. 1989; Raney et al. 1990, 1991, 1994). The preS2 promoter, although functioning preferentially in hepatocytes, is constitutively active in many cell types (Chang and Ting 1989; Dubois et al. 1980; Faktor et al. 1988; Standring et al. 1984; Stenlund et al. 1983). The preS2 promoter is responsible for the production of a set of transcripts of about 2.1 kb in length with heterogeneous 5' ends and a common 3' end. These transcripts encode the middle and small envelope proteins. It has been reported that the preS2 promoter is more robust than the preS1 (Schaller and Fischer 1991; Shaul 1991; Siddiqui 1991; Yen 1993), which is consistent with the excess synthesis of the small envelope protein over the large and middle envelope proteins. The X promoter is only weakly active and produces the shortest mRNA which codes for the X protein (Antonucci et al. 1989).

In general, the known functional viral RNA transcripts are in two major classes: (1) genomic transcripts of 3.5 kb in length and (2) subgenomic transcripts of 2.4, 2.1 and 0.9 kbs in length (Figure 1-4), which are used as mRNAs. However, one (pgRNA) of the genomic RNAs is bifunctional, serving as an mRNA for both core protein and polymerase protein and as a template for reverse transcription after being encapsidated via interaction with its two gene products. All of these transcripts are 5'

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capped, unspliced, and with a common polyadenylation signal possessing a hexanucleotide sequence (TATAAA) within the beginning of the C gene. Small amounts of spliced viral RNAs, however, have been observed in some transfected cell systems (Chen et al. 1989; Su et al. 1989; Suzuki et al. 1989; Terre et al. 1991; Wu et al. 1991) although they appear dispensable for viral replication.

Recently, several research groups have identified a post-transcriptional regulatory element (PRE), containing multiple binding sites for nuclear factors, which seems to be required for transport of the unspliced viral RNA from the nucleus to the cytoplasm (Huang and Liang 1993; Huang and Yen 1994, 1995; Huang et al. 1996).

4.   Translation of viral proteins

Hepadnaviruses employ an array of strategies to efficiently utilize their compact genome. In addition to using overlapping open reading frames in their gene organization, they also involve multiple initiation codons for translation of these genes to generate structurally related but functionally distinct proteins. However, in general the initiation of translation at internal positions mediated by the ribosomal scanning is not efficient (Kozak 1989). In order to overcome this, hepadnaviruses generate mRNAs with 5' ends next to each AUG except the polymerase mRNA (pgRNA) and thus the ribosomes have access to these internal initiation codons efficiently (Cattaneo et al. 1983). The viral gene products and their biological functions are summarized in Table 1-2.

Table 1-2 HBV gene products[a]

Gene	mRNA		Protein products		Function
	Name	Size (kb)	Name	Size (kDa)[b]	
preC/C	pgRNA	3.5	Core protein	p21	Structural protein of capsid
	preC mRNA	3.5	HBeAg	p17 (serum)	Inducing immunologic tolerance
				p22 (virion)	
Polymerase	pgRNA	3.5	DNA polymerase	p94	Replication
Envelope	preS1 mRNA	2.4	Large (preS1)protein	p39, gp46	Virion assembly and receptor binding
	preS2 mRNA	2.1	Middle (preS2) protein	p31, gp33	Receptor binding
	preS2 mRNA	2.1	Small (S) protein	p24, gp27	Major structural protein of envelope
X	X mRNA	0.9	X protein	p17	Trans-activation

[a] Data are obtained from Ganem 1996, Lau and Wright 1993 and Raney and McLachlan 1991.

[b] Molecular weights indicated for p (protein), gp (glycoprotein).

preC protein/HBeAg

The precore protein is translated from the longer genomic transcripts and then is targeted to the endoplasmic reticulum membrane via its N-terminus signal sequence (Garcia et al. 1988; Ou et al. 1986; Standring et al. 1988). The protein is transported through the vesicular transport system and finally secreted from the cell after the cleavage of its basic C-terminal region as a 17 kDa protein known serologically as e antigen (HBeAg) (Jean-Jean et al. 1989a, 1989b; Schlicht 1991; Wang et al. 1991). It is believed that the HBeAg is dispensable for viral replication since a virus with a nonsense mutation in preC grows normally in both cultured cells and experimental animals (Chang et al. 1987; Chen et al. 1992; Schlicht et al. 1987; Schneider et al. 1991; Tong et al. 1991).

However, the role of the HBeAg as a tolerogen has been demonstrated. In neonates, most HBV infections become persistent possibly because the immune systems of newborn children recognize HBeAg as a self-antigen, since HBeAg is able to cross the placental membrane (Arakawa et al. 1982). It was also observed that perinatal infection with HBeAg negative mutant virus displays severe acute forms of diseases (Terazawa et al. 1991). Moreover, HBeAg transgenic mice induced immunologic tolerance to HBeAg in nontransgenic progeny (Milich et al. 1990). Despite it being nonessential in viral replication, HBeAg as a tolerogen might have evolved to function in giving a selective advantage to the virus.

Core/nucleocapsid protein

Core protein is translated from the shortest genomic transcripts/pgRNA which also contain the polymerase ORF overlapping the 3' end of the upstream core ORF. This protein is the primary structural component of the nucleocapsid, into which the

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pgRNA is encapsidated along with the viral polymerase (see replication cycle for details). The basic arginine-rich C terminus of the core protein possesses sequence-nonspecific nucleic acid-binding properties. However, capsid formation, independent of pgRNA packaging, is mediated via the interaction of the C-termini of core dimer subunits (Seifer et al. 1993).

The C-terminus of the core protein is phosphorylated *in vivo* by cellular kinase(s) and the phosphorylation of the protein is required for the progression of the viral DNA synthesis, which suggests that the phosphorylation state of the nucleocapsid may serve as a maturation signal (Pugh et al. 1989; Yu and Summers 1994). The C-terminus of the core protein may also play a role in specific interactions with the surface protein during viral assembly. The C-terminus of the core protein contains a nuclear localization signal, therefore it is postulated that the core protein may also have a role in shuttling the nucleocapsid between the cytoplasm and the nucleus.

Polymerase (P/Pol)

The mechanism for polymerase expression from the shortest genomic RNA/pgRNA is incompletely understood. Genetic mutation studies have indicated that viral polymerase expression is mediated by *de novo* synthesis with internal entry of ribosomes rather than a ribosomal frameshifting process (Chang et al. 1989; Chang et al. 1990; Jean-Jean et al. 1989b; Roychoudhury and Shih 1990; Schlicht et al. 1989). However, the mechanism by which ribosomes gain access to this internal AUG remains an open question and efforts to identify ribosome entry sequences have failed. Recent studies have suggested that the alternative ribosomal scanning mechanism, in which ribosomes scan from the cap site at 5' end of the pgRNA until the ribosomes reach and translate the C gene, allows the ribosomes to scan to the out of frame AUG

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(+1 position related to the C ORF) of the polymerase (Fouillot et al. 1993; Lin and Lo 1992).

Polymerase protein is the critical viral replication enzyme (see encapsidation and DNA replication below) with multifunctional domains: the N-terminal protein domain, the centrally located DNA polymerase/RT domain and the C-terminal RNase H domain (Bartenschlager and Schaller 1988; Chang et al. 1990; Radziwill et al. 1990; Schodel et al. 1988). In addition, a region between the terminal protein domain and the DNA polymerase domain appears to be tolerant of a wide range of mutations, and thus has been designated as spacer/tether (Chang et al. 1990; Li et al. 1989; Radziwill et al. 1990). The functional significance of the polymerase has been validated by genetic mutation studies (Chang et al. 1990; Faruqi et al. 1991; Radziwill et al. 1990).

Surface/envelope proteins

The viral surface polypeptides contain three components: the Small (S), Middle (M) and Large (L) polypeptides (Table 1-2). The three proteins share a common C-terminal S domain and differ mainly by the N-terminal length and structure (Stibbe and Gerlich 1983). All of the proteins have glycosylated isomeric counterparts (Gerin et al. 1971; Heermann et al. 1984). The N-terminus of the large proteins is further modified by a myristic acid group (Persing et al. 1987). These three surface proteins are the common structural components of the three types of viral related particles: 22-nm diameter spheres and filaments and 42-nm Dane particles. However, the distribution of these three proteins is not balanced among these various particles. The small surface protein is the major component and the middle and large protein are minor components of all three particles. There is little or no large surface protein in the subviral particles. The preS proteins (large and middle) play an important role as ligands for receptor

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binding in viral infection. The large surface protein also plays a role in viral assembly and export. The envelope protein is highly immunogenic and can efficiently induce a neutralizing anti-HBs antibody response (Koff and Galambos 1987).

X protein

The X protein is the smallest viral functional protein translated from the X ORF of the mammalian hepadnavirus DNA. The function of this protein has not been completely understood. Recent studies, however, suggest that this protein has strong and broad-spectrum *trans*-activation activity in enhancing the expression of several viral and cellular genes in various cultured cells (Balsano et al. 1991; Rossner 1992; Seto et al. 1988; Spandau and Lee 1988; Twu and Schloemer 1987; Twu et al. 1989). This regulation takes place mainly at the level of transcription (Balsano et al. 1991; Colgrove et al. 1989) via modification of transcriptional factors or via protein-protein interaction (Maguire et al. 1991). It has been shown that X protein does not bind DNA (Yen 1993) and none of the targets have consensus transcriptional factor-binding DNA sequences (Rossner 1992). Consistent with this, recent studies have shown that the X protein can activate protein kinase cascades, by which the X protein may phosphorylate transcriptional factors and therefore stimulate gene expression (Kekulé et al. 1993). Nonetheless, the X protein itself does not possess protein kinase activity. As a *trans*-transcriptional activator, the X protein may have a role in oncogenesis (Kim et al. 1991; Koike et al. 1994), however, other studies gave contradictory results (Lee et al. 1990). It has been also reported that the X protein may be required for viral infection as WHV with a nonsense mutation in the X gene failed to generate a WHV infection (Chen et al. 1993; Zoulim et al. 1994).

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5. *Encapsidation of pregenomic RNA*

The encapsidation of the pgRNA marks the initiation step of viral genomic replication. Viral encapsidation is dependent on three viral components, the polymerase, core protein and pgRNA. Viral core protein is required for the formation of the icosahedral capsid structure (Hirsch et al. 1990; Lavine et al. 1989). Capsid particles assemble cooperatively once core protein dimer intermediates have reached a threshold concentration, which perhaps occurs immediately post translation (Chang et al. 1994; Seifer et al. 1993; Zhou et al. 1992). However, capsid formation is not dependent on pgRNA packaging since intact capsids can be formed with isolated core gene expression in heterologous systems (Birnbaum and Nassal 1990; Cohen and Richmond 1982; Roosinck et al. 1986; Zhou et al. 1992; Zhou and Standring 1992).

The *P* gene product is also required for pgRNA encapsidation (Bartenschlager et al. 1990; Chiang et al. 1990; Hirsch et al. 1990). Genetic studies have revealed that the structurally intact polymerase is needed for binding pgRNA to initiate encapsidation, however, the activity of the viral polymerase is not required for viral encapsidation (Bartenschlager et al. 1990; Hirsch et al. 1990 Roychoudhury et al. 1991). The polymerase appears to have a preference for encapsidating its own mRNA, since the pgRNA expressing a full polymerase is selected as the template for DNA synthesis rather than the pgRNA expressing a truncated polymerase (Bartenschlager et al. 1992; Hirsch et al. 1990).

Interestingly, only the shortest genomic RNA is selected from the heterogeneous population of viral RNAs and cellular mRNAs for encapsidation into core particles (Enders et al. 1987). This is because a *cis*-element, termed epsilon (ε), present on the 5' end of the pgRNA is responsible for the exclusive encapsidation of

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the pgRNA (Chiang et al. 1992; Junker-Neipmann et al. 1990). The second ε copy on the 3' end of the pregenomic RNA is not required for pgRNA encapsidation (Hirsch et al. 1991) which may explain the selective encapsidation of genomic RNA. The subgenomic RNA only contains the nonfunctional 3' copy ε and cannot be encapsidated (Enders et al. 1987; Hirsch et al. 1991). The longer genomic RNA also contains a 5' epsilon, however it also has the preC AUG upstream of the 5' epsilon. Studies indicate that translation through this region prevents the recognition of this ε, thus the longer genomic RNA is not encapsidated. In the case of DHBV, an additional noncontiguous *cis*-element downstream from the ε element has been also reported to be required for genome encapsidation (Calvert et al. 1994; Hirsch et al. 1991). In accordance with this, this region completely overlaps with our proposed *trans*-repression element which has been suggested by our laboratory as the regulatory element for the viral polymerase translational inhibitory effect (see Chapter 3 and 4 for details).

Using a computer analysis, a conserved stem-loop structure has been predicted in the ε region of all of the hepadnaviruses (Junker-Niepmann et al. 1990) (Figure 1-5) and this secondary structure has been confirmed by chemical analysis of the HBV pgRNA (Knaus and Nassal 1993; Pollack and Ganem 1993). Further, the functional importance of the ε stem-loop structure for viral encapsidation has been examined (Knaus and Nassal 1993; Pollack and Ganem 1993; Tong et al. 1993). These studies have revealed that the specific sequences in the loop, the base pairing in the lower and some upper stem regions and the bulge structure are critical for encapsidation. Recently, Pollack and Ganem have provided direct evidence for the specific interaction

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Figure 1-5 Computer prediction of a stem-loop secondary structure in the HBV epsilon region. The secondary structure with lower and upper stems, a six nucleotide bulge and a six nucleotide loop is shown. This secondary structure was generated from the epsilon sequence of HBV subtype ayw. The altered nucleotide sequence of HBV subtype adw is also presented. (Adapted from Ganem, 1996).

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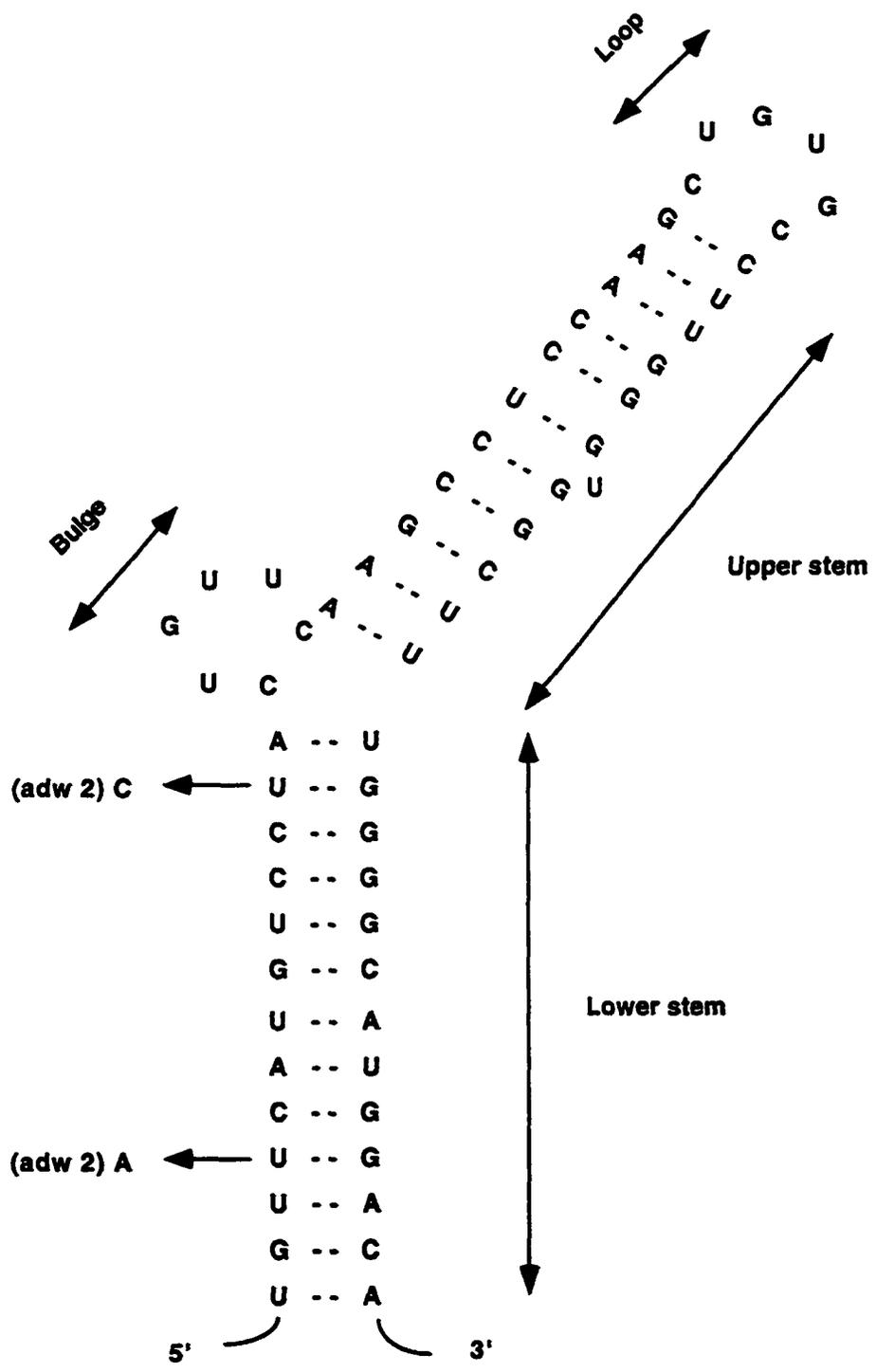
of the viral polymerase with the ε stem-loop. This interaction has proven to be critical for viral encapsidation, since mutations in the ε stem-loop block the interaction of P and pgRNA *in vitro* and also inhibit encapsidation *in vivo* (Pollack and Ganem 1993).

The subsequent steps of the assembly of the P-pgRNA complex with the core subunit are not completely clear. Some indirect information suggests that the C-terminus of the polymerase may interact with the core subunit while the N-terminus binds the pgRNA, although there is no direct evidence for the interaction of polymerase and core protein (Hirsch et al. 1990; Pollack and Ganem 1994).

Recently, it was demonstrated that nucleocapsidation is also dependent on a heat shock protein 90 (hsp90), because antihsp90 antibodies or a hsp90 inhibitor, Geldanamycin, prevents the formation of P-pgRNA complexes *in vitro* and encapsidation *in vivo* (Hu and Seeger 1996). It was suggested that the hsp acts as a molecular chaperone to facilitate formation of the different structures of polymerase needed to fulfil its complex roles in viral replication (Hu et al. 1997).

6.   *Synthesis of minus strand DNA*

The complex mechanism of viral DNA replication, by which a single strand pgRNA is converted to a partially double-stranded DNA genome, is now well understood. A summarized view of this reaction is presented in Figure 1-6.

Viral DNA replication takes place within the nucleocapsid following the pgRNA encapsidation mediated by viral polymerase. The polymerase serves as a protein primer to initiate the minus strand synthesis (Wand and Seeger 1992) within the bulge region

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Figure 1-6 Schematic diagram of viral DNA synthesis. The pgRNA, a template for reverse transcription with large terminal redundant sequences (R), epsilon stem-loop and direct repeats (DR1 and DR2), is shown by a dashed line (1). The polymerase (shaded oval) interacts with the 5' stem-loop on the pgRNA to prime minus strand DNA synthesis, generating a short oligo of three or four nucleotides covalently linked to a tyrosine residue on the polymerase (2). The polymerase and the covalently attached short oligo are translocated and annealed to the homologous sequences of the 3' DR1 where minus strand DNA synthesis continues toward the 5' end of pgRNA (3). During minus strand synthesis, the pgRNA is concomitantly degraded by the RNase H activity of the polymerase (4). The RNase H activity of the polymerase leaves an RNA oligomer containing short terminal redundant sequences (r) and DR1 (5). This RNA oligomer is transferred to the DR2 site on the minus strand DNA to prime the plus strand synthesis (6). Once the plus strand synthesis has reached the 5' end of the minus strand DNA, an intramolecule template switch facilitated by the complementary r region of both minus and plus strands is required for continuation of plus strand synthesis to yield a relaxed circular and partially double-stranded DNA (7). (Modified from Ganem 1996).

1. pgRNA

2. Priming (-)

3. Transfer of primer

4. (-) Synthesis

5. Completion of (-)

6. Priming (+)

7. (+) Synthesis

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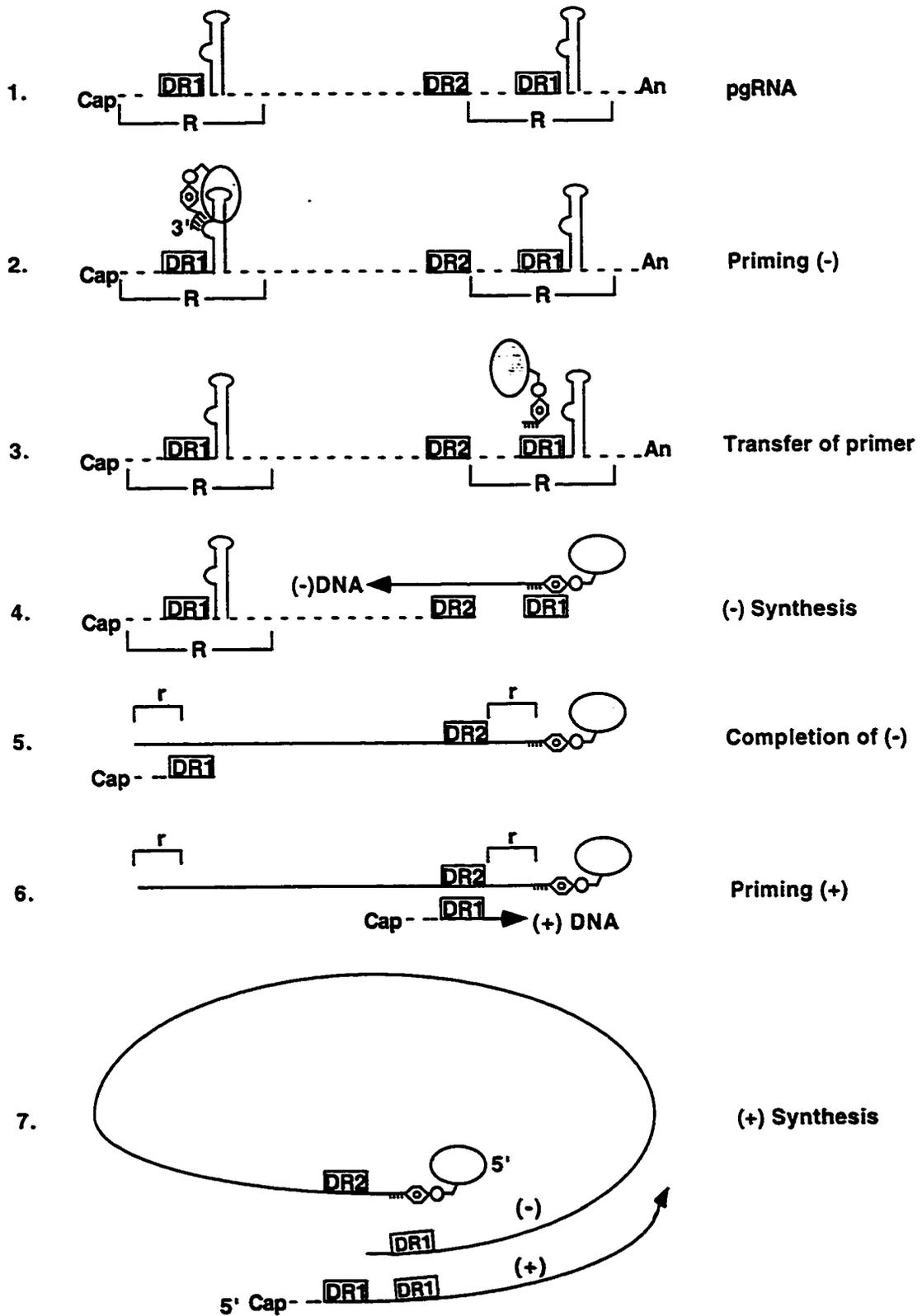
of the 5' end epsilon stem-loop of the pgRNA (Tavis and Ganem 1993; Tavis et al. 1994; Wang and Seeger 1993). These studies demonstrated that DHBV polymerase expressed *in vitro* became labeled by the covalent attachment of ^{32}P-dNTPs and this reaction was inhibited by a mutation at the polymerase active site. In addition, deletion of the stem-loop or even the bulge region of the stem-loop on the pgRNA template completely abolished the reverse transcription. Other researchers reported that this protein-primed reaction resulted in a covalent linkage of a *de novo* synthesized short oligomer (3 to 4 nucleotides) to a tyrosine residue in the N-terminal domain of the polymerase (Bosch et al. 1988; Gerlich and Robinson 1980; Lanford et al. 1995; Molnar et al. 1983; Weber et al. 1994; Zoulim and Seeger 1994). The newly synthesized oligomer with the covalently linked polymerase is then transferred to the DR1 on the 3' end of the pgRNA, which is facilitated by the four homologous nucleotides between the oligomer and the DR1 regions (Seeger and Maragos 1990). There the minus strand DNA is elongated toward the 5' end of the pgRNA to generate a minus strand DNA with short terminal redundant sequences (approximately 8 nucleotides) termed as r (Seeger et al. 1986; Will et al. 1987).

7. Synthesis of plus strand DNA

During the synthesis of the minus strand DNA, the reverse transcribed pgRNA template is concomitantly degraded by the RNase H activity of the viral polymerase (Chang et al. 1990; Miller et al. 1984; Radziwill et al. 1990; Summers and Mason 1982), except for a short capped oligoribonucleotide sequence (including DR1 and six nucleotides to the DR1) at the 5' end of pgRNA (Lien et al. 1986). The specific cleavage site is determined by measurement from the end of the pgRNA, not by specific sequences at the cleavage site (Loeb et al. 1991; Seeger and Maragos 1989; Staprans et

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al. 1991). Following the completion of minus strand synthesis, plus strand synthesis begins by the translocation and annealing of the short capped oligomer to a homologous sequence site (DR2) on the 5' end of the minus strand (Lien et al. 1986; Molnar-Kimber et al. 1984; Seeger et al. 1986; Will et al. 1987). How the ribooligomer is transferred remains elusive. It is thought, however, that the translocation event may not be passive and may be facilitated by other proteins. Occasionally, the riboprimer fails in the translocation and extends at the original position. This results in the production of a fully duplex linear viral DNA, rather than a circular duplex molecule (Staprans et al. 1991). Following RNA priming at the DR2 site on the minus strand, the plus strand is elongated toward the 5' end of the minus strand. When the synthesis reaches the 5' end of the minus strand an intramolecular template switch, facilitated by the homologous r sequences in both minus and plus strands, is required for continuous plus strand synthesis and results in the formation of the circular genome. Plus strand synthesis does not proceed to completion (Lien et al. 1987; Molnar-Kimber et al. 1984). Why the synthesis terminates prematurely is not clear; presumably this relates to viral assembly and secretion. As virus buds into the ER its access to the cytoplasmic dNTP pool may be limited and thus the plus strand synthesis terminates.

8.   *Virion assembly and release*

Electron microscopic (EM) studies of HBV infected cells have revealed that final viral or subviral particles assemble within the endoplasmic reticulum (Huovila et al. 1992; Patzer et al. 1986). This was subsequently confirmed by biochemical studies which revealed that viral envelope proteins are modified by the addition of ER specific mannose oligosaccharide chains (Patzer et al. 1984). The viral particles are then released via the constitutive pathway (Kelly 1985).

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When envelope protein production increases, nucleocapsids acquire envelope proteins and are released from the cell. All of the three envelope proteins accumulate in ER and form aggregates with mixed arrays of large, middle and small envelope proteins for virion assembly (Chisari et al. 1987). Mutation studies which selectively prevent L, M and S protein production indicate that both large and small surface proteins but not the middle protein are required for virion assembly (Bruss and Ganem 1991; Santantonio et al. 1992). An interaction of the nucleocapsid with large envelope proteins, on the cytoplasmic face of the ER membrane (Ostapchuck et al. 1994), may trigger the incorporation of the mature core into the viral envelope (Bruss and Thomssen 1994). Following the viral budding event, it is likely that the large envelope proteins undergo a dramatic rearrangement to allow proteins to be disposed on the surface of the virion since the preS1 domain of the large protein is largely exposed on the virion surface (Kuroki et al. 1990; Ostapchuck et al. 1994). Mutation analysis of myristylation of the large envelope protein indicated that the myristylation of the protein is not required for virion assembly but may have an important role in subsequent viral infection (Bruss and Ganem 1991; Macrae et al. 1991).

The 20-nm subviral particles usually contain middle and small envelope proteins but lack detectable large envelope protein. The 20-nm filaments contain relatively higher amounts of large envelope proteins (Heermann et al. 1984; Stibbe and Gerlich 1983). However, subviral particles can be released with only the small envelope proteins since the small envelope protein contains all the information necessary for the export process. Interestingly, the secretory ability of the middle and small proteins is inhibited by the N-terminus of the large envelope protein (Chisari et al. 1986; Kuroki et al. 1989; Persing et al. 1986; Standring et al. 1986).

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9. Maintenance of cccDNA

As noted before, the nucleus of each infected cell contains 10 to 20 or more copies of cccDNA, which are involved in viral persistence. Several possible mechanisms could account for the maintenance of a pool of cccDNA. Density labeling studies, however, demonstrated that the reverse transcription pathway or the reimportation of newly generated nucleocapsids to the nucleus, rather than semi-conservative replication of cccDNA, are involved in amplification of cccDNA (Tuttleman et al. 1986). Subsequent studies also indicated that the reinfection of cells by extracellular virus did not account for the formation of cccDNA, because amplification of the cccDNA within an infected cell was not blocked by inhibitors of viral infection (Wu et al. 1990). The production of cccDNA was in fact enhanced in DHBV mutants lacking the large envelope protein, which are unable to produce progeny virus for horizontal spread of infection to surrounding cells (Lenhoff and Summers 1994; Summers et al. 1990, 1991). The amplification of cccDNA may also require the partial dephosphorylation of the core protein on the nucleocapsid to facilitate the migration of the capsid to the nucleus (Pugh et al. 1989).

In general, the low concentration of large envelope proteins allows the amplification of the cccDNA early in infection; however, late in infection, the increased production of the large envelope limits the accumulation of cccDNA by facilitating the assembly of the virion and the export of the nucleocapsid as virion.

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D. HBV infection

1. Host range and tissue tropism

The host range of HBV is highly restricted (Gust et al. 1986). It is known that HBV primarily infects human and chimpanzees (Acs et al. 1987; Barker et al. 1973; Hirschman et al. 1969; Lichter 1969; Maynard et al. 1972; Sureau et al. 1988; Vaudin et al. 1988), although there have been reports that HBV can be transmitted to other primates, such as Gibbons (Bancroft et al. 1977), rhesus monkeys (Barker et al. 1975; London et al. 1972), wooly monkeys (Barker et al. 1975), and African Green monkeys (London et al. 1970). Current studies indicate that the viruses are typically restricted to species close to that of the natural host and preventing cross-species transmission is at the level of virus entry into the hepatocyte or at the level of the cellular receptor (Galle et al. 1988; Shih et al. 1989). Despite the substantial sequence homology within the avian hepadnaviruses, the heron hepatitis B virus only infects herons, not ducks nor chickens. However, if the entry pathway is bypassed by transfecting HHBV DNA into heterologous cells, the viral replication proceeds normally. In addition, an HHBV/DHBV chimera, in which the HHBV is enveloped by the DHBV envelope proteins which mediate the binding of the specific cellular receptor, can efficiently infect DHBV hepatocytes.

The primary replication site of HBV is the hepatocyte, although viral replication has been observed in other nonhepatic tissues, such as peripheral blood mononuclear cells (Hadchouel et al. 1988; Laure et al. 1985; Lie-Injo et al. 1983; Noonan et al. 1986; Shen et al. 1986; Yoffe et al. 1986); pancreatic acinar cells (Shiraki et al. 1977); bile duct epithelial, endothelial and smooth muscle cells (Blum et al. 1983); skin and kidney cells (Dejean et al. 1984); lymph nodes, spleen, bone marrow stem cells, thyroid gland, gonads and adrenal glands (Elfassi et al. 1984; Romet-Lemonne et al.

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1983). The factors responsible for viral tissue tropism may include a need for liver-specific transcription factors, such as HNFs and C/EBP (see transcription), for viral gene expression. Studies have suggested that the enhancers and the core promoter direct preferential gene expression in hepatocytes (Seeger et al. 1989), whereas the small envelope proteins are expressed in a range of cultured cell lines (Dubois et al. 1980; Standring et al. 1984; Stenlund et al. 1983). In addition, viral hepatotropism can be circumvented by expressing the viral genes under heterologous promoters rather than the viral core promoter (Junker et al. 1987; Seeger et al. 1989), which suggests that the prevention of the replication in the nonhepatocyte is mainly at the level of regulation of genomic transcription.

2. Routes of infection

The transmission of HBV occurs primarily parenterally by exposure to virus-containing blood, blood products or body fluids, such as semen. An infection can occur via either vertical or horizontal routes. Vertical transmission of an HBV infection from an infected mother to her child is particularly important in high endemic areas, such as China and the Far East (Perrrillo 1993). Horizontal transmission of HBV infections through sexual contacts and percutaneous contacts (e.g., drug injection) is the major route in North America and Europe (Szmuness et al. 1975). Regardless of the route by which the virus gains access to human circulation, it needs to contact hepatocytes to initiate its life cycle.

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3. Clinical consequences of infection

The clinical features of hepatitis B infections range from an healthy asymptomatic carrier state to acute or fulminant hepatitis and chronic liver diseases, including liver cirrhosis and hepatocellular carcinoma (HCC). The consequences of the infection are dependent on several factors. (1) The age at the time of infection is a major determinant. In infections of newborns or infants, more than 90% are asymptomatic but persist; on the other hand, more than 90% of infections of adults are symptomatic but resolve. (2) The type of virus is the other determinant. It is reported that HBV variants with mutations in the core gene are frequently found in chronic hepatitis (Ehata et al. 1992; Bertoletti et al. 1994). In addition, variants with mutations in the pre-core region are frequently found in patients with severe hepatitis (Ehata et al. 1993; Aye et al. 1994; Lai et al. 1994; Sato et al. 1995). (3) Host factors (i.e. viral mediated host immune response) are the most important determinants in producing the tissue lesions (Chisari and Ferrari 1995).

In acute hepatitis B, inflammation and necrosis of the liver are associated with the HBV infection. The first evidence of the infection is the detection of HBsAg in serum. This is followed by the appearance of HBV DNA and HBeAg and then anti-HBc in the serum. By 30–50% patients suffer from malaise with elevated liver transaminases in their serum. However, 50–70% of HBV infections may never produce clinical evidence of liver disease (asymptomatic). The decline of clinical symptoms is followed by a rise in anti-HBs and -HBe antibodies, which indicates resolution of the infection. About 90–98% of patients recover completely from the infection (Holliger 1996).

Approximately 5–10% of the HBV infected patients will develop chronic hepatitis which is characterized by the persistence of the HBV infection either

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associated with inflammation and hepatocyte necrosis (10–30%) or asymptomatic (70–90%, healthy carriers) (De Franchis et al. 1993). While some patients eventually recover completely from chronic infections with the development of anti-HBs, others do not and ultimately develop cirrhosis and hepatocarcinoma.

II.   Antiviral Therapy

For therapy of chronic hepatitis B infections, two broad classes of agents: antiviral nucleoside analogues and immunological response enhancing agents, e.g., lamivudine and interferon-α have been shown clinically useful. However, additional antiviral agents may be of value for combination antiviral therapy to improve clearance of chronic HBV infections and to slow the development of resistance.

A.   Immunomodulating agents

The natural clearance of an HBV infection is dependent on the recognition and lysis of the infected cells by the cytotoxic T lymphocytes (CTLs) (Ando et al. 1994). This response is also amplified by cytokines and neutralizing antibodies (Gilles et al. 1992).

Interferon alpha (IFN-α) is the mainstay of therapy for chronic HBV infection, especially in patients with moderate or severe antiviral immune responses, in whom IFN-α may shift a partially effective immune response to a potent immune response to facilitate the immune clearance of the virus. Studies have shown that IFN-α amplifies the immune response in several ways. It induces the expression of major histocompatibility complex (MHC) class I molecules, hence enhancing the presentation of the viral antigens (Harris and Gill 1986). It may activate the CTLs and induce the expression of interleukin-12 (IL-12) receptor, therefore, augmenting the CD4^{+} T helper lymphocyte (Th1) response (CTL response) (von Hoegan 1995). It can also increase

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the activity of natural killer cells (Ortaldo et al. 1984) and induce the humoral immune response (Marinos et al. 1995; Waters et al. 1995).

Despite the activity of the IFN-α in the control of the viral replication, current studies indicate that only 20–40% of carefully selected patients, who have low initial levels of HBV DNA, HBeAg and elevated transaminases, respond to IFN-α treatment (Feinman et al. 1922; Hoofnagle et al. 1988; Tine et al. 1993; Woo and Burnakis 1997). Patients with minimal hepatitis, usually infected at birth with subsequent immune tolerance, are generally nonresponsive. This may be because IFN-α can only amplify an existing immune response, but cannot break immune tolerance. Side effects of IFN-α are common. Relapse with return of viremia and hepatitis occurs in up to 50% of patients treated with IFN-α (Hoofnagle and Lau 1996).

Other important immunomodulating agents are interleukin 2 (IL-2), tumor necrosis factor-alpha (TNF-α) and IL-12, which can enhance a Th1 response and facilitate IFN-α antiviral activity (Gille et al. 1992; Guilhot et al. 1993; Rossol et al. 1996). These agents may be important in the future for use in combination with other antiviral agents.

B. Nucleoside analogues

Treatment with nucleoside analogues is another approach to clearing the HBV infections by inhibiting viral replication until the infected cells are cleared by the natural senescence process of the liver cell. This may need long term therapy (Shaw and

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Locamini 1995). Lamivudine (3TC) and famciclovir have been shown to be clinically useful (Tyrrell et al. 1993; Dienstag et al. 1995; Brown et al. 1996).

Famciclovir (oral form of penciclovir) is a purine nucleoside analogue. Famciclovir is changed to penciclovir in the small intestine, serum and liver. The active form of the phosphorylated penciclovir has a long half-life and potent antiviral activity (Boyd et al. 1993; Vere Hodge 1993). Penciclovir blocks the protein priming step of viral reverse transcription (Zoulim et al. 1995). This agent has a wide therapeutic margin and no significant toxicity (Shaw and Locamini 1995; Shaw et al. 1996; Vere Hodge 1993). Efficacy studies of penciclovir in HBV infected patients have demonstrated significant reduction of HBV DNA levels (Kruge et al. 1994; Main et al. 1996).

The antiviral activity of penciclovir is dependent on the host phosphorylation enzyme, since HBV does not encode enzymes capable of catalyzing phosphorylation of penciclovir. Cellular phosphorylation variability accounts for a variable response to penciclovir, therefore, penciclovir has not received strong endorsement by clinicians for treatment of chronic HBV.

Lamivudine/thiacytidine (3TC) is a pyrimidine analogue with a 3'-thio substitution in the ribose ring. It has recently been licensed for the treatment of chronic HBV infection. The L (–) enantiomer has been shown to have greater antiviral activity and less toxicity than its D (+) enantiomer counterpart (Van Draanen et al. 1994). Phosphorylated 3TC acts as a competitive inhibitor resulting in the premature termination of HBV DNA chain elongation, since it lacks a 3'-hydroxyl group on the ribose ring (Severini et al. 1995). Recent clinical studies have shown that 3TC promptly reduces viral DNA levels and, over a period of 4–6 weeks, decreases viral protein levels and normalizes alanine aminotransferase (ALT) (Dienstag et al. 1995; Lai

et al. 1994; Tyrrell et al. 1993). However, viremia rebounds to pretreatment DNA levels on cessation of treatment (Tyrrell et al. 1993). Lamivudine appears to improve liver histology, including reductions in necroinflammation and fibrosis (Lai et al. 1998).

The problem of monoantiviral therapy with 3TC or famciclovir is the development of resistant HBV mutants. Lamivudine resistance was predicted and the first lamivudine resistant mutant was produced in DHBV in our laboratory by site-directed mutagenesis at the conserved YMDD (tyrosine, methionine, aspartate, aspartate) motif of the viral polymerase (Fischer and Tyrrell 1996). Subsequently, resistant mutants to 3TC have been isolated from patients on long term lamivudine therapy (Bartholomew et al. 1997; Tipples et al. 1996). Mutations at the YMDD motif in the catalytic (C) domain of the viral polymerase have been shown to reduce sensitivity to 3TC and may produce total drug resistance or resistance to other drugs, such as famciclovir. A C domain dominant mutation has been shown to be associated with a B domain mutation that reduces the sensitivity to famciclovir (Tipples et al. 1996).

HBV resistant viruses were also obtained from patients who has been treated with famciclovir. Three mutations in the B domain of the viral polymerase were found, associated with increased viremia (Aye et al. 1997). The emergence of mutants with reduced sensitivity to antiviral agents over the long-term is a major concern for single agent therapy.

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C. Combination chemotherapies

The development of combination therapy with agents that act by different mechanisms appears justified. Strategies of combining immunomodulating agents with nucleoside analogues, two different nucleoside analogues or nucleoside analogues with other potential new viral inhibitors, such as a glycosidase inhibitor which prevents the formation of infectious virus (Block et al. 1998; Lu et al. 1997; Mehta et al. 1997), may offer effective treatment for persistence HBV infections. These would minimize the rate of development of drug resistant variants since not only might different target specific antiviral agents be synergistic, but they work by different mechanisms to delay the development of resistance (Colledge et al. 1997; Korba 1995; Fontana and Lok 1997). This has clearly been true in the improvement of antiviral agents for human immunodefficiency virus (HIV).

III. Difficulties in Viral Polymerase Expression

Early inferences that the *P* ORF encoding the viral polymerase was involved in viral replication were supported by alignment of this coding region with that of retroviral reverse transcriptase (Toh et al. 1983) and observations that mutations in *P* ORF inactivated viral DNA synthesis (Bavand et al. 1989). In order to obtain more detailed information about the polymerase and its activity, several laboratories have attempted to express the polymerase in a variety of heterologous systems. However, these numerous researchers have been frustrated in their attempts to produce sufficient amounts of full-length polymerase which remains enzymatically active polymerase. A summary of the different recombinant expression systems is discussed below.

A)   Prokaryotic expression systems – *E. coli*

Attempts to express the hepadnaviral polymerases in prokaryotic systems have been largely disappointing, although retroviral polymerases have been successfully expressed in bacterial systems (Hostomsky et al. 1992; Kotewicz et al. 1985). Several researchers from different laboratories have tried to express HBV polymerase in an *E. coli* system by fusion of truncated or near full-length polymerase fragments in frame to the 3' end of the *E. coli* β-galactosidase gene (*LacZ*), bacteriophage MS2 polymerase or *E. coli* Trp E (Chang et al. 1989; Köchel et al. 1991; Stemler et al. 1988). These polymerase fusion proteins are immunogenic, since anti-polymerase-containing sera from HBV infected patients or chimpanzees specifically interact with these fusion proteins. None of them, however, demonstrated enzymatic activity, although one of the fusion proteins was reported to have specific HBV RNA binding properties (Köchel et al. 1991).

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B)   Recombinant vaccinia virus expression system – HepG2

A vaccinia virus expression system was also used to examine HBV polymerase (Bartenschlager et al. 1992). Full-length and truncated C- and N-terminus polymerase proteins were synthesized and detected by the polymerase specific antibodies in HepG2 cells infected by the recombinant vaccinia virus. An unusually short half life (40 min) of the free polymerase products within HBV infected cells was observed. The N-terminal protein had much longer half life while the C-terminal protein had very short or below detectable half life. This suggested that the C-terminus of polymerase was more susceptible to degradation. In contrast, the viral core protein expressed in the same system had a half life of longer than 22 hs. All of the recombinant proteins were tested for enzymatic activities, but no enzymatic functions were demonstrated as measured by incorporation of radioactivity into DNA products or by activity gel analysis.

C)   Rabbit reticulocyte lysate expression system

An enzymatically active DHBV polymerase has been expressed *in vitro* (Howe and Tyrrell 1992; Wang and Seeger 1992). The recombinant polymerase mRNA contains an epsilon sequence, which is required for initiation of minus strand DNA synthesis and plays an important role for the activity of the polymerase (Tavis et al. 1994, 1996, 1998; Wang et al. 1994; Wang and Seeger 1993), at the 3' end of the polymerase transcript (Wang and Seeger 1992). An authentic viral polymerase activity was demonstrated, in which the recombinant polymerase possessed protein priming activity and synthesized a partial minus strand of approximately 500 nucleotides beginning from the viral replication origin.

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D)   Yeast expression system

An enzymatically active DHBV polymerase has also been expressed as a fusion protein of polymerase with yeast retrotransposon Ty1 in yeast cells (Tavis et al. 1993). The recombinant polymerase mRNA contains the epsilon sequence at the 3' end of the transcript. An authentic viral polymerase activity was demonstrated in yeast cells and *in vitro*, in which minus strand synthesis was initiated at the replication origin of viral RNA and was covalently linked to the viral polymerase. Recently, the yeast retrotransposon Ty1 system was also employed to express HBV polymerase protein (Qadri and Siddiqui 1999). Recombinant HBV polymerase synthesized minus strand DNA which was protein-primed, however, the template and DNA products of the polymerization reaction were not characterized. The recombinant construct did not include the epsilon sequence.

E)   *Xenopus* oocyte expression system

An active HBV polymerase was expressed in *Xenopus* oocyte lysate (Seifer and Standring 1993). In this system, the recombinant HBV polymerase plasmid contains no epsilon sequence. Polymerase activity was demonstrated *in vitro* with the production of the DNA products which bound to the recombinant polymerase, however the template and DNA products of the polymerization reaction have not been fully characterized.

F)   Recombinant baculovirus expression system

Recombinant baculovirus expression systems also have been utilized to express HBV polymerase (Ayola et al. 1993; McGlynn et al. 1992; Noonan et al. 1991). Full

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length and various truncated forms of the HBV polymerase were expressed with the recombinant baculoviruses in insect cells. Each polymerase protein was immunogenic and reacted with specific anti-HBV polymerase peptide antibodies. A high polymerase expression was obtained in a *Trichiplusia ni* (Tn) insect cell line (Ayola et al. 1993). Two proteins with approximate molecular masses of 93 and 72 kDa were expressed from a preC mRNA-like transcript and detected in the *Spodoptera frugiperfa* (Sf9) insect cell line by the mAb against the polymerase (McGlynn et al. 1992). The larger protein probably represents the full length HBV polymerase product, whereas the smaller product may represent a specific cleavage product of the larger polypeptide or an internal initiation product. Neither product was enzymatically active.

Recently, an active HBV polymerase was expressed in a recombinant baculovirus system (Lanford et al. 1995). In this system, the epsilon sequence required for the polymerase activity was included in the 3' end of the polymerase ORF and an affinity tag was cloned in frame at the N-terminus of the polymerase for rapid purification of the tag-polymerase fusion protein. Protein priming and reverse transcriptase activities were demonstrated in this system.

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IV. Translational regulation of gene expression

Translation of an eukaryotic gene is a complex process involving an array of translational protein factors that function in cooperation with ribosomes and tRNAs to decode an mRNA and to generate the coded polypeptide chain (Merrick and Hershey 1996). The translation process can be divided basically into three stages: (1) initiation, in which the 80S ribosome assembles at the proper initiation (AUG) codon of an mRNA. For most mRNA species the scan for the initiation codon starts at the 5' cap structure. This stage ends with the formation of the first peptide bond; (2) elongation, which involves translocation of the ribosome down the coding region and addition of amino acids to form a polypeptide chain; and (3) termination, during which a stop codon (UAA, UGA, UAG) signals the release of the polypeptide chain from the ribosome and the ribosome from mRNA. Initiation is considered to be the rate-limiting step in the translation process and, therefore, it is often subject to regulation.

Regulation of translation, although still less well characterized than the regulation of gene transcription, has now become increasingly clear (Pain 1996). Translational control relies on the specific interaction of the *cis*-acting mRNA element and the *trans*-acting protein. Such interactions can occur in all regions of an mRNA, i.e. the 5' or 3' untranslated regions (UTRs) or within the coding region or the 5' cap structure or the 3' poly (A) tail of an mRNA. The mRNA-binding regulatory protein seems mostly to act as a negative regulator, binding to a specific site on an mRNA resulting in interference with translational events, especially the initiation of translation.

An increasing number of mRNAs are now being recognized as subject to translational regulation. In addition to controlling the constitutive level of translation of a specific transcript, the 5' mRNA motifs can also have a regulatory role on the translation of a specific transcript. The 5' mRNA secondary structural element can

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provide binding sites for regulatory proteins. Such an interaction of the 5' *cis*-acting element and the *trans*-regulatory protein can, by steric hindrance, impede the binding of the 43S ribosomal preinitiation complex to the 5' cap structure and of other translational factors (e.g., RNA helicase eIF4A, 4B) to the 5' UTR. This interaction can also act as a barrier to scanning by the 43S ribosomal complex by stabilizing the structural element even when it is not inhibitory to initiation. A well characterized example of such translational regulation is ferritin mRNA, which contains a stem-loop motif known as an iron responsive element (IRE) in its 5' UTR. A 100 kDa iron regulatory protein (IRP) binds the IRE on the ferritin mRNA with high affinity when intracellular concentrations of iron are low, which results in inhibition of ferritin mRNA translation (Goossen et al. 1990). The IRE must be cap proximal for effective regulation, which suggests that the interaction of IRP and IRE blocks the early initiation of translation.

The translation of human thymidylate synthase mRNA is also regulated by the interaction of mRNA with a regulatory protein. In this case, the enzyme itself acts as a regulatory protein to bind its own mRNA and mediate an autoinhibition of translation (Chu et al. 1991). The specific binding and inhibition are abolished by addition of the ligands that associate with this enzyme. The binding sites have been mapped to a region around the initiation codon which forms a secondary structure and to a second site in the coding region of the thymidylate synthase mRNA (Chu et al. 1993). Numerous other examples of translational repression by the interaction of 5' UTR of an mRNA with a regulatory protein have been described, such as dihydrofolate reductase mRNA translation (Chu et al. 1993) and ribosomal protein mRNA translation (Levy et al. 1991).

It is becoming increasingly clear that the 3' UTR of an mRNA can have a role equally important to that of the 5' UTR in translational regulation of gene expression. Examples of such translational regulation include erythroid 15-lipoxygenase (LOX)

mRNA, which is synthesized at the bone marrow cell stage, but remains as untranslated mRNA until just before reticulocytes mature into erythrocytes. This translational inhibition is thought to be due to a specific interaction between a 48 kDa regulatory protein and a pyrimidine-rich repeat motif in the 3' UTR of the LOX mRNA (Ostareck-Lederer et al. 1994). It is clear that inhibition is not due to degradation of the mRNA. Although the exact mechanism of translation regulation by such RNA and protein interactions is not clear, several models have been proposed. In the first model, it is speculated that specific binding of a regulatory protein to the 3' UTR results in hindrance of the 5' cap structure either by direct association of the 3' UTR binding protein or by indirect association of another protein with the 5' UTR. In an alternative model, it is postulated that the binding of a specific protein to its 3' UTR site acts as a nucleation signal for the binding of other proteins to the mRNA to prevent access of the translation machinery to the mRNA. Additional examples of translational control by the interaction of a 3' UTR and a regulatory protein are protamine mRNA translation in developing spermatids (Kwon and Hecht 1991) and the translation of ribonucleotide reductase and cyclin A mRNAs in immature oocytes (Standart 1992).

In conclusion, the importance of translational regulation on gene expression is becoming increasingly clear. This translational regulation exists in a wide range of biological systems.

V. Rationale and Objectives

Investigation of hepadnavirus replication has mostly relied on studies in the DHBV system. In contrast to its HBV counterpart, DHBV polymerase can be expressed in an enzymatically active form either *in vitro* or in a yeast retrotransposon Ty1 system (Howe et al. 1992; Tavis and Ganem 1993; Wang and Seeger 1992). Although numerous attempts have been made, HBV polymerase could not be synthesized in the similar recombinant expression systems for a long time. Information on HBV polymerase has largely come from studies with infected tissues and transfection of a cloned HBV genome into cell lines. The expression of a functional HBV polymerase in both *Xenopus* oocyte and recombinant baculovirus systems have been reported (Seifer and Standring 1993; Lanford et al. 1995). Recently, the yeast retrotransposon Ty1 system was also successfully employed to express enzymatically functional HBV polymerase (Qadri and Siddiqui 1999). However, an *in vitro* translation system has not been successfully employed for the expression of HBV polymerase.

My first objectives were to:

(1) Clone and express a full-length recombinant HBV polymerase protein *in vitro* (Chapter 2).

(2) Demonstrate and characterize the enzymatic activities of recombinant HBV polymerase (Chapter 2).

Hepadnaviral polymerase plays a pivotal role in encapsidation and DNA synthesis steps of viral replication. In addition, previous findings in our laboratory demonstrated that the viral polymerase may have a regulatory role in viral gene expression in the DHBV system (Howe and Tyrrell 1996). Anita Howe, a graduate

student in the Dr. Tyrrell's laboratory, had observed that synthesis of the DHBV core protein was specifically inhibited in the presence of the DHBV polymerase-containing lysate. Second, the polymerase lysate contained both the DHBV polymerase mRNA and its translation product HBV polymerase. In order to exclude the possibility that the polymerase mRNA was involved in the repression of the core mRNA translation, a nonsense mutation of the polymerase expression construct was built. This mutant construct was not able to produce any protein products. The reduction of the core protein synthesis was not detected in the presence of the lysate translated from this nonsense mutated polymerase construct, indicating that the DHBV polymerase mRNA was insufficient to repress the DHBV core protein synthesis. When the lysate contained both polymerase mRNA and polymerase protein, repression was seen. Based on this observation, it was believed that the polymerase protein and not polymerase mRNA was involved in repression of core expression. Third, deletion analysis done by Dr. Howe indicated that sequences from nucleotides 140 to 828 at the 3' periphery of the core ORF were required for the polymerase-mediated translational inhibition. Fourth, on further analysis, sequences from nucleotides 401 to 870 were sufficient to transfer the susceptibility of polymerase-mediated inhibition into a heterologous mRNA (human CD4).

In my research, I wanted to determine if translational control of the viral polymerase is a conserved mechanism in members of the hepadnaviral family and to further map the repression sequences.

My second objectives were to:

(3)   **Demonstrate translational regulation in the HBV system and cross species translational regulation in the hepadnaviral family (Chapter 3).**

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(4) Map the translational regulatory cis-acting element using silent mutations of the regulatory sequences from nt 137 to 830 and examine effects of the silent mutations on the translational control of gene expression by the viral polymerase (Chapter 4).

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CHAPTER 2*

CLONING, EXPRESSION AND CHARACTERIZATION OF *IN VITRO* EXPRESSED HUMAN HEPATITIS B VIRUS POLYMERASE

I.   Introduction

Human hepatitis B virus (HBV) causes both acute and chronic hepatitis and is associated with the development of hepatocellular carcinoma (HCC) (Beasley 1988; Hoofnagle 1981; Koff and Galambos 1987).   HBV is a member of the family hepadnaviridae characterized by a partially double-stranded DNA genome with a size of 3.2 kb (Summers et al. 1975).   Genome replication involves the reverse transcription of a viral pregenomic RNA (pgRNA) by the viral polymerase (Pol) which is encoded by the polymerase open reading frame (*P* ORF) (Ganem and Varmus 1987; Summers and Mason 1982).

The hepadnavirus polymerase contains four domains, listed from amino to carboxyl termini: terminal protein (TP), space/tether, DNA polymerase/reverse transcriptase (Pol/RT), and RNase H (Radziwill et al. 1990).   The initial step of viral genome replication is the recognition and binding of a viral pgRNA by the viral polymerase. The pgRNA-Pol complex is then encapsidated (Bartenschlager et al. 1990).   Within the nucleocapsid, minus-strand DNA synthesis is initiated by the protein

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priming activity of the polymerase using, as a template, a bulge sequence in an epsilon stem-loop structure near the 5' end of the pgRNA (Tavis et al. 1994; Wang and Seeger 1993). The priming reaction covalently links a dGTP nucleotide (nt) to a tyrosine residue (tyrosine 63 in the case of HBV Pol) within the amino-terminal domain of the viral polymerase (Bartenschlager and Schaller 1988; Gerlich and Robinson 1980; Lanford et al. 1997; Molnar-Kimber et al. 1983; Weber et al. 1994; Zoulim and Seeger 1994). A short oligodeoxyribonucleotide of approximately four bases is synthesized. The nucleotide-Pol complex is then translocated to a complementary direct repeat (DR1) sequence at the 3' end of the pgRNA and minus-strand DNA is elongated (Molnar-Kimber et al. 1984; Seeger et al. 1986; Will et al. 1987). Plus-strand DNA is synthesized, after being primed by a capped RNA oligomer, from the 5' end of the pgRNA hybridized to the DR2 sequence on the minus-strand DNA template (Lien et al. 1986; Seeger et al. 1986; Will et al. 1987). Viral replication results in a noncovalently closed, partially duplex and circular DNA molecule with the minus-strand attached at its 5' end to the priming protein of the viral polymerase (Summers and Mason 1982).

Efforts to study the viral polymerase have been limited by difficulties in producing a fully active polymerase from viral particles or in heterologous expression systems. Duck hepatitis B virus (DHBV) polymerase with its priming and reverse transcriptase activities has been expressed in a rabbit reticulocyte lysate system (Howe et al. 1992; Wang and Seeger 1992) and in a yeast retrotransposon Ty1 expression system (Tavis and Ganem 1993). HBV polymerase expressed in a Xenopus oocyte system has shown priming and reverse transcriptase activities (Seifer and Standring 1993), but the template and products of this system have not been fully characterized. Recently, HBV polymerase has been expressed in a baculovirus expression system. Some of the polymerase products exhibited template-dependent priming and reverse transcriptase activities (Lanford et al. 1995). More recently, HBV polymerase has been

expressed in a yeast retrotransposon Ty1 expression system (Qadri and Siddiqui 1999). The recombinant polymerase synthesized the minus strand that is protein-primed. However, the template and DNA products of the polymerization reaction were not characterized.

In this study, I have expressed the HBV polymerase in a rabbit reticulocyte lysate-coupled transcription/translation system. The full-length molecule and the N-terminal portion of the viral polymerase demonstrated protein priming activity in an *in vitro* priming assay. This polymerase is also active in an *in vitro* polymerase assay. Both protein priming and polymerization activities of this enzyme are dependent on an RNA sequence bearing HBV DR1/epsilon stem-loop motifs known to be essential for viral replication.

II. Materials and Methods

Construction of the recombinant HBV polymerase

The circular HBV genomic sequence of subtype adw is numbered using the unique *Eco*R I site as reference for the first nucleotide (Galibert et al. 1979). A complete HBV polymerase gene, with flanking sequences, of the strain HBV 991 subtype adw was constructed from three DNA fragments. A 2297 base pair (bp) restriction fragment (from *Bsp*E I site at nt 2332 to *Bam*H I site at nt 1408) containing most of the HBV polymerase gene was isolated from an HBV replicating dimer plasmid pKSVHBV1 (Seifer et al. 1990). The other two fragments, the 5' end sequence (134 nt with 21 nt located in the *P* ORF) and the 3' end sequence (269 nt with 220 nt located in the *P* ORF) of the HBV polymerase gene, were polymerase chain reaction (PCR) amplified from the same plasmid by *Pyrococcus furiosis* (Pfu) DNA polymerase (Stratagene). Cloning sites, a *Pst* I site at the 5' end and a *Xma* I site at the 3' end, were incorporated into the appropriate PCR primers (see Appendix for PCR primers). A complete HBV polymerase gene (2538 nt) with flanking sequences was obtained by ligating the three fragments. The HBV polymerase gene was subsequently inserted into an *in vitro* expression vector pTZ19R (Pharmacia Biotech) under the control of a bacteriophage T7 RNA polymerase promoter. The resulting recombinant plasmid was used to transform *E. coli* competent cells. The construct of the recombinant plasmid designated pTZHBVP was verified by restriction mapping and DNA sequence analyses across the ligation sites.

A template containing the HBV DR1/epsilon stem-loop sequence from nt 1679 to nt 1910 of strain HBV 991 subtype adw under the control of a bacteriophage T7 RNA polymerase promoter was produced by PCR amplification from plasmid

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pKSVHBV1 (see Appendix for PCR primers). Transcription from this template in a reticulocyte lysate-coupled transcription/translation system produced a 231 nt RNA bearing the DR1/epsilon stem-loop sequence.

Polymerase chain reaction

Amplification of DNA fragments was achieved by PCR using Pfu DNA polymerase and the method of Sambrook with some modifications (Sambrook et al. 1989). PCR was used mainly to add cloning restriction sites or to obtain DNA sequence for subcloning. The PCR reaction was performed on a Single Block™ System (Ericomp Inc.) or a PTC-100™ Programmable Thermal Cycler (MJ Research). A typical reaction contained 100–300 ng of DNA template, 50 pmol of primers, 0.2 mM dNTPs and 2.5 units of Pfu DNA polymerase in 1× Pfu polymerase buffer (Stratagene) and was conducted at 94°C for 5 min for one cycle; 94°C for 1 min, 50–60°C for 1 min, 72°C for 1 min for 20–30 cycles; 94°C for 1 min, 72°C for 5–10 min for one cycle. The PCR products were separated from primers by use of 30,000 NMWL MC Ultrafree® filters (Millipore Corporation) or a 0.8% low melting point (LMP) agarose gel. This purified DNA was ready to be used for subcloning. The primers were made on an Applied Biosystems 391 DNA synthesizer (ABI).

Site-directed PCR mutagenesis of the recombinant HBV polymerase

Mutagenesis of the second initiation codon AUG of the polymerase gene at amino acid residue 113 to GUG was achieved by a two step PCR-based mutagenesis involving three oligonucleotide primers (Picard et al. 1994): the 5' flanking primer

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(pMVU) 5'-TTATCAACACTTCCGGAAAC upstream of the mutation site, the 3' flanking primer (pMVD) 5'-CCAAGAATATGGTGACCCGC downstream of the mutation site and the mutagenic primer (pMV) 5'-TTGAAATTAATTGTGCCTGCTAGAT. The underlined sequence of the mutagenic primer is the substituted nucleotide (A to G). Megaprimers (209 nt) were synthesized by PCR amplification with plasmid pTZHBVP primed with pMV and pMVD. Mutant DNA fragments (522 nt) were then PCR amplified with plasmid pTZHBVP primed with the pMVU and the megaprimer. Mutant DNA fragments were digested with BspE I and BstE II, purified and directly inserted into the plasmid pTZHBVP previously cut with the same enzymes. The new construct was designated pTZHBVPM. The presence of the mutated sequence was confirmed by DNA sequencing. PCR was performed according to a standard protocol (Sambrook et al. 1989) on a PTC-100™ Programmable Thermal Cycler. Oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer.

Transformation of *E. coli* with DNA by classical and electroporation methods

In the classical method, after ligation of gene fragments with vector at room temperature for 1–2 h or at 15°C overnight, 10 μl (~ 10 ng DNA) was mixed gently with 100–200 μl of calcium chloride competent *E. coli* cells (prepared in a salt solution and frozen in a salt solution containing 10% glycerol) prethawed on ice. After incubation on ice for 15 min, this mixture was heat shocked at 42°C for 90 sec. Following another incubation on ice for 5 min, the transformed cells were grown in 1–2 ml of 1× LB media at 37°C for 30–90 min in tubes on a roller wheel and plated out on

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1× LB/100 µg/ml ampicillin selective plates which were incubated at 37°C overnight.

Transformed colonies were picked from the plates directly or after screening colonies by hybridization with a specific probe. Transformant DNA was harvested by an alkaline lysis miniprep method.

In the electroporation method, the ligation mixture was spin-dialyzed in distilled deionized water (ddH$_2$O) to removing any salts. The salt free-ligated DNA (up to 5 µl, 50–500 ng) was mixed with 40 µl of electroporation competent *E. coli* cells (prepared in pure water and frozen in pure water containing 10% glycerol) and transferred into a prechilled cuvette of the electroporation unit (Bio-Rad). The cells were exposed to the 2.5 kV electric pulse and immediately transferred to an incubation tube containing 2 ml of SOC media and the cells were grown at 37°C for 90 min. The plating of transformant cells, picking the interesting colonies and preparation of transformant DNA were carried out as described for the classical method.

DNA sequencing

DNA sequencing was performed utilizing a dsDNA Cycle Sequencing System (Life Technologies) according to the manufacturer's instructions.

1. End-labeling of primer: In this kinase forward reaction, a total volume of 5 µl mixture contained 1 µl 5× kinase buffer, 1 µl [γ-^{32}P]ATP (NEN, 3000 Ci/mmol, 10 mCi/ml), 1 µl T4 polynucleotide kinase (1 U/µl), 1 pmol of sequencing primer. The mixture was gently mixed, spun briefly and incubated at 37°C for 10 min, then at 55°C

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for 5 min to terminate the reaction. The reaction mixture was briefly centrifuged and placed on ice.

2. Prereaction mixture: To 5 µl of the primer labeling reaction described above 0.1–0.2 µg DNA template, 4.5 µl 10× Taq (*Thermus aquaticus*) sequencing buffer and 0.5 µl Taq DNA polymerase (2.5 units) were added. The reaction was brought up to a total volume of 36 µl by ddH₂O. The reaction was mixed and kept on ice.

3. Performing the sequencing reaction: Four 0.5-ml microcentrifuge tubes were labeled A, C, G and T. Two microliters of termination mix-A, C, G and T were added into each tube, respectively, followed by addition of 8 µl of the prereaction mix from step 2 into each of the four sequencing reaction tubes and mixed well by pipetting. One drop of silicone oil was added to each tube and the samples were centrifuged and placed on ice. After placing the four tubes into the Thermal Cycler preheated to 95°C, the amplification was performed by 20 cycles of a denaturation step at 95°C for 30 sec, an annealing step at 40–55°C for 30 sec and an extension step at 70°C for 60 sec, followed by 10 cycles of a denaturing step at 95°C for 30 sec and an extension step at 70°C for 60 sec. The reaction was stopped by adding 5 µl of stop solution to each tube and the completed reactions were stored at –20°C until further analysis.

4. Gel electrophoresis: The sequencing reactions were analyzed on 6% polyacrylamide/TBE-urea gels. The samples were heated at 90°C for 5 min before loading. Gels were run at 32–42 watts constant power until the bromophenol blue moved just off the bottom. After fixing in a 10% glacial acetic acid and 10% methanol solution for 15 min, gels were dried at 80°C for 45 min and analyzed by autoradiography.

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Expression of the recombinant HBV polymerase

HBV polymerase was expressed from plasmid pTZHBVP (1 µg/50 µl reaction) in a rabbit reticulocyte lysate-coupled transcription/translation system in the presence of [^{35}S]methionine (Amersham, final concentration: 0.8 mCi/ml, 1000 Ci/mmol) at 30°C for 90 min, as recommended by the supplier (Promega). The ^{35}S-labeled proteins were denatured in four volumes of electrophoresis buffer containing 2% sodium dodecyl sulfate (SDS) and 8% 2-mercaptoethanol by heating to 96°C for 5 min. Protein products were analyzed on SDS-10% polyacrylamide gels, which were stained with Coomassie blue, dried and subjected to autoradiography.

In vitro polymerase priming assay of the recombinant HBV polymerase

The plasmid pTZHBVP was expressed in a coupled transcription/translation system under conditions similar to those described for expression of the recombinant polymerase but with cold methionine. The *in vitro* polymerase priming assay was modified from the previously reported study (Wang and Seeger 1992). During translation, an *in vitro* protein priming buffer containing [α-^{32}P]dGTP (NEN, final concentration: 0.2 mCi/ml, 3000 Ci/mmol), the other three dNTPs (30 µM each), 100 mM Tris-HCl (pH 7.5) and the salts (30 mM NaCl, 20 mM MgCl$_2$) was added. Templates of the HBV DR1/epsilon stem-loop sequence under the control of a phage T7 promoter (final concentration: 18 nM) were also included in this *in vitro* protein priming reaction. The reaction was incubated at 37°C for 60 min. Samples of this reaction mixture in 4 volumes of 2% SDS protein loading buffer were heated at 96°C

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for 5 min, analyzed by SDS-10% polyacrylamide gel electrophoresis (PAGE) and subjected to autoradiography.

In vitro polymerase assay of the recombinant HBV polymerase

HBV polymerase was expressed from plasmid pTZHBVP under conditions similar to those described above. The *in vitro* polymerase assay was performed under optimal conditions after dose response curves were completed for substrates and DR1/epsilon template. During translation, an *in vitro* polymerization buffer containing [α-^{32}P]dCTP (NEN, final concentration: 0.2 mCi/ml, 3000 Ci/mmol), the other three dNTPs (200 μM each), 10 mM Tris-HCl (pH 7.5), salts (10 mM NaCl, 60 mM MgCl$_2$) and 10 mM dithiothreitol (DDT) was utilized. Template (36 nM) of the HBV DR1/epsilon sequence under the control of a phage T7 promoter was included for the polymerase assay. For Southern hybridization analysis, the 4 unlabeled dNTPs at final concentrations of 200 μM each were used. Reactions were incubated at 37°C for 60 min. The polymerization activity of the recombinant HBV polymerase was analyzed by measuring the radioactivity (cpm) of [α-^{32}P]dCTP incorporated into trichloroacetic acid (TCA) precipitable polynucleotides and by detecting DNA products through electrophoresis and Southern blotting.

DNA extraction, electrophoresis and Southern blotting

DNA products from *in vitro* polymerase reactions were treated with proteinase K (1 mg/ml) in TES buffer (10 mM Tris [pH 7.8], 5 mM EDTA, 0.5% SDS) for 2 h at

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42°C. DNA was extracted first with phenol/chloroform, then with chloroform, and ethanol precipitated. DNA products were separated on 1% agarose gels and transferred to a nylon membrane. The membrane was subjected to autoradiography for detecting radiolabeled DNA or hybridized to an HBV specific minus-strand DR1/epsilon stem-loop riboprobe for detecting unradiolabeled DNA. The riboprobe was made by *in vitro* transcription with [α-^{32}P]UTP (NEN, final concentration: 0.5 mCi/ml, 3000 Ci/mmol) and the other three NTPs using a MAXIscript™ Kit T7 (Ambion). The template for the transcription was generated by PCR, which appended a T7 promoter sequence at the 5' end of the HBV DR1/epsilon stem-loop sequence (from nt 1679 to nt 1910, HBV subtype adw).

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III. Results

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

To develop an *in vitro* system for analysis of HBV polymerase, I cloned HBV polymerase cDNA into an *in vitro* expression vector pTZ19R downstream of a phage T7 promoter, designated pTZHBVP (Figure 2-1). Plasmid pTZHBVP was expressed in a reticulocyte lysate-coupled transcription/translation system in the presence of [^{35}S]methionine. Protein products were analyzed by SDS-PAGE followed by autoradiography. Three predominant ^{35}S-labeled products with molecular masses of approximately 94, 81 and 40 kDa were produced (Figure 2-2, lane 1). These protein products were not produced in the transcription/translation of luciferase plasmid pPOL(A)-luc(T7) (Promega), lysate or pTZ19R vector templates (Figure 2-2, lanes 2, 3, 4). The 94 kDa component corresponds to the full-length HBV polymerase protein and is consistent with a predicted MW for 845 amino acids. Since internal initiation at the second methionine 113 of the polymerase would give a protein product of 81 kDa, site-directed mutagenesis was used to change the second methionine to valine at this site. This mutation abolished the production of the 81 kDa product, confirming that the 81 kDa protein is generated through an internal initiation of protein translation at amino acid residue 113 of the polymerase (Figure 2-3A, lane 2). Digestion of polymerase cDNA with *Eco*R V at the position of amino acid 651, to truncate the C-terminus of the polymerase, resulted in the loss of the 94 and 81 kDa proteins and the production of smaller products, but did not affect the 40 kDa protein (Figure 2-3B, lane 2). This

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Figure 2-1 HBV polymerase expression plasmid. An HBV polymerase gene is shown with TP, spacer, RT/Pol, and RNase H domains indicated as open rectangles. The closed rectangles represent HBV sequences flanking the polymerase gene. The phage T7 promoter used for the *in vitro* transcription of HBV polymerase gene is located upstream of the polymerase gene. The positions of the first two methionines, the stop codon, the TYR 63 and the amino acid positions at the boundary of polymerase functional domains are indicated at the bottom of the figure. The nucleotide positions shown at the top of the figure were determined using the unique *Eco*R I site as reference for the first nucleotide.

5' pTZ19R T7 | TP | Spacer | RT/Pol | RNase H | 3'

2197 2310   1626 1678

AUG 1   TYR 63   AUG 113   178   336   680   UGA 846

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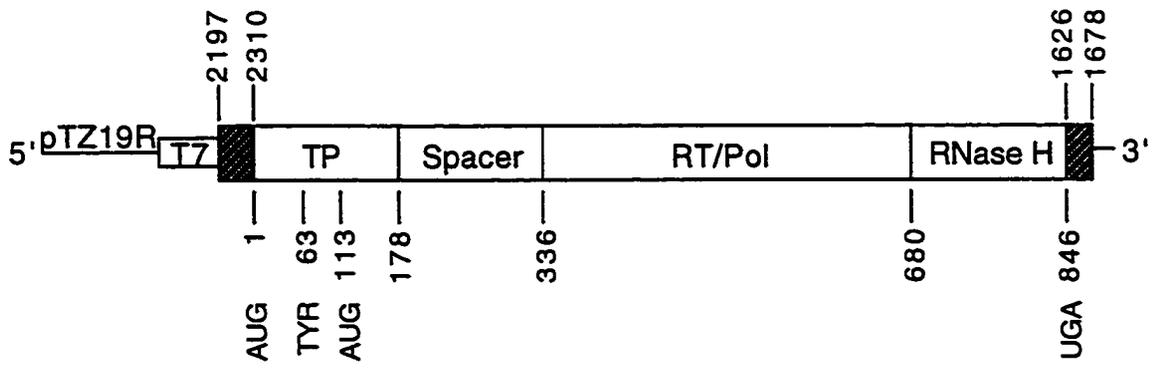
Figure 2-2   Expression of HBV polymerase in an *in vitro* coupled transcription/translation system. The HBV polymerase was expressed from the plasmid pTZHBVP in the presence of ^{35}S-methionine. The translation products were analyzed by SDS-PAGE. The three major ^{35}S-labeled products are indicated in lane 1. The products of transcription/translation of luciferase plasmid pPOL(A)-luc(T7) (lane 2), lysate alone (lane 3), and pTZ19R vector template (lane 4) are shown.
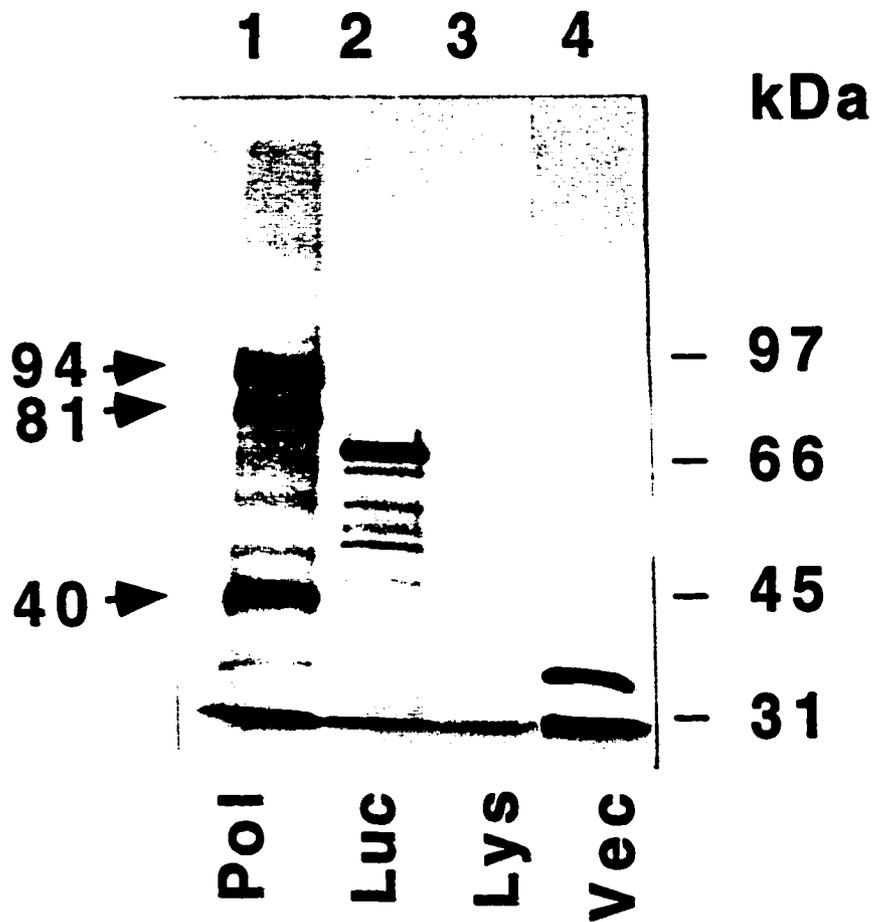
Figure 2-3 Characterization of the *in vitro* expressed HBV polymerase. (A) Site-directed mutagenesis study of HBV polymerase gene. The second methionine at amino acid 113 of the polymerase was changed to valine by PCR-based mutagenesis. The mutated plasmid pTZHBVPM was expressed and analyzed as described in the legend for Figure 2-2. The translation products of the mutant plasmid are shown in lane 2. The translation products of pTZHBVP and lysate alone are shown in lanes 1 and 3, respectively. (B) Truncation analysis of HBV polymerase gene. The HBV polymerase expression plasmid pTZHBVP was truncated at amino acid 651 by digestion with *Eco*R V. The C-terminus truncated plasmid pTZHBVP was expressed and analyzed as described in the legend of Figure 2-2. The results of translation of the truncated polymerase gene are shown in lane 2. The translation products of the plasmid pTZHBVP and the lysate control are shown in lanes 1 and 3, respectively.

B.

A.
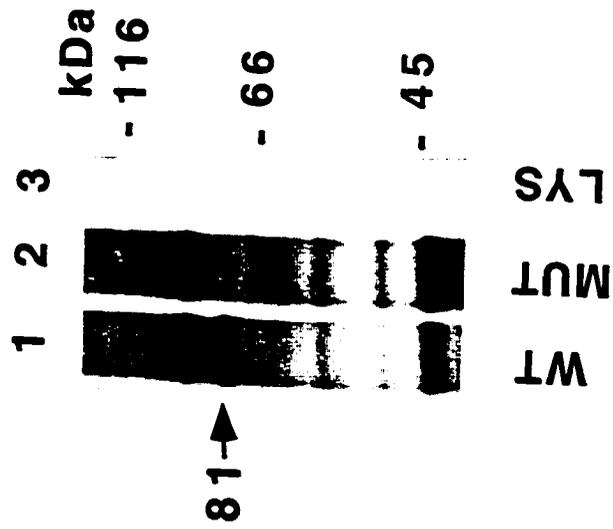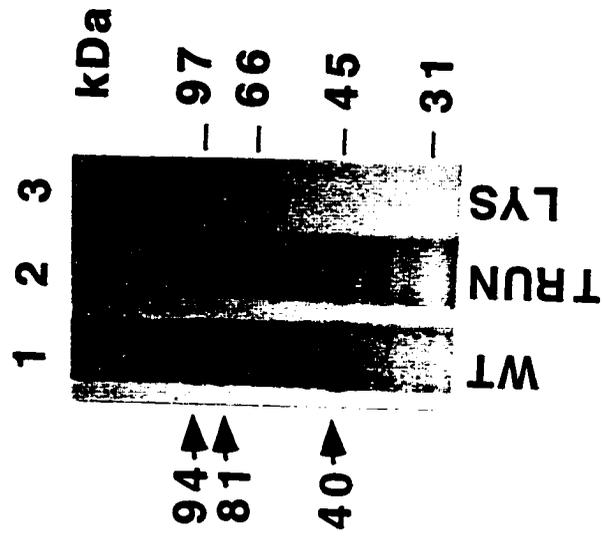
suggested that the 40 kDa species likely arose from the amino-terminal portion of the protein.

[α-^{32}P]dGTP labeling of recombinant HBV polymerase

To test the protein priming activity of the recombinant HBV polymerase, plasmid pTZHBVP was expressed in the coupled transcription/translation system in the absence of ^{35}S-methionine. During translation, *in vitro* protein priming buffer containing [α-^{32}P]dGTP and the other three dNTPs (30 μM each) was added. Templates (18 nM) of the HBV DR1/epsilon stem-loop sequence under the control of a phage T7 promoter were also included for the protein priming reaction. The transcription of this template in the coupled transcription/translation system produced a 231 nt RNA sequence bearing the HBV DR1/epsilon stem-loop. The DR1 and epsilon stem-loop sequences are known to be important for the initiating viral genome replication and for the activity of the polymerase (Lanford et al. 1997; Tavis and Ganem 1994, 1996; Tavis et al. 1998; Wang et al. 1993, 1994). The protein-priming products were analyzed by SDS-10% PAGE. Autoradiography revealed that the 94 kDa protein corresponding to the full-length polymerase was labeled with [α-^{32}P]dGTP (Figure 2-4, lane 2). In addition, a 40 kDa protein, which probably arose from the amino-terminus of the polymerase, was also labeled (Figure 2-4, lane 2). However, the 81 kDa protein arising from internal initiation at the second methionine of the polymerase was not labeled in the priming assay (Figure 2-4, lane 2). This is very likely due to the lack of tyrosine residue 63, which is known to be the site involved in the covalent linkage of the polymerase to the first deoxyribonucleotide (e.g., ^{32}P-dGMP) in the

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Figure 2-4 [α-^{32}P]dGTP labeling of recombinant HBV polymerase in an *in vitro*

polymerase priming reaction. HBV polymerase was expressed from plasmid

pTZHBVP in a coupled translation/transcription system in the absence of

[^{35}S]methionine. During translation, *in vitro* protein priming buffer containing [α-

^{32}P]dGTP and the other three dNTPs was added. Template RNA bearing the HBV

DR1/epsilon stem-loop sequence was also included in this *in vitro* protein priming

reaction. Aliquots of this reaction in 4 volumes of 2% SDS protein loading buffer were

heated at 96°C for 5 min, and analyzed by SDS-10% PAGE and autoradiography. The

results of the *in vitro* priming reaction are shown in lane 2. The products of the control

reactions, without polymerase expression plasmid (lane 3) and without DR1/epsilon

stem-loop RNA sequences (lane 4), are also presented. The translational products of

the plasmid pTZHBVP in the presence of [^{35}S]methionine are shown in lane 1.

1 2 3 4   kDa

94→   − 97
81→   − 66

       − 45

40→   − 31

35S   32P
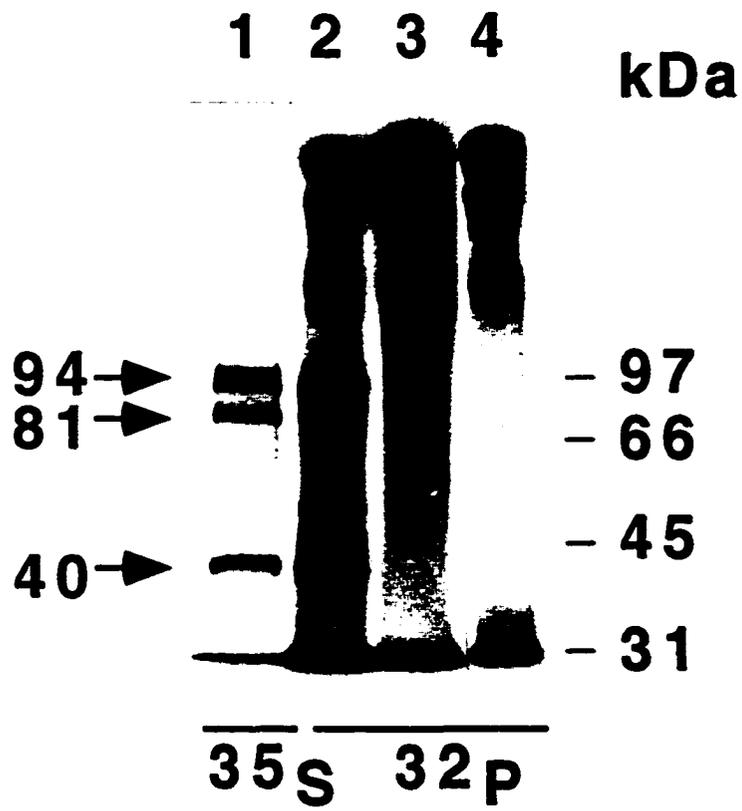
protein priming step (Lanford et al. 1997). There was no [α-^{32}P]dGMP labeling of polymerase polypeptides in the absence of recombinant polymerase (Figure 2-4, lane 3, lysate control) or in the absence of DR1/epsilon stem-loop templates (Figure 2-4, lane 4). The radioactivity appearing at the top of the gel could be due to the nonspecific binding of radioisotope to proteins in the lysate (Figure 2-4, lanes 2, 3, 4). The polymerase priming reaction was also performed with increasing amounts of a phage T7 promoter-driven DR1/epsilon stem-loop template. The amounts of ^{32}P-labeled polymerase polypeptides increased as the amount of DR1/epsilon stem-loop template was increased (Figure 2-5, lanes 2, 3, 4, 5). These *in vitro*-expressed polymerase polypeptides were likely labeled through covalent linkage to [α-^{32}P]dGMP because the polypeptides remained labeled after boiling in 2% SDS protein loading buffer (Figure 2-4). The results indicate that the human hepadnaviral polymerase expressed in the rabbit reticulocyte lysate system has protein priming activity. This activity resides in the N-terminus of the polymerase, presumably due to the N-terminus of the polymerase containing the tyrosine 63 priming site, which is known to be covalently linked to [α-^{32}P]dGMP during the priming. In addition, this *in vitro* protein priming reaction is dependent on the *in vitro*-expressed HBV polymerase and an RNA fragment bearing the HBV DR1/epsilon stem-loop sequence.

Template-dependent polymerization activity of the recombinant HBV polymerase

To assay for the reverse transcription activity of recombinant HBV polymerase, HBV polymerase was expressed from plasmid pTZHBVP under conditions similar to those described above. During translation, an *in vitro* polymerase buffer containing

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Figure 2-5 Effects of HBV DR1/epsilon stem-loop template on HBV polymerase priming activity. An *in vitro* protein priming assay was performed and analyzed as in Figure 2-4 except with increasing amounts of T7 driven HBV DR1/epsilon stem-loop template. [α-^{32}P]dGMP labeled HBV polymerase in an *in vitro* priming reaction in the absence of DR1/epsilon templates (lane 2) and in the presence of 18 nM DR1/epsilon element (lane 3), 36 nM DR1/epsilon element (lane 4), 54 nM DR1/epsilon element (lane 5), and lysate with 54 nM DR1/epsilon (lane 6) are shown. ^{35}S-methionine labeled HBV polymerase expressed in the coupled transcription/translation system is in lane 1.

^{35}S ^{32}P

Epsilon[nM] 0   0   18   36   54   54   kDa

94▶

81▶   −97

     −66

40▶   −45

     −31

1   2   3   4   5   6

[α-^{32}P]dCTP as well as the other three dNTPs (200 μM each) was added. For the

Southern blot study, four cold dNTPs were used in the reaction. The template (36 nM)

of HBV DR1/ epsilon stem-loop sequence under the control of a phage T7 promoter

was included for the polymerase activity assay. Nucleotide incorporation by the

recombinant polymerase, estimated by measuring the ^{32}P-radioactivity in the TCA

precipitable fraction, was $1.2 \times 10^5 \pm 3.7 \times 10^3$ cpm, about 50-fold higher than the

control (Figure 2-6). The products from the *in vitro* polymerase reactions were

digested with proteinase K, extracted with phenol/chloroform, ethanol precipitated,

separated on a native gel and analyzed by Southern blot. Radiolabeled single-stranded

DNA was detected on an autoradiogram. The polymerase produced radiolabeled

ssDNA products with sizes between 100 to 500 nt (Figure 2-7A. Lane 1). Unlabeled

ssDNA was detected by hybridization with HBV minus-strand specific riboprobes.

Our experiments showed that the unlabeled DNA, with molecular weights similar to the

labeled DNA species, hybridized to minus-strand specific DR1/epsilon stem-loop

riboprobes (Figure 2-7B, lane 2). The DNA products did not hybridize with a pTZ19R

vector control probe (data not shown). In addition, there was no detection of

radiolabeled DNA nor unlabeled DNA in the absence of recombinant polymerases (Fig.

2-7A, B, lane 3, lysate control). In the polymerase reaction without DR1/epsilon stem-

loop template no DNA synthesis was detected by measuring the radioactive counts of

^{32}P-dCTP incorporation into TCA precipitable polynucleotides $(4.9 \times 10^3 \pm 1.4 \times 10^2$

cpm), which was similar to the negative control (lysate alone). Treatment with DNase I

but not with pancreatic RNase prior to electrophoresis completely abolished the

polymerization products. Reduced amounts of DNA product were detected without

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Figure 2-6 *In vitro* polymerase activity of the recombinant HBV polymerase. Plasmid pTZHBVP or negative control (lysate alone) was expressed as described in the legend to Figure 2-4. During translation, *in vitro* polymerase buffer containing [α-^{32}P]dCTP and the other three dNTPs (200 μM each) was added. A T7 driven HBV DR1/epsilon sequence (36 nM) was included as a template. The polymerase activity was estimated by measuring the radioactivity counts (cpm) of [α-^{32}P]dCTP incorporated into TCA-precipitable polynucleotides.

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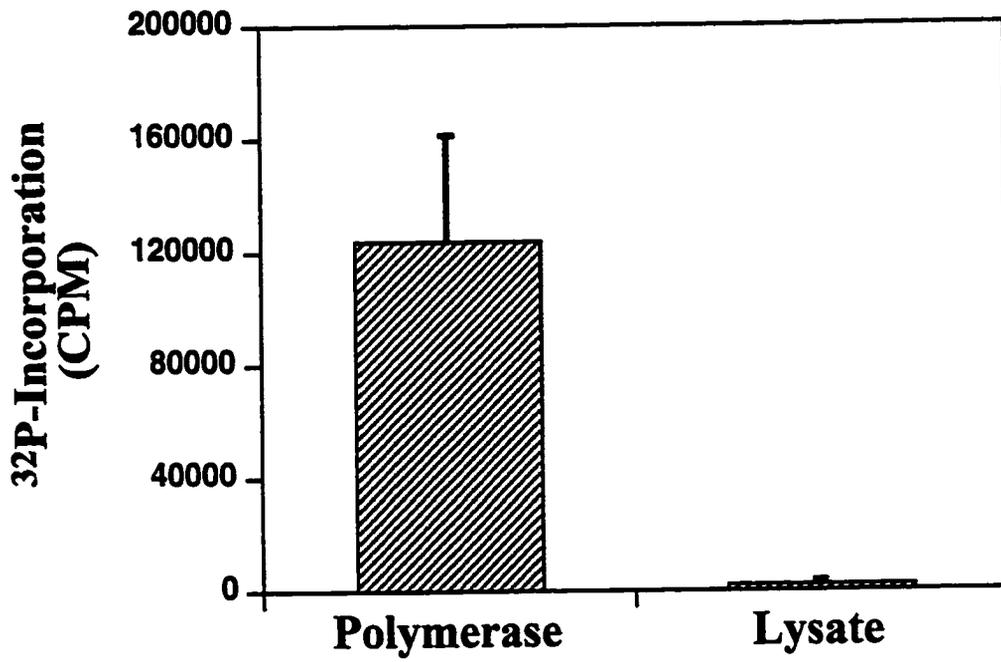
Figure 2-7 Template-dependent polymerase activity of the recombinant HBV polymerase. The *in vitro* polymerase assay was performed as described in the legend to Figure 2-6. The DNA products of this reaction were digested with proteinase K, extracted with phenol/chloroform and ethanol precipitated. DNA was then subjected to electrophoresis on agarose gels and transferred to a nylon membrane. The membrane was subjected to autoradiography to detect radiolabeled DNA (Figure 2-7A, lane 1, radiolabeled DNA; lane 2, unradiolabeled DNA; lane 3, lysate). The same membrane was then hybridized with HBV specific minus-strand riboprobes containing the DR1/epsilon stem-loop sequence for the detection of HBV specific DNA synthesis (Figure 2-7B, lane 1, radiolabeled DNA; lane 2, unradiolabeled DNA; lane 3, lysate).

B.

		bp
		738
		615
		492
		369
		246
		123

3   2   1

POL   -   +   +
32P   +   -   +

A.

		bp
		738
		615
		492
		369
		246
		123

3   2   1

POL   -   +   +
32P   +   -   +
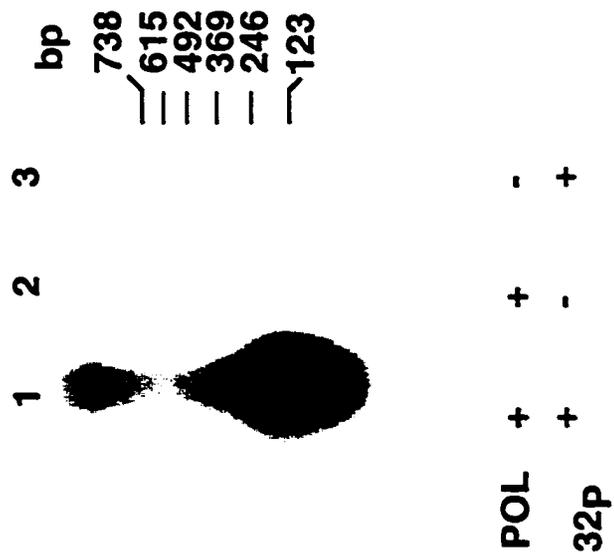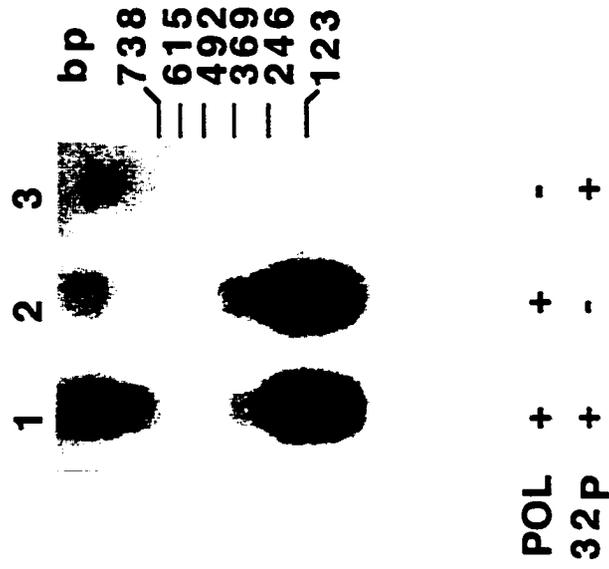
proteinase K treatment prior to phenol extraction (Addendum 2-1). Taken together, our data indicate that HBV polymerase expressed *in vitro* possesses true polymerization activity. The recombinant polymerase utilizes the HBV DR1/epsilon stem-loop RNAs as template for its reverse transcription to synthesize DNA products with sizes of approximately 100–500 nt. Some of these DNA products are linked to the recombinant polymerase, since DNA products that are linked to polymerase were removed from the aqueous phase by phenol extraction unless the DNAs were first treated with proteinase K (Addendum 2-1).

Addendum 2-1 Analysis of DNA products by proteinase K treatment. The *in vitro* polymerase assay was performed as described in the legend to Figure 2-6. The DNA products of this reaction were digested with or without proteinase K, extracted with phenol/chloroform and ethanol precipitated. DNA was then subjected to electrophoresis on 15% denaturing polyacrylamide gels. The gel was subjected to autoradiography to detect radiolabeled DNA (Addendum 2-1, lane 1, proteinase K treatment; lane 2, without proteinase K treatment).

Proteinase K

+   −

bs

369 —

246 —

123 —

1   2
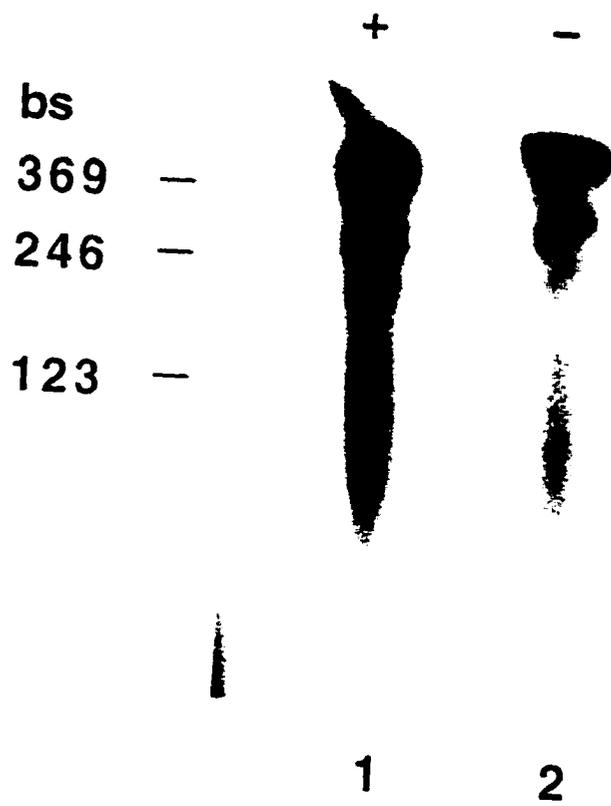
VI. Discussion

I have expressed a full-length and two smaller species of HBV polymerase proteins in reticulocyte lysates in the absence of the viral nucleocapsid protein and the *cis* DR1/epsilon stem-loop sequence. In an attempt to produce an enzymatically active HBV polymerase, I translated HBV polymerase in the presence of a phage T7 promoter-driven HBV DR1/epsilon stem-loop sequence, which will produce a transcription product with an expected length of 231 nt, provided in *trans*. The interaction between the viral epsilon stem-loop and Pol during or shortly after translation was able to induce or activate Pol enzymatic activities, possibly via a structural alteration of Pol (Lanford et al. 1997; Tavis and Ganem 1996; Tavis et al. 1998; Wang et al. 1994).

This study provides the first evidence that HBV polymerase produced in a reticulocyte lysate system can correctly initiate reverse transcription *in vitro* in the absence of other viral proteins and the stem-loop in *cis*. It is now clear that the encapsidation of core protein is not required for the initiation step of viral replication. The results suggest that recombinant polymerase can recognize the replication initiation site of the epsilon stem-loop sequence in *trans* and serve as a protein primer for *in vitro* priming and polymerase reactions, even though hepadnaviral polymerase *in vivo* employs its own mRNA (pgRNA) with a *cis* DR1/epsilon stem-loop as template for the protein priming and reverse transcription functions (Hirsch et al. 1991; Junker-Niepmann 1990). Furthermore, the protein derived from the amino-terminus of the polymerase was covalently labeled by $[\alpha\text{-}^{32}\text{P}]$dGMP in an *in vitro* priming reaction in the study. Both terminal protein and Pol/RT domains of the viral polymerase are required for the protein priming activity since mutations in either domain will prevent priming activity (Lanford et al. 1997; Weber et al. 1994; Zoulim and Seeger 1994). I

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speculate that full-length polymerase transcomplements the priming activity of the terminal protein. Consistent with this, Lanford recently observed that the terminal protein and reverse transcription domains expressed separately in a baculovirus system are able to complement each other in the presence of the epsilon stem-loop sequence (Lanford et al. 1997).

The recombinant polymerase expressed in the reticulocyte lysate generated DNA products in an *in vitro* polymerase reaction. Further analysis indicates that the recombinant polymerase uses the *trans* HBV DR1/epsilon stem-loop sequence as a template for its polymerization activity rather than using the Pol mRNA lacking a *cis* epsilon stem-loop. This suggests that HBV polymerase initiates DNA synthesis solely with a template of the DR1/epsilon stem-loop sequence without the polymerase mRNA sequence being involved in the initiation. These findings are in accord with the observation that the Pol recognizes and initiates the minus-strand synthesis at a bulge region of the epsilon stem-loop, the replication origin for the virus (Tavis et al. 1994; Wang and Seeger 1993). Moreover, the *in vitro* polymerization reaction produced HBV-specific DNA molecules with minus polarity, demonstrating the correct reverse transcription activity of HBV polymerase. The *in vitro* polymerase reaction generated a majority of DNA product of ~200 nt, but a minor species of ~400 nt was also detected. The smaller product corresponds to the single-length and the larger product corresponds to double-length HBV DR1/epsilon stem-loop template. I hypothesize that the major species of DNA is a product of reverse transcription of the 231 nt RNA template containing the epsilon stem-loop. The minor product which is double the size of the major product could result from a hairpin mechanism which allows continuous synthesis of DNA from the newly synthesized minus-strand DNA to generate a product with a length twice of the original RNA template (Figure 2-8).

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Figure 2-8   Schematic diagram of DNA synthesis by the recombinant viral polymerase using an RNA template bearing the HBV DR1/epsilon stem-loop motif. (A) HBV polymerase uses the bulge region sequence as a template to initiate minus-strand DNA synthesis and produces a short oligodeoxyribonucleotide. (B) The polymerase synthesizes minus-strand DNA until it reaches the 5' end of the template and falls off. (C) The polymerase uses a hairpin-like mechanism to synthesize products longer than the template.

A.

5' ———————————— DR1 ———— 3'
RNA

B.

(-) DNA

3' ◄———————————— RNA

DR1

C.

(-) DNA

5' ———————————— DR1 ———— 3'
RNA

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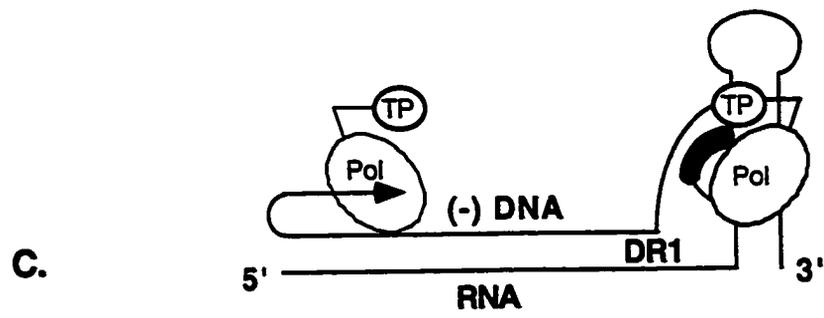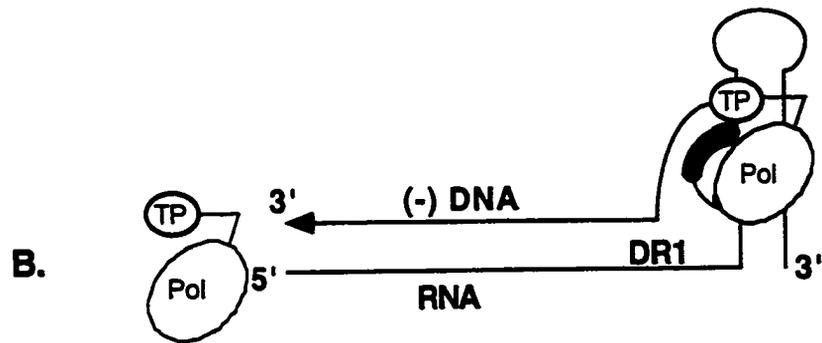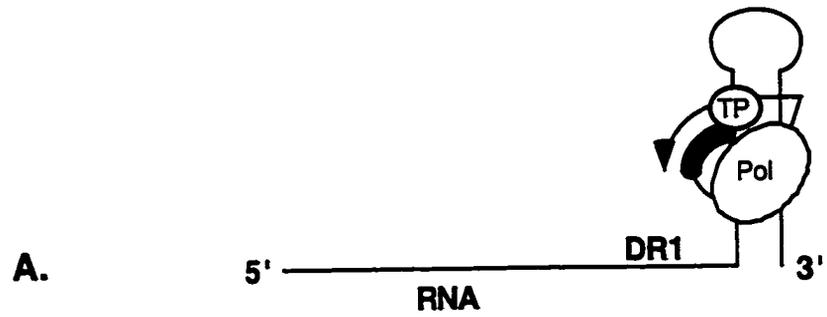
The creation of an *in vitro* translated HBV polymerase in a reticulocyte lysate system that possesses both protein priming and reverse transcription activities has provided us with a novel and simple approach to the study of HBV polymerase functions. In addition, this *in vitro* assay system will be useful in studying the mechanism of action of antiviral compounds inhibiting HBV replication. For example, purine analogues such as 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR) may selectively inhibit the protein priming step of this *in vitro* assay (Howe et al. 1996), whereas (–)-ß-L-2',3'-dideoxy-3'-thiacytidine-5'-triphosphate (3TC-TP) would be expected to be a chain terminator substituting for dCTP (Severini et al. 1995).

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CHAPTER 3[*]

HEPADNAVIRALPOLYMERASE SUPPRESSES THE TRANSLATION OF VIRAL CORE mRNA *IN VITRO*: A CONSERVED MECHANISM ACROSS SPECIES

I. Introduction

Hepadnaviruses are a family of small, enveloped, relaxed circular and partially duplex DNA viruses that replicate through reverse transcription of an RNA intermediate or pregenomic RNA (pgRNA) in the viral core particle located in the cytoplasm of infected cells (Summers and Mason 1982). Well characterized members of this family are the hepatitis B viruses of humans (HBV) (Gust et al. 1986), woodchucks (WHV) (Summers et al. 1978), ducks (DHBV) (Mason et al. 1980) and ground squirrels (GSHV) (Marion et al. 1980). All hepadnaviruses share similar features of virion size and ultrastructure, polypeptide and antigenic composition, genome size and genetic organization and unique replication strategies.

Following virus entry into a liver cell, a relaxed and partially duplexed viral genome is converted into a covalently closed circular DNA (cccDNA) that serves as a template for the transcription of RNA of genomic and subgenomic length. The shortest genome transcript or pgRNA is bifunctional, serving as an mRNA for both core protein and polymerase protein as well as a pgRNA to be encapsidated together with

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polymerase into a nucleocapsid. The encapsidation is initiated by a high affinity binding of viral polymerase to an epsilon sequence (a stem-loop structure containing the core gene initiating AUG codon) at the 5' end of pgRNA (Chiang et al. 1992; Hirsch et al. 1991; Junker-Niepmann et al. 1990; Knaus and Nassal 1993; Pollack and Ganem 1993). A sequence of approximately 900 nucleotides downstream from the epsilon is also required for effective encapsidation in the DHBV system (Calvert and Summers 1994; Hirsch et al. 1991). How the pgRNA is switched from its mRNA role, where the presence of translational machinery on the pgRNA would interfere with the encapsidation of pgRNA, to its pgRNA role for replication is not clear.

Previous work by Dr. Howe in our laboratory revealed that translation of the DHBV core mRNA was specifically inhibited by the DHBV polymerase-containing lysate *in vitro* (Howe and Tyrrell 1996). The polymerase-lysate-mediated repression of the DHBV core synthesis was attributed to the DHBV polymerase protein but not the polymerase mRNA, since the translation lysate from a mutant of polymerase expression construct that eliminated the polymerase translation could not inhibit the translation of DHBV core mRNA. Deletion analysis of the core expression plasmid indicated that sequences extending from nt 140 to 828 were required for the polymerase-mediated repression and sequences from nt 401 to 870 were sufficient for transferring the polymerase-mediated inhibition onto a heterologous mRNA.

As a high degree similarity of structure and replication strategies remains despite the divergence of the different species specificity amongst members of the hepadnaviral family, the objective of this chapter is to look at the conservation of translational repression of core mRNA by the viral polymerase. These studies were done in rabbit reticulocyte lysates similar to the homologous studies completed by Dr. Howe. The results of this study demonstrated that translational repression by the viral polymerase

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protein is conserved in the hepadnaviridae family. The implication of this translational

inhibition of the hepadnaviral polymerase in the viral replication will be discussed.

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II. Materials and Methods

Construction of expression plasmids

The molecular manipulations described below are standard cloning procedures used to create the *in vitro*-expression plasmids needed for the study (Sambrook et al. 1989). The sequence of HBV subtype adw is numbered using the first nucleotide of an unique *Eco*R I site as the first nucleotide. An *in vitro*-expression plasmid pTZHBVP of HBV polymerase was constructed from three DNA fragments (see Chapter 2, Materials and Methods). Cloning sites, a *Pst* I site at the 5' end and a *Xma* I site at the 3' end, were incorporated into appropriate polymerase chain reaction (PCR) primers (see Appendix for PCR primers). A complete HBV polymerase gene (2538 bp) with flanking sequence was obtained by ligation of the above three fragments and subsequently inserted into an *in vitro*-expression vector pTZ19R (Pharmacia Biotech) under the control of a bacteriophage T7 RNA polymerase promoter. The ligated DNA was transformed, harvested and the recombinant plasmid was verified by restriction mapping and DNA sequence analyses across the sites of ligation as described in Chapter 2, Materials and Methods.

The *in vitro* HBV core expression plasmid pBSHBVCL, containing an entire HBV core protein open reading frame (ORF, 558 bp) and a 941 bp sequence downstream from the core ORF, is controlled by a bacteriophage T7 promoter. The cloning process was completed in two steps. First, to construct a pBSHBVC plasmid, the 558 bp cDNA of the core ORF was PCR amplified as described in Chapter 2, Materials and Methods from a plasmid pKSVHBV1 (Seifer et al. 1990). The cloning sites, an *Eco*R I site at the 5' end and a *Bam*H I site at the 3' end, were incorporated

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into the appropriate PCR primers (see Appendix for PCR primers). The amplified fragment was digested with *Eco*R I and *Bam*H I and ligated with an *in vitro*-expression vector pBluescript SK⁻(Stratagene) which had been previously cut with corresponding enzymes. Second, to construct a pBSHBVCL plasmid, a 1070 bp fragment, containing the C-terminal sequence (129 bp) of the core ORF and the downstream sequence (941 bp), was excised from the plasmid pTZHBVP by restriction enzymes *Bsp*E I and *Avr* II and directly inserted into the pBSHBVC plasmid previously cut with *Bsp*E I and *Spe* I enzymes. The constructs were transformed, purified and confirmed as described in Chapter 2, Materials and Methods.

Plasmids pTZ19Rcore and pTZ19Rpol, which are T7 promoter driven DHBV core and polymerase expression constructs, respectively, were provided by Anita Y. M. Howe, a graduate student who completed her Ph.D. in our laboratory in 1995. Plasmid pT7-7HisWHVcore, which contains an entire WHV core ORF under the control of a T7 promoter, was provided by Rajan George, a research associate in our laboratory. Plasmids pSP72Tat and pSP72T4, which have an entire human immunodeficiency virus (HIV) Tat and a human CD4 ORF cloned downstream from a T7 promoter, respectively, were provided by L.-J. Chang (University of Florida, Gainesville, FL, USA).

In vitro transcription

Plasmids pTZ19Rpol, pTZHBVP, pSP72Tat, pBSHBVCL, pT7-7HisWHVcore and pSP72-T4 were linearized with *Eco*R I, *Xma* I, *Sph* I, *Nco* I and *Bam*H I, respectively. The linearized plasmids were purified by phenol/chloroform extraction and ethanol precipitation. One microgram of each linearized plasmid was transcribed into mRNA in 50 μl of transcription mixture using a T7 Ambion

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MEGAscript kit following the manufacturer's protocol (Ambion Inc.). After 4 h of incubation at 37°C, all transcripts were evaluated on an 1% agarose gel to verify their integrity and the transcription efficiency. The resulting transcripts were treated with RNase-free DNase, phenol/chloroform extracted and ethanol precipitated. The mRNAs were dissolved in diethyl pyrocarbonate (DEPC)-treated water to a final concentration of 1 μg/μl and stored at −20°C in aliquots.

In vitro translation

The transcripts of pTZ19Rpol, pTZHBVP and pSP72Tat plasmids were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine (Amersham, final concentration of 0.8 mCi/ml, 1000 Ci/mmol) and methionine-free amino acids and incubated at 37°C for 60 min as recommended by the manufacturer (Promega Co.). Twenty-five microliters of translation mixture, containing [^{35}S]methionine, methionine-free amino acids and transcripts of pBSHBVCL, or pTZ19Rcore, or pT7-7HisWHVcore or pSP72-T4, were translated with equal or various amounts of hepadnaviral DNA polymerase or HIV Tat protein-containing lysate. The final volume of the reaction mixture was brought up to 50 μl by the addition of lysate containing [^{35}S]methionine and methionine-free amino acids. The translation products were treated in 2% sodium dodecyl sulfate (SDS) and 8% 2-mercaptoethanol sample treatment buffer, boiled for 5 min and analyzed by SDS-12.5% polyacrylamide gel electrophoresis (PAGE). Unincorporated label was removed by soaking the gel in 40% (vol/vol) methanol/10% (vol/vol) glacial acetic acid for 2 h. The translation products were identified by autoradiography after drying at 80°C for 2 h.

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Immunoprecipitation

Fifty microliters of each *in vitro*-translated product of HBV core mRNA in the absence or presence of HBV polymerase were incubated on ice with 150 µl of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl [pH 8.0]) and 2 µl of polyclonal rabbit anti-HBcAg antibodies (DAKO corporation, Dimension Laboratories, Inc.). After 1 h of incubation, 100 µl of 10% (vol/vol) protein A-Sepharose (Pharmacia Biotech.) in RIPA buffer were added. The suspension was rocked at 4°C for 1 h and then transferred to a Spin-X tube (Fisher Scientific), in which the immunoprecipitated complex was collected and washed three times with RIPA buffer. After being boiled in 40 µl of 2% SDS sample treatment buffer, the denatured protein was separated from protein A-Sepharose by spinning at room temperature for 20 sec in a microfuge and the supernatant was analyzed by SDS-12.5% PAGE and the ^{35}S labeled product detected by autoradiography.

Isolation of mRNA and Northern blot

The mRNAs were extracted with TRIzol™ reagent (GIBCO BRL Life Technologies) according to the protocol described by the manufacturer. *In vitro* translations of lysate alone, or DHBV mRNA or DHBV mRNA in the presence of DHBV polymerase were carried out as described in *in vitro* translation above. After translation, 1 ml of TRIzol™ reagent was added to the translation mixture. The mRNAs were extracted with 0.2 ml of chloroform per 1 ml of TRIzol™ reagent used. The mRNAs in the aqueous phase were transferred to an Eppendorf tube and

precipitated by equal volumes of ice-cold isopropanol and incubated at room temperature for 10 min. The supernatant was removed by centrifugation at 12,000 rpm for 10 min at 4°C in a microfuge. The RNA pellets were washed with cold 75% ethanol in DEPC-treated water (≥ 1 ml of 75% ethanol per 1 ml of TRIzol™ reagent) and centrifuged at 7,500 rpm at 4°C for 5 min in a microfuge. The air-dried RNA pellets were dissolved in TE (pH 7.6) in DEPC-treated water.

One third of each RNA sample was separated on a 0.8% agarose-2.2 M formaldehyde gel as described by Sambrook et al. 1989. The RNAs were transferred to a Hybond-N membrane (Amersham Life Science). The RNA blot was prepared and hybridized with a ^{32}P-labeled DHBV core DNA specific probe following a standard protocol (Sambrook et al. 1989). The RNAs were visualized by autoradiography.

III. Results

HBV polymerase inhibits the translation of HBV core mRNA *in vitro*

To translate the HBV core protein *in vitro*, I constructed an expression plasmid pBSHBVCL containing the HBV core ORF with a T7 promoter (Figure 3-1). The plasmid was transcribed *in vitro* prior to translation. One microgram of HBV core mRNA was translated in a final volume of 50 µl of the rabbit reticulocyte lysate translation mixture containing [^{35}S]methionine. Two microliters of the translation product were analyzed by SDS-12.5% PAGE and the translation products were identified by autoradiography. A protein product resolving at approximately 21 kDa was observed, corresponding to the molecular weight of HBV core protein (Figure 3-2, lane 2). A shorter product was also obtained which probably represents a degradation product. The translation of the reticulocyte lysate in the absence of exogenous mRNA did not yield any protein products (Figure 3-2, lane 1).

To investigate the effects of HBV polymerase on the HBV core mRNA translation, HBV polymerase expression plasmid pTZHBVP was first translated *in vitro*. The translation efficiency of the polymerase was verified by measuring the [^{35}S]methionine incorporation and visualizing the translation product by SDS-PAGE. Twenty-five microliters of the translation mixture containing HBV core mRNA were translated with an equal amount of HBV polymerase-containing reticulocyte lysate translated previously. The translation product of HBV core mRNA in the presence of polymerase was examined by SDS-12.5% PAGE. The translation of HBV core mRNA was almost completely inhibited by the addition of HBV polymerase at a ratio

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Figure 3-1 Schematic diagram of HBV pgRNA and the HBV core expression construct. A. Diagram of pgRNA with its stem-loops and ORFs. The pgRNA, with the 5' copy and 3' copy stem-loop, 5' cap and 3' poly (A) tail, is shown by a linear cartoon. The core and polymerase ORFs are indicated by open boxes. The initiation codon (AUG) of the core ORF residing within the 5' stem-loop is depicted by a small triangle. The position of the nucleotides at the boundaries of the core and polymerase ORFs are noted at the bottom of the figure. B. HBV core expression construct. The HBV DNA is shown as a hatched box and the HBV core gene is indicated by an open box. The T7 promoter sequence is shown as an arrow. The numbers of the nucleotides at the boundaries of the core and HBV sequences are marked at the bottom of the figure.

A.

B.

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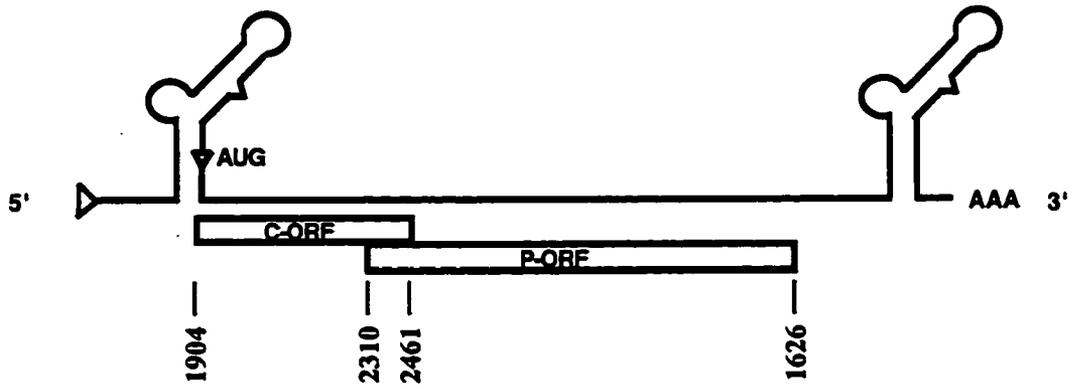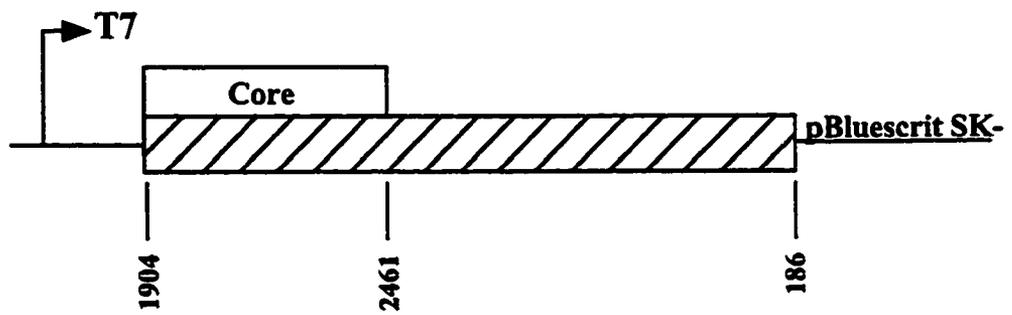
Figure 3-2 Expression of HBV core mRNA in the absence or presence of HBV polymerase-containing lysates. Reticulocyte lysates were translated with no additional mRNA, with HBV core mRNA or with HBV core mRNA in the presence of HBV polymerase-containing lysate. The translation products labeled with [^{35}S]methionine were analyzed by SDS-PAGE and detected by autoradiography. Lane 1, reticulocyte lysate (LYS) translation alone; lane 2, HBV core mRNA (HC mRNA) translation; lane 3, HBV core mRNA translation in the presence of HBV polymerase lysate (HC mRNA + HP) are shown. Arrows indicate the migration of HBV polymerase (HP) and HBV core protein (HC).
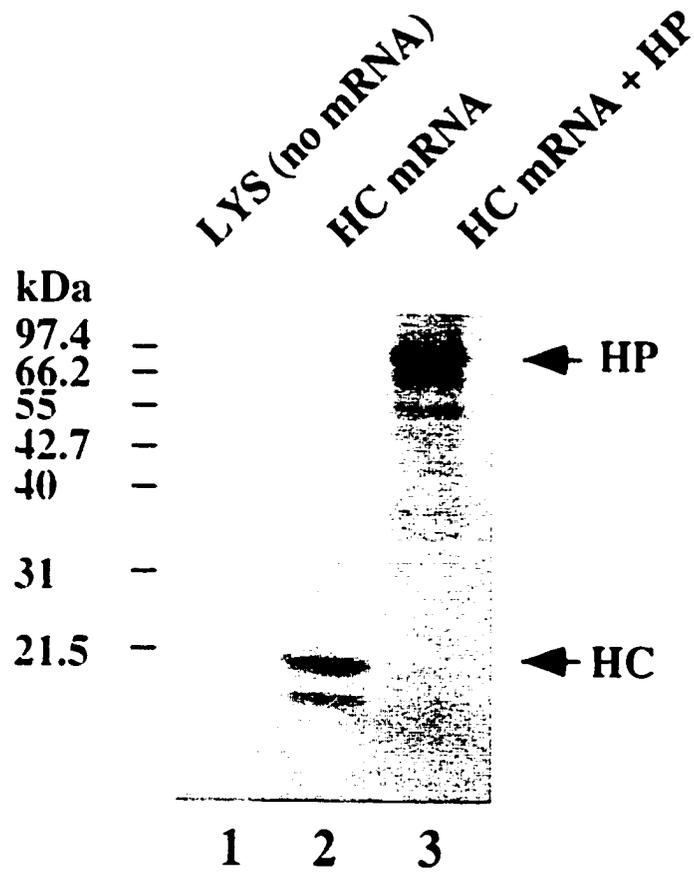
of one volume of core protein translation mixture to one volume of polymerase-containing lysate (Figure 3-2, lane 3).

The dose-dependent effect of HBV polymerase on the translation of HBV core mRNA was also determined. In this experiment, various amounts of HBV polymerase-containing reticulocyte lysate (0, 5, 10, 15, 20, 25 µl) translated previously were added to 25 µl of HBV mRNA translation mixture. The final volume of each reaction was brought up to 50 µl by addition of reticulocyte lysate containing [^{35}S]methionine and methionine-free amino acids. The final reaction products were analyzed by SDS-12.5% PAGE. The results shown in Figure 3-3 revealed that there was a progressive decrease in HBV core protein synthesis with the increase in polymerase-containing lysate. There was no change in the amount of luciferase protein in each lane, used for normalization of sample loading (Figure 3-3). Therefore, the results clearly indicate that HBV polymerase inhibits the translation of HBV core mRNA and this inhibition is proportional to the amount of polymerase added.

Immunoprecipitation of HBV core protein translated in the presence and absence of HBV polymerase

To verify that the 21 kDa protein product represented the HBV core protein, an immunoprecipitation analysis using polyclonal rabbit anti-HBcAg antibodies was carried out. Each *in vitro*-translated product of HBV core mRNA in the absence or presence of various amounts of polymerase-containing lysate was immunoprecipitated with anti-HBcAg antibodies. The immunoprecipitated protein was analyzed by SDS-

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Figure 3-3 Dose-dependent effect of HBV polymerase-containing lysate on the translation of HBV core mRNA *in vitro*. *In vitro*-produced HBV core mRNA (1 µg) was translated in 25 µl of translation mixture lysate in the absence or presence of increasing amounts (0, 5, 10, 15, 20, 25 µl) of HBV polymerase-containing lysate translated previously *in vitro*. Two microliters of ^{35}S-labeled translation product were electrophoresed on SDS-12.5% PAGE and detected by autoradiography. Arrows indicate HBV polymerase (HP), HBV core protein (HC) and luciferase (Luc). ^{35}S-labeled luciferase protein was added into each sample for controlling of sample loading.

	0	5	10	15	20	25	HP (µl)
	+	+	+	+	+	+	HC mRNA

kDa
97.4 —
66.2 —
55 —
42.7 —
40 —

31 —
21.5 —
14.4 —

← HP
← Luc

← HC
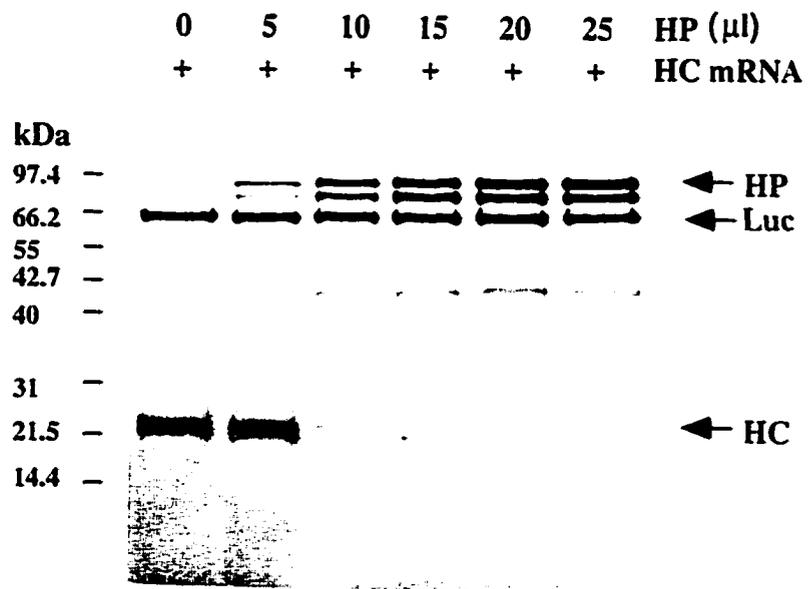
12.5% PAGE and detected by autoradiography. The 21 kDa product and the smaller product were immunoprecipitated by the HBV core protein specific antibodies as shown in Figure 3-4. With increasing amounts of polymerase-containing lysate, HBV core protein synthesis was progressively inhibited as illustrated in Figure 3-4. Thus, we concluded that the 21 kDa protein was in fact the translational product of the HBV core mRNA.

HBV polymerase specifically inhibits the HBV core mRNA translation *in vitro*

In order to demonstrate that the translational inhibition of HBV core mRNA in the presence of HBV polymerase was not a general inhibitory effect in this system, HBV core mRNA was expressed in the presence of HBV polymerase-unrelated protein such as HIV Tat protein. In this study, one microgram of HBV core mRNA was translated in a 25 μl reticulocyte translation mixture in the absence or presence of equal amounts of HIV Tat protein-containing lysate translated previously. The results are presented in Figure 3-5A, lane 1 and clearly indicate that HBV core mRNA translation was not inhibited by the HIV Tat protein. The HBV core mRNA translation was further investigated by experiments in which other protein-containing lysates, such as luciferase and *Xenopus* elongation factor 1 protein, were included in the translation mixture of core mRNA. No inhibition of core mRNA translation was detected (Addendum 3-1). On the basis of the above evidence, it is reasonable to conclude that the inhibition effect of HBV polymerase on the translation of HBV core mRNA is specific.

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Figure 3-4 Immunoprecipitation of HBV core mRNA translation products. *In vitro-*translated HBV core proteins, in the absence or presence of 5 to 25 µl of lysate containing HBV polymerase protein, were immunoprecipitated with polyclonal rabbit anti-HBcAg antibodies and subjected to SDS-12.5% PAGE analysis and autoradiography. The migration of HBV core (HC) is indicated by the arrow.

	0	5	10	15	20	25	HP (μl)
	+	+	+	+	+	+	HC mRNA

kDa

42.7 —
40 —

31 —
21.5 —   ←— HC

14.4 —
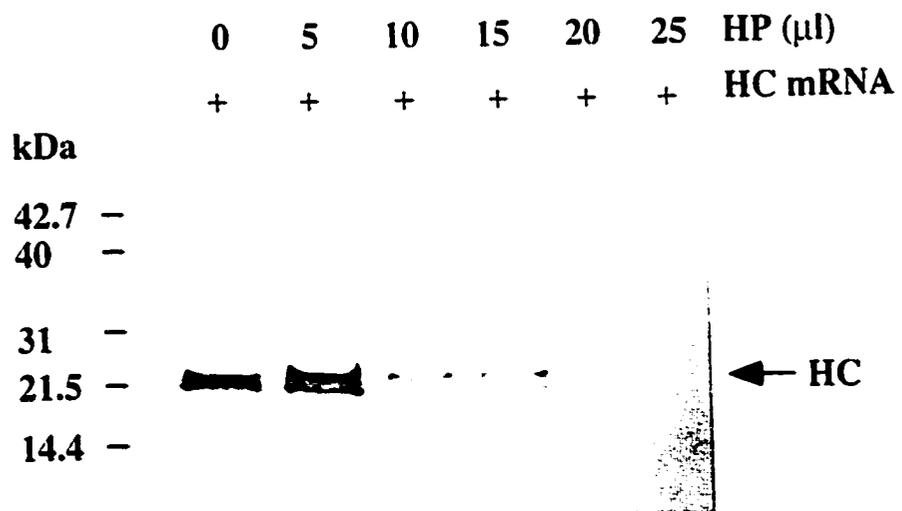
Figure 3-5 Analyses of the inhibitory specificity of HBV polymerase on HBV core mRNA translation. A. Translation of HBV core mRNA in the presence of HIV Tat protein. *In vitro*-translated HBV core protein in the absence or presence of HIV Tat protein was analyzed on SDS-15% PAGE and detected by autoradiography. The migration of HBV core (HC) and HIV Tat protein (Tat) is indicated by the arrows. B. Translation of human CD4 mRNA in the presence of HBV polymerase. *In vitro*-translated CD4 protein, in the absence or presence of HBV polymerase protein, was subjected to SDS-12.5% PAGE and detected by autoradiography. The positions of HBV polymerase (HP) and human CD4 protein (CD4) are indicated by the arrows.

B.

CD4 mRNA + HP

CD4 mRNA

kDa
97.4
66.2
55
42.7
40

31

HP

CD4

1.   2

A.

HC mRNA + Tat

HC mRNA

HC

Tat

kDa
40
31

21.5

1   2
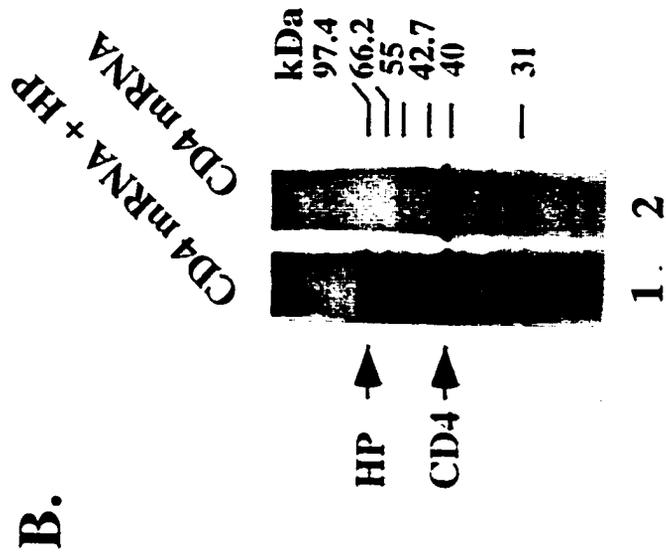
Addendum 3-1   Specific inhibition of HBV core synthesis by HBV polymerase protein. A. Translation of luciferase and *Xenopus* elongation factor 1 mRNAs in the presence of HBV polymerase protein. *In vitro*-translated luciferase and *Xenopus* elongation factor 1 proteins in the absence or presence of HBV polymerase protein (POL) was analyzed on SDS-12.5% PAGE and detected by autoradiography. The migration of luciferase (LUC) and *Xenopus* elongation factor 1 (Xefl 1) is indicated by the arrows. B. Translation of HBV core mRNA in the presence of luciferase or *Xenopus* elongation factor 1 proteins. *In vitro*-translated HBV core protein, in the presence or absence of luciferase or *Xenopus* elongation factor 1 proteins, was subjected to SDS-15% PAGE and detected by autoradiography. The position of HBV core protein is indicated by the arrow.
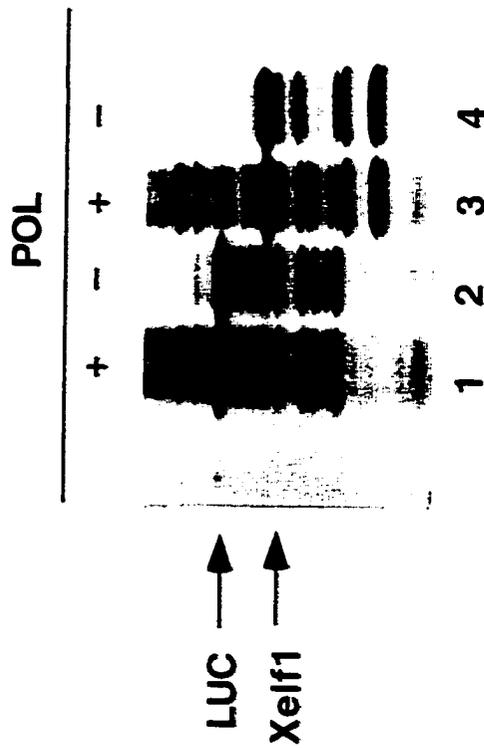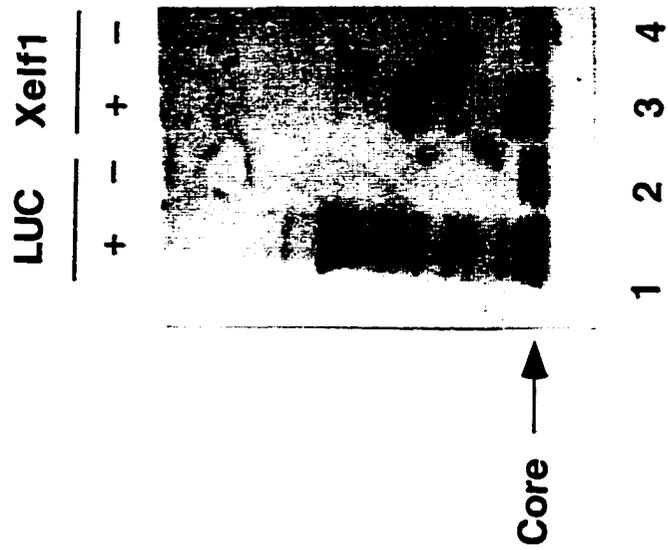
The exclusive effect of HBV polymerase on HBV core mRNA translation was also examined. In this case, 1 μg of human CD4 mRNA was translated in a 25 μl reticulocyte translation mixture in the absence or presence of equal amounts of HBV polymerase-containing lysate translated previously. The results are shown in Figure 3-5B, lane 1. No reduction of the CD4 mRNA translation was noted in the presence of polymerase. In addition, the effects of HBV polymerase on the translation of luciferase and *Xenopus* elongation factor 1 mRNAs were investigated. There was no inhibition of the synthesis of these proteins in the presence of the polymerase (Addendum 3-1). Taken together, the results of our study demonstrate that the inhibition of HBV core mRNA translation mediated by the viral polymerase is a specific event and this inhibition is limited to viral core mRNA.

Cross-species inhibition of core mRNA translation by viral polymerase in hepadnaviridae

Since all hepadnaviruses use very similar replication mechanisms, we asked whether there would be a cross-species inhibition of core mRNA translation by viral polymerase within the hepadnaviral family. To address this question, the *in vitro* translation of HBV core, DHBV core or WHV core mRNAs was conducted in the present of the viral polymerase of other species. For example, 1 μg of HBV core mRNA was translated in the absence or presence of equal amounts of lysate containing previously translated DHBV polymerase. The reaction products were analyzed by SDS-PAGE and autoradiography as described for Figure 3-2. Complete inhibition of HBV core mRNA translation in the reaction containing DHBV polymerase

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Figure 3-6 In vitro expression of core mRNAs with hepadnaviral polymerases of other species of this family. A. HBV and DHBV core proteins, translated *in vitro*- in the absence or presence of DHBV or HBV polymerase, were analyzed on SDS-12.5% PAGE and detected by autoradiography. The positions of hepadnaviral polymerases (P), DHBV core protein (DC) and HBV core protein (HC) are indicated by the arrows. B. WHV core protein translated *in vitro*- in the absence or presence of DHBV or HBV polymerases was subjected to SDS-12.5% PAGE and detected by autoradiography. The positions of hepadnaviral polymerases (P) and WHV core protein (WC) are indicated on the right.

B.

WC dsRNA + HP

WC dsRNA

WC dsRNA + DP

WC dsRNA

P

←WC

kDa
97.4
66.2
55
42.7
40
31
21.5

1 2 3 4

A.

WC dsRNA

WC dsRNA + DP

WC dsRNA

WC dsRNA + HP

kDa
97.4
66.2
55
42.7
40
31
21.5

1 2 3 4
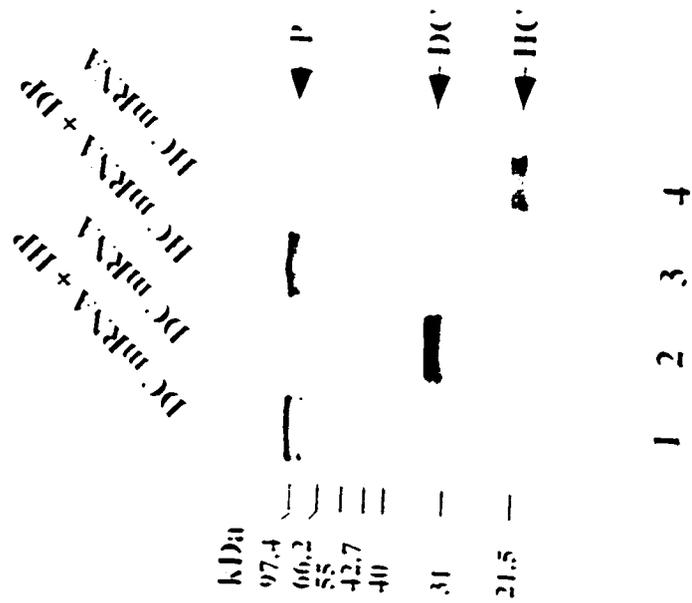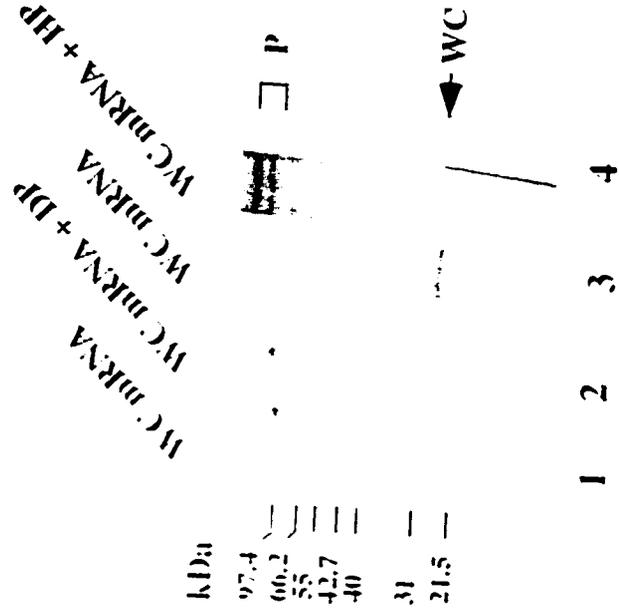
lysate was observed (Figure 3-6A, lane 3). Likewise, the translation of DHBV core protein mRNA was studied in the absence or presence of equal amounts of lysate containing previously translated HBV polymerase. The results as presented in Figure 3-6A, lane 1 revealed that the DHBV core mRNA translation was significantly inhibited by HBV polymerase. WHV core protein mRNA was translated in the absence or presence of equal amounts of lysate containing either DHBV or HBV polymerases. The results shown in Figure 3-6B, lanes 2, 4 demonstrated that WHV core mRNA translation was inhibited by either DHBV or HBV polymerase. These results support the hypothesis that the translational repression of viral core mRNA by the viral polymerase is a conserved mechanism in the hepadnaviridae family.

Northern blot analysis of core translation in the presence of polymerase

Hepadnaviral polymerase has RNase H activity, therefore, it is critical to examine whether the polymerase can degrade the core mRNA. This question was addressed by a Northern blot analysis of the core mRNA from the translation mixtures of the *in vitro* translation of DHBV mRNA, DHBV mRNA in the presence of polymerase and lysate alone as control. The results of the Northern hybridization revealed that the level of DHBV mRNA remained relatively stable and the size of the DHBV mRNA did not change in the presence of polymerase, as shown in Figure 3-7, lane 2 and lane 3. Based on this result, I concluded that the polymerase has no effect (e.g., stability or degradation) on the core mRNA and the RNase H activity of the polymerase does not account for the translation inhibition of core mRNA.

Figure 3-7 Northern blot detection of DHBV core mRNA. The mRNA was extracted from the *in vitro* translation of lysate alone (control, lane 1), DHBV mRNA (lane 2) and DHBV mRNA in the presence of polymerase (lane 3). The RNA samples were separated on a 0.8% agarose-2.2 M formaldehyde gel and transferred onto a nylon membrane. The RNA blot was hybridized with a ^{32}P-DHBV core DNA probe. The position of DHBV mRNA is shown by an arrow.

Lysate   DHBV mRNA   DHBV mRNA + DHBV Pol

← Core mRNA

1   2   3
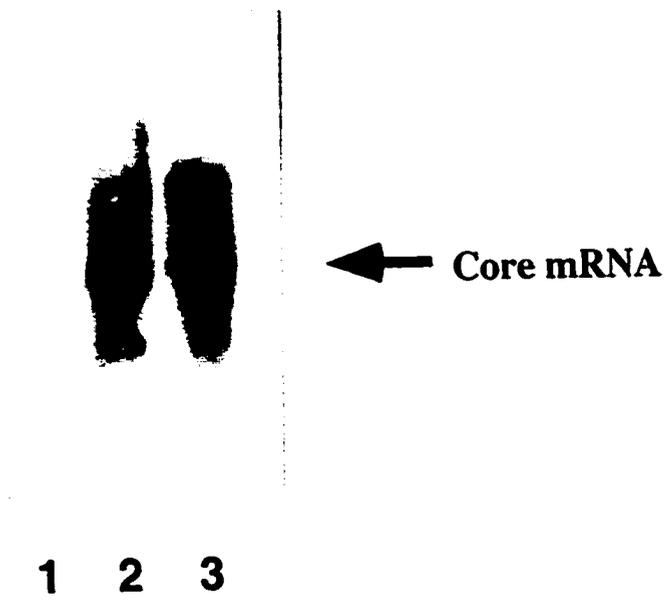
IV. Discussion

This study demonstrated that the *in vitro* translation of the HBV core mRNA was inhibited by the HBV polymerase-containing lysate and Dr. Howe's study indicated that this inhibition which was mediated by the polymerase-containing lysate was attributed to the polymerase protein not its mRNA in the lysate. By uncoupling the transcription step from the translation step in this system, I have shown that the inhibition effect takes place at the level of translation. Degradation of the core mRNA did not likely account for the decreased core protein synthesis in the presence of the polymerase. This inhibition is specific in that the translation of other unrelated mRNA transcripts remains unaffected by HBV polymerase. More importantly, our data provide the first evidence for a cross-species translational inhibition of core synthesis mediated by viral polymerase in the hepadnaviridae family. HBV polymerase inhibited the translation of core mRNAs of DHBV and WHV. Similarly, DHBV polymerase inhibited the translation of core mRNAs of HBV and WHV. This supports the concept that a conserved mechanism is involved in this regulation. Translational inhibition was predicted by Nassal et al. (1990). The results in the present study have confirmed the inhibition of translation of DHBV core mRNA by viral polymerase reported by our laboratory earlier (Howe and Tyrrell 1996). The results in the current study demonstrated cross species translational repression.

It is becoming increasingly apparent that translational control plays an important role in the regulation of gene expression. Recent studies have shown that a specific interaction between a *cis*-acting element of mRNA and a *trans*-acting factor is directly responsible for the translational control of ferritin (Rouault et al. 1988), thymidylate synthase (Chu et al. 1991), dihydrofolate reductase (Chu et al. 1993), erythroid 15-lipoxygenase (LOX) mRNA (Ostareck-Ledderer et al. 1994) and protamine mRNA of

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round spermatids (Kwon and Hecht 1991). Although the mechanism by which the HBV polymerase inhibits the translation of core mRNA is not completely known, all available information suggests that the regulation of core protein synthesis is likely the result of interaction of the polymerase directly or indirectly to the core mRNA/pgRNA. This interaction of polymerase with core mRNA could occur at several target sites. It has been noted that polymerase specifically interacts with a region of 90 nt bearing an epsilon stem-loop on the 5' end of the pgRNA during the process of encapsidation (Bartenschlager and Schaller 1992; Kochel et al. 1991; Pollack and Ganem 1994). Although polymerase could act at a single target site on the pgRNA, a second docking site downstream of the 5' end of the pgRNA for polymerase during the process of encapsidation in DHBV was also suggested (Calvert and Summers 1994; Hirsch et al, 1991; Howe and Tyrrell 1996). Deletion studies of the region located at the 3' periphery of the core ORF on the DHBV core expression plasmid indicated that the region from nt 140 to 828 is involved in translational regulation and the sequence transfer analysis suggested that the residues from nt 401 to 870 are sufficient to transfer the polymerase-mediated translational inhibition onto a heterologous mRNA (Howe and Tyrrell 1996). None of the core expression constructs in our study contained the 5' epsilon stem-loop structure known to interact with the viral polymerase, but these results do not exclude the possibility that the 5' epsilon stem-loop is involved in the regulation *in vivo*. Further defining the *cis* element involved in translational suppression will enhance our understanding of the polymerase-mediated repression of gene expression.

Overall, the evidence obtained in this *in vitro* study system supports conserved translational control of core mRNA expression by polymerase within the hepadnaviridae family. This may be the mechanism by which the role of pgRNA is switched from messenger for both core protein and polymerase to encapsidation and as

a template for genomic replication. Early in replication, pgRNA serves as mRNA for translation of core and polymerase protein (Figure 3-8A). We hypothesized that soon after it has been translated, the polymerase binds to the stable secondary structure(s) on the pgRNA and very likely functions as a translational repressor to shut off the translation of pgRNA, thus to release the pgRNA from the translational machinery to facilitate the viral core assembly (Figure 3-8B).

Polymerase ORF is the second cistron in the bicistronic mRNA, pgRNA. No internal ribosome entry site elements as seen in picornaviruses were found in HBV which could be attributed to the polymerase translation, however, an alternative scanning mechanism was proposed for the polymerase translation (Fouillot et al. 1993). Ribosomes scan from the 5' end of the bicistronic pgRNA and translate the first cistron core ORF. The translation of core ORF allows ribosomes to bypass and translate the out of frame polymerase ORF. The translation initiated at the internal position mediated by the ribosomal scanning is inefficient (Kozak 1989) and this is believed to account for the low production of polymerase protein in HBV infected cells. We believe that while polymerase inhibits the core translation, it inhibits its own translation as well. Taken together, this may explain how two proteins, core and polymerase, translated from the same mRNA produce two different amounts of proteins (240 copies of core molecule compared to 1–2 copies of polymerase molecules per virion). It will be important also to determine the biological relevance of the regulation observed in the studies. These initial studies provide attractive insights into the viral replication mechanism and contribute to the growing knowledge of regulation of gene expression at the posttranscriptional level.

Figure 3-8   Schematic representation of a model for *trans*-repression of the hepadnaviral core mRNA expression by viral polymerase. A. The pgRNA functions as mRNA for the synthesis of viral core and polymerase proteins.   B. The viral polymerase interacts with pgRNA at the 5' stem-loop and/or the other region, displaces the translation machinery and exposes the pgRNA for encapsidation.

A.
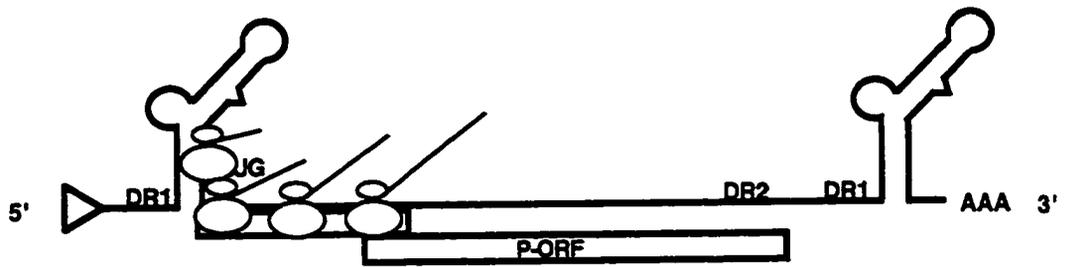
B.
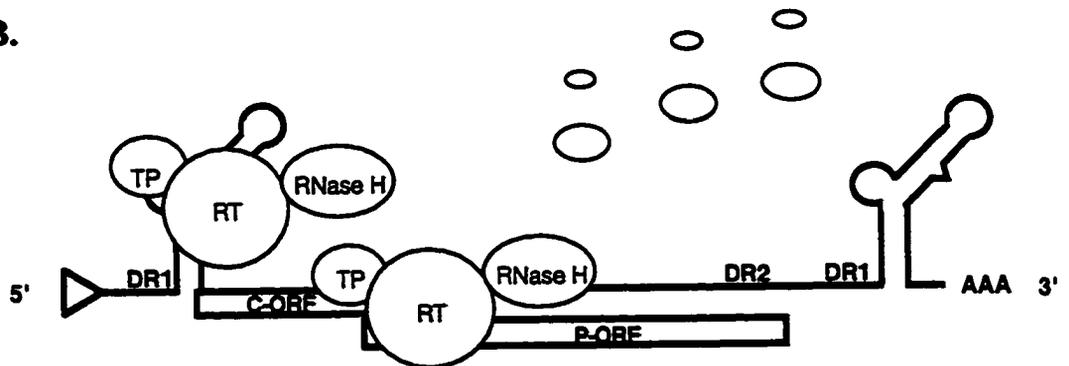
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CHAPTER 4*

EFFECT OF SILENT MUTATIONS IN THE *TRANS*-REPRESSION DOMAIN ON THE INHIBITORY EFFECT OF POLYMERASE

I.   Introduction

As previously noted, Dr. Howe's study has demonstrated that DHBV polymerase induces translational repression of DHBV core mRNA. This polymerase-mediated repression of core protein synthesis is dependent upon the sequences from nt 140 to 828 at the 3' periphery of the core ORF. However, the sequences from residue 401 to 870 are also sufficient to render the polymerase-induced translational repression onto a heterologous mRNA expression. I further confirmed and extended the polymerase mediated translational inhibition of the gene expression. I have shown that HBV polymerase-mediated reduction of the HBV core protein synthesis *in vitro* and demonstrated that the degradation of the core mRNA does not account for the inhibition of the core mRNA expression. I also discovered that the hepadnaviral polymerase-associated translational repression of the viral core production is conserved amongst hepadnaviruses. DHBV polymerase inhibited the translation of HBV and WHV core mRNAs. Similarly, HBV polymerase inhibited DHBV and WHV core mRNA translation.

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* Most of the data presented in this chapter will be submitted for publication in J. of Virol. (2000).

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The mechanism of the polymerase-associated translational repression has not yet be defined. Dr. Howe performed a series of 3' terminal truncations of the DHBV core expression construct and found that sequences of approximately 700 nt at the 3' periphery of the core ORF are required to complete the polymerase-mediated translational inhibition. Some of this sequence can transfer the polymerase translational inhibition to a heterologous system. In this study, I wanted to further map the sequences from nt 137 to 830, the *trans*-repression domain or TR. We have produced silent mutations in the putative TR region that change the primary sequences and secondary structures of the RNA while keeping the amino acid sequences unchanged. We have examined the effects of the silent mutations on the polymerase-mediated translational inhibition of core protein synthesis. Second, I developed a reporter system to study the polymerase induced posttranslational inhibition. I have transferred wild and silent mutated TR DNA sequences into a bacterial chloramphenicol acetyltransferase (CAT) expression system. These TR sequences were cloned into a CAT expression plasmid downstream from the CAT ORF and the chimeric plasmids were examined for the translation efficiency and CAT activity in the presence and absence of DHBV polymerase-containing lysate.

II. Materials and Methods

Construction of recombinants

A CAT protein expression recombinant series, containing wild-type or silent mutations of DHBV-TR sequences downstream of a *cat* reporter gene (Gorman et al. 1982), were cloned. The wild-type DHBV-TR sequence from *Hinc* II to *Aat* II sites (693 bp, nt 137–830) was polymerase chain reaction (PCR) amplified, as described in Materials and Methods of Chapter 2, from a DHBV core protein expression plasmid pTZ19Rcore (Howe and Tyrrell 1996) using primers DHBVsphI and DHBVxbaI (see Appendix for primers). The amplified TR DNA was digested with *Sph* I and *Xba* I enzymes and cloned into a CAT protein expression plasmid pCMVCAT-1.3.DNA, kindly provided by K. Fischer, which had been cut with the same enzymes downstream of the CAT ORF. This resulting chimeric plasmid was designated pCMVCAT-TR. A derivative plasmid pCMVCAT.TRmHAXB of the plasmid pCMVCAT-TR, containing silently mutated sequence at the 5' end of DHBV TR between the sites of *Hinc* II and *Bgl* II (254 bp, nt 137–391), was made by Dr. R. George. Two other DHBV polymerase expression mutant plasmids pTZ19RCore.TRmHAXB and pTZ19RCore.mutB/A derivatived from the plasmid pTZ19RCore, containing the silent mutations at the 5' end of TR from *Hinc* II to *Bgl* II and at the 3' end of TR from *Bgl* II to *Aat* II (439 bp, nt 391 to 830), respectively, were also made by Dr. R. George. Nucleotide numbering was done according to the convention of Mandart et al, beginning at the unique *Eco*R I site of DHBV-16 (Mandart et al. 1984). Multiple silent mutation oligonucleotides, chemically synthesized by Dr. R. George using an Applied Biosystems 391 DNA synthesizer (ABI), were used to obtain the mutations of the DHBV-TR sequences. The mutations were confirmed by DNA sequencing (Biochemistry Department, University of Alberta).

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Preparation of transcripts *in vitro*

Plasmid pCMVCAT-1.3.DNA, containing CAT ORF, was linearized by *Hpa* I digestion to produce an mRNA (CAT-VEC) with CAT-coding sequences and approximately 770-bp backbone sequences downstream of the CAT ORF. The chimeric plasmid pCMVCAT-TR bearing CAT ORF and TR was linearized by *Sph* I and *Xba* I, respectively, to generate a mRNA with the CAT ORF only (CAT) and an mRNA with the CAT ORF plus the TR sequence (CAT-TR). The mutant plasmid pCMVCAT-TRmHAXB containing CAT ORF and mutated TR was linearized by *Xba* I digestion. An *in vitro* expression plasmid pTZ19RPol having DHBV polymerase ORF was linearized by *Eco*R I digestion (Howe and Tyrrell 1996). The DHBV core expression plasmid pTZ19RCore and its mutants pTZ19RCore.TRmHAXB and pTZ19RCore.mutB/A were linearized by *Aat* II digestion. Linearized plasmids were transcribed *in vitro* by a T7 RNA polymerase following the manufacturer's recommendation (Promega) (see Materials and Methods of Chapter 3 for details of the procedure).

In vitro translation

In vitro translation was performed as described previously in Materials and Methods of Chapter 3, using the rabbit reticulocyte lysate from Promega. A typical reaction contained 1 μg of transcripts and was translated in 50 μl of a rabbit reticulocyte translation mixture. In the translational inhibition experiment, the translational mixture containing the target mRNA was translated in the presence of an equal volume of the polymerase-containing lysate.

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CAT assays

The CAT assay was based on one described previously (Ausubel et al. 1996; Sambrook et al. 1989; Shaw and Brodsky 1968). CAT mRNAs from different recombinants were translated *in vitro* using rabbit reticulocyte lysate according to the manufacturer's recommendation (Promega). The translation lysate was assayed for enzyme activity. To ensure that the assay conditions corresponded to the linear kinetic range of the response curve, pilot assays with serially diluted lysates were performed. An optimal amount of CAT protein-containing lysate was mixed with 1M Tris-HCl (pH 7.8) to a total volume of 125 μl. Twenty microliters of 3.5 mg/ml acetyl coenzyme A (Sigma) and 3 μl of [^{14}C]chloramphenicol (46.9 mCi/mmol, 0.1 mCi/ml, Amersham) were then added. The reaction was incubated at 37°C for 30–90 min depending on the activity of CAT protein. The reaction was subsequently stopped and extracted by adding 1 ml of cold ethyl acetate. The organic phase, containing chloramphenicol, was transferred to a fresh Eppendorf tube and dried in a Savant SpeedVac (Savant Instruments Inc.). The resulting product residue was resuspended in 30 μl of ethyl acetate, spotted on siliconized thin-layer glass plates (Whatman International Ltd.) and separated using chloroform:methanol (19:1) as a mobile phase (thin-layer chromatography or TLC). The converted products were quantitated using a phosphoimager (BAS1000, Fuji) and autoradiography. CAT activities are presented as percentages of the conversion [(product density × 100)/(substrate density + product density)].

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Secondary structure prediction of mRNA

Secondary structure of the mRNA was predicted from primary sequences using algorithm and thermodynamic method (Le, S.-Y. and Zuker, M. 1991; Mathews et al. 1999; Zuker et al. 1999) through an mfold software. The mfold software package is available online (http://www.ibc.wustle.edu/~zuker/rna/form1.cgi) linked to M. Zuker's homepage. The algorithm and thermodynamic based RNA folding for finding the energetically most favorable structure (i.e. minimum free energy conformation) has been significantly improved. Any personal computer with a web browser can use mfold software for predicting RNA secondary structure for sequences of considerable length.

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III. Results

Translation of chimeric CAT mRNAs in the presence of DHBV polymerase

To further test whether the hepadnaviral *trans*-repression domain was responsible for the inhibitory effect of the hepadnaviral polymerase, a reporter chimeric plasmid pCMVCAT-TR bearing CAT ORF and wild-type DHBV-TR sequence (from nt 137 to 830), which is required for the polymerase-mediated repression according to Dr. Howe's study, was constructed (Figure 4-1A, b). The mRNAs containing only CAT ORF (CAT) or containing CAT ORF and TR sequence (CAT-TR) were transcribed *in vitro* from the chimeric plasmid pCMVCAT-TR linearized by *Sph* I and *Xba* I, respectively. The mRNA bearing CAT ORF and with the downstream backbone sequence (CAT-VEC) was prepared *in vitro* from the plasmid pCMVCAT-1.3.DNA (Figure 4-1A, a) linearized by *Hpa* I. Each of these mRNAs was translated in the rabbit reticulocyte lysate in the absence or presence of DHBV polymerase. The translation efficiency of the mRNAs is shown in Figure 4-2. The translation of CAT-TR mRNA in the presence of DHBV polymerase-containing lysate was reduced by 95% (Figure 4-2, lanes 5–6). In contrast, the translation of CAT mRNA was inhibited only 20% (Figure 4-2, lanes 1–2). Similarly, a small decrease in the translation of CAT-VEC mRNA was observed in the presence of the polymerase (Figure 4-2, lane 4). As a positive control, translation of the DHBV core mRNA added in each of the above reactions was completely inhibited in the presence of DHBV polymerase protein (Figure 4-2, lanes 2, 4, 6). CAT activities produced by the translation of CAT and CAT-TR mRNAs were also assayed. A representative autoradiogram of a TLC plate of CAT assays is shown in Figure 4-3. The results of CAT assays of the translation

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Figure 4-1 Schematic structure of the expression constructs. Details of constructing these plasmids are in Materials and Methods. (A) CAT expression plasmid series. Plasmids are depicted linearized at the junction of the promoter and vector sequence. CMV and T7 promoters are shown as arrows. CAT-coding sequences are shown as open rectangular boxes labeled CAT. Plasmid pCMVCAT-1.3.DNA (a) is a parental CAT expression construct. Plasmid pCMVCAT-TR (b) is a derivative plasmid of pCMVCAT-1.3.DNA constructed by inserting 693-bp wild-type DHBV-TR sequence from nt 137 to 830 at the *Sph* I and *Xba* I sites. Plasmid pCMVCAT-TRmHAXB (c) is identical to the chimeric plasmid pCMVCAT-TR except that it has silent mutation sequences between the *Hinc* II and *Bgl* II sites from nt 137 to 391 (see Figure 4-5A for silent mutated sequences). (B) DHBV core protein expression plasmid series. Plasmids are depicted linearized at the junction of the promoter and vector sequences. T7 promoters are shown as arrows. DHBV core-coding sequences are shown as open rectangular boxes labeled Core. DHBV wild-type sequences are indicated by hatched boxes. The construct pTZ19RCore (a) is a parental DHBV core expression plasmid; plasmids pTZ19RCore.TRmHAXB (b) and pTZ19RCore.mutB/A (c) are identical to plasmid pTR19RCore but with silent mutations between the *Hinc* II to *Bgl* II sites from nt 137 to 391 and *Bgl* II to *Aat* II sites from nt 391 to 830, respectively (see Figure 4-5 for silent mutation sequences).

A.

a. pCMVCAT-1.3.DNA

b. pCMVCAT-TR

c. pCMVCAT.TRmHAXB

B.

a. pTZ19RCore

b. pTZ19RCore.TRmHAXB

c. pTZ19RCore.mutB/A

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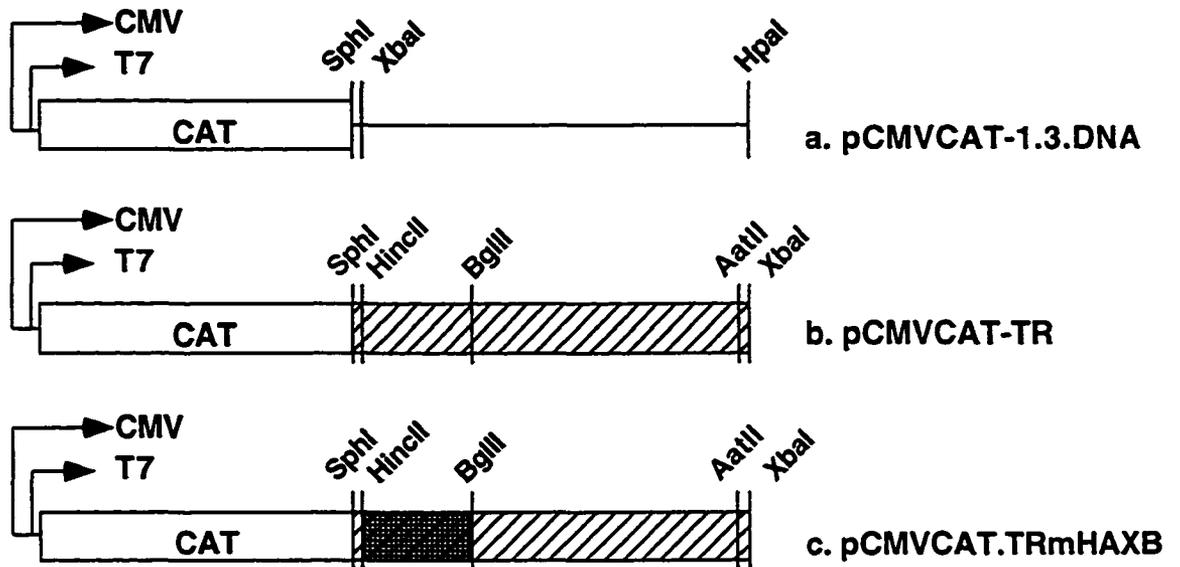
Figure 4-2 *In vitro* translation of CAT and CAT chimeric mRNAs in the presence of DHBV polymerase. The translation products were analyzed by polyacrylamide gel electrophoresis (PAGE). In lanes 1, 3 and 5, 5 µl of translation mixture, containing 1 µg CAT mRNA (CAT), CAT-VEC mRNA (CAT-VEC) and CAT-TR mRNA (CAT-TR), respectively, were cotranslated with 1 µg DHBV core mRNA in the absence of DHBV polymerase-containing lysate. In lanes 2, 4, and 6, 5 µl of translation mixture, containing 1 µg CAT, CAT-VEC and CAT-TR mRNAs, respectively, were cotranslated with 1 µg DHBV core mRNA in the presence of previously translated DHBV polymerase-containing lysates. The DHBV core protein (Core) and CAT protein (CAT) are indicated at the right.
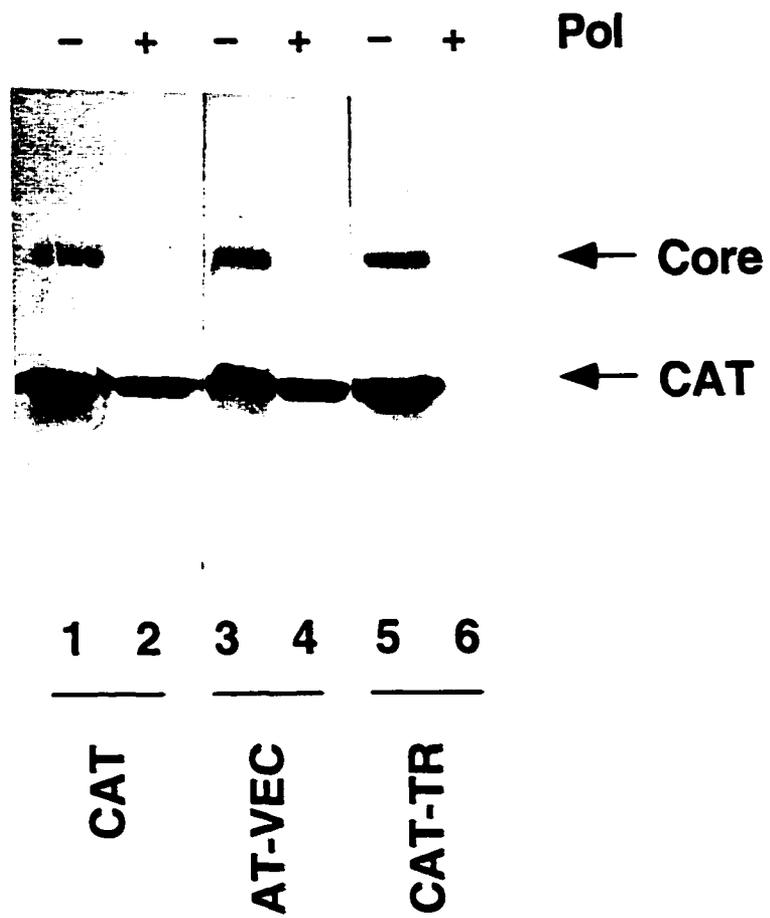
Figure 4-3   CAT activities of CAT proteins translated from CAT and CAT-TR mRNAs in the presence of DHBV polymerase. The figure shows an autoradiogram of a TLC plate used for CAT assay of CAT-containing lysates translated from CAT mRNA (CAT) without or with DHBV polymerase-containing lysates (Pol) in lanes 1, 2 and from CAT-TR mRNA (CAT-TR) without or with DHBV polymerase-containing lysates in lanes 3, 4. Details of the assay are in Materials and Methods. 3Ac-Cm and 1Ac-Cm mark the position of the two monoacetylated products 3-acetylchloramphenicol and 1-acetylchloramphenicol and Cm marks the position of the modified [^{14}C]chloramphenicol substrates after TLC. Conversion rates of each reaction, in percentages, are shown at the bottom of the figure.
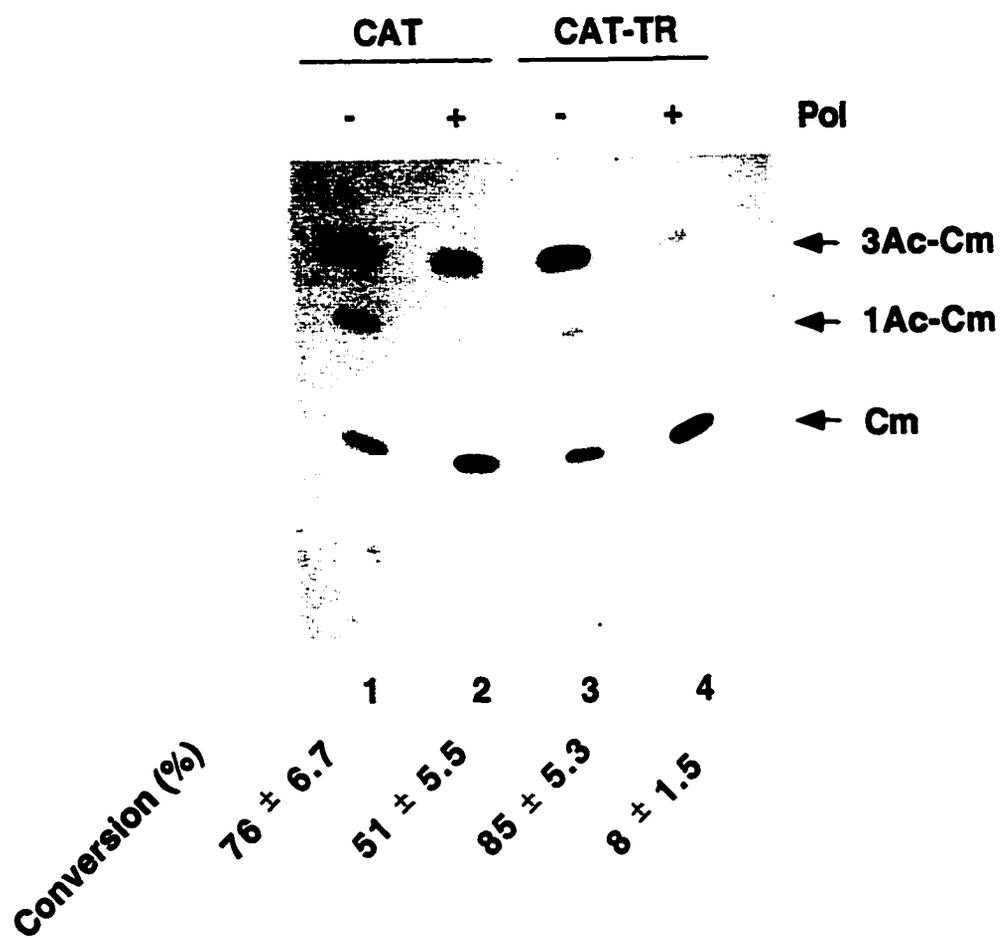
lysates from CAT and CAT-TR mRNAs are in accord with the repression effects of DHBV polymerase on the translation products of these mRNAs. The CAT activity of the translation lysate from the chimeric CAT-TR mRNA was 90% lower in the presence of polymerase (Figure 4-3, lanes 3 and 4). However, the CAT activity of the translation lysate from CAT mRNA was only marginally repressed in the presence of polymerase (Figure 4-3, lanes 1 and 2). The CAT activities of CAT-containing lysates translated from CAT-TR, CAT and CAT-VEC mRNAs in the presence of increasing amounts of DHBV polymerase-containing lysate were also examined. With increasing amounts of polymerase-containing lysate, a progressive reduction of the CAT activity translated from the CAT-TR mRNA was observed; whereas increasing the amounts of polymerase lysate had no inhibitory effect on CAT activities translated from CAT or CAT-VEC mRNAs (Figure 4-4). These experimental results clearly demonstrated the regulatory effect of the DHBV polymerase on the reporter CAT gene expression and that the inhibition is associated with the hepadnaviral *trans*-repression domain. We have confirmed Dr. Howe's results that the *trans*-repression domain and repression effect of the hepadnaviral polymerase on the gene expression can be transferred to a heterologous system.

Silent mutation of the *trans*-repression domain

To ascertain the importance of the putative TR region on the polymerase-mediated translational inhibition of gene expression, silent mutations of the DHBV-TR region were made by Dr. R. George. These silent mutation sequences were generated by multiply mutated oligonucleotides. Two silent mutation fragments from the sites of *Hinc* II to *Bgl* II and *Bgl* II to *Aat* II of the DHBV-TR were obtained as shown in

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Figure 4-4 CAT activities of CAT-containing lysates translated from CAT and CAT chimeric mRNAs with increasing amounts of DHBV polymerase. CAT activities of CAT-containing lysates, translated from 5 µl CAT translation mixture with CAT, CAT-VEC and CAT-TR mRNAs with increasing amounts (0, 2.5, 5, 10, 20 µl) of DHBV polymerase-containing lysates, were examined. Details of the *in vitro* translation and the CAT assay are described in Materials and Methods. After TLC and autoradiography, the density of the products and substrates was determined by a phosphoimager. The CAT activities were calculated by determining the percentage conversion.

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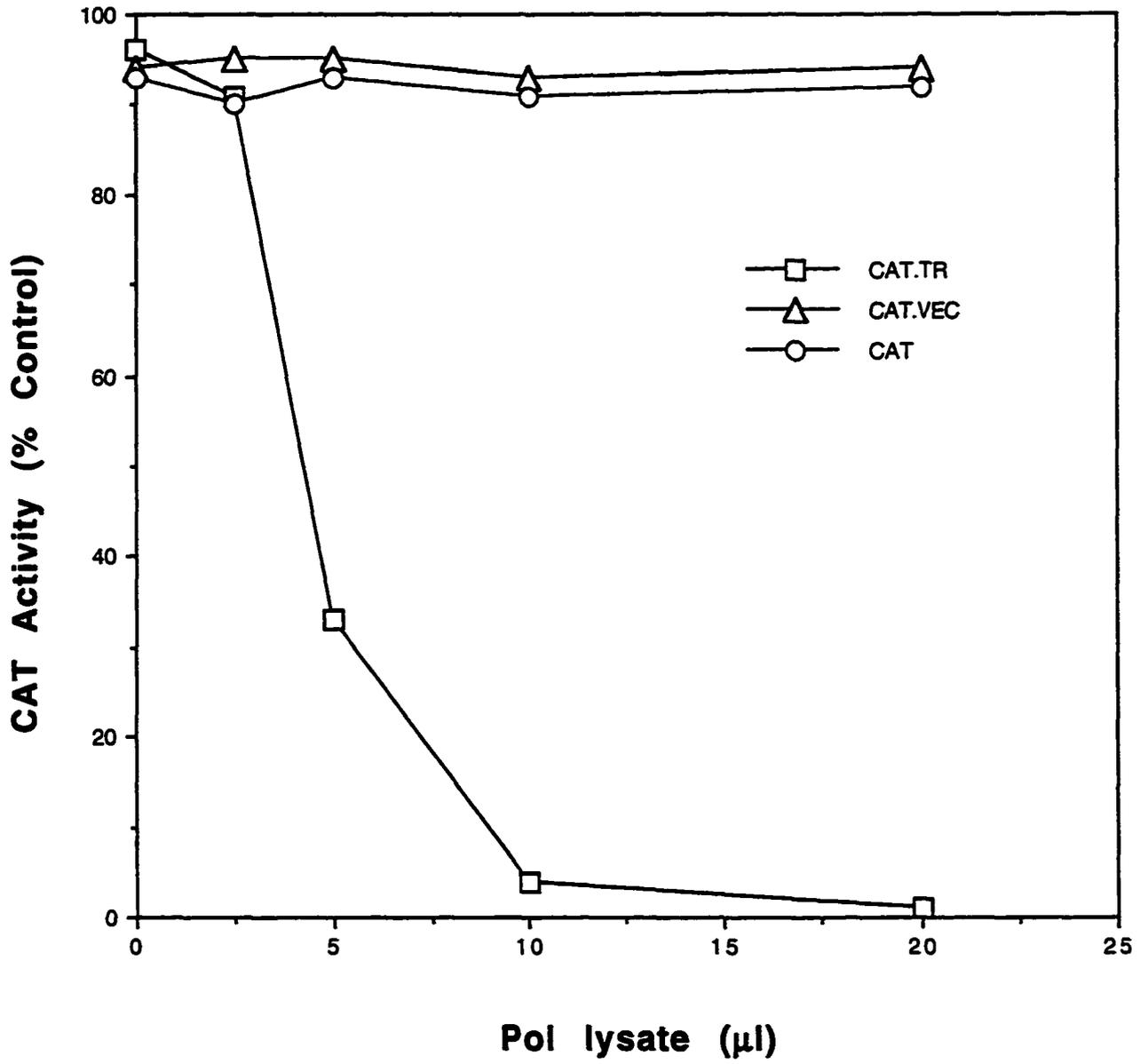
Figure 4-5. These two silently mutated fragments were cloned into the DHBV core expression plasmid pTZ19RCore at the *Hinc* II and *Bgl* II sites as well as *Bgl* II and *Aat* II sites, respectively, to replace the counterpart wild-type sequences of the DHBV-TR (Figure 4-1B, b, c). The mutated fragment between the sites of *Hinc* II and *Bgl* II was also cloned into the chimeric CAT expression plasmid pCMVCAT-TR at the sites of *Hinc* II and *Bgl* II to replace the corresponding wild-type sequences of the DHBV-TR (Figure 4-1A, c). The effects of these silent mutations on the polymerase-mediated translational inhibition of gene expression were examined.

Silent mutations of the TR region reversed the inhibitory effect of the hepadnaviral polymerase

Plasmids with silently mutated TR region were transcribed *in vitro*. Transcripts of each mutant were translated in rabbit reticulocyte lysate in the absence or presence previously translated DHBV polymerase-containing lysates. Translation efficiencies were determined by PAGE or CAT assay. DHBV core mRNA from the plasmid pTZ19RCore.mutB/A, having mutated sequences between the *Bgl* II and *Aat* II sites in the 3' TR, was translated slightly more efficiently than was the wild-type mRNA in the presence of the polymerase (Figure 4-6B, lanes 2, 4). However, DHBV core mRNA from the plasmid pTZ19RCore.TRmHAXB, having mutated sequences between the *Hinc* II and *Bgl* II sites of the 5' TR, was translated approximately 18-fold more efficiently than was wild-type mRNA in the presence of the polymerase (Figure 4-6A, lanes 2, 4). Similarly, CAT mRNA from the mutant plasmid pCMVCAT.TRmHAXB, having mutated sequences between the *Hinc* II and *Bgl* II sites in the 5' TR, was translated again much more effectively than was the wild-type CAT mRNA in the

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Figure 4-5 Silent mutations of DHBV-TR sequences. A. Mutation sequences between the sites of *Hinc* II and *Bgl* II. B. Mutation sequences between the sites of *Bgl* II and *Aat* II. The sequences of wild-type (WT), amino acid codons (AA) and silent mutations (Mut) are listed. The site-specific mutation sequences are indicated by asterisks. The underlined sequences indicate the area of the predicted epsilon stem-loop like structure. These mutations were kindly produced by Dr. R. George.

A.

<u>*Hinc II*</u>

```
G TCA ACT CCT GAG AAA TAT AGA GGT AGA GAT GCC CCG ACC ATT GAA GCA ATC ACT AGA CCA ATC CAG
    S   T   P   E   K   Y   R   G   R   D   A   P   T   I   E   A   I   T   R   P   I   Q
G TCG ACC CCC GAA AAG TAC CGT GGC CGT GAC GCT CCT ACG ATC GAG GCT ATT ACA CGT CCT ATT CAA
    *   *   *   *   *   *   * *   * * *   *   *   *   *   *   *   *   *   * * *   *   *   *


  GTG GCT CAG GGA GGC AGA AAA ACA ACT ACG GGT ACT AGA AAA CCT CGT GGA CTC GAA CCT AGA AGA
   V   A   Q   G   G   R   K   T   T   T   G   T   R   K   P   R   G   L   E   P   R   R
  GTT GCC CAA GGG GGT CGT AAG ACT ACG ACA GGA ACA CGT AAG CCC AGA GGT CTG GAG CCA CGT CGT
   *   *   *   *   * * *   *   *   *   *   *   *   * *   *   * * *   *   *   *   * * * * *


  AGA AAA GTT AAA ACC ACA GTT GTC TAT GGG <u>AGA AGA CGT TCA AAG TCC CGG GAA AGG AGA GCC CCT</u>
   R   K   V   K   T   T   V   V   Y   G   R   R   R   S   K   S   R   E   R   R   A   P
  CGT AAG GTC AAG ACT ACT GTC GTA TAC GGA CGT CGT AGA AGC AAA TCC CGG GAG CGT CGT GCT CCC
  * *   *   *   *   *   *   *   *   *   * * * * * * * * * ***   *      * * * * * *   *   *


                                                                        <u>*Bgl II*</u>
  <u>ACA CCC CAA CGT GCG GGC TCC CCT CTC CCA CGT AGT TCG AGC</u> AGC CAC CAT AGA TCT CCC TCG CCT
   T   P   Q   R   A   G   S   P   L   P   R   S   S   S   S   H   H   R   S   P   S   P
  ACT CCT CAG AGA GCT GGA AGC CCC TTG CCT AGA TCA AGT TCA TCA CAT CAC AGA TCT CCC TCG CCT
   *   *   * * *   *   * **   * * *   * * ***  ***  ***  ***   *   *   *   *   *   *   *


  AGG AAA TAA        *WT*
   R   K   end       *AA*
  AGG AAA TAA        *Mut*
```

B.

<u>*BgI II*</u>

```
ATA GAT CTC CCT CGC CTA GGA AAT AAA TTA CCT GCT AGG CAT CAC TTA GGT AAA TTG TCA GGA CTA
 I   D   L   P   R   L   G   N   K   L   P   A   R   H   H   L   G   K   L   S   G   L
ATA GAT CTG CCA AGA CTC GGG AAC AAG CTC CCA GCA CGA CAC CAT CTC GGA AAG CTC AGC GGG TTG
         *       *   *   *       *       *       *   *   *       *   *   *   *       * * *     *   *   *


TAT CAA ATG AAG GGC TGT ACT TTT AAC CCA GAA TGG AAA GTA CCA GAT ATT TCG GAT ACT CAT TTT
 Y   Q   M   K   G   C   T   F   N   P   E   W   K   V   P   D   I   S   D   T   H   F
TAC CAG ATG AAA GGG TGC ACG TTC AAT CCT GAG TGG AAG GTG CCT GAC ATA AGC GAC ACC CAC TTC
 *   *       *   *   *   *   *   *   *   *   *       *   *   *   *   * * *   *   *   *   *


AAT TTA GAT GTA GTT AAT GAG TGC CCT TCC CGA AAT TGG AAA TAT TTG ACT CCA GCC AAA TTC TGG
 N   L   D   V   V   N   E   C   P   S   R   N   W   K   Y   L   T   P   A   K   F   W
AAC TTG GAT GTC GTC AAC GAA TGT CCA AGC AGG AAC TGG AAG TAC CTC ACC CCT GCT AAG TTT TGG
 *   *       *   *   *   *   *   *   * *   * *       *   *   *   *   *   *   *   *


CCC AAG AGC ATT TCC TAC TTT CCT GTC CAG GTA GGG GTT AAA CCA AAG TAT CCT GAC AAT GTG ATG
 P   K   S   I   S   Y   F   P   V   Q   V   G   V   K   P   K   Y   P   D   N   V   M
CCT AAA TCC ATA AGC TAT TTC CCA GTA CAA GTC GGA GTA AAG CCT AAA TAC CCA GAT AAC GTA ATG
 *   *   * *   *   * *   *   *   *   *   *   *   *   *   *   *   *   *   *   *       *


CAA CAT GAA TCA ATA GTA GGT AAA TAT TTA ACC AGG CTC TAT GAA GCA GGA ATC CTT TAT AAG CGG
 Q   H   E   S   I   V   G   K   Y   L   T   R   L   Y   E   A   G   I   L   Y   K   R
CAG CAC GAG AGC ATT GTT GGA AAG TAC CTC ACT CGT TTA TAC GAG GCT GGT ATT CTC TAC AAA AGA
 *   *   *   * * *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   * *


ATA TCT AAA CAT TTG GTC ACA TTT AAA GGT CAG CCT TAT AAT TGG GAA CAG CAA CAC CTT GTC AAT
 I   S   K   H   L   V   T   F   K   G   Q   P   Y   N   W   E   Q   Q   H   L   V   N
ATT TCC AAG CAC CTC GTT ACC TTC AAG GGA CAA CCC TAC AAC TGG GAG CAA CAG CAT CTC GTT AAC
 *   *   *   *   *   *   *   *   *   *   *   *   *   *       *   *   *   *   *   *   *
```

<u>*Aat II*</u>

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CAA CAT CAC ATT TAT GAT GGG GCA ACA TCC AGC AAA ATC AAT GGA CGT CAG ACG          WT
 Q   H   H   I   Y   D   G   A   T   S   S   K   I   N   G   R   Q   T          AA
CAG CAC CAT ATA TAC GAC GGT GCT ACT TCT TCC AAG ATT AAC GGA CGT CAG ACG          Mut
 *   *   *   *   *   *   *   *   *   *   * *   *   *   *
```

Figure 4-6 In vitro translation of wild-type and mutant DHBV core expression plasmids in the presence of DHBV polymerase. Translation products were assayed by PAGE. (A) Translation of wild-type mRNA (WT) from the plasmid pTZ19RCore in lanes 1 and 2 and mutant mRNA (MutH/B) from the plasmid pTZ19RCore.TRmHAXB in lanes 3 and 4 in the absence or presence of DHBV polymerase-containing lysates, respectively. (B) Translation of wild-type mRNAs (WT) from the plasmid pTZ19RCore in lanes 1 and 2 and mutant mRNA (MutB/A) from the plasmid pTZ19RCore.mutB/A in lanes 3 and 4 in the absence or presence of DHBV polymerase-containing lysates, respectively. The position of the DHBV polymerase (Pol) and DHBV core protein (Core) are shown at the right.

presence of increasing amounts of DHBV polymerase-containing lysate (Figure 4-7). The CAT activity of the CAT protein translated from the mutant and wild-type chimeric CAT-TR mRNAs in the presence of an equal amount of DHBV polymerase-containing lysate was also analyzed. The results, shown in Figure 4-8, are in agreement with the inhibitory effects of the DHBV polymerase on the CAT translation. The CAT activity of the CAT-containing lysate translated in the presence of DHBV polymerase from the mutant CAT-TR mRNA was 22-fold higher than that from the wild-type mRNA (Figure 4-8, lanes 2, 4). Thus, it is conceivable that the inhibitory effect of the hepadnaviral polymerase on viral core protein translation is due to the TR domain. The most crucial element for the repressive effects of the polymerase is the region between the *Hinc* II and *Bgl* II sites.

Prediction of an epsilon like stem-loop secondary structure in the TR region

I have demonstrated that the polymerase-mediated translational inhibition phenomenon is conserved amongst hepadnaviruses. I wanted to further examine whether any conserved motif was involved in the translation control. The sequence from nt 137–830 of the 3' periphery of the DHBV core ORF, which is responsible for the negative effect of the DHBV polymerase protein on the translation of DHBV core protein suggested by Dr. Howe's study, was analyzed using an RNA mfold program (Le, S.-Y. and Zuker, M. 1991; Mathews et al. 1999; Zuker et al. 1999). The TR-corresponding sequences at the 3' end periphery of the HBV and WHV core ORFs from nt 2309 to 2809 and from nt 2427 to 2927, respectively, were also analyzed by the RNA mfold program (Figure 4-9). This program predicted many secondary

Figure 4-7 *In vitro* translation of wild-type and mutant CAT chimeric plasmids with increasing amounts of DHBV polymerase. One microgram of wild-type or mutant CAT chimeric mRNAs, transcribed from plasmids pCMVCAT-TR or pCMVCAT.TRmHAXB respectively, was mixed in 50 μl of rabbit reticulocyte translation mixture containing [^{35}S]methionine and a methionine-free amino acid mixture. Five microliters of the translation mixture, containing wild-type mRNAs (WT) in lanes 1 to 4 or mutant mRNAs (Mut) in lanes 5 to 8, were translated with increasing amounts (0, 2.5, 5, 10 μl) of previously translated DHBV polymerase-containing lysates. *In vitro* translated products were analyzed on PAGE. The translation efficiency of the CAT protein was determined by a phosphoimager and presented as a percentage of the control.

kDa $\quad$ <u>WT</u> $\quad$ <u>Mut</u>

$\quad$ 1 2 3 4 5 6 7 8

66.2 —
55 —
40 —

31 —

$\qquad$ ←CAT

21.5 —

Pol

% Translation: $\quad$ 100 65 15 5 100 97 95 85
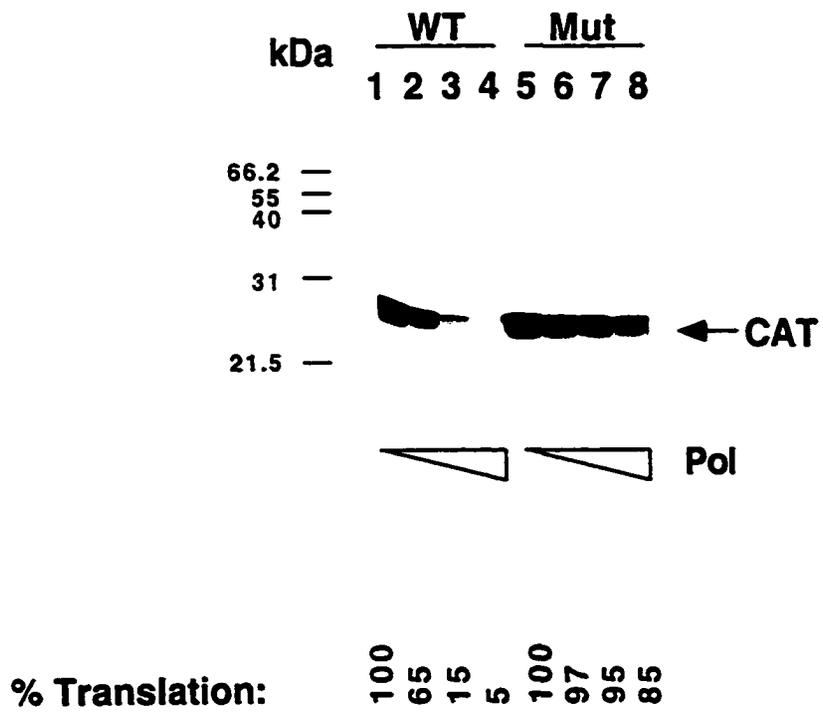
Figure 4-8   CAT activities of wild-type and mutant CAT chimeric constructs translated in the presence of DHBV polymerase.   Plasmids of pCMVCAT-TR and pCMVCAT-TRmHAXB were transcribed *in vitro* to generate the wild-type and mutant chimeric CAT mRNAs (WT and Mut), respectively.   CAT activity of CAT-containing lysate, translated from 1 μg mRNAs of WT (lanes 1, 2) and Mut (lanes 3, 4) in the absence or presence of DHBV polymerase-containing lysates, respectively, was performed.   CAT activity was analyzed on a TLC plate followed by autoradiography and quantitation.   The monoacetylated products 3-acetylchloramphenicol (3Ac-Cm), 1-acetylchloramphenicol (1Ac-Cm) and chloramphenicol (Cm) are shown at the right.

	WT		Mut		Pol
	-	+	-	+	
					◄ 3Ac-Cm
					◄ 1Ac-Cm
					◄ Cm
					◄ Origin
	1	2	3	4	

Conversion (%) 84.6   3.3   88.6   76.7
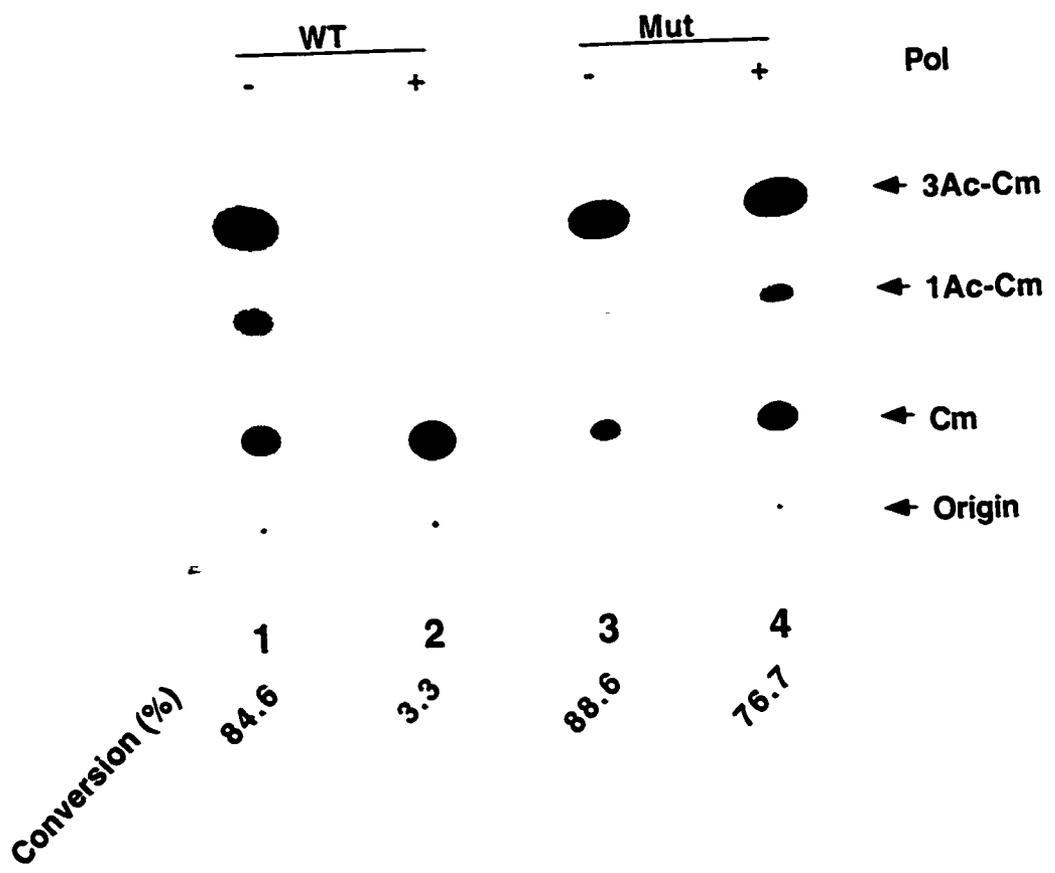
Figure 4-9 Schematic diagram showing regions analyzed by an RNA mfold program. The locations of core ORFs, TR regions and stem-loop structures of DHBV, HBV and WHV are shown. Nucleotide numbering is beginning at the unique *EcoR* I site of each genome.

A.

2647 · 411 · C-ORF — DHBV core mRNA

137 · Hinc II · Bgl II · 391 · 830 · Aat II — TR

301 377 — Stem-loop

B.

1903 · 2461 · C-ORF — HBV core mRNA

2309 · 2809 — TP

2514 2590 — Stem-loop

C.

2021 · 2587 · C-ORF — WHV core mRNA

2427 · 2927 — TR

2478 2522 — Stem-loop

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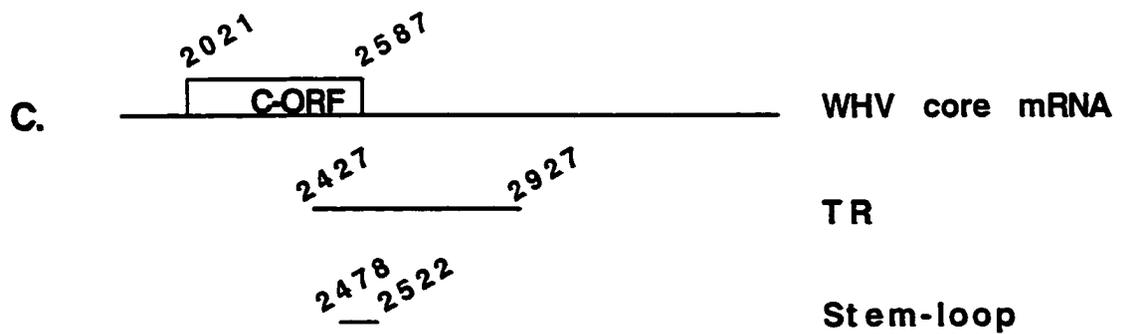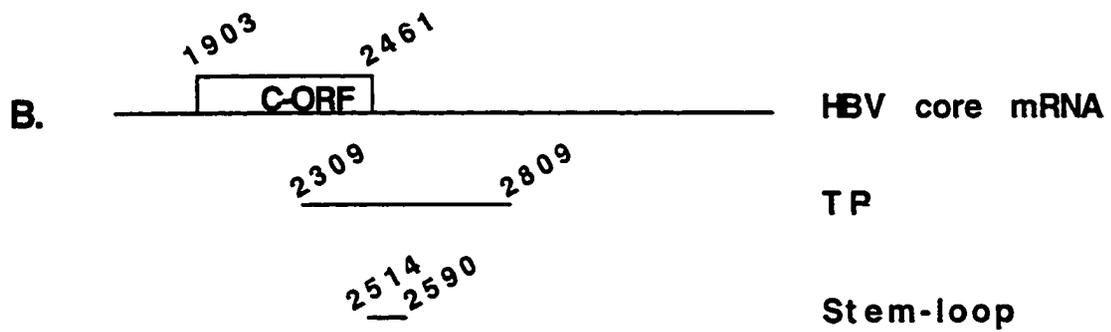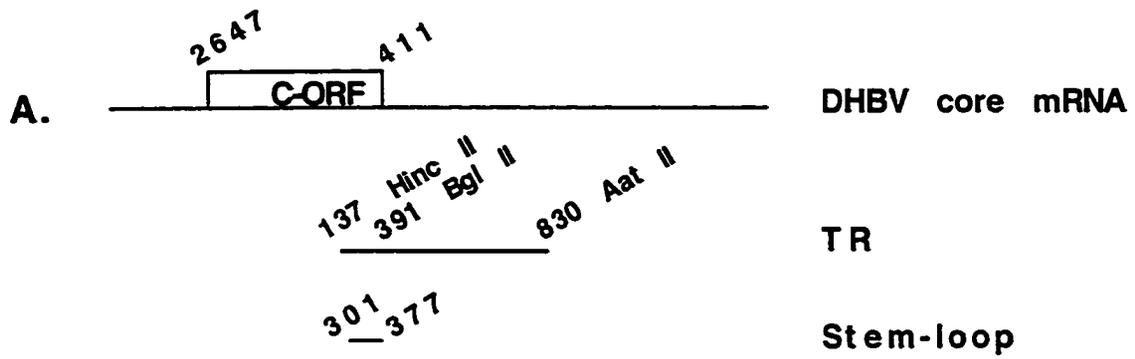
structures, however, an epsilon like stem-loop secondary structure containing a small loop, a small bulge, and upper and lower stems was predicted in the region from nt 301 to 377 of the DHBV mRNA and a similar stem-loop structure was also predicted in the region from nt 2514 to 2590 and from nt 2478 to 2522 of the HBV and WHV genomes, respectively, as shown in Figures 4-9 and 4-10. It is worthy to note that the epsilon like stem-loop structures are not as conserved as the epsilon stem-loop structures, in length or structure, between viruses. Nonetheless, the region of the secondary structure stem-loop in the DHBV TR coincides with the region of the most critical sequences (137–391) for the polymerase-mediated translational inhibition indicated by our CAT and DHBV core silent mutation studies. Silent mutations of the sequence from nt 137 to 391 completely eliminated the epsilon like stem-loop structure (Figure 4-11) and these mutations reversed the polymerase-mediated translational inhibition of the DHBV core and CAT protein synthesis (Figures 4-6, 4-7, 4-8). The implication of the epsilon like stem-loop of the TR region will be discussed.

Figure 4-10 Comparison of the epsilon stem-loop structures of the pgRNAs to the epsilon like stem-loop structures of the viral core mRNAs. A. The epsilon stem-loop structures with six nucleotide loops and bulges as well as upper and lower stems of DHBV, HBV and WHV pgRNAs. B. Predicted epsilon like stem-loop structures in the TR regions of DHBV, HBV and WHV showing similar secondary structures.

A.

Epsilon Stem-loop Structures

DHBV	HBV	WHV

-14.8	-25.2	-26.7

Free Energy (kcal/mole)

B.

Epsilon Like Stem-loop Structrues

DHBV	HBV	WHV

-26.7	-8.7	-14.5

Free Energy (kcal/mole)

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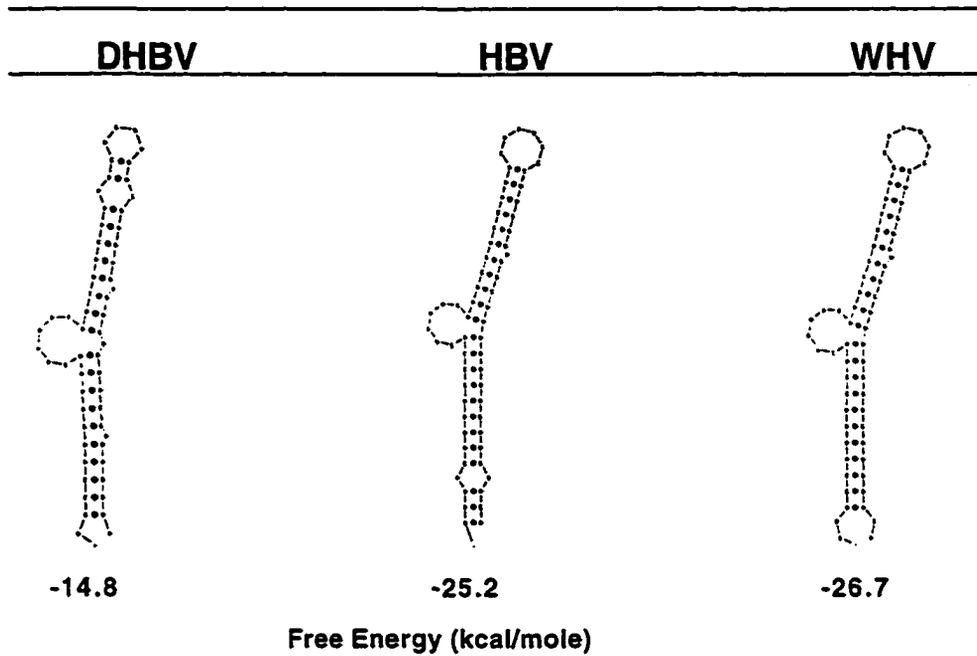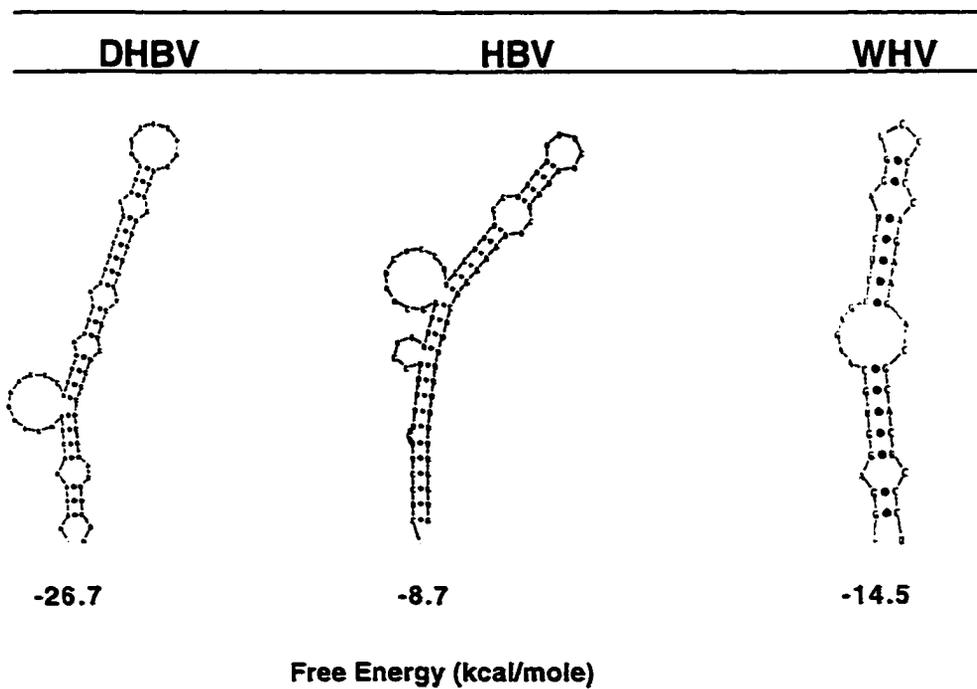
Figure 4-11 Predicted secondary structures between the *Hinc* II and *Bgl* II sites. (A) Epsilon like stem-loop structure of the wild-type DHBV-TR sequence between the *Hinc* II and *Bgl* II sites and DHBV epsilon structure. (B) Alterative secondary structures of the silent mutation sequences between the *Hinc* II and *Bgl* II sites of DHBV-TR.

A. Epsilon Like Stem-loop of WT in the TR Region
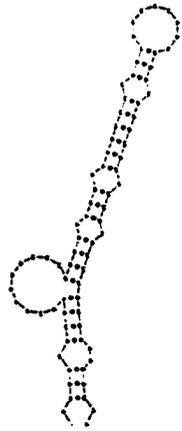
Epsilon Stem-Loop at the 5' End of pgRNA

-26.9

-14.8

Free Energy (kcal/mole)

B. Two Alterative Structures as a Result of Silent Mutations in the Stem-loop of the TR Region
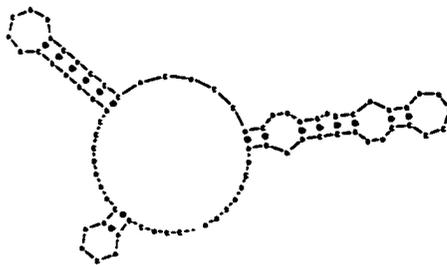
-16.5
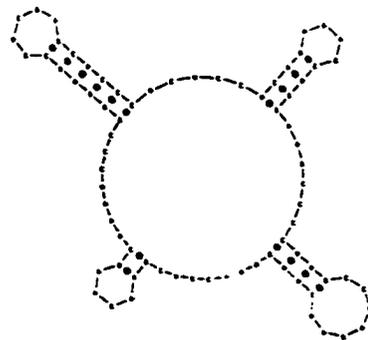
-17.5

Free Energy (kcal/mole)

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IV. Discussion

The hepadnaviral polymerase gene product is a multifunctional protein which is involved in the initiation of reverse transcription and in viral DNA synthesis (Gerlich and Robinson 1980; Bartenschlager and Schaller 1988; Radziwill et al. 1990; Seeger et al. 1986). The polymerase protein was also found to be an essential structural component for the encapsidation of the pregenome RNA /pgRNA (Bartenschlager et al. 1990; Hirsch et al. 1990). The nucleoencapsidation step requires a specific and high affinity binding of the viral polymerase with the 5' epsilon stem-loop structure of the pgRNA (Pollack and Ganem 1993, 1994; Tavis et al. 1994). The interaction of the polymerase with the pgRNA at the 5' epsilon stem-loop and/or other region is probably also responsible for the translational repression of the core protein synthesis (see results in Chapter 3; Howe and Tyrrell 1996; Nassal et al. 1990). Our evidence shows that hepadnaviral core mRNAs were translationally inhibited by the hepadnaviral polymerase (Chapter 3). We have identified that a regulatory element (*trans*-repression domain/TR) at the 3' periphery of the hepadnaviral core protein mRNA is involved in this translational control.

To further evaluate the proposed role of the TR sequences, we asked whether the presence of this *trans*-repression domain would be sufficient to transfer the regulatory capacity of the hepadnaviral polymerase to a heterologous mRNA. Dr. Howe has shown that the sequences from nt 140 to 828 are involved in the polymerase-mediated translation inhibition and sequences from nt 401 to 870 are sufficient for transferring this polymerase induced translational inhibition to another RNA (human CD4). We wanted to develop a reporter system that would improve upon Dr. Howe's study. We constructed a chimeric plasmid pCMVCAT-TR by cloning the DHBV-TR sequences (nt 137 to 830) downstream from the CAT ORF. A translational inhibitory

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effect of DHBV polymerase on the expression of this chimeric plasmid was demonstrated (Figure 4-2, lanes 5–6). In contrast to the chimeric plasmid, DHBV polymerase did not significantly inhibit the translation of CAT expression plasmids that did not contain DHBV-TR sequences or contained DHBV-TR unrelated sequences as shown in Figure 4-2, lanes 1–4. This supports that the hepadnaviral TR sequence was necessary for the inhibitory effect that viral polymerase has on the gene expression. The TR region was sufficient to transfer the translational regulation to a heterologous mRNA system.

Changing the primary sequence, would likely change the secondary structure of the *trans*-repression domain and release the inhibitory effect of the polymerase on the gene expression. To examine this hypothesis, silent mutations in the TR region were designed and chemically synthesized. The TR containing the silent mutations was introduced into the DHBV core expression and the chimeric CAT expression plasmids. The mutant Core and CAT expression plasmids were examined for their translation efficiency in the presence of the DHBV polymerase. As shown in Figure 4-6B, lane 4, silent mutation sequences at the 3' end of the TR from nt 391 to 830 only marginally affected the translational inhibition of DHBV core mRNA in the presence of the polymerase, showing that this region is not crucial for the polymerase dependent translational inhibition. However, DHBV core and chimeric CAT expression constructs with silent mutations in the region between the *Hinc* II and *Bgl* II sites (nt 137–391) in the 5' TR dramatically reduced the effect of the polymerase on the expression of these genes (Figure 4-6A, lane 4 and Figure 4-7, lanes 5–8). This suggests that the sequence between the *Hinc* II and *Bgl* II sites of the TR domain is critical for the translational repression of core expression by viral polymerase.

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Using the online mfold algorithm and thermodynamic based RNA secondary structure folding computer program, we analyzed DHBV, HBV and WHV mRNAs and predicted an epsilon stem-loop like structure located at the 3' periphery of all these core mRNAs as presented in Figures 4-9 and 4-10. The mfold computer software has been advanced for efficient and accurate prediction of secondary structure. The program predicted an epsilon like stem-loop structure in the region which was critical for the polymerase induced-translational inhibition, the same region where silent mutations eliminated the stem-loop structure and released the polymerase-mediated inhibition on the gene expression. Hence, I believe that this epsilon like stem-loop structure is essential for this polymerase induced-translation. The polymerase may recognize and interact directly or indirectly with the stem-loop structure on the core mRNA/pgRNA resulting in the translational repression.

In conclusion, we have confirmed that the 3' terminal sequences of the hepadnaviral core mRNA are responsible for the inhibitory effect of the hepadnaviral polymerase on the gene expression. We have suggested that the sequence between the *Hinc* II and *Bgl* II, bearing a putative conserved epsilon stem-loop like structure, is the primary regulatory element for the translational inhibition of the hepadnaviral polymerase. This corresponds to Dr. Howe's deletion study showing that the region from nt 141 to 391 removed the most translational repression of the polymerase. However, Dr. Howe also demonstrated in transfer studies that the sequences from nt 401 to 870 also mediated polymerase-induced inhibition in a heterologous system (human CD4). From my results, I am surprised by Dr. Howe's results as I could not demonstrate the polymerase induced-repression with these sequences.

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CHAPTER 5

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

I.   Overall Summary and Discussion

The hepadnaviral polymerase is a multifunctional enzyme. It is well known to have roles in viral DNA synthesis and pgRNA encapsidation (Bartenschlager and Schaller 1992; Summers and Mason 1982). There are also suggestions that it may have other functions such as translational regulation (Howe et al. 1996; Nassal et al. 1990). Further study and understanding of the biology, synthesis and function of the polymerase is necessary.

The objectives of the work described in this thesis were first to establish an *in vitro* expression system for HBV polymerase. It will help us better characterize the polymerase and improve our understanding of the mechanism of action of antiviral agents to facilitate rational design of new antiviral agents. Secondly, we aimed to investigate the possible downregulation properties of the polymerase on core protein expression which we believe will lead to deeper insights into the mechanism of viral replication and gene expression and possibly enable us to express significant quantities of the polymerase.

In Chapter 2, I described a system used to clone and express an enzymatically active HBV polymerase. This project has been particularly challenging. I and other researchers from laboratories around the world have tried to express HBV polymerase in heterologous systems, such as *E.coli*, recombinant baculovirus and vaccinia virus

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expression systems, and have achieved only limited success over many years (Ayola et al. 1993; Bartenschlager et al. 1992; Chang et al. 1989; Köchel et al. 1991; McGlynn et al. 1992; Noonan et al. 1991; Stemler et al. 1988). Failure to express a full-length, enzymatically active polymerase was first believed to partially due to the toxicity of the polymerase to the biological host (Chang et al. 1989; Foster et al. 1991). In order to circumvent this, I employed a cell free system in which a full-length and two smaller species of HBV polymerase proteins were expressed in a transcription-translation-coupled reticulocyte lysate system. Since the viral epsilon stem-loop RNA sequence is required for the function of the polymerase in DNA synthesis (Lanford et al. 1997; Tavis and Ganem 1996; Tavis et al. 1994; 1998; Wang and Seeger 1993; Wang et al. 1994) and this sequence is not present in our polymerase expression construct, the HBV polymerase was always translated in the presence of a T7 driven epsilon stem-loop sequence, which will itself produce a transcription product of 231 nucleotides in length. The enzymatic activities of the *in vitro* translated HBV polymerases were tested. Protein priming activity in which the full-length and truncated terminal domain of the recombinant polymerase became covalently labeled by [α-^{32}P]dGMP was demonstrated in an *in vitro* priming assay. Furthermore, I demonstrated that minus polarity DNA products ranging from 200 to 400 nucleotides in length were synthesized by the recombinant polymerase using the epsilon stem-loop sequence as the template. These demonstrations of protein priming and template-dependent polymerization activities clearly indicate the authentic activities of the recombinant polymerase. The expression of HBV polymerase *in vitro* provides us with a novel and simple approach to the study of the biology of the polymerase and the mechanism of action of the antiviral agents.

In the process of attempting to express considerable quantities of polymerase by cotranslating core and polymerase genes, we obtained some evidence of translational

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inhibition by the polymerase on core expression in the DHBV system (Howe and Tyrrell 1996). The sequence from nt 140 to 828 at the periphery of the core ORF was reported to be required for the inhibitory regulation in the DHBV system. This regulatory property of the polymerase was also suggested by others (Nassal et al. 1990). Since hepadnaviruses utilize similar replication mechanisms, this inhibitory effect of the DHBV polymerase led us to investigate the possible inhibitory effects of the polymerases of other members in the family. I used an *in vitro* expression system to demonstrate that the HBV core mRNA translation was specifically inhibited by the HBV polymerase. By uncoupling the transcription step from the translation step in this system, I have shown that the inhibitory effects take place at the level of translation. Northern blots indicate that degradation of the mRNA was not involved in this translational repression. More importantly, I have provided the first evidence in the hepadnaviral family for a cross-species translational inhibition of core synthesis mediated by viral polymerase. The conservation of the mechanism involved in this downregulation indicated that it may be important in viral replication.

To further evaluate the putative role of the 3' periphery sequences of the core mRNA as *trans*-repression elements/TR for the inhibitory effects of the polymerase, I used the CAT gene as a reporter gene. I constructed a chimeric plasmid by cloning the DHBV-TR sequence from nt 137 to 830 into a CAT expression plasmid downstream from the CAT ORF, and analyzed it for its expression efficiency in the presence of the DHBV polymerase protein. The translation of the chimeric plasmid and the CAT activity of the translation product were dramatically reduced in the presence of DHBV polymerase. This confirmed Dr. Howe's study that the TR region is responsible for the downregulation of the polymerase and that the TR region can transfer the translation regulation of the polymerase to a heterologous mRNA. The TR region was further targeted for silent mutations which change the primary sequence and the secondary

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structure in this region to evaluate the effect of these changes on the inhibitory effect of the polymerase. The *in vitro* analysis of the translation efficiency of the mutants in the presence of the polymerase has been quite revealing. Silent mutations at the 3' end of the TR region between the sites of the *Bgl* II and *Aat* II (nt 391–830) only slightly released the translational inhibition of the polymerase on DHBV core mRNA translation while silent mutations between the *Hinc* II and *Bgl* II sites in the 5' end of the TR (nt 137–391) resulted in a dramatic relief of the negative effects of the polymerase on gene expression as seen in the level of the core and CAT protein productions and the activities of the CAT translation products. This result is consistent with Dr. Howe's deletion study and indicated that the sequence from *Hinc* II to *Bgl* II was mainly responsible for the inhibitory activity of the polymerase.

A computer program was used to analyze the folding of core mRNAs of HBV, DHBV and WHV and predicted an epsilon like stem-loop structure located at the 3' periphery of all these mRNAs. Specifically, the epsilon like stem-loop structure was predicted in the region between the sites of *Hinc* II and *Bgl* II of the DHBV core mRNA and our study demonstrated that this region was critical element for the polymerase-mediated inhibition. The silent mutations at the sites of *Hinc* II and *Bgl* II completely changed the epsilon like stem-loop and these silent mutations eliminated the polymerase-mediated inhibition. It is known that during the process of viral encapsidation, the viral polymerase interacts with the 5' epsilon stem-loop structure. However, prior to the encapsidation, pgRNA functions as a mRNA for core and polymerase translation and it is covered by ribosomes, which will interfere with the interaction of the polymerase with the 5' epsilon encapsidation signal. We believe that as soon as the polymerase has been synthesized, it probably functions as a translational repressor to interact with the downstream epsilon like stem-loop on the pgRNA. This interaction may block the translation of the pgRNA and help release of ribosomes from

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the pgRNA, therefby facilitating encapsidation by freeing the 5' epsilon stem-loop structure of the pgRNA to interact with polymerase. Although this model is very attractive, other data indicated that a region further downstream other than the epsilon like stem-loop region is required for the polymerase induced translational inhibition and encapsidation (Calvert and Summers 1994; Howe and Tyrrell 1996).

Since core and polymerase are translated from the same transcript pgRNA, polymerase may also interfere with its own translation. Such autoregulation as well as the inefficient internal AUG translation of the polymerase may contribute to the regulation of protein synthesis which results in 240 core protein molecules and 1–2 polymerase protein molecules for each virion. This polymerase-mediated translational inhibition may well account for the difficulties that several laboratories have encountered in trying to express the polymerase in heterologous systems.

II. Future Directions

I have expressed HBV polymerase with the protein priming and reverse transcription activities in a transcription-translation-coupled *in vitro* system. Therefore, this novel and simple system could be used to determinate the mechanism of action of antiviral nucleoside analogues such as penciclovir or 2,6 diaminopurine 2',3'-dideoxyriboside (ddDAPR), a prodrug of ddG, which are priming blocking agents (Howe et al. 1996; Zoulim et al. 1995).

Regarding the downregulatory property of the polymerase, I have shown the negative effect of the polymerase on gene expression and evaluated the putative *cis*-element with an epsilon stem-loop-like structure involved in the activity. However, it is still not clear whether the function of the polymerase is required for the inhibitory regulation and whether the primary or secondary structure or both within the TR is involved in the regulation. Site-directed mutations at the conserved tyrosine priming site (Lanford et al. 1997) or at the conserved YMDD motif in the catalytic domain (Kamer and Argos 1984) of the polymerase would inactivate the polymerase priming or polymerization activities, respectively. Evaluating translational inhibition effects of these polymerase mutants on the translation efficiency of the viral core and CAT-TR chimeric mRNAs may be considered. The effect of TR region on the polymerase induced translational regulation could also be examined with silent mutations which only change the primary sequences but retain the epsilon like stem-loop structure, although these mutations may be very difficult to perform. The results of such studies should help us to understand the precise molecular mechanisms of the negative effects of the polymerase on the gene expression.

Although I have demonstrated translational inhibition of the polymerase on the core expression, I have not examined control by the polymerase protein on its own

translation. Further studies are required to elucidate any autoregulatory mechanism, for example, the effects of silent mutations in TR on the translation of the polymerase. Such information may be useful for us to overcome the difficulties we have experienced in attempting to express polymerase and allow us to express the polymerase protein in large quantities. High expression of the polymerase will facilitate purification of the enzyme and therefore allow us to further study of the enzyme, such as kinetics studies and even crystal structure analysis of the polymerase protein.

III. Bibliography

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APPENDIX I

Sequence of Primers

Table AI-1 Primers

Primer designation	Sequence (5'–3')
HBV polymerase construction	
Pst I-HBV 5'	AACTGCAGAAATGCCCCTATCTTATCAAC
BspE I-HBV 5'	GTAGTTTCCGGAAGTGTTGA
BamH I-HBV 3'	CGGGATCCTTCGCGGGACGTCC
Xma I-HBV 3'	TCCCCCCGGGGGGAGTCCAAGAGTCCTC
HBV DR1/epsilon construction	
T7 DR1/epsilon 5'	TCCCCCCGGGAATACGACTCACTATAGAGCAAT GTCAACGACCGAC
T7 DR1/epsilon 3'	CCCCATGGCGCCGGCGACAGGTACGGGGTTTC GG
Mutagenesis of HBV polymerase gene	
pMVU	TTATCAACACTTCCGGAAAC
pMVD	CCAAGAATATGGTGACCCGC
pMV	TTGAAATTAATTGTGCCTGCTAGAT
HBV core construction	
pCi	CCGGAATTCATGGACATTGACCCTTAT
pCt	CGCGGATCCCTAACATTGTGATTCCCG

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CAT-TR construction

DHBVsphI   ACATGCATGCGTTTACTGTGGGGAGAAGCT

DHBVxbaI   CGAGATCTCTGCAGGTAACTAAAACGAC

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RECOMBINANT PLASMID CONSTRUCTS
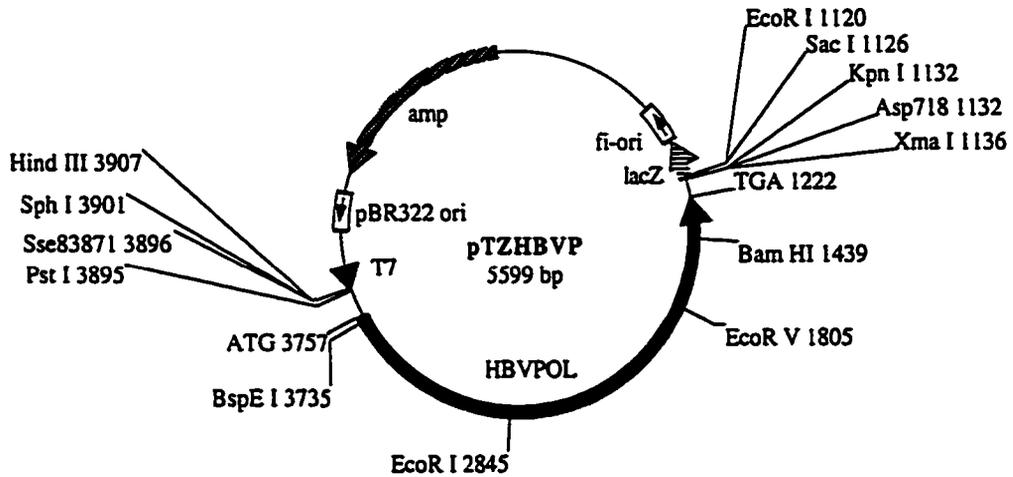
Plasmid name: pTZHBVP
Plasmid size: 5599 bp
Constructed by: Z. Li
Construction date: 1994
Comments/References: A comolete HBV polymerase with flanking sequences was isolated from an HBV replicating dimer plasmid pKSVHBV1 (Seifer et al., Virology 1990) and subcloned into an in vitro expression vector pTZ19R (Pharmacia Biotech) downstream from a T7 promoter.

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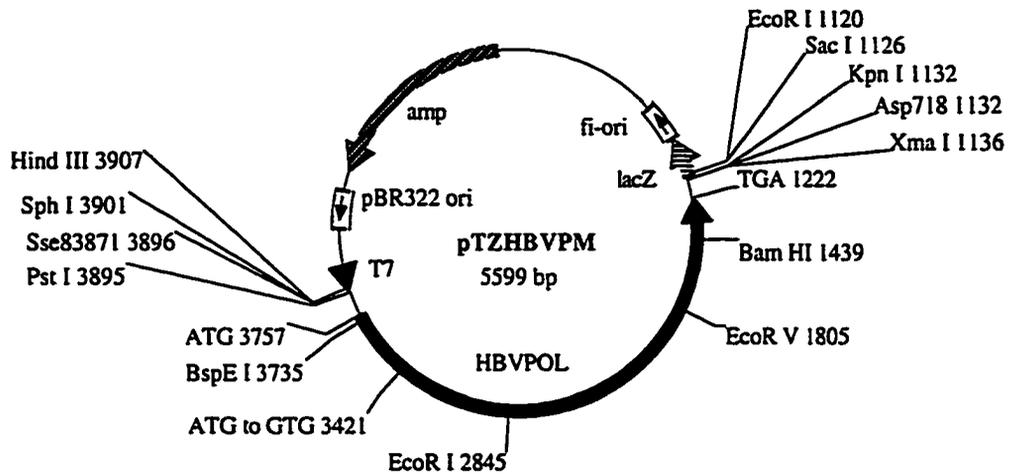
Plasmid name: pTZHBVPM
Plasmid size: 5599 bp
Constructed by: Z. Li
Construction date: 1996
Comments/References: The HBV polymerase with flanking sequences from a plasmid pKSVHBV1 (Seifer et al., Virology 1990) was subcloned into the pTZ19R (Pharmacia Biotech). Site-directed mutagenesis was used to change the 2nd methionine to valine at 113 a.a. of the polymerase.

234
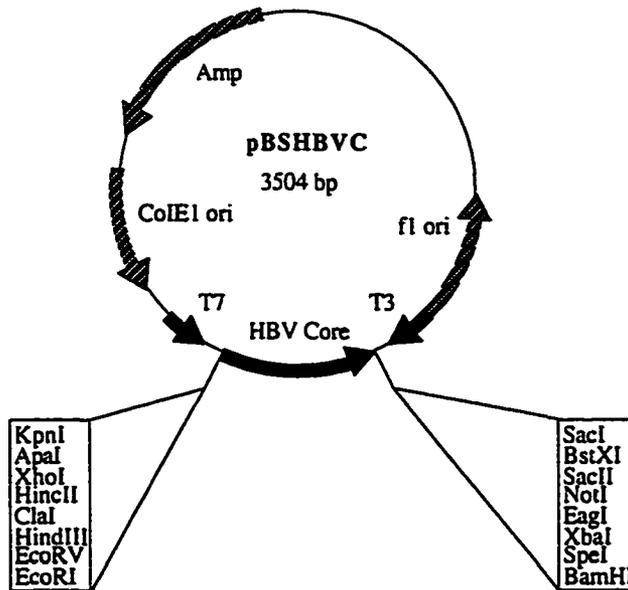
Plasmid name: pBSHBVC
Plasmid size: 3504 bp
Constructed by: Z. Li
Construction date: 1997
Comments/References: A 558 bp cDNA of HBV core ORF was PCR amplified from a plasmid pKSVHBV1 (Siefer et al. 1990) and subcloned into the EcoRI and BamHI sites of pBluescript SK-.

235
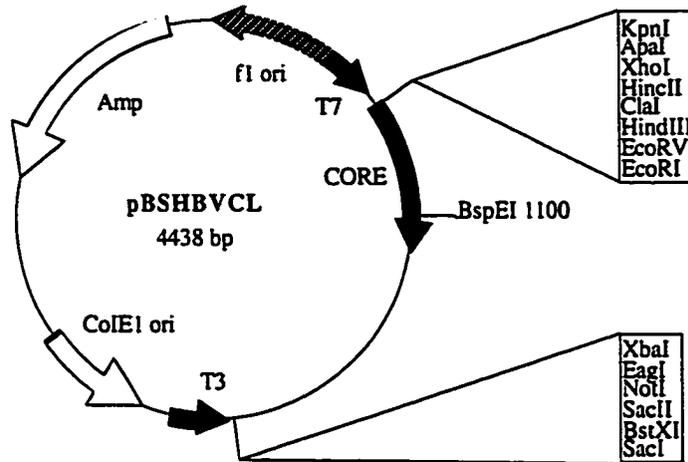
Plasmid name: pBSHBVCL

Plasmid size: 4438 bp

Constructed by: Z. Li

Construction date: 1997

Comments/References: pBSHBVCL was derived from plasminds pBSHBVC and pTZHBVP. An 1070 bp fragment was released by digesting pTZHBVP with BspEI and AvrII and cloned into the sites of BspEI and SpeI of pBSHBVC.

236
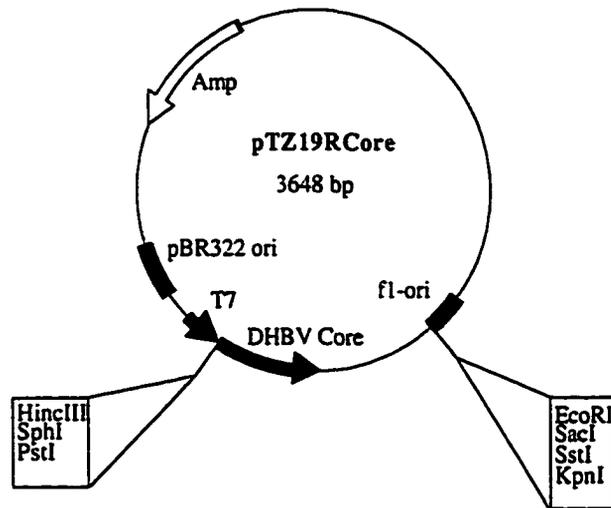
Plasmid name: pTZ19RCore
Plasmid size: 3648 bp
Constructed by: A.Y.M. Howe
Construction date: 1993
Comments/References: DHBV core ORF and 548 bp downstream sequence are PCR
amplified and cloned into the PstI and SmaI sites of pTZ19R. The SmaI site is destroyed
(Howe A.Y.M. and Tyrrell D.L.J., J. Virol 1996; 70:5035–5042).

237
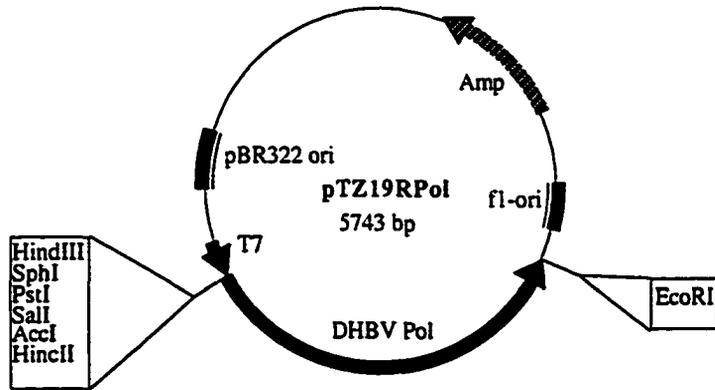
Plasmid name: pTZ19RPol
Plasmid size: 5743 bp
Constructed by: A.Y.M. Howe
Construction date: 1992
Comments/References: DHBV polymerase ORF is inserted into the pTZ19R vector
at the HincII and EcoRI sites (Howe A.Y.M. and Tyrrell D.L.J., J of Virol.1996; 70:5035-5042).

238
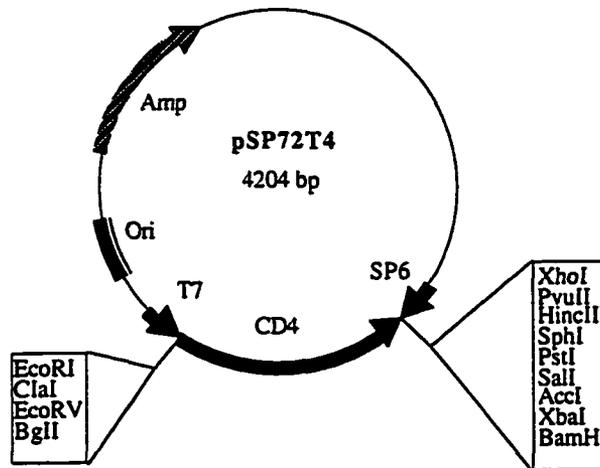
Plasmid name: pSP72T4
Plasmid size: 4204 bp
Constructed by: L.-J. Chang
Construction date: 1993
Comments/References: Human CD4 gene was cloned under the T7 promoter at the
EcoRI and BamHI sites of the Sp72 vector.

239
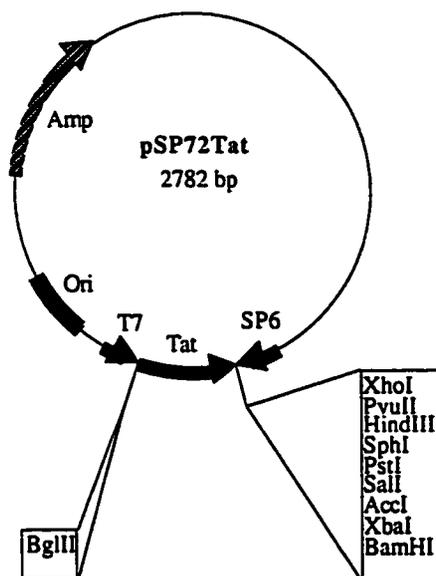
Plasmid name: pSP72Tat
Plasmid size: 2782 bp
Constructed by: L.-J. Chang
Construction date: 1992
Comments/References: The entire HIV Tat gene was obtained by PCR amplification
with primers incorporated approciate BamHI and BglII sites at the 5' and 3' ends,
respectively. The Tat cDNA was cloned into the BglII and BamHI sites of pSP72 vector.

240
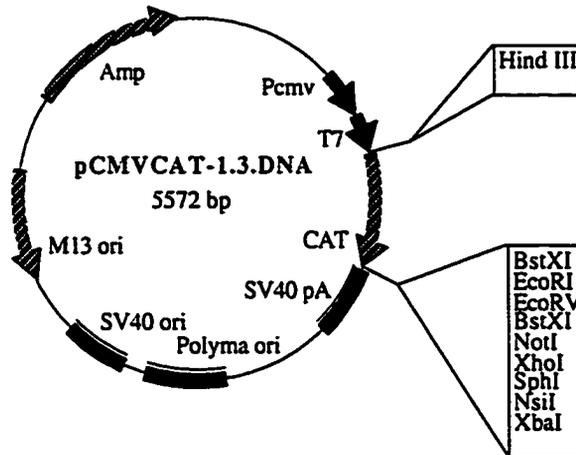
Plasmid name: pCMVCAT-1.3.DNA
Plasmid size: 5572 bp
Constructed by: K. Fischer
Construction date: 1996
Comments/References: The CAT gene was released from a plasmid pSV2CAT (Gorman et al., Mol. Cell. Biol. 1982; 2:1044–1051) with Hind III and Sau3A blunted by Klenow and cloned into a vector pDNA1.1/Amp (Invitrogen) at the sites of Hind III and BamHI-blunted.

241
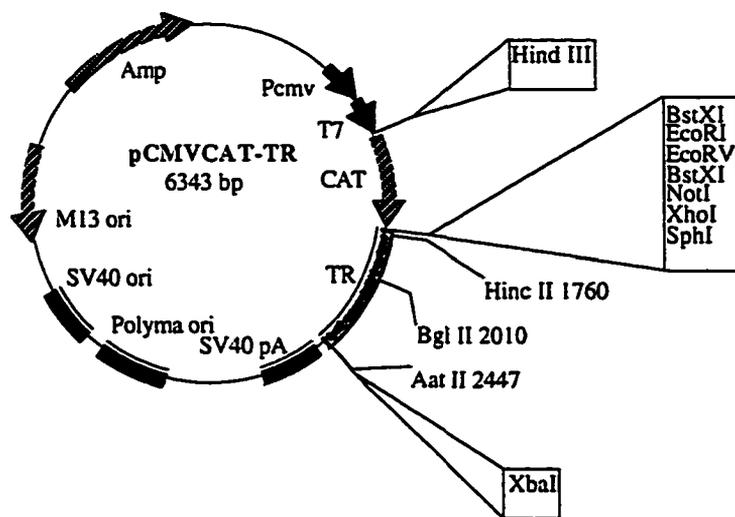
Plasmid name: pCMVCAT-TR
Plasmid size: 6343 bp
Constructed by: Z. Li
Construction date: 1998
Comments/References: The DHBV-TR sequence from Hinc II to Aat II sites was
 PCR amplified from pTZ19Rcore plasmid with primers DHBVsphI and DHBVxbaI and subcloned
 into plasmid pCMVCAT-1.3.DNA at the sites of SphI and XbaI.

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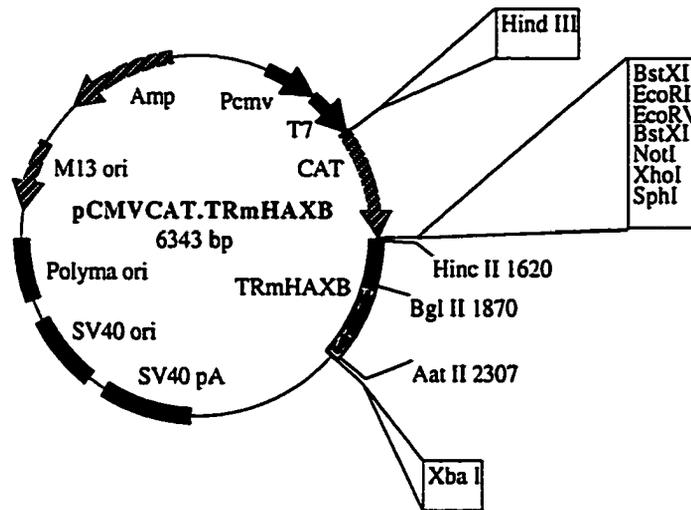
Plasmid name: pCMVCAT.TRmHAXB
Plasmid size: 6343 bp
Constructed by: R. George
Construction date: 1998
Comments/References: This clone was derived from pCMVCAT-TR by replacing the
sequences between the Hinc II to Bgl II with silently mutated sequences, which was
obtained by ligation of mutiple mutated oligonucleotides.

243
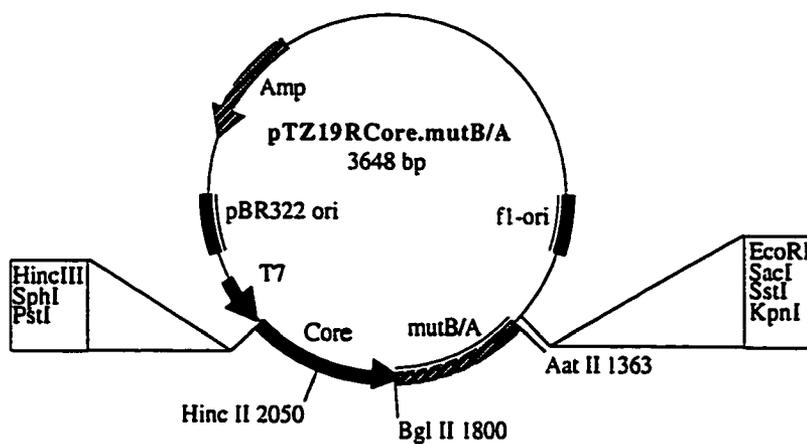
Plasmid name: pTZ19RCore.mutB/A
Plasmid size: 3648 bp
Constructed by: R. George
Construction date: 1998
Comments/References: This clone was derived from pTZ19RCore plasmid by replacing the sequences between the Bgl II and Aat II sites with silently mutated sequences, which was obtained by ligation of multiple mutated oligonucleotides.

244
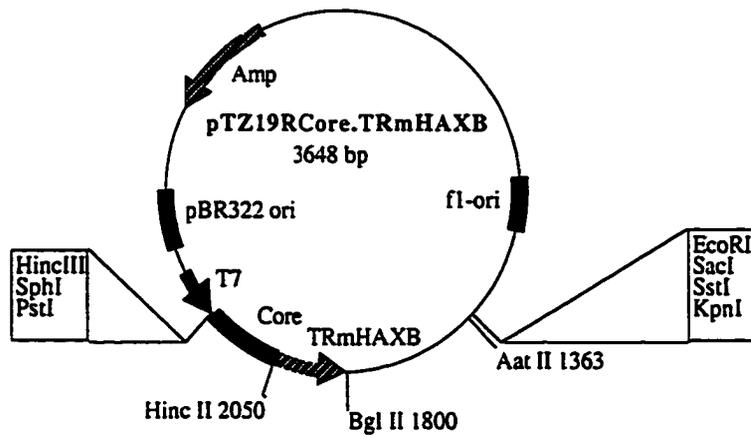
Plasmid name: pTZ19RCore.TRmHAXB
Plasmid size: 3648 bp
Constructed by: R. George
Construction date: 1998
Comments/References: This clone was derived from pTZ19RCore plasmid by replacing the sequences between the Hinc II and Bgl II sites with silently mutated sequences, which was obtained by ligation of multiple mutated oligonucleotides.

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