THE CLONING AND EXPRESSION OF THE HEPATITIS B POLYMERASE GENE IN *ESCHERICHIA COLI*

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Doctor of Philosophy

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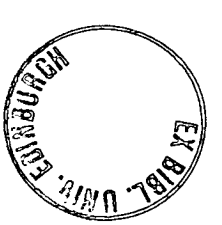

September 1987.

DECLARATION:

I hereby declare that I alone have composed this thesis and that, except where stated, the work presented within is my own.

September, 1987.

TO MY FATHER AND MOTHER

ABSTRACT

The Hepatitis B virus (HBV) possesses a partially double-stranded circular DNA genome. The virions carry an endogenous polymerase that uses the minus strand as a template to fill in the single-stranded gap. Since attempts to purify this enzyme have been unsuccessful the origin of this DNA polymerase has not been established. It may be virally encoded or a host polymerase which is encapsulated during maturation of the virion. Sequence analysis of the viral genome revealed the presence of a long open reading frame (L.ORF) with the coding capacity of 93kd, the expected size of a DNA polymerase. The predicted amino acid sequence of the L.ORF revealed the existence of amino acid homologies with the reverse transcriptase of some other viruses. The size, and the belief that HBV replication involves a reverse transcriptase activity of the endogenous polymerase led to the assignment of the long ORF as the putative polymerase gene.

Segments of the putative polymerase gene were fused in frame to the 3' end of the β-galactosidase gene of Escherichia coli, and expressed as fusion proteins. Antisera raised to these fusion proteins recognise distinct epitopes in the amino and central regions of the putative polymerase gene product. Using immunoblotting, convalescent sera from chimpanzees infected with HBV were shown to contain antibodies that cross-reacted with the fusion proteins. These antibodies were found only transiently, and reached a maximum titre about 25 weeks after inoculation with the virus at which stage the animals still exhibited HBe antigenaemia, but were also producing antibodies to all the HBV antigens. Antibodies that cross-reacted with the fusion protein were not found in the serum of a chimpanzee that had been vaccinated prior to inoculation with HBV. Furthermore,

antibodies raised to HBV surface, core, e and X antigens did not react with the β-galactosidase-polymerase fusion product.

Extracts of E.coli expressing the gene fusion products were also shown to exhibit a polymerase activity with the characteristics of the endogenous Hepatitis B polymerase.

These experiments show that the HBV long ORF encodes a protein with polymerase activity; also the gene is expressed during viral infection and antibodies to its product are found in convalescent sera during HBV infection. These findings leave little doubt that the endogenous polymerase associated with HBV is indeed encoded by the long ORF and is not a host polymerase.

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A	:	Adenosine
A	:	Ampere
AMPS	:	Ammonium persulphate
ATP	:	Adenosine 5' triphosphate
bp	:	Base pair
BSA	:	Bovine serum albumin
c	:	Cytosine
^{o}C	:	Degree Celsius
Ci	:	Curie (1 Ci = 2.2 x 10^{12} dpm)
D	:	Dalton (1/12 of the mass of one atom of nuclide ^{12}C, i.e. 1.663 x 10^{-24}g)
(d)dATP	:	2'(3'-di) deoxy adenine-5'-triphosphate
(d)dCTP	:	2'(3'-di) deoxycytidine-5'-triphosphate
(d)GTP	:	2'(3'-di) deoxyguanosine-5'-triphosphate
(d)TTP	:	2'(3'-di) deoxythymidine-5'-triphosphate
(d)NTP	:	2'(3'-di) deoxynucleotide-5'-triphosphate
dH_2O	:	Distilled water
DMSO	:	Dimethyl sulphoxide
DNA	:	Deoxyribonucleic acid
DNase	:	Deoxyribonuclease
dpm	:	Decompositions per minute
DTT	:	Dithiothreitol
EDTA	:	Ethylene-diamine-tetracetic acid.
G	:	Guanosine
g	:	Gram
g	:	Acceleration due to gravitiy (9.81 $m.s^{-2}$)
HEPES	:	4-(2-hydroxyethyl)-1-peperazine-ethanesulphonic acid
h	:	hour

IPTG	:	Isopropyl β-D-thiogalactoside
k	:	Kilo (10^3). e.g. kg: kilogram
kb	:	Kilobase of double-stranded DNA or of single-stranded RNA
l	:	Litre
M	:	Molar
m	:	Metre
m	:	Milli (10^{-3}). e.g. ml : millilitre
min	:	Minute
MOPS	:	4-Morpholine-propane-sulphonic acid.
mRNA	:	Messenger ribonucleic acid
Mw	:	Molecular mass
n	:	Nano (10^{-9}) e.g. nm : nanometre
NP40	:	Nonidet P-40
OD.600nm	:	Optical density with respect to light of wavelength 600nm
ORF	:	Open reading frame
p	:	Pico (10^{-12}). e.g. pM : picomolar
^{32}P	:	A β-emitting radioactive isotope of phosphorus
pCp	:	Cytidine 3', 5'-bisphosphate
PEG	:	Polyethylene-glycol
pH	:	Hydrogen ion concentration - $\log_{100}$
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
rpm	:	Revolutions per minute
rRNA	:	Ribosomal ribonucleic acid
.s	:	Second (time)
^{35}S	:	A β-emitting radioactive isotope of sulphur
SDS	:	Sodium dodecyl sulphate
T	:	Thymidine

TCA	:	Tricholoro acetic acid
TEMED	:	N, N, N', N'-Tetramethyl-ethylenediamine
Tris	:	2-Amino-2-hydroxymethyl-propane-1,3-diol
tRNA	:	Transfer ribonucleic acid
Tween 20	:	Polyoxyethylene(20)-sorbitan-monolaurate
U	:	Uracil
U.V.	:	Ultra-violet light
V	:	Volt
v/v	:	Volume per unit volume
w/v	:	Weight per unit volume
μ	:	micro (10^{-6}). e.g. μl : microlitre

STANDARD AMINO ACID ABBREVIATIONS

A	:	Alanine
C	:	Cysteine
D	:	Aspartic acid
E	:	Glutamic acid
F	:	Phenylalanine
G	:	Glycine
H	:	Histidine
I	:	Isoleucine
K	:	Lysine
l	:	Leucine
M	:	Methionine
N	:	Asparagine
P	:	Proline
Q	:	Glutamine
R	:	Arginine
S	:	Serine
T	:	Threonine
V	:	Valine
W	:	Tryptophan
X	:	Any amino acid
Y	:	Tyrosine

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Chapter 1

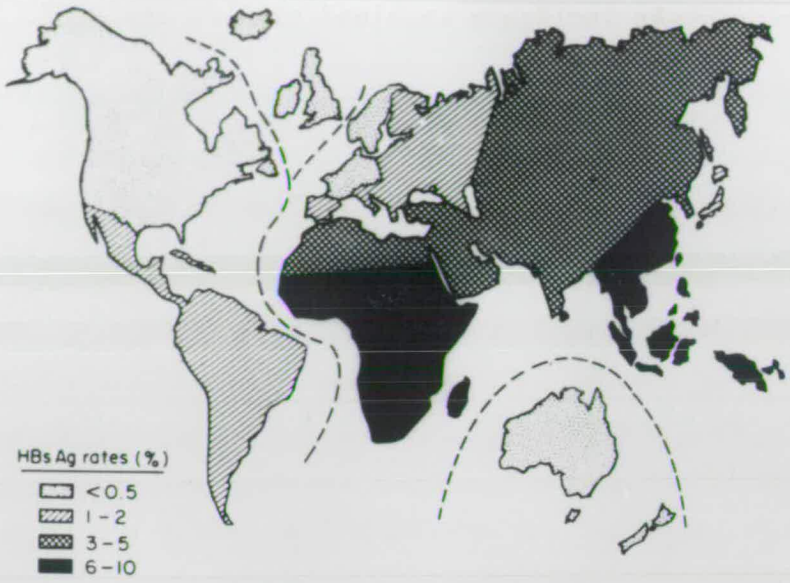
The first reference to epidemic jaundice has been ascribed to Hippocrates (460-375 BC). Many epidemics have been described since. However, it was not until 1940 that epidemic jaundice was found to be caused by inflammation of the liver, i.e. hepatitis. A virus was later discovered by Blumberg to be the causative agent of this severe form of hepatitis (Blumberg, et al., 1967). This virus is now known as Hepatitis B virus (HBV).

Evidence of infection with HBV has been obtained in every population in the world irrespective of geographical location, but the prevalence of HBV infection varies according to the region of the world (Fig.1.1a). In many African and Asian countries infection is almost universal. In South America, North Africa, Middle East and South Eastern Europe around 1%-5% of the population is infected while the lowest incidence of HBV infection (0.3% of the population) is found in the developed countries of Western Europe, U.S.A., Canada, Australia, New Zealand and the Scandinavian countries.

1.1. The Virus

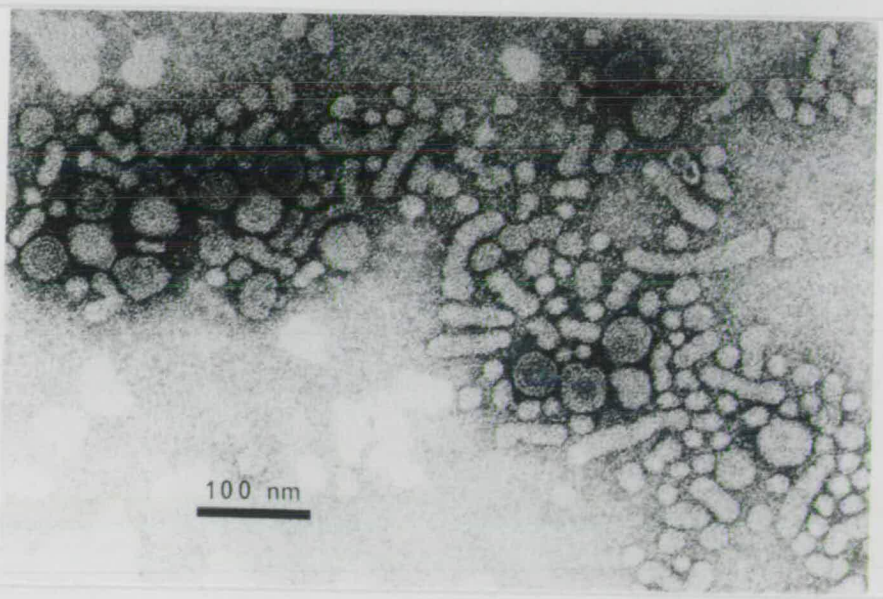
Three distinct structures can be consistently detected in the serum of HBV individuals infected with HBV: a) small spherical particles approximately 22nm in diameter, b) tubular structures that are approximately 22nm in diameter and vary in length, and c) enveloped particles with a diameter of approximately 42nm (Fig.1.1b). This 42nm enveloped particle was later shown to be the infectious agent (also known as the Dane particle). It possesses an inner nucleocapsid structure, approximately 28nm in diameter, which contains the genetic information of the virus (a partially double-stranded, partially single strand circular DNA genome), and an endogenous polymerase activity (Fig.1.1c).

A.

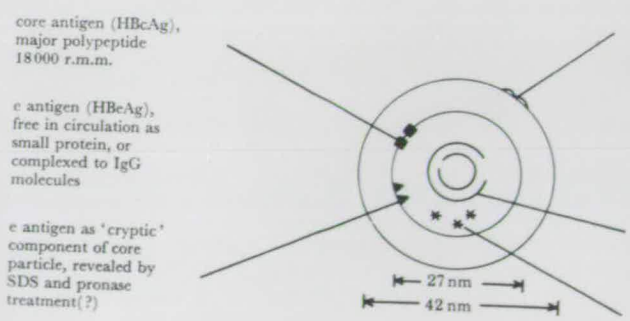


A schematic map of worldwide distribution of HBsAg.

B.



C.



(Robinson 1977, Kaplan et al., 1973). The smaller particles and tubular structures associated with infection contain no viral genetic material and result from the over-production, during infection, of the viral coat proteins which subsequently aggregate to form these structures.

1.2 Components of the Virus Particle

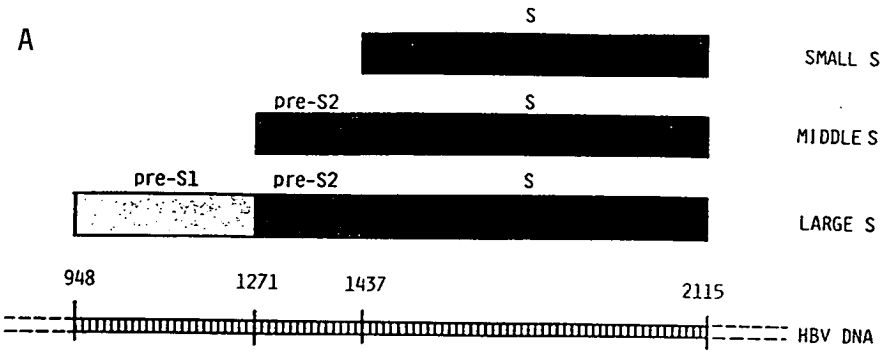
1.2.1 The envelope and surface structure.

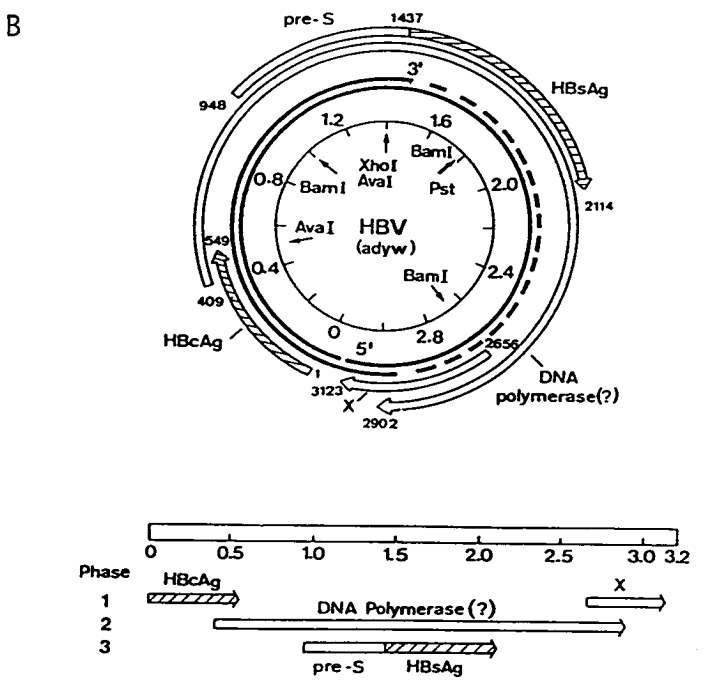
The virion is surrounded by an envelope, which is proposed to originate from the endoplasmic reticulum (Persing et al., 1986). The surface antigen protein and the glycoproteins are embedded in the lipid bilayer.

The three surface proteins of the virion, and of the spherical and tubular structures are pre S1 (p39), pre S2 (p31) and the major surface protein (p24). These 3 proteins share the same carboxy terminus but have different amino termini reflecting the different translational start sites used within the surface ORF, which has 3 inframe AUG initiation codons at the beginning of the surface gene (Fig.1.2a). The distribution of these 3 proteins is not equal among the circulating forms of HBsAg. The pre S1 protein is found in higher abundance in viral particles and filaments than in the more numerous 22nm subviral particles, while the converse is true of the more abundant preS2 and S proteins. These findings suggest that pre S1 determinants may be important in virus assembly and/or infectivity (Persing et al., 1987). The major surface protein is 226 amino acids in length. It has two forms; a protein of 24kd (p24) and a glycosylated form with a molecular weight of 27kd (GP27). The latter possesses a complex N-linked glycan at ASN146. HBsAg is an extremely hydrophobic molecule rich in cysteine

FIGURE 1.2(a) Structure of the HBV surface antigen showing the location and sizes of the three major domains (Harrison et al., 1986).

FIGURE 1.2(b) The genome of HBV. Heavy lines denote the DNA strands, the broken line showing the region of variable length of the short strand. Arrows represent the four open reading frames (as coding sequences) with the numbers of initiation and termination triplets in the system adopted by Pasek et al., (1979).


A
S
SMALL S
pre-S2
S
MIDDLE S
pre-S1
pre-S2
S
LARGE S
948
1271
1437
2115
HBV DNA


B
pre-S
1437
3'
HBsAg
948
1.2
1.6
BamI
XhoI
AvaI
0.8
BamI
Pst
2.0
2114
AvaI
HBV
(adyw)
549
0.4
BamI
2.4
409
0
5'
2.8
HBcAg
1
2656
DNA
polymerase(?)
3123
X
2902
0
0.5
1.0
1.5
2.0
2.5
3.0
3.2
Phase
HBcAg
X
1
DNA Polymerase (?)
2
3
pre-S
HBsAg

and proline residues. The transmembrane region of the protein is between amino acid residues 80-100 (Valenzuela et al., 1982). The central part of the protein contains 2 hydrophilic domains at positions 45-80 and 110-150, the latter region containing the main group epitope and subtype determinants. HBsAg is a conformational antigen, the dimer linked by disulphide bridges representing the structural unit that bears full HBsAg antigenicity (Tiollais et al., 1985).

The pre S2 protein is 281 amino acids long with a molecular weight of 31kd (p31) composed of the 24kd surface protein and an additional 55 amino acids of the pre S region. It can undergo two types of glycosylation to produce either GP33 which has an additional glycan unit at residue 4 or GP36 which has a second additional glycan attached near the amino terminus. Both glycosylated forms also contain the glycan that occurs at ASN146 in GP27 (Machida et al., 1983). An immunodominant epitope resides on the hydrophillic 55 amino acid region of the pre S2 protein and antibodies which react with this determinant have been found in both humans and chimpanzees recovering from HBV infection (Neurath et al., 1985).

The pre S protein is composed of the pre S1, pre S2 and surface region. The length of the protein varies according to the subtype of virus. It can be either 389 or 400 amino acids long and with a corresponding molecular weight of 39kd (p39) or 42kd which represents the glycosylated form (Tiollias et al., 1985). The most variable region between hepadna viruses occurs at the N-terminal region of the pre S1 protein, and has been implicated in attachment of HBV to hepatocytes (Neurath et al., 1985).

1.2.2 The Nucleocapsid

The nucleocapsid of the virus encloses the viral DNA, and has associated with it the DNA polymerase activity and a protein kinase activity (Robinson 1975; Kaplan *et al.*, 1973 and Albin and Robinson, 1980).

The nucleocapsid of the virion is composed of one major polypeptide, the core antigen HBcAg, p22, which has a molecular weight of 22kd. The deduced polypeptide sequence of the core gene is interesting as the carboxy terminal region (residues 150-183) has extensive homology with protamines; 16 out of 34 amino acids are arginines (Pasek *et al.*, 1979). This feature infers that the C-terminal region binds the HBV DNA within the nucleocapsid while the rest of the protein participates in other structural roles. The core protein contains four cysteine residues with the potential for both intra- and inter-molecular disulphide bridge formation, which offers a possible explanation for some of the higher molecular weight values observed for core antigen. The major core protein p22 is also phosphorylated by the protein kinase associated with the nucleocapsid. Determination of the complete viral DNA sequence revealed the presence of a second inframe initiation site 87 nucleotides upstream from the initiating AUG of the major core protein. This region, known now as the pre core region,encodes a largely hydrophobic amino acid sequence resembling a peptide leader sequence (Pasek *et al.*, 1979). This was shown to be cleaved after synthesis of the pre core protein *in vitro* (Enders *et al.*, 1985). Expression studies in bacteria (Stahl *et al.*, 1982) and in eukaryotic cells (Will *et al.*, 1984) have shown that this region is not necessary for the production of the core antigen or for assembly of core particles which are structurally and morphologically

similar to the virus core particles (Cohen and Richmond,1982), although these core particles have not been shown to interact with other viral components in the assembly of virus particles.

In the sera of infected patients there is no circulating core antigen. There is, however, a soluble antigen distinct from the surface and core antigen known as the HBeAg. The origin of this antigen was unknown for a long time.The presence of HBeAg was not detected on the surface of the virion and anti HBeAg was shown not to precipitate the virions (Takahashi et al., 1979). However, when the nucleocapsid of the virion was treated with either proteolytic enzymes or SDS, the presence of HBeAg was detected. (Ohari et al., 1979). This demonstrates that HBeAg existes in a cryptic form on the nucleocapsid of the virions. The definitive experiment that showed that HBeAg was found on the core protein was performed by Mackay et al., (1981). They found that HBcAg produced by bacterial cells could be converted to HBeAg by treatment with proteolytic enzymes or reducing agents thereby showing HBeAg is a cryptic form of HBcAg. Two distinct e epitopes have been localized on the HBcAg polypeptides which are exposed when the molecule is unfolded (Ferns and Tedder, 1986). These are called HBeAg/1 and HBeAg/2.

HBeAg resides on a polypeptide of 15.5kd (Takahashi et al., 1979). Amino acid sequencing of the C-terminal sequence revealed that the C-terminus of this protein lies 33 amino acids from the carboxy end of the major core polypeptide (Tiollais et al., 1985). Miller (1977) proposed that HBeAg may be produced by proteolytic self-cleavage of the core protein as a protease-like amino acid sequence was identified at the amino terminus of the core sequence. As well as the two HBeAg epitopes in this polypeptide there is also a distinct epitope

for the core antigen.

HBeAg in the serum occurs either as a 15.5kd polypeptide or in association with IgG. Analyses of HBeAg purified from the Dane particles and the serum have shown that they are practically identical in amino acid composition and antigenic determinants (Takahashi et al., 1979). Despite their close relationship, HBeAg has been accepted as an entity separate from HBcAg since HBeAg may be detected in sera that contain anti-HBcAg in high titres. High levels of HBeAg are accepted as an indication of high titres of virions and of relative infectivity (Zuckerman,1982).

HBe antigen is postulated to be secreted from the infected cells via the pre core region. Ou and Rutter (1985) expressed the core protein with and without the pre core region in eukaryotic cells, and showed that the pre-core region is not required for expression of core antigen or HBe antigen. However, it is required for the secretion of HBeAg.

The nucleocapsid core also contains a DNA polymerase activity (Kaplan et al., 1973). This DNA polymease will fill in the single-stranded gap in the viral genome in vitro when supplied with deoxy-nucleotide triphosphates (dNTP). The origin of this polymerase has not yet been determined. Attempts to purify the polymerase from the virus particle have failed and comparative studies of the properties of the HBV and mammalian polymerases are inconclusive (Hirschman and Garfinkel, 1977(b); Hess et al., 1981; Goto et al., 1984).

An uncharacterized protein is bound to the 5' terminus of the long strand (Gerlich and Robinson, 1980) which has been proposed to be encoded by the X gene. Recent experiments have shown that this gene is expressed by the virus and antibodies directed against it are

produced during HBV infection. (Gough and Murray 1982, Pugh et al., 1986; Moriarty et al., 1985; Kay et al., 1985, Meyers et al., 1986). Although these experiments have demonstrated that the virus uses this gene, its function remains unknown.

1.3 Structure of the Viral Genome and its Genetic Organisation

HBV is the prototype of a family of viruses called Hepadnaviruses. Other members of this family include: Woodchuck Hepatitis Virus (WHV) (Summers et al., 1978), Ground Squirrel Hepatitis Virus (GSHV) (Marion and Robinson, 1983), Duck Hepatitis B Virus (DHBV) (Mason et al., 1980) and Tree Squirrel Hepatitis B Virus (THBV) (Feitelson, et al., 1986). All viruses of the family have a narrow host range and are not readily propagated in cell culture. They possess a partially double-stranded, partially single-stranded circular DNA genome (see Fig.1.2b) (Marion et al., 1980). Neither strand is a covalently closed circle, but the circular configuration is maintained by a 5' complementary sequence at the termini of the two strands. By heating under the appropriate conditions, the circular genome can be converted to a linear form with single-stranded cohesive ends (Sattler and Robinson, 1979). The single-stranded portion varies in length from approximately 15-50% of the circle length with a preferred minimum length of 650-700 nucleotides. The shorter of the two strands has a fixed 5' terminus and a variable 3' terminus (Delius et al., 1983). In HBV the longer of the two strands is approximately 3200 nucleotides in length and has a nick at a unique site, with a protein bound at its 5' terminus (Gerlich and Robinson, 1980). At both sides of the cohesive ends there is an 11bp direct repeat, DR1 and DR2. The nucleotide sequence of cloned HBV defined the major viral coding regions (Pasek et al., 1979). The

organisation of the genetic information is compact, with four open reading frames (ORF) found in the longer of the two strands (minus strand). The assignment of the ORFs to the viral proteins was based on partial amino acid sequencing of purified HBsAg (Peterson et al., 1977) and the expression of HBcAg in Escherichia coli (Burrell et al., 1979). As the virion carries an endogenous polymerase (Kaplan et al., 1973) and there is a long ORF coding for a protein that is the expected size for a polymerase, this has been assumed to be the polymerase gene. The last and smallest ORF is referred to as the X gene. The short strand has one small open reading frame but this is not conserved in all sub-types, or across the hepadna virus family.

1.4 Mode of Transmission

HBV is present in blood and other body fluids, including saliva, seminal fluids, menstrual and vaginal discharges. Transmission of infection has been associated with transfusion of contaminated blood, sexual intercourse, and the use of contaminated syringes and hypodermic needles. Transmission occurs through the inoculation of blood with either blood or body fluids contaminated with the virus. Infection has also been shown to be transmitted vertically, from infected mother to child shortly after birth (Gust and Crowe, 1986).

The primary site of replication of HBV in the infected host is the liver. However, in both ducks and humans infected with DHBV and HBV respectively, all forms of viral DNA and RNA which are characteristic of active synthesis of the virus have also been found in the pancreas and kidneys (Halpern et al., 1983, Dejean et al., 1984).

1.5 Disease

The incubation period of HBV varies from 14-180 days.

There are two major forms of HBV infection; the first is short-lived and is known as acute hepatitis; the second, chronic hepatitis, is defined as a persistent infection (Zuckerman, 1982).

An acute infection with HBV is marked by the appearance of the following in the serum: surface antigen appears first (see Fig.1.3a), followed by the viral polymerase activity and the e antigen. Detection of these in the serum precedes an increase in the level of amino transferase and the development of jaundice. Antibodies against core antigen can be detected 2-4 weeks after the appearance of surface antigen and is followed by the production of antibodies against e antigen and finally, antibodies against surface antigen (Burrell, et al., 1980).

Chronic hepatitis (carrier state) develops from acute hepatitis in approximately 10% of infected adults and 98% of infected new-born children (see Fig.1.3b) (Lever and Thomas, 1986). This form of infection is characterised by the persistence of surface antigen in the serum and absence of antibodies against surface antigen (Hoofnagle et al., 1973). The carrier state can be life-long and is associated with liver damage, ranging from minor changes in the nuclei of hepatocytes to chronic persistent hepatitis (CPH), chronic active hepatitis (CAH) and cirrhosis (Wright, 1980). In total, 50% of carriers will die of a liver-related disease and 5% will develop primary hepato-cellular carcinoma (Lever and Thomas, 1986).

Within the population, there are well defined groups which have a tendency to develop chronic infection. These 'risk' groups include the following:

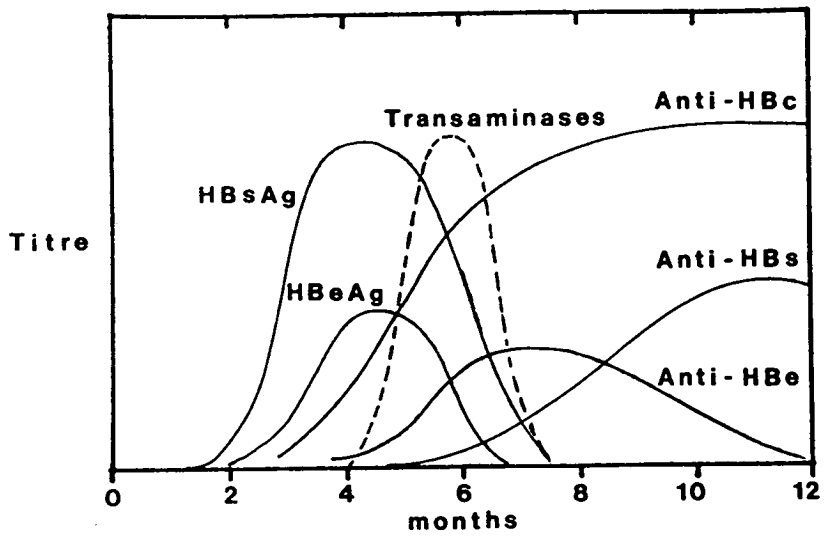


FIGURE 1.3 (a) Serological markers presented in the serum, during acute Hepatitis B.

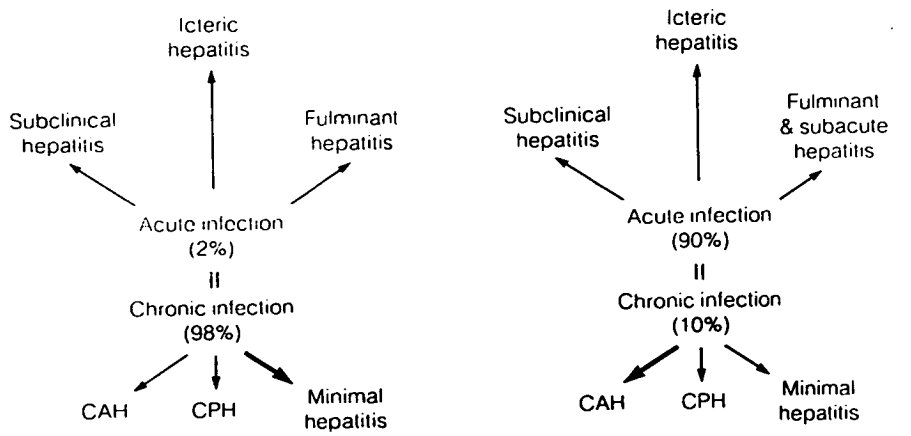


FIGURE 1.3 (b) Clinical manifestations of HBV infection. CAH - chronic active hepatitis; CHP - chronic persistent Hepatitis.

those infected at a young age, and those who suffer from natural or acquired immune deficiencies. Figures have also revealed that males are more likely than females to develop chronic hepatitis, the ratio of male to female carriers being 2:1 (Beasley and Hiwang, 1984).

Sero-epidemiological surveys reveal that in the world today there are at least 200 million carriers of Hepatitis B (Zuckermann, 1982). These carriers represent a serious medical problem as they act as a large reservoir for the infectious virus. Approaches to eradicate this virus require an understanding of its life cycle at a molecular level. However, progress has been hindered until recently by the lack of a cell culture system for HBV. Nevertheless, some of the basic strategies employed by the virus during its life cycle are now understood and are discussed below.

1.6 Life Cycle of the virus

1.6.1 Attachment and Entry

The first step in the life cycle of HBV is the attachment of the virus to the hepatocyte. Several theories have been proposed to explain the mechanism of attachment.

It was shown by several groups that the 55 amino acids at the N-terminus of the pre S2 protein have the capacity to bind polymerised human serum albumin (pHSA). This protein was shown to bind only polymerised albumin from animals susceptible to HBV. (Machida *et al.*, 1983; Tiollais *et al.*, 1985). It was also found that hepatocytes have the ability to bind pHSA (Machida *et al.*, 1983). Hence it was proposed that HBV attaches to the hepatocyte via a polymeric albumin bridge, and the HBV polyalbumin complex is then internalised by endocytosis.

Recently, however, Ishihara et al., 1987 have demonstrated that the interaction between HBsAg particles and pHSA is inhibited by physiological concentrations of mature serum albumin. Also, Neurath et al., 1985 found that antibodies to pHSA did not affect binding of HBsAg (HBV) to a human hepatocyte cell line. These latter results indicate that the ability of pre S2 to bind to pHSA may not play a role in virus uptake as has previously been suggested. Neurath et al., 1986, showed that antibodies to the pre S region, but not to the surface region, inhibited the binding of HBV to Hep G2 cells (human hepatoma cells), indicating that the pre S region may be essential for recognition of the Hep G2 cell receptor. Further investigation, using synthetic peptides from pre S1 and pre S2 regions, showed that the antisera against synthetic peptides from pre S1 region very strongly inhibited binding of HBV, while antisera to synthetic peptides from pre S2 inhibited binding by only 50%. This would suggest that pre S1 has a region essential for HBV binding hepatocytes while pre S2 sequence has only an ancilliary role in HBV binding to the cells.

Interestingly, there is very little amino acid sequence homology between pre S regions of the different hepadna viruses. In contrast, the major surface gene between these viruses is very well conserved. This may be the region that affords the tight host specificity within the hepadna virus family. At present some groups are trying to make viable recombinants between GSHV and WHBV which will be very useful in discovering the region of the virus responsible for species specificity.

Once the virus is attached to the surface of the hepatocyte, penetration of the virus could occur via two mechanisms. Firstly, by endocytosis of intact virions with subsequent release from endosomes, or secondly, by fusion of the viral envelope and liver cell plasma

membrane with penetration of the nucleocapsid into the cytoplasm. As yet, no evidence supporting either mechanism exists.

1.6.2 Transcription

After the virus enters the hepatocyte the DNA reaches the nucleus where transcription can occur. The template for transcription is a supercoiled viral genome produced by conversion of the partially double-stranded DNA into a supercoiled form by the endogenous DNA polymerase activity (Kaplan et al., 1973). The genomic structure of the virus with the single-stranded region and the nick has been shown to be unnecessary for initiating HBV infection. This was demonstrated by Catteneo et al., (1984a) who were able to initiate acute viral hepatitis in chimpanzees when they injected liver cells transformed with the cloned double-stranded HBV molecules directly into their livers.

The mammalian hepadna viruses contain coding information for at least seven distinct proteins. The HBV sequence provides some information on potential transcription signals such as 5'-TATTAAA-3' boxes and AATAAA directed polyadenylation sites. However, these are not sufficient to define the actual viral transcription units. The exact location of HBV promoters has been hampered by the lack of a tissue culture system for the propagation of HBV although the recent demonstration of the production of virus in transformed hepatoma cell lines could solve this problem (Surrea et al., 1986). Various approaches, including the transcription of HBV DNA in cell-free transcription systems and the introduction into mammalian cells of HBV DNA via viral vectors or by co-transformation have led to an increased understanding of the expression of HBV and the tentative identification of some promoters.

The first attempts to analyse the expression of HBsAg were performed in cell lines that were found to express the surface antigen. One particular cell line PLC/PRF/5 (Alexander et al., 1976), has been extensively used to analyse expression of the surface gene, a 2.3 kb HBV-specific minus strand transcript, which hybridised to the HBsAg gene, has been assigned as the HBsAg messenger RNA (mRNA) (Edman et al., 1980; Pourcel et al., 1982; Chakrabarty et al., 1980). Further analysis has shown that two classes of mRNA exist for HBsAg. One class initiates upstream of pre S1 and can encode the 39 kd pre S1 protein. The other initiates within the pre S2 region and can encode both the pre S2 and major surface proteins (Fig.1.4).

Gough and Murray (1982) and Pourcel et al., (1982) using permanent fibroblast cell lines transformed with HBV, mapped the 5'initiation site of HBsAg mRNA and found that it initiated upstream of the pre S1 region. The results from in vitro experiments agreed with these results and showed that HBsAg mRNA initiated upstream of the pre S1 region between nucleotides 900-910 in accordance with the numbering system of Pasek et al.,(1979), Schaul et al.,(1983), Rall et al., 1983) (Fig.1.4). This mRNA has the potential to produce the 39kd pre S1 protein. Sequence analysis of the region upstream from this mRNA start site identified a consensus 5'-TATTAAA-3' box at position 876 which could form part of the promoter which expressed this mRNA. However, mapping experiments performed by Cattaneo et al.,(1983) with HBsAg mRNA isolated from infected chimpanzee livers and from rat cell lines transformed with HBV disagreed with the above findings suggesting that the 5' initiation start site for HBsAg mRNA was at nucleotide position 1256, i.e. within the pre S2 region (Fig.1.4). Standring et al.,(1984), Enders et al.,(1985) and Buscher et al., (1985), using HBV,

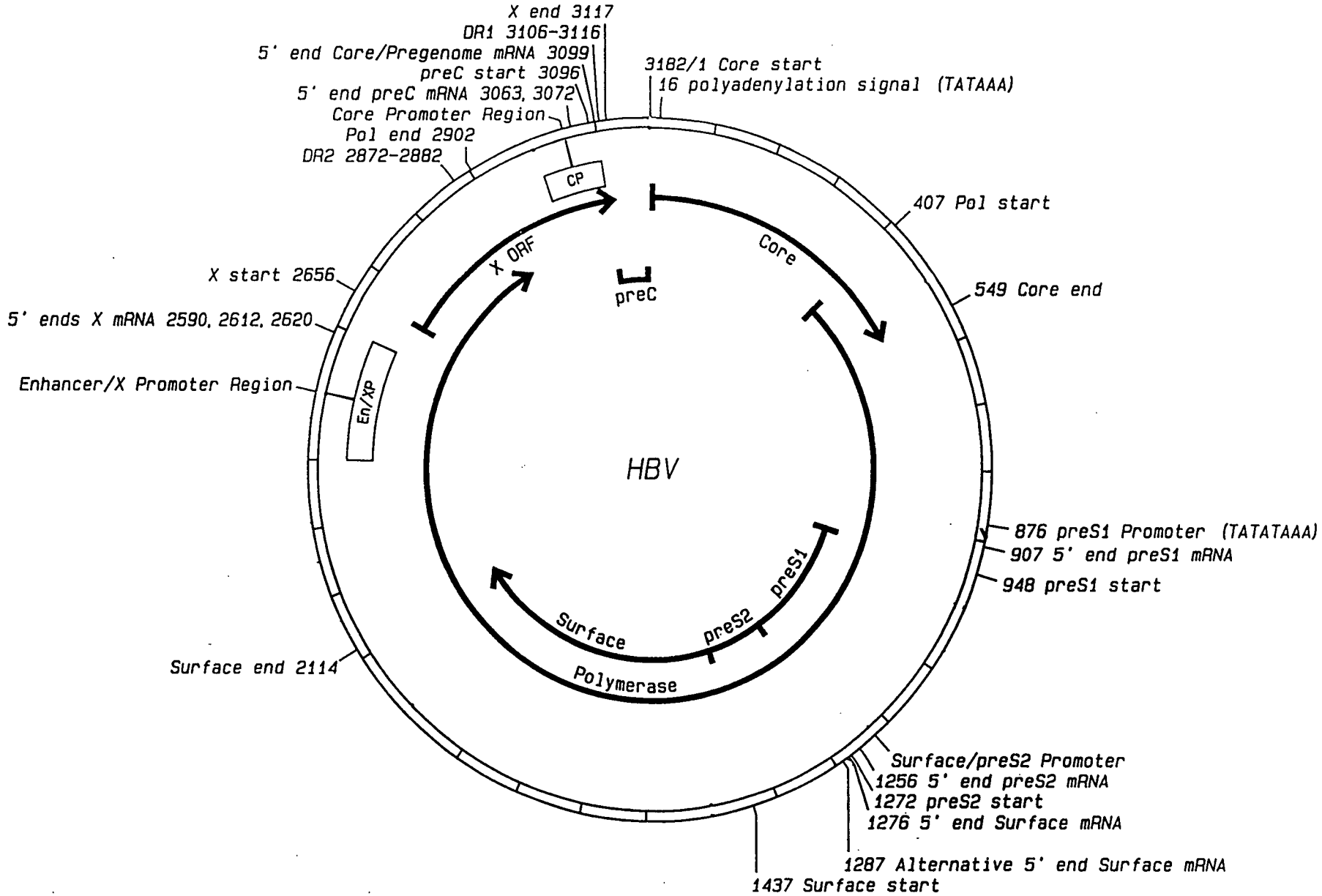


FIGURE 1.4 Transcription map of HBV.(drawing by R. Jackson).

GSHV and DHBV systems respectively, all showed the presence of a mRNA species initiating within the pre S2 region and also demonstrated that this mRNA displayed 5' heterogeneity. This mRNA could produce the pre S2 (p31).Upstream from this 5' start site there were no TATA box-like sequences. However, at position 1215 there is a sequence which bears strong homology with the late promoter of Simian virus 40. This promoter controls the transcription of a major structural protein of SV40 and interestingly also exhibits 5' heterogeneity for initiation of transcription (Brady, 1982). Perhaps these regions represent an unusual viral control sequence.

The fact that the 5' heterogeneity exhibited by the HBsAg mRNA, initiating around the p31 AUG, occurs in all systems and for all hepadna viruses studied tends to support the theory that variability in initiation of this mRNA has functional significance for hepadna virus gene expression.

Siddiqui et al., (1987), by putting the promoter sequences upstream of the bacterial chloramphenicol acetyl transferase (CAT) marker gene and measuring the relative levels of CAT produced, showed that both the promoters described for HBsAg mRNA are equally active and show little cellular specificity. However, these results may not reflect the relative activities of these promoters in the normal infected cell as the promoters have been separated from their normal products and other viral gene products which may exert control on these promoters. This is supported by the findings of Ou and Rutter (1985) who found that only 2% of HBsAg mRNA isolated from PLC/PRF/5 originated upstream of pre S1 which may indicate that it is a weaker or more controlled promoter than the promoter at 1215.

In summary, there appear to be two distinct promoter regions associated with the HBsAg gene; one positioned upstream from the second conserved ATG which could produce the major surface and pre S2 protein (1215), the other lies upstream of pre S1 (876) and could produce the pre S1 protein.

It is now generally accepted that the mRNA for the surface antigen is not spliced although Simonsen and Levinson (1983) and Cattaneo et al., (1983) have reported the presence of a spliced species of HBsAg mRNA, but these are believed to be artefacts of the system used.

Both classes of HBsAg mRNA terminate and are polyadenylated approximately 20 nucleotides downstream from a variant polyadenylation signal at nucleotide position 16 TATAAA 21 which is situated within the core gene (Cattaneo et al., 1983).

A second class of HBV specific poly A^+ minus strand transcripts has also been described in both in vitro and in vivo experiments. These transcripts are greater than genomic length. Rall et al., (1983) showed, using in vivo and in vitro experiments, that the 5' initiation site of this mRNA mapped upstream of the core gene. Gough (1983), using rat cells transformed with HBV DNA, proved that these large mRNAs are required for HBcAg and HBeAg expression as cells without these messages did not express the core protein. The large mRNAs were present in the livers of ducks, humans and ground squirrels infected with DHBV, HBV and GSHV respectively (Enders et al., 1985; Cattaneo et al., 1983, 1984; Buscher et al., 1985). More detailed analysis using S 1 nuclease and primer extension experiments revealed that this message covers the complete genome plus an additional sequence of 120 nucleotides. This message also displays the same 5' heterogeneity as the surface messenger RNA (Yaginuma et al., 1987). 5'

mapping has revealed that GSHV, DHBV and HBV transcripts from infected cells, have three core-specific messenger RNA initiation sites distributed over a 30-nucleotide region (Enders et al., 1985, Moroy et al., 1985 and Yaginuma et al., 1987). In the case of HBV the mRNAs were shown to initiate at nucleotide 3063 and 3072 (upstream of the pre-core region and therefore able to express the pre-core region) and at 3099 downstream of the pre-core initiating AUG, but still has the capability to code for mature core protein. Zelent et al., (1987) demonstrated that without the latter message no core protein was produced even when the larger message was present. It has also been proposed that the larger messenger RNAs are used as a template for the synthesis of viral minus strands as part of the replicative process.

The production of these larger than genome messages would require read through of the polyadenylation and termination signals within the core gene during the first round of transcription with termination and polyadenylation occurring during the second round of transcription. This is similar to the strategy adopted by polyoma virus to transcribe its late messenger RNAs (Tooze et al., 1973).

No mRNA has been detected that contains the first AUG codon in the polymerase frame as its 5' proximal AUG indicating that such a transcript may only be present in very low amounts if at all. Translation of the polymerase frame may start at an internal AUG of the core mRNA (and/or longer mRNA) as is known for other polycistronic mRNAs (Kozak, 1986). Alternatively, the polymerase may be translated as a fusion protein with the core protein. This would involve a frameshift during translation and the subsequent cleavage of the polymerase from the polyprotein precursor. Such strategies are used for the production of the polymerase gene from retroviruses (Beveren et

al., 1985). Will et al (1986) have inferred the presence of a polymerase-core fusion in a hepatoma carcinoma. As discussed previously, the NH_2 terminus of the core protein has protease-like sequences which may be active in the cleavage of HBcAg to HBeAg. This may also act in the processing of core-polymerase fusion proteins (Miller, 1987). It was postulated that due to the absence of the core protein NH_2 terminus in the core-polymerase fusion protein described by Will et al., (1986), the viral protease is absent and thus fusion polyproteins would be able to accumulate.

The presence of a small poly(A)$^+$ minus strand message, which hybridises specifically to the X region in cell lines transformed with HBV, has been described by Gough (1983) and Treinin and Laub (1987), showed that this message is produced from its own independent promoter positioned within the region 2524-2629 (Treinin and Laub, 1987).

The 5' end of this messenger displayed heterogeneity with multiple start sites at 2590, 2612 and 2620. The X gene promoter, like the internal HBV surface promoter, lacks a TATA box-like sequence. The discovery of a new promoter located upstream from the X ORF provides more evidence that these sequences represent a functional gene which is expressed from its own transcription unit.

In summary, the two major classes of viral mRNA for the core and surface genes and the less abundant X message are unspliced, polyadenylated co-terminal and display 5' heterogeneous ends that encode proteins with different amino termini. Hence one could conclude that the heterogeneity displayed must have a functional significance for hepadna viral gene expression.

These experiments have identified active promoters but they do not indicate how these promoters are regulated. The contrast between the

weak promoter activity in non-primate and non-hepatic cell types and the production of large amounts of core antigen during HBV infection in humans prompted the investigation of whether an HBV enhancer influences promoter activity.

Using the CAT gene system, Shaul et al., (1985) defined a HBV transcriptional enhancer element. This enhancer element was located 450 bp upstream of the HBcAg gene (at position 2279-2529) and was shown to increase the promoter activity of both the core and the X gene. The enhancer was shown to have strict host and tissue specificity (Jameel and Siddiqui, 1986) and was functional only in the liver cells of human origin. This enhancer activity functioned independently of HBV gene products present in the cell lines tested. From their data it could easily be assumed that some component present within the human liver cells is required to activate this enhancer. Using a nitrocellulose filter-binding assay and DNAse I footprinting techniques, Shaul and Ben-Levey (1987) indeed found that specific liver cell nucleic proteins are bound to the enhancer and its adjacent sequence. Other DNA binding proteins have been discovered which modulate promoter activity by binding near the promoter, e.g. the SV40 large T antigen promoter (Dynan and Tijan, 1985). One particular factor, nuclear factor 1 (NF1) is required for efficient activity of the S gene promoter which binds within the pre S1 region. Standring et al., (1983) have shown that a short ORF (700 HBV) in the short strand is transcribed by RNA polIII in an in vitro transcription system. This transcript could encode a polypeptide of 164 amino-acids starting with a methionine start codon. However, the sequence of theTiollais group (Tiollais et al., 1985) predicts an open reading frame truncated to 86 amino acids by an extra stop codon, while the equivalent reading frame in Woodchuck

virus contains no methionine start codon. Hence a protein encoding region appears unlikely for 700 HBV. As this RNA is complementary to the minus strand messenger RNA it may serve a regulatory or processing function.

To determine whether hormonal or physiological conditions affect the expression of the HBV genome, Babinet et al., (1985), produced transgenic mice that had HBV DNA (except for the core gene) inserted into their genome. These mouse strains had high levels of HBsAg in their serum and HBV-specific mRNA in their livers. Analysing the amount of HBsAg and HBsAg mRNA at different times during development and maturation revealed that HBsAg expression varied. This variation was shown to be due to the direct effect of sex hormones on HBV expression as the levels rose and fell during development. Interestingly, it was found that in adult male mice, HBsAg levels were 5-10 times higher than in adult females (Farza et al., 1987). The expression of HBsAg appears to be controlled at the transcriptional level as HBsAg mRNA levels rose, HBsAg production was increased but a more detailed analysis is required before this can be proven. The above results may explain at a molecular level epidemiological data which reveals that males have an increased risk of becoming a carrier of HBV than females have. (Beasley and Hiwang, 1984).

In conclusion, hepadna viruses exploit the genetic information which is encoded in its small genome by using a mixture of heterogeneous transcription and translation initiation start signals to increase its coding capacity. HBV expression appears to be under strict specific cellular control and is regulated by both trans-activating proteins and hormonal control.

1.6.3 Replication

Once HBV has expressed the necessary viral proteins, the virus can begin to replicate.

Summers and Mason (1982) isolated viral core-like particles from the cytoplasm of hepatocytes of ducks infected with DHBV. these particles were shown to incorporate deoxynucleoside triphosphates into DNA. This was found to be a different polymerase reaction from that observed with the mature virions, as these particles synthesized the viral DNA minus strand as well as the plus strand. Plus strand and not minus strand synthesis was sensitive to actinomycin D which was consistent with plus strand synthesis from a DNA template and minus strand synthesis from an RNA template. Analysis of the products and template of liver cores at various times after infection revealed that the minus strand DNA was synthesised by copying viral RNA which was rapidly degraded behind the growing point, possibly by a ribonuclease H activity. Therefore, replication of DHBV involves a reverse transcription step. Evidence that this is a common mechanism for hepadna viral replication has come from analyses of DNA from HBV, WHV, DHBV and GSHV infected liver using specific hybridization probes. All these investigations revealed the presence of DNA or RNA intermediates of replication (Monjardino et al., 1982; Blum et al., 1983; Weiser et al., 1983; Mason et al., 1982). Miller et al., (1984) have also isolated cores from human livers that appear to synthesise HBV minus strands from an RNA template and hence resemble those particles isolated from duck livers.

The poly(A)$^+$ minus strand transcript found in the liver core particles is known as the pre-genome, it is longer than the minus strand, and has a terminal redundancy of approximately 120 nucleotides.

The template for transcription of this pre-genome is most likely to be the supercoiled viral genome present in the infected hepatocyte. As neither chimpanzees infected with HBV, nor ducks infected with DHBV have been shown to exhibit integrated viral sequences as an immediate consequence of infection, it is assumed that unlike retroviruses, hepadna viruses do not require an integration step for replication.

Once the full length pre-genome is transcribed, it is proposed that it is packaged with the endogenous polymerase within core-like structures referred to by Summers and Mason (1982), as immature cores (Fig.1.5).

The RNA might be packaged before reverse transcription for several reasons. Since the pre-genome RNA serves as both a template for reverse transcription and as a mRNA for the core antigen and since it is unlikely that both processes can occur simultaneously on one strand, it may be more efficient to separate the mRNA required for viral replication from the pool of other messengers. Reverse transcription may also be more efficient in a compartment separated from the cytoplasm as it allows close association of template and enzyme.

Analysis of the 5' ends of packaged RNA revealed which of the three large mRNAs transcribed from the core promoter is used as the template for reverse transcription. This showed that the shortest core message, which initiates within the pre-core region, is present within the core particles while the other two longer messages are found associated with polyribosomes (Yaginuma et al., 1987; Enders et al., 1987). These findings suggest that there exists a packaging system capable of specifically selecting which mRNA species is packaged-based on different 5' termini.

Using primer extension techniques on deproteinised DNA, the 5' end

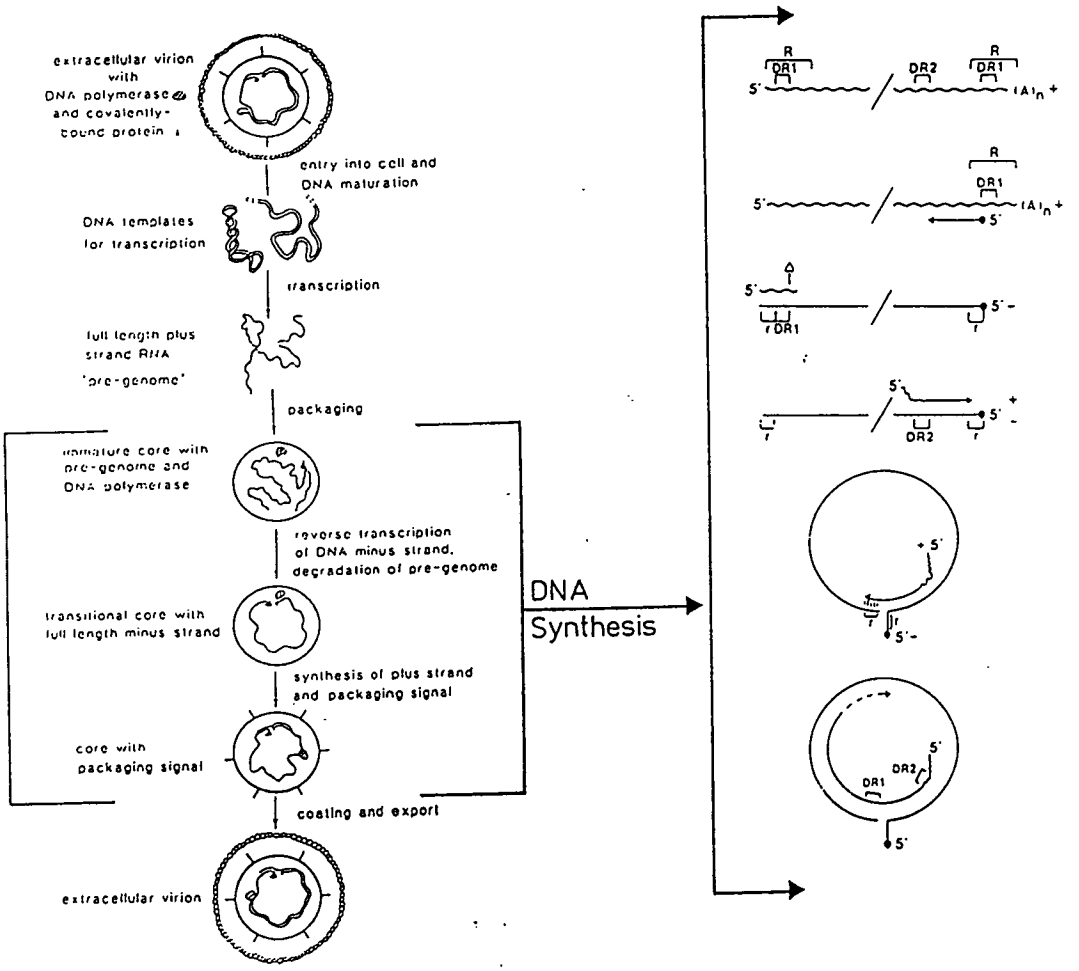


FIGURE 1.5 A scheme for the replication of HBV. From Summers and Mason (1982) and Seeger et al. (1986)

of the minus strand was mapped to the direct repeat DR1 (Seeger et al., 1986; Will et al., 1987). However, as there are two copies of DR1 in the pre-genome, initiation could occur either at the 3' end or the 5' end of the pre-genome (see Fig.1.5). Initiation at the 5' end would allow only synthesis of a few nucleotides of minus strand DNA and then require an early template switch, whereas initiation at the 3' end would not require any switch for a complete DNA minus strand to be synthesised. Reverse transcription could proceed up to the 5' end of the pre-genome and produce a full length DNA minus strand. The latter possibility appears to occur as the minus strand seems to be terminated at the 5' end of of the template RNA (Will et al., 1987). The fact that even the shortest minus strands in infected livers (Weiser et al., 1983), or in isolated core particles (Molner-Kimber et al., 1984) are linked to protein, suggests that this protein may prime minus strand synthesis. This protein has not been characterised but it may be the same protein attached to the 5' end of the minus strand in mature virions. Very little plus strand synthesis is seen until the minus strand is completed, indicating that plus strand uses the minus strand for its template. The 5' end of DHBV and HBV plus strand has been shown to map to the 5' side of DR2 (Will et al., 1987; Seeger et al., 1986). Lien et al., 1986 demonstrated that a small 20bp RNA is covalently linked to the 5' end of DHBV plus strand which suggests that this is the primer for second strand synthesis. Sequencing of the oligoribonucleotide showed that this RNA is derived from the 5' end of the pre-genome RNA which contains the DR1 sequence. It was therefore proposed that an oligomer containing DR1 from the 5' end of the pre-genomic RNA was translocated to the DR2 site of the minus strand DNA to

prime synthesis of plus strand DNA (See Fig.1.5). After transfer of the primer to DR2, DNA plus strand synthesis can only proceed up to the 3' end of the DNA minus strand. For further elongation, a template switch has to take place which could use the short terminal redundancy of the minus strand. This would allow an intramolecular template switch to take place resulting in the circular DNA conformation of the HBV genome. The DNA plus strand could then be elongated. The initiation of plus strand synthesis may cause a structural change in the core particles that allows it to be packaged regardless of whether the plus strand is completed. Following packaging, the nucleocapsid is coated with surface Ag from the host cell.

1.6.4 Assembly and Export of the Virus

At some point before the completion of plus strand synthesis the nucleocapsid interacts with the surface antigen and is exported from the infected cell. The pre-core region of the core protein may be important for this. Experiments by Ou et al., (1986) have shown that the pre-core region apparently facilitates the transportation of the core antigen from the cytoplasm into the endoplasmic reticulum where interaction between the surface coat and its nucleocapsid occurs.

The pre S1 region has also been implicated in viral morphogenesis. Pre S1 proteins, unlike the pre S2 and surface proteins, are not secreted into the culture medium despite the presence of secretory information (Persing et al., 1986). Furthermore, when surface and pre S1 proteins are synthesised together, secretion of the surface proteins is strongly and specifically inhibited. Persing et al., (1987) found that the pre S1 protein but not the other surface proteins have been modified at the N-terminus by the addition of myristic acids. They

postulated that myristillation may anchor the pre S1 protein in the endoplasmic reticulum thus preventing it from being spontaneously secreted and thereby allowing it to interact with the nucleocapsid. However, by what mechanism the pre S1 protein inhibits HBsAg secretion remains unknown. As yet the exact mechanism of viral assembly is not understood but perhaps experiments in which both the pre-core, core, pre S1, pre S2 and surface proteins are expressed in hepatoma cell lines will allow some insight into the interactions between the viral components. Using this system it will also be possible to use site-directed mutagenesis and deletion studies to gain a better understanding of viral morphogenesis.

1.6.5 Integration

A number of hepatoma cell lines and liver biopsies from chronic carrier patients have been analysed and found to contain integrated HBV DNA in their genome (Chakrabarty et al., 1980; Edman et al., 1980). However, as neither chimpanzees infected with HBV nor ducks infected with DHBV have been shown to contain integrated viral sequences as an immediate consequence of infection, it would appear that an integration step is not an essential requirement for the propagation of hepadna viruses. It is more likely that integration of DNA is a consequence of high levels of viral sequences present in the cell for long periods of time.

1.7 HBV and Hepatocellular Carcinoma

HBV carriers have been shown to be at a much greater risk of developing primary hepatocellular carcinoma (HCC) compared to non-carriers. In contrast to the very good epidemiological data linking

HBV with liver cancer, molecular biological investigations have not revealed any particular function or effect of HBV infection which could prove that HBV causes liver cancer.

Evidence for the integration of HBV DNA into the genomic DNA in the livers of carrier patients and HCC patients originally led to the speculation that integration of HBV sequences may be the catalyst for oncogenesis. However, from extensive analysis of cell lines particularly from the human hepatoma cell line PLC/PRF/5 (Ziemer et al., 1985) of the integrated sequences it was concluded that there was no specificity in either HBV DNA or host DNA at or near the integration site.

It was therefore proposed that HBV might encode a protein with oncogenic potential analogous to retroviruses. These viruses are known to assimilate pieces of cellular genes, oncogenes, into their genomes, which have transforming functions. If this were the case with HBV, it would mean that one particular gene would have to be present in all the hepatomas investigated. The only HBV gene that fulfils these criteria is the gene for HBsAg. However, it seems extremely unlikely that HBsAg has oncogenic activity as it is expressed for many years by chronic carriers without producing tumours. Moriarty et al., (1985) proposed that the X gene may be an oncogene as they found that patients with HCC had a high titre of antibody to its gene product although recent evidence reported by Weber et al., (1987) queries these findings. Using computer-assisted DNA and protein sequence analyses, Miller and Robinson (1986), found that both the X gene and the enhancer region have the same codon usage as genes from eukaryotic cells, in contrast with the other hepadna viral genes which clearly share the

same codon usage for virus genes (Siddiqui et al., 1987) thus indicating that perhaps both the enhancer and the X gene, like the retroviral oncogenes, were acquired recently from the host cell. It is still unlikely that the HBV has an oncogenic function as viruses that carry oncogenes result in rapid tumour formation in contrast to HCC which develops over a long period, often taking many years.

Another possibility is that one of the HBV genes may have a transactivating function capable of activating cellular genes which may cause tumour formation over a long period in a similar fashion to the pX gene of HIV I, II and III. P. Hofschneider (personal communication) has performed experiments indicating that the X gene of HBV has trans-activating functions. Interestingly, like pX, this gene is also located at the 3' end of the genome (Miller and Robinson, 1986). Alternatively, perhaps the presence of HBV DNA within the genome may cause enhanced mutability in the region of the inserted DNA, or perhaps the viral promoters and/or the enhancer may cause regulatory genes to be switched on. At this stage one can only speculate how HBV may induce tumour formation. It may be possible that the progression from chronic liver disease to liver cancer is due to the continual presence of foreign material in the body which eventually wears down the immune system such that the immune surveillance breaks down, no longer destroying the abnormal cells and thus allows them to grow and multiply.

1.8 Approach to the Eradication of Hepatitis B

HBV is still a major problem in the world. With the existence of over 200 million carriers world-wide. The effect of this virus and the suffering it causes, produces a very large burden both economically and medically on the social services. Several approaches have been

suggested to eradicate HBV and these fall roughly into two directions. Firstly, a successful vaccination programme to protect those people most at risk would interrupt the transmission cycle. Secondly, the development of suitable drugs to terminate the carrier state would destroy the large infectious reservoir for HBV. Research into both these areas has reached an advanced stage and is discussed below.

1.8.1 Vaccination In the developed world a limited programme of vaccination has been initiated for people in high risk groups. The vaccine presently used is a purified preparation of the 22nm particles isolated from the serum of infected individuals (Zuckerman, 1980). These particles are treated with heat and formalin and have been shown to be effective and safe. The vaccine has disadvantages as it relies upon a supply of infected human serum and requires very elaborate purification and safety checks. This makes it very costly to produce and is too expensive for Third World countries, which have the highest incidence of HBV, other types of vaccine have been investigated.

Valenzuela et al., (1982) were the first of many groups to synthesise HBsAg gene in yeast. The HBsAg purified from these yeast cells has been shown to be an effective vaccine (Murray et al., 1984) and is now commercially available.

The use of live vaccines has also been explored. Vaccinia virus recombinants, which are able to produce HBsAg, have been used in trials with chimpanzees and shown to almost completely protect chimpanzees from HBV (Smith et al., 1983). These trials indicate the feasibility of using a recombinant virus as a vaccine. The advantage of vaccinia virus recombinant vaccines include low cost and ease of administration all of which is critically important for the Third World. However,

vaccinia is known to have some adverse effects in some populations and its adoption is at present fraught with both technical and political problems .

Experiments performed by Milich et al (1985) and Neurath et al., (1985) have shown that epitopes residing on the pre S region elicit a very strong immune response. It was also found that if the pre S region is presented with HBsAg to animals that had previously not responded to HBsAg, antibodies were produced to both the major surface and pre S proteins. Therefore, the inclusion of the pre S region allowed the animal to be converted from a non-responder to a responder. As these regions are also known to be involved in hepatocyte receptor recognition, it has been proposed that the pre S region should be included in present HBV vaccines. However, recent reports suggest that patients in control studies make antibodies against their own livers (G. Better, personal communication).

The low titre of HBV virus in carriers producing antibodies against HBeAg suggests that HBeAg may also be usefully incorporated into a vaccine. Murray et al., (1984) inoculated preparations of HBcAg and HBeAg into two chimpanzees. These were subsequently challenged with HBV. One was found to be partially protected and the other, which had low titres of HBeAg antibodies, was completely protected. Similar results were seen by Stephen et al., (1984). These results imply that antibodies to HBeAg and HBcAg protect the animals partly or completely from HBV infection. These antigens are thought to stimulate a cell-mediated response and may play a role in future vaccination programmes.

Preliminary data from Zuckerman (1986) shows that vaccination has been successful in the interruption of HBV infection in some areas.

It will be interesting, when a more widespread vaccination campaign is undertaken to determine whether the levels of primary hepatocellular carcinoma fall along with reduction in the incidence of HBV infection.

1.8.2 Chemotherapy

Several types of chemotherapy have been used to treat patients with chronic hepatitis; most of these use drugs which inhibit the endogenous DNA polymerase activity, which may act in inhibiting viral replication. Hess et al., (1981) showed that the arabinoside AraA, (vidarabine) a nucleoside analogue, inhibited DNA polymerase. In clinical trials of the drug, 40% of the patients treated serconverted from HBeAg positive to anti-HBe positive (Bassendine et al., 1981). However, in other studies the results were not as convincing though combined treatment with AraA and Leucocyte A Interferon did convert some chronic persistent patients from HBV DNA positive to HBV DNA negative (Scullard et al., 1981; Smith et al., 1983).

Acyclovir which has been so successful with the herpes virus was also tried but although it initially decreased the titres of hepatitis DNA in the serum, as soon as treatment was stopped levels in the serum rose again (Weller et al., 1982).

The use of α Interferon has shown some promising results in limited studies reported with 50% of treated patients showing loss of HBV DNA and HBeAg for prolonged follow-up periods (Thomas and Scully, 1985).

So far the most successful treatment reported is treatment with the steroid Prednisolon and then subsequent treatment with AraA or Interferon (Omata et al., 1985). This combined treatment appears to give a higher success rate for clearing the virus from the chronic

carrier's system. All of these treatments have a higher success rate if the patient is treated within two years of the onset of infection; This may reflect the ability to eliminate the virus from the patient before it can integrate into the host genome.

As yet no universal treatment is employed in the treatment of chronic carriers. Although most of the drugs discussed are targeted against the endogenous polymerase, not enough is known about this enzyme as it has not yet proved possible to purify and completely characterise this endogenous polymerase. Perhaps when this polymerase has been more extensively analysed, it may be possible to develop better and more specific drugs, which could interfere with the enzyme's action.

1.9 DNA Polymerase of HBV

In 1972, Paul Kaplan and co-workers found that a DNA polymerase activity was present in the serum of hepatitis B carriers (Kaplan et al., 1973). Further investigation established that this polymerase activity was associated with the virions. Fractionation of the serum showed that this enzyme activity was found in the density range characteristic of Dane particles on a sucrose gradient, and after removal of the surface coat (with NP40), it was found within the density range of the core particles. Electron microscopy of these fractions revealed that the enzyme activity was co-sedimenting with the core particles. The DNA polymerase activity could be precipitated from the infected serum using antibodies to the surface antigen and after NP40 treatment using antibodies to the core antigen (Robinson and Greenman 1974).

These findings were consistent with the DNA polymerase activity being a component of the nucleocapsid. The DNA polymerase reaction

was found to be dependent on the presence of magnesium and the four deoxynucleoside triphosphates. The inhibition of the enzyme activity by actinomycin D indicated that the template was DNA and not RNA. Several pieces of evidence pointed to not only the DNA polymerase activity being an internal component of the nucleocapsid but the template and the reaction product being so as well. Treatment with DNAse and RNAse had no effect on the template or the product. However, when these were extracted from the nucleocapsid and then treated with DNAse and RNAse, they were found to be completely degraded by DNAse but insensitive to RNAse. The addition of a wide variety of polynucleotides which are accepted as primer/templates by other DNA polymerases were not utilised by the endogenous polymerase activity, suggesting that either the enzyme could not utilise the templates or that the enzyme had no access to them.

So what is the nature of the template and primer, and what is the structure of the product?

To identify the primer for DNA synthesis Robinson and Lutwick (1976) examined the sedimentation of the virion DNA in alkali sucrose gradients after being labelled for very short reaction times. They found the radioactive DNA product was attached to a molecule with a sedimentation coefficient close to that of the double-stranded region of the circular molecule before the reaction. This suggested that the open strand of the circular DNA molecule serves as the primer for the DNA polymerase reaction rather than a smaller DNA or RNA primer.

The amount of DNA synthesised during the endogenous DNA polymerase reaction has been determined using COT analysis, electron microscopy and variation in electrophoretic mobility (Lutwick and Robinson 1977; Hruska et al., 1977). This established that the DNA synthesised

corresponded to approximately a quarter of the viral genome. Summers et al., (1978) demonstrated that the reaction product corresponded to the filling in of the single-stranded gap. Using the reverse transcriptase from avian myoblastosis virus (AMV), and DNA extracted from Dane particles as a template in an in vitro polymerase reaction, it was shown that the nucleotides were incorporated in the same restriction fragments as in the in vivo polymerase reaction by the HBV associated enzyme (Summers et al., 1978). These fragments corresponded to the region of the single-stranded gap. In neither reaction did the filling in of the single-stranded gap produce a closed circular molecule as detected by S1 sensitive sites.

In conclusion, the hepatitis B particles have an endogenous DNA polymerase which uses the minus strand as the template to fill in the single-stranded gap. The biological advantage of a virus having a single-stranded gap is not clear.

1.9.1 The Origin of the Endogenous DNA Polymerase

The origin of the HBV associated DNA polymerase activity has not been established. The small size of the HBV genome originally led to the belief that the virus would not have the coding capacity to encode its own polymerase but instead would utilise the available eukaryotic polymerase. However, sequence analysis revealed the presence of a long open reading frame (L.ORF) coding for a protein with a predicted mol. wt. of 93kd, the expected size of a DNA polymerase (Pasek et al., 1979). The assignment of this L.ORF as the gene encoding the endogenous DNA polymerase and the characterisation of this polymerase has been difficult for two reasons. Firstly, attempts to purify the polymerase have been unsuccessful and so biochemical studies have been

restricted to purified virion particles, and secondly, since no cell culture system was available until very recently for HBV (Surrea et al., 1986), the source of the virus particles was limited to infected humans and chimpanzees. Despite these difficulties, however, the evidence for the DNA polymerase being encoded by the long ORF is now convincing.

(a) The Long ORF

The protein encoded by this open reading frame is within the size range expected for a DNA polymerase. The long ORF and the potential products encoded by it are highly conserved across the hepadna virus family. The size and the conserved nature of this gene leaves little doubt that it does have a coding function.

Using computer-assisted protein sequence analysis, it was found that the predicted amino acid sequence of the long ORF contains regions of homology with retroviral reverse transcriptase but not with bacterial or eukaryotic polymerases (See Fig.1.6) thereby implying that this homology is indicative of a protein with reverse transcriptase activity (Mandart et al., 1984; Toh et al., 1983, 1985).

Examples	Reverse Transcriptase Motif
RSV	YMDDLLL
MMLV	YVDDLLL
HTLVI	YMDDLLL
HTLVIII	YMDDLYV
ATLV	YMDDILL
CAMV	YVDDILV
HBV	YMDDVVL

(From Feutterer and Hohn et al., 1987)

As hepadna viruses replicate via an RNA intermediate this requires an enzyme that has RNA-dependent DNA polymerase activity, an activity not normally found in eukaryotic cells. It was thus proposed that the L.ORF does encode a DNA polymerase and that replication of HBV uses a reverse transcriptase activity of this protein. It should be noted that the putative polymerase gene does not appear to have any homologous sequences associated with the ribonuclease H, endonuclease and protease activities of the polymerase genes of retroviruses (Johnson et al., 1986; Feutterer and Hohn, 1987).

(b) Biochemical Analysis:-

The endogenous polymerase has been extensively characterised in vitro by several groups using purified virions, and its properties have been compared with those exhibited by host polymerases and the reverse transcriptases of several retroviruses. These results are summarised in Table 1.

Higher eukaryotic cells contain three distinct polymerases, α, β and γ. These enzyme activities can be separated easily on the basis of their chromatographic properties, molecular weights, sensitivity to N ethylmaleimide (NEM) salts and their ability to copy various templates:-

α - is ubiquitous in growing cells and is believed to be the principal replicating enzyme. It is a large enzyme with an approximate molecular weight of 150kd and is associated with smaller proteins forming a hetero oligomer. The enzyme is specific for polydeoxy-nucleotide templates and shows little activity with synthetic ribo-polymers such as poly (A) or RNA. It has optimal activity with gapped DNA and will use either deoxy or ribo primers. α has an absolute

Table 1.
Physical and Chemical Properties of HBV, α, β, Polymerases and Reverse Transcriptase
(N.T. = not tested)
(+ = inhibition)
(- = no inhibition)

Physical and Chemical Props.	HBV	α	β	γ	RT
Mol.Wt. (kd) (Native)		220	43	119	MuLv-84 AMV C 65 AMV B 95
Aphidocolin	-	+	-	-	-
PFA	+	-	-	-	+
PAA	-	-	-	-	+
ddTTP	+	-	+	+	+
Ara CTP	+	+	-	-	+
Ara A	+	N.T.	N.T.	N.T.	N.T.
Act. D	+	+	+	+	- +
N.E.M.	-	+	-	+	+
Cationic Reqs., with	Mg^{2+}				
a) activated DNA		Mg^{2+}	Mg^{2+}	Mg^{2+}	Mn^{2+}
b) synth. polymers		Mn^{2+}	Mn^{2+}	Mn^{2+}	Mg^{2+}
High Ionic Strength	Stimul.	Inhib.	Stimul.	Stimul.	Stimul.
Template(T)/Primer(P) Complex:					
a) Homopolymers:					
deoxy(T)/deoxy(P)	(daT inhib.)	Yes	Yes	Yes	(dA inhib.)
deoxy(T)/ribo(P)	No	Yes	No	No	Yes
ribo(T)/deoxy(P)	No	No	Yes	Yes	Yes
poly C(T)/dG_{12}(P)	No	No	No	No	Yes
2'-O-methyl citidylate(T)/oligo dG_{12}(P)	N.T.	No	No	No	Yes
b) Natural:					
activated d.s. gapped DNA	No	Yes	Yes	Yes	Yes
Native DNA	No	No	No	No	Yes - poor

Table 1 cont.

	HBV	α	β	γ	RT
RNA primed DNA	No	Yes	No	No	Yes
Inhibition by					
Abs Against:					
a) α	N.T.	Yes	No	No	No
b) β	N.T.	No	Yes	No	N.T.
c) γ	N.T.	No	No	Yes	N.T.
d) rev.trans.	N.T.	No	No	No	Yes
Orthophenanthraline	±	+	+	+	+
Eth. Br.	+	+	+	+	+
Misincorporation	High	$1:10^{-4}$	$1:10^{-4}$	$1:10^{-4}$	High
pH Optimum	7.2-8.0	7.2	8.5-9.0	8.0	8.3
Associated DNase enzyme activities:					
a) 3'---5' exo.	N.T.	No	No	No	No
b) 5'---3' exo.	N.T.	No	No	No	No
c) RNase H	N.T.	No	No	No	Yes
d) endonuclease	N.T.	No	No	No	Yes

requirement for Mg^{2+} or Mn^{2+} and is inhibited by high salt concentrations. The requirement for sulphydryl groups for activity makes it very sensitive to NEM, a sulphydryl alkylating agent. It is resistant to ddTTP but is very sensitive to Aphidicolin, a fungal antibiotic, and Ara CTP, an arabinosyl nucleoside (Kornberg, 1980).

β - the level of β activity is a tenth of α activity and is associated with repair and recombination functions. It is a small protein of 43kd and is located in the nucleus. β copies gapped duplex DNA efficiently and, to a lesser extent, can copy poly(A) template primed with oligo(dT). However, it does not use this polymer in the presence of Mg^{2+} ions or in the presence of high concentrations of phosphate buffer. β has no requirement for sulphydryl groups and is therefore insensitive to NEM. Unlike α it is not inhibited by Ara CTP or Aphidicolin (Kornberg, 1980).

_ - is found in the nucleus and in the mitochondria and is postulated to be responsible for the replication of mitochondrial DNA. It is a large enzyme with a molecular weight of 119kd. The enzyme is distinguished by its ability to copy ribohomopolymers at higher rates than gapped duplex DNA. However, both β and are inactive on any natural RNA used as a template whether primed or unprimed. They also cannot use poly 2'-O-methylcytidylate, a very specific template for a reverse transcriptase (Gerrard et al., 1974). Neither of these polymerase activities is inhibited by antibodies to reverse transcriptase. Therefore these enzymes are not reverse transcriptases but the synthetic templates in the presence of Mn^{2+} can form a structure which both of these enzymes can utilise. requires sulphydryl groups for maximum activity and is inhibited by NEM. It is, like β, insensitive to Ara CTP and Aphidicolin (Kornberg, 1980).

Reverse Transcriptase

An RNA-dependent DNA polymerase can be found in the virions of retroviruses and infected cells (Temin and Baltimore, 1972). The reverse transcriptase of murine retroviruses is a monomeric protein of approximately 80kd (Roth et al., 1985), while AMV reverse transcriptase is a heterodimer of two subunits α and β. The α sub-unit is the cleavage produce of β, releasing a polypeptide of 32kd. The alpha subunit exhibits the DNA polymerase activity while the p32 protein contains a specific nuclease. The β subunit contains both of these activities and is thought to help stabilise the enzyme template reactions (Tanese et al., 1986).

There are three enzymatic activities associated with reverse transcriptase:

(i) A ribonuclease H (RNAse H) activity which degrades the RNA strand of a DNA:RNA hybrid but does not degrade either single-stranded or double-stranded RNA (Johnson et al., 1986).

(ii) A DNA-polymerase capable of copying RNA on a DNA template.

(iii) A DNA endonuclease/integrase function.

The DNA polymerase and RNAse H activity resides on the same polypeptide but have different functional sites. Reverse transcriptase is active on a wide variety of RNA and DNA templates. Gerrard et al., (1974) showed that the enzyme can utilise poly 2'-O-methylcytidylate oligo (dG) very efficiently. Reverse transcriptase is sensitive to Ara, CTP, ddTTP and NEM. The RNA:DNA synthesis is resistant to actinomycin D, but the DNA:DNA synthesis is sensitive to it.

Hepatitis B polymerase

The endogenous polymerase of HBV was characterised in a similar fashion to the other DNA polymerases described. The endogenous

hepatitis B polymerase is dependent on the presence of magnesium, though the optimal concentration was slightly higher than for other polymerases [20-200m mol/l] (Hess et al., 1981). The enzyme had a strict preference for magnesium and had only very little activity when magnesium was replaced by other divalent cations i.e. manganese and zinc (Hess et al., 1981). It was active at remarkably high concentrations of salt (0.4M - 0.8M KCL) and this allowed the development of an assay that could measure HBV associated DNA polymerase activity in a mixture of bacterial DNA poll and α polymerases (Hirschman and Garfinkel, 1977b). HBV DNA polymerase was found to be highly sensitive to organic solvents (Nath et al., 1982) and to be insensitive to NEM. The insensitivity of this polymerase to the zinc-chelating agent 1,10 phenathroline suggests that it is not a zinc metaloenzyme. (Goto et al., 1984). In the search for anti-viral drugs against Hepatitis B, various compounds have been tested to discover whether or not they inhibit the endogenous DNA polymerase of HBV. The arabinoside analogues Ara ATP and Ara CTP wereshown to inhibit this enzyme activity (Hess et al., 1981. Nordenfelt et al., (1979) demonstrated that although HBV-DNA polymerase is resistant to the anti-viral drug phosphonoacetate (PAA), which is a pyrophosphate analogue, it is very sensitive to phosphonoformate(PFA). Interchelating agents, ethidium bromide and actinomycin D were also inhibitory as was ddTTP (Hirschman and Garfinkel, 1977).

The above results show that the HBV-DNA polymerase has a different biochemical profile from α and polymerases. It is similar in some respects to β, the repair enzyme of the host, as it operates under high salt concentrations, is insensitive to NEM, and is resistant to PAA. Interestingly, it is also very similar to the reverse transcriptase enzyme.

1.10 The Objective of the Present Study

In conclusion, the studies performed on the HBV-associated polymerase cannot prove or disprove that this polymerase is an encapsulated eukaryotic polymerase. The definitive experiment would be to express this long ORF in either bacteria or eukaryotic cells and show that the protein it encodes has a DNA polymerase activity with the same properties as the endogenous polymerase.

The aim of the work presented in this thesis was to determine whether or not the long open reading frame, present in HBV, is expressed during the course of viral infection and if so what is the function of the gene product.

CHAPTER 2

MATERIALS and METHODS

2.1 Suppliers of Laboratory Reagents:-

Restriction endonucleases were purchased from Amersham International, P.O. Box 16, Amersham, Buckinghamshire, NP7 9LL.

Boehringer Mannheim, Boehringer Mannheim House, Lewes, East Sussex, BN7 1LG.

New England Biolabs or Pharmacia, Pharmacia House, 351 Midsummer Boulevard, Central Milton Keynes, MK9 3YY.

E.coli DNA polymerase I (Klenow fragment and holoenzyme) was purchased from Boehringer Mannheim.

Deoxynucleoside triphosphates were purchased from Bethesda Research Laboratories.

Dideoxynucleoside triphosphates were purchased from P-L Biochemicals.

Radiochemicals were purchased from Amersham International.

Ultra-pure agarose and low melting point agarose were purchased from Sigma.

Standard Laboratory reagents were purchased from BDH, Fisons or Sigma.

2.2 Solutions:-

TE	Tris-HCl (10mM, pH 7.5), EDTA (1mM)
Sequencing TE	Tris-HCl (10mM, pH 8.0), EDTA (0.1mM)
10 x TBE	Tris-base (0.89M), boric acid (0.89M), EDTA (10mM)
DNA gel sample buffer	EDTA (10mM), ficoll (1%, w/v), bromphenol blue (0.025%, w/v), xylene cyanol FF (0.05%, w/v)
4 x Nick Translation buffer	Tris-HCl (210mM, pH 7.5), $MgCl_2$ (21mM), BSA (20µg/ml), β-mercaptoethanol (140mM), dGTP, dATP, dTTP (0.08mM each)
OLB	Solution O: $MgCl_2$ (0.125M), Tris-HCl (1.25M, pH 8.0) Solution A: 0.95ml of solution O was added to the following mixture: β-mercaptoethanol (18µl, 14M), dATP (25µl,20mM), dGTP (25µl, 20mM), and dTTP (25µl, 20mM) Solution B: Hepes (2M, pH 6.6) Solution C: Hexadeoxyribonucleotides (Pharmacia) resuspended in TE to give a final concentration of 90 OD units/ml Solution OLB was made by mixing solutions A, B and C in the ratio 2:5:3 and was stored at -20°C

T4 DNA Ligase Buffer	Tris-HCl (50mM, pH 7.8), Mg Cl_2 (10mM), DTT (20mM), ATP (10mM)
20 x SSC	NaCl (3M), trisodium citrate (300mM), adjusted to pH 7.0 with NaOH
50 x Denhardt's Reagent	Ficoll (1%, w/v), polyvinyl pyrrolidine (1%, w/v), BSA (1%, w/v)
Denatured salmon sperm DNA	Was made up as 5mg/ml stock and sonicated (50 seconds, Soniprobe, Dawe Instruments)
X-gal (BCIG)	5-bromo-4-chloro-3-indoyl-β-galactoside (20mg/ml in dimethyl formamide)
IPTG	Isopropyl-β-D thiogalactoside (20mg/ml)
10 x MOPS	MOPS (0.2M), Na acetate (50mM), EDTA (10mM), adjusted to pH 7.0 with 5M NaOH.
Formamide sample buffer	Formamide (50%, v/v), formaldehyde (7.5%, v/v), MOPS (x 1)
RNA loading buffer	Glycerol (50%,v/v), EDTA (1mM), bromophenol blue (0.4%, w/v), xylene cyanol FF (0.4%, w/v).
40% Acrylamide stock	Acrylamide (38%, w/v), N-N-methylene bisacrylamide (2%, w/v). Filtered and stored at $4^{o}C$.

TM	Tris-HCl (100mM, pH 8.0), $MgCl_2$ (100mM)
Formamide loading buffer	Xylene cyanol (0.3%, w/v), bromophenol blue (0.3%, w/v), EDTA (10mM), in 100% formamide
TEMED	NNN'N'-tetra-methyl-1, 2 diamino-ethane (BDH)
AMPS	Ammonium persulphate (10%, w/v)
30% Acrylamide stock	Acrylamide (30%, w/v), N-N'-methylene bis-acrylamide (0.2%, w/v), filtered and stored at $4^{o}C$
1 x Protein gel buffer	Tris-base (50mM, pH 6.8), glycine (384mM), SDS (1%, w/v)
LTB	Tris-HCl (20mM, pH 7.9), $MgCl_2$ (20mM), EDTA (1mM)

2.3 Microbiological Strains and Media.

Microbiological Strains.

All bacterial strains, plasmid vectors and bacteriophage vectors used in this study are described in Section 2.3 to 2.5. Bacterial stocks were preserved by drying, using the Stamp method (Stamp et al., 1947) and were also maintained on the appropriate plates. M13 bacteriophage were stored as DNA in sequencing TE at -20°C or in LTB at 4°C.

Media

Media were sterilised by autoclaving. Unless otherwise stated, all quantities below refer to 1 litre of solution. Where appropriate, antibiotics were added to plates and media: ampicillin (100µg/ml), tetracycline (50µg/ml), or kanamycin (25µg/ml).

Luria broth (LB)	Difco Bacto Tryptone (10 g), Difco Bacto yeast extract (5g), NaCl (5g); pH 7.2
Luria agar (L-agar):	Agar (15g, Difco) added to LB (1L)
BBL top agar	Trypticase (10g, Baltimore Biological Laboratories), NaCl (5g) and agar (6.5g, Difco)
Spizizen minimal salts	$(NH_4)_2\ SO_4$ (10g), K_2HPO_4 (20g), KH_2PO_4 (30g),tri-sodium citrate (5g), $MgSO_4$(1g)
Minimal agar	Spizizen minimal salts (80ml), glucose (4ml, 20%, w/v), Vitamin B_1 (0.2ml,1mg/ml), Difco Bacto agar (6g); made up to 400 mls with water

2.4 Bacterial strains

Strain	Genotype	Reference
HB101	F⁻ *hsdS20* *recA13* *ara14* *proA2* *lacY1*, *galK2* *rpsL20* (SM^r) *xyl5* *mtl-1* *supE44* λ^-	Boyer and Roulland-Dussoix, 1969
NM522	*hsdΔ*($M^-S^-R^-$) *Δlac* *Δpro* *supE* *thi* F' *lacI*^{q} *lacZΔM15*	Gough, 1983
NF1	K12*ΔH1Δtrp* *lacZ* *am* *λNam7* *Nam53* *cI857ΔH1*	Stanley and Luzio, 1984
WL542	F⁻ *dapDT6*^{S}*minA* *purE*λ^- *pdxc* *minB* *his* *str*^{r} $T3^N$ *xyl* *ilv* *cycA*^{N} *cycB*^{N} *met*	Manson *et al.*, 1986
SG935	F⁻*lac(am)* *trp(am)* *pho(am)* *supC* (ts) *rpsL* *mal(am)* *htpR(am)* *tsx*::TN10 *lonΔ100*	S. Goff(unpublished)

2.5 Plasmid and Bacteriophage Vectors.

Vector	Description	Source/Reference
pEX1/2/3	Amp^r(β-galactosidase-fusion protein expression vector)	Stanley and Luzio, 1984.
ptac322	Amp^r (Expression vector using tac promoter)	Stahl (unpublished)
M13mp10, 11, 18 and 19	E.coli bacteriophage M13-based vectors; lacZ gene	Norrander et al., (1983)

2.6 Other Plasmids Used.

Plasmid	Description	Source/Reference
pR1-11	Amp^r (contains HBV core gene under the control of the Lac promoter)	Stahl, et al., 1982
pHBVCB	Tet^r (contains the HBV genome inserted into PstI site of pBR322).	Gough and Murray, 1982.

pHPL-1	Amp^r (contains the sequences encoding the first 28 amino acids of HBV core antigen fused to the sequences encoding residues, 40-210 of the HBV polymerase gene.	McGarvie (unpublished)

2.7 General Nucleic Acid Methods.

2.7.1 Deproteinisation of nucleic acids.

DNA or RNA solutions were deproteinised by extraction with an equal volume of phenol. This was accomplished by vortexing until a white homogeneous emulsion was formed. The phases were then separated by centrifugation (17,500 g, 2 min.) and the upper aqueous layer recovered. The aqueous layer was extracted three times with ether to remove residual phenol and the nucleic acid was then precipitated with ethanol (see below).

2.7.2 Precipitation of Nucleic Acids with Ethanol

DNA was precipitated from aqueous solutions by the addition of sodium acetate (0.1 vol., 3M, pH 5.8) and ethanol (2.5 vol.). The solution was cooled (-70^{o}C, 15 min.) and the DNA pelleted by centrifugation (17,500 g, 15 min.). The supernate was removed and the pellet dried in a vacuum desiccator before being redissolved in TE.

2.7.3 Quantification of Nucleic Acids

The optical density at 260nm was used to quantify nucleic acids. An OD_{260} of 1.0 is equivalent to 50µg/ml for DNA or 40µg/ml for RNA.

2.7.4 Gel photography

Nucleic acid was visualised in gels stained with ethidium bromide using an ultraviolet transilluminator (254nm). Photographs were taken through a red (A1) filter on Ilford HP5 film (f 4.5, 20s). Films were developed in Ilford microphen (5 min.), stopped in acetic acid (3%, v/v, 30s), and fixed in Ilford Hypam (5 min.) at room temperature. Films were washed well in water, dried, and the relative mobilities of the bands measured directly from the negative (distance migrated is inversely proportional to $\log_{10}$ M wt.).

2.7.5 Autoradiography

Autoradiography was performed using Cronex 4 X-ray film and cassettes. For ^{32}P, films were preflashed and exposed at -70°C using intensifying screens. Films were developed in an Agfa x 1 automatic film processor.

2.7.6 Measurement of Radioactivity incorporated into Nucleic Acids

The efficiency of incorporation of radiolabel into nucleic acids was estimated by measuring acid-precipitable counts. Aliquots (2 µl) were transferred onto Whatman GF/C glass fibre discs and dried, then placed in a beaker containing ice-cold TCA (5%, w/v) and incubated for 10 min. on ice. Following this the filters were washed three times with ice-cold TCA (5%,w/v) and twice with 100% ethanol and dried. As a control, another aliquot (2µl) was spotted directly onto a Whatman GF/C filter and dried. Each filter was transferred into a scintillation vial, immersed in scintillant (butyl-PBD, 6g/L in toluene) and counted in a liquid scintillation spectrometer. The percentage incorporation of label into nucleic acid was estimated by comparing the two values.

2.8 Nucleic Acid Methods

2.8.1 Small-scale Preparation of Plasmid DNA

(Modified from Birnboim and Doly, 1974).

Small-scale plasmid DNA preparations were obtained by a modification of the rapid extraction method of Birnboim and Doly (1974). Cells were grown overnight in 2 mls of LB-ampicillin or LB-tetracycline, pelleted by centrifugation (17,500 g, 2 min.), resuspended in 1 ml of Tris-HCl (10mM, pH 8.5), EDTA (1mM) and pelleted as above.

The cells were resuspended in 150µl sucrose mix; sucrose (15%, w/v), Tris-HCl (50mM, pH 8.5), EDTA (50mM), lysozyme (0.25mg), and incubated at room temperature (30 min.), then at 4°C (30 min.). Water (400µl, 4°C) was then added, mixed and incubated at 4°C (10 min.). Following this, the Eppendorf tube was incubated at 70°C (15 min.) and the cell debris and chromosomal DNA pelleted by centrifugation (17,500 g, 15 min.). The supernate was transferred to another microcentrifuge tube and DNA precipitated by the addition of $NaClO_4$ (75µl, 5M) and isopropanol (200µl). The DNA was pelleted by centrifugation (17,500 g, 15 min.) and the supernate removed. The DNA was resuspended in Na acetate (100µl, 0.3M, pH5.8) and reprecipitated by the addition of ethanol (300µl). Following centrifugation (17,500, 15 min.) the DNA pellet was resuspended in TE (80µl).

2.8.2 Large-scale Preparation of Plasmid DNA

(Modified from Maniatis et al., 1982)

A single bacterial colony was used to inoculate L-broth (10ml, with added antibiotics) and grown to stationary phase (normally at 37°C). The culture was diluted 1:50 in L-broth (with added antibiotics) and

grown at 37°C to A_{650}=1. Chloramphenicol was added (final concentration of 150µg/ml) and the culture grown for a further 12 h. Cells were pelleted by centrifugation (4,000g, 10 min, 4°C) and resuspended in 6ml of sucrose solution [sucrose (25%, w/v), Tris-HCl (50mM, pH 8.1) and EDTA (40mM)]. Lysozyme (1ml, 10mg/ml, in sucrose solution) and EDTA (0.5ml, 0.5M, pH 8.1) were added. Following incubation (4°C, 5min.), 13ml of triton mix [Triton X-100 (0.1%, v/v), EDTA (62.5mM, pH 8.1), Tris-HCl (50mM, pH 8.1)] were added, mixed and left at 4°C (10 mins.). Cell debris was then pelleted by centrifugation (31,000g, 30 min., 4°C) and the supernate recovered. CsCl (9g) and ethidium bromide (0. 8ml, 10mg/ml) were added to supernate (9ml) and the mixture transferred to a polyallomer centrifuge tube (1.6 x 7.6 cm, Beckman). Following centrifugation (95,000g, 60 h, 20°C) DNA was visualised with long wavelength UV light and plasmid DNA (the lower of the two fluorescent bands) recovered through the side of the tube with a 19-gauge needle and syringe. Ethidium bromide was removed by repeated extraction with an equal volume of butan-2-ol (equilibrated with TE). The CsCl was then removed by dialysis against several changes of TE (4°C), and the DNA precipitated with ethanol.

2.8.3 Agarose gel Electrophoresis of DNA

DNA was fractionated according to size by agarose gel electrophoresis. Molten Sigma agarose (0.75 - 1.5%, w/v in 1 x TBE gel buffer) was cooled to 60°C and ethidium bromide (final concentration of 0.5µg/ml) added. The mixture was poured into horizontal gel formers.

Set gels were submerged in Bethesda Research Laboratories electrophoresis tanks containing 1 x TBE gel buffer. DNA gel sample buffer (0.25 vol.) was added to the DNA sample and then applied to the sample well. Electrophoresis was performed at various voltages (5-100 V/cm) until the required separation was achieved.

2.8.4 Digestion of DNA with Restriction Endonucleases.

DNA was incubated with a three-fold excess of restriction endonuclease in a microcentrifuge tube for 1 h. (using the manufacturers' recommended conditions). Digestion was terminated by extraction with an equal volume of phenol or by heating.

2.8.5 Modification of the 3' and 5' termini of DNA.

Treatment with the Klenow fragment of E.coli DNA polymerase I will convert a 3' recessed terminus to a blunt-ended terminus. DNA (2-10µg) was incubated (37°C, 60 min.) with 5-15 units of the Klenow fragment in the following: Tris-HCl (10mM,pH 8.0), $MgCl_2$ (10mM) and dATP, dCTP, dGTP and dTTP (0.25mM each).

Treatment with S1 nuclease will remove single-stranded tails from DNA fragments to produce blunt ends. DNA (2-10µg) was incubated at 4°C, (1 h.) with 4 to 8 units of S1 nuclease (Sigma) in S1 buffer; Na acetate (30mM,pH 4.8), zinc acetate (3mM), Na Cl (300mM) (final volume 100µl).

2.8.6 Ligation of DNA

Linearised vector and insert DNA bearing complementary cohesive ends were mixed at a molar ratio of 1:3 (final vector concentration of 5-30μg/ml) in a volume of 10μl containing 1 x T4 DNA ligase buffer and 1 unit of T4 DNA ligase. The reaction was incubated at 16°C for 18 h. For ligation of DNA molecules with a blunt end, reactions were carried out in a similar fashion to those with cohesive ends, but with higher concentrations of DNA (200-500μg/ml).

2.8.7 Extraction of DNA from low melting point Agarose.

The required DNA fragment was excised from the gel and transferred into a microcentrifuge tube. An equal volume of 1 x TBE, NaCl (0.2M) was added, and the tube incubated at 65°C for 15 min. The mixture was extracted twice with phenol at room temperature (saturated with 1 x TBE, NaCl (0.1M)). To remove residual phenol and reduce the volume to 200μl the aqueous phase was extracted several times with butan-2-ol. Finally the DNA was precipitated from sodium acetate/ethanol, pelleted and re-dissolved in TE.

2.8.8 Transformation of E.coli with DNA

E.coli strains were made competent for the uptake of plasmid DNA by the following method:

Cells were grown at the appropriate temperature to stationary phase in L broth, diluted 1:10 in L-broth and grown to A_{650}=0.5 (A_{650}=0.25 for NM522). Cells were pelleted by centrifugation (4,000 g 10 min., 4°C), resuspended in $CaCl_2$ (0.5 vol., 100mM) and left on ice for 30 min. Cells were pelleted as above and resuspended in $CaCl_2$ (0.05 vol., 100mM). After incubation (30 min., 4°C) cells were then transformed with DNA.

Competent cells (0.2ml) were added to the DNA sample (<100ng) in glass tubes and left on ice for 30 min. The tubes were transferred to 42°C for 4 minutes, 1ml of L-broth was added and the mixture shaken for 45 min. at 37°c or 30°c. Aliquots of the mixture were plated onto selective media.

Labelling of DNA

2.8.9. Nick Translation (Rigby et al., 1977).

Plasmid DNA (1µg) was incubated at 14°C for 1-3h. with 2 x 10^{-5}µg of DNase I and 0.5 unit of E.coli DNA polymerase I in 20µl of 1 x nick translation buffer containing ^{32}P dCTP (final concentration of 10m Ci/ml).

Labelled DNA was separated from unincorporated nucleotides by two precipitations from sodium acetate/ethanol with the addition of 5µg E.coli tRNA.

2.8.10 Random-priming (Feinberg and Vogelstein 1983, 1984)

The desired DNA fragment was excised from a low melting point gel, weighed and placed in a 1.5ml microcentrifuge tube. Water was added (3ml/g of agarose) and the tube incubated at 100°C (7 min.), to melt the agarose and denature the DNA. The reaction was set up by adding the following:

OLB (10µl), BSA (2µl, 10mg/ml), DNA (60ng), Klenow fragment of E.coli DNA polymerase I (5 units), and dCTP (final concentration of 10m Ci/ml). The reaction was incubated at room temperature for 2 h.

2.8.11 Labelling DNA Termini

The termini of DNA fragments were labelled when the appropriate (^{32}P)dNTP was used in the repair of recessed 3' termini using the Klenow enzyme as described in section 2.8.5.

2.8.12 Colony Blotting (Grunstein and Hogness, 1975).

Transformed E.coli colonies were arranged in a grid formation on L-agar containing the appropriate antibiotic and grown overnight at the appropriate temperature. Plates were placed at 4^{o}C for 30 min. then a nitrocellulose membrane (0.4 μm pore size, Schleicher and Schull) was gently laid on top and left for 2 min. The membrane was lifted off gently and cells were lysed and DNA bound to the nitrocellulose membrane by the following method: The membranes were placed, colony-side up, onto filter paper soaked in NaOH (0.5M) for 7 min. then neutralised by transferring the nitrocellulose to filter paper soaked in Tris-HCl (1M, pH 7.4) for 4 min. The nitrocellulose filters were then transferred onto filter paper soaked in Tris-HCl (0.5M, pH 7.4) containing NaCl (1.5M), for 4 min. The membranes were dried and baked at 80^{o}C for 90 min. under vacuum.

2.8.13 Southern Blotting (Southern et al 1975).

After electrophoresis of DNA through an agarose gel, the gel was soaked in HCl (0.25M, 15 min.) to partially depurinate the DNA, then rinsed in water. The gel was then immersed in NaOH (0.5M) containing NaCl (1.5M) for 30 min. to denature the DNA and then in NaOH (0.02M) containing ammonium acetate (1M) to neutralise the gel. To allow bidirectional transfer, two sheets of nitrocellulose (Schleicher and Schull) were soaked in the neutralising solution described above. These were placed on both sides of the gel and sandwiched with 3MM Whatman

filter paper. After leaving for 8 to 18 h. the nitrocellulose membrane was removed and rinsed in 2 x SSC then baked for 90 min. at 80°C under vacuum.

2.8.14 Hybridisation of Membrane Filters with Radioactive Probes

Filters were prehybridised in 10-30 mls of hybridisation solution [1 x Denhardt's reagent, 4 x SSC, formamide (50%, v/v), sonicated salmon sperm DNA (100µg/ml)] at 37°C for 30 min. with constant agitation. The filters were then incubated overnight (37°C) in hybridisation solution (10ml) containing the denatured radiolabelled probe. Following hybridisation, filters were washed twice in the following solution: 2 x SSC, SDS (0.1%, w/v) then in a buffer containing 1 x SSC, SDS (0.1%, w/v) for a further two hours. Filters were then dried and exposed to X-ray film.

2.8.15. RNA Preparation (Squires et al., 1981)

A single bacterial colony was used to inoculate a 10ml culture in L-broth (with antibiotics as required) and grown to stationary phase. The culture was diluted 1:50 in L-broth (with antibiotics) and grown to A_{650}=1.0. Cells were harvested by centrifugation (4,000 g, 10 min.) washed in 50ml of TE and harvested again as above.

Cells were resuspended in 6.5ml of the following solution: KCl (10mM), $MgCl_2$ (5mM), Tris-HCl (10mM, pH 7.3) containing 2mg of lysozyme. Following incubation at -70°C for 30 min. the suspension was allowed to thaw and SDS (0.9ml, 10%, w/v) was added. After incubation (64°C, 5-10 min.) sodium acetate (0.33ml, 2.5M, pH 5.2) was added and the solution mixed with hot phenol (64°C) and shaken at 64°C for 4 min. Following centrifugation (2000g, 10 min.) the aqueous phase was removed and NaCl (1g) was added.

2.8.16 Electrophoresis of RNA

RNA was fractionated according to size by electrophoresis through a 1.3% agarose gel. Agarose (1.95g) was melted in 109 mls of H_2O and allowed to cool to 60°C, 10 x MOPS (15 ml) and formaldehyde (26 ml) were added and the mixture poured into horizontal gel formers. RNA samples were added to formamide sample buffer, incubated at 60°C for 5 min. and cooled immediately on ice. Following addition of RNA loading buffer (0.25 vol.) the samples were subjected to electrophoresis (5V/cm for 18 h. in 1 x MOPS gel buffer at room temperature). Gels were stained for 20 min. with ethidium bromide (5µg/ml) in ammonium acetate (0.1M) at room temperature and destained for 2 h. in ammonium acetate (0.1M). Xenopus borealis 18S and 28S RNA were used as size markers.

2.8.17 Northern Blotting (Thomas, 1983).

Following electrophoretic separation of RNA, the gel was directly assembled into a capillary blot. For this purpose, a glass plate was placed over a reservoir of 20 x SSC and a large double sheet of Whatman 3MM paper (pre-soaked in 20 x SSC) positioned on the plate with its ends dipping into the reservoir. The gel was then placed on top of this, followed by a nitrocellulose sheet (cut to the exact dimensions of the gel) and several sheets of 3MM Whatman paper (soaked in 20 x SSC). Twenty sheets of dry, Whatman 3MM paper were placed on top of this assembly and held in place by a large weight. Blotting was carried out for at least 16 h. after which the nitrocellulose membrane was removed, dried and baked at 80°C for 90 min. under vacuum. The nitrocellulose membrane was treated for hybridisation as described previously (see section 2.8.14).

2.9 DNA Sequencing by the Dideoxynucleotide Chain Termination Method (Sanger et al., 1977).

2.9.1 Preparation of Double-Stranded Replicative Form (RF) DNA of the Bacteriophage M13.

A single blue plaque was used to inoculate a 2.5ml culture of NM522 (A_{650}=0.25) and shaken at 37°C for 6 h. Following growth, 1ml of this culture was added to 2 x 250ml cultures of NM522 (A_{650}=0.25) and grown for a further 4 h. at 37°C with constant agitation. The RF form of the bacteriophage M13 DNA was then isolated as described in Section 2.8.2.

2.9.2 Cloning into M13mp 10, 11, 18 and 19

DNA to be sequenced was purified as a convenient restriction fragment from a low melting point agarose gel and sub-cloned into M13 vectors. The α fragment of the β-galactosidase coding region, along with its operator and promoter regions have been inserted into the intergenic region of M13. Many unique restriction sites have been engineered into this region to facilitate sub-cloning of DNA fragments with many different termini. Insertion of DNA into this poly-linker region results in the inactivation of the α fragment and provides a convenient screening procedure for the identification of recombinant plaques. Insertional inactivation prevents the cleavage of X-gal. Hence recombinant phages show up as "white" plaques and non-recombinants as blue plaques in the presence of X-gal and IPTG.

2.9.3 Plaque Hybridisation (Benton and Davis, 1977).

"White" plaques were picked, using a toothpick, and transferred into 50μl of LTB. Aliquots of the mixture were placed in a grid formation on

minimal plates overlaid with 3 mls BBL top agar containing 0.2mls NM522 (A_{650}=0.25), IPTG (20µl, 20mg/ml) and X-gal (20µl, 20mg/ml), and incubated at 37°C (16 to 18 h.). The plates were then incubated at 4°C for 30 min. and a nitrocellulose membrane was placed in contact with the plaques (2 min.). The membrane was then gently lifted off and placed sequentially, plaque side up, onto filter paper soaked in NaOH (0.5M), NaCl (1.5M) for 2 min. then Tris-HCl (0.5M, pH 7.4), NaCl (3M) for 5 min. The filters were then washed twice (5 min. each) in 2 x SSC, dried and baked at 80°C for 90 min. under vacuum. The filters were then hybridised as described previously (section 2.8.14).

2.9.4 Preparation of Template DNA

Single-stranded template DNA was prepared for sequence analysis by the following method. Cultures of NM522 (1.5ml, A_{650}=0.25) were inoculated with individual recombinant plaques. The cultures were grown for 4 to 5 h. at 37°C and then transferred to microcentrifuge tubes and cells pelleted by centrifugation (17,500g, 5 min.). The supernate was transferred to a fresh tube and the phage precipitated by the addition of 200µl of: PEG (20%, w/v), NaCl (2.5M). Following incubation (30 min., room temperature) phage were pelleted by centrifugation (17,500g, 5 min.). The supernate was discarded and the pellet re-suspended in sequencing TE (100µl). To remove the capsid proteins, phenol (50µl) was added, vortexed and left at room temperature for 30 min. After centrifugation (17,500g, 5min.) single-stranded template DNA was precipitated from the aqueous phase by ethanol precipitation (twice) and the pellet redissolved in sequencing TE (50µl) and stored at -20°C.

2.9.5 Sequencing Reactions

Annealing

The yield of the single-stranded template DNA prepared as described above was approximately 5µg. To template DNA (8µl), in a 1.5ml micro-centrifuge tube, TM (1µl) and M13 univeral primer (1µl,0.2 pmol/µl, New England Biolabs) were added. This mixture was incubated at 60°C for 60 min. then briefly centrifuged to drive any condensation to the bottom of the tube.

2.9.6 Primed Synthesis

All sequencing reactions were carried out in 1.5ml uncapped Sarstedt tubes in plastic 10-hole centrifuge racks. Aliquots (2µl) of the annealed mix were dispensed into the 4 sequence reaction tubes containing 2µl of dideoxy A/G/C/T nucleotide mixes respectively (see Table 2.1). Finally, 2µl of Klenow mix [1µ Ci [α^{35}S]-dATP (400 Ci/m mol), Klenow enzyme (0.4 units),Tris-HCl (10mM, pH 8.5), DTT (10mM)] were added to each reaction tube. After 20 min. at room temperature, 2µl of sequencing chase (dATP, dTTP, dCTP, dGTP, 0.25mM) were added to each tube, centrifuged briefly and left for a further 15 min. at room temperature. Sequencing reactions can be stored at -20°C at this stage. Prior to gel electrophoresis,2µl of formamide loading dye were added to each tube and centrifuged again. Racks were placed into a boiling water bath for 3 min. and reaction products analysed by polyacrylamide gel electrophoresis (PAGE).

TABLE 2.1 Composition of ddNTP Solutions.

(all volumes in microlitres)

	"T" mix	"C" mix	"G" mix	"A" mix
50mM dTTP	-	2.5	2.5	2.5
50mM dCTP	2.5	-	2.5	2.5
50mM dGTP	2.5	2.5	-	2.5
10mM ddTTP	30	-	-	-
10mM ddCTP	-	7.5	-	-
10mM ddGTP	-	-	15	-
1mM ddATP	-	-	-	15
0.5mM dTTP	12.5	-	-	-
0.5mM dCTP	-	12.5	-	-
0.5mM dGTP	-	-	12.5	-
Sequencing TE	500	500	500	250
H_2O	500	500	500	750

2.9.7 Separation of DNA Fragments in Acrylamide Gels

The Standard Gel System

Electrophoresis was performed on a 0.3mm x 20cm x 40cm gel, cast between flat glass plates. The notched plate was siliconised on the inner surface before assembly. The glass plates were assembled by placing 0.5cm x 40cm x 0.3mm spacers along both 40cm edges between the plates, and then the sides and bottom were sealed with PVC tape. Each gel required approximately 35ml of gel mix, [acrylamide stock (6%, v/v), 1 x TBE, urea (7M)]. Polymerisation of this mixture was initiated by the addition of AMPS (240µl,10%, w/v) and TEMED (35µl) then poured into the prepared glass plates and a well-former placed in position. After the gel was set, the tape was removed, the gel mounted on the gel tank, and the reservoirs filled with 1 x TBE.

Samples were prepared as described above and applied to the sample wells. The gel was run at constant power (40 watts).

Following electrophoresis gels were fixed in acetic acid (10%, v/v), methanol (10%, v/v), for 15 min. The gels were then transferred to Whatman 3MM paper, covered in Saran Wrap and dried in a vacuum gel drier. When the gel was dry, the Saran Wrap was removed and the gel subjected to autoradiography (see section 2.7.5).

2.9.8 Buffer Gradient Gels

The gradient gel was prepared as follows: 40ml of 0.5 x TBE gel mix [urea (17g), sucrose (2g), 10 x TBE (2ml), acrylamide stock (6ml) made up to 40 ml with dH_2O], AMPS (240µl, 10%, w/v) and TEMED (35µl) were mixed. 10ml 2.5 x TBE gel mix [urea (4.25g), acrylamide stock (1.5ml), 10 x TBE (2.5ml), made up to 10ml with dH_2O], AMPS (70µl, 10%, w/v) and TEMED (7µl) were mixed: 4ml of the polymerising 0.5 x TBE gel mix were taken up in a 10ml pipette, then 6ml polymerising 2.5 x TBE gel mix were taken up in the same pipette, a crude gradient was formed by introducing 4 air bubbles then pipetting the mixture between the glass plates assembled as described above. The rest of the polymerising 0.5 x TBE gel mix was added, a well-former was inserted and the gel allowed to set. When the gel had set,the tape was removed and the gel mounted on the vertical gel tank. The top reservoir was filled with 0.5 x TBE and the bottom reservoir was filled with 1 x TBE. The rest of the procedure was as described above.

2.10 Protein and Antibody Techniques

2.10.1 Lowry Protein Assay.

Protein concentrations were estimated as described by Lowry et al., (1951). The reagents used were as follows: Solution A anhydrous Na_2Co_3 (2%, w/v in 0.1M NaOH), Solution B $CuSO_4.5\ H_2O$ (0.5%, w/v) in sodium citrate (1%, w.v), Solution C (1ml Solution B plus 50ml Solution A); Solution D (Folin and Ciocalteau's phenol reagent diluted with dH_2O, 50:50).

Various concentrations of BSA,ranging from 0 to 300µg/ml (total volume of 0.4ml) were used to provide a standard protein concentration curve. Test samples were diluted to 1:50, 1:100 and 1:200 in 400µl dH_2O. Each sample was mixed with 2ml of Solution C in a glass tube and the mixture incubated for 10 min. at room temperature. Solution D (0.2ml) was then added, and the mixture incubated for a further 30 min. The absorbance of these samples was measured at A_{550nm} and the absorbance related to protein concentration using the standard protein concentration curve.

2.10.2 Electrophoresis of Proteins in Polyacrylamide Gels

Polyacrylamide gel electrophoresis (PAGE) was carried out using the discontinuous buffer system of Laemmli (1976). Stacking gels of 170mm x 150mm x 1.5mm were polymerised between glass plates separated by perspex spacers. The composition of the separating and stacking gel is described in Table 2.2. The gel mould was sealed by placing it in a trough in which 10ml of the separating gel solution had been polymerised by the addition of AMPS (150µl,10%, w/v) and TEMED (10µl). After 15 min. the polyacrylamide in the trough had set and the remainder of the separating gel, following the addition of SDS (400µl,10%, w/v), AMPS (200µl,10%, w/v)

and TEMED (20µl) was poured. The polymerising gel was overlaid with dH_2O and allowed to set (2 hours). The water was then poured off and the gel left to drain (2 min.). SDS (150µl, 10%, w/v), AMPS (10µl, 10%, w/v) and TEMED (10µl) were added to the stacking gel which was then poured above the running gel. A well-former was placed in position and the gel was allowed to set. To protein samples were added an equal volume of 2 x loading buffer. [2 x contains Tris-HCl (2ml, 0.6M, pH 6.8), glycerol (2ml) SDS (4ml, 10%, w/v), H_2O (2ml)] and DTT to a final concentration of 0.06M and heated to 100^0C for 5 min. before loading. Gel electrophoresis was carried out at 2-12 V/cm for 12-16 h. in 1 x protein gel buffer.

TABLE 2.2 Composition of SDS-Polyacrylamide Gel Mixes (All volumes in ml)

Stock Solutions	Stacking Gel Mix (3.75% acrylamide)	Separating Gel Mix 7% acrylamide	Separating Gel Mix 10% acrylamide
30% (w/v) Acrylamide	1.875	11.6	16.67
0.2% (w/v) bis-Acrylamide	1.875	11.6	16.67
3M Tris-HCl pH 8.8	-	6.25	6.25
1M Tris-HCl pH 6.8	1.875	-	-
dH_2O	9.35	20.5	10.4

2.10.3 Staining of Protein Gels

Following electrophoresis, gels were stained with Coomassie Brilliant Blue R to reveal protein bands. This was carried out by gently agitating the gel for 30 min. at room temperature in staining solution: Coomassie Brilliant Blue R (2g/L), methanol (45%, v/v), glacial acetic acid (10%, v/v), distilled water (45%, v/v). The gel was then destained by gentle agitation at room temperature in repeated changes of the above solution, lacking Coomassie Brilliant Blue R.

2.10.4 Protein molecular Weight Markers

To calibrate SDS-PAGE, high and low molecular weight markers (Pharmacia) were loaded on one track of each gel. For a Coomassie Brilliant Blue R stained gel, one fifth of a high and one tenth of a low molecular weight marker kit vial was loaded per track. One high molecular weight marker kit vial contained: ferritin (220K and 18.5K, 50μg), albumin (67K, 40μg) catalose (60K, 36μg) and lactate dehydrogenase (30K, 48μg). One low molecular weight marker kit vial contained: phosphorylase b (94K, 64μg) albumin (67K, 83μg), ovalbumin (43K, 147μg) carbonic anhydrase (30K, 83μg), trypsin inhibitor (20.1K, 80μg) and α-lactalbumin (14K, 121μg).

2.10.5 Small-scale Preparation of Protein from E.coli

For rapid screening procedures, a single colony was used to inoculate L-broth (with the appropriate antibiotic added). After growth to stationary phase the culture was diluted 1:50 in L-broth and grown at 37°C or 30°C to A_{650nm}=0.5. At this stage the cells carrying the recombinant plasmids were induced according to the respective promoters. For Tac and Lac promoters, transcription was induced by adding IPTG to a final concentration of 2mM for 1 hour. For the rightward promoter of lambda, induction was produced by incubating the cells at 42°C for 30 min. Cells were harvested from a 1ml culture by centrifugation (17,500g, 2 min.), the pellet resuspended in sample loading buffer (40μl), lysed by incubation at 100°C (5 min.) and loaded onto the gel.

2.10.6 Large-scale Preparation of Fusion Protein from E.coli

A single colony was used to inoculate L-broth (with the appropriate antibiotics added) and the culture grown until it reached stationary phase. The culture was diluted 1:50 in 500ml of L-broth and grown at 30°C until A_{650} = 0.5. Cells were incubated at 44°C for 45 min. with constant agitation and then returned to 30°C for a further 30 min. Cells were harvested by centrifugation (4,000g, 10 min.) and the bacteria resuspended in 2mls of 1 x PBS or 2mls of TEN [Tris-HCl (50mM, pH 7.5), EDTA (0.5mM), NaCl (0.3M)]. The mixture was sonicated 5 times (10 seconds each time, Soniprobe, Dawe Instruments type 7530A), then centrifuged (4,000g, 10 min.). The pellet, which contained the majority of the insoluble fusion protein was resuspended in 2mls of 1 x PBS or 2mls of TEN.

2.10.7 Analysis of Plasmid Gene Products expressed by E.coli Minicells

The minicell-producing strain, WL542, carrying the plasmid of interest was grown to stationary phase in 2 Litres of L-broth from a 2ml inoculum. Cells were pelleted by centrifugation (4,000g, 3 min.) and the supernate collected and centrifuged again (8,200g, 10 min.). The pellet was resuspended in 18ml of L-broth then agitated vigorously for 10 min. using a magnetic stirrer (at 4°C). The mixture was layered onto 4 x 35ml sucrose gradients (in 50ml polycarbonate tubes). These gradients were prepared by making 20% (w/v) sucrose in M9 glucose (M9 glu) [water 100ml, M9 x 4 (25ml), glucose (1ml,20%, w/v), $MgSO_4$ (0.1ml, 1M), Vitamin B_1 (0.025ml, 0.8mg/ml)] freezing (-20°C) and then thawing (at 4°C overnight) prior to use. The gradients were centrifuged (4,000g, 20 min., 4°C), and the minicell band, which lies midway down the gradient, was removed with a syringe and a needle (which had been bent at an angle of 90°). The minicells were harvested by centrifugation (20,000g,

10 min.), and the pellets were resuspended in a total of 5ml M9 glu. This mixture was layered onto 2 x 35ml sucrose gradients and the minicell band collected and pelleted as above. The pellet was resuspended in 2.5ml M9 glu and layered on one 35ml sucrose gradient and the minicell band collected as described above. The minicells were harvested finally by centrifugation (8,000g, 10 min.). The pellet was resuspended in 1ml M9 glu, glycerol (30%, v/v), and the minicells were frozen at -70°C.

2.10.8 Labelling Protein Products expressed by Minicells

The minicell preparation was thawed at room temperature, the preparation was diluted with M9 glu to A_{600}=0.2 and cells harvested by centrifugation (17,500g, 2 min.). The pellet was resuspended in M9 glu (0.1ml) and incubated at 37°C for 60 min. Methionine [^{35}S] (20 μ Ci; 1,000 Ci/m mol:Amersham) in 20μl of methionine assay medium (Difco) was added and incubated for 45 min. at 37°C. This was followed by the addition of unlabelled methionine (5μl, 8mg/ml) and incubation for a further 5 mins. Cells were collected by centrifugation (17,500g, 2 min.) washed with Tris-HCl (1ml, 0.05M, pH 6.8), pelleted as above and resuspended in sample loading buffer (40μl). Samples were heated at 100°C for 5 min. and subjected to SDS-PAGE. After electrophoresis the gel was stained, destained, dried and exposed to X-ray film.

2.10.9 Immunisation of Rabbits

A sample of the required fusion protein was prepared by electrophoresis of 1.5ml of the induced E.coli cell extract on the whole width of a protein gel. Three narrow longitudinal strips were cut from the gel, one from each side and one from the middle. These were stained with Coomassie Blue and destained. With reference to the stained strips,

the region of unstained gel that contained the protein band of interest was excised with a scalpel blade and homogenised in 3-5 volumes of PBS.

The rabbits used in this study were New Zealand Whites. Before any injection, 5ml of blood was taken from the ear as a preimmune control. The protein solution (400µl, 100µg protein) was mixed with Freund's complete adjuvant (400µl) for the first injection and with Freund's incomplete adjuvant for the second and subsequent injections. At intervals of one month the preparation was injected subcutaneously at several locations on a single rabbit, and 8-9 days after each injection approximately 10ml of blood was collected from the ear vein. Collected blood was transferred to a glass tube, incubated at room temperature for 30 min. and the clot removed. After incubation at 4°C for 6-15 h.,serum was decanted into centrifuge tubes, cleared by centrifugation (17,500g, 30 seconds) and stored at -20°C.

2.10.10 Purification of Antiserum

A single colony of NF1 carrying the vector was used to inoculate a 10ml culture of L-broth (with added ampicillin), and grown to stationary phase, diluted 1:50 in 500ml of L-broth and grown to A_{650}=0.5. Following induction of expression by incubation at 42°C (45 min.), the cells were incubated at 30°C (30 min.) and harvested by centrifugation (4,000g, 10 min.). The pellet was resuspended in TS [NaCl (0.15M), Tris-HCl (10mM, pH 7.4)], and added to SDS (final concentration of SDS was 1%, w/v), prior to heating (100°C, 5 min.). This lysate was then diluted 1:10 with TS. Equal volumes of the lysed cell mixture and antiserum were mixed by rotation for 12-16 h. (4°C). This was centrifuged (17,500g, 15 min.), supernate transferred to a fresh tube, and an equal volume of lysed cell mixture added. The procedure was repeated 4 times and the supernate stored at -20°C.

2.10.11 Western Blotting

Proteins were electrophoretically transferred from SDS-polyacrylamide gels to nitrocellulose filters by the Western blotting procedure of Towbin et al., (1979). A gel sandwich was constructed in a Bio-Rad transfer cassette. All components were first soaked in transfer buffer, and layered onto the cassette in the following order: Scotchbrite pad, 3 sheets of blotting paper cut to the same size as the gel, the gel, nitrocellulose (0.45µm Schleicher and Scheul) the same size as the gel, 3 more sheets of blotting paper, and finally another Scotchbrite pad. The cassette was closed and placed in a Bio-Rad electrotransfer kit, (nitrocellulose towards the anode), containing transfer buffer [Tris base (12.11g), glycine (55.8g), methanol (1 litre) and dH_2O (4 litres)]. Proteins were transferred at 60V for 5 hours at 4^oC. After transfer, the nitrocellulose membrane was stained with Ponceau S (0.5%, w/v), in TCA (3%), and washed in dH_2O to visualise the transferred proteins.

2.10.12 Immunological detection of Antigens Bound to Nitrocellulose

This procedure was used to detect antigens bound to nitrocellulose membrane. Following transfer of proteins to the nitrocellulose membrane,unoccupied binding sites were blocked by agitation for at least 1 hour at room temperature in TS containing ovalbumin (5%, w/v) (Sigma) (OTS). Immunological probingwas carried out by agitation at room temperature with antibody diluted in OTS (for 12-16h.). Before and after each antibody was applied, the nitrocellulose was washed in 5 changes of TS over a 30 minute period. After washing the filter, it was incubated for 2 hours at room temperature with either affinity purified goat anti-human IgG conjugated to peroxidase or goat anti-rabbit IgG when rabbit antisera was used. Both IgG fractions were diluted 500-fold in OTS.

After extensive washing in TS at room temperature, the colour was developed by incubation in imidazole (0.01M, pH 7.4), dianisidine (250µg/ml),hydrogen peroxide (0.3% v/v); this reagent was prepared immediately prior to use. Brown bands appeared after approximately 5 min. at positions where the first antibody had bound. The reaction was stopped by washing in water. Filters were blotted dry and stored in the dark at room temperature.

2.11 Reverse Transcriptase Assays

2.11.1 Preparation of Crude Lysates of E.coli for Reverse Transcriptase Assays

Crude lysates were prepared by a modification of a method described by Kleid et al., 1981. E.coli NF1 cells carrying the plasmids were grown at 30°C to stationary phase in L-broth (containing ampicillin) diluted 1:50 in L-broth (500 mls) and grown at 30°C to A_{650} = 0.4. Shifting the temperature to 42°C induced expression. Cells were harvested by centrifugation (4,000g, 10 min.). The bacteria were resuspended in 1:200th vol. of TEN: Tris-HCl (50mM, pH 7.5), EDTA (0.5mM), NaCl (0.3M). The suspension was then sonicated (5 times, 10 seconds each time, Soniprobe, Dawe Instruments, type 7530 A) and 4.5ml of the following: NaCl (1.5M), $MgCl_2$ (12mM), and deoxyribonuclease (final concentration of 2µg/ml), was added and stirred on ice for 1 hour. This treatment produced an E.coli lysate in which the β-galactosidase fusion proteins were largely insoluble. These were recovered by centrifugation (4,000g, 10 min.) and washed three times by resuspension in TEN (2 mls) and centrifugation. The final pellet was resuspended in 1:200th the original culture volume in TEN.

2.11.2 Assay for Reverse Transcriptase Activity

Crude lysates were adjusted to; 50mM Tris-HCl (pH 8.3), 50mM KCl, 10mM $MgCl_2$, 0.1% NP40, 90µg/ml poly rC:oligo $dG_{(12-18)}$ (Sigma), 5mM DTT, 14.2 µM [^{3}H]-dGTP (16.9 Ci m mole^{-1} Amersham) (Takatsuji et al., 1986). After incubation (37^{o}C, 60 min.), acid precipitable material was collected by adding sonicated salmon sperm DNA (100µl, 1mg/ml), Na pyrophosphate (100µl, 0.1M) and TCA (1ml, 10%, w/v, 4^{o}C), incubating on ice (10 min.) and centrifugating (17,500g, 10 min.). The pellet was washed twice with TCA (1ml, 10%, w/v, 4^{o}C), once with ethanol (1ml, 100%, v/v),dried and counted in a liquid scintillation spectrometer after addition of scintillation fluid (Butyl PBD, 0.5ml, 6g/L in toluene).

2.11.3 Inhibition of Reverse Transcriptase Activity by Phosphonoformate (PFA)

PFA, a gift from Astra Ltd., was prepared as an aqueous solution (2%, w/v), stored at 4^{o}C and adjusted to pH 7.4 with HCl (2M) prior to use. PFA was mixed with template and reaction buffer (as described in Chapter 6) before addition of crude lysates.

CHAPTER 3

EXPRESSION OF THE HEPATITIS B POLYMERASE GENE IN *ESCHERICHIA COLI*

Page

Chapter 3

INTRODUCTION

Expression of the large open reading frame (L.ORF) of HBV in bacterial cells could provide an abundant and safe source of the protein encoded by this gene and, it is hoped, allow detailed analysis of its product.

Synthesis of a protein at high rates in Escherichia coli (E.coli) depends upon frequent transcription of its gene and efficient translation of the transcript. In contrast to the expression of cloned bacterial genes, those from eukaryotes are expressed poorly, if at all, in E.coli. In order to express efficiently in bacteria, cloned eukaryotic genes must be placed under the control of bacterial transcription and translation signals.

Transcription of a gene is controlled by the binding of RNA polymerase to the promoter, normally located upstream of the gene. In E.coli it has been shown that a promoter contains, at the nucleotide level, two highly conserved regions. The first is located approximately 35 base pairs upstream from the transcription initiation site (-35 region), the second is found 10 base pairs upstream of the initiation site (Pribnow box or "core" recognition region). The consensus sequence of these two regions is 5'-TTGACA-3' and 5'-TATAAT-3' respectively (Rosenberg and Court, 1979). Promoters from the lactose (Lac) and tryptophan (Trp) operons (Backman and Ptashne, 1978, and Hershfield et al., 1974), the left hand promoter of the bacteriophage lambda (P_L) (Moir and Brammar, 1976) and a synthetic hybrid of the Trp and Lac promoters (Tac) (de Boer et al., 1982) have been incorporated into cloning vectors for this purpose. The choice of vector is limited, to some extent, by the restriction enzyme sites used to place the gene under the control of the promoter. For the expression of the recombinant

HBV long ORF, vectors that carried the bacterial promoters Lac and Tac were used.

The Lac promoter is subject to two forms of control; positive regulation by the catabolite gene activator (CAP) system, and negative control by the Lac repressor (Reznikoff and Abelson, 1978). The version of the Lac promoter most widely used in cloning vectors is the promoter Lac UV5. Lac UV5 carries the regulatory region of the Lac operon and contains the L8 mutation conferring CAP independence and the UV5 "up-promoter" mutation which enhances the rate of transcription (Backman et al., 1976). This has been used successfully to express a number of viral and eukaryotic genes in E.coli (Table 3.1).

Promoter strength has been shown to be directly proportional to the degree of similarity with the consensus sequence of the -35 region and Pribnow Box (Russell and Bennett 1982). Therefore a hybrid promoter has been constructed, which consists of the optimal consensus sequence, by fusing the -35 region of the Trp promoter to the -10 region of the Lac promoter. This promoter, known as the Tac promoter, has been used for the expression of the Human Growth Hormone (HGH) (Table 3.1) (de Boer et al., 1982).

Table 3.1 Expression of viral and Eukaryotic Proteins in E.coli.

Protein (mol.wt.)	Promoter	Construction	Level of Expression (% Total cell protein)	Reference
Rat proinsulin	Lac	β-gal fusion	Low	Talmadge et al. 1980.
Human proinsulin	Lac	β-gal fusion	12mg/322g wet cells	Wetzel et al. 1981.
β-endorphin	Lac	β-gal fusion	5%	Shine et al., 1980.
Chicken Ovalbumin	Lac	Short β-gal fusion	1-1.5%	Fraser and Bruce, 1978.
Hepatitis B virus core antigen.	Lac	β-gal fusion	Low	Stahl et al., 1982.
Hepatitis B virus surface antigen.	Lac (in phage)	Long β-gal fusion	0.05%	Charnay et al., 1980.
Rous Sarcoma virus protein kinase (Src)	Lac	8 amino acid β-gal fusion	0.3%	Gilmer and Erikson, 1981.
HSV thymidine kinase (TK)	Lac	Short β-gal fusion	0.2-0.3%	Garapin et al., 1981.
Polyoma Small t antigen	Lac	β-gal linker fusion	0.15%	Horwich et al., 1980.
SV40 t ag	Lac	rbs fusion	0.8% $1\text{-}5 \times 10^3$ mol/cell	Roberts et al. 1979.
Human Growth Hormone (HGH)	Tac	rbs fusion		de Boer et al., 1982.

Inducible control is important when over-expressing genes in E.coli as the resulting gene product may be toxic to the cells.

An example of this was found by Shimatake and Rosenberg (1981) when they tried to express large amounts of the λcII protein using the P_L promoter. They found that clones carrying λcII could transform a λ lysogen (carrying a ts cI857 gene) very well but could not, however, transform cells that did not make any λ repressor, demonstrating that the λcII protein is toxic to E.coli. Expression of λcII could be induced by growing the cells at 42°C.

Both the Lac and Tac promoters are regulated by the Lac repressor. However, when these promoters are carried on multicopy plasmids, they over-titrate the Lac repressor within the cell resulting in constitutive transcription. These promoters can be repressed in strains which over-produce the Lac repressor, i.e. Lac I^q (Backman et al., 1976; O'Farrel et al., 1978) and then induced by the presence of lactose or by the addition of a non-metabolisable inducer isopropyl-thiogalactosidase (IPTG) which binds to the repressor and removes it from the operator.

Efficient translation of mRNA in prokaryotic cells requires the presence of an efficient ribosome-binding site. For most E.coli messenger RNAs the ribosome-binding site consists of two components; the initiation codon AUG, and a Shine Dalgarno sequence (SD). The SD sequence is found 3-12 bases upstream of the initiation codon and is complementary to the 3' end of the 16S ribosomal RNA (Shine and Dalgarno, 1975).

A common strategy to ensure translation of a gene normally expressed in E.coli is to generate a fusion protein by joining the initial portion of a gene which is efficiently translated to the gene

whose expression is sought. The ribosome-binding site and initiating AUG of the E.coli gene provide the start signals for translation which proceeds into the cloned gene producing a fusion protein. This method has been used successfully in expressing the HBcAg in E.coli(Table 3.1) (Stahl et al., 1982).

To detect the expression of the cloned genes one can either use immunological methods, or phenotypic selection to detect the synthesis of the gene product. However, to detect the expression of cloned genes when the function is not known, or when a specific antibody is not available, is more difficult. In these cases one can directly analyse the protein if expressed at a high level, or if not, use mutant E.coli cells in which the background of the host proteins is minimised, e.g. minicells or alternatively an in vitro transcription translation system could be used.

Minicells are small, spherical cells which are produced throughout the growth cycle of minicell-producing mutant strains of E.coli. They contain RNA and protein but little or no chromosomal DNA. Because of their size difference, minicells and normal cells can be separated easily on sucrose gradients. Plasmid DNAs segregate into minicells, and purified minicell preparations allow the labelling of plasmid-encoded proteins in the absence of any background from chromosomal proteins (Frazer and Curtis, 1975), and hence are ideal for detecting the expression of novel proteins carried by recombinant plasmids.

In vitro transcription and translation systems are very useful for two reasons. Firstly, incorporation of radioactive label into protein in vitro is much more efficient than in vivo thus increasing its sensitivity. Secondly, the apparent inability to express some genes in E.coli has been due to proteolytic degradation of the foreign

gene product. This problem can be overcome using an *in vitro* transcription translation system. An example of this was described by Mellado and Murray (1983) when they cloned histone genes under the control of a bacterial promoter and were unable to detect the presence of any histone protein *in vivo*. However, using an *in vitro* transcription translation system, histones expressed from the recombinants were visualised.

In conclusion, using the methods and approaches discussed, this chapter describes the construction and expression of the HBV long open reading frame in *E.coli*.

3.1 The origin of HBV Genomic Clones

HBV DNA isolated from Dane particles from a single HBsAg and HBcAg donor was labelled with ^{3}H-dTTP using the endogenous polymerase. This DNA was then digested with *Eco*RI or *Bam*HI and hybrid plasmids constructed by insertion of these HBV DNA fragments into pBR322 at the PstI cleavage site via a 3' oligo dG and oligo dC tail (Burrell *et al.*, 1979). Two of these clones, pHBV130 and pHBVCB, carried inserted fragments with slightly over a genome length of viral HBV DNA (Fig.3.1a) (Gough and Murray, 1982). Unfortunately, neither of these constructs contained the long open reading frame in an intact form,thus the cloning of this gene involved the reconstruction of this long ORF piece by piece using several fragments (Fig.3.1b).

3.2 Construction of pR1-pol8 and pTac-pol8

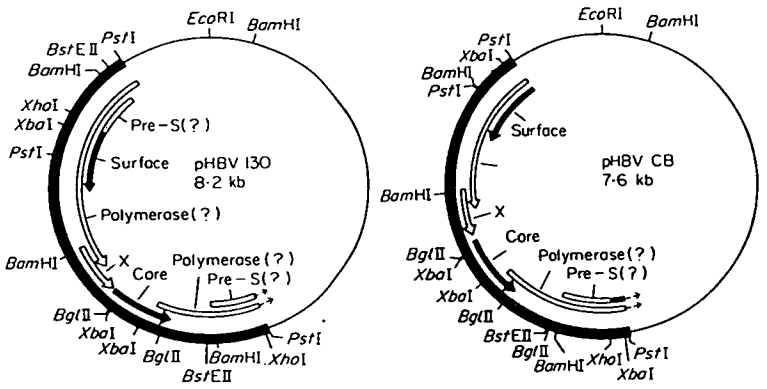
The plasmid pR1-11, expresses the core antigen of HBV as a fusion with 8 amino acids of β-galactosidase, using the Lac promoter to drive expression (Stahl *et al.*, 1982)(Table 3.1). This plasmid contains 1kb

FIGURE 3.1(a)

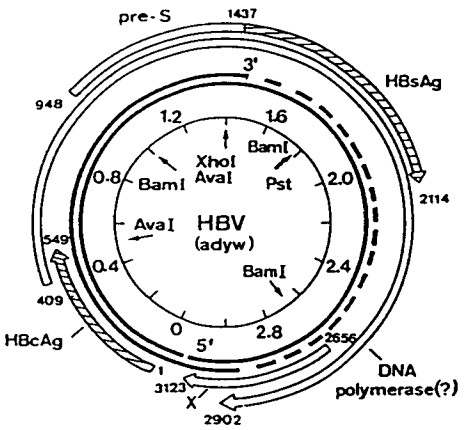
pHPV130, and pHBVCB. The heavy lines represent HBV sequences and the thin lines represent pBR322 sequences. The location and direction of open translational reading frames within the HBV genome are indicated by arrows. The surface antigen and putative polymerase genes are interrupted by pBR322 sequences (Gough and Murray, 1982).

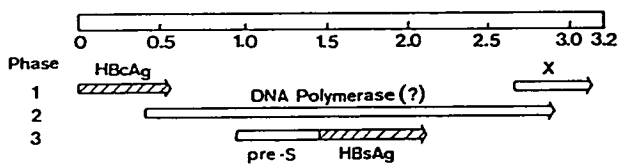
FIGURE 3.1(b) The genome of HBV. Heavy lines denote the DNA strands, the broken line showing the region of variable length of the short strand. Arrows represent the four open reading frames (as coding sequences) with the numbers of initiation and termination triplets in the system adopted by Pasek et al., (1979).

A


EcoRI
BamHI
PstI
BstEII
BamHI
XhoI
XbaI
PstI
Pre-S(?)
Surface
pHBV 130
8·2 kb
Polymerase(?)
BamHI
X
Core
Polymerase(?)
Pre-S(?)
BglII
XbaI
XbaI
BglII
BamHI
XhoI
PstI
BstEII
EcoRI
BamHI
PstI
XbaI
BamHI
PstI
Surface
pHBV CB
7·6 kb
BamHI
X
Core
BglII
XbaI
Polymerase(?)
Pre-S(?)
XbaI
BglII
BstEII
BglII
BamHI
XhoI
PstI
XbaI

B


pre-S
1437
3'
HBsAg
948
1.2
1.6
BamI
XhoI
AvaI
0.8
BamI
Pst
2.0
2114
AvaI
HBV
(adyw)
549
0.4
BamI
2.4
409
0
5'
2.8
HBcAg
2656
DNA
polymerase(?)
1
3123
X
2902


0
0.5
1.0
1.5
2.0
2.5
3.0
3.2
Phase
1
2
3
HBcAg
X
DNA Polymerase (?)
pre-S
HBsAg

of HBV DNA. Removal of a 439bp BglII fragment, which deletes most of the core gene, results in a fused product consisting of the first 84 nucleotides (equivalent to the N-terminal 28 amino acids of the core antigen gene fused to the polymerase gene at a position corresponding to amino acid residue 40). This construct, pHPL-1, was kindly given by Dr. Michael McGarvie (Fig.3.2).

The reading frame of the polymerase component of pHPL-1 was extended by the insertion of a 1678 bp BamHI fragment from pHBV130. pHBV130 was digested with BamHI and the resulting restriction fragments separated by agarose gel electrophoresis. The desired fragment was purified by electro-elution and then added to BamHI linearised pHPL-1 in a ligation reaction containing T4 DNA ligase. Following ligation, the mixture was used to transform competent E.coli W110I^{q} cells and plated out in the presence of ampicillin. Plasmid DNA was prepared from overnight cultures of ampicillin-resistant transformants using a rapid isolation technique. Recombinant plasmids were identified on the basis of size by agarose gel electrophoresis. A recombinant containing the insert in the correct orientation was then identified by restriction mapping; this construct is called pR1-130.

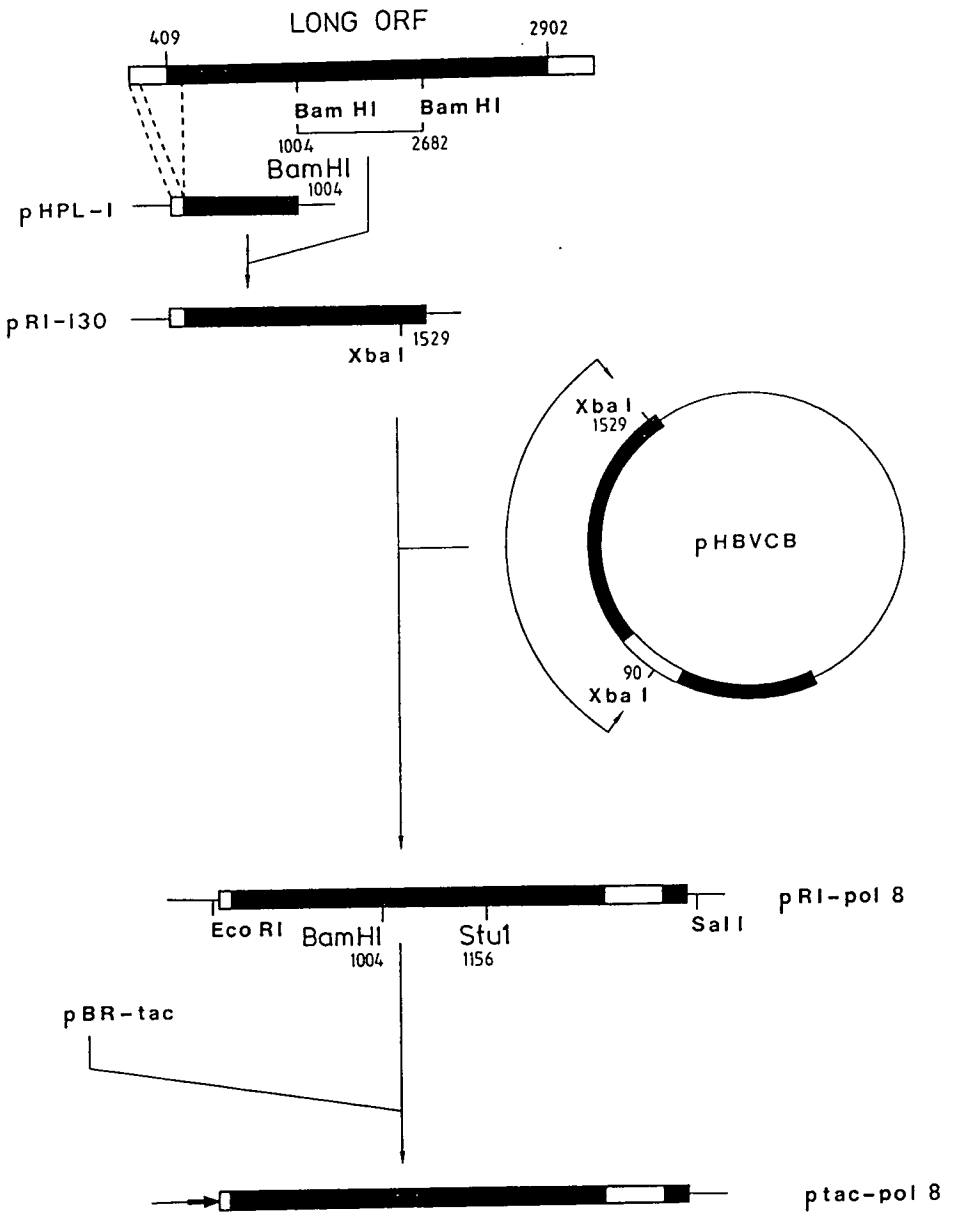
pR1-pol8 was constructed in a manner analogous to that used for pR1-130, i.e. by ligating an XbaI fragment from pHBV CB into XbaI-digested pR1-130, thereby introducing the rest of the coding region including the translation termination signal of the long ORF. The identity of this construct was confirmed by restriction mapping

Conservation of the reading frame at the BglII deletion was verified by sequencing across the junction. This was achieved by sub-cloning the EcoRI-BamHI fragment covering the junction sequence of the recombinant plasmid into M13 mp11 and sequencing by the dideoxy chain

FIGURE 3.2 The construction of pR1-pol8 and ptac-pol8.

1) pHPL-1 contains HBV DNA sequences equivalent to the first 28 amino acids of the core protein fused in frame to amino acids 40 to 201 of the polymerase gene product. 2) A BamHI fragment encoding the internal sequences of the putative polymerase gene was inserted into the unique BamHI site of pHPL-1 to form pR1-130. 3) The rest of the coding information, including the translation termination signal of the long ORF, was inserted into an XbaI fragment from pHBVCB to form pR1-pol8. 4) EcoRI and SalI sites flank the polymerase region of pR1-pol8. This plasmid was restricted with these enzymes and the fragment containing the polymerase gene was inserted into the unique EcoRI and SalI sites of the expression vector pBR-Tac to produce ptac-pol8.

Key: filled area, HBV Long ORF.


LONG ORF
409
2902
Bam HI
Bam HI
1004
2682
BamHI
1004
pHPL-I
pRI-130
1529
Xba I
Xba I
1529
pHBVCB
90
Xba I
pRI-pol 8
Eco RI
BamHI
1004
Stu1
1156
Sal I
pBR-tac
ptac-pol 8

termination procedure. Likewise, an XhoI-PstI fragment was sub-cloned into M13 mp19 and sequenced to confirm that insertion of the XbaI fragment from pHBVCB into pR1-130 did not disrupt the reading frame.

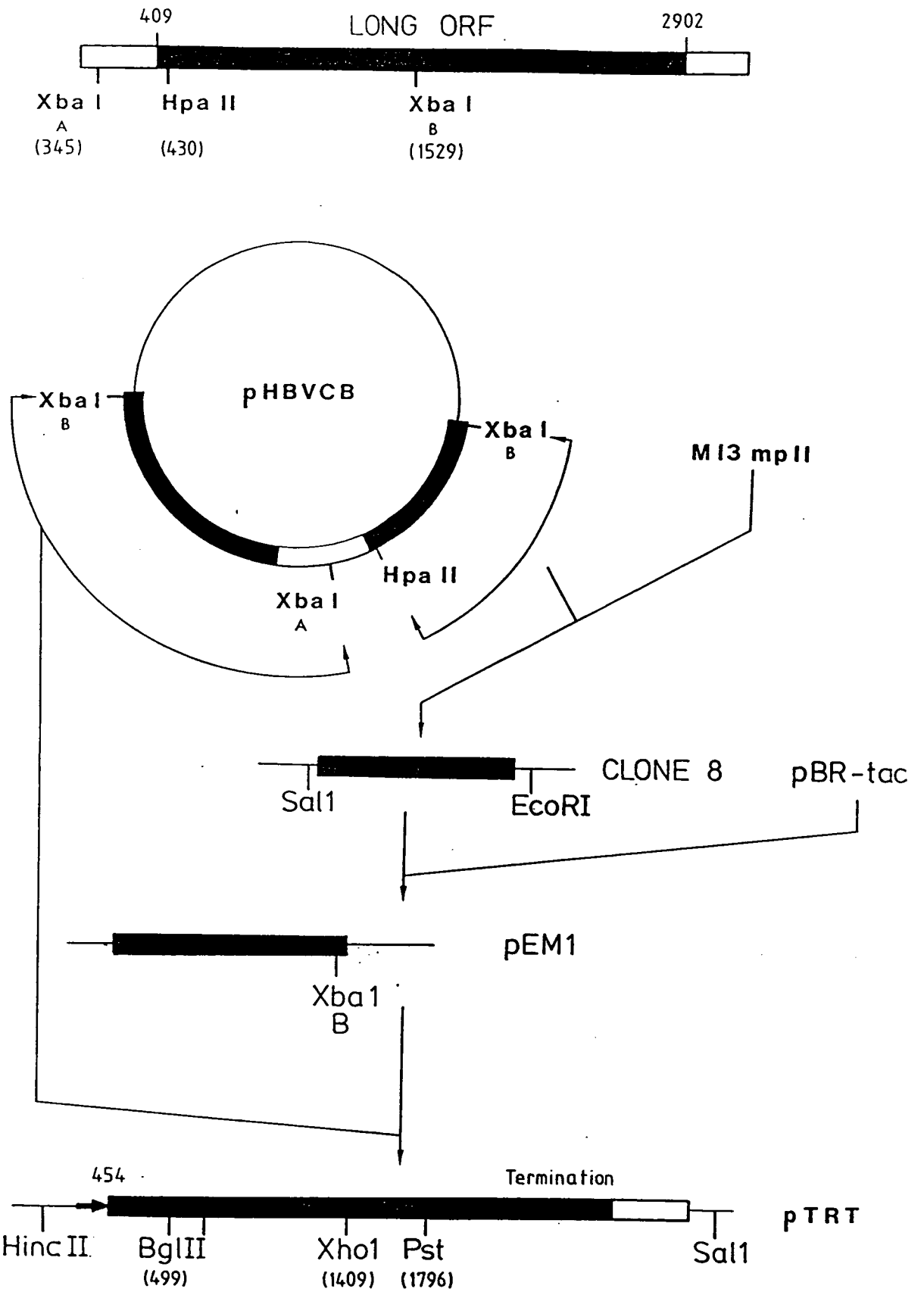
pBR Tac1 is a vector that carries the tac1 promoter inserted into pBR322. It has unique EcoRIand SalI sites. EcoRI and SalI sites flank the polymerase region of pR1-pol8. Restriction of this plasmid with these enzymes and isolation of the appropriate fragment allowed the polymerase recombinant gene to be inserted between the EcoRI and SalI sites of pBR tac1. This construct, called pTac-pol8,a derivative of pR1-pol8, would express the recombinant polymerase gene under the control of the Tac promoter. Its identity was confirmed by restriction mapping and,like pR1-pol8, was made and propagated in W110I^{q} cells. Both pTac-pol8 and pR1-pol8 have all but the first 38 amino acids of polymerase fused to the N-terminal 28 amino acids of core (HBV core Ag)

3.3. Construction of pT.R.T

A third plasmid was constructed (Fig. 3.3) which contained all the coding information of the long ORF of HBV except for the first N-terminal 11 amino acids which were replaced by 8 amino acids from the N-terminal β-galactosidase protein (Fig. 3.3).

The plasmid pHBVCB which contains HBV DNA inserted into pBR322 (see Section 3.2) was restricted with HpaII and treated with S1 nuclease. The reaction was stopped by phenol extraction and then ethanol-precipitated. The products were then digested with XbaI and separated on a 1% low melting point (L.M.P.) agarose gel. The purified fragment containing the N-terminal region of the long ORF was ligated to M13 mp11, digested with SmaI and XbaI. Following ligation, the reaction was used to transform competent NM522 cells. White plaques

FIGURE 3.3 The construction of pT.R.T. (1) The plasmid pHBVCB was restricted with HpaII treated with S1 nuclease, and digested with XbaI. 2) The purified fragment containing the N-terminal region of the long ORF was inserted into M13 mp11, and digested with SmaI and XbaI to form clone 8. 3) Clone 8 was opened at the EcoRI site, treated with the Klenow fragment in the presence of dTTP and dATP, and digested with SalI. A 1075bp fragment containing the long ORF region was isolated. pBR-tac was restricted with EcoRI, treated with Klenow in the presence of dTTP and dATP, digested with SalI, and the 1075 bp fragment was inserted to produce pEM1. The remainder of the coding information was supplied by inserting an XbaI fragment from pHBVCB. This construct is called pT.R.T. Key: Filled area, HBV long ORF.


409
LONG ORF
2902
Xba I
A
(345)
Hpa II
(430)
Xba I
B
(1529)
pHBVCB
Xba I
B
Xba I
B
M13 mpII
Hpa II
Xba I
A
Sal1
CLONE 8
EcoRI
pBR-tac
pEM1
Xba1
B
454
Termination
pTRT
Hinc II
BglII
(499)
Xho1
(1409)
Pst
(1796)
Sal1

were picked and purified and a battery of recombinants was sequenced to determine the extent of S1 nuclease digestion. One recombinant, clone 8 (nucleotide position 454) was selected for further use (Fig. 3.3).

Clone 8 was opened at the EcoRI site and treated with the Klenow fragment of E.coli DNA poll in the presence of dTTP and dATP to maintain the reading frame restricted with SalI and the digestion products separated on a 1% L.M.P. agarose gel. A 1075bp fragment containing the putative polymerase region was isolated. pBR tacl was restricted with EcoRI and the recessed end was made blunt by treating with the Klenow fragment as described above after which it was digested with SalI and the 1075bp fragment was inserted using T4 DNA ligase. Competent W110I^{q} cells were transformed with the ligation reaction mixture and plated out in the presence of ampicillin. Ampicillin-resistant, tetracycline-sensitive transformants were characterised and a recombinant plasmid identified by restriction mapping. This construct is called pEM1.

The remainder of the coding information, including the translation termination signal of the long ORF, was supplied as described for the construct pR1-pol8 by inserting an XbaI fragment (nuleotide position 1529-90) from pHBVCB. This construct is called pT.R.T. and has been characterised by restriction mapping.

Conservation of the reading frame at the point of fusion of the vector and inserted sequence was confirmed by sequencing across the junction. This was achieved by transferring the HincII-BglII fragment into M13 mp18 and M13 mp19 and sequencing by the dideoxy chain termination procedure. An Xho-PstI fragment from pT.R.T. was sub-cloned into M13 mp19 and sequenced to ensure that the reading frame had not been disrupted by the insertion of the XbaI fragment from pHBV CB into pEM1.

3.4 Expression of the recombinant long ORF in E.coli

The minicell system was used to identify novel proteins expressed from the recombinant plasmid in the absence of an antibody specific for the translation product from the long ORF. The expected molecular weight from the computer predicted amino acid sequence for the recombinants as follows:

Table 3.2. Predicted Molecular Weight of the Recombinant Proteins

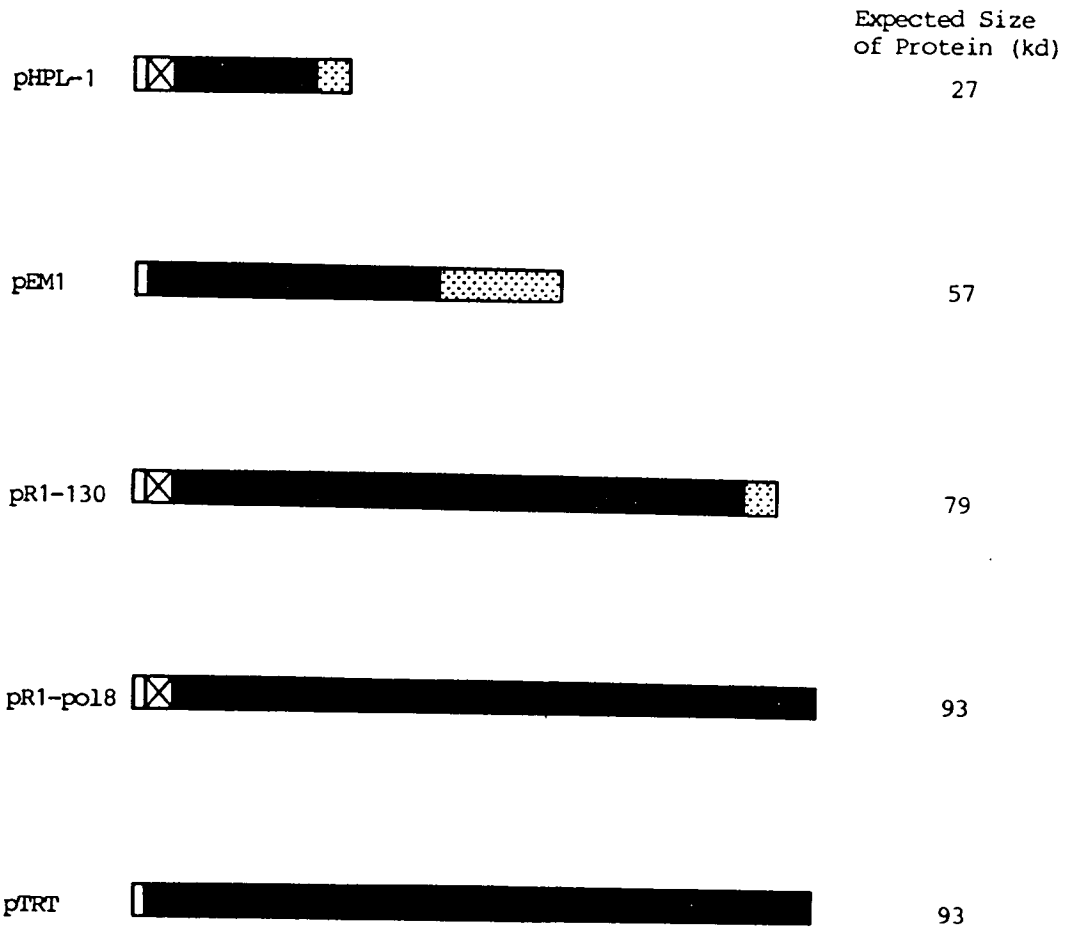
Recombinant Plasmids	Molecular Weight (Kd)
pR1-11	22
pHPL-1	27
pEM1	57
pR1-130	79
pR1-pol8	93
pTac-pol8	93
pT.R.T.	93

The composition of these proteins is illustrated in Figure 3.4.

Recombinant plasmids were used to transform the competent, minicell-producing strain W1542. Transformants were selected as ampicillin-resistant colonies and minicells were prepared from them.

FIGURE 3.4 The composition and molecular weight of the translation gene products expressed by the recombinant plasmids.

Key: Open area, β-galactosidase polypeptide; crossed area, N terminal, 28 amino acids of the core protein; filled area, HBV polymerase polypeptide; dotted area, amino acids encoded by the pBR322 sequence.


Expected Size
of Protein (kd)
pHPL-1
27
pEM1
57
pR1-130
79
pR1-pol8
93
pTRT
93

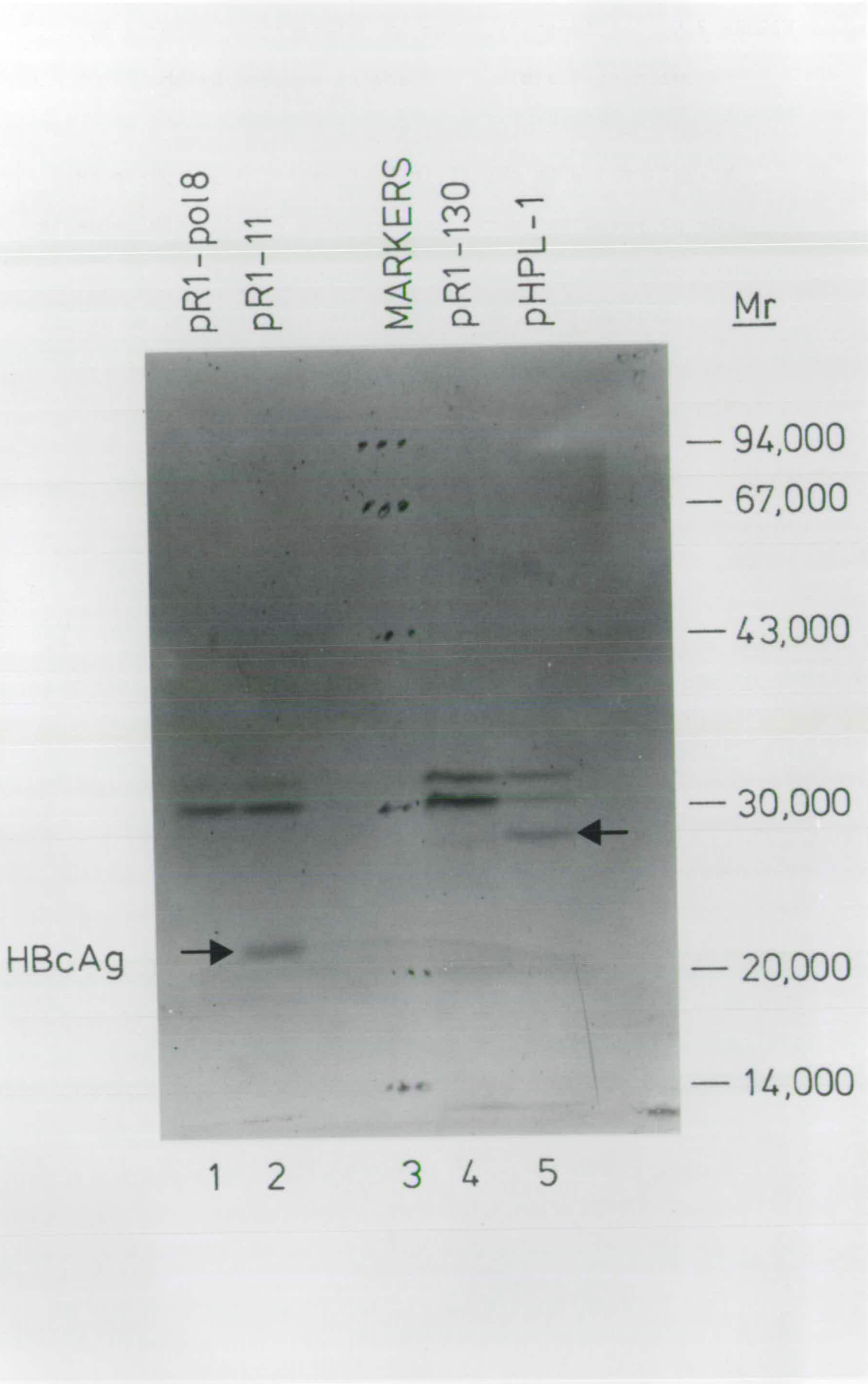
Plasmid-encoded proteins were labelled for 30 minutes by incubation of the minicells in a medium containing [^{35}S]-L-methionine, amino acids and 2mM IPTG then analysed by electrophoresis in SDS polyacrylamide gels. Figure 3.5 shows that the recombinant plasmid, pR1-11, directs the synthesis of a novel protein of about 22kd, which has been previously identified as the HBcAg (Stahl et al., 1982). pHPL-1 also expresses a novel protein of the expected molecular weight. However, neither pR1-130 nor pR1-pol8 appeared to express any novel proteins. Similarly, polypeptide produced by pEM1, pT.R.T., and pTac-pol8 were analysed. It was found that none of these plasmids appeared to produce a novel protein. These experiments were repeated several times using various induction times ranging from 10 minutes to 3 hours, but the expected translation products could not be detected.

In conclusion, although HBcAg could be readily detected in minicells, there was no evidence for the synthesis of polypeptides of the anticipated size in minicells carrying pR1-130, pR1-pol8, pEM1 and pT.R.T., or indeed, significant quantities of any polypeptide that did not appear in cells harbouring the vector alone.

The constructs which do not produce the expected translation products may not be transcribed, but this is unlikely as they are under the inducible control of the strong bacterial promoters Tac and Lac. It is unlikely that failure to detect the anticipated synthesis of the fused β-galactosidase-polymerase polypeptides results from a lack of sequences necessary for translation of the corresponding messenger RNA, as this ribosomal binding site was shown to direct efficent translation when fused to the coding sequence for HBcAg, and the first 160 amino acids of the putative polymerase gene.

It is possible that the secondary structure of the messenger RNA

FIGURE 3.5 Identification of polypeptides encoded by recombinant plasmids. Proteins encoded by the recombinant plasmids were detected in minicells of WL542 transformed with pR1-11, pHPL-1, pR1-130 and pR1-pol8. The polypeptides were labelled with [^{35}S]L-methionine in the presence of IPTG and separated on a 10% (w/v) polyacrylamide gel containing SDS, and visualised by autoradiography. 1) pR1-pol8; 2) pR1-11; 3) markers; 4) pR1-130; and 5) pHPL-1.


pR1-pol8
pR1-11
MARKERS
pR1-130
pHPL-1
Mr
94,000
67,000
43,000
30,000
20,000
14,000
HBcAg
1
2
3
4
5

transcribed from pEM1 or pT.R.T. leads to very inefficient translation (Inserentant and Fiers, 1980) or, in the case of pHPL-1, when additional sequences are inserted the resulting messenger RNA may form undesirable secondary structures thus reducing the efficiency of translation. Alternatively, the messenger RNA produced may be labile, or the polypeptides produced may be particularly sensitive to E.coli proteases.

In order to establish that the recombinant DNA sequences were being transcribed, RNA from these clones was extracted and analysed. The predicted secondary structure of messenger RNA produced by these constructs was also deduced and using an in vitro-coupled transcription translation system, the polypeptides from the recombinant plasmid were analysed in vitro.

3.5 Analysis of RNA produced by pEM1, pTac-pol8, pT.R.T. etc.

E.coli cells (strain W1101^{q}) harbouring the recombinants were grown in the presence of ampicillin to A_{650} = 0.5. Cells were then induced by the addition of 2mM IPTG and grown for a further 60 minutes. The RNA was extracted and fractionated by electrophoresis on a 1.3% agarose formaldehyde-denaturing gel, and transferred to a nitro-cellulose filter. The filter was then baked and hybridized with an HBV genome-specific probe (3.2kb XhoI fragment from HBV130) which had been labelled with ^{32}P, using the random oligonucleotide method. The HBV-specific probe hybridised to mRNA produced by cells carrying either pEM1 or pTac-pol8. The same probe did not hybridise to mRNA produced by cells carrying the vector pBRtac alone (Fig. 3.6a).

Treating the RNA with RNAse and DNAse confirmed that the probe was hybridising to RNA transcripts and not to any contaminating plasmid DNA

FIGURE 3.6(a) Identification of long ORF-related transcripts in E.coli transformed with the recombinant plasmids. RNA was prepared as in Methods Section and separated on 1.3% (w.v) agarose gel containing formaldehyde. The RNA was transferred to nitrocellulose and probed with a ^{32}P-labelled HBV-specific probe. The transcripts which hybridised to the probe were visualised by auto-radiography. Markers were yeast ribosomal RNA. The samples were as follows: tracks 2 and 3, R1-11, 10µg and 16µg respectively of RNA; tracks 4 and 5, ptac-pol8, 10µg and 16µg respectively of RNA; tracks 6 and 7, pEM1, 10µg and 16µg respectively of RNA; tracks 8 and 9, pBR-tac, 10µg and 16µg respectively of RNA.

FIGURE 3.6(b) Northern blot analysis of the recombinant plasmid transcripts using a ^{32}P-labelled HBV-specific probe. 10µg of each of the RNA samples were loaded in the following order: Lane 1, yeast ribosomal markers; Lane 2, pHBV CB digested with BamHI; Lane 3, R1-11; Lane 4, ptac-pol8; Lane 5, pEM1; Lane 6, pBR-tac; Lane 7, pEM1 treated with DNase 1 before loading; Lane 8, pR1-11 treated with DNase 1 before loading; Lane 9, ptac-pol8 treated with RNase before loading; Lane 10, pR1-11 treated with RNase before loading; Lane 11, ptac-pol8 treated with RNase before loading; Lane 12, pEM1 treated with RNase before loading.

A
MARKERS
pR1-11
pR1-11
ptac-pol8
ptac-pol8
pEMI
pEMI
pBR-tac
pBR-tac
4·4S
1·9S
1 2 3 4 5 6 7 8 9
B
MARKERS
pHBVCB
pR1-11
ptac-pol8
pEMI
pBR-tac
pEM1-DNASE
pR1-11 DNASE
ptac-pol8 DNASE
pR1-11 RNASE
ptac-pol8 RNASE
pEM1 RNASE
4·4S
1·9S
1 2 3 4 5 6 7 8 9 10 11 12

(Fig. 3.6b). The fact that the RNA from R1-11 did not hybridise to the probe is puzzling as the plasmid expresses HBcAg; perhaps this mRNA has a very short half-life.

This experiment shows that the recombinant plasmids are being transcribed within E.coli. Transcription is initiated at nucleotide position -22 in both the Lac and Tac promoter region but it is not clear where the transcription terminates.

As the HBV DNA has been cloned into the Tet^r gene of pBR322, it is reasonable to expect that RNA polymerase will recognise the transcription termination signals used in the normal expression of the Tet^r gene (Stuber et al., 1981). If this were so then transcripts of approximately 4kb and 1kb ought to be produced from pTac-pol8 and pEM1 respectively. However, single bands on the Northern blot, corresponding to such species of mRNA, were not detected though smears were identified which did not extend beyond approximately 4kb for pTac-pol8 and 1kb for pEM1.

Two observations led to the conclusion that the RNA has not been degraded during extraction. Firstly, on ethidium bromide-stained agarose gels of the RNA from the recombinant plasmids and the vector, the E.coli ribosomal bands are clearly visible, although as these RNAs have a very strong secondary structure they are not as susceptible to degradation. Secondly, RNA extracted in the presence of RNAse inhibitors still displayed the same pattern of hybridisation. Smearing may be the result of premature termination, or rapid degradation within the cell, or alternatively the product of partial transcription which is interrupted by extraction.

In conclusion, these experiments show that the recombinant plasmids are being transcribed but they do not give any indication of the processing or stability of these transcripts.

3.6 Messenger RNA Secondary Structure

Initiation of translation involves interaction between an activated 30S ribosomal subunit and the 5' terminal region of a messenger RNA which is already folded in a specific secondary structure. Greater efficiency of translation occurs if the initiated codon and, to a lesser extent, the Shine Dalgarno sequence is freely accessible to the 30S ribosomal subunits (Iserentant and Fiers, 1980). The predicted secondary structure for mRNA of the β-galactosidase gene and the recombinant plasmids pT.R.T and pTac-pol8 around the initiation codon was determined (Fig. 3.7) and the thermodynamic stability of each structure was calculated following the rules of Tinocco et al., 1973.

The initiation codon AUG in pTac-pol8 is freely accessible in a hairpin loop. In the case of pT.R.T, a third of the initiation codon is in a hairpin loop while the other two bases are involved in base pairing within a stem structure. However, this is identical to the environment of the initiation codon of the β-galactosidase messenger RNA which is translated very efficiently. Therefore, the predicted secondary structure of the mRNA produced by the recombinant plasmids shows that the initiation codon and the Shine Dalgarno sequence are in a favourable position for the initiation of translation.

3.7 Coupled in vitro-Transcription Translation Systems

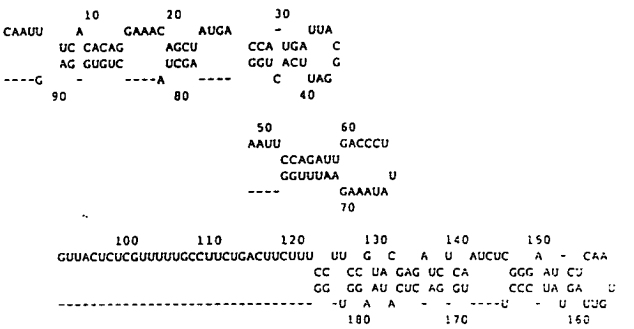
The previous experiments showed that the recombinant plasmids are being transcribed and that these transcripts should be translated. The translation products from these transcripts were analysed using an in vitro-coupled transcription-translation system. As incorporation of label into proteins is more efficient in vitro, and the presence of E.coli proteases is reduced in this system, it was hoped that

FIGURE 3.7 Postulated secondary structure of the mRNA synthesised by the recombinant plasmids. The thermodynamic stability of each structure was calculated following the rules of Tinoco et al., 1973.

A.

ptac-pol8

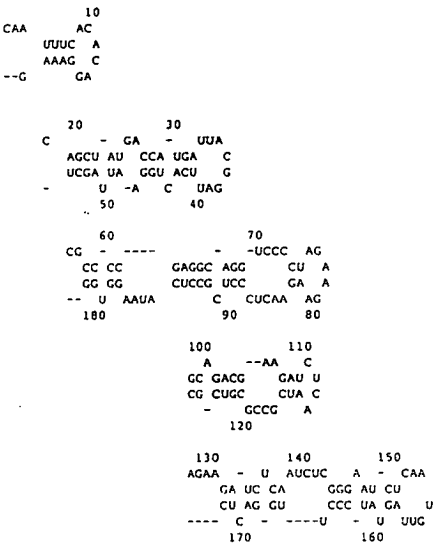
Energy = -49·1



B.

pT.R.T.

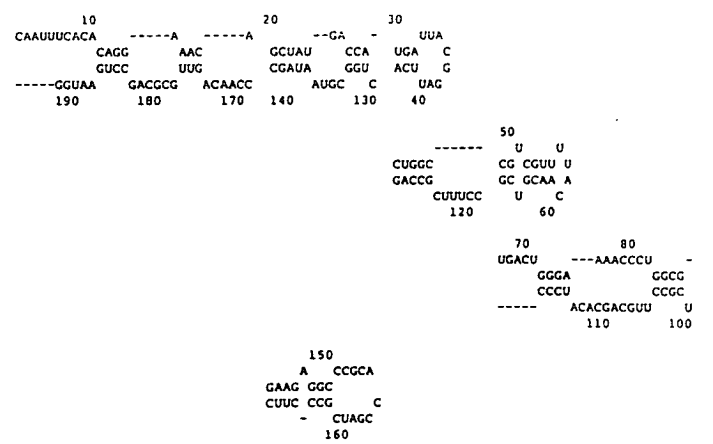
Energy = -56·5



C.

β-galactosidase

Energy = -61·0



previously unidentified proteins expressed from these constructs would be detected.

DNA from the recombinant plasmids and vector controls were used as templates. The polypeptides synthesized were labelled with [^{35}S]-L-methionine and analysed by gel electrophoresis and autoradiography (Fig. 3.8).

The protein which was detected in minicells harbouring pHPL-1 was again found *in vitro*. A very faint novel polypeptide of approximately 80kd was seen when pR1-130 was used as a template. High molecular weight polypeptides were synthesised uniquely when pTac-pol8 was used as a template though these were not found with pR1-pol8. Using pEM1 as a template, a novel protein of approximately 60kd, the expected size of a translation product from this construct, was synthesised efficiently. A series of weak, high molecular polypeptides was also detected when pT.R.T. was used as a template.

In conclusion, novel proteins within the size range expected for translation products were synthesised by the recombinant plasmids (with the exception of pR1-pol8) which had not been detected *in vivo*. Disappearance of the proteins from the gel, if the insert DNA is cleaved, would confirm that the novel protein products were coded for by HBV DNA. This experiment, however, proved to be technically difficult as a linear template was transcribed so poorly that it did not allow comparison of products from the linear and supercoiled templates. Alternatively, ^{35}S-labelled polypeptides could be purified and characterised via Edman degradation but with the exception of pEM1 and pHPL1, the proteins of interest are not expressed in high enough quantities to allow such analysis.

FIGURE 3.8 Proteins encoded by the recombinant detected by transcription and translation of plasmid DNA in a cell-free system from E.coli. The polypeptides were labelled with [^{35}S]L-methionine and separated on a 16% (w/v) polyacrylamide gel containing SDS and visualised by auto-radiography. The samples were as follows: 1) pR1-11; 2) ptac-core; 3) pR1-pol8; 4) ptac-pol8; 5) pHPL-1; 6) pR1-130; 7) pEM1; 8) pT.R.T; 9) pBR-tac; 10) ptac-core; 11) pBR-322; 12) markers. (Gel by Mohamed El Alhama, Biogen, S.A.).

β-lactamase
HBcAg
1 2 3 4 5 6 7 8 9 10 11 12
pR1-11
ptac-CORE
pR1-pol8
ptac-pol8
pHPL-1
pR1-130
pEM1
pT.R.T.
pBR-tac
ptac-CORE
pBR-322
MARKERS
Mr
30,000
21,500
12,500

In summary, novel proteins of the expected size are being synthesised by the recombinant plasmids. This allows one to tentatively conclude that these polypeptides are being expressed from these constructs and represent β-galactosidase-polymerase fusion proteins. Why these proteins have not been detected *in vivo* is not clear, particularly the abundant protein expressed by the construct pEM1. As RNA has been shown to be produced from these constructs (Section 3.5), and the RNA is most likely to be translated, the problem with the synthesis of these proteins *in vivo* may be due to some translational event within the cellular environment, and the simplest explanation is that hybrid β-galactosidase polymerase molecules expressed from pR1-130, pTac-pol8, pR1-pol8, pEM1 and pT.R.T. are susceptible to attack by one or more of the proteases that are present in *E.coli*.

If the instability of the newly synthesised polypeptides is indeed the reason for the failure to detect these novel proteins, transferring these recombinants into protease-deficient strains may allow the β-galactosidase polymerase fusion to be expressed *in vivo*.

3.8 Expression of the Recombinant Plasmids in Protease-minus Strains of *E.coli*

S. Goff and co-workers have constructed an *E.coli* cell line, SG935, which is deficient in La, an ATP-dependent protease encoded by the *lon* gene (Goff and Goldberg, 1985). This protease has been shown to play an important role in the degradation of abnormal proteins and *lon* mutants have been found to degrade abnormal proteins two or four times more slowly than the wild-type cell.

pDM1, a plasmid which carries the I^q gene of the lac operon, and

the kanamycin resistance gene, is compatible with pBR322 (D. Simmons, personal communication). Competent SG935 cells were transformed with this plasmid and kanamycin-resistant cells were selected; these were then made competent and were transformed with the recombinant plasmids. kanamycin-resistant, ampicillin-resistant transformants were characterised. Cells harbouring the recombinant plasmids were grown to stationary phase in L-broth containing kanamycin and ampicillin (LB.amp/kan) diluted 1:50 in LB.amp/kan and grown to A_{650}=0.2. Cells were induced by adding IPTG to a concentration of 2mM and grown for a further 60 minutes. Cells were harvested and the proteins analysed by electrophoresis on an SDS-polyacrylamide gel (Fig. 3.9)

Figure 3.9, track 3 shows that cells harbouring pEM1 synthesise a novel protein of approximately 60kd, corresponding to the protein detected during *in vitro*-transcription translation of this construct and also is the correct size for the translation product expected from this recombinant plasmid. A smaller, unique protein migrating at approximately 45kd is also seen in these cells and is presumably a breakdown product of the larger protein. The 27kd protein, which is shown to be expressed by pHPL-1 *in vivo* and *in vitro*, is also synthesised in these protease-minus strains. The translation product from pHPL-1 is obviously present in greater abundance in the Lon-minus strains as this protein can be visualised, by staining with Coomassie blue, from extracts of the protease-minus strains, but is not detected by this method using minicells. The recombinant plasmids which should express longer segments of the long ORF did not synthesise any novel polypeptides. Perhaps the additional polypeptide sequence contains a site which is particularly sensitive to proteolytic degradation.

FIGURE 3.9 Expression of the recombinant plasmids in protease-minus strains of E.coli. The bacteria were grown to OD.4. After inducing for 1 hour at 37°C with 2mM IPTG the proteins were separated on a 10% (w/v) polyacrylamide gel containing SDS. The gel was stained with Coomassie blue. Lane 1, pT.R.T; Lane 2, pHPL1; Lane 3, pEM1; Lane 4, pBR-tac; Lane 5, pR1-pol8; Lane 6, ptac-pol8 and Lane 7, pR1-130.

pTRT
pHPL-1
pEM1
pBR-tac
pR1-pol8
ptac-pol8
pR1-130
Mr
94,000
67,000
43,000
30,000
20,000
1
2
3
4
5
6
7

3.9 Dicussion

A number of proteases are present in E.coli which are used to degrade abnormal and normal proteins throughout the life cycle of the bacteria. These are also involved in processing secretory and membrane proteins. At present seven proteolytic activities have been isolated which are capable of hydrolysing polypeptides to acid-soluble material; these are - Do, Re, Mi, Fa, So, La and Pi, and these are found in different locations within the cell; in the cytoplasm, the periplasmic space, and the cell membrane (Goldberg et al., 1982). These proteases have also been found to rapidly breakdown proteins when the cells are in a stationary phase or are starved (Goldberg and St. John, 1976).

Proteolytic degradation appears to involve an initial endo proteolytic hydrolytic incision rather than exoproteolytic cleavage. This would imply that a specific recognition signal is required for a protein to be degraded by proteases. This is supported by the work of Bachmair et al.,(1986) who found that the half lives of different proteins can be correlated with the amino-terminal amino acid of the protein (N- end Rule) ranging from proteins with methionine, serine, alanine, threonine, valine and glycine at their N-terminus which have half lives of more than 20 hours to proteins with half lives of 2 minutes which have an arginine N-terminus. Rogers et al (1986) have also proposed that selective proteolytic degradation is a result of a "signal sequence". Using a eukaryotic system, it was found that proteins which contain one or more regions rich in proline (P), glutamic acid (E), serine (S) and threonine (T) in PEST sequences are subject to rapid intracellular degradation.

Eukaryotic proteins expressed in E.coli have been shown to be very

susceptible to proteolytic degradation (Davis et al., 1981). The problem of degradation of foreign proteins may be difficult to resolve. The observation that hybrid sequences can be used to transport insulin into the periplasmic space which increased its half life by a factor of ten, allows one to build constructs designed to ensure secretion (Talmadge et al., 1982; Talmadge and Gilbert, 1982). Alternatively, the gene of interest could be fused to various amounts of a bacterial gene. Heiland and Gething (1981) obtained low levels of haemoglutin activity (HA) in E.coli cells with constructs carrying the Lac promoter directing transcription of short β-galactosidase HA fusion. However, high levels of expression of HA (5-7% of total cell protein) were achieved by using long β-glactosidase fusions (Davis et al., 1981). It was found that these longer fusions are insoluble and thereby partially protected from proteolysis (Stanley and Luzio, 1984).

The use of bacterial cells lacking one or more of the normal complement of proteins may be another way of slowing down degradation of eukaryotic proteins. The ATP-dependent protease La, the product of the lon gene, has been shown to play an important role in the degradation of abnormal proteins. Interestingly, it was found that the presence of the abnormal proteins increases transcription of the lon gene via positive induction of a heat-shock regulatory gene htpR. E.coli mutants are now available which are deficient for the function of htpR and lon (Goff and Goldberg, 1985). Simon et al.,(1984) showed that the bacteriophage T4 carries a gene which causes the decrease in the degradation of abnormal proteins. This gene was identified and is called Pin, protease inhibition gene. It was found that the labile eukaryotic proteins, e.g. fibroblast interferon, expressed in E.coli is stabilised in cells in which the T4 gene is expressed.

At present there are few studies on the stability of the analogue of eukaryotic messenger RNA in E.coli. Structural features, but not the size of the messenger RNA, determine its susceptibility to decay (Belasco et al., 1986). Protection of the messenger RNA by 3' terminal structures act as barriers against nucleolytic attack and a site-specific incision will generate an exposed 3' terminus. Three enzymes appear to be involved in degradation. RNAse III appears to be an endonuclease and RNAseII and polynucleotide phosphorylse act as 3' exonuclease. Messenger RNA from a eukaryotic source has been stabilised in strains lacking polynucleotide phosphorylase and the expression of dehydroquinase from N.crassia and insulin has been increased.

In conclusion, it is highly probable that the N-terminus of the protein encoded by the long ORF does not contain a region which is particularly sensitive to degradation, or if it does, it lies in a conformation not readily accessible. Addition of the rest of the protein may introduce such a site, or alternatively could cause unfolding, and hence exposure of this region thus causing the protein to be rapidly degraded.

The inability to express the mature form of the HBV long open reading frame in E.coli has also been reported in other laboratories: G. Darby, Wellcome, England, and W. Gerlich, Hygiene Institut, University of Gottingen, West Germany, (personal communication).

In an effort to obtain greater expression of the N-terminal region and to express other regions of the long ORF, recombinant plasmids were made in which various segments of the long ORF were fused to practically all the β-galactosidase genes. (This is discussed in Chapter 4).

CHAPTER 4

GENERATION OF ANTISERA TO THE PUTATIVE POLYMERASE GENE PRODUCT

Page

Chapter 4

INTRODUCTION

Segments of the long open reading frame of HBV were fused in frame to a truncated cro-β-galactosidase gene carried on expression vectors which were constructed by Stanley and Luzio (1984) (Fig.4.1). These vectors contain all the necessary signals for transcription and translation of foreign DNA fragments in E.coli. The righthand promoter (P_R) of the bacteriophage λ directs the expression of the cloned DNA, which can be inserted in all three reading frames via a polylinker at the 3' end of the LacZ gene. Immediately downstream of this polylinker are transcription and translation stop signals. The fusion proteins should be expressed with equal efficiencies as inserting the fragment at the 3' end of the gene should not affect the 5' secondary structure of the messenger RNA. Expression of the desired product can be controlled by using a bacterial strain which expresses the temperature-sensitive lambda repressor. The E.coli strain NF1 (Stanley and Luzio, 1984) carries the λcI857 gene on a defective lambda prophage. Amplifying the plasmids at 30°C blocks transcription from the P_R promoter and by shifting the temperature to 42°C, transient expression of the fusion protein can be induced.

This chapter describes the expression of the hybrid cro-LacZ-L.ORF gene (LacZ-pol gene) in E.coli and the generation of antiserum to the cloned gene product.

4.1 Construction of the recombinant Plasmids; pRCT, pRCD and pRCJ

The recombinant plasmids pHPL-1 and pR1-130 (see Section 3.2) contain DNA which codes for 28 amino acids of the core gene fused to either 160 or 719 amino acids respectively of the putative polymerase gene product. EcoRI and SalI sites flank the polymerase regions of

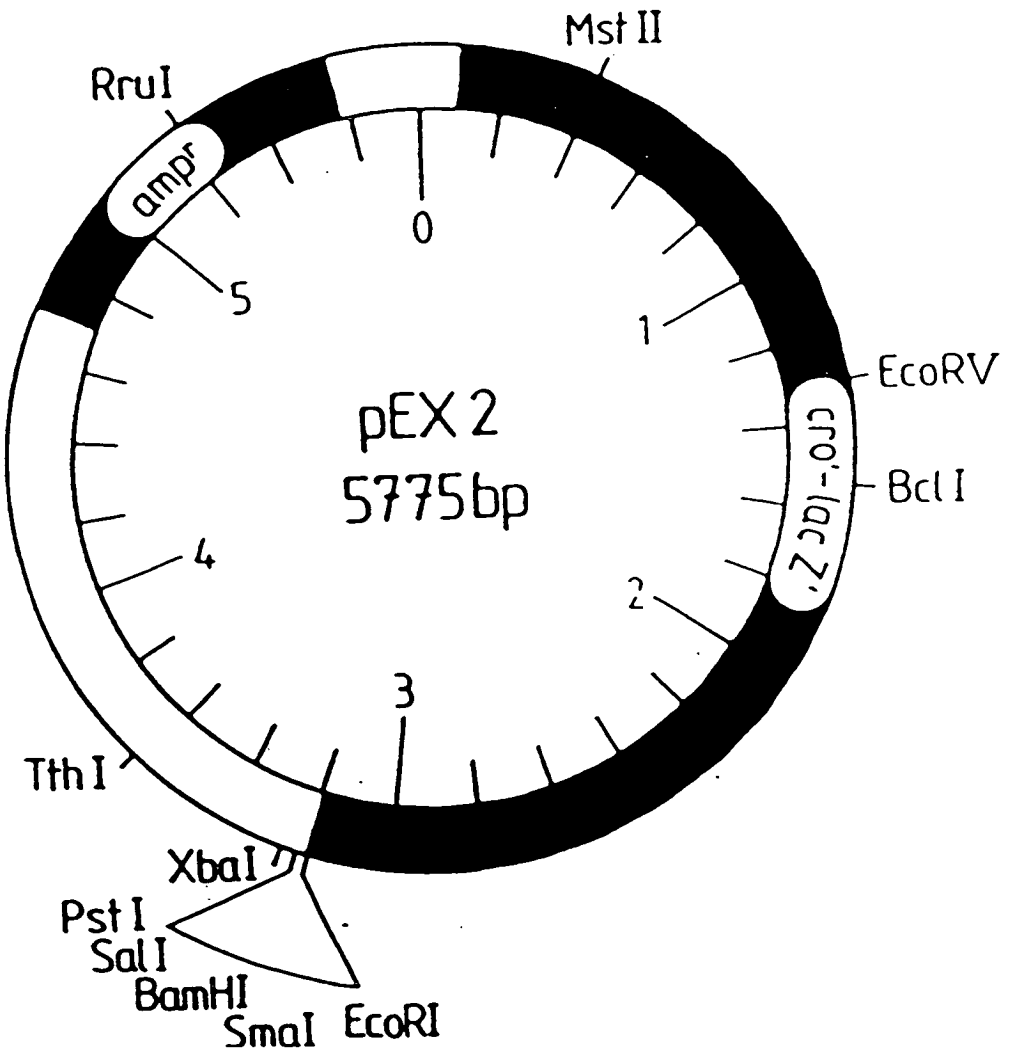


FIGURE 4.1 Map of pEX2. Unique restriction enzymes sites are shown.

both pHPL-1 and pR1-130 (Fig. 4.2). The plasmids were restricted with these two enzymes and the resulting fragments separated on a 1% low melting point gel. The appropriate fragments were purified and ligated to EcoRI and SalI digested pEX2. The ligated mix was used to transform competent E.coli NF1 cells and plated out in the presence of ampicillin and grown overnight at 30°C. Plasmid DNA was prepared from overnight cultures (grown at 30°C) of ampicillin-resistant transformants and recombinant plasmids were identified by restriction mapping. The two resulting recombinant plasmids are called pRCT and pRCD. pRCJ was constructed in a similar manner to that used for pRCT and pRCD. Plasmid pHBVCB carries the HBV genome (Section 3.1). This was digested with BglII and PstI to produce a fragment that codes for 424 amino acids of polymerase and none of the core antigen (Fig. 4.3). This fragment was isolated as described above and inserted between the BamHI and PstI sites of pEX3 to give the recombinant plasmid pRCJ which was characterised by restriction mapping.

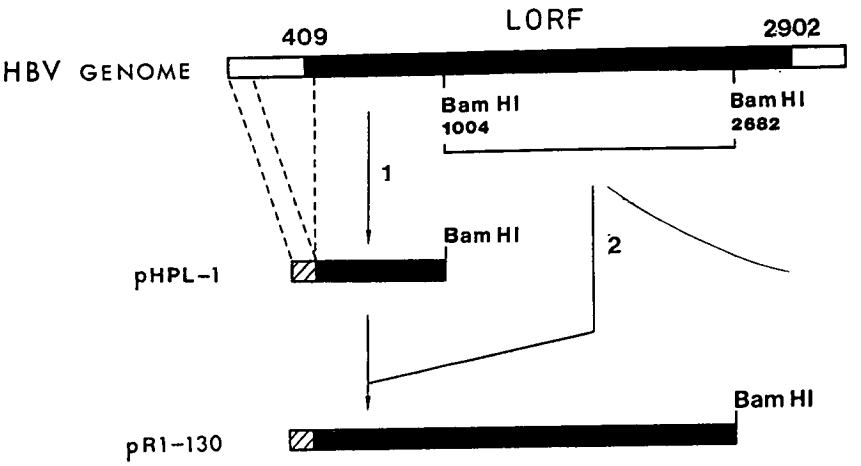
Conservation of the reading frame at the point of fusion between vector and insert DNA was confirmed by sequencing across the junction. This was achieved for pRCT and pRCD by transferring the EcoRV-BglII fragments covering the junction sequences of the recombinant plasmids into M13 mp18 and for pRCJ by cloning the SmaI PstI fragment covering the LacZ-pol fusion into M13 mp19. The junctions were then sequenced by the dideoxy chain termination procedure.

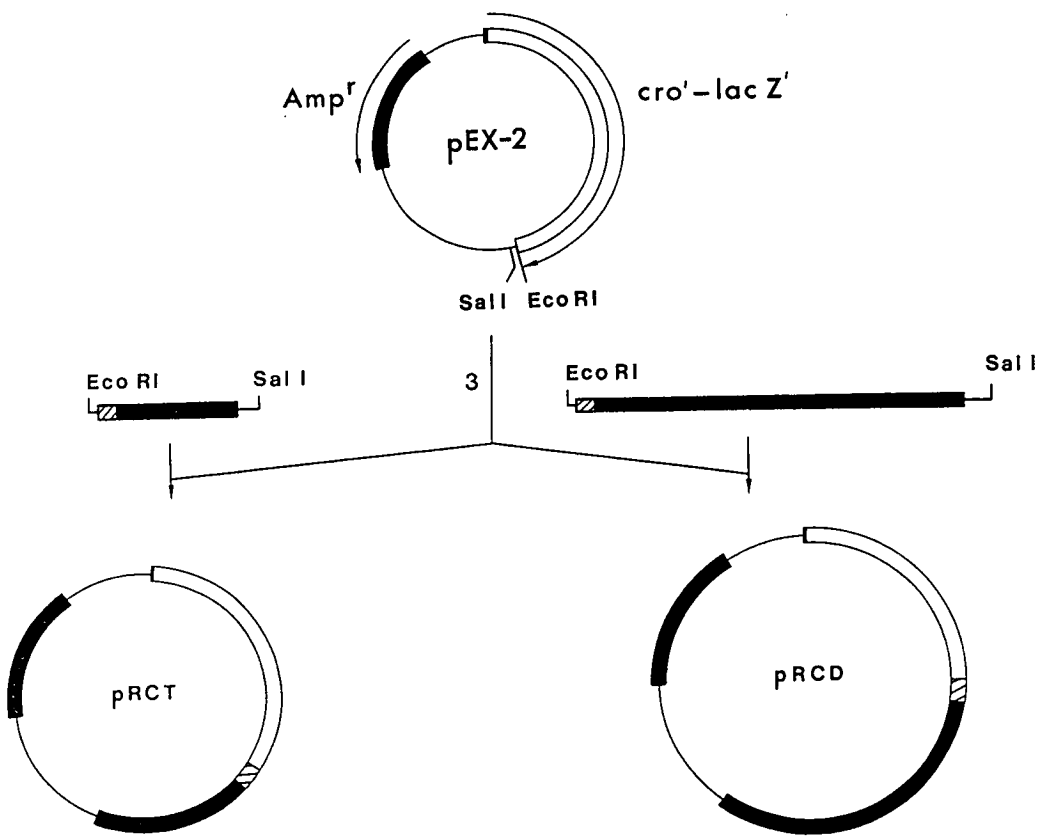
4.2 Analysis of Proteins encoded by pRCT, pRCD and pRCJ

E.coli NF1 cells transformed with pRCT, pRCD or pRCJ were grown at 30°C to a stationary phase in L-broth containing ampicillin and grown at 30°C to A_{650} = 0.4. Cells were induced by shifting the

FIGURE 4.2 The construction of pRCT and pRCD. 1) PHPL-1 contains HBV DNA sequences equivalent to the first 28 amino acids of the core protein fused in frame to amino acids 40 to 201 of the polymerase gene product. 2) A BamHI fragment encoding the internal sequences of the polymerase gene was inserted into the unique BamHI site of pHPL-1 to form pR1-130. 3) pR1-130 and pHPL-1 were digested with EcoRI and SalI, the fragments carrying HBV sequences recovered, and transferred to the expression vector pEX2.

Key: hatched area, HBV core gene; filled area, HBV polymerase gene; open area, λ-cro-lacZ.


HBV GENOME
409
LORF
2902
Bam HI
1004
Bam HI
2682
1
Bam HI
pHPL-1
2
Bam HI
pR1-130


Amp^r
pEX-2
cro'-lac Z'
Sal I
Eco RI
Eco RI
Sal I
3
Eco RI
Sal I
pRCT
pRCD

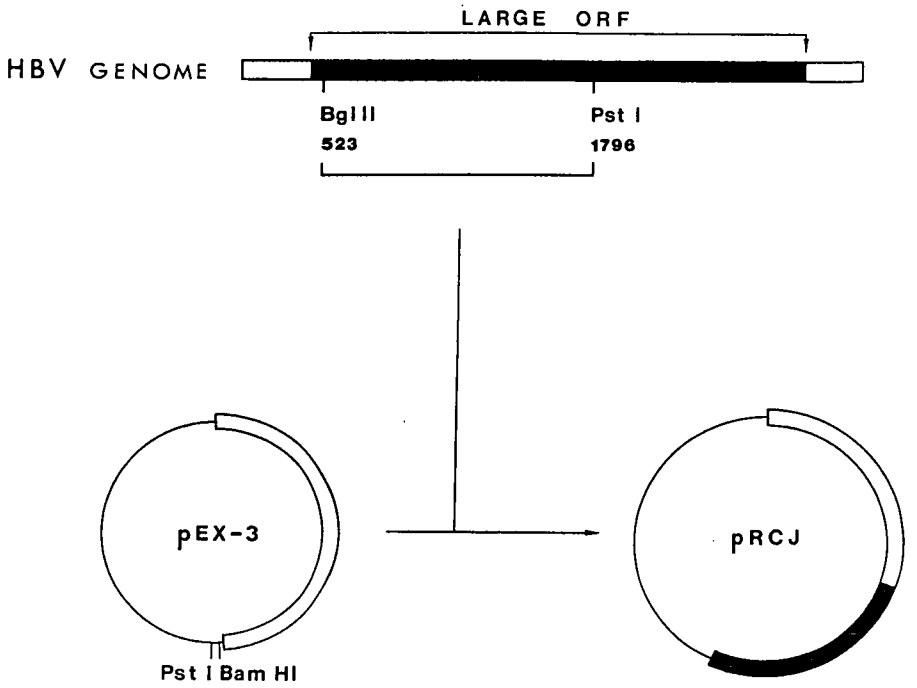


FIGURE 4.3 The construction of pRCJ. 1) a Bg1II-PstI fragment encoding sequences of the polymerase gene was inserted into the BamH1-PstI sites of the vector pEX3.
Key: filled area, polymerase gene; open area λ-cro-lacZ gene.

temperature to 42°C for 45 minutes, then returning to 30°C for a further 30 minutes. Cells were harvested and resuspended in protein loading buffer, boiled for 5 minutes and the proteins separated by electrophoresis on an SDS-polyacrylamide gel. The gel was then stained with Coomassie blue to visualise the proteins. Novel proteins were synthesised in cells harbouring pRCD, pRCT or pRCJ with molecular weights of 143,000, 195,000 and 158,000, respectively, which correspond to the size of the expected β-galactosidase polymerase fusion products (Fig. 4.4). As cells harbouring the recombinant plasmid only synthesise the novel proteins under conditions in which transient expression from P_R is induced, one can conclude that this promoter is driving the expression of these unique polypeptides.

Interestingly, pRCD, which is a derivative of pR1-130, was shown previously to synthesise no novel proteins in vivo (Section 3.4). However, when the polymerase region of pR1-130 is preceded by a large portion of the β-galactosidase protein (as in pRCD), the fusion protein of the expected size is produced. Thus it would appear that the β-galactosidase polypeptide stabilises or protects the foreign gene product from degradation. Using various induction times it was shown that the fusion protein synthesised in cells harbouring pRCD was sensitive to proteolytic degradation. Figure 4.5, tracks 7 and 8, shows that after induction at 42°C for 30 minutes the fusion protein is synthesised in high amounts by pRCD. However, if it is induced for a further 60 minutes, the fusion protein is completely degraded. Although there is a slight reduction in the amount of fusion protein synthesised by pRCT, under these conditions there is not a dramatic degradation as seen with pRCD. Interestingly, pRCT is a derivative of pHPL-1 which does produce a novel protein in vivo (Section 3.4).

FIGURE 4.4 Polypeptides encoded by pRCT, pRCD and pRCJ. Extracts from E.coli cells were fractionated on 7% (w/v) polyacrylamide gel containing SDS. The gel was stained with Coomassie blue. The arrows identify the β-galactosidase and fusion proteins produced by pEX2, pRCT, pRCD, and pRCJ, respectively. The samples were the following: 1) NF1 cells; 2) pEX2 at 30°C; 3) pEX2 induced for 30 min at 42°C; 4) pRCT at 30°C; 5) pRCT induced for 30 min at 42°C; 6) pRCD at 30°C; 7) pRCD induced for 30 min at 42°C; 8) pRCJ at 30°C; 9) pRCJ induced for 30 min at 42°C.

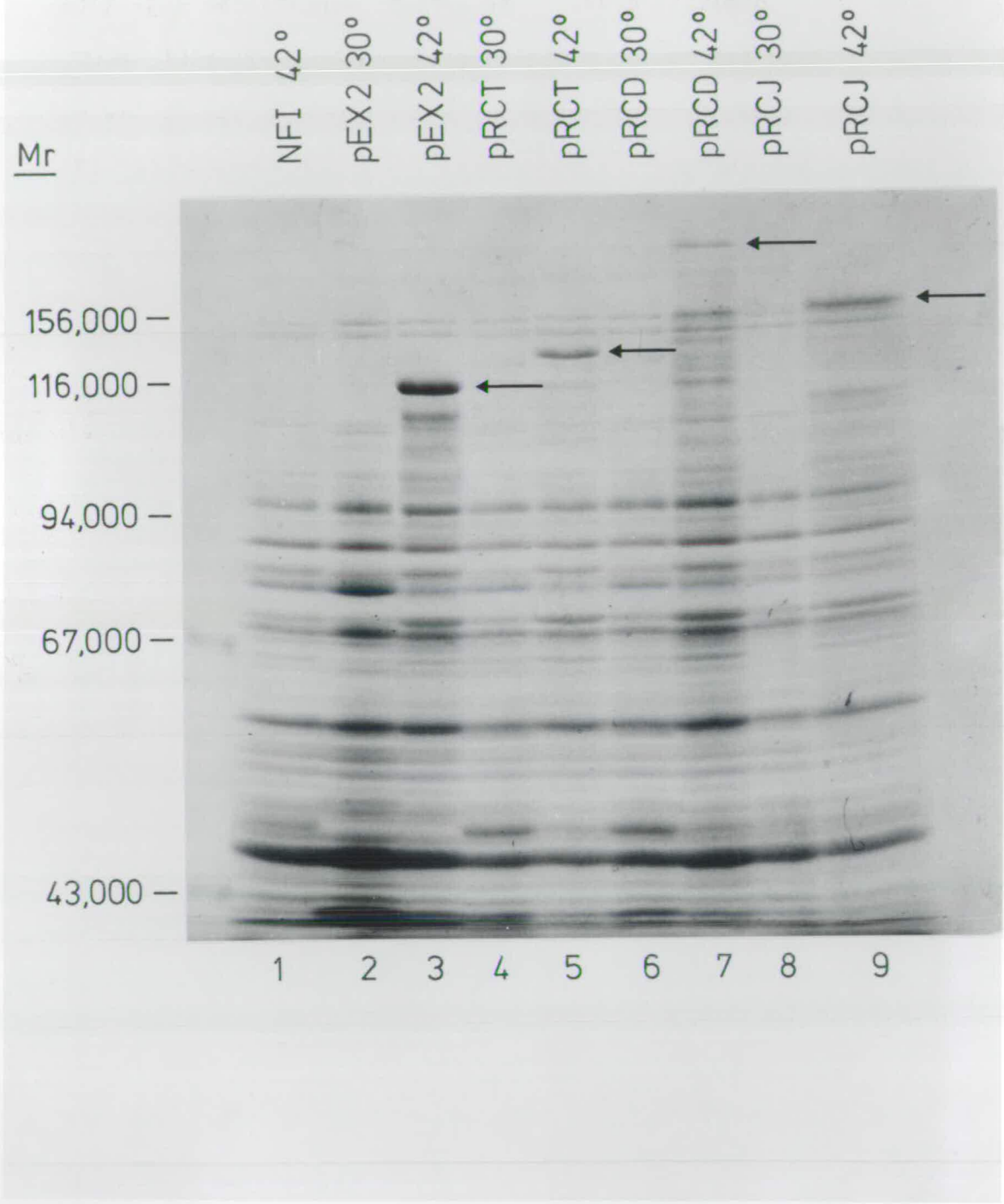

Mr
NFI 42°
pEX2 30°
pEX2 42°
pRCT 30°
pRCT 42°
pRCD 30°
pRCD 42°
pRCJ 30°
pRCJ 42°
156,000
116,000
94,000
67,000
43,000
1
2
3
4
5
6
7
8
9

FIGURE 4.5 Analysis of polypeptides encoded by pRCT and pRCD at various times of induction. Extracts from E.coli cells were fractionated on a 7% (w/v) polyacrylamide gel containing SDS. The gel was stained with Coomassie blue. The arrows identify the β-galactosidase and fusion proteins produced by pEX2, pRCT and pRCD respectively. The samples were the following: 2) NF1 cells; 3) pEX2 induced for 90 min at 42°C; 4) pRCT induced for 10 min at 42°C; 5) pRCT induced for 30 min at 42°C; 6) pRCT induced for 90 min at 42°C; 7) pRCD induced for 30 min at 42°C; 8) pRCD induced for 90 min at 42°C; 9) pRCT induced for 90 min at 42°C.

Mr
MARKERS
NF1 42° 90'
pEX2 42° 90'
pRCT 42° 10'
pRCT 42° 30'
pRCT 42° 90'
pRCD 42° 30'
pRCD 42° 90'
pRCT 42° 90'
116,000
94,000
67,000
1 2 3 4 5 6 7 8 9

This observation suggests that when the cells reach stationary phase, during the extended induction times, the proteolytic activity which is stimulated under these conditions (Goldberg and St. John, 1976) is recognising a particular sequence or conformation present in the polymerase region encoded by pRCD but not pRCT.

Protein extracts from cells harbouring the recombinant plasmids and vector control were separated electrophoretically and transferred to nitrocellulose membranes. The identity of these proteins as products of the gene fusion was confirmed by immuno-blotting these membranes with antiserum raised against β-galactosidase (Fig. 4.6).

4.3 Preparation of Antiserum to the Putative Polymerase Gene Product

To prepare antiserum that should recognise a wide spectrum of epitopes on the putative polymerase protein, three types of immunisation procedures were performed, using different antigen preparations. Isolating the recombinant β-galactosidase polymerase fusion proteins from an SDS-polyacrylamide gel will expose linear sequential determinants, purification of the fusion proteins using sonicated cell extracts, and gel exclusion chromatography will allow the polypeptide to be isolated such that the conformational epitopes will be exposed and lastly, by using HBV core preparations treated with non-ionic and ionic detergents a wide variety of antigens of the in vivo product will be exposed.

The 143,000 and 195,000 Dalton proteins synthesised by cells containing pRCT and pRCD respectively were used for the preparation of antiserum for two reasons; both are present at higher levels than the novel proteins synthesised by either pHPL-1 or pEM1, and since they are high molecular weight proteins, they migrate to a region of the gel

FIGURE 4.6 Reaction of β-galactosidase antiserum with polypeptides encoded by recombinant plasmids. Western blot analysis of E.coli cells NF1 transformed with recombinant plasmids. Samples were run on a 7% (w/v) polyacrylamide gel containing SDS. After transfer to nitrocellulose the samples were probed with antiserum to the denatured β-galactosidase protein. The reactions were visualised by addition of peroxidase-conjugated donkey anti-rabbit serum and stained in 0.01M imidazole pH7.4, dianisidine (250 μg/ml, 0.3% (v/v) hydrogen peroxide. The arrows identify the β-galactosidase and fusion proteins produced by pEX2, pRCT, pRCD and pRCJ respectively. The smaller sized species of immunoreactive proteins are breakdown products of the larger fusion proteins. 1) pRCJ induced for 30 min at 42°C; 2) pRCJ at 30°C. 3) pRCD induced for 30 min at 42°C; 4) pRCD at 30°C; 5) pRCT induced for 30 min at 42°C; 6) pRCT at 30°C; 7) pEX2 induced for 30 min at 42°C; 8) pEX2 at 30°C; 9) NF1 induced for 30 min at 42°C.

pRCJ 42°
pRCJ 30°
pRCD 42°
pRCD 30°
pRCT 42°
pRCT 30°
pEX2 42°
pEX2 30°
NFI 42°
1 2 3 4 5 6 7 8 9

where there is less likelihood of contamination with other E.coli proteins. The fusion proteins were fractionated from an E.coli lysate by electrophoresis in a 7.5% polyacrylamide gel in SDS and identified by staining strips cut from the edge of the gel with Coomassie blue. The corresponding band was cut from the remainder of the gel and homogenised in an equal volume of PBS. Samples of the homogenate, containing about 100µg of protein, were emulsified with Freund's complete adjuvant and injected into rabbits. The immunisation procedure is described in more detail in the Materials and Methods Section. Cultures of cells containing pRCT or pRCD were grown at 30°C to A_{650} = 0.4 induced by raising the temperature to 42°C, as described previously, harvested and then sonicated in 0.5% of the original culture volume of TEN buffer. The membrane and supernatant fraction were separated by centrifugation. Repeated attempts to purify the fusion proteins to homogeneity were unsuccessful because of the insolubility of the hybrid proteins which precipitated with the pellet fraction. These pellets were therefore used as antigens and emulsified with an equal volume of Freund's complete adjuvant. As the fusion proteins are synthesised at high levels in the cells, it was hoped that an antibody response would still be mounted against these polypeptides as was reported by Burrell et al., (1979) who injected whole E.coli cell extracts of clones producing minute quantities of HBsAg and induced an anti HBsAg response.

Hepatitis B core particles were isolated from the liver at the autopsy of a patient suffering from chronic hepatitis B virus. These core preparations were treated with 1% SDS and 5% NP40 and incubated at 37°C for 30 minutes. The mixture was diluted 1:10 with PBS and emulsified with an equal volume of Freund's adjuvant and injected into rabbits as before.

4.4 Analysis of Antisera

Antisera raised against the fusion proteins extracted from an SDS polyacrylamide gel, will react with numerous epitopes present on these, including the highly immunogenic β-galactosidase moiety. In order to determine whether the antisera contain antibodies which react with the polymerase region of the fusion proteins, antibodies against β-galactosidase were first removed. Removal of such antibodies was carried out by incubating the antisera with cellular extracts of E.coli, containing the vector pEX2. The resulting immunoprecipitate was removed by centrifugation. This process of adsorption was repeated three more times and allowed the detection of antibodies in the antisera that react specifically with the polymerase moiety of the fusion proteins (Fig. 4.7 and Fig. 4.8). However, while the antiserum against the fusion product encoded by pRCD also cross-reacted strongly with the gene fusion product from pRCJ, cross-reaction with the pRCT fusion product was weak (Fig. 4.8). Antibodies against the fusion protein synthesised by pRCT also cross-reacted weakly against the two heterologous antigens (Fig. 4.7). These results suggest that at least two distinct epitopes are located in the amino terminal and central regions of the gene product.

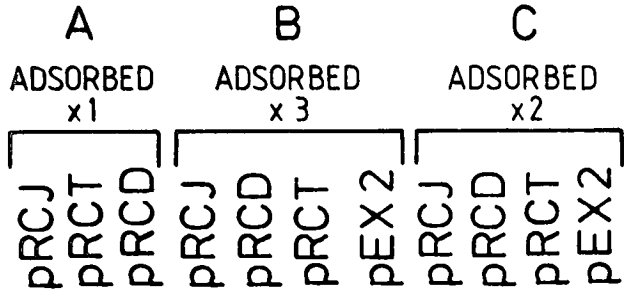
The antiserum raised to the pellet fraction from crude lysates of pRCD and pRCT, barely recognised the fusion protein using the Western blot technique. Likewise, the rabbits that were exposed to core preparations treated with NP40 and SDS, produced anti HBcAg, and anti HBe antibodies respectively (detected by using radioimmuno- assays) but not antibodies that cross-react with the fusion protein.

These results suggest that the antibodies produced by the latter two procedures recognise, perhaps, only conformational epitopes and so

FIGURE 4.7 Reaction of monospecific antiserum with polypeptides encoded by recombinant plasmids. Extracts from cells bearing pEX2, pRCT, pRCD and pRCJ were separated electrophoretically and transferred to nitrocellulose membranes. After transfer samples were probed with antiserum raised to the β-galactosidase-polymerase fusion protein synthesised by pRCT. The antiserum was preabsorbed with extracts from cells carrying the vector the following number of times: panel A, once, panel C twice, and panel B, three times. The blot was hybridised and developed as described in Section 2.10.12. Lane 1, pRCJ; Lane 2, pRCT; Lane 3, pRCD; Lane 4, pRCJ; Lane 5, pRCD; Lane 6, pRCT, and Lane 7, pEX2. The samples in Lanes 8-11 are duplicates of those in Lanes 4-7.

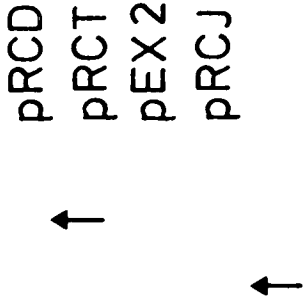
FIGURE 4.8 Reaction of monospecific antiserum with polypeptides encoded by recombinant plasmids. Samples were run on a 7% (w/v) polyacrylamide gel containing SDS. After transfer to nitrocellulose the samples were probed with antiserum raised to the β-galactosidase-polymerase fusion protein synthesised by pRCD. The antiserum had been previously adsorbed three times, with extracts from cells carrying the vector. The blot was hybridised and developed as described in Section 2.10.12. Lane 1, pRCD; Lane 2, pRCT; Lane 3, pEX2, and Lane 4, PRCJ.

Figure 4·7



1 2 3 4 5 6 7 8 9 10 11

Figure 4·8



1 2 3 4

would not react with the fusion proteins in Western blots. To determine whether or not the antisera produced against cell pellet fractions, or Dane particles treated with detergents, recognise conformational antigens on the fusion proteins, one would have to use the techniques of either radioimmunoassay or immunoprecipitation. Due to the lack of purified antigen and the presence of a large variety of antibodies capable of cross-reaction with E.coli proteins, it has not yet been possible to carry out such experiments. Further work, involving purification of the fusion proteins and removal of non-specific antibodies, will have to be carried out before the antibodies raised against the crude preparations of fusion proteins and Dane (core) preparations can be properly analysed.

4.5 Discussion

In conclusion, segments of the long ORF of HBV were fused in frame to the β-galactosidase gene of E.coli. Expression of the recombinant plasmids in E.coli produced fusion proteins which were sufficiently abundant and stable for direct detection after electrophoresis of the total bacterial proteins. The fusion proteins contained regions of the putative polymerase polypeptide which had not been previously synthesised. Rabbit antisera were raised to the hybrid proteins and this was shown to contain antibodies that recognised epitopes on the putative polymerase protein. Cross-reaction experiments tentatively identified at least two distinct epitopes located in the central and amino terminal region of this HBV polypeptide.

This rabbit antisera can now be used as a probe to confirm that the protein encoded by the HBV long ORF is present within the virion particle and expressed during the course of the virus life cycle. At

present Professor Eddleston (King's College, London) is using this antiserum to detect any cross-reactivity with proteins from liver biopsies of patients infected with HBV. In future experiments this antiserum will be used to analyse Dane particles and cell lines transformed with HBV for the presence of the putative polymerase gene product. The polymerase gene fusion product synthesised in E.coli also provides a reagent for the detection of antibodies to HBV polymerase.

CHAPTER 5

THE DETECTION OF ANTIBODIES, TO THE PUTATIVE POLYMERASE GENE PRODUCT, IN CONVALESCENT SERA

Page

Chapter 5

The polymerase gene fusion products provide a reagent which allows one to determine firstly, if and how this gene is expressed during the life-cycle of HBV, and secondly, whether or not the infected host mounts an antibody response to it.

It may be expected that a viral polymerase, normally an internal component, would not induce an antibody response. However, it has been shown that, in the course of infection of both human immunodeficiency virus (HIV) and Moloney murine Leukaemia virus (M-MuLV), an antibody response against the viral polymerase is induced. Antibodies to M-MuLV reverse transcriptase and some but not all antibodies to HIV reverse transcriptase have also been shown to inhibit enzyme activity in disrupted virions (Aaronson et al., 1971; Oroszlan et al., 1977; Laurence et al., 1987). Likewise, Cappel et al., (1976) reported the presence of antibodies to the endogenous HBV DNA polymerase in the sera of patients infected with HBV. These antibodies, which appeared transiently, were detected by their ability to inhibit the repair of the single-stranded gap in the HBV genome by the endogenous polymerase. They were found to be specific in that they did not inhibit Rous sarcoma or bacterial polymerases (Cappel et al., 1977). However, it is not obvious how these antibodies could inhibit the endogenous DNA polymerase within the intact core particle. This inhibition of the HBV polymerase by antibodies has not been observed by others (Hess et al., 1980. Therefore, the inhibitory antibodies observed by Cappel and his co-workers have yet to be proven unequivocally to be specific to the hepatitis B associated polymerase.

To investigate this phenomenon immunoblotting experiments with

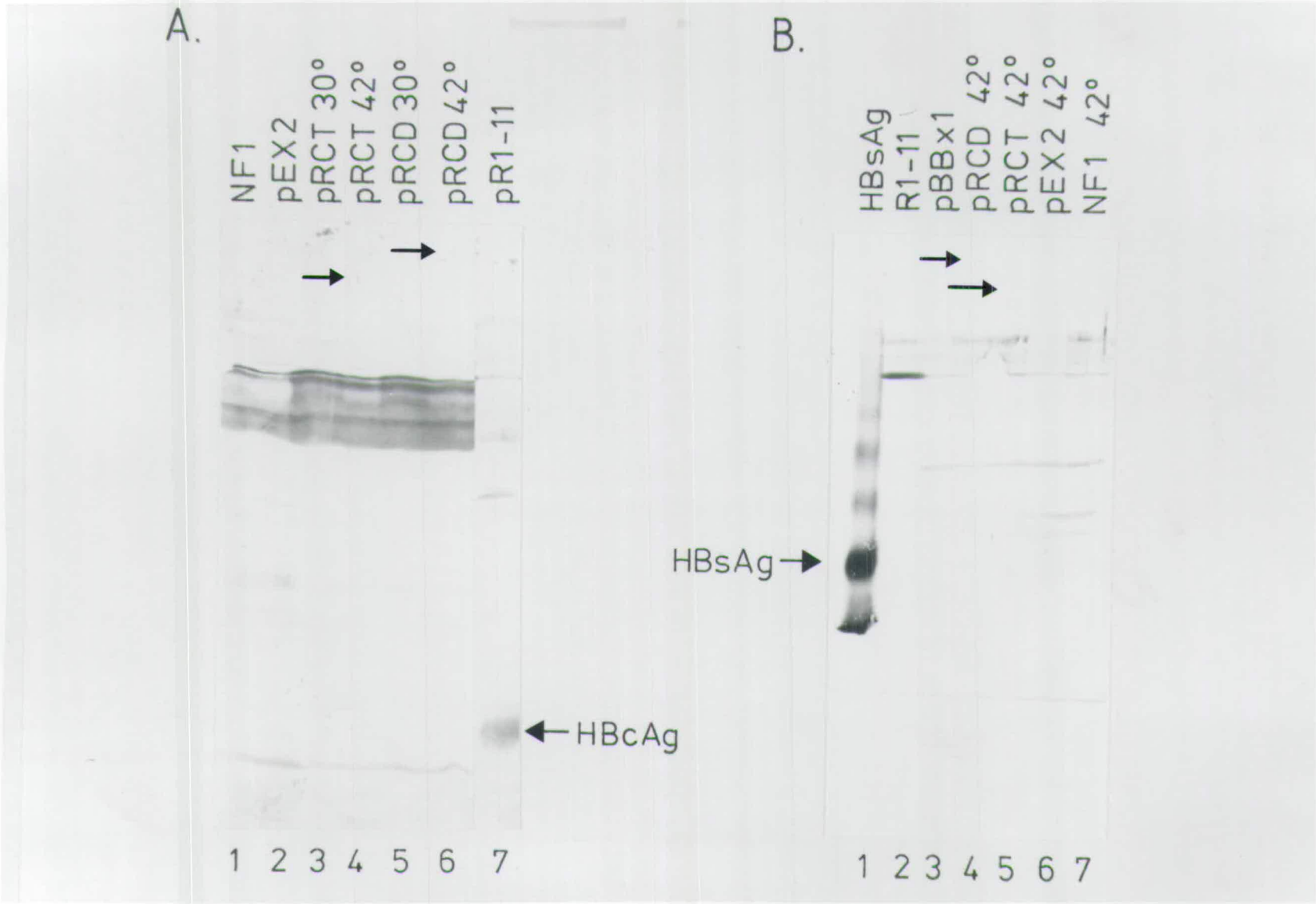
bacterial extracts carrying the β-galactosidase polymerase fusion protein were performed and are discussed in this chapter. The results showed that convalescent sera from chimpanzees infected with HBV contain antibodies that cross-react with the β-galactosidase polymerase fusion but with no other polypeptide encoded by HBV.

5.1 Detection of Antibodies to the Polymerase Gene Product in Convalescent sera.

Before trying to detect antibodies against the putative polymerase gene product in convalescent sera, using the fusion proteins produced by pRCD and pRCT as antigens, it was necessary to show that the N-terminal segment of HBcAg, present in both of the fusion proteins, did not show detectable cross-reaction with antibodies against either HBcAg or HBeAg. Crude extracts from cells carrying the recombinant plasmids or vectors were fractionated electrophoretically on an SDS polyacrylamide gel and transferred onto nitrocellulose membranes. Serum samples from nine separate rabbits that were anti-HBcAg positive after being immunised against various native or denatured core preparations were then tested against the proteins bound to nitrocellulose (Fig. 5.1a). Bound antibodies were detected by incubating with either alkaline phosphatase or peroxidase conjugated to anti-rabbit/human IgG and stained as described in Materials and Methods section. No antibodies that cross-react with either of the fusion protein were found. In addition, the antiserum generated to the fusion proteins showed no reactivity in immunoblotting experiments with HBcAg or HBeAg. Hence one can conclude from the data that in the first 28 amino acids of the core polypeptide there is no epitope for HBcAg or HBeAg, which agrees with extensive epitope mapping of the HBeAg carried out by R. Tedder

FIGURE 5.1(a) Antibodies against HBcAg do not cross-react with fusion proteins. Proteins from cell lysates harbouring pR1-11, pEX2, pRCD and pRCT were fractionated by electrophoresis in a 10% polyacrylamide gel containing SDS and transferred electrolytically to a cellulose nitrate membrane. This membrane was probed with HBcAg antibodies. The reactions were detected by addition of peroxidase-conjugated donkey anti-rabbit serum and stained as described previously. The arrows identify where the fusion protein migrates. Cross-reacting polypeptides across all tracks may be due to the serum (raised against a bacterial preparation of HBcAg) containing antibodies to these E.coli proteins. 1) NF1; 2) pEX2 induced at 42°C for 30 min; 3) pRCT at 30°C; 4) pRCT induced at 42°C for 30 min; 5) pRCD at 30°C; 6) pRCD induced at 42°C for 30 min; 7) Purified HBcAg expressed by R1-11.

FIGURE 5.1(b) Antibodies against HBsAg do not cross-react with fusion proteins. Purified HBsAg expressed by yeast and crude lysates of cells carrying the plasmids of interest were run on a 10% (w/v) polyacrylamide gel containing SDS, then transferred onto nitrocellulose. The blot was probed with HBsAg antibodies and developed as described in Section 2.10.12. The arrows identify where the fusion proteins migrate. 1) HBsAg; 2) pR1-11; 3) pBBX-1 expressing HBxAg; 4) pRCD induced at 42°C for 30 min; 5) pRCT induced at 42°C for 30 min; 6) pEX2 induced at 42°C for 30 min; 7) NF1.


A.
NF1
pEX2
pRCT 30°
pRCT 42°
pRCD 30°
pRCD 42°
pR1-11
HBcAg
1 2 3 4 5 6 7
B.
HBsAg
R1-11
pBBx1
pRCD 42°
pRCT 42°
pEX2 42°
NF1 42°
HBsAg
1 2 3 4 5 6 7

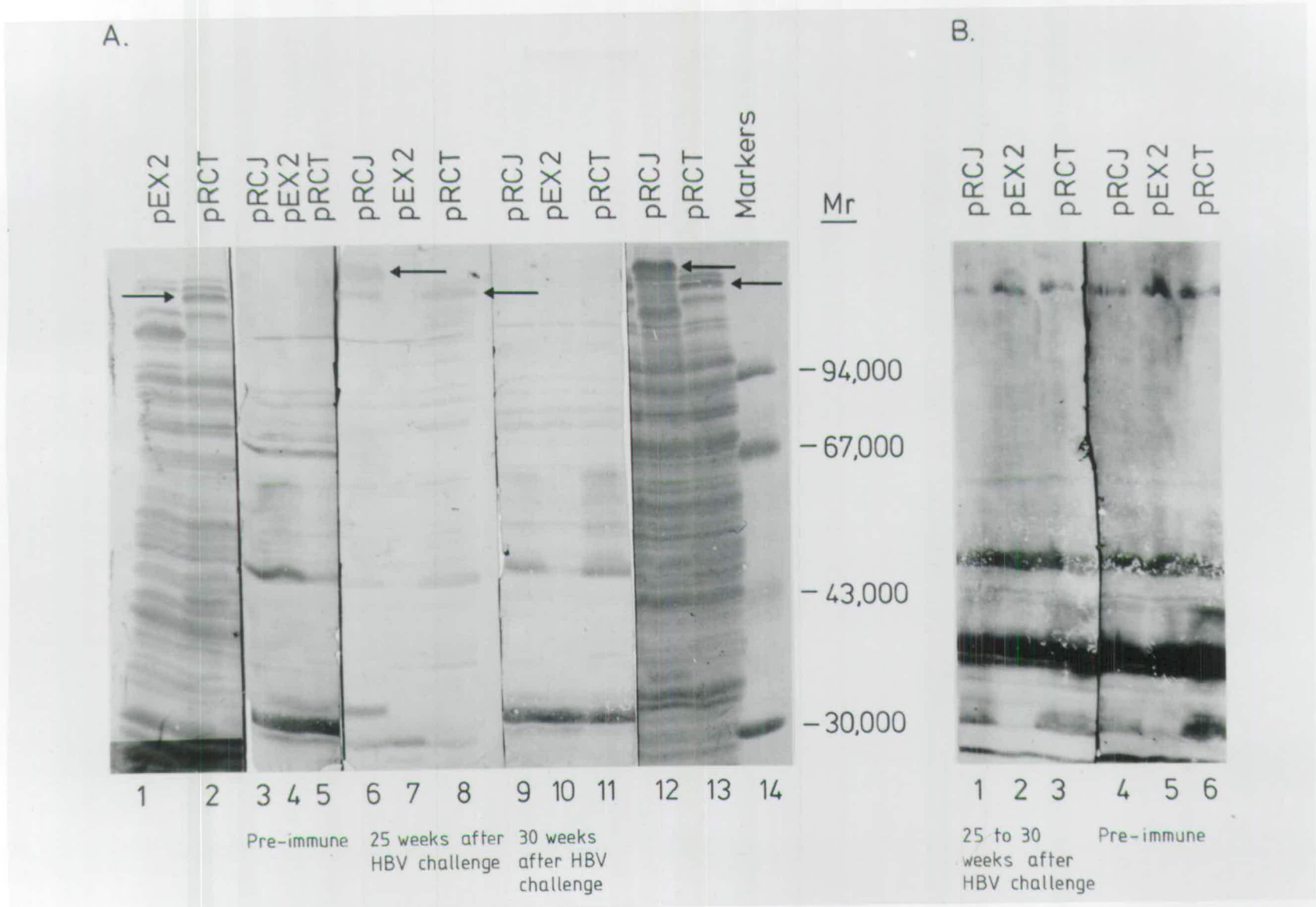
(personal communication). It also shows that the fusion proteins, synthesised by pRCT, pRCD and pRCJ, the latter of which has no core sequences, can be used as antigens to detect antibodies to the gene product of the long ORF.

As the levels of the HBeAg and the endogenous Hepatitis B polymerase activity rise and fall at the same time during the course of HBV infection it was postulated that an antibody response to the endogenous polymerase would correlate with the appearance of anti-HBeAg. Therefore, serum samples taken serially from three chimpanzees recovering from HBV infection, which were anti-HBeAg positive, were tested against the fusion proteins bound to nitrocellulose. Cross-reacting antibodies were found in the serum of the infected animals (Fig. 5.2 and 5.3), but these antibodies were present only transiently and reached a maximal level 25 weeks after inoculation of the virus, which correlates exactly with the maximal levels of anti-HBe.

Similar cross-reacting antibodies were not found in the serum of chimpanzees that had been vaccinated prior to inoculation with HBV (Fig.5.2b). Furthermore, antibodies raised against HBsAg (Fig.5.1b), as well as HBcAg and HBeAg (Fig. 5.1a), did not react with the β-galactosidase-polymerase fusion products. Hence these results clearly show that during HBV infection a specific antibody response, directed against the gene product of the long ORF, is elicited.

A panel of human sera from patients infected with HBV was examined for the presence of antibodies to the putative polymerase gene product. None of the human sera contained antibodies which cross-reacted with the β-galactosidase-polymerase fusion proteins. The negative results for the human serum may reflect the small panel of blood samples (five) used and the stage of infection when they were taken; late acute and the

FIGURE 5.2 Detection of antibodies that cross-react with the fusion proteins expressed by pRCT and pRCJ. Extracts from cells bearing pEX2, pRCT and pRCJ were separated electrophoretically and transferred to nitrocellulose membranes. These membranes were either stained with Coomassie blue, or incubated with chimpanzee serum samples listed below. Membranes incubated with serum were then washed and incubated with goat anti-human serum conjugated with horse radish peroxidase for staining with peroxidase as described in the Methods section. A) Membranes incubated with serum samples from a convalescent chimpanzee following acute hepatitis B infection. Lanes 1,2,12,13 and 14, correspond to pEX2, pRCT, pRCJ, pRCT and molecular weight markers stained with Coomassie blue. Lanes 3-5 are pRCJ, pEX2 and pRCT, respectively, probed with pre-immune serum. The samples in Lanes 6-8 are duplicates of those in Lanes 3-5, but incubated with serum taken 25 weeks after HBV challenge. Lanes 9-11 are duplicates of the two previous groups, but probed with serum taken 30 weeks after HBV challenge. B) Membranes probed with serum from a chimpanzee vaccinated (with purified HBsAg synthesised in yeast) prior to inoculation with HBV. The samples in Lanes 4-6 contain pRCJ, pEX2 and pRCT incubated with pre-immune serum. The samples in Lanes 1-3 are duplicates of those in Lanes 4-6 probed with serum taken 25-30 weeks after challenge with the virus.


A.
pEX2
pRCT
pRCJ
pEX2
pRCT
pRCJ
pEX2
pRCT
pRCJ
pEX2
pRCT
pRCJ
pRCT
Markers
Mr
94,000
67,000
43,000
30,000
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Pre-immune
25 weeks after HBV challenge
30 weeks after HBV challenge
B.
pRCJ
pEX2
pRCT
pRCJ
pEX2
pRCT
1 2 3 4 5 6
25 to 30 weeks after HBV challenge
Pre-immune

carrier state. As the antibodies present in the chimpanzees were shown to occur only transiently, it is possible that such antibodies have been produced but are no longer present in the sera. At present a larger panel of human sera, consisting of over 50 samples (some of which had been taken very early in the course of the disease), are being tested for the presence of the cross-reacting antibodies. To investigate whether or not the antibodies found in the chimpanzee serum also react with the homologous polymerase gene products of Moloney murine Leukaemia virus (M-MuLV), and Avian murine Leukaemia virus (AMV), samples of the purified reverse transcriptase from both these viruses were separated electrophoretically on an SDS polyacrylamide gel and transferred onto nitrocellulose membranes. These membranes were then probed with serum samples which cross-reacted with the β-galactosidase-polymerase fusion proteins. It was found that the convalescent chimpanzee sera showed no cross-reaction with either reverse transcriptase and hence is specific for the putative polymerase of HBV (Fig. 5.4).

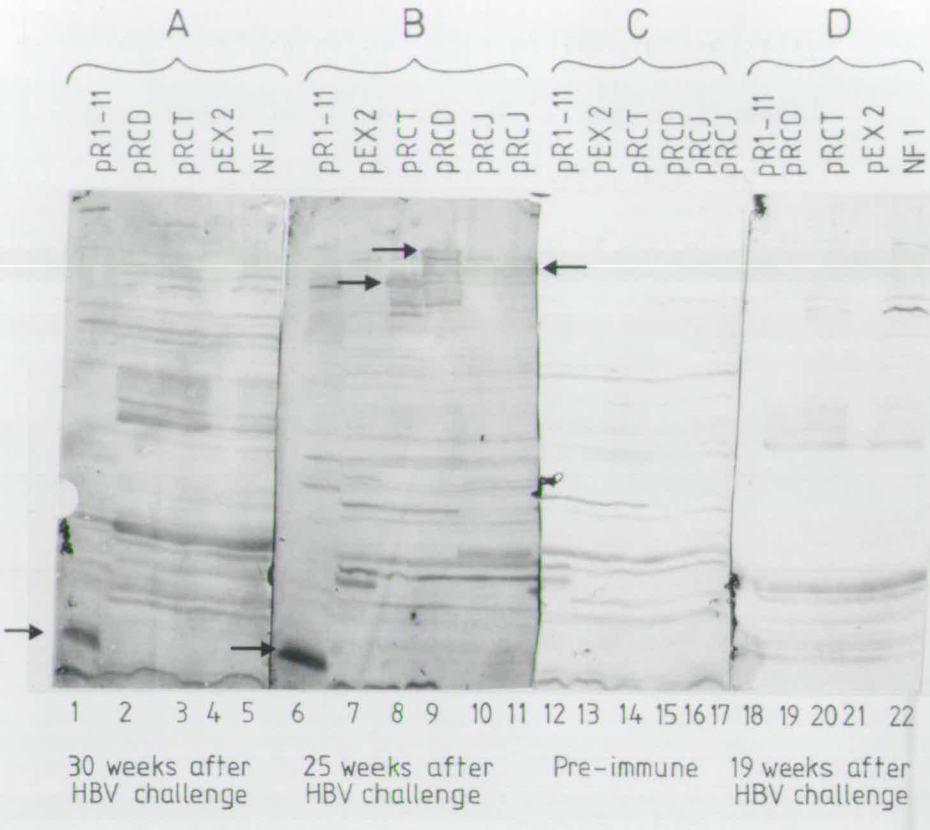
5.2 Discussion

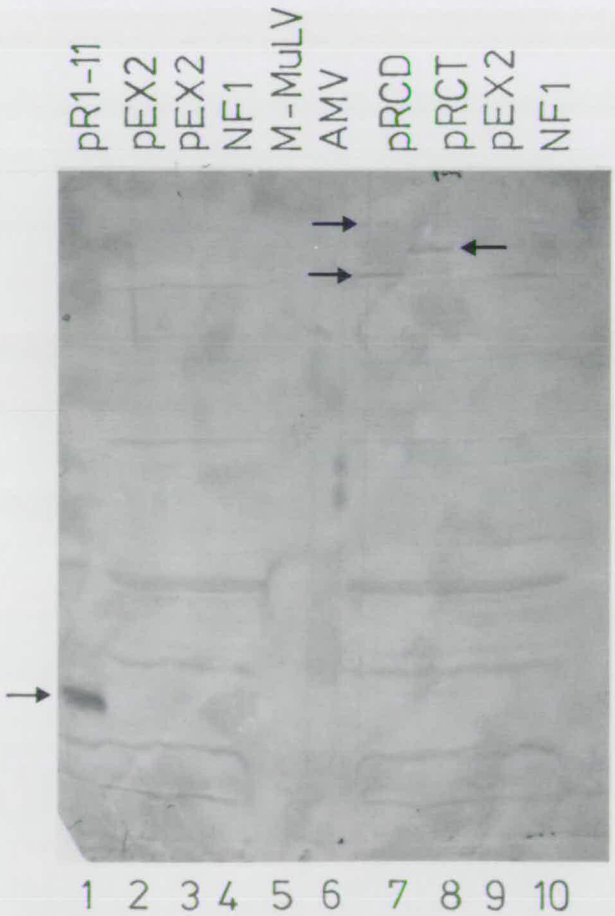
These experiments provide clear evidence that the putative polymerase gene of HBV is expressed and its product exposed to the immune system of the host during normal viral infection. They also show that the two epitopes tentatively identified at the amino terminal and central regions of the gene product (Section 4.4) elicit an antibody response during HBV infection.

Whether the antibodies described by Cappel *et al.*, (1976), which inhibited the endogenous DNA polymerase, are related in any way to the antibodies generated to the translation product of the long ORF remains

FIGURE 5.3 Demonstration of antibodies to the β-galactosidase-polymerase fusion proteins in serum by immunoblotting. Proteins were separated by SDS-PAGE on a 10% gel and transferred onto nitrocellulose strips. These strips were incubated with chimpanzee serum samples as described below. The reactions were visualised by the addition of goat anti-human serum conjugated with peroxidase and stained as described in the Materials and Method Section. Lanes 1-5; extracts from cells carrying pR1-11, pRCD, pRCT, pEX2 and NF1, respectively probed with serum taken 30 weeks after HBV challenge. The samples in Lanes 18-22 were duplicates of those in lanes 1-5 but incubated with serum taken 19 weeks after HBV challenge. Lanes 6-11; extracts from cells carrying pR1-11, pEX2, pRCT, pRCD, pRCJ and pRCJ respectively, incubated with serum taken 25 weeks after HBV challenge. Finally, Lanes 12-17 are duplicates of those samples in Lanes 6-11, probed with pre-immune serum serum.

FIGURE 5.4 Antibodies which cross-react to the recombinant fusion protein do not recognise either AMV or M-MuLV reverse transcriptase. Crude extracts from cells carrying the recombinant plasmids and purified reverse transcriptase from AMV and M-MuLV were separated on a 10% (w/v) polyacrylamide gel. Proteins were Western-blotted onto nitrocellulose and probed with antiserum from a chimpanzee taken 25 weeks after HBV challenge. The blot was developed as described in Section 2.10.12. 1) pR1-11; 2) pEX2; 3) pEX2; 4) NF1; 5) M-MuLV; 6) AMV; 7) pRCD 8) pRCT.


A
B
C
D
pR1-11
pRCD
pRCT
pEX2
NF1
pR1-11
pEX2
pRCT
pRCD
pRCJ
pRCJ
pR1-11
pEX2
pRCT
pRCD
pRCJ
pRCJ
pR1-11
pRCD
pRCT
pEX2
NF1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
30 weeks after HBV challenge
25 weeks after HBV challenge
Pre-immune
19 weeks after HBV challenge


pR1-11
pEX2
pEX2
NF1
M-MuLV
AMV
pRCD
pRCT
pEX2
NF1
1 2 3 4 5 6 7 8 9 10

to be determined. It is of interest to note, however, that both sets of antibodies appeared transiently in the early stages of infection. The transient nature of the antibodies showing cross-reaction with the fusion proteins may reflect the system used. The Western blot method will only detect antibodies which recognise linear epitopes rather than conformational epitopes. Antibodies raised to sequential determinants are known to be shorter lived (Weber et al., 1987). Perhaps, by using immunoprecipitationmethods,a more persistent class of antibody to the gene product of the long ORF could be identified.

The production of antibodies against the X antigen has been observed in the same group of animals (Weber et al., 1987). This is interesting as both the endogenous polymerase and the HBxAg are believed to be internal components of HBV and the appearance of antibodies may reflect exposure of both of these antigens to the immune system. Perhaps at a particular stage in HBV morphogenesis these proteins are exposed due to their presence in the cell membrane. This is the method by which the core antigen is proposed to elicit a humoral response. The polymerase of M-MuLV, which induces an antibody response, has also been detected on the membrane of cells infected with mutant M-MuLV, which are unable to complete the latter stages of viral assembly (Aaronson et al., 1971; Witte and Baltimore, 1978). Alternatively, the cellular components may be exposed as a result of host cell lysis.

In conclusion, it remains to be seen whether or not antibodies against the polymerase protein are produced in humans, and if so, whether or not such antibodies play a protective role.

CHAPTER 6

EXPRESSION OF THE RECOMBINANT β-GALACTOSIDASE POLYMERASE GENE IN *E.COLI* RESULTS IN THE SYNTHESIS OF A UNIQUE DNA POLYMERASE ACTIVITY

Page

Chapter 6

INTRODUCTION

In the previous chapter it was shown that the long ORF of HBV is expressed by the virus during HBV infection. As discussed earlier, this long ORF is thought to encode the endogenous polymerase. However, to definitively assign this function to the long ORF it is necessary to prove that the translation product has DNA polymerase activity.

The polymerase gene of both M-MuLV and HIV have been expressed in E.coli either as a fusion or native protein and shown to induce the synthesis of reverse transcriptase activity. In the case of M-MuLV reverse transcriptase, part of this gene, when fused to the TrpE gene, was shown to produce high levels of reverse transcriptase activity even though the amino and carboxy termini of the protein product were not present (Tanese et al., 1985)

It was also found that when the HIV polymerase gene was expressed as a fusion protein with TrpE much of the polymerase gene could be removed without affecting activity but the TrpE portion of the construct was essential for good activity (Tanese et al., 1986).

As a result of the above observations, it was decided to test extracts of cells expressing the β-galactosidase HBV fusion proteins for a novel DNA polymerase activity. Although originally described as a DNA-dependent polymerase (Kaplan et al., 1973), the HBV polymerase is now believed to function as a reverse transcriptase in the generation of an RNA intermediate during viral replication (Summers and Mason, 1982). Accordingly an assay for reverse transcriptase was adopted. The assay measures the incorporation of radioactively labelled dGTP into acid precipitable material in a reaction that uses poly r(C) as template and oligo d(G) as primer (Takatsuji et al., 1986). The

results presented in this chapter show that extracts of E.coli expressing the HBV gene fusion products exhibit a polymerase activity with the characterisatics of a reverse transcriptase.

6.1 The β-galactosidase-Polymerase Fusions induce Reverse Transcriptase Activity

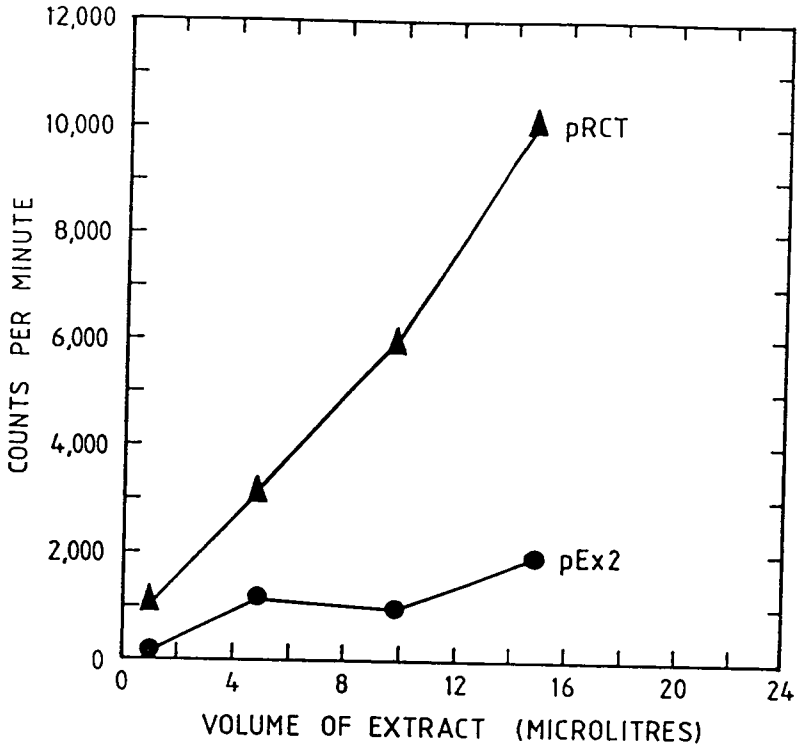
Cultures of E.coli NF1 transformed with the vector pEX2 and the three recombinant plasmids, pRCT, pRCJ, and pRCD, were grown at 30°C to A_{650}=0.4 induced for 45 minutes at 42°C, harvested and resuspended in Tris EDTA NaCl buffer (T.E.N.) (See Methods Section). The cells were lysed by sonication and treated with deoxyribonuclease 1. Cell debris was pelleted by centrifugation and the supernatant and pellet fractions were separated. The pellet was washed three times by resuspension in TEN and centrifugation, and used in various assay reactions. Aliquots of the crude lysate mixture from cells carrying pRCT, pRCD and pRCJ, and the vector were added to the following:- the reaction buffer, poly r(C) oligo $dG_{(12-18)}$, and radioactive labelled ^3H dGTP. After incubation at 37°C for 60 minutes, acid-precipitable material was collected and counted (see Methods Section).

Extracts from cells carrying the vector exhibited small but significant activity in this assay, but equivalent extracts from cells carrying the plasmids pRCD, pRCJ or pRCT consistently gave significantly higher activity in the polymerase assay than cells carrying the vector. The highest specific activity was obtained repeatedly with extracts of pRCT (Fig.6.1a) and cells transformed with this plasmid were therefore used for further experiments. Extracts from cells harbouring pRCD and pRCJ had polymerase activities approximately twice that of cells carrying the vector.

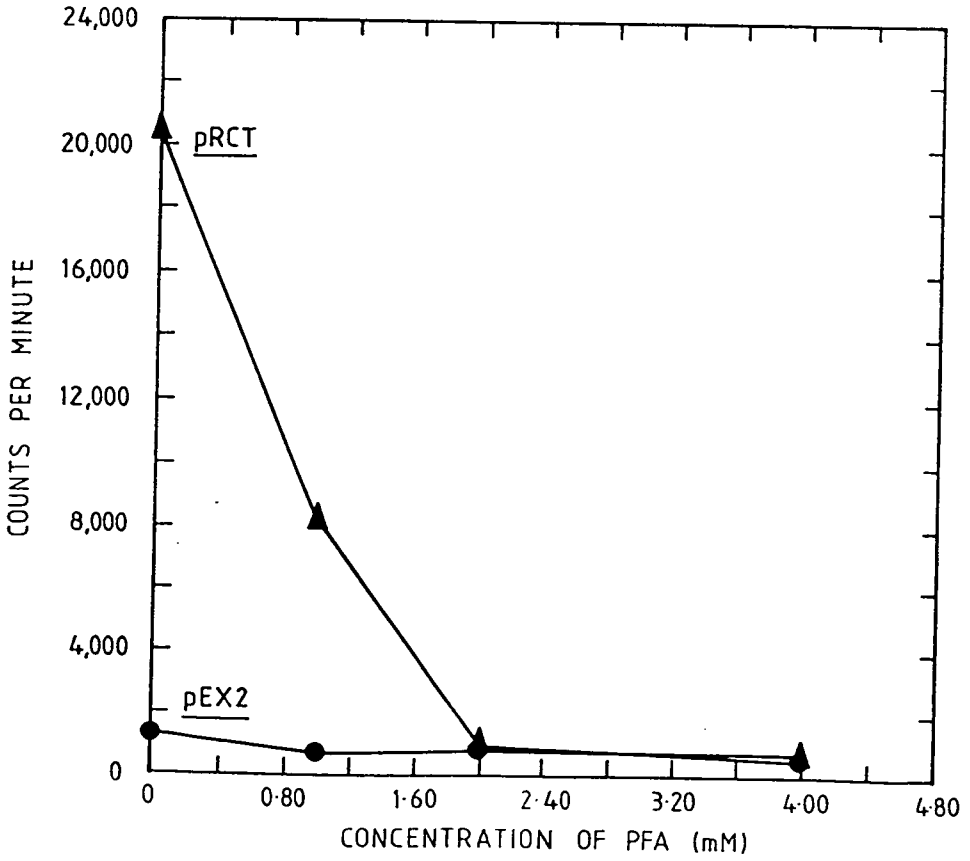
FIGURE 6.1(a) Lysates prepared from E.coli NF1 cells carrying pEX2 and pRCT assayed for polymerase activity.

FIGURE 6.1(b) Lysates prepared from E.coli NF1 cells carrying pEX2 and pRCT assayed for polymerase activity in the presence of PFA.

A. Polymerase activity



B. Inhibition with PFA



The polymerase activity of cells carrying pRCT was for the most part associated with the insoluble material which made purification very difficult. The crude lysate mixture was therefore used for all the studies of enzyme activity.

The basal polymerase activity of the E.coli cells carrying pEX2 could be due to the ability of E.coli DNA polymerase I (DNA poll) to carry out either DNA or RNA directed DNA synthesis and its ability to utilise the template poly r(C) oligo $dG_{(12-18)}$ (Karkas, 1973; Loeb et al., 1973). The polymerase activity of crude extracts from cells carrying pEX2 and pRCT were therefore compared (Table 1).

Table 1

Assay Conditions	dGTP incorporated (Cpm/60 min.)		Specific Activity (Cpm/µg protein/60 min.)		Specific Activity (pmoles/ng protei	
	pEX2	pRCT	pEX2	pRCT	pEX2	pRC
(a)	703	12466	5.6	99	0.17	3.
(b)	149	1042	1.2	8.2	0.04	0.
Heat (c)	164	154	1.3	1.8	0.04	0.
No Mg^{2+}	229	0	1.9	0	0.05	0
Mn^{2+}(d)	2326	5575	18.0	44	0.53	1.

(a) Lysates were prepared from E.coli NF1 cells carrying pEX2 and pRCT were assayed for polymerase activity (see Methods). In all the reactions, unless otherwise stated, 10µl of the preparation was used

(b) These reactions were as in (a), but with 1µl of the preparation.

(c) Crude lysates were heated for 15 min. at 70°c, cooled on ice then assayed.

(d) Mn^{2+} at 2mM replaced Mg^{2+} (10mM) in the assay conditions described in the Methods Section.

The polymerase activity of both lysates could be abolished by heat treatment and was shown to be dependent on the presence of a divalent cation. The activity from the vector extracts was stimulated when manganese replaced magnesium, but the activity from cells carrying pRCT was markedly reduced in the presence of manganese. This is of twofold interest. Firstly, it supports the hypothesis that DNA poll is responsible for the basal polymerase activity of the vector cells as the reverse transcriptase activity of DNA poll is stimulated by manganese (Karkas, 1973), and secondly, the loss of polymerase activity from pRCT in the presence of manganese reflects the cation requirement for the polymerase activity associated with HBV (Goto et al., 1984).

6.2 Inhibition of the Polymerase Activity by Phosphonoformate

Phosphonoformate (PFA), a pyrophosphate analog, is an inhibitor of reverse transcriptase and other viral polymerases (Nordenfelt et al., 1980; Hess et al., 1980). It has also been shown to inhibit HBV polymerase and has in fact been used to treat patients with fulminant Hepatitis (Heden et al., 1986).

Treatment of extracts of E.coli carrying pRCT with PFA inhibited the polymerase activity while the addition of PFA to extracts of E.coli carrying pEX2 had little effect on the polymerase activity (Fig. 6.1B). The sensitivity of the polymerase activity in cells carrying pRCT is therefore similar to the endogenous HBV polymerase.

6.3 Discussion

It is interesting that cell extracts carrying the smallest of the recombinant gene fusion, pRCT, has the highest polymerase activity and

that extension of the 3' end of the gene results in a dramatic loss in enzyme activity. This is analogous to the situation found with M-MuLV reverse transcriptase gene fusions. It was found that progressive deletions at the 3' end of the gene resulted in increased levels of reverse transcriptase activity in cell extracts carrying these deletions (Roth et al., 1985). This may reflect the fact that these smaller fusion proteins are more soluble than the parental fusion proteins and therefore more accessible to the substrates, or alternatively the template or triphosphate binding sites are in a more favourable conformation for efficient synthesis.

To try to specifically inhibit the DNA polymerase activity in cells carrying pRCT, cell extracts synthesising the β-galactosidase-polymerase fusion protein were incubated with rabbit and chimpanzee sera that had previously been shown to cross-react with this protein in immunoblotting experiments. These experiments were unsuccessful which may be a correct indication of cross-reaction without blocking enzyme activity as has been observed in other systems(e.g. several antibodies that cross-reacted with HIV reverse transcriptase were found not to inhibit its enzyme activity (G.Derby, personal communication). The sensitivity of the polymerase activity in extracts of cells carrying pRCT to PFA, and the marked preference for magnesium, leads to the conclusion that the major component of the polymerase activity is due to the polymerase activity encoded by the segment of the long ORF of HBV. The limited tests carried out so far show that the biochemical profile of the cloned enzyme activity is similar to that of the endogenous Hepatitis B polymerase and the gene product of the long ORF does have polymerase activity.

CHAPTER 7

OVERALL DISCUSSION

Page

Chapter 7

In the world today there are some 200 million carriers of HBV. The prognosis for any patient carrying HBV is not good. Although some carriers spontaneously revert, and eliminate the virus, more than 50% will die of a liver-related disease. At present no universal chemotherapy is employed in the treatment of HBV carriers. The main approach is to use drugs that have been shown to inhibit the endogenous DNA polymerase with the assumption that this will stop viral replication. The major limitation in this approach is that the drug is being analysed with respect to the repair function of the endogenous polymerase and it may or may not affect the reverse transcriptase activity of this enzyme which is believed to be involved in the replication of HBV (Summers and Mason, 1982). Hence it is important that more is known about this enzyme, in particular the origin of this encapsulated polymerase.

The main purpose of this study was to determine whether the long ORF of HBV, previously assigned as the putative polymerase gene is expressed during the course of viral infection and whether its product has polymerase activity. The long term aim was to provide a safe and abundant source of the HBV polymerase, using recombinant DNA techniques thereby allowing detailed analysis of the DNA- and RNA-dependent polymerase activities of the enzyme with the hope of providing an effective and specific drug against the HBV polymerase.

7.1 Expression of the long ORF in E.coli

Several fragments of the putative polymerase gene were expressed in E.coli under the inducible control of the Lac and Tac promoters. It was found that the only construct that synthesised a detectable novel protein of the expected size was one that expressed 160 amino acids

from the N-terminal putative polymerase gene product. Synthesis of novel proteins by E.coli cells carrying plasmids with extended polymerase coding information could not be detected. Although the translation products could not be detected it was shown, using Northern blotting, that plasmids were producing HBV-specific transcripts.

The predicted secondary structure of the messenger RNA showed that the initiation codon and the Shine dalgarno sequence were in a favourable position for the initiation of translation. These observations indicated that the failure to detect novel proteins was possibly due to instability of the protein products as opposed to any block during expression. This was confirmed by using a coupled in vitro transcription translation system. Using this system, novel proteins of the predicted size were expressed by the recombinants which had not been detected in vivo.

Therefore, in an attempt to overcome this problem in vivo, the plasmids were transferred to a protease-deficient strain of E.coli (SG935). This bacterium is deficient in the protease La which plays an important role in the degradation of abnormal or foreign proteins (Goff and Goldberg, 1985). Using this strain it was possible to detect a novel protein with the expected molecular weight in cells carrying pEM1. It was also found that the level of the translation product expressed by pHPL-1 was dramatically increased. However, the larger constructs, pR1-130, pR1-pol8, ptac-pol8 and pT.R.T., which should have expressed the rest of the putative polymerase gene product, did not produce any novel polypeptides. Therefore it is highly probable that the central or carboxy region of the translation product from the long ORF carries a region that is particularly sensitive to proteolytic degradation, e.g. the PEST sequence (Rogers et al.,

1986). Similarly, the polymerase gene products of HIV and M-MuLV, have been found to be sensitive to proteolytic degradation when synthesised in bacteria (Tanese, et al., 1985, 1986; Kotewicz et al., 1985). For M-MuLV, stable expression of its reverse transcriptase was achieved by the removal of DNA from the 3' terminus of the gene (Roth et al., 1985). Likewise when the reverse transcriptase of HIV was expressed in a mature form, most of its protein product was present in smaller sized species (Farmierie et al., 1987). The use of Lon mutants to express the M-MuLV recombinant, doubled the enzyme activity previously seen in Lon^+ cells (Kotewicz et al., 1985).

The fusion of a eukaryotic or viral sequence to a bacterial gene has often led to the stable synthesis of the hybrid fusion protein whereas the expression of the same recombinant gene fused to only a short bacterial polypeptide has been unsuccessful.

Therefore, in an effort to obtain greater expression of the N-terminal region and to express other regions of the long ORF stably in bacteria, recombinant plasmids were constructed in which various segments of the long ORF were fused in-frame to practically all of the β-galactosidase genes of E.coli.

Expression of the β-galactosidase-polymerase fusion genes in E.coli produced proteins that were sufficiently abundant and stable for direct detection after electrophoresis of the total bacterial proteins. Interestingly, this included regions of the putative polymerase gene, the product of which had previously been undetectable in vivo. However, during extended induction times constructs containing the central and carboxy termini of the putative polymerase protein were selectively degraded. This suggests that when the cells are allowed to reach stationary phase, the proteolytic activity

stimulated under these conditions recognises a particular sequence or conformation encoded at the central or carboxy terminus of the fusion proteins (Goldberg and St. John, 1976). However, constructs containing only the N-terminus of the putative polymerase protein express their translation product in a native form in E.coli without such specific proteolytic degradation.

7.2 Analysis of Antisera that cross-react with the Putative Polymerase Gene Product

Rabbit antisera were raised to the hybrid fusion proteins. The antiserum recognised specific epitopes on the putative polymerase protein. Cross-reaction experiments with this antiserum and the various fusion proteins demonstrated that at least two distinct epitopes were located in the central and amino terminal regions of the putative polymerase.

The polymerase gene fusion product synthesised in E.coli provided a reagent for the detection of antibodies to HBV polymerase. Cross-reacting antibodies that recognise these fusion proteins were found in the sera of chimpanzees recovering from acute Hepatitis B infection. The presence of these antibodies was found to be transient; they were not observed in the serum of a chimpanzee that had been vaccinated prior to inoculation with HBV. Furthermore, antibodies raised to HBV surface, core, e and X antigen showed no cross-reaction with the β-galactosidase polymerase fusion proteins.

These experiments provide clear evidence that the putative polymerase gene of HBV is in fact expressed and antibodies to its protein product are produced during HBV infection.

At present it still remains to be estabished whether or not antibodies that cross-react with the putative polymerase gene product are produced in humans following HBV infection. However, it is very likely that they are, as antibodies against HBxAg which have been observed in the same group of animals have also been observed in humans (Weber et al., 1987). It is not clear whether antibodies to the putative polymerase gene product would play a protective role. In the case of HIV, antibodies to the endogenous polymerase have been shown to interfere with virion assembly, and budding in vitro (Wong et al., 1987).They may also reflect a particular point in the course of viral infection. Laurence et al., (1987) showed that asymptomatic HIV sero positive carriers with high titres of antibodies to reverse transcriptase lost these antibodies prior to the development of the acquired immune deficiency syndrome (AIDS). However, it is more likely that the antibodies seen to the putative HBV polymerase gene product and also the X antigen, are actually surrogate markers for a protective cellular immune response. In this way both HBcAg and HBeAg have been shown to play a protective role in stopping HBV infection by stimulating cell mediated response (Vento et al., 1985; Murray et al., 1984). It is possible that both the HBV polymerase and HBxAg may also be involved in this process if only to a limited extent.

7.3 The Protein Product of the Long ORF has Polymerase Activity

Extracts of E.coli expressing the recombinant β-galactosidase polymerase fusions had significantly higher polymerase activity than cells bearing the vector. Interestingly, the smallest gene fusion alone, expressing 160 amino acids of the N-terminal polymerase polypeptide, gave the highest specific activity. This may be due to

the increased solubility of the smaller fusion product and greater accessibility to the substrate. This portion excludes the region of strong homology which exists between the reverse transcriptases of a number of viruses located in the centre of the polypeptide (Toh et al., 1983, 1985); this particular motif is homologous with an amino acid sequence in the α-subunit of the E.coli DNA-directed RNA polymerase which has been suggested to have a role in template of primer binding (Kamar and Argos, 1987).

For HIV reverse transcriptase it has also been shown recently that the amino terminal region of the polypeptide is essential for polymerase activity. By changing an asparagine residue to a glutamine residue within this region, enzyme activity was completely lost. (Larder et al., 1987). These experiments pinpoint a small area of the putative polymerase protein which carries several important regions for the polymerase activity. Using site-directed mutagenesis of pRCT it should be possible to pinpoint the amino acid residues that are responsible for polymerase activity.

7.4 Inhibition of the Polymerase Activity by Phosphonoformate

Since the polymerase activity produced by pRCT is sensitive to PFA, a site of action of this drug must reside within amino acid residues 40 to 201 of the Hepatitis B polymerase. Interestingly, the N-terminal region of the reverse transcriptase of HIV has been shown to be essential for inhibition by PFA. Hence pRCT provides a useful substrate for the study of PFA action. Using site-directed mutagenesis of pRCT it should be possible, by creating drug-resistant mutants, to identify a site of drug action. Hence by understanding

drug interaction at this level it should also be possible to design more specific drugs which would interact and interfere with the function of the polymerase activity encoded by the HBV long ORF. Although one can use the recombinant polymerase expressed in E.coli to some extent to identify drugs that inhibit the enzyme activity, this approach is limited as the clone produces only part of the polymerase protein and is expressed in a bacterial environment. A more comprehensive approach to screening would be to express the polymerase gene in eukaryotic cells. To this end the long ORF of HBV had been cloned into a vaccinia virus. The recombinant virus should express the polymerase gene under the control of vaccinia transcription signals. At present the products of this recombinant virus are being analysed by Dr. M. Mackett, Paterson Laboratory, Manchester.

7.5 Conclusion

Segments of the long ORF of the Hepatitis B virus genome have been fused to the E.coli β-galactosidase gene and expressed as fusion proteins. Antisera raised to these fusion proteins recognise two distinct epitopes in the amino and central regions of the putative polymerase gene product. Using immunoblotting, convalescent sera from chimpanzees infected with HBV were shown to contain antibodies that cross-reacted with the fusion proteins. Extracts of E.coli expressing the gene fusion products were also shown to exhibit a polymerase activity with the characteristics of the authentic endogenous Hepatitis B polymerase.

Thus, the long ORF of HBV is expressed during the course of viral infection and its product has polymerase activity (McGlynn and Murray, 1987).

Indeed from these findings there is little doubt that the endogenous polymerase associated with HBV is encoded by the long ORF and is not a host polymerase encapsidated during maturation of the virus. The antibodies that specifically recognise the polymerase could be used to detect the presence of this protein in hepatomas, Dane particles, and cell lines transformed with HBV. The biochemical characteristics of the polymerase and the action of antivirals upon it can now be investigated using the fusion proteins. Information of this nature will be of fundamental interest and may lead to the development of more effective drugs against hepatitis B virus.

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