

TABLE OF CONTENT

	Page
TITLE PAGE.....	i
DECLARATION.....	ii
DEDICATION.....	iii
PUBLICATION AND PRESENTATIONS.....	iv
ACKNOWLEDGMENT.....	vi
ABSTRACT.....	viii
TABLE OF CONTENTS.....	xii
LIST OF FIGURES.....	xvi
LIST OF TABLES.....	xix
LIST OF ABBREVIATIONS.....	xxi
 CHAPTER 1.0: INTRODUCTION.....	 1
1.1.1. THE FAMILY <i>HEPADNAVIRIDAE</i>	5
1.1.1. VIRAL STRUCTURE.....	6
1.1.2. VIRAL GENOME.....	7
1.1.3. HBV TRANSCRIPTS.....	8
1.1.4. VIRAL GENE PRODUCTS.....	11
1.1.4.1 PRECORE AND CORE GENE PRODUCTS.....	11
1.1.4.2 SURFACE PROTEINS.....	12
1.1.4.3 POLYMERASE.....	15
1.1.4.4 HB _x PROTEIN (X PROTEIN).....	16
1.1.5. VIRAL <i>CIS</i> - ELEMENTS.....	17
1.1.5.1 PROMOTERS AND ENHANCERS.....	17
1.1.5.2 THE ENCAPSIDATION SIGNAL.....	18
1.1.5.3 POLYADENYLATION SIGNAL.....	18
1.1.6. HBV LIFE CYCLE.....	19
1.1.7. IMMUNO-PATHOGENESIS OF HBV INFECTION.....	24
1.1.7.1 TRANSMISSION.....	24
1.1.7.2 PATHOGENESIS OF HBV INFECTION.....	24
1.1.7.3 SELF-LIMITED (ACUTE) HBV INFECTION.....	25
1.1.7.4 PERSISTENT (ASYMPTOMATIC) HBV INFECTION.....	26
1.1.7.5 HEPATOCELLULAR CARCINOMA (HCC).....	28
1.1.8. HBV GENOTYPES: MOLECULAR CHARACTERISTICS AND GEOGRAPHIC DISTRIBUTION.....	30
1.1.8.1 GENOTYPES AND SUBGENOTYPES OF HBV.....	34
1.1.9. HBV MUTANTS: DISTRIBUTION AMONG GENOTYPES AND INFLUENCE ON OUTCOME OF HBV INFECTION.....	39
1.1.9.1 PRECORE/ CORE GENE MUTANTS.....	39
1.1.9.2 BASIC CORE PROMOTER (BCP) MUTANTS.....	42
1.1.9.3 X GENE MUTANTS.....	43
1.1.9.4 S GENE MUTANTS.....	43

1.1.9.5	POLYMERASE GENE MUTANTS.....	45
1.1.10	GENOTYPES AND OUTCOME OF HBV INFECTION.....	46
1.2	AIMS AND ORGANIZATION OF THE STUDY.....	50
CHAPTER 2.0: DISTINCTIVE SEQUENCE CHARACTERISTICS OF SUBGENOTYPE A1 ISOLATES OF HBV FROM SOUTH AFRICA.....		53
	SUMMARY.....	53
2.1	MATERIALS AND METHODS.....	55
2.1.1	SUBJECTS AND SERUM SAMPLES.....	55
2.1.2	DNA EXTRACTIONS, AMPLIFICATION, CLONING AND SEQUENCING.....	55
2.1.2.1	DNA EXTRACTION.....	55
2.1.2.2	DNA AMPLIFICATION.....	55
2.1.2.3	DETECTION OF AMPLIFIED PRODUCT.....	58
2.1.2.4	CLONING.....	59
2.1.2.5	SEQUENCING.....	60
2.1.3	PHYLOGENETIC ANALYSIS.....	60
2.2	RESULTS	
2.2.1.	AMPLIFICATION, CLONING AND SEQUENCING.....	64
2.2.2.	PHYLOGENETIC ANALYSIS.....	64
2.2.3.	COMPARISON OF AMINO ACID SEQUENCES OF SUBGENOTYPE A1 TO THOSE OF SUBGENOTYPE A2 AND OTHER GENOTYPES..	73
2.2.4.	COMPARISON OF THE NUCLEOTIDE SEQUENCES OF <i>CIS</i> -ACTING ELEMENTS OF SUBGENOTYPE A1 TO THOSE OF SUBGENOTYPE A2 AND OTHER GENOTYPES.....	78
2.3	DISCUSSION.....	79
2.4	CONCLUSION.....	86
CHAPTER 3.0: EFFECT OF THE G1888A MUTATION OF SUBGENOTYPE A1 ON THE TRANSLATION OF THE CORE PROTEIN...		87
	SUMMARY.....	87
3.1	INTRODUCTION.....	89

3.2	MATERIAL AND METHODS	92
3.2.1	AMPLIFICATION OF THE PRECORE AND CORE GENE.....	92
3.2.2	<i>IN VITRO</i> -TRANSCRIPTION/TRANSLATION.....	92
3.2.3	CONSTRUCTION OF GFP FUSION PLASMIDS.....	93
3.2.4	SITE DIRECTED MUTAGENESIS.....	95
3.2.5	TISSUE CULTURE AND TRANSFECTION.....	97
3.2.6	FLOW CYTOMETRY.....	98
3.2.6.1	PREPARATION OF CELLS FOR FLOW CYTOMETRY.....	98
3.2.6.2	FLOW CYTOMETRY ANALYSIS.....	99
3.3	RESULTS	100
3.3.1	DISTRIBUTION OF THE G1888A SUBSTITUTION AMONG VARIOUS GENOTYPES.....	100
3.3.2	THE EFFECT OF G1888A ON THE ΔG OF THE ENCAPSIDATION SIGNAL.....	101
3.3.3	THE EFFECT OF THE G1888A MUTATION WAS NOT DETECTABLE IN COUPLED TRANSCRIPTION/ TRANSLATION EXPERIMENTS <i>IN VITRO</i>	102
3.3.4	MEASUREMENT OF TRANSLATION USING GFP EXPRESSING VECTOR AND FLOW CYTOMETRY.....	102
3.3.5	G1888A RESULTS IN A DECREASE IN CORE PROTEIN PRODUCTION. THIS COULD BE DETECTED UPON IN-FRAME FUSION OF THE COMPLETE HBV CORE GENE TO THE GFP –ENCODING SEQUENCE.....	108
3.3.6	LEAKY SCANNING ACCOUNTS IN PART, FOR THE DECREASE IN CORE PROTEIN PRODUCTION.....	109
3.3.7	STALLING OF RIBOSOMES AT THE STOP CODON OF THE α ORF MAY INTERFERE WITH TRANSLATION INITIATION AT THE CORE AUG.....	113
3.4	DISCUSSION	117
3.5	CONCLUSION	121
	CHAPTER 4.0: ANALYSIS OF GENOTYPE D ISOLATES FROM SOUTH AFRICA	122
	SUMMARY	122
4.1	RESULTS	123

4.1.1	PHYLOGENTIC ANALYSIS.....	123
4.1.2	COMPARISON OF AMINO ACID SEQUENCES OF D3 TO THOSE OF OTHER GENOTYPE D MEMBERS AND OTHER GENOTYPES.....	131
4.1.3	COMPARISON OF THE NUCLEOTIDE SEQUENCES OF CIS-ACTING ELEMENTS OF D3 TO THOSE OF OTHER GENOTYPE D MEMBERS AND OTHER GENOTYPES.....	132
4.1.4	ANALYSIS OF THE PRE-S1 REGION OF GENOTYPE D ISOLATES FROM GENE BANK.....	138
4.2	DISCUSSION	141
4.3	CONCLUSION	144
 CHAPTER 5.0: INTEGRATION OF HBV SUBGENOTYPE A1 DNA INTO CHROMOSOMAL DNA DURING ACUTE HEPATITIS...		145
	SUMMARY	145
5.1	INTRODUCTION	147
5.2	MATERIALS AND METHODS	150
5.2.1	PATIENTS STUDIED.....	150
5.2.2	DNA EXTRACTION, AMPLIFICATION, CLONING AND SEQUENCING.....	150
5.3	RESULTS	152
5.4	DISCUSSION	159
5.5	CONCLUSION	165
 CHAPTER 6.0: GENERAL DISCUSSION AND CONCLUSION		166
APPENDICES		170
REFERENCES		176

LIST OF FIGURES

Figure 1.1	Geographical distribution of chronic hepatitis B virus infection...	3
Figure 1.2a	Diagrammatic representation of the complete HBV virion.....	7
Figure 1.2b	Incomplete filamentous viral form.....	7
Figure 1.2c	Incomplete spherical form.....	7
Figure 1.3	Organization of the HBV genome.....	10
Figure 1.4	Model of the HBsAg showing the second hydrophilic region.....	14
Figure 1.5	A diagrammatic representation of the replication process of HBV	21
Figure 1.6	HBV life cycle.....	23
Figure 1.7	Spectrum of liver diseases after HBV infection.....	29
Figure 1.8	HBV genotype distribution across Africa.....	33
Figure 1.9	‘a’ determinant of HBsAg showing the prototype vaccine escape mutant.....	44
Figure 2.1	Dendrogram based on 30 genotype A complete genomes and representative sequences from the remaining 7 genotypes.....	68
Figure 2.2	Dendrogram based on the polymerase gene of 30 genotype A isolates and representative sequences from the remaining 7 genotypes.....	69
Figure 2.3	Dendrogram based on the complete S gene of 30 genotype A isolates and representative sequences from the remaining 7 genotypes.....	70
Figure 2.4	Dendrogram based on the precore gene of 30 genotype A isolates and representative sequences from the remaining 7 genotypes.....	71

Figure 2.5	Dendrogram based on the X gene of 30 genotype A isolates and representative sequences from the remaining 7 genotypes.....	72
Figure 2.6	Comparison of amino acid residues of S, polymerase and X ORFs of subgenotype A1 isolates with amino acids residues of subgenotype A2 and other HBV genotypes.....	75
Figure 2.7	Comparison of the nucleic acid sequences of the cis-acting elements of subgenotype A1 isolates with sequences of subgenotype A2 and other HBV genotypes.....	77
Figure 2.8	HBV polymerase represented as a ribbon diagram.....	76
Figure 3.1	Diagrammatic representation of primer design for site directed mutagenesis.....	97
Figure 3.2	The nucleotide sequence and predicted secondary structure of the HBV encapsidation signal of subgenotype A1 and the introduction of a start codon at position 1888 caused by the G-to-A substitution.....	101
Figure 3.3	<i>In vitro</i> translation of precore and core plasmids.....	103
Figure 3.4	Flow cytometry analysis of GFP expression of the Negative Control, pWay 19, and UpWay19.....	106
Figure 3.5	Expression of GFP in HuH7 cells transfected with GFP-expressing plasmid pWay19 and UpWay19 the average of three experiments is shown.....	107
Figure 3.6	Flow cytometry analysis of GFP expression of coreA1, coreA2, and coreA1+Opt.....	111
Figure 3.7	Expression of GFP in HuH7 cells transfected with core/GFP-expressing plasmid with and without G1888A substitution measured in arbitrary units. The average of three experiments is shown.....	112
Figure 3.8	Flow cytometry analysis of GFP expression of G1888(A5), G1888A(15), G1888A(60), and G1888A(100).....	115
Figure 3.9	Expression of GFP in HuH7 cells transfected with core/GFP-expressing plasmids with G1888A substitution and varying distances of the uORF stop codon from the core AUG. The average of three experiments is shown.....	116

Figure 4.1	Dendrogram based on 34 genotype D complete genomes and representative sequences from the remaining 7 genotypes.....	126
Figure 4.2	Dendrogram based on the polymerase gene of 34 genotype D isolates and representative sequences from the remaining 7 genotypes.....	127
Figure 4.3	Dendrogram based on the complete S gene of 34 genotype D isolates and representative sequences from the remaining 7 genotypes.....	128
Figure 4.4	Dendrogram based on the precore gene of 34 genotype D isolates and representative sequences from the remaining 7 genotypes.....	129
Figure 4.5	Dendrogram based on the X gene of 34 genotype D isolates and representative sequences from the remaining 7 genotypes.....	130
Figure 4.6	Comparison of amino acid residues of S, polymerase and X ORFs of Clade D1 isolates with amino acids residues of clade D2 and other HBV genotypes.....	135
Figure 4.7	Comparison of the nucleic acid sequences of the <i>cis</i> -acting elements of D1 isolates with sequences of D2 and other HBV genotypes.....	137
Figure 4.8	Phylogram of the pre-S1 region of 76 Genotype D isolates from GeneBank.....	140
Figure 5.1	Ethidium bromide stained 1% agarose gel showing pPCR-Script Amp SK(+) plasmid containing amplicons restricted with <i>PvuII</i>	153
Figure 5.2	Schematic representation of the HBV DNA integrant amplified from the serum of acute hepatitis patient (#0962) using primers 455(+) and 1800(-).....	157

LIST OF TABLES

Table 1.1	HBV transcripts.....	11
Table 1.2	Distribution of serological subtypes and genotypes of HBV.....	30
Table 1.3	The serological subtypes of HBV.....	31
Table 2.1	PCR Primers and conditions used for amplification.....	62
Table 2.2	Primers used for sequencing.....	63
Table 2.3	Mean nucleotide divergence (%) of complete genome and individual open reading frame (ORF) sequences of HBV genotype A obtained using DAMBE.....	66
Table 2.4	Mutations within the <i>cis</i> -regulatory elements found predominantly in subgenotype A1.....	78
Table 2.5	Different types of mutations at 1809/1811/1812 affecting HBeAg synthesis.....	83
Table 2.6	Representation of MT mutations in subgenotype A1 isolates.....	84
Table 3.1	Plasmid used for <i>in vitro</i> translation.....	93
Table 3.2	Primers used for plasmid construction.....	94
Table 3.3	Primers sets used for site directed mutagenesis.....	96
Table 3.4	Distribution of the G1888A mutation in different genotypes of HBV.....	100
Table 4.1	Mean nucleotide divergence (%) of complete genome and individual open reading frame (ORF) sequences of HBV genotype D obtained using DAMBE.....	133
Table 4.2	Mutations within the <i>cis</i> -regulatory elements found predominantly in D1.....	138
Table 5.1	HBV “specific” primers and chromosomes complementary to these primers.....	152
Table 5.2	Summary of cloning and sequencing results.....	155

Table 5.3	Liver function test performed on patient with acute hepatitis.....	158
Table 7.1	Flow cytometry analysis of wild-type GFP and uORF + GFP....	174
Table 7.2	Flow cytometry analysis of core/GFP fusion plasmids with or without the G1888A mutation.....	174
Table 7.3.	Flow cytometry analysis of core/GFP-expressing plasmids with G1888A substitution and varying distances of the uORF stop codon from the core AUG.....	174

ε	Encapsidation signal
aa	Amino acid
ALT	Aspartate aminotransferase
ASHV	Arctic squirrel hepatitis B virus
AST	Alanine amino transferase
BCP	Basic core promoter
BQW	Best quality water
cccDNA	covalently closed circular DNA
CURS	Core upstream regulatory sequence
DCPD	Duck carboxypeptidase
DHBV	Duck hepatitis B virus
DNA	Deoxyribonucleic acid
DNAML	DNA maximum likelihood
DR1	Direct repeat one
DR2	Direct repeat two
EnhI	Enhancer one
EnhII	Enhancer two
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
GRE	Glucocorticoid-responsive element
GSHV	Ground squirrel hepatitis B virus
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B virus e antigen

HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular Carcinoma
HHBV	Heron hepatitis B virus
HNF	Hepatocyte nuclear factor
IPTG	isopropyl-beta-D-thiogalactopyranoside
LB	Luria-Bertini medium
LHBs	Large surface proteins
MHBs	Medium surface protein
MHC	Major histocompatibility complex
mRNA	messenger RNA
NEIGHBOR	Neighbor-joining
ORF	Open reading frame
PCR	Polymerase chain reaction
pgRNA	Pregenomic RNA
PHYLP	Phylogeny inference package
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
SHBs	Small surface proteins
STHBV	White stork hepatitis B virus
TP	Terminal protein

uORF	upstream open reading frame
URR	Upstream regulatory region
UV	ultraviolet
WBSCR	William-Beuren syndrome critical region
WHV	Woodchuck hepatitis B virus
WMHBV	Woolly monkey hepatitis B virus
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
YMDD	Tyrosine, Methionine, Aspartic acid, Aspartic acid.