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Effects of a Naturally Occurring Mutation in the Hepatitis B Virus Basal Core Promoter on Precore Gene Expression and Viral Replication

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The basal core promoter (BCP) of hepatitis B virus (HBV) controls the transcription of both the precore RNA and the core RNA. The precore RNA codes for the secreted e antigen, while the core RNA codes for the major core protein and the DNA polymerase and also is the pregenomic RNA. The double mutation of nucleotides 1762 and 1764 in the BCP from A and G to T and A, respectively, is frequently observed in HBV sequences isolated from chronic patients. Several papers have reported conflicting results regarding whether this double mutation is important for e antigen expression. In order to address this issue, we have introduced this double mutation into the HBV genome and studied its effects on HBV gene expression and replication. Our results indicate that the mutated BCP can no longer bind a liver-enriched transcription factor(s) and that the transcription of only precore RNA and, consequently, the expression of e antigen were reduced. The reduction of precore gene expression was accompanied by an increase in progeny virus production. This increase was found to occur at or immediately prior to the encapsidation of the pregenomic RNA. Thus, the results of our in vitro study resolve the discrepancy of previous clinical observations and indicate that this double mutation suppresses but does not abolish the e antigen phenotype. The implications of these findings in the pathogenesis of HBV are discussed.

Hepatitis B virus (HBV) is a small DNA virus with a 3.2-kb genome. The HBV genome contains four open reading frames coding for core, surface, polymerase, and X gene products (for a review, see reference 37). The basal core promoter (BCP) has been mapped to nucleotides (nt) 1744 to 1804 (39, 40) and controls the production of both core RNA and precore RNA. The core RNA has multiple functions: it codes for the core protein, which is the major capsid protein; it serves as the pregenomic RNA, which is packaged into viral cores; and it codes for the DNA polymerase, which reverse transcribes the pregenomic RNA into the partially double-stranded DNA genome. The precore RNA codes for the precore protein which is the precursor of the e antigen. The precore protein contains the entire in-frame coding sequence of the core protein plus a leader sequence which contains a signal sequence required for the secretion of the e antigen (22). The signal sequence is removed from the precore protein sequence by signal peptidase in the endoplasmic reticulum to generate the precore protein derivative p22 (9). p22 is further cleaved at it carboxy terminus by protease(s) in the post-endoplasmic reticulum compartment to generate the mature e antigen for secretion (22, 33).

The e antigen is thought to play a role in the induction of immunological tolerance (18). It has been demonstrated that there is a correlation between e antigen expression and the establishment of chronic infection in the babies of mothers infected with HBV (20). Similarly, in the highly related woodchuck hepatitis virus, e antigen has been shown to be important for the development of chronic infections following perinatal infections (6). Interestingly, despite its importance in the establishment of chronic infections, e antigen is not essential for viral replication (5, 6).

HBV mutants unable to produce e antigen often become the dominant viral quasispecies in the viral population present in the infected individual (3). The mechanism for their increased fitness may involve selection via the immune response (3) or by enhancement of viral replication rates (8). The mutations associated with the loss of e antigen production in patients frequently include stop codons and frameshift mutations within the precore leader sequence (4, 12, 25, 31). Two recent analyses of the mutations occurring in the BCP of Japanese patients with fulminant hepatitis revealed a correlation between the e antigen-negative phenotype and a frequently observed double mutation of A to T (A→T) at nt 1762 and G→A at nt 1764 (21, 27). The implication of this observation was that this double mutation in the BCP could also prevent e antigen expression. In contrast to this finding, Laskus et al. (15) have recently examined the BCP in HBV-infected individuals from the United States and found that while this double mutation was frequently observed, it was not correlated with the e antigen status of patients. Interestingly, by studying another cohort of Japanese patients, Takahashi et al. (30) suggested that this double mutation suppressed but did not abolish e antigen expression and hence have termed this mutation an e-suppressive mutation. Thus, there is a discrepancy concerning the effect of this BCP double mutation on the production of e antigen.

DNase I protection analysis of the BCP performed in our and others' laboratories has demonstrated that several DNA-binding proteins including a liver-enriched factor(s) directly cover the sites of this double mutation (11, 17). A liver-specific factor(s) binding to this region has also been characterized by electrophoretic mobility shift assays (EMSA) (40). As such,

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this double mutation may produce a change in the binding pattern of transcription factors in the BCP and affect precore RNA transcription and e antigen expression. To investigate whether and how this double mutation of A→T and G→A at nt 1762 and 1764, respectively, in the BCP affects precore RNA transcription and e antigen production, we constructed a mutant HBV with these changes and examined the effects of this double mutation on protein binding to the BCP, on transcription of the precore and core RNAs, on the production of e antigen, and on viral replication. In this report we demonstrate that this double mutation prevents the binding of a liver-enriched transcription factor to the BCP. Furthermore, this mutation reduces, but does not abrogate, the transcription of precore RNA and the synthesis of e antigen but does not affect the production of core (pregenomic) RNA and the synthesis of the core protein. In addition, this mutation leads to increased production of mutant progeny virus relative to that of the wild type, possibly by enhancing the packaging efficiency of the pregenomic RNA. Thus, our findings are in agreement with the observations of Takahashi et al. (30) and also provides an explanation of why HBV with this double mutation prevails over the wild-type virus during chronic infections.

MATERIALS AND METHODS

Plasmids. The wild-type HBV construct pWTD contains a head-to-tail dimer of 3.2-kb HBV *adw*2 DNA (32) inserted via its unique *Eco*RI site into the *Eco*RI cloning site of pUC19 (New England Biolabs). Oligonucleotide M1 has the sequence AGGAGATTAGGTTAATGATCT. This sequence corresponds to HBV *adw*2 (nt 1750 to 1770) with the mutations of nt 1765 (A→T) and nt 1767 (G→A) which correspond to the nt 1762 (A→T) and the nt 1764 (G→A) mutations previously described for other HBV strains (15, 21, 27, 30). We will use the map numbers nt 1762 and nt 1764 in this paper to avoid confusion. Mutant plasmid pM1D was constructed by M13-based site-directed mutagenesis procedures with the M1 oligonucleotide (23). Thus, this plasmid is identical to pWTD except that nt 1765 and 1767 of both genomic copies have been mutated from A and G to T and A, respectively. The mutagenesis was verified by restriction digest analysis and direct sequencing. pWTH contains one copy of the wild-type HBV sequence but with a *Hin*dIII site engineered at nt 1820. This plasmid was used in EMSA and methylation interference experiments. The following NF-κB double-stranded oligonucleotide from the immunoglobulin kappa light chain enhancer (nt 3937 to 3957) (28) was used as a nonspecific competitor for competition experiments:

5′-CAGAGGGGACTTTCCGAGAGG-3′
3′-GTCTCCCCTGAAAGGCTCTCC-5′

pXGH5 expresses human growth hormone under the control of the mouse methallothionein promoter. This plasmid was used as an internal transfection control by measuring the amount of secreted growth hormone (Nichols Institute). pRVL$^-$ is a plasmid which contains the HBV genome with a 5-bp insertion in the large surface open reading frame and does not release virion particles (2). pECE-PC′, a precore protein expression plasmid, has been described previously (29). The expression of the precore protein in this plasmid is under the control of the simian virus 40 early promoter. pCMV-core expresses the core protein. The construction of this plasmid has been previously described (36).

Cell culture and DNA transfections. HeLa cervical carcinoma cells were grown in Dulbecco's modified essential medium (DMEM) with 10% fetal bovine serum. Huh7 human hepatoma cells were grown in a 1:1 ratio of DMEM and F12 plus 5% fetal bovine serum. Cells were transfected by the calcium phosphate coprecipitation method (10).

EMSA and methylation interference assays. EMSA were conducted with ^{32}P-end-labeled probes derived from pWTH or pM1D. Reaction mixtures contained 5 μg of nuclear extract (1), 2.5 μg of poly(dI-dC), 4 μl of 5× Stefan's binding buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 5 mM EDTA, 25% glycerol, 1.5 mg of bovine serum albumin per ml, 5 mM dithiothreitol), and H_2O to 20 μl. These mixtures were incubated on ice for 10 min, radioactive probes were added, and the mixtures were incubated for a further 20 min on ice. Samples were electrophoresed on a 4% nondenaturing polyacrylamide gel (26). Methylation interference was carried out according to previously published procedures (11), with the *Hin*dII-*Hin*dIII (nt 1688 to 1820, labeled at nt 1820) or *Stu*I-*Hin*dIII (nt 1705 to 1820, labeled at nt 1705) probe isolated from pWTH.

Northern blot and primer extension analyses of HBV RNA. RNA was isolated by the guanadinium thiocyanate method (24), and Northern (RNA) blotting was performed with a random-primed X gene probe (nt 1386 to 1988) (26). Primer extensions for core and precore RNAs were carried out as described previously (41), with RNA extracted with RNAzolB (Biotecx) and the oligonucleotide 5′-GGTGAGCAATGCTCAGGAGACTCTAAGG-3′ of HBV (nt 2051 to 2024) as a primer. The HBV RNA in core particles was analyzed in the same manner except that the RNA was obtained by lysing cells with phosphate-buffered saline plus 0.1% Nonidet P-40, by removing the nuclei with a 2-min centrifugation at 14,000 × *g*, by pelleting the core particles in the cytoplasmic lysates for 3 h at 70,000 × *g*, and then by extracting the RNA from the core particle pellet with RNAzolB.

Radioimmunoprecipitation of core protein and e antigen. For analysis of core protein and e antigen production, 48 h after plasmid DNA transfection, Huh7 cells were starved for 2 h in methionine-free medium and labeled for 3 h with [^{35}S]methionine in methionine-free medium (200 μCi per 60-mm-diameter plate). After labeling, the media were harvested and cells were lysed with RIPA buffer (10 mM Tris-HCl [pH 8], 150 mM NaCl, 1% Triton X 100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]). These media and cell lysates samples were incubated overnight with 1 μl of rabbit anti-core/e antigen antibody (23). The immune complexes were precipitated with 15 μl of Pansorbin (ICN), which had been preblocked overnight with 1 ml of fetal bovine serum. The precipitates were washed four times with RIPA and finally suspended in 15 μl of Lammli buffer (26). The samples were heated for 5 min at 100°C and electrophoresed on a denaturing SDS–12.5% polyacrylamide gel with a 4.5% stacking gel (26). Gels were fixed in 50% methanol plus 10% acetic acid, enhanced with Enlightening (Dupont), dried, and exposed to autoradiographic film. Southern analysis of excreted viral particles was performed with Duralon-UV blotting membranes (Stratagene) with a random-primed, ^{32}P-labeled, whole-genome-length HBV DNA probe as described previously (16, 34). All experiments were repeated at least three times.

RESULTS

Effect of double mutation of A→T at nt 1762 and G→A at nt 1764 on protein factor binding to BCP. As a first step in the analysis of the protein factors which bind to the BCP, we performed EMSA of the complete BCP (nt 1688 to 1820). Using nuclear extract from the human hepatoma cell line Huh7, we observed three protein-BCP complexes, which we called complexes I, II, and III (Fig. 1A). The signals of complexes I, II, and III were removed by an unlabeled specific competitor (nt 1688 to 1820) but not by a nonspecific oligonucleotide containing the NF-κB site. A nonspecific band which was not removed by the specific competitor was also observed (denoted by an asterisk in Fig. 1A). When nuclear extract from HeLa human cervical carcinoma cell line was used in the EMSA, no significant amount of complex III was detected (Fig. 1A). This finding suggests that complex III is generated by a liver-enriched factor(s). This complex may correspond to the liver-specific complex previously detected by DNase I footprint analysis (11, 17) and EMSA (40).

To investigate how the double mutation of A→T at nt 1762 and G→A at nt 1764 affects the binding of protein factors to the BCP, an EMSA was performed with a BCP fragment which contains these two point mutations. As shown in Fig. 1B, while the formation of complexes I and II was not significantly affected by this double mutation, complex III was almost entirely lost. These results indicate that the double mutation at nt 1762 and 1764 can prevent complex III formation.

Localization of protein factor binding site of complex III. To precisely define the protein factor binding site of complex III, we next performed methylation interference analysis. The BCP DNA fragment was methylated with dimethyl sulfate, and an EMSA was performed with the methylated DNA. Free probe and complex III were isolated from the gel, cleaved with piperidine, and electrophoresed on a sequencing gel. As shown in Fig. 2A, the methylation of G at residues 1759, 1760, 1766, and 1767 on one strand and G-1769 on the other strand interfered with complex III formation. Thus, the protein factor binding site of complex III includes nt 1759 to 1769, which directly cover the sites of the double mutation. The sequence of this region of the BCP is shown in Fig. 2B.

Effects of introduced mutation on HBV precore gene expression. To investigate whether this double mutation affects HBV RNA transcription, we performed Northern blot analysis. Fig-

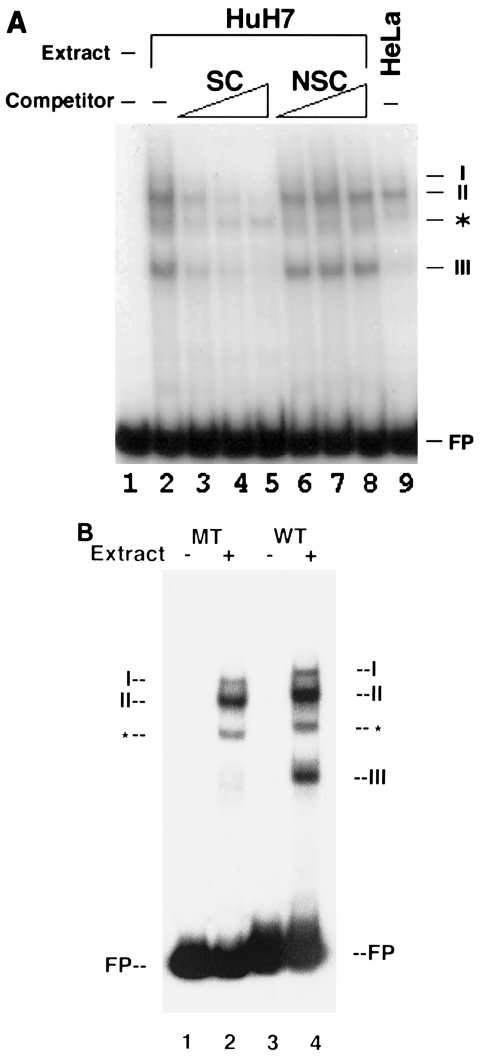


FIG. 1. Analysis of double mutation effects of A→T at nt 1762 and G→A at nt 1764 on the binding of protein factors to the BCP. (A) The complete BCP (nt 1688 to 1820) was used as a probe. This DNA fragment was end labeled with [γ-^{32}P]ATP, incubated with 5 μg of Huh7 or HeLa nuclear extract, and electrophoresed on a 4% nondenaturing polyacrylamide gel. Lanes: 1, probe alone without nuclear extract; 2, probe and Huh7 nuclear extract; 3 to 5, probe and Huh7 nuclear extract with 10, 30, and 75 ng of nonlabeled specific competitor (SC) (nt 1688 to 1820) respectively; 6 to 8, probe and Huh7 nuclear extract with 10, 30, and 75 ng of nonspecific competitor (NSC) (NF-κB site), respectively; 9, probe and HeLa nuclear extract. (B) Wild-type (WT; nt 1688 to 1820 of pWTH) or mutant (MT; nt 1688 to 1806 of pM1D) probe was electrophoresed as described for panel A without (−) or with (+) Huh7 nuclear extract. I, II, and III mark the locations of complexes I, II, and III, respectively. The asterisk denotes the location of a nonspecific band. FP, free probe.

ure 3 is a representative result of Northern blotting of RNA extracted from Huh7 cells transfected with the pUC19 control, the pWTD wild type, or the pM1D mutant HBV head-to-tail genomic dimer construct. No major difference in the expression of viral transcripts was observed, indicating that this double mutation did not grossly affect HBV RNA transcription. Because of their similar sizes, the precore and core RNA could not be resolved by Northern blotting. For that reason, we next analyzed the expression of these two transcripts by performing primer extension analysis. As shown in Fig. 4, while the amount of core RNA produced was the same for both wild-type pWTD-transfected and mutant pM1D-transfected cells, the production of precore RNA in mutant-transfected cells was only about one-third that of wild-type-transfected cells. The primer extension product of the core RNA migrated with a size of 230 nt, corresponding to a start site at nt 1821, while that of the precore RNA gave a product with a size of 260 nt, corresponding to the major start site at nt 1791. Both start sites were the same as those previously observed (13, 35, 41). Therefore, the mutation did not affect the selection of start sites for the transcription of either the core or the precore RNA but did specifically reduce the transcription level of precore RNA.

Since the precore RNA specifically gives rise to the e antigen, the expression of e antigen in Huh7 cells transfected with the mutant and wild-type HBV genomic DNA was also analyzed by radioimmunoprecipitation. The results are shown in Fig. 5. As expected from the RNA results, the secretion of e antigen by the mutant pM1D DNA was correspondingly reduced relative to that expressed by the wild-type pWTD DNA. The expression of the core protein, on the other hand, was not affected (Fig. 5).

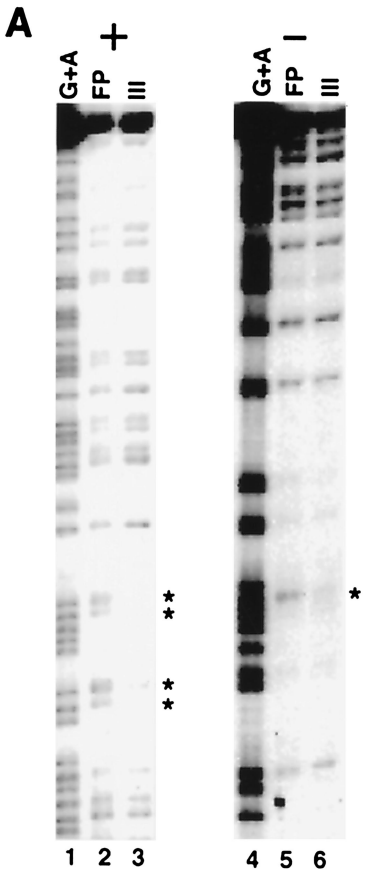
Effects of the double mutation in the BCP on virion production. When virus particles were precipitated from the supernatant of transfected cells with polyethylene glycol and subjected to Southern blot analysis, the effect of the mutation on the secretion of HBV virions was also evident (Fig. 6). Two- to threefold more virions were consistently observed in media harvested from mutant pM1D-transfected cells than in media harvested from wild-type pWTD-transfected cells. This difference of virus production was not due to variation of transfection efficiency. As shown in Fig. 6B, the transfection efficiency of pM1D in various experiments, as determined by the growth hormone activity expressed by the internal control plasmid, pXGH5, was almost identical to that of pWTD.

To examine at which stage of the viral life cycle the double mutation of nt 1762 and 1764 enhanced HBV virion production, the core particles in the cytoplasm of wild-type pWTD- or mutant pM1D-transfected cells were isolated, and the amount of packaged RNA was analyzed by primer extension (41). As demonstrated in Fig. 7, significantly more core (pregenomic) RNA was detected in core particles isolated from pM1D-transfected than from pWTD-transfected cells. This result demonstrated that the double mutation in the BCP most likely enhanced the progeny virus production at or immediately prior to the step of pregenomic RNA encapsidation, as both wild-type and mutant viruses produced similar amounts of core (pregenomic) RNA for encapsidation.

DISCUSSION

There has been controversy regarding whether the double mutation of A→T at nt 1762 and G→A at nt 1764 in the HBV genome is responsible for the loss of e antigen expression in patients. In order to resolve this controversy and to understand how these nucleotides might regulate e antigen expression, we introduced the mutation of these two nucleotides into the HBV genome. As shown in Fig. 1, our results indicated that this double mutation prevented the binding of a liver enriched protein factor(s) to the HBV DNA fragment containing the BCP. By a methylation interference assay, we were able to map the liver-enriched protein factor binding site to nt 1759 to 1769, which directly cover the location of this double mutation (Fig. 2). This region has previously been found by us and others to be bound by a liver-enriched factor (11, 17, 40).

The introduction of this double mutation into the HBV genome reduced the precore RNA level and correspondingly the e antigen protein level to approximately one-third that of wild-type HBV (Fig. 4 and 5). This finding is in good agree-



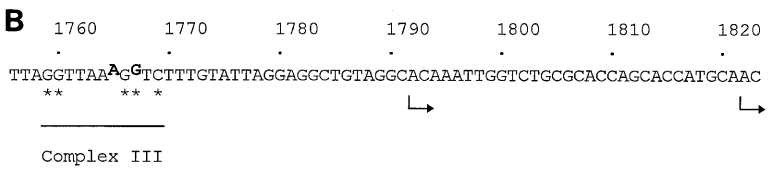


FIG. 2. Methylation interference analysis of the protein factor binding site of complex III. (A) Methylated ^{32}P-end-labeled probe of the *Stu*I-*Hin*dIII (+; nt 1705 to 1820, labeled at nt 1705) or the *Hin*dII-*Hin*dIII (−; nt 1688 to 1820, labeled at nt 1820) DNA fragment from pWTH was incubated with 5 μg of Huh7 nuclear extract and electrophoresed on a 4% nondenaturing polyacrylamide gel. Complex III was isolated, eluted from the gel, subjected to piperidine cleavage, and run on a 6% sequencing gel. FP, free probe; III, complex III; G+A, G+A sequencing ladder; +, positive strand of the HBV genome; −, negative strand of the HBV genome. (B) Nucleotide sequence of the protein factor binding site in the BCP. Asterisks mark the location of G residues at which methylation interferred with complex III formation. The raised boldface letters mark the location of nt 1762 and nt 1764. Arrows indicate the transcription start sites of the precore (nt 1791) and core (nt 1821) RNA. Nucleotide sequence numbering in the figure is based on HBV adw2.

ment with the finding of Takahashi et al. (30), who suggested that this mutation would not abrogate e antigen production but would reduce e antigen expression level by an average of 70% in patients. This double mutation had no effect on the transcription of the core RNA or other HBV transcripts (Fig. 3 and 4). As such, the liver-enriched transcription factor which binds to nt 1759 to 1769 of the BCP appears to be important solely for the production of the precore RNA, and indicating that the mechanism regulating the transcription of the precore RNA may be separate from that regulating the transcription of the core RNA, as suggested by Chen et al. (7).

As revealed in the methylation-interference assay, a single methylation event was sufficient to prevent the binding of the protein factor to nt 1759 to 1769. Indeed, we have noticed that a single mutation of A→T at nt 1762 or G→A at nt 1764 was sufficient to reduce the formation of complex III (data not shown). Thus, unresolved is the question of why this double mutation is observed with such high frequency when a single mutation or other mutations in this region might equally well disrupt complex III formation. One possible explanation is that the single mutation or other mutations may create lethal mutations to the overlapping X protein coding sequence. In this regard, the double mutation which changes Lys to Met at codon 130 and Val to Ile at codon 131 may be required to maintain a functional X protein. Another explanation is that any BCP mutation other than this double mutation might also affect core RNA transcription. Insight into the process by which these mutations arise would shed valuable light on this intriguing phenomenon.

Along with the reduced production of precore RNA and e antigen caused by the double mutation at nt 1762 and 1764 was a concomitant increase in the synthesis of progeny virus (Fig. 6). This increase was not due to the increased expression of other HBV genes since the levels of the core RNA and the surface RNAs were not affected by the double mutation. In our preliminary studies, we found that the precore gene expressed by a heterologous promoter could reduce the replication efficiency of the HBV mutant carrying the double mutation in a *trans*-complementation experiment (data not shown). This result, which is in accordance with previous reports that precore transcription could suppress viral progeny synthesis (14) and that some e antigen-negative mutants had higher than wild-type levels of replication (38), indicates that the increase of progeny virus production caused by the double mutation could be at least partially attributed to the reduction of precore gene expression. In order to understand how the double mutation at nt 1762 and 1764 increased viral progeny production, we analyzed the levels of pregenomic RNA encapsidated in core particles. Despite our observation that equal levels of the pregenomic (core) RNA were produced by both the mutant and the wild-type HBV genome (Fig. 4), we found more pregenomic RNA encapsidated in the core particles from mutant-transfected cells than from wild-type-transfected cells (Fig. 7). Therefore, the increase in progeny virus production most likely occurred at or immediately prior to the pregenome encapsidation step of viral assembly. It is unlikely that the precore RNA suppressed the encapsidation process by competing with the almost identical pregenomic RNA for the core protein, since it has been demonstrated that the packaging signal ε is sup-

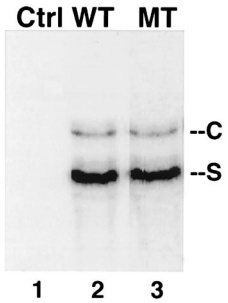


FIG. 3. Northern blot analysis of HBV RNA from wild-type or mutant HBV DNA-transfected cells. RNA was collected from Huh7 cells (100-mm-diameter plate) transfected with 20 μg of pUC19 (Ctrl), pWTD (WT), or pM1D (MT) DNA, electrophoresed on a 1% agarose gel, blotted to nitrocellulose, and probed with a ^{32}P-labeled X gene fragment. The locations of the core (C) and surface (S) gene transcripts are indicated.

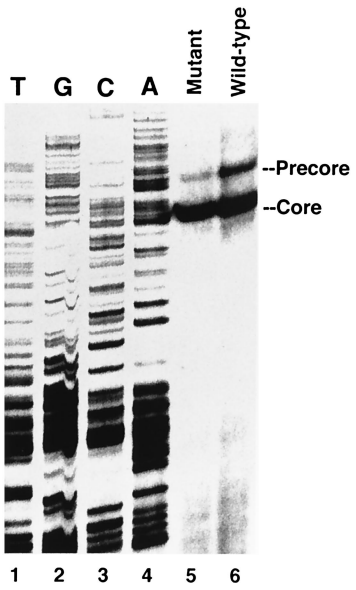


FIG. 4. Primer extension analysis of RNA isolated from wild-type and mutant HBV DNA-transfected cells. RNA was collected from pWTD (wild-type) and pM1D (mutant)-transfected Huh7 cells (10 μg of DNA per 60-mm-diameter plate) and analyzed by primer extension for precore and core transcripts. Lanes: T, G, C, and A, sequencing reaction mixtures of HBV DNA with the same primer. The positions of the precore and core transcripts are indicated on the right.

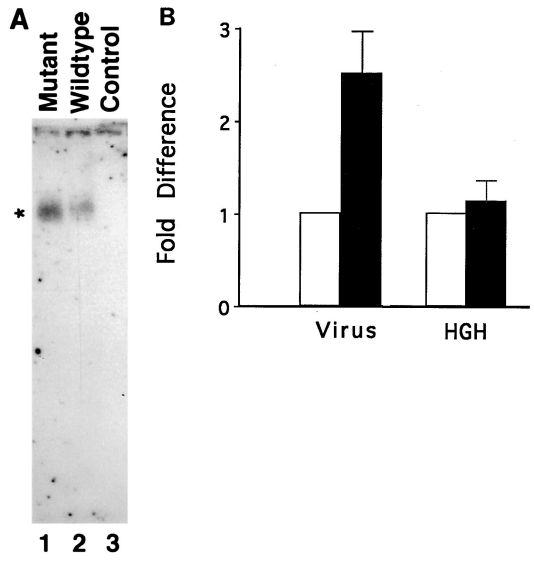


FIG. 6. Quantitative analysis of virus particles secreted from transfected cells. One 60-mm-diameter plate of Huh7 cells was transfected with 10 μg of pRLV$^-$ (control), wild-type (pWTD), or mutant (pM1D) plasmid. In each transfection experiment, 0.2 μg of pXGH5 was also included as an internal control for monitoring transfection efficiency. At 48 h after transfection, an aliquot of the incubation medium was used for determining the growth hormone activity and the rest was precipitated with polyethylene glycol. HBV particles were resuspended and electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and probed with a ^{32}P-labeled HBV wild-type whole genome fragment. (A), Representative result of Southern blotting. *, position of HBV virion DNA. (B), Statistical analysis of transfection results. Virus, comparison of virus yield; HGH, comparison of transfection efficiency. Open bars, wild type; closed bars, mutant. To calculate the relative virus yield, autoradiograms as the one shown in panel A were analyzed with an LKB Broma ULTROSCAN XL laser densitometer, and the fold difference was calculated by dividing the density of the mutant signal by that of the wild type after adjustment of the signal relative to transfection efficiency. To calculate the transfection efficiency, the value for growth hormone activity expressed by pM1D-transfected cells was divided by that for pWTD-transfected cells. The results shown are the means obtained from six independent transfection experiments; error bars indicate standard deviations.

pressed in the precore RNA (19), and that some e antigen-negative mutants which were still capable of producing normal levels of precore RNA showed an increased replication rate (38). We have previously found that a small fraction of the e antigen precursor p22 is not translocated into the endoplasmic reticulum lumen but could be released back into the cytosol after the removal of the signal sequence (9, 23). Thus, it is perhaps more likely that the cytosolic p22, which is almost identical to the core protein, interacts with the core protein and/or pregenomic RNA and interferes with RNA encapsidation. Further experiments will be required to confirm the mechanism by which this enhancement of viral production occurs. Regardless of the molecular mechanism, this phenomenon provides an explanation for the predominance of this viral quasispecies in the majority of the chronic hepatitis patients (30). The increased replication efficiency will allow this quasispecies to become the predominant viral species after multiple rounds of replication in patients.

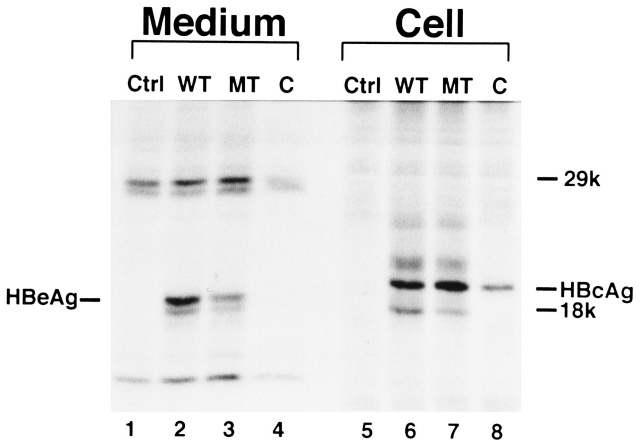


FIG. 5. Immunoprecipitation of precore and core proteins from wild-type and mutant HBV DNA-transfected cells. Medium and cell samples from ^{35}S-labeled Huh7 cells (60-mm-diameter plate) transfected with 10 μg of pUC19 (Ctrl), pWTD (WT), pM1D (MT), or pCMV-core (C) DNA were immunoprecipitated with anti-core (HBcAg) and anti-e antigen (HBeAg) antibody and run on an SDS–12.5% gel. The migration positions of prestained molecular weight standards (Gibco-BRL) are shown on the right.

The double mutation of A→T at nt 1762 and G→A at nt 1764 has been found to positively correlate with the serum alanine aminotransferase levels in patients (30). While only 5% of patients with an alanine aminotransferase level of less than 10 Karmen U/liter were found to carry HBV with this double mutation, 60% of patients with an alanine aminotransferase level of >35 U/liter were found to carry this HBV mutant. The increase of this hepatitis symptom could be due to the reduction of e antigen expression, which enhances the immune response to infected hepatocytes (3) and/or the increased replication rate of the virus which may be cytopathic to infected cells.

In summary, we have analyzed the effects of the frequently observed BCP mutation of an A→T at nt 1762 and a G→A at nt 1764 on HBV gene expression and replication. The mutated BCP no longer was able to bind a liver-enriched transcription factor(s), and this lack of factor binding was found to be associated with a reduction in the transcription of only precore

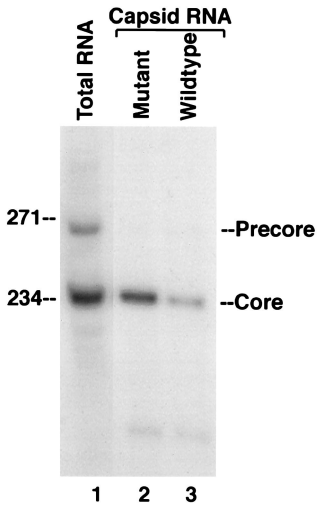


FIG. 7. Analysis of encapsidated pregenomic RNA. Intracellular core particles were isolated from pM1D (mutant)- or pWTD (wild-type)-transfected Huh7 cells. RNA was isolated from these core particles and used for primer extension. Lanes: 1, primer-extension products of a total RNA sample isolated from pWTD-transfected Huh7 cells; 2, primer-extension product of RNA isolated from the core particles of pM1D-transfected cells; 3, primer-extension product of RNA isolated from the core particles of pWTD-transfected cells. Note the selective encapsidation of core (pregenomic) RNA. The sizes (in nucleotides) of the DNA size markers are indicated on the left.

RNA and consequently a reduced production of e antigen. The mutated BCP was also found to increase the amount of pregenomic RNA packaged and progeny virus produced. This increase in the replication rate, which appears to be due to the reduction of precore gene expression, could explain why the HBV mutant carrying this double mutation often prevails over the wild-type virus during chronic infection.

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