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Coordinate Regulation of Replication and Virus Assembly by the Large Envelope Protein of an Avian Hepadnavirus

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We have used linker scanning and site-directed mutagenesis in an attempt to distinguish among the known functions of the duck hepatitis B virus large envelope protein, p36. We found that linker-encoded amino acid substitutions in at least one region of the pre-S envelope protein p36 produced defects in both the production of enveloped virus and the regulation of covalently closed circular DNA (cccDNA) synthesis. Most linker substitutions, typically in the 5′ two-thirds of the pre-S region of the p36 gene did not affect either cccDNA regulation or enveloped virus production but did destroy the infection competence of the enveloped particles produced. Single amino acid substitutions of residues 128 and 131 demonstrated a similar correlation between defects in the ability of p36 to support enveloped virus production and to control cccDNA levels. We concluded from these studies that virus production and cccDNA regulation probably require a common activity of p36.

Duck hepatitis B virus (DHBV) is an animal model for the human hepatitis B virus (8). The extracellular virus is a 40- to 42-nm spherical particle with an outer envelope containing two viral-encoded envelope proteins, p36 and p17 (11, 13, 17). Each envelope protein is produced from a specific mRNA by translational initiation at different start codons in the envelope open reading frame (1). The small envelope protein (p17) is highly hydrophobic, consists of 166 amino acids, and comprises 75 to 90% of the viral protein in the viral lipid envelope (13, 10). The large protein (p36) contains all the amino acids found in the small protein and has an additional unique sequence of 162 N-terminal amino acids referred to as the pre-S domain. P36 is myristylated at the N terminus (9, 13, 15), and this modification is required for the initiation of infection by DHBV (15).

The large envelope protein has at least three functions in the viral life cycle. Mutational analysis has established that p36 (i) inhibits the synthesis of viral transcriptional templates (covalently closed circular DNA [cccDNA] molecules in the nucleus) that are produced from viral DNA synthesized in the cytoplasm, (ii) participates, along with the small protein, in the formation of the viral envelope and is required for production of enveloped DNA-containing particles, and (iii) is required for infectivity of enveloped viral particles (14, 15). Previous genetic studies did not clearly differentiate the functions of p36 required for the regulation of the size of the pool of cccDNA from those required for enveloped virus production. We sought, therefore, to distinguish these two functions by linker scanning and site-directed mutagenesis.

The results indicated that the genetic elements that specifically control cccDNA levels and those that control the production of enveloped virus were tightly linked within the pre-S coding region of the p36 gene and were distinct from those that control infectivity of enveloped virus. In addition, these two regions within the pre-S half of the p36 gene could act independently of each other since p36 genes with mutations at either locus could complement each other for the production of infection-competent virus particles.

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MATERIALS AND METHODS

Plasmids and mutations. The wild-type p36 expression plasmid (puc119CPS) consisted of pUC119 in which the cytomegalovirus (CMV) immediate early promoter was positioned to drive expression of a pre-S message from a segment of DHBV DNA, beginning at nucleotide 719 of the DHBV genome (7) and ending at the *Eco*RI site at nucleotide position 3021 (Fig. 1, top). Linker substitution mutations starting with this wild-type pre-S expression plasmid were constructed as depicted in Fig. 1. Nuclease *Bal* 31 was used to create two sets of deletions extending into the pre-S region of the pre-S gene in opposite directions. Deletions from the 5′ end of pre-S were made from the unique *Sac*I site in a polylinker located between the DHBV DNA and the CMV promoter in pUC119CPS. Deletions from the 3′ end of the pre-S region were made from the *Kpn*I restriction enzyme site at nucleotide position 1280 in DHBV. A specific palindromic linker (5′-GGGCCCTGC AGCGCGCTGCAGGGCCC-3′) was ligated to the *Bal* 31-digested ends after they were made blunt with the large fragment of DNA polymerase I (Klenow fragment). Excess linker copies were removed by digestion with the restriction enzyme *Bss*HII (restriction site GCGCGC at center of the linker) followed by religation to regenerate a single copy of the complete linker. The 5′- and 3′-deletion libraries were transformed into the DH5α strain of *Escherichia coli*, and individual deletion mutants were subsequently isolated and sequenced to determine the extent of the *Bal* 31 deletion. Selected deletion mutants from each library were then matched to produce in-frame linker substitutions of approximately wild-type size by cleavage with either *Bss*HII, *Pst*I, or *Apa*I sites in the linker and mixed religation of appropriate fragments from each plasmid. Plasmid pUC119C containing pUC119 and the CMV promoter but no DHBV DNA was used in control experiments.

Single amino acid substitutions were generated by site-directed mutagenesis as previously described (15) by using the method of Kunkel et al. (5). Each mutagenized restriction fragment (*Bgl*II-*Kpn*I) was subcloned into an infectious plasmid (pUC119.CMV.DHBV) in which transcription of the DHBV pregenome-encoding sequence was driven by the CMV promoter (15).

Plasmid pSPDHBV5.1(2×)1165A consisted of a dimer of *Eco*RI-cut DHBV DNA cloned into pSP65. The DHBV

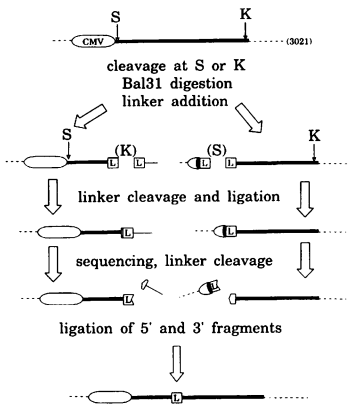

FIG. 1. Construction of linker substitution mutations in the pre-S region of the p36 gene. Detail of the 5′ region of the p36 expression vector, pUC119CPS, is shown at the top. The DHBV sequences subjected to linker mutagenesis are shown as a thick line extending from a *Sac*I site (S) in a synthetic polylinker to the *Kpn*I site (K) at nucleotide position 1285 just downstream of the pre-S region of the p36 gene. Deletions from the 3′ end of the region were made by digestion with *Kpn*I and *Bal* 31 (left side), and deletions from the 5′ end were made by digestion with *Sac*I and *Bal* 31 (right side). Appropriate 5′ and 3′ fragments were ligated to produce in-frame substitution of the linker, as shown.

genome contained a single nucleotide change, which created a translational terminator in pre-S mRNA (15).

Cell culture and transfection. The avian hepatoma cell line, LMH (2, 4) was seeded at 3.5×10^6 cells per 60-mm-diameter dish in F12-Dulbecco's modified Eagle's medium (1:1) supplemented with 10% fetal bovine serum. Transfections were carried out by the calcium phosphate method (3), with 10 μg of DNA per dish as previously described (15). For transfections with the 1165A genome-containing plasmid and a p36 expression plasmid, 5 μg of each plasmid was transfected. If no p36 was required in the transfection, 5 μg of the puc119C plasmid was used as a nonactive carrier along with 5 μg of the 1165A genome. The culture medium containing the calcium phosphate-DNA mixture was replaced with fresh medium 18 h posttransfection. At 2 and 3 days posttransfection, medium was harvested for virus isolation. At 3 days posttransfection, cells were washed once with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (HBS; 2 mM HEPES [pH 7.4], 150 mM NaCl) and harvested for analysis of intracellular viral DNA.

Hepatocytes were prepared and cultured as previously described (10, 16) from 3- to 7-day-old ducklings obtained from Metzer Farms (Redlands, Calif.).

Assay of transfected cells for cccDNA. cccDNA was assayed in transfected LMH cells by the method of Yu and Summers (18). At 3 days posttransfection, the LMH cells were washed once with HBS, 1 ml of cccDNA isolation buffer (10 mM Tris [pH 7.5], 10 mM EDTA, 1% sodium dodecyl sulfate [SDS]) was added to each plate, and the mixture was incubated for 5 min at 37°C. KCl (0.25 ml of 2.5 M solution) was then added to the lysate, and the lysate was briefly vortexed and chilled on ice for 5 min. After removal of the detergent-protein precipitate by centrifugation, the supernatant was extracted with an equal volume of phenol and the nucleic acids were precipitated with ethanol. The dried pellet was dissolved in 40 μl of TE (10:1) (10 mM Tris-HCl, 1 mM EDTA [pH 7.4]).

To eliminate transfected plasmids that usually contaminate the viral cccDNA fraction, the nucleic acids were digested with *Dpn*I, which carries out methylation-dependent cleavage at multiple sites in the plasmid. Residual fragments of plasmid DNA were further digested with exonuclease III. Briefly, 10 μl of the dissolved nucleic acid was incubated with 5 U of *Dpn*I and 25 U of exonuclease III in restriction buffer containing 10 mM Tris (pH 7.5), 10 mM magnesium acetate, 50 mM NaCl, 1 mM dithiotheitol, and 0.01% Nonidet P-40 at 37°C for 3 h. One-half of the viral cccDNA digest was assayed directly by agarose gel electrophoresis and Southern blot hybridization.

Assay of transfected cells for release of DNA-containing enveloped virus. Culture fluids were harvested at 2 and 3 days posttransfection, clarified by centrifugation at 10,000 rpm for 10 min, and stored at 4°C. Precipitation of the virus by adding polyethylene glycol to a final concentration of 10% was followed by incubation at 4°C for 1 h. The precipitates were collected by centrifugation at 925 × *g* for 20 min and dissolved in 120 μl of hepatocyte culture medium containing 10 mM magnesium acetate, and residual DNA from the original transfection was removed by incubation with 100 μg of DNase I per ml at 37°C for 1 h. One hundred microliters of the solution was used to infect a single 60-mm-diameter plate of primary hepatocytes.

DNA-containing virus particles in the sample were assayed by adding 5 μl of the remaining solution to 15 μl of TE (10:1) containing 750 μg of pronase per ml and incubating the mixture for 1 hour at 37°C. We found that this digestion was sufficient to release viral DNA contained in free capsids but not sufficient to release viral DNA from enveloped particles (see Results and Fig. 3). Viral DNA released by the pronase digestion was then removed by the addition of 1 mg of DNase I (type II; Sigma) per ml and incubation for 30 min at 37°C.

The level of enveloped virus was determined either by analysis of viral particles by gel electrophoresis, blotting, and hybridization or by extraction of DNA from the sample, followed by gel electrophoresis and Southern blot hybridization. For direct assay of viral particles, the sample was subjected to electrophoresis through a 1% agarose gel, with the use of standard DNA electrode buffer with recirculation. Virus particles in the gel were transferred to a nylon filter by blotting with TNE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 150 mM NaCl). The filter was thoroughly dried, and the DNA-containing particles were denatured by soaking the filter for 30 s in 0.2 M NaOH containing 1.5 M NaCl. The filter was neutralized with 0.2 M Tris-HCl containing 1.5 M NaCl, washed in TNE, and dried. Viral DNA was detected by hybridization of the filter with a ^{32}P riboprobe specific for detection of a viral minus strand.

For the analysis of viral DNA forms that were present in the pronase-resistant particles, nucleic acids were released by the addition of SDS (final concentration 0.5%) and pronase (500 μg/ml). Viral DNA was analyzed by agarose gel electrophoresis and Southern blot hybridization with a riboprobe specific for the detection of the viral minus-strand nucleic acids.

Assay for total envelope particles in culture fluids. Culture fluids were concentrated by polyethylene glycol precipitation, and the pellets were dissolved in hepatocyte culture medium as described above. Samples corresponding to the precipitate from 0.5 ml of culture fluids were fractionated by electrophoresis through nondenaturing agarose gels as described above and transferred to a nitrocellulose filter by blotting with TNE. The filter was washed in TNE and dried. Bound p36-containing particles were detected by incubation of the filter with rabbit antiserum reactive with p36, followed by ^{125}I-protein A labeling and autoradiography.

Infection of primary hepatocyte cultures. Culture supernatants from transfected LMH cells were concentrated as de-

TABLE 1. Locations and amino acids of linker substitutions

Mutant[a]	Amino acid changes[b]	Location[c]
5-L-15	P5-GPCSALQGGP-E15	A815-L(26)-A842
18-L-27	E18-RALQRAAGP-R27	A855-L(26)-A879
25-L-37	A25-GPCSALQGP-S37	C875-L(26)-G908
32-L-44	G32-GPCSALQGP-D44	G896-L(26)-A929
42-L-51	T42-RALQGP-Q51	C927-L(16)-G950
48-L-58	D48-RALQGP-T58	C945-L(16)-C971
58-L-67	T58-RALQGP-A67	C975-L(16)-T998
67-L-77	A67-GALQRAAGP-N77	G1002-L(26)-A1029
80-L-90	P80-RALQG-P90	C1041-L(16)-C1070
93-L-101	D93-RALQRAAGP-R101	C1080-L(26)-C1101
104-L-115	Q104-GALQRAAGP-P115	G1113-L(26)-C1143
116-L-127	P116-SGPAGP-G127	C1150-L(16)-G1179
118-L-126	S118-PGPAG-P126	C1156-L(16)-G1179
118-L_A-128	S118-PGPAARCRAR-D128	C1156-L(26)-G1180
118-L_B-128	S118-GPCRAR-D128	C1154-L(16)-G1180
126-L-137	P126-GPCRAP-L137	C1178-L(16)-C1207
129-L-137	D129-PGPAARCRAL-L137	C1189-L(26)-T1210
132-L-145	L132-GPAARCRA-Q145	G1198-L(26)-A1234
157-L-167	P157-GPCSALQGP-G167	C1271-L(26)-C1298

[a] Numbers indicate the wild-type amino acid positions flanking the linker.
[b] Amino acids encoded by the linker.
[c] Wild-type nucleotides flanking the linker. The number in parentheses indicates the length of the linker in base pairs.

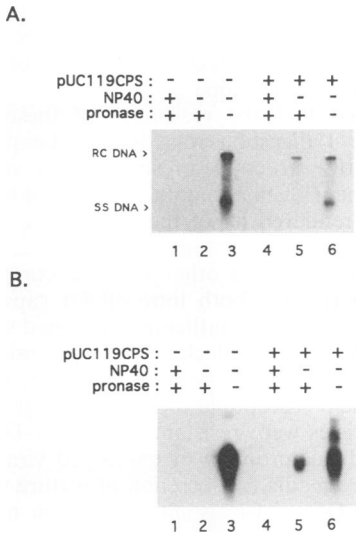

FIG. 2. Selective assay for enveloped virus in culture fluids of transfected LMH cells. Particles from the culture fluids were concentrated and assayed by extraction of nucleic acids, gel electrophoresis, and Southern blot hybridization (A) or by electrophoresis of intact particles in a nondenaturing agarose gel, blotting, denaturation, and hybridization (B). All transfections contained the p36-negative 1165A genome with or without the envelope protein expression vector, pUC119CPS, as indicated. Treatment with Nonidet P-40 (NP40) or pronase was done as indicated. All samples were subsequently digested with DNase I prior to nucleic acid extraction (A) or agarose gel electrophoresis (B). RC DNA, relaxed circular DNA; SS DNA, single-stranded DNA.

scribed above, and the pellets were dissolved in hepatocyte culture medium. Virus precipitated from 8 ml of the LMH cell culture fluid was used to infect a 60-mm-diameter dish of duck hepatocytes. Infection of hepatocytes was carried out at 2 days postplating by the addition of the concentrated virus to the hepatocyte culture medium and incubation for 24 h at 37°C. Subsequently, the medium was changed daily (6 ml per 60-mm-diameter dish) for the course of the experiment.

Analysis of replicative intermediates in primary hepatocytes. Primary hepatocytes were collected 10 days postinfection, and viral replicative intermediates were assayed as previously described (14). Briefly, cells were lysed in a buffer containing 1% SDS, and protein-bound replicative forms were precipitated in a protein-detergent complex by the addition of KCl to a final concentration of 0.5 M. DNA was solubilized from the pellet by the addition of 1 ml of TE containing 0.5 mg of pronase per ml and incubation at 45°C for 90 min with occasional vigorous mixing. The total nucleic acid fraction was then purified by extraction with phenol and precipitated with ethanol. Typically, 1/10th of each cell lysate was analyzed by electrophoresis through a 1% agarose gel, followed by transfer to a nylon membrane and hybridization with ^{32}P-labeled RNA specific for the detection of minus-strand nucleic acids.

RESULTS

Linker substitutions. Linker-encoded amino acid substitutions of up to 10 residues were constructed in the pre-S domain of the large envelope protein of DHBV (p36) as described in Materials and Methods and depicted in Fig. 1. The amino acid substitutions described in this paper are listed in Table 1. The majority of the pre-S region of p36 was covered by the mutations that we constructed. For the sake of brevity, only about half of the linker substitutions that were made and tested were included in Table 1. The additional substitutions which are not shown in Table 1 covered duplicate regions of pre-S, and the results of analyses of these additional mutants were consistent with the conclusions of the study reported here.

Virus particle assay. In order to determine the effects of pre-S amino acid substitutions on the secretion of enveloped virus particles, we developed an assay that would distinguish between authentic enveloped virus and free capsids which could be found in the culture medium of transfected cells. We found that free capsids were present in the culture medium of cells transfected with a replication-competent viral genome that could not produce enveloped DNA-containing particles. In the experiment for which the results are presented in Fig. 2, we concentrated particles from the culture medium of cells transfected with the mutant genome 1165A (defective in the synthesis of p36) in the presence or absence of a p36 expression vector, pUC119CPS. The particles were analyzed by electrophoresis through a nondenaturing agarose gel. Fractionated particles were assayed for the presence of viral DNA following capillary transfer to a nylon membrane, denaturation, and hybridization to a riboprobe specific for the detection of minus-strand viral nucleic acids (Fig. 2B).

While a single hybridizing species was present in the absence of the envelope protein (Fig. 2B, lane 3), two species were observed in the cotransfection with the envelope protein expression vector (lane 6). The same samples were assayed by extraction of total DNA, agarose gel electrophoresis, and Southern blotting (Fig. 2A, lanes 3 and 6). Particles produced in the absence of envelope protein contained viral DNA typical of intracellular viral DNA replicative intermediates (lane 3), and these particles could be completely destroyed by prior treatment of the sample with pronase and DNase I, resulting in the loss of all virus-specific DNA (Fig. 2, lanes 2). In contrast, while the total DNA pattern of particles from the culture fluid of the cotransfected cells was similar (Fig. 2A, lane 6), treatment of these particles with pronase-DNase I revealed the presence of a resistant fraction containing only viral relaxed circular double-stranded DNA (Fig. 2, lanes 5). Since relaxed

circular double-stranded DNA is the only viral DNA species present in enveloped virus, the result suggested that the resistant fraction represented that fraction of virus capsid particles that had an envelope.

We confirmed that the resistance of these particles to pronase-DNase I digestion was due to the presence of a detergent-sensitive structure, presumably the viral envelope, since pretreatment of this sample with a nonionic detergent, Nonidet P-40, rendered all of the viral DNA in the sample sensitive to pronase-DNase I digestion (Fig. 2, lanes 4). We concluded from these and other reconstruction experiments (data not shown) using both intracellular capsids extracted from transfected cells and authentic enveloped virus from the serum of DHBV-infected ducks that free capsids lacking an envelope were sensitive to pronase digestion, rendering the DNA within them susceptible to DNase I degradation, while enveloped particles were resistant to pronase-DNase I treatment. We used this property of enveloped virus particles to assay the culture media for secretion of mature virus by assay of the pronase-DNase I-resistant particles in nondenaturing agarose gel electrophoresis.

We noted that digestion of samples containing enveloped virus with pronase increased the mobility of virus particles in that sample in nondenaturing agarose gels. For example, in Fig. 2B, the mobility of virus particles from the pronase-DNase I-digested sample (lane 5) was increased over that of particles in the untreated sample (lane 6) and fortuitously was similar to that of free capsids in the undigested sample. This effect could be traced to a retarding effect of nonspecific soluble proteins in the sample on virus particle mobility in nondenaturing gels (unpublished observations). This retarding effect was alleviated by the removal of soluble proteins in the sample by pronase digestion.

Effects of linker substitution on cccDNA levels, enveloped virus production, and infectivity. In order to eliminate the effects of linker substitution that were due to mutations in the overlapping polymerase open reading frame, we assayed p36 function by its ability to complement a replication-competent p36-defective genome for three known properties of p36, i.e., enveloped virus particle production, infectivity, and control of cccDNA amplification. A plasmid containing a dimer of the mutant 1165A DHBV genome was cotransfected into LMH cells with a plasmid that expressed either mutant or wild-type p36 from a transcript driven by the CMV immediate early promoter. Each complementation was assayed for enveloped virus production and cccDNA levels and for the infectivity of enveloped virus in primary duck hepatocytes. The results of these assays for each of the p36 linker substitution mutants are represented in Fig. 3 and 4.

The level of enveloped virus produced in the culture fluids of transfected LMH cells is shown in Fig. 3B. Most mutations that disrupted virus production were clustered in a region of the p36 gene between, and including, amino acid codons 117 and 136. Those mutations that destroyed enveloped virus production also produced high levels of cccDNA (Fig. 3A), indicating that the ability of the p36 protein produced by these mutants was coordinately affected. The majority of mutations did not affect either function of p36.

Linker substitutions outside the region of codons 117 through 136 occasionally were seen to result in high cccDNA levels because of the production of abnormally low levels of p36 (data not shown). In addition, three other mutants that produced detectable levels of p36 (mutants 5-L-15, 25-L-37, and 158-L-167 [numbers indicate the amino acid positions flanking the linker, L]) produced intermediate to high levels of cccDNA as well as enveloped virus in the culture fluids,

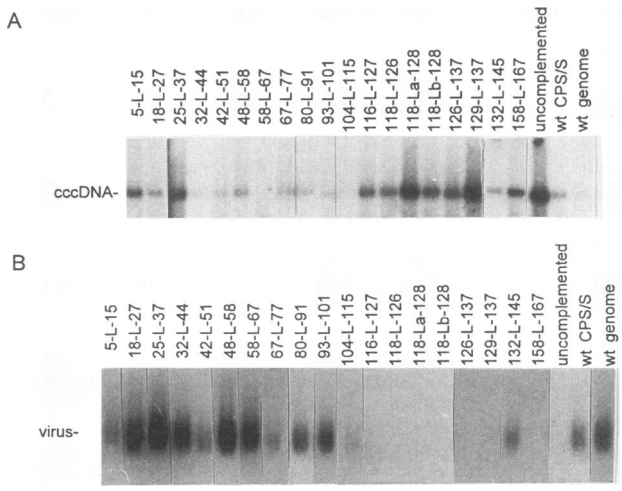

FIG. 3. Levels of cccDNA (A) or enveloped virus (B) produced by transfected LMH cells. LMH cells were transfected with the 1165A mutant genome either uncomplemented or complemented with the mutant or wild-type (wt) pre-S expression vector. Viral cccDNA in the transfected cells and enveloped virus in the culture fluids were assayed as described in Materials and Methods. CPS/S, CMV pre-S expression vector. La and Lb, two different linker substitutions for codons 118 to 126.

indicating that the activities of p36 in addition to its function in enveloped virus production were required for regulation of wild-type levels of cccDNA.

Primary hepatocytes were infected with virus particles isolated from the medium of cotransfected LMH cells and assayed for the production of viral DNA replicative intermediates in hepatocyte cultures. Figure 4 shows the level of replicative intermediates in hepatocytes for some representative mutants. Only one of the 19 mutants (132-L-145) could produce infection-competent particles although most mutations did not destroy enveloped virus production. The single linker substitution mutant that produced infectious particles was altered in a small region of p36 whose function has previously been reported to be insensitive to insertions and amino acid substitutions (6). From this study we concluded that infectivity required the integrity of a large 5′-coding region of the p36 gene.

Complementation between p36 mutants. Incorrect folding of an altered protein could result in defects in both cccDNA

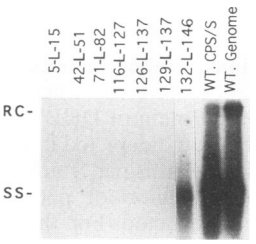

FIG. 4. Infectivity of virus particles containing mutant pre-S proteins. Virus particles from culture fluids of LMH cells transfected with the 1165A mutant genome uncomplemented or complemented with wild-type (WT) or mutant pre-S expression vector were used to infect primary duck hepatocytes. At 10 days postinfection, the hepatocytes were assayed for viral DNA replicative intermediates by Southern blot hybridization. CPS/S, CMV pre-S expression vector.

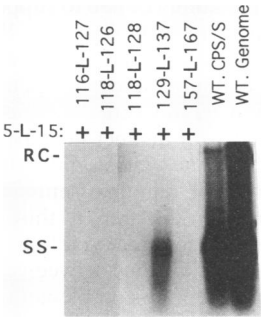

FIG. 5. Intragenic complementation between two p36 pre-S mutants. LMH cells were transfected with the 1165A mutant genome complemented with 5-L-15 in the presence of the indicated mutant envelope protein expression vectors. Culture fluids from the transfections were used to infect primary duck hepatocytes which were analyzed for viral DNA replicative intermediates at 10 days postinfection. CPS/S, CMV pre-S expression vector.

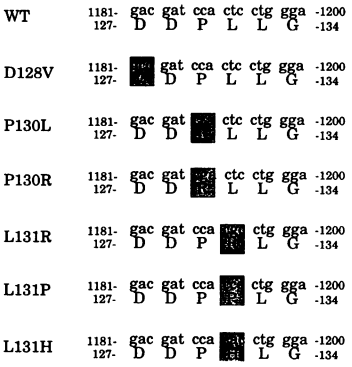

FIG. 7. Missense mutations in p36. Single nucleotide substitutions introduced by site-directed mutagenesis and the resulting amino acid replacements are shaded. The nucleotides and amino acid sequences of the wild-type and mutant p36 genes in the region of mutation are shown. Numbers indicate the positions of nucleotides (6) or amino acids flanking the region shown.

regulation and virus production that were due to protein instability or a failure to be inserted in the appropriate cell membrane. We therefore attempted to identify which of the p36 mutants that showed coordinate defects in cccDNA regulation and virus production were nevertheless able to produce a properly folded protein. Our approach was to test these mutants for their abilities to complement a second p36 linker substitution mutant that was wild type in cccDNA regulation and virus production but which released noninfectious particles (5-L-15). Six linker substitution mutants covering the region between codons 116 and 137 and one downstream mutant (157-L-167) were tested in this way, and the results of one complementation experiment are shown in Fig. 5. In all, only two of the seven mutants defective for enveloped virus production were found to be able to complement the envelope protein produced by mutant 5-L-15 and provide the enveloped virus particle with the p36-specific functions required for infectivity. We concluded that these two linker substitution mutants, 126-L-137 and 129-L-137, produced correctly folded proteins that were specifically defective in a distinct function required both for assembly or export of enveloped DNA-containing particles and for cccDNA regulation. Furthermore, our data indicated that these two mutants were not defective in the production of extracellular viral envelopes, as shown in Fig. 6. This result suggested that mutants 126-L-137 and 129-L-137 were specifically defective in the assembly of DNA-containing capsids into viral envelopes.

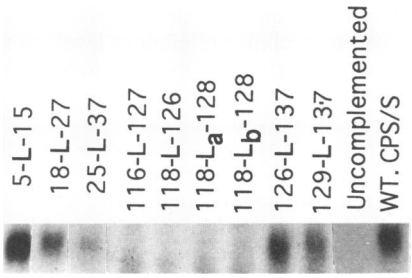

FIG. 6. Production of viral-specific envelope particles by linker substitution mutants. LMH cells were transfected with the 1165A mutant genome either uncomplemented or complemented with the indicated pre-S envelope protein expression vectors. Total particles reactive with a rabbit antiserum against the DHBV envelope proteins were detected by nondenaturing agarose gel electrophoresis and immunoblot analysis, as described in Materials and Methods. CPS/S, CMV pre-S expression vector.

Failure to detect empty envelope particle production in four other mutants defective in cccDNA regulation and virus production, shown in Fig. 6, was consistent with the inability of these mutants to complement 5-L-15 for infectivity (Fig. 5); however, we cannot rule out that these mutations may have destroyed a major epitope detected by our antiserum. The failure of mutant 157-L-167 either to produce enveloped virus or to complement mutant 5-L-15 for infectivity cannot be explained by a failure to be incorporated into viral envelopes, since we detected p36-containing envelope particles in culture fluids of transfected LMH cells. This mutant, which produces intermediate levels of cccDNA, apparently could not supply a function required for infectivity to 5-L-15-containing virus particles, suggesting either that it was not incorporated into DNA-containing particles or that its incorporation into such particles did not complement the defect of the 5-L-15 protein.

Single amino acid substitution mutations. Single amino acid substitutions in the region between amino acids 127 and 136 of p36 resulted in coordinate defects in enveloped virus production and control of cccDNA levels. Substitutions in this region were produced by site-directed mutagenesis of codons 128, 130, and 131. The mutations and the resulting amino acid substitutions are shown in Fig. 7. Mutations were designed to leave the coding in the overlapping P open reading frame unaltered. The mutated segment was cloned into a plasmid in which pregenome transcription was driven by the immediate early CMV promoter, and the mutant genomes were tested for enveloped virus production and cccDNA levels after transfection into LMH cells. Figure 8 shows the results of these assays.

Four mutants involving substitutions at two amino acid residues (D-to-V mutation at position 128 [D128V], L131R, L131P, and L131H) showed defects in enveloped virus production (Fig. 8, lower panel). In these four mutants, cccDNA levels were increased (upper panel). Two mutants involving a single amino acid residue (P130R and P130L) showed no defect in enveloped virus production, and cccDNA levels in both of these mutants were normal. A similar concurrence of mutant or wild-type phenotypes for these two properties of p36 was observed in these and all other mutants isolated at this locus (data not shown), supporting the hypothesis that control

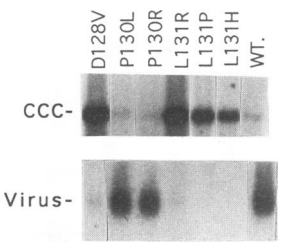

FIG. 8. Effects of missense mutations on production of enveloped virus and cccDNA. LMH cells were transfected with infectious plasmids containing the indicated mutations and incubated for 4 days. cccDNA (ccc) in the transfected cells (top) and enveloped virus in the culture fluids (bottom) were assayed as described in the legend to Fig. 3. WT, wild type.

of cccDNA levels was due to the same p36 activity that was required for enveloped virus production.

DISCUSSION

We have used linker scanning and site-directed mutagenesis in an attempt to distinguish among the known functions of the DHBV large envelope protein, p36. We found that the production of enveloped virus and the regulation of cccDNA synthesis were both sensitive to mutations in at least one coding region of the pre-S p36 gene. Two mutations that produced coordinate defects specifically in these two functions resulted from linker substitutions in the region of codons 127 through 136. Furthermore, most linker substitutions, typically in the 5′ two-thirds of the pre-S region of the p36 gene, did not affect either cccDNA regulation or enveloped virus production but did destroy the infection competence of the enveloped particles produced. The results of our analyses of the linker substitution mutants are summarized in Table 2.

Single amino acid substitutions were made within the region identified by linker substitution as required for virus production and cccDNA regulation. Amino acid replacements at residues 128 and 131 further demonstrated a correlation between defects in the ability of p36 to support enveloped virus production and to control cccDNA levels. We concluded from these studies that virus production and cccDNA regulation probably require a common activity of p36.

TABLE 2. Phenotypes of linker substitution mutants[a]

Mutant	Virus production	cccDNA regulation	Infectivity
5-L-15	+	±	−
18-L-27	+	+	−
25-L-37	+	±	−
32-L-44	+	+	−
42-L-51	+	+	−
48-L-58	+	+	−
58-L-67	+	+	−
67-L-77	+	+	−
80-L-90	+	+	−
93-L-101	+	+	−
104-L-115	+	+	−
116-L-127	−	−	NA
118-L-126	−	−	NA
118-L_A-128	−	−	NA
118-L_B-128	−	−	NA
126-L-137	−	−	NA
129-L-137	−	−	NA
132-L-145	+	+	+
157-L-167	−	±	NA

[a] +, wild-type phenotype; −, defective phenotype; ±, intermediate between wild-type and defective phenotypes or not consistent between experiments; NA, not applicable (defect in infectivity due to lack of virus production).

Linker substitution within three regions of the pre-S p36 gene were associated with distinct defects. In addition to the large region of the gene whose integrity is essential for infectivity and the small region essential for both cccDNA regulation and virus production, a third series of defects associated with the production of stable high levels of protein resulted from linker substitutions between codons 116 and 126. These mutants were unable to complement defects in infectivity, to regulate cccDNA levels, or to support enveloped virus production, presumably because of defects in protein production or stability, since few or no protein products of these mutant genes were found either in the culture fluids (Fig. 6) or in the cells (data not shown). The function of this region of the protein is not known, but it may be involved in primary protein folding or configuration of the protein in the lipid membrane or envelope of the virus. Alternatively, these proteins might have been antigenically altered by the substituted amino acids and rendered undetectable by our antiserum.

Using complementation analysis, we identified two correctly folded mutant proteins that were defective for enveloped virus production and cccDNA regulation but which could nevertheless provide the functions required for infectivity of enveloped virus. These results suggest that at least two independent functions of the pre-S protein exist and that a defect in either function does not prevent the defective proteins from being coassembled in a functional form into the same viral envelope.

The genetic data reported here are consistent with our earlier model for regulation of cccDNA amplification, i.e., that assembly of nucleocapsids into virus particles and their utilization for cccDNA synthesis are competing pathways that are modulated by the availability of p36, which directs nucleocapsids into a pathway for enveloped virus production and thereby inhibits cccDNA synthesis (14, 15). A specific alteration of the ability of p36 to direct assembly of nucleocapsids into this pathway would be predicted by the model to cause a coordinate alteration in its ability to regulate cccDNA amplification, as was shown in this study. These two activities of p36 could be mediated through a physical interaction of the protein with mature nucleocapsids, although such an interaction was not shown by this study.

High levels of cccDNA, however, were not always accompanied by defects in virus production. Examples of this phenotype were previously described for N terminally truncated p36 molecules (15), and the phenotype was ascribed to abnormally rapid p36 secretion requiring higher levels of cccDNA to maintain normal intracellular regulatory levels of the protein. In this study, two linker substitutions showing high levels of cccDNA with enveloped virus production were located near the N terminus of p36, and therefore, these proteins might have been defective in intracellular retention, protein stability, or some other property that impaired the ability of the protein to achieve effective intracellular regulatory levels. In any case, our results indicate that activities of p36 in addition to those that mediate virus production may be required for cccDNA regulation.

During these studies we found that the linker substitution 129-L-137, when it was present in an intact viral genome, did not inhibit viral DNA replication. Moreover, we observed that much of the polymerase gene in the region that overlaps the pre-S p36 genic region could be removed in frame without severely affecting P protein function (5a). The insensitivity of the polymerase open reading frame to amino acid substitutions

in this region, the spacer region (12), is well-known and suggests that the conserved functions for viral replication encoded in this region might be primarily those carried out by p36. If sequence variation in the pre-S region is not constrained by coding requirements of the P open reading frame, then it is possible that spontaneous variants with altered cccDNA regulation may arise and be propagated. Our experiments demonstrated that single nucleotide changes resulting in missense mutations in the p36 gene could produce such changes in p36 function. Although defects in the ability of p36 to inhibit cccDNA synthesis caused reduced virus production in transfected LMH cells, the enhanced synthesis of p36 resulting from partial loss of cccDNA regulation might be expected to compensate for partial defects in its function in hepatocytes, in which all viral gene expression depends on cccDNA rather than on transfected DNA. Such variants in p36 might be expected to display altered levels of virus replication, virulence, or persistence in an infected animal or in a population of susceptible hosts.

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