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Proteolytic Self-Cleavage of Hepatitis B Virus Core Protein May Generate Serum e Antigen

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A model is proposed to explain the presence of the e antigen (HBeAg) of hepatitis B virus (HBV) in the serum of individuals infected with this virus. The e antigen, which has only recently been characterized, is a fragment of the virus core, or nucleocapsid, protein. Serum HBeAg is a valuable clinical marker for active HBV infection because its appearance correlates both with virus replication in the liver and with the presence of circulating virions. In this study a protease-like amino acid sequence was identified at the amino terminus of the core protein sequence. Experimental evidence indicates that HBeAg may be produced by proteolytic self-cleavage of the core protein.

THREE ANTIGEN COMPLEXES ARE ASsociated with infection by hepatitis B virus (HBV). The outer viral coat protein is detected as surface antigen (HBsAg) and the inner nucleocapsid protein as core antigen (HBcAg). The serum of infected individuals contains intact virions (Dane particles) and two viral antigens not associated with infectious particles: HBsAg and e antigen (HBeAg). Free HBsAg circulates as spherical and tubular particles: these noninfectious forms have been used to produce the serum-derived HBV vaccine. In contrast, HBeAg occurs as a soluble protein in the serum. The relationship of HBeAg to HBV has only recently been established. The finding of HBeAg reactivity in virus particles only when they were disrupted in vitro implied that HBeAg was present in the whole virus, but in a cryptic form. Further analysis demonstrated that both HBeAg and HBcAg reactivities were associated with the viral core protein and that HBeAg was, in fact, a fragment of the HBcAg molecule (*1, 2*). Analysis, by amino acid mapping, positioned HBeAg at the amino (NH_2) terminal half of the core molecule. The vast majority of soluble HBeAg in serum is not derived from HBcAg released from disrupted virions. It is, for an unknown reason, synthesized in excess during virus replication in hepatocytes and released from these cells as a soluble protein. Data presented here suggest that HBeAg may be produced by proteolytic self-cleavage of HBcAg in infected hepatocytes.

Human HBV is the prototypic member of the hepadnavirus family, which includes similar viruses which infect ground squirrels, woodchucks, and ducks. Recent studies show that the hepadnaviruses and retroviruses may be genetically related because they share a novel mechanism of genome replication involving reverse transcription of RNA (*3*), and because the nucleotide and predicted amino acid sequences of the genomes of these virus families share sequence homology (*4, 5*). Recent data also indicate that hepadnaviruses may express the polymerase protein as a nucleocapsid polymerase polyprotein, as do retroviruses (*6*). The protease gene sequence of retroviruses has been identified, and the putative active site has considerable homology with cellular acid, or carboxyl, proteases. However, no such protease gene sequence or protease activity has been identified in hepadnaviruses.

In this report I present evidence for a protease-like sequence in the predicted amino acid sequences of hepadnaviruses. The amino acid sequences of the hepadnavirus gene products (core, surface, polymerase, and X genes) were examined for sequences homologous with those of retroviral and cellular proteases. This analysis revealed the presence of a protease-like sequence located at the NH_2 terminus of the viral core protein sequence. This is likely to be a biologically

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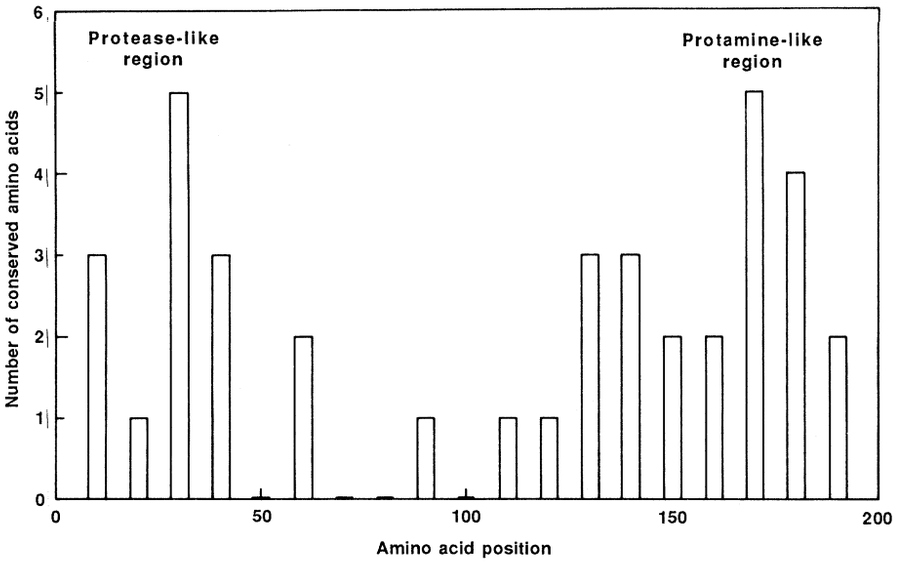

Fig. 1. Histogram of the location of conserved amino acids in the hepadnavirus core protein sequence. The predicted amino acid sequences of 14 hepadnavirus core genes [nine HBV with surface antigen subtypes adr, adw, adyw, and ayw; two duck hepatitis B viruses (DHBV); one ground squirrel hepatitis virus; and two woodchuck hepatitis viruses (*5*)] were aligned, and the number of identical amino acids per block of ten amino acids was plotted against the position of the amino acid group from the NH_2 terminus (No. 10) to the COOH terminus (No. 190) of the molecule. Therefore, the score of 3 at position 10 signifies that three amino acids were identical among all viruses at the same position in the first block of ten consecutive amino acids at the NH_2 terminus of the molecule. Thus, the maximum score for any block of ten amino acids is 10. Basic amino acids (H, K, and R; see legend to Fig. 2) were scored as equivalent in this analysis. It should be noted that pre-core protein sequences were not included in the analysis, and that the core protein sequence in the two DHBV genomes analyzed contains an additional 80 amino acids inserted in the central region of the molecule that were not included in the histogram.

important domain on the core protein molecule because the amino acid sequence of this region is highly conserved among all 14 hepadnaviruses analyzed (Fig. 1). The protease-like sequence is most homologous to the protease sequence of the human immunodeficiency virus (HIV) of the retrovirus sequences examined. One 85–amino acid region of the HIV protease sequence is 45% homologous to the protease-like sequence of HBV (Fig. 2A). Even more striking is the homology between a highly conserved 11–amino acid domain of the hepadnavirus protease-like sequence and the functionally important amino acids located at the active site of cellular carboxyl proteases (Fig. 2B). All sequences share an invariant aspartic acid necessary for the enzymatic function of proteases of this class (*7*). Therefore, this protease-like sequence of hepadnaviruses may represent the gene sequence encoding the protease activity necessary for cleavage of nucleocapsid polymerase polyproteins during viral replication. Development of a specific inhibitor of the protease function may lead to an antiviral therapy that is effective in inhibiting viral replication.

The protease gene sequence of all known retroviruses and retrovirus-like genetic elements (such as the Ty element of yeast) is located at the carboxyl (COOH) terminus of the nucleocapsid gene sequence. Thus, the position of the protease-like sequence of hepadnaviruses on the NH_2 terminus of the nucleocapsid protein is unusual. Analysis indicates that this sequence may have been acquired by recombination with a retrovirus (*8*). One likely candidate is HIV, or a closely related retrovirus, because of the high degree of homology and the fact that both viruses replicate in lymphocytes (*9*). The location of the protease on the core protein

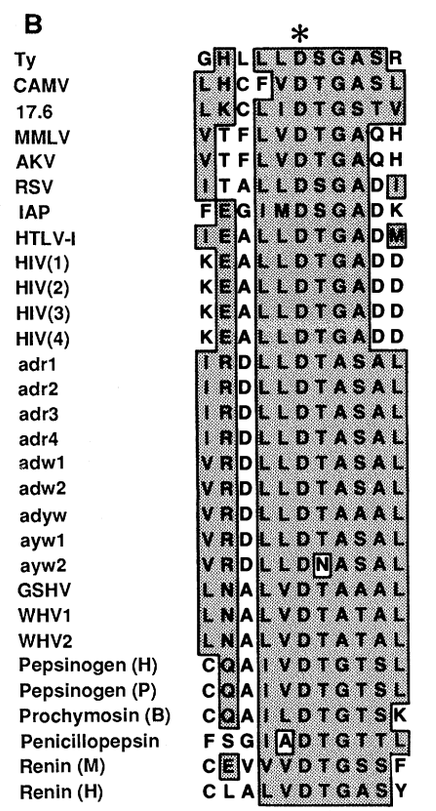

Fig. 2. Protease-like sequence of hepadnaviruses. (**A**) Homology between the protease-like sequence of hepadnaviruses and the protease sequence of retroviruses. The woodchuck hepatitis virus (WHV) protease-like sequence is compared to the protease sequence of three retroviruses: Moloney murine leukemia virus (MMLV), Rous sarcoma virus (RSV), and human immunodeficiency virus (HIV). Dashes represent gaps in the sequence inserted to maximize alignment. (**B**) Putative active site of the hepadnavirus protease. Comparison of the 11 amino acids at the NH_2-terminal active site of cellular carboxyl proteases [bottom (*7*)] with homologous amino acids at the putative active site of the protease gene sequence of retroviruses and retrovirus-like genetic elements [top (*7*)], and the protease-like sequence of hepadnaviruses [center (*5*)]. Shown, from top to bottom, are: yeast transposable element (Ty), cauliflower mosaic virus (CAMV) of plants, *Drosophila* transposable element 17.6, Moloney murine leukemia virus (MMLV), AK virus (AKV), Rous sarcoma virus (RSV), intracisternal A particle (IAP) of mice, human T-cell leukemia virus type I (HTLV-I), four sequences of human immunodeficiency virus (HIV), human hepatitis B virus of surface antigen subtype adr (four sequences), adw (two sequences), adyw, and ayw (two sequences), ground squirrel hepatitis virus (GSHV), two sequences of woodchuck hepatitis virus (WHV), human (H) and porcine (P) pepsinogen, bovine (B) prochymosin, penicillopepsin, and murine (M) and human (H) renin. The asterisk denotes the invariant aspartic acid (D) residue found at the active site of carboxyl proteases. Identical and similar amino acids are enclosed in shaded boxes. Amino acid similarities are those of Toh *et al.* (*4*). Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

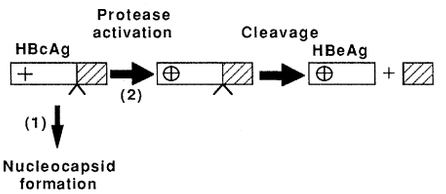

Fig. 3. Model of self-cleavage of HBcAg to produce HBeAg. The hepadnavirus core protein (left) is shown with the protease-like sequence (+) at the NH_2 terminus and the cleavage recognition site, (^) near the COOH terminus of the molecule. (The cleavage recognition site is not known, but may contain the amino acid sequence threonine-valine since these residues are found at the COOH terminus of HBeAg by amino acid mapping, and are conserved among all hepadnavirus core sequences.) The shaded region represents the protamine-like domain of the core protein that is rich in basic amino acids and is utilized in binding nucleic acid prior to nucleocapsid formation. There are two possible pathways: in pathway 1, without protease activation the core protein molecule remains intact, the protamine-like region at the COOH terminus binds a viral nucleic acid genome, and the molecule becomes a subunit of the growing nucleocapsid structure (small arrow); in pathway 2, with protease activation (large arrow), the enzymatic activity on the NH_2 terminus of the molecule cleaves at the protease recognition site (^) dividing the core protein into at least two smaller proteins: HBeAg (unshaded box) and a molecule containing the protamine-like region of the core protein (shaded box). The size of the arrows is approximately proportional to the percentage of molecules following each pathway.

suggests that the enzyme may also cleave the core protein itself into smaller molecules. I hypothesize that the HBeAg molecule is produced in such a reaction. Figure 3 shows a diagram of this model. After the core protein is synthesized in infected hepatocytes, two pathways are available. In the first pathway, the protease is in an inactive form. A cleavage reaction does not take place; the core protein molecule remains intact and is incorporated into nucleocapsid structures. In the second pathway the protease becomes activated (by post-translational modification, for example). This enzymatic activity is utilized to cleave the nucleocapsid polymerase polyprotein. However, the core protein itself is cleaved at a site near the COOH terminus of the molecule. This reaction produces HBeAg (from the NH_2 terminus) and a small protein containing the protamine-like region of the core protein (from the COOH terminus). Since the HBeAg molecule lacks the nucleic acid binding region present on the intact core protein (the protamine-like region), it is useless in nucleocapsid assembly and is exported from the cell.

There is experimental evidence supporting this hypothesis. In a study defining the endogenous protein kinase activity of HBV, Albin and Robinson (*10*) found that after an autophosphorylation reaction the HBV core protein (20.6 kD) was converted into at least two smaller proteins with apparent molecular sizes of 14.7 and 6.0 kD. These are the approximate sizes of the e and protamine-like proteins expected from self-cleavage of the core protein (Fig. 3). In addition, experiments on the in vitro expression of the HBV core protein suggest that a self-cleavage reaction may take place. If the hepadnavirus core protein contains a protease activity, which cleaves at a recognition site on the molecule itself, then it should be difficult to produce the intact core protein in recombinant DNA expression systems. This has been the case both in the synthesis of core protein with the use of mammalian cell expression vectors (*11–14*) and in transfection of mammalian cells with recombinant HBV DNA (*15–17*). Although small quantities of core protein have been detected, the major protein produced has been the HBeAg fragment of the core protein molecule. Recent data indicate that inclusion of pre-core sequences results in production of up to tenfold more e than core protein in these systems. It is interesting that the intact core protein, with little or no HBeAg appearing, has been synthesized in bacterial (*18, 19*) and yeast (*20, 21*) expression systems. Thus the problem in expressing the intact molecule appears peculiar to mammalian cells. One explanation is that these cells possess a protease that recognizes and cleaves the core protein. Another is that a protease on the molecule itself is active only in mammalian and not other cells. If the latter interpretation is true, it is likely that some type of specific post-translational processing of the core protein, found in mammalian cells (for example, glycosylation or phosphorylation) is necessary to generate an active viral protease.

Evidence for the activity of a viral, rather than a cellular, protease in cleavage of viral proteins comes from recent data characterizing HBV fusion polypeptides. This work also suggests that the protease is located at the NH_2 terminus of the core protein molecule. Will *et al.* (*6*) have demonstrated the presence of HBV nucleocapsid polymerase polyproteins in hepatocellular carcinoma (HCC) tissues by serological screening of HCC tissue containing integrated HBV genomes. Detailed analysis of the fusion proteins reveals that they consist of the middle and COOH-terminal region of the core protein fused to the polymerase protein. The NH_2 terminus of the core protein is absent, presumably because the HBV genome integrated in the DNA of these tissues in such a way that the NH_2 terminus of the core gene was deleted or its sequence interrupted. Screening of normal infected liver tissue did not detect the presence of fusion polyproteins. Thus, fusion proteins do not accumulate in infected hepatocytes, presumably because they are subjected to proteolytic cleavage by either a cellular or a viral protease soon after synthesis. However, fusion proteins do accumulate in HCC tissues that contain the same cellular proteases. It is probable, therefore, that the accumulation of fusion proteins in HCC is due to the absence of a viral protease. Indeed, the nucleocapsid polymerase polyproteins in HCC lack the protease-like amino acid sequences at the NH_2 terminus of the core sequence. Thus, the data of Will *et al.* (*6*) can be explained by the existence of a viral protease at the NH_2-terminal region of HBcAg that cleaves the fusion polyprotein during normal infection but is missing in the fusion polyprotein sequence of HCC cells.

It would then appear that the production of HBeAg during replication occurs as an unnecessary side reaction that is tolerated by the virus because the involved protease fulfills a separate, essential function: cleavage of the nucleocapsid polymerase polyproteins to release an active polymerase molecule for genomic replication. As long as enough intact core protein is produced for nucleocapsid production, the replication cycle can be completed. This may be accomplished by inhibiting the action of the protease (via a lack of post-translational processing of some molecules), by immediate binding of the cleavage recognition site to viral genomic nucleic acid (and hence protection from proteolytic cleavage), or by synthesis of core proteins without the amino acids at the NH_2 terminus of the core, or pre-core, protein (*12–14*). Apparently, synthesis of HBeAg, like the formation of HBsAg spherical and filament-like particles of serum, represents a by-product of virus replication. It remains to be seen whether the production of these proteins is advantageous to the virus in any way. In any event, these serum antigens have played an invaluable role in the serological diagnosis of HBV infection.

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8. Hepadnaviruses have two protease-like sequences that are homologous to retrovirus protease sequences. One, as described in the text, is located at the NH_2 terminus of the core protein. The other is located at the NH_2 terminus of the polymerase gene sequence, in a position analogous to the protease gene of retroviruses. The latter protease-like sequence may represent the original protease. However, it lacks the aspartic acid necessary for active

catalytic activity of the carboxyl proteases and may be inactive or only weakly active. The ancestor of the hepadnaviruses may have acquired a second protease, at the NH_2 terminus of the core gene, to compensate for the mutation at the active site of the original protease.
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Calcium-41 Concentration in Terrestrial Materials: Prospects for Dating of Pleistocene Samples

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Calcium-41 has been suggested as a new tool for radiometric dating in the range of 10^5 to 10^6 years. The concentration of cosmogenic calcium-41 in natural samples of terrestrial origin has now been determined by high-sensitivity accelerator mass spectrometry after pre-enrichment in calcium-41 with an isotope separator. Ratios of calcium-41 to total calcium between 2×10^{-14} and 3×10^{-15} were measured for samples of contemporary bovine bone and from limestone deposits. Some prospects for the use of calcium-41 for dating Middle and Late Pleistocene bone and for other geophysical applications are discussed.

THE MEASUREMENT OF NATURAL concentrations of ^{41}Ca (half-life $t_{1/2} \cong 1.0 \times 10^5$ years) (*1*) has been proposed as a new tool for radiometric dating (*2, 3*) and as a geological solar-neutrino detector (*4*). The major interest in ^{41}Ca stems from its potential for establishing the date of calcium-containing materials to earlier times than is possible with ^{14}C ($t_{1/2}$ = 5730 years). Particular interest arises from its prospects as a means of dating Middle and Late Pleistocene bone that contains significant amounts of calcium and that is found at many sites of paleoanthropological interest. The ability to provide an isotopic method of assigning age directly to bone samples in this age range would help clarify uncertainties about chronological relationships among important fossil hominids for a time period during which major events in hominid biological and cultural evolution occurred. However, the natural $^{41}Ca/Ca$ ratio in terrestrial samples is expected to be very low, on the order of 10^{-14} or less. (Throughout this report the term "$^{41}Ca/Ca$ ratio" signifies the isotopic abundance of ^{41}Ca in total calcium.) The long half-life of ^{41}Ca and its decay by electron capture to the ground state of ^{41}K (with only soft x-rays and Auger electrons with energies of about 3 keV as detectable radiation) make decay counting infeasible. We have therefore used the highly sensitive method of accelerator mass spectrometry (AMS) in an attempt to detect ^{41}Ca at natural levels. We pre-enriched the samples with an isotope separator to measure for the first time $^{41}Ca/Ca$ in natural terrestrial samples (*5*). For contemporary bovine bone we measured a value of $^{41}Ca/Ca$ of $(2.0 \pm 0.5) \times 10^{-14}$. For limestone from the surface and depths of 11 m, values of 8×10^{-15} and 3×10^{-15}, respectively, were observed.

The AMS measurements were performed with the Argonne Tandem Linac Accelerator System (ATLAS). The AMS technique makes use of the fact that ions that are accelerated to sufficient kinetic energy (typically a few million electron volts per nucle-

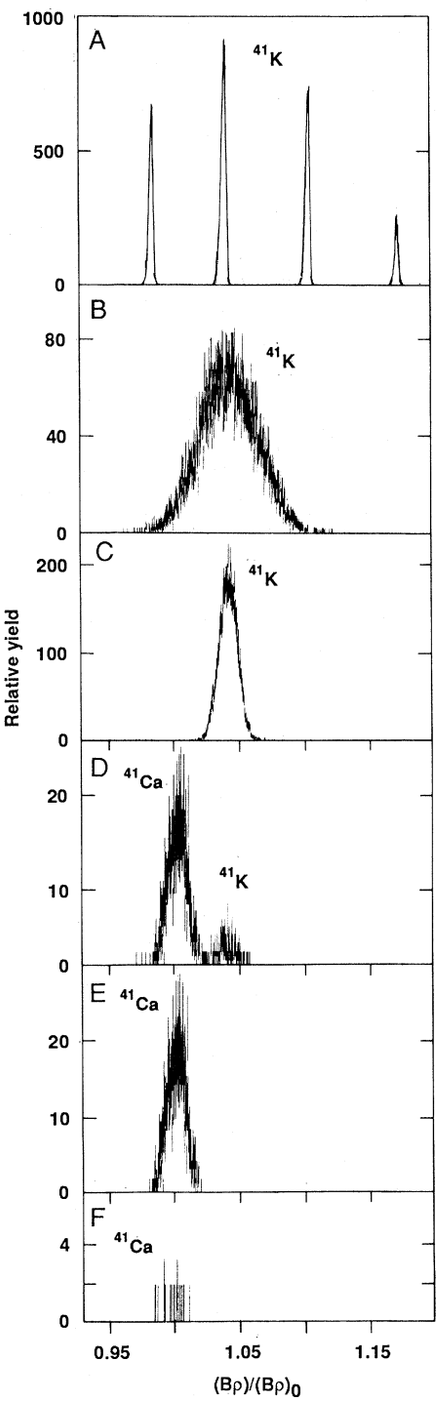

Fig. 1. Position spectra (converted to relative magnetic rigidity $B\rho$, where B is the magnetic field and ρ the bending radius) from the focal plane detector of the spectrograph for ^{41}Ca and ^{41}K yields from various sample materials are shown. The magnetically dispersed charge-state spectrum of ^{41}K ions (**A**) obtained from the passage through a thin gold foil collapsed to a single line when nitrogen gas at pressures of 1.0 torr (**B**) or 8.0 torr (**C**) was introduced into the magnetic field region. The yields for the gas-filled device at 8.0 torr from a calibration sample with known ^{41}Ca concentration are shown in (**D**) and (**E**), and for contemporary bovine (long leg) bone in (**F**). In (E) and (F) additional gating from the detector total-energy and energy-loss signals was applied. For a blank sample no ^{41}Ca counts were detected after a measuring time that was three times as long as that used for the bone sample (see text).

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