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Expression of Hepatitis B Virus Core Antigen Gene in *Saccharomyces cerevisiae*: Synthesis of Two Polypeptides Translated from Different Initiation Codons

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Two recombinant plasmids were constructed that allow expression of the hepatitis B core (HBc) antigen gene in the yeast *Saccharomyces cerevisiae* under the control of the repressible acid phosphatase promoter. One plasmid was designed to produce polypeptide I, which consists of 183 amino acids, and the other plasmid was designed to produce polypeptide II, which has an additional 29-amino-acid sequence at the amino terminus of polypeptide I. The viral genome may code for either one or both of these two polypeptides, depending upon the selection of initiation codons. Both polypeptides produced in yeast cells reacted with anti-HBc antibody and were assembled into spherical particles approximately 27 nm in diameter. Particles made of polypeptide I were stable, whereas those made of polypeptide II readily dissociated when exposed to high salt levels. The antigenicity of the HBc (as defined by its reactivity to anti-HBc antibody in the reversed passive hemagglutination assay) disappeared as the particle dissociated, leaving materials that sedimented slowly and that reacted to anti-hepatitis B e antibody. These observations strongly suggest that native viral cores are mostly (if not all) made of polypeptide I, because it is reasonably stable, and that the N-terminal portion of this polypeptide has some, but not a profound, influence on the assembly of polypeptides into particles.

Hepatitis B virus causes serious liver disorders and is linked to later development of hepatoma. Despite its crucial role in human health problems, there is only limited knowledge of its modes of replication, integration, and tumor induction because the virus multiplies only in human and chimpanzee livers.

Recently this viral genome has been cloned and sequenced (3, 4, 15, 29), allowing us to understand a great deal of the molecular biology of this virus. An example is the research on hepatitis B surface antigen, whose chemical, physiochemical, and immunological properties are under intensive investigation, now that the gene has been cloned and expressed in yeast cells (7, 13, 28).

In this paper we present the results of studies involving a similar approach, with the hepatitis B core antigen (HBcAg) expressed in *Saccharomyces cerevisiae* cells. HBcAg is the major viral nucleocapsid protein consisting of a 19- to 20-kilodalton polypeptide(s). It is assembled into approximately a 27-nm particle that covers the viral genome, and the core particle itself is enveloped by the hepatitis B surface antigen. In no stage of virus propagation has this antigen been detected in the sera of patients, although they often develop antibody against this protein, suggesting that HBcAg is released at a very early stage of infection. HBcAg is thought to be related to hepatitis B e antigen (HBeAg), which often appears in the sera of acute hepatitis patients. Because HBcAg has been obtained only from infectious Dane particles or from liver specimens of special patients, our knowledge about this important viral component has been severely limited.

Sequencing studies have shown that HBcAg has a protaminelike amino acid sequence in the carboxy-terminal portion of the polypeptide (3, 4, 15, 25, 29) and relatively neutral, hydrophilic amino acids at the amino-terminal portion. The sequencing data have shown, in addition, that three of the four cloned viral genomes have two initiation codons separated by 87 base pairs (bp) in the same reading frame (3, 4, 16, 29). The polypeptide produced by translating from the upstream or downstream initiation codon consists of 212 or 183 amino acids, respectively. This difference cannot be detected in molecular weight estimations of the HBcAg obtained from in vivo specimens. It is possible that either one of the two polypeptides is predominantly used or both could be produced equally in vivo. One cloned hepatitis B virus genome happened to have a small deletion, resulting in loss of the upstream initiation codon (15). This, however, does not prove that the smaller polypeptide is the natural HBcAg protein.

We attempted to solve this problem by constructing a yeast expression system that allows separate production of the small (I) and large (II) polypeptides. The schemes for constructing the recombinant plasmids are outlined in Fig. 1. A 735-bp DNA fragment covering the putative HBcAg gene was extracted by digesting the hepatitis B virus genome with *Rsa*I (3). The *Rsa*I fragment includes, in addition to the common coding region for polypeptides I and II, a 42-bp region upstream from the initiation codon for polypeptide II and a 54-bp region downstream from the common termination codon for polypeptides I and II. *Xho*I linkers were added to the ends of this fragment, which was cloned into the single *Xho*I site of pACYC177 (1) to yield pAHBC (Fig. 1). The *Xho*I fragment was extracted from pAHBC and inserted at the unique *Xho*I site in the expression vector, pAM82 (13). The resulting plasmid, pAC301, was expected to allow production of polypeptide II. To prepare polypeptide I, the upstream initiation codon was eliminated as follows: pAHBC was converted into pHBC1 (by converting the upstream *Xho*I site into an *Eco*RI site) and the corresponding

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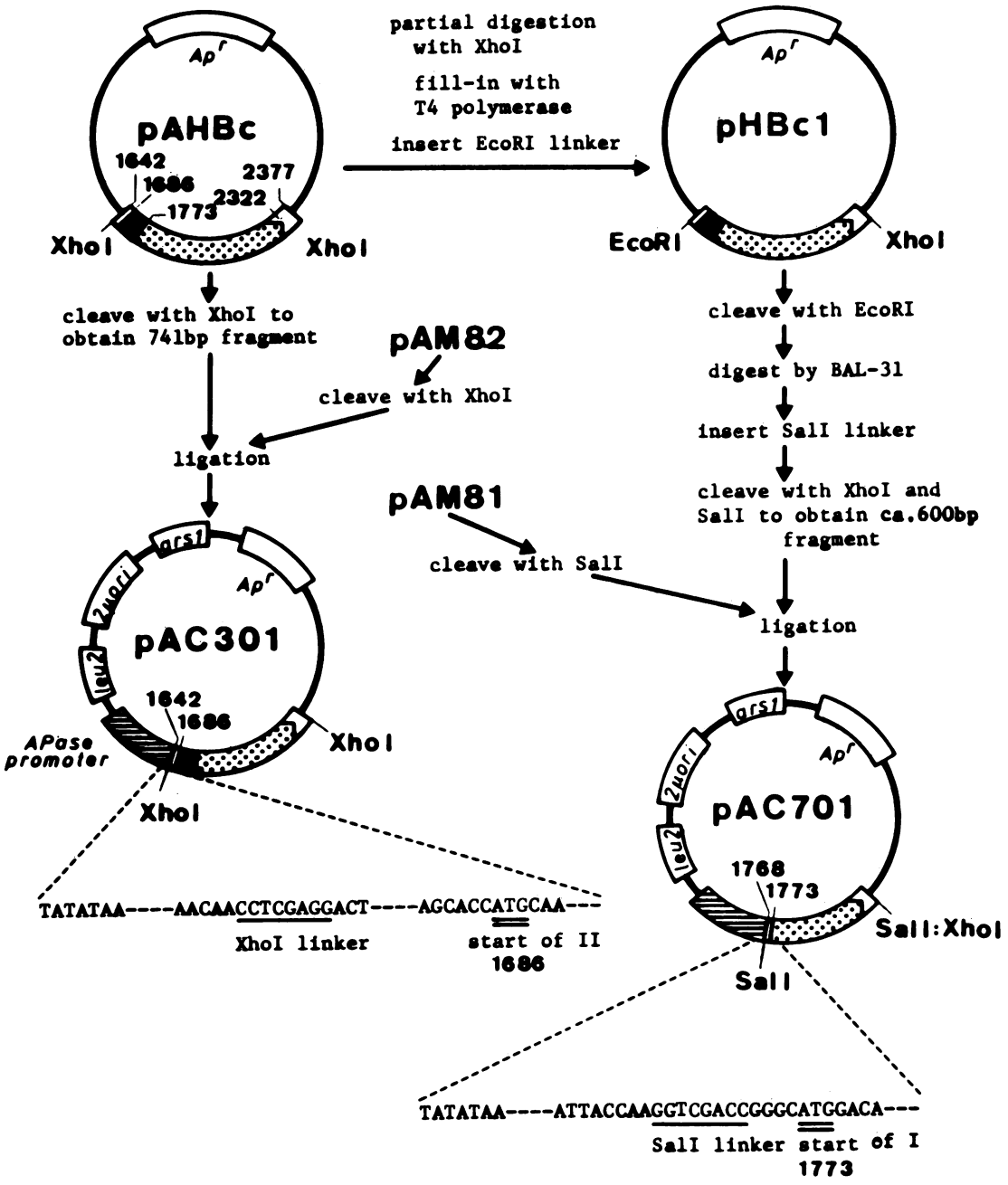

FIG. 1. Construction of plasmids pAC701 and pAC301, which allow expression of polypeptides I and II, respectively. For explanations, see text. Plasmids pAM81 and pAM82 are identical shuttle vectors except for a single *SalI* or *XhoI* cleavage site. The plasmids carry the yeast repressible acid phosphatase (APase) promoter and both replicators in yeast and *E. coli* (13). pAHBc is described in the text. The sequences around the junction of the acid phosphatase promoter and the HBcAg gene in each plasmid are shown. ▬, Region between the upstream initiation codon for polypeptide II and the downstream initiation codon for polypeptide I; ▭, coding region for polypeptide I; ▨, acid phosphatase promoter.

region was cleaved by *Eco*RI and then digested with exonuclease BAL 31. After being sealed in the presence of the *Sal*I linker, the *Xho*I-*Sal*I fragment (ca. 600 bp) was extracted and inserted into the *Sal*I site of an another expression vector, pAM81. This expression vector is identical to pAM82, except that the *Xho*I site has been converted into a *Sal*I site. One of the resulting plasmids, pAC701 (Fig. 1), was chosen. The initiation codons in pAC301 and pAC701 are located 112 bp and 108 bp, respectively, downstream from the TATA sequence of the yeast acid phosphatase promoter.

The recombinant plasmid DNAs propagated in *Escherichia coli* were transformed into a yeast recipient strain, AH22 (6), or into its acid phosphatase constitutive derivative, AH22*pho80* (6, 13), by the lithium acetate procedure (8). The Leu$^+$ transformants were selected on Bulkholder

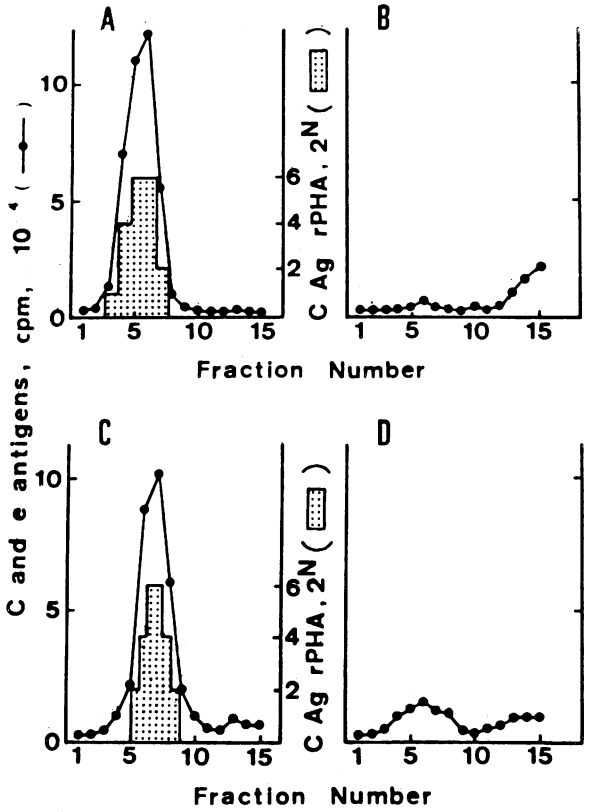

FIG. 2. Sucrose linear gradient centrifugation of HBcAg. An extract of cells carrying pAC701 was prepared and incubated for 2 h at 30°C in the presence (B) or absence (A) of 0.1% SDS–0.1% 2-mercaptoethanol. An extract of cells carrying pAC301 was prepared similarly, except that SDS treatment was omitted. The extract was dialyzed against 1 M NaCl. Samples were taken before (C) and after (D) dialysis. An aliquot (1 ml) of each sample was layered on top of a 5-ml linear sucrose gradient (20 to 60% [wt/wt]) in TEN buffer (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA, 0.1 M NaCl) and centrifuged at 30,000 rpm for 14 h at 4°C with an RPS40T rotor (Hitachi). Fractions were collected and assayed for materials that react with antisera against HBcAg plus HBeAg with an HBeAg radioimmunoassay kit (Abbott Laboratories), which reacts with both HBeAg and HBcAg indiscriminately (●). Also, a reversed passive hemagglutination assay (rPHA) was done for HBcAg (▨). This assay kit prepared by our laboratory does not cross-react with HBeAg. Samples B and D demonstrate the presence of a slow-sedimenting component that must be HBeAg, since it does not react with anti-HBc. A small amount of the fast-sedimenting component in sample D represents the undissociated particles.

TABLE 1. Relative levels of HBcAg gene expression in yeast[a]

Host yeast strain	Plasmid	Codes for polypeptide:	HBcAg and HBeAg (cpm) in yeast cells[b]	
			Induced	Noninduced
AH22	pAC301	II	110,000	510
	pAC701	I	93,000	2,100
	pAM81	none	1,100	500
AH22*pho80*	pAC301	II	110,000	100,000
	pAC701	I	88,000	77,000
	pAM81	none	1,200	480

[a] Yeast cells carrying the plasmid as indicated were grown to 4 × 10^6 cells per ml in minimal medium at 30°C and then were transferred into phosphate-free medium to induce them or into phosphate-containing medium to serve as the noninduced control. When the cell density reached 2 × 10^7 cells per ml, the cells were collected by centrifugation and cell lysates were prepared (13).

[b] Each cell lysate was assayed for HBeAg with an Abbott HBe radioimmunoassay kit, which also reacts with HBcAg.

minimal medium plates supplemented with histidine (20 μg/ml) at 30°C, were grown in liquid medium, and were induced in phosphate-free medium as described previously (13). After 48 h the cells were collected, lysed, and subjected to radioimmunoassay, which detected both HBcAg and HBeAg (18). The results are shown in Table 1.

Cells carrying pAC301 or pAC701 yielded substantial amounts of materials that react with HBc and HBe antibodies, whereas control cells carrying pAM81 expressed only the background activity of the HBcAg and HBeAg. The data with and without induction show that expression was under the control of the yeast acid phosphatase promoter (9, 10, 17, 24, 26).

The extracts from the two systems were examined by sedimentation through a linear sucrose gradient. Each extract carried a fast-sedimenting component, which reacted with anti-HBc antibody (Fig. 2A and C). Electron microscopic examinations revealed spherical particles with diameters of 27 nm (Fig. 3). Both samples displayed particles that were indistinguishable in size and shape. Thus, these polypeptides can self-assemble in the absence of liver components. The polypeptide I produced in *E. coli* or *Bacillus subtilis* has also been reported to assemble into particles (2, 5, 19). The size of these and of those produced in the livers of patients is 27 nm. The number of these particles produced in yeast was roughly 4 × 10^3 per cell. Assuming that about 150 HBc polypeptide molecules are assembled into a particle, one yeast cell may have synthesized about 6 × 10^5 polypeptide molecules under the conditions imposed. Replacing the promoter with glyceraldehyde 3-phosphate dehydrogenase enabled us to obtain 100-fold more HBc polypeptides (data not shown).

The fast-sedimenting peak disappeared upon treatment of the extracts with 0.1% sodium dodecyl sulfate (SDS)–0.1% 2-mercaptoethanol. By contrast, a slow-sedimenting component reactive with anti-HBe antibody appeared (Fig. 2B) in accordance with the notion that HBc antigenicity is associated with core particles, which give rise to HBeAg after dissociation (14).

The SDS-treated polypeptide I and II samples were subjected to SDS-polyacrylamide gel electrophoresis, followed by Western blotting (27) with anti-HBc and -HBe antibodies. The results in Fig. 4 demonstrate that polypeptides I and II have molecular weights of 21,500 and 24,500, respectively, in complete agreement with the values expected from the DNA sequence.

The production of HBcAg in yeast cells will enable us not

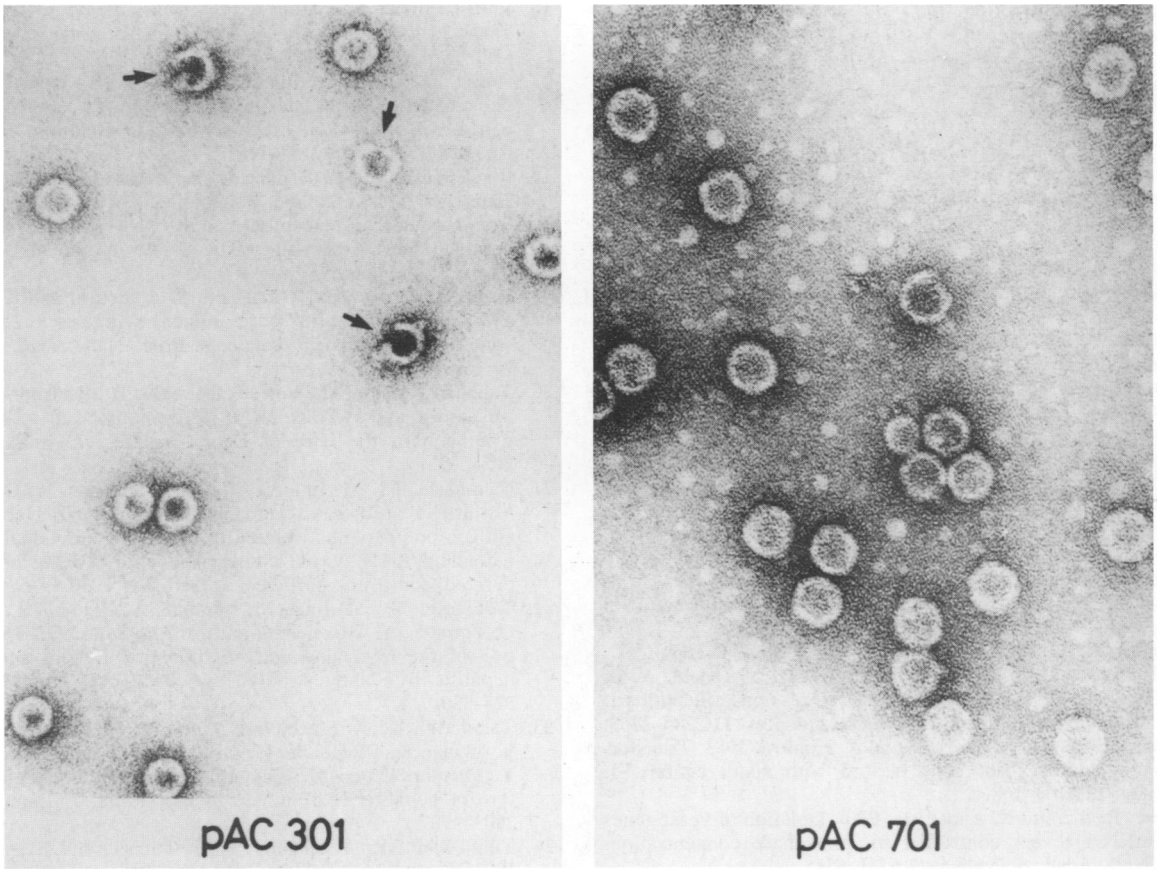

FIG. 3. Electron micrographs of HBcAg particles produced by yeast cells carrying plasmids pAC701 (producing polypeptide I) and pAC301 (producing polypeptide II). Each 6 ml of cell extract was layered on top of a 32-ml linear sucrose gradient (20 to 60% [wt/wt]) in TEN buffer, followed by centrifugation in an SW28 rotor (Beckman Instruments, Inc.) at 25,000 rpm for 21 h. Peak fractions of HBcAg plus HBeAg activity (see Fig. 2) were collected and stained with uranyl acetate. Arrows indicate ruptured particles.

only to provide large amounts of materials but also to open new methods for further study of the conversion of HBcAg into HBeAg and to confirm the biological implications of such methods, which so far have been inferred (12, 14, 20–23).

Some particles consisting of polypeptide II are ruptured (Fig. 3), as though they are less stable than those composed of polypeptide I. This inference was supported by the observation that treatment of the same sample with 1 M NaCl, followed by sedimentation, resulted in the disappearance of the fast-sedimenting component (Fig. 2D). Particles consisting of polypeptide I did not show this behavior.

The results demonstrate that the presence of the extra 29 amino acids at the amino-terminal portion of the polypeptide does not affect production and accumulation of the HBcAg polypeptides and their self-assembly into particles. However, the particles made of polypeptide II are less stable and are readily dissociated by salt. It will be interesting to see whether the extended polypeptide is located inside or outside of the particles.

Core particles produced in liver cells are not so sensitive to salt treatment (14, 20). These observations strongly suggest that the downstream initiation codon is preferred over the upstream one for use in vivo, though this argument does not rule out the possibility that polypeptide II is produced in vivo to some extent. If the downstream initiation codon is preferred, it poses the interesting question of how it is selectively chosen in the liver cell. Polypeptide II could also

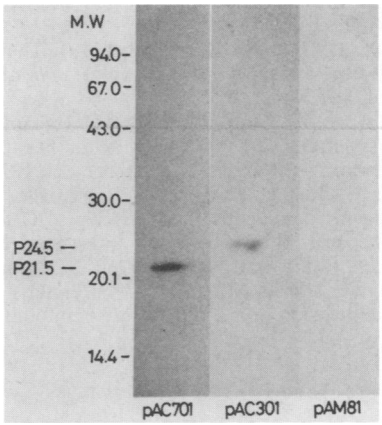

FIG. 4. SDS-polyacrylamide gel electrophoresis and Western blot analysis of HBcAg protein produced in yeast. Antigens in extracts of cells carrying the indicated plasmid were partially purified by sucrose linear gradient centrifugation as described in the legend to Fig. 2. The peak fractions of HBcAg plus HBeAg activity were collected and treated with 0.2% SDS–0.2% 2-mercaptoethanol (11) and electrophoresed on a 15% polyacrylamide gel. The immunoreactive polypeptides were identified by electrophoretic transfer from polyacrylamide gel to nitrocellulose paper, followed by exposure of the nitrocellulose to ^{125}I-labeled anti-HBe (Abbott) and autoradiography for 24 h. P21.5, Polypeptide I; P24.5, polypeptide II; M.W, molecular weight markers.

be synthesized, but since the polypeptide is less stable in assembled form, it could be released preferentially as HBeAg from the cell. Further investigation is needed to elucidate these problems.

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