Hepatitis B Virus (HBV)-specific Structures found in Cytoplasmic Extracts of Cells producing HBV Surface Antigen (HBsAg) in vitro

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(Accepted 18 July 1980)

SUMMARY

Two kinds of hepatitis B virus-specific particles are present in cytoplasmic extracts of hepatoma cells synthesizing hepatitis B virus (HBV) surface antigen. One class of particle contains the surface antigen of the virus, is 20S in size and has a buoyant density (in CsCl) of 1.2 g/ml. The second class of particle is a deoxyribonucleoprotein (DNP) with 1.3 g/ml buoyant density (in CsCl) and is 30S in size, the DNA of which contains HBV sequences thus proving virus specificity.

INTRODUCTION

Synthesis of hepatitis B virus (HBV), or its components, has not been studied extensively due to lack of cultured cells which are permissive for the virus. All the knowledge gained has been based on the isolation of virus from the blood of chronic carriers. Since HBV has some unique characteristics as a virus (Robinson, 1977), and is an important pathogen, extensive research on synthesis of virus components is important for both molecular virology and for application of the information to clinical medicine. Such research, however, faces substantial difficulties which have been overcome by two recent developments. First, identification of a hepatoma cell line in which at least one virus gene is expressed (Alexander et al. 1978); second, cloning of part (Burrell et al. 1979; Pasek et al. 1979) or the entire (Charnay et al. 1979; Galibert et al. 1979; Shinsky et al. 1979; Valenzuela et al. 1979) HBV DNA in bacteria, making it possible to isolate large amounts of HBV DNA. Using these clones, different fragments of HBV DNA (Pasek et al. 1979; Valenzuela et al. 1979), as well as the entire genome (Galibert et al. 1979), have been sequenced.

We took advantage of having both the cells expressing the surface antigen (HBsAg) gene of the virus and plasmid clones containing HBV DNA fragments. It was shown earlier (Hofschneider et al. 1979) that two kinds of particles could be labelled with radioactive amino acids in the cytoplasm of a hepatoma cell line producing the HBsAg. One class of particle contained HBsAg as determined by solid radioimmunoassay (Alexander et al. 1978; Hofschneider et al. 1979) and was therefore virus-specific. The nature of the second class of particle was unclear, and here we have investigated these particles in more detail. We have found that the particles are deoxyribonucleoproteins (DNPs), the DNA of which contains HBV sequences.

METHODS

Cells and radioactive labelling. HBsAg-producing hepatoma cells (Alexander et al. 1978), line PLC/PRF/5, were cultivated in Eagle's minimum essential medium (MEM) supplemented with 10% heated (56 °C for 40 min) foetal calf serum and antibiotics. Labelling the

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0022-1317/80/000-4273 $02.00 © 1980 SGM
cells with amino acids (³⁵S-methionine, 360 to 450 Ci/mmol, 10 to 20 μCi/ml or ³⁵S-cystine, 420 to 480 Ci/mmol, 10 to 20 μCi/ml) was performed in media containing dialysed serum and reduced amounts (20%) of the appropriate non-labelled amino acid as described earlier (Hofschneider et al. 1979). Labelling the cells with ³³H-thymidine (50 Ci/mmol, 15 to 25 μCi/ml) was performed as for labelled amino acids, except that standard MEM was used.

_Treatment of cell monolayers._ The monolayers (60 to 80% confluency) were rinsed with isotonic phosphate buffer solution (PBS), treated with 0.01% EDTA-0.125% trypsin mixture, washed with PBS, resuspended in a hypotonic buffer (HB) containing 10 mM-tris-HCl pH 7.5, 10 mM-KCl and 1.5 mM-magnesium acetate and homogenized in a Dounce tissue homogenizer. Homogenates were centrifuged at 600 g for 10 min, the procedure being repeated with the pellet after this centrifugation. Combined supernatants were centrifuged at 15000 g for 20 min, and particulate material was isolated from post-mitochondrial supernatants (S₁₀) by centrifugation through a 10% sucrose cushion (prepared in HB) in an SW₅₀.₁ rotor (45000 rev/min for 5 to 6 h at 4°C). The resulting pellet (P₂₅₀) was resuspended in 10 mM-tris-HCl pH 7.5, 150 mM-NaCl, 1 mM-EDTA containing 0.5 mg/ml bovine serum albumin (TNEB), and stored at −20°C either as such, or after adding NP₄₀ and 2-mercaptoethanol to 1% and 0.1% respectively.

_Virus and antigens._ HBsAg was measured in 50 μl samples by solid-phase radioimmunoassay (RIA: Ausria II, Abbott Laboratories) according to Ling & Overby (1972).

HBV DNA used for labelling was extracted by phenol from a preparation of partially purified HBV (Kaplan et al. 1973) isolated from the sera of chronic carriers. Presence of the virus in sera was monitored by the presence of HBV core antigen, HBVe antigen and endogenous DNA polymerase activity in high speed pellets. The plasmids used in the study for either labelling (by nick translation) or cRNA synthesis _in vitro_ were pBR 322 and its derivative, pHBV 138 which contains a 1940 nucleotide-long HBV DNA fragment (Pasek et al. 1979). The isolation of the plasmid has been described earlier (Burrell et al. 1979).

_Radioactive labelling of DNA._ Labelling of either HBV DNA or DNA extracted from P₂₅₀ was done by nick translation according to Rigby et al. (1977). Reaction mixtures (50 μl) contained 50 mM-tris-HCl pH 7.8, 5 mM-MgCl₂, 10 mM-2-mercaptoethanol, 50 μg/ml bovine serum albumin, 15 mM each of three non-labelled dNTPs, 5 μM-α-³²P-dNTP (either α-³²P-dTTP or α-³²P-dGTP with sp. act. of 300 to 400 Ci/mmol), 2 μg of an appropriate DNA and 80 to 100 units DNA polymerase I (Boehringer, Mannheim, West Germany). After incubation at 15°C for 90 min the reaction was stopped with phenol, and DNA was extracted. Radioactive DNA was separated from free dNTPs on a 1 ml Sephadex G-50 column and precipitated with ethanol. The final product routinely had a sp. act. of about 10⁶ ct/min/μg DNA.

_RNA synthesis._ _In vitro_ RNA synthesis was carried out in a reaction vol. of 100 μl containing 30 mM-tris-HCl pH 7.9, 30 mM-MgCl₂, 120 mM-NaCl, 1 mM each of ATP, GTP and CTP, 0.05 mM-³²P-UTP (37.5 Ci/mmol), 5 to 10 μg DNA (either pBR 322 or pHBV 138) and 40 μg RNA polymerase. After incubation at 37°C for 60 min the reaction mixture was treated with DNase and the RNA was extracted with phenol. Radioactive RNA was separated from nucleotides on a 1 ml Sephadex G-50 column and precipitated with ethanol. The final product routinely had a sp. act. of about 10⁶ ct/min/μg DNA.

_DNA-RNA hybridizations._ These were performed under conditions similar to those described by Verma (1978). Nucleic acids were melted at 107°C in 400 to 600 mM-NaCl, 20 mM-tris-HCl pH 7.5, 2.5 mM-EDTA and 0.1% SDS. The samples were both under- and overlaid with paraffin oil and incubated in sealed capillary tubes (siliconized) for 4 to 18 days at 67°C. The materials were layered on to Cs₂SO₄ solutions (density 1.55 g/ml) containing 10 mM-tris-HCl pH 7.5 and 2.5 mM-EDTA and centrifuged in an SW₅₀.₁ rotor for 68 to
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Fig. 1. \(^{35}\text{S}\)-methionine- and \(^{35}\text{S}\)-cystine-labelled cytoplasmic structures in hepatoma cells producing HBsAg. The cells were cultivated, labelled and fractionated as described in Methods. Postmitochondrial pellets (P\(_{200}\)) containing either (a) \(^{35}\text{S}\)-methionine- or (b) \(^{35}\text{S}\)-cystine-labelled materials were analysed in 1.15 to 1.45 g/ml preformed CsCl gradients prepared in HB. Centrifugation was done in a SW41 rotor at 35,000 rev/min and 4°C for 36 h. Gradients were fractionated into 21 fractions each, and TCA-insoluble radioactivity as well as binding of antibodies against HBsAg in samples of each fraction were measured. Peak fractions 10 to 12 and 7 from panel (b) were dialysed and analysed by velocity sedimentation in 15 to 30% sucrose gradients (c and d respectively) prepared in HB using gradient-purified \(^3\text{H}\)-uridine-labelled HeLa ribosomes as a marker. Centrifugation was done in an SW50.1 rotor at 45,000 rev/min and 4°C for 3 h. ○—○, \(^{35}\text{S}\) radioactivity; □—□, \(^{125}\text{I}\)-labelled antibodies against HBsAg bound; ■—■, buoyant density.

RESULTS

Amino acid-labelled particles in cytoplasmic extracts of HBsAg-producing hepatoma cells

Labelling PLC/PRF/5 cells with radioactive amino acids revealed two distinct classes of particles in cytoplasmic extracts (Hofschneider et al. 1979). The buoyant density values of these classes suggest that the first one (\(\rho=1.2\) g/ml), which we designated alpha, is represented by proteins only (in the simplest case) whereas the second one (\(\rho=1.3\) g/ml), which we designated beta, contains in addition a denser component. We therefore compared these two kinds of particles in order to see whether or not the beta particles could be candidates for virus deoxyribonucleoproteins (DNPs). Fig. 1(a, b) shows the distribution in CsCl gradients of particulate material from cytoplasmic extracts after the cells were labelled with \(^{35}\text{S}\)-methionine and \(^{35}\text{S}\)-cystine respectively. It can be seen that ‘heavy’ particles are labelled with either amino acid, and they are clearly separated from ‘light’ particles containing HBsAg. Fig. 1(c) shows that alpha particles [dialysed fractions 10 to 12 from panel (b)] are 20S in size as compared to 80S ribosomes. A relatively heterogeneous distribution of beta particles [dialysed fraction 7 from panel (b)] is seen in Fig. 1(d). The heterogeneous distribution in the gradient could be a result of some kind of damage of the particles due to the conditions employed (the high ionic strength during centrifugation in CsCl gradients, etc.) and this possibility was then studied.
Fig. 2. Velocity sedimentation of formaldehyde-treated beta particles. P_{60S} from ^35S-methionine-labelled cells was analysed as in the legend to Fig. 1(a), except that tris-HCl in HB was replaced by triethanolamine-HCl (10 mM, pH 7.5), and 3.8% formaldehyde pH 6.8 was present in the CsCl gradient. The peak fraction was dialysed against modified HB containing formaldehyde and analysed in 15 to 30% sucrose gradients (containing formaldehyde). EDTA-dissociated ^3H-uridine-labelled formaldehyde-treated ribosomes were analysed in a parallel gradient. Centrifugation was done in an SW50.1 rotor at 45000 rev/min and 4 °C for 3 h.

Size of the beta particles

Two different experiments were carried out to investigate the possible damage of the beta particles. The first approach was based on findings that aldehyde treatment prevents deproteinization in CsCl of structures containing both proteins and nucleic acids (Spirin et al. 1965; Baltimore & Huang, 1968). Therefore, centrifugation in CsCl was performed in the presence of formaldehyde with subsequent analysis of the peak fractions in sucrose gradients containing formaldehyde. Fig. 2 shows that beta particles now sediment as a relatively homogeneous-sized 30S population. The result described in Fig. 2 suggests that the distribution of beta particles shown in Fig. 1(d) was probably due to partial degradation (deproteinization?) of the structures. In the second experiment, individual fractions of the peak from a CsCl gradient with no formaldehyde added, were investigated by velocity sedimentation in isokinetic gradients. The experiment was arranged as that in Fig. 1, except that fractions 5, 6 and 7 of the peak (Fig. 3a) were dialysed and centrifuged in isokinetic gradients separately (Fig. 3b, c and d respectively). It can be seen that all the fractions of the peak show different patterns in glycerol gradients ranging from completely heterogeneous distribution (panel b) to rather homogeneous sedimentation (panel d). Moreover, some radioactivity is seen in the top fractions of Fig. 3(b) and (c), but not in Fig. 3(d), supporting the suggestion of possible deproteinization of beta particles in high salt.

Beta particles are deoxyribonucleoproteins

The buoyant density value of beta particles suggests the presence of a material which should have a density higher than that of proteins. We therefore investigated whether or not the beta particles contain DNA. The cells were labelled with ^3H-thymidine and the high-speed pellets obtained by centrifuging the mitochondria-free cytoplasmic extracts were analysed in formaldehyde-containing CsCl gradients. Fig. 4(a) shows a clear radioactivity peak in fraction 6 (buoyant density 1.32 g/ml), although some radioactivity is present in the
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Fig. 3. Velocity sedimentation of non-treated beta particles. P250 from 35S-methionine-labelled cells was analysed as in the legend to Fig. 1 (a), except that HBsAg was not tested (a). Fractions 5, 6 and 7 were dialysed and analysed in 15 to 30% glycerol gradients prepared in a buffer containing 10 mM-tris-HCl pH 7.5, 10 mM-KCl and 2.5 mM-EDTA (b, c and d respectively). EDTA-dissociated 3H-uridine-labelled HeLa ribosomes were used as a marker in a parallel gradient. Centrifugation was done in an SW50.1 rotor at 45,000 rev/min and 4°C for 3 h. O--O, Radioactivity; I—■, buoyant density.

Fig. 4. Isopycnic and isokinetic analysis of formaldehyde-treated cytoplasmic structures containing DNA. Cells were labelled with 3H-thymidine (50 Ci/mmol, 15 μCi/ml) as with amino acids except fresh maintenance medium with normal concentration of constituents was used. P250 was analysed in a CsCl gradient containing formaldehyde (a), as in the legend to Fig. 2. Part of the P250 was analysed in 15 to 30% sucrose gradients (with formaldehyde) using HeLa ribosomes as a marker (SW50.1 rotor, 45,000 rev/min, 4°C, 3 h) (b). ▲—▲, Radioactivity; I—■, buoyant density.
Fig. 5. Analysis of DNA extracted from P,

\[ \text{Fraction no.} \]

\[ 0 \ 1 \ 2 \ 3 \ 4 \ 5 \ 6 \ 7 \ 8 \ 9 \ 10 \ 11 \ 12 \ 13 \ 14 \ 15 \ 16 \ 17 \ 18 \ 19 \ 20 \ 21 \ 22 \ 23 \ 24 \ 25 \]

\[ \text{ct/min \times 10}^5 \]

beta particles contain DNA since thymidine-labelled and amino acid-labelled structures are identical in both size and density. Fig. 5 shows that the DNA extracted from the post-mitochondrial pellet is 11S. In another experiment non-labelled DNA was fractionated in sucrose gradients and 19 to 24S, 9 to 12S and < 7S materials were analysed for their competence as templates for *Escherichia coli* DNA polymerase. Only 9 to 12S DNA was found to be nick-translated (not shown) which confirms the estimate given in Fig. 5.

**Virus-specific nature of beta particle DNA**

The finding that beta particles contain DNA raises the question of whether or not this DNA (or part of it) consists of HBV sequences. This was investigated by hybridizing DNA of beta particles to 3H-labelled cRNA transcribed from pHBV 138 DNA and the resulting material was analysed by isopycnic centrifugation in CsCl gradients for density shifts of hybrid molecules (Fig. 6). All the DNAs in this experiment were labelled by nick translation in the presence of α-32P-dGTP. It can be seen in panel (a) (negative control) that hybridization of non-related nucleic acids, cRNA of pBR 322 and beta particle DNA, results in clear separation of 3H (RNA) and 32P (DNA) during isopycnic centrifugation: they are found in fractions of the gradient with densities of 1.6 and 1.4 g/ml respectively. Hybridization of nucleic acids sharing identical sequences (HBV DNA and pHBV 138 cRNA) is shown in panel (b) where both labels are found also in fractions with intermediate densities (1.45 to 1.55 g/ml). Panel (c) shows hybridization of 32P-DNA of beta particles to pHBV 138 3H-cRNA. It can be seen that both 32P and 3H radioactivity have been shifted. It was concluded from this experiment that the beta particle DNA contains HBV sequences.

**DISCUSSION**

It was shown earlier that two new kinds of cytoplasmic particles, termed alpha and beta, can be isolated from the HBsAg-producing hepatoma cell-line PLC/PRF/5 (Hofschneider *et al.* 1979). Here we show that both particles are related to HBV. The alpha particle is
Fig. 6. Hybridization of DNA extracted from P_{250} to cRNAs transcribed in vitro from pBR 322 or pHBV 138. Extraction of DNA from P_{250} or HBV, nick translation of these DNAs, in vitro RNA synthesis on either pBR 322 or pHBV 138 (containing an HBV DNA fragment), hybridization procedures and separation of hybrid materials in Cs_{2}SO_{4} gradients were done as described in Methods. (a) Hybridization of ^{32}P-DNA of beta particles to ^{3}H-pBR 322 cRNA; (b) hybridization of ^{32}P-HBV DNA to ^{3}H-pHBV 138 cRNA; (c) hybridization of ^{32}P-beta particle DNA to ^{3}H-pHBV 138 cRNA. ▲—▲, ^{3}H-radioactivity (RNA); △—△, ^{32}P-radioactivity (DNA); ■—■, buoyant density.

represented by 20S structures of 1.2 g/ml buoyant density in CsCl which are low in methionine content (Fig. 1); the virus-specific nature of alpha particles is proven by the presence of HBsAg. The particles are not identical, however, to those isolated from the
blood of chronic carriers of the virus. The 'natural' particles, although having the same density, are 40S in size (Burrell et al. 1976). Furthermore, natural particles contain methionine in an amount comparable to that of cystine (Vyas et al. 1972) while alpha particles show a large difference as can be calculated from 35S ratios of methionine: cystine in the 1.3 to 1.2 g/ml peaks (Fig. 1a, b). The latter result shows the disadvantage of using 35S-methionine for labelling HBsAg synthesized in PLC/PRF/5 cells or in a cell-free system using mRNAs from these cells.

The beta particle is represented by 30S structures (Fig. 3) of 1.3 g/ml buoyant density in CsCl (Fig. 1a, b). The DNA detected in this particle (Fig. 4) is 11S in size (Fig. 5) and, as shown by hybridization experiments (Fig. 6c), it consists, at least in part, of HBV-specific sequences. Additional studies using plasmids which contain distinct fragments of HBV DNA (Pasek et al. 1979) will show whether virus DNA is represented partially or completely within beta particle DNA. Experiments of this kind are in progress and preliminary results show the presence of the HBsAg DNA sequences in the beta particle DNA (O. Marquardt, unpublished data). Another interesting question is the relationship of beta particle DNA to HBV-specific DNA integrated into chromosomal DNA (Marion & Robinson, 1980).

DeLap et al. (1978) reported that small circular DNAs of unknown function are found in the cytoplasm of mammalian cultured cells. Beta particle DNA may belong to this type of DNA. If this is so, the presence of a virus marker could be a useful tool with which to study replication and function of small circular DNAs.

Initial studies on proteins of beta particles (V. Zaslavsky et al., unpublished data) have revealed a transient polymerase activity. In the product of an endogenous reaction HBV-specific DNA sequences can be obtained which show the potential ability of beta particle DNA to be replicated in cytoplasm. Further studies must show whether the observed enzymic activity is related to the polymerase of HBV and whether or not the particles contain any other HBV-specific protein, as one would expect if they are defective virus particles.

We are grateful to Dr G. G. Frösner (Munich, West Germany) for HBsAg assays, to Dr W. Zillig (Martinsried, West Germany) for a gift of RNA polymerase preparation and to Dr E. Eisenburg (Munich, West Germany) and Dr R. Müller (Hannover, West Germany) for providing us with sera containing HBV. We wish to thank also Ms Marianne Kauzmann, Ms Elisabeth Bürgelt and Mrs I. Dick for excellent technical assistance. The results of this study were partially presented at the Cold Spring Harbor Symposium, September 1979.

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(Received 28 May 1980)