Evidence for Non-chromosomal Hepatitis B Virus Surface (HBsAg)- and Core Antigen (HBcAg)-specific DNA Sequences in a Hepatoma Cell Line

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SUMMARY

As demonstrated previously, a 'beta particle' fraction isolated from the cytoplasm of PLC/PRF/5 cells contains hepatitis B virus (HBV)-specific DNA. Here, further evidence is provided that the specificity of the DNA for HBV is represented at least by sequences coding for the surface and core antigen (HBsAg and HBcAg). This was shown by two different hybridization techniques. One of them, the technique of Southern, distinguished these hybrid molecules formed from those containing HBV DNA integrated into chromosomes. The HBV-specific beta particle DNA forms two distinct bands separate from the high molecular weight cellular DNA.

It has been shown recently that post-mitochondrial supernatants of hepatitis B virus surface antigen (HBsAg)-producing hepatoma cultured cells (PLC/PRF/5) contain deoxyribonucleoprotein particles with HBV-specific DNA sequences (beta particles; Zaslavsky et al., 1980). Thus, HBV-specific DNA is present not only in nuclei of the cells (Marion et al., 1980; Chakraborty et al., 1980; Brechot et al., 1980; Edman et al., 1980; Koshy et al., 1981) but also in a non-chromosomal form. Since chromosomal DNA of these cells contains integrated virus genes not only for HBsAg (which is synthesized in the cells) but very probably the complete virus genome (Marion et al., 1980), one could envisage the presence of additional HBV sequences also in the beta particle DNA. The size of the latter (11S), measured by velocity sedimentation (Zaslavsky et al., 1980), would lend support to this idea.

Cloned HBV DNA fragments containing different parts of the virus genome offer an experimental approach to investigate this idea. We have used this approach, and the data presented in the report show that the low mol. wt. DNA of the beta particles hybridizes to sequences specific for both HBsAg and hepatitis B virus core antigen (HBcAg).

The PLC/PRF/5 line of hepatoma cells (Macnab et al., 1976) was used. Cultivation of cells as well as their fractionation into nuclei, mitochondria and post-mitochondrial supernatant have previously been described in detail (Zaslavsky et al., 1980). Particulate material from the latter was further concentrated by pelleting through a 10% sucrose cushion and is subsequently referred to as beta particles. The beta particles as well as fractions containing nuclei or mitochondria were submitted to a gentle DNA extraction procedure (Koshy et al., 1981). Two recombinant plasmids (Burrell et al., 1979), pHBV 20 [with a 650 base pair (bp) insert specific for HBsAg] and pHBV 8.2.51 (with a 550 bp insert specific for HBcAg), have been kindly provided by Dr K. Murray, Heidelberg, F.R.G. Recloning and isolation of the plasmids were performed according to the conventional method (Tanaka & Weisblum, 1975).

Sequence homology of nucleic acids can be demonstrated using several methods. One of them, isopycnic Cs₂SO₄ gradient centrifugation, separates RNA, DNA and RNA/DNA hybrids according to their difference in buoyant density. An experiment of this kind was performed after transcription of both strands of each of the recombinant plasmids, pHBV 20 (1.5 x 10⁵ ct/min/50 ng) and pHBV 8.2.51 (3 x 10⁵ ct/min/50 ng), into cRNA with
Fig. 1. HBsAg- and HBeAg-specific RNA/DNA hybrids in Cs2SO4 gradients. 32p-labelled beta particle DNA was hybridized to 3H-labelled cRNA, either prepared from pHBV 20 (a, b) or from pHBV 8.2.51 (c, d). Half of each reaction volume was mixed directly with the Cs2SO4 solution (a, c); the other was first incubated with S1 nuclease (b, d). Centrifugation and analysis of the gradients were done as described earlier (Zaslavsky et al., 1980). △, 3H; ●, 32P; ——, density. Arrows indicate RNA (1.6 g/ml Cs2SO4) or DNA (1.4 g/ml) position.

DNA-dependent RNA polymerase (kindly provided by Dr W. Schulz, Martinsried, F.R.G.) in the presence of one 3H-labelled and three non-labelled rNTP. Each 3H-labelled cRNA preparation was hybridized to beta particle DNA which was 32P-labelled (1.5 x 10^6 ct/min/150 ng) by the nick translation procedure (Rigby et al., 1977). 3H- and 32P-labelled nucleic acids had been purified by precipitation with cetyltrimethylammonium bromide from low mol. wt. radioactivity (Reitz et al., 1972) prior to use. The hybridization was performed for 70 h at 67 °C in a reaction volume of 20 μl containing 20 mM-potassium phosphate pH 7.5, 5 mM-EDTA, 400 mM-NaCl and 0.1% SDS following preincubation at 110 °C for 2 min. Half of each hybridization reaction was digested with 20 units S1 nuclease at standard conditions, and both digested and non-digested materials were analysed in Cs2SO4 isopycnic gradients (1.55 g/ml in 2 x SSC = 300 mM-NaCl, 30 mM-sodium citrate pH 7) for density shifts of either label and for S1 resistance.

Fig. 1 shows the results of this experiment. Panels (a) and (c) demonstrate the presence of 3H-labelled RNA in fractions with buoyant densities lower than 1.6 g/ml and the presence of 32P-labelled DNA in fractions with buoyant densities heavier than 1.4 g/ml and, therefore, formation of RNA/DNA hybrids. Panels (b) and (d) show that the hybrids are S1-resistant and, therefore, specific and of considerable length. Since 3H-labelled cRNA present in the hybrids has been transcribed from pHBV 20 (Fig. 1a, b, fractions 11 and 16) as well as from pHBV 8.2.51 (Fig. 1c, d, fractions 12 and 13) and since no such hybrids could be observed when 3H-labelled cRNA was prepared from the cloning vector alone (data not shown), it can be concluded that beta particle DNA from PLC/PRF/5 cells shares common sequences with the HBV inserts but not with the vector plasmid. Since the HBV specificity of the inserts
Fig. 2. HBsAg- and HBcAg-specific DNA/DNA hybrids on Southern blots. (a to f) Ethidium bromide-stained DNA in agarose gels: (a) pHBV 20; (b) pHBV 8.2.51 (both linearized with restriction enzyme EcoRI); (c) undigested plasmid pSF 2124 (1.5 µg); (d) lambda DNA (1.6 µg) digested with 5 units of restriction enzyme PstI under conditions described for EcoRI; (e) undigested beta particle DNA (5 µg) from PLC/PRF/5 cells; (f) undigested DNA extracted from the mitochondrial fraction (5 µg). The DNA was blotted on to nitrocellulose membranes (the gel containing a to d was cut in track d from top to bottom, so that one-half only of slot d was blotted) and that containing tracks (a) to (d) was hybridized to 32P-labelled beta particle DNA, whereas that containing tracks (e) and (f) was hybridized to 32P-labelled pHBV 20 DNA. The corresponding autoradiographs are shown in (a') to (f'). The size markers (kb) refer to linearized pBR322 and to the HBV-specific insert of pAO1 HBV (Cummings et al., 1980) which were applied to the same gel (data not shown).

concerns HBsAg and HBcAg, it can be concluded further that the hybridization to beta particle DNA is dependent on the presence of HBsAg- as well as HBcAg-specific sequences. The S1-resistant 3H-labelled peaks with a low content of 32P-label are considered to be dsRNA, the formation of which is possible due to transcription of dsDNA into RNA.

The method of Southern (1975) was also used to investigate hybrid formation between sequences coding for HBsAg and HBcAg and beta particle DNA. For that purpose, DNA from pHBV 20 and pHBV 8.2.51 (0.6 µg of each) was linearized with 5 units of the restriction enzyme EcoRI (Boehringer, Mannheim) in a reaction vol. of 20 µl containing 10 mM-tris pH 7.5, 6 mM-MgCl₂, 100 mM-NaCl and 6 mM-β-mercaptoethanol for 1 h at 37 °C and subsequently electrophoresed in an agarose gel (1% agarose, 36 mM-tris, 30 mM-phosphate buffer pH 7.5, 10 mM-EDTA in a horizontal chamber for 16 h at 100 mA). For hybridization experiments gels were treated following the protocol of Southern (1975) to transfer the DNA to nitrocellulose membranes. They were then incubated overnight at 67 °C
in 5 × SSC, 50 mM-potassium phosphate pH 6.5, 0.1% each of bovine serum albumin (BSA), Ficoll and polyvinylpyrrolidone and 250 μg/ml of sonicated and melted calf thymus DNA. For hybridization the nitrocellulose membranes were immersed in 10 ml 50% formamide, 10% dextran sulphate, 5 × SSC, 20 mM-potassium phosphate pH 6.5, 0.02% each of BSA, Ficoll and polyvinylpyrrolidone and 100 μg/ml of sonicated and melted calf thymus DNA. The probe for hybridization was nick-translated beta particle DNA (1 × 10^7 ^32 P ct/min/1.5 μg), which was purified away from low mol. wt. radioactivity, melted at 110 °C for 2 min, chilled in ice and added to the nitrocellulose membrane. The hybridization was done at 37 °C for 50 h and was stopped by transferring the membranes to 2 × SSC buffer (250 ml, prewarmed to 67 °C for 2 to 4 h). Following eight changes of wash buffer, the membrane was air-dried and submitted to autoradiography in a cassette equipped with intensifying screens for up to 5 days at −70 °C. The results are shown in Fig. 2. Tracks (a) to (d) show the distribution of electrophoresed DNAs in a gel, while tracks (a') to (d') demonstrate the results of the hybridization experiment. It can be clearly seen that the ^32 P-labelled DNA hybridizes to both pHBV 20 (track a') and pHBV 8.2.51 (track b'), while no hybridization can be seen with non-related DNAs (track e' and d').

The finding that HBV DNA sequences are observed in a concentrate of material extracted from the cytoplasm of PLC/PRF/5 cells can be explained either by the presence there of HBV-specific beta particles or by assuming contamination with chromosomal DNA which is known to contain HBV DNA in an integrated form. Therefore, an experiment was done to rule out one of the possibilities. For that purpose, DNA from a beta particle preparation and from the mitochondrial fraction were electrophoresed in an agarose gel, blotted on to a nitrocellulose membrane and hybridized as described above to ^32 P-labelled pHBV 20 DNA (1 × 10^7 ct/min/1.5 μg). If hybridization is due to the presence of HBV sequences integrated into chromosomal DNA contaminating the beta particle preparation, it should be observable with non-degraded high mol. wt. DNA. Such contaminating high mol. wt. DNA should be present in the DNA extracted from the mitochondrial fraction also, which therefore serves as control. Attempts have been made to avoid nuclease activities or random degradation of DNA during preparation. In fact, DNA extracted from the mitochondrial fraction formed two bands in a CsCl gradient, one of which represented closed circular DNA (data not shown). Therefore, the DNA samples are considered to be non-degraded. Even assuming random degradation of any high mol. wt. DNA contaminating beta particle DNA, such DNA cannot be expected to be concentrated by electrophoresis in distinct bands in a gel, but rather to be randomly distributed, giving rise to background hybridization all over the track. On the other hand, if hybridization is due to HBV-specific sequences present in beta particle DNA, it should be observable at areas representing 11S material, the known size of the DNA (Zaslavsky et al., 1980).

The results are shown in Fig. 2. While the only DNA stainable by ethidium bromide in tracks (e) and (f) is apparently of high mol. wt. it shows an increased background rather than hybridization (tracks e' and f', uppermost third). The hybridization evident in Fig. 2(e'), however, corresponds to two areas representing considerably lower mol. wt. DNA of remarkable homogeneity. No hybridization was detectable in the mitochondrial fraction (track f'). It was concluded from these results that there exist two homologous low mol. wt. components with HBV specificity in DNA extracted from a concentrate of material present in the cytoplasm of PLC/PRF/5 cells. The DNA used in the study was neither enzymically nor randomly degraded. Therefore, the two homogeneous hybridization signals are not due to HBV DNA integrated into chromosomes. They are due to the DNA released from beta particles (Zaslavsky et al., 1980) which may be defective hepatitis B virions. Since very little is known about structure and shape of beta particle DNA, its size in the gel cannot be deduced from the positions of linear size markers of dsDNA (pBR322 and pAO1);
Cummings et al., 1980). In any case, it is different in size from HBV sequences integrated into chromosomes.

Our finding that HBV-specific low mol. wt. DNA is present in PLC/PRF/5 cells appears to contradict results published by others, but in fact does not. The material we used in the study consisted of concentrated particles from the post-mitochondrial supernatant of the cells, while others either used one of the bands from total cellular DNA formed in CsCl gradients (Marion et al., 1980) or purified nuclei (Chakraborty et al., 1980; Koshy et al., 1981) or total cellular DNA (Brechot et al., 1980; Edman et al., 1980). Therefore, the results cannot be compared.

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