Human non-hepatocytes support hepadnaviral replication and virion production

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The competence of non-hepatocytes to support hepatitis B virus (HBV) gene expression and replication was studied by transient transfection of various human cell lines with a head-to-tail dimer of HBV DNA. Independent of their neuroectodermal, mesenchymal or epithelial origin, all non-hepatocyte cell lines tested synthesized and secreted hepatitis B surface antigen (HBsAg) and hepatitis B core/e antigen (HBc/eAg). Further analyses of two of these cell lines (LS 180 and COLO 320) identified the two major HBV transcripts of 3-6 and 2-2/2-4 kb length, respectively. LS 180 cells were permissive for HBV and duck hepatitis B virus (DHBV) DNA replication and secretion of infectious virions. COLO 320 cells also supported HBV DNA replication, but did not appear to export complete viral particles. These findings provide direct evidence that both HBV and DHBV can replicate in non-hepatic tumour cell lines, one of which is shown also to produce infectious virions.

Recent advances in the molecular biology of hepatitis B virus (HBV) have identified viral DNA, RNA or proteins not only in hepatocytes but also in a number of extrahepatic sites, such as lymphoblastoid cells, lymph nodes, bile ducts and vascular elements in the liver (Blum et al., 1983; Romet-Lemonne et al., 1983; Yoffe et al., 1990). Recent reports demonstrated HBV-specific transcripts in transiently transfected murine fibroblasts (Seifer et al., 1990) and viral replication after stable transfection of murine fibroblasts with an HBV construct linked to the simian virus 40 enhancer/early promoter (Zelent et al., 1987). To date, however, extrahepatic human tissues have not been unequivocally shown to support HBV replication. We therefore analysed the competence of different human cell lines to support HBV gene expression and replication.

In an initial set of transient transfection experiments we assessed the expression and secretion of hepatitis B surface antigen (HBsAg) and hepatitis B core/e antigen (HBc/eAg) from the following cell lines: the human hepatoma cell line HuH-7 (Nakabayashi et al., 1982), the two human colon adenocarcinoma cell lines LS 180 (Tom et al., 1976) and CaCo2 (Fogh et al., 1977), the human rectal adenocarcinoma cell line SW837 (Leibovitz et al., 1976), the human APUDoma cell line COLO 320 (Quinn et al., 1979) and the human osteosarcoma cell line Saos2 (Fogh et al., 1977). The cells were cultured in Eagle's MEM (Sigma) containing 10% foetal bovine serum. All cell lines were transfected at a confluence of 60 to 90% with 20 μg DNA per 100 mm dish, using the calcium phosphate precipitation technique (Chen & Okayama, 1987). For transfection experiments, a head-to-tail dimer (HTD) of HBV DNA HBsAg subtype adw (adw HTD) was constructed (Blum et al., 1991a). Using a plasmid carrying the β-galactosidase gene (Morgenstern & Land, 1990; Price et al., 1987), transfection efficiency was found to be about 3% and was similar for all cell lines used. After transfection, culture media aliquots were removed and analysed for the presence of viral proteins. HBV-encoded proteins were identified by radioimmunoassays as shown in Table 1. All five cell lines tested secreted HBsAg and HBc/eAg into the cell culture medium with particularly high levels for the APUDoma cell line COLO 320, approaching the values obtained for the hepatoma cell line HuH-7.

The two cell lines COLO 320 and LS 180 were selected for a more detailed analysis of gene expression and replication. Viral transcripts were analysed 36 h after transfection. Cytoplasmic RNA was prepared by the guanidinium isothiocyanate method and analysed by
Fig. 1. Northern blot analysis of HBV RNA isolated from HuH-7 (lane 1), COLO 320 (lane 2) and LS 180 (lane 3) cell lines 2 days after transfection with 20 μg adw HTD DNA per 100 mm plate. Hybridization with full-length, 32P-labelled HBV DNA. Autoradiographic exposure time at -80°C was 4 h for lanes 1 and 2, and 1 day for lane 3.

Table 1. HBsAg and HBc/eAg in cell culture medium 4 days after transfection with HBV DNA

<table>
<thead>
<tr>
<th>Human cell line</th>
<th>HBsAg</th>
<th>HBc/eAg</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuH-7</td>
<td>710</td>
<td>20</td>
</tr>
<tr>
<td>COLO 320</td>
<td>570</td>
<td>20</td>
</tr>
<tr>
<td>CaCo2</td>
<td>46</td>
<td>9</td>
</tr>
<tr>
<td>LS 180</td>
<td>26</td>
<td>10</td>
</tr>
<tr>
<td>Saos2</td>
<td>72</td>
<td>3</td>
</tr>
<tr>
<td>SW837</td>
<td>36</td>
<td>7</td>
</tr>
</tbody>
</table>

* Ratio of c.p.m. for medium from transfected cells to c.p.m. for medium from non-transfected cells. The positive cutoff value is 2.2 for both tests. HBsAg and HBc/eAg were determined by commercially available radioimmunoassays (Centocor and Abbott Laboratories).

Northern blot hybridization (Ausubel et al., 1990; Davis et al., 1986). The analysis of the RNA isolated from the HuH-7, COLO 320 and LS 180 cell lines (Fig. 1) revealed two major transcripts of 2.2/2.4 and 3.6 kb length. While HuH-7 and COLO 320 cells produced substantial amounts of subgenomic 2.2/2.4 kb RNAs, similar to the finding of Zelent et al. (1987), the predominant species in LS 180 cells were pre-genomic full-length 3.6 kb transcripts, similar to findings in murine fibroblasts (Zelent et al., 1987). These findings indicate that the APUDoma and the colon adenocarcinoma cell lines tested are competent to synthesize the major HBV transcripts (Blum et al., 1991a; Cattaneo et al., 1984; Su et al., 1989; Yoffe et al., 1990).

The replication competence of non-hepatocytes was assessed by Southern blot hybridization of core particle-associated HBV DNA. Cell culture medium was collected and cells were harvested 4 days after transfection. Cell lysate was prepared by adding 3 ml of lysis buffer to one 100 mm plate (Blum et al., 1991b). The lysate was passed twice through a 21-gauge needle. Cell culture media and lysates were centrifuged for 45 min at 50000 g at 20 °C. To remove input DNA, MgCl2 was added to the supernatant to 10 mM final concentration and incubated for 1 h at 37°C with 1 μg DNase I per ml. The DNase-treated solution was layered over 30% sucrose and centrifuged for 3 h at 178000 g at 4°C. The pellet was digested for 2 h at 55°C in 500 μl STE buffer containing 500 μg proteinase K (Boehringer Mannheim) per ml and 1% SDS. After phenol extraction and ethanol precipitation, the DNA species were fractionated through a 1-25% agarose gel, transferred to a Nytran membrane and hybridized with a full-length HBV DNA probe 32P-labelled by random priming. Prehybridization and hybridization were carried out according to Sambrook et al. (1989).

As shown in Fig. 2, for the COLO 320 (lane 2) and LS 180 (lane 3) cells, replicating viral DNA sequences are present in cell culture media after transfection with adw HTD. These replicating viral DNA species in non-hepatocyte cell lines are similar to those observed after transfection of the human hepatoma cell line HuH-7 (Fig. 2, lane 1).

To substantiate the core particle association of the replicating HBV DNA species observed, we analysed endogenous DNA polymerase activity (EPA) in viral core particles (Kaplan et al., 1973). Core particles were prepared from lysates of transfected cells. The pellet was incubated for 16 h at room temperature with a solution containing 50 μCi [α-32P]dCTP (3000 Ci/mmol), 30 μl labelling buffer (Multiprime DNA labelling kit, Amersham) and 120 μl H2O. The reaction continued in the presence of all four dNTPs (each 0.8 mM) for 10 h at room temperature. DNA was extracted as described above.
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(a) (b)
Fig. 3. (a) Southern blot analysis (lane 1) and endogenous DNA polymerase activity (lane 2) of core particles isolated from HBV DNA-transfected HuH-7 cells. (b) Endogenous DNA polymerase activity of HBV core particles isolated from HuH-7 (lane 1), COLO 320 (lane 2) and LS 180 (lane 3) cell lines 4 days after transfection with 20 μg adw HTD DNA per 100 mm plate. Autoradiographic exposure time was 3 h at -80°C.

Fig. 4. Southern blot analysis of DHBV DNA isolated from cell lysate (lanes 1) and culture medium (lanes 2) of HuH-7 (a), and LS 180 cells (b) 4 days after transfection with 20 μg DHBV HTD DNA per 100 mm plate. Autoradiographic exposure time was 2 days at -80°C.

and fractionated by agarose gel electrophoresis. As shown for HuH-7 cells, the single-stranded and partially double-stranded DNA species identified by Southern blot hybridization (Fig. 3a, lane 1, hybridization and autoradiography), were converted to full-length double-stranded molecules as a result of radiolabelled dCTP incorporation (Fig. 3a, lane 2, autoradiography only). In a similar manner to the EPA of core particles from transfected HuH-7 cells (Fig. 3b, lane 1), core particles isolated from COLO 320 and LS 180 cells showed EPA with incorporation of radiolabelled dCTP as demonstrated by autoradiography (Fig. 3b, lanes 2 and 3, respectively).

These data demonstrate that HBV replication is not restricted to hepatocytes. Since woodchuck hepatitis virus and duck hepatitis B virus (DHBV) have previously been identified in cells other than hepatocytes (Korba et al., 1989; Walter et al., 1991), we analysed the competence of LS 180 cells to support DHBV replication. From a full-length DHBV clone (Mattes et al., 1990) a head-to-tail dimer (DHBV HTD) was constructed in pGEM7-Zf(+) as described previously for the HBV adw HTD construct (Blum et al., 1991a). HuH-7 and LS 180 cells were then transfected and analysed as described above. Replicative DHBV DNA species were observed not only in HuH-7 cells, similar to the finding reported by Hirsch et al. (1988), but also in LS 180 cell lysate and culture medium (Fig. 4). Hence, for the first time, an avian hepadnavirus has been shown to replicate in a non-hepatic human cell line.

To characterize viral particle assembly in non-hepatocytes, cell culture media were subjected to caesium chloride density gradient centrifugation (Tong et al., 1991). After addition of 1-5 g caesium chloride per 4.5 ml of culture medium and centrifugation at 178 000 g for 60 h at 4°C, 350 μl fractions were collected from the top. The density was determined by weight. HBsAg and HBC/eAg were determined as described above. For the detection of HBV DNA, caesium chloride gradient fractions were pooled. DNA extraction and Southern blot analysis were carried out as described above. Fig. 5(a) (HuH-7), 5(b) (COLO 320) and 5(c) (LS 180) indicate that HBsAg and HBC/eAg were detectable at densities of about 1-16 g/ml and 1-25 g/ml, respectively, as has been previously reported by Yaginuma et al. (1987). These findings demonstrate that viral antigens produced by non-hepatocytes have physical properties similar to those produced by hepatoma cell lines in vitro (Blum et al., 1991a; Sells et al., 1987; Yaginuma et al., 1987), as well as by liver cells in vivo (Kaplan et al., 1976). As further shown in Fig. 5, viral DNA in culture media was associated with HBC/eAg-positive fractions in the three cell lines studied (Fig. 5a to c, lane 3). Moreover, in media from HuH-7 and LS 180 cells HBV DNA was associated with HBsAg, presumably representing complete Dane particles (Fig. 5a and c, lane 2). However, in culture medium from COLO 320 cells, complete Dane particles were not detected (Fig. 5b, lane 2), although these cells support HBV DNA replication and encapsidate HBV DNA species into core particles.

To assess the biological significance of extrahepatic replication and virion production, the infectious potential of Dane particles produced and secreted by human non-hepatocytes was studied. HuH-7 and LS 180 cells
Fig. 5. Caesium chloride gradient analysis of culture medium from (a) HuH-7, (b) COLO 320 and (c) LS 180 cells 4 days after transfection with adw HTD DNA. Left side of each panel: radioimmunoassays for HBsAg (□) or HBeAg (◆) (20 µl aliquot per assay). Right side of each panel: Southern blot analysis of DNA extracted from cell culture medium prior to CsCl gradient centrifugation (lane 1), CsCl gradient HBsAg-positive fractions 20 and 21 (lane 2) and HBeAg-positive fractions 25 and 26 (lane 3). Single-stranded and relaxed circular forms of DNA are indicated.
were transfected with DHBV HTD. At day 4 after transfection 10 ml culture medium was collected and concentrated over 3 ml of 30% sucrose cushion by centrifugation at 100,000 g for 16 h at 20°C and resuspended in 100 µl TE buffer. Three days after hatching, ducklings (Anas domesticus) were inoculated intraperitoneally with the concentrated cell culture medium. Blood was taken from the foot vein and tested for the presence of DHBV DBA by dot blot hybridization (Walter et al., 1987), prior to and 7, 14 and 30 days after inoculation. Sera from ducklings inoculated with cell culture medium from the DHBV DNA transfected HuH-7 and LS 180 were positive for DHBV DNA after 7 and 14 days, respectively (data not shown). In addition, 30 days after inoculation DNA was extracted from liver tissues (Walter et al., 1991). Southern blot analysis of liver DNA revealed replicating DHBV DNA species: single-stranded (below 2-0 kbp) and partially double-stranded (smear below 3-0 kbp) as well as relaxed circular molecules (Fig. 6). Taken together, these data demonstrate that the human LS 180 cell line is competent to produce infectious DHBV particles.

In summary, the data demonstrate that HBV can replicate in non-hepatic cells. Replication of DHBV was shown to occur in non-avian and non-hepatic cells and DHBV particles secreted from the non-hepatic human LS 180 cell line are infectious. In light of recent evidence for extrahepatic HBV infection (for review see Omata et al., 1990), we can speculate that non-hepatocytes play an important role in the natural course of hepadnavirus infection; non-hepatocytes may be a site of infection and virion production and may represent a reservoir from which liver cells are infected. Further investigation should identify the non-hepatic cell population involved in natural HBV infection and their pathobiological contribution in man.

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References


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