though there are some reports about in vitro infection of HBV to HepG2 [5; 6].

As HBV infection systems, human normal hepatocytes and a human hepatocellular carcinoma originated cell line have been available on the commercial base [7; 8] and uPA/SCID mice with human hepatocytes have been developed [9; 10; 11]. These systems are very useful for HBV and hepatitis C virus (HCV) research indeed but they are very expensive and inconvenient for daily HBV research. Thus, there is still a high hurdle to expand knowledge about HBV.

Actually, many studies about HBV and its related diseases such as acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma have been performed so far [12; 13; 14; 15]. Such studies, however, were very limited because of absence of a useful and convenient *in vitro* infection system for HBV as mentioned. In order to overcome such a big problem, we need very useful, convenient and inexpensive systems for HBV infection to understand a true HBV life cycle and pathogenesis of HBV related diseases, and then to develop specific drugs and means to treat such diseases.

Identification of an HBV receptor is indispensable to establish *in vitro* and *in vivo* or a convenient animal infection model. Information on the HBV receptor has

been reported, including non-human hepadnaviruses such as duck hepatitis B virus [16; 17; 18] and such seems not to be a real and complete HBV receptor, and has not reached establishing an HBV infection system in culture system. HBV can infect primary hepatocytes for sure whose preparation is still troublesome, and although there are several human hepatocytes originated hepatocellular carcinoma cell lines as noted, we do not know why such cell lines becomes insusceptible to HBV infection, which might mean that differentiation status of hepatocyte could be critical for the expression and/or the activity of an HBV receptor [19; 20].

It should be very difficult to identify an HBV receptor and thus we must design a revolutionary method for the identification. Pseudotype viruses could be very useful not only to transfer and express some genes exogenously but also to monitor their infectivity [21]. VSV-G based pseudotype containing retroviral vectors and lentiviral VSV-G pseudotype vectors have been proved to be extremely useful to transduce heterologous genes into cells and in case of the latter [22; 23], even into resting cells such as well-differentiated neuronal cells [24]. On the other hand, specific viral enveloped pseudotype viruses could be helpful to test what kinds of cells and how such

viruses can infect. Though HCV pseudotype particles (HCVpp), in which lentiviral capsids are enveloped by HCV membrane proteins, has been successfully generated and utilized without any knowledge of viral particle maturation and egress [25]. Thus, the bottleneck is dependent on whether such pseudotype particles really can be generated or not.

Here, we challenged to develop HBV pseudotype particles (HBVpp), which consist of HBV membrane particles outside and murine leukemia virus (MLV) originated capsids and genomes insides. Therein, we prepared a packaging cell system, where the cells produce MLV gag-pol fusion polypeptides and a retroviral genome containing an *EGFP* gene and a hygromycin resistant gene (hyg^R) in addition to murine leukemia virus packaging signal ψ. Pantropic retroviruses with vesicular stomatitis virus G proteins were successfully generated in our generated packaging system and infected the other cell lines, meaning that the packaging cell lines were practicable. Introducing an HBV membrane protein expression vector into the packaging cells, which expresses three HBV membrane proteins, large S (LS), middle S (MS) and small S (SS) enabled release of particles enveloped by HBV membrane proteins.

Immunoprecipitation with anti-S antibodies followed by RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) targeted the *EGFP* gene revealed the designed retroviral genome inside. Physico-chemical studies using CsCl-density gradient ultracentrifugation followed by RNA extraction and RT-PCR targeted the *EGFP* gene also revealed that the particles focused around 1.22~1.25 g/ml density zone, which was consistent with the HBV particle density, and the retroviral genomes were inside.

Successful formation of an HBVpp seems to be very useful and practical for identification of an HBV receptor since we are able to establish an assay system based on the viral infectivity by using versatile cell culture systems, though our system is an MLV based packaging system, which depends on cell growth for their integration.

Materials and Methods

Cells.

HEK293, a human embryonic kidney cell line, and its derivatives, GP2-293 (Clontech-Takara Bio), in which an MMLV gag-pol gene was stably integrated for its expression, were maintained in Dulbecco's modified essential medium (DMEM) (nakalai tesquec) supplemented with 10 IU/ml penicillin G, 10 μg/ml streptomycin and 10 % fetal bovine serum (FBS), and BC3, a human primary effusion lymphoma [5] cell line in RPMI1640 (nakalai tesque) supplemented with the same antibiotics and 20 % fetal bovine serum. All cells were cultured in 5 % CO2 atmosphere. For the selection, hygromycin B (Wako Chemicals Japan) was added into each medium at 0.5 mg/ml.

Plasmids.

A retroviral expression vector, pHyTc-GFP was consisted of 5' and 3' LTR, a ψ packaging signal, a hygromycin resistant gene (hyg^R) at the gag-pol position, the cytomegalovirus (CMV) enhancer-promoter region followed by an *EGFP* gene from pEGFP C1 (Clontech) at the env position (see Fig. 1 A). pCEP4 LS-S was an HBV membrane protein expression vector and contained BstEII to EcoRV fragment of the cloned HBV DNA subtype adw2 (Accession No. X02763.1, GI: 59418) at a PvuII site of pCEP4 vector (Invitrogen).

Transfection and establishment of packaging cell lines.

pHyTc-GFP was transfected to GP2-293 cells (Clontech-Takara Bio) with a transfection reagent, TransIT LT1 (Takara Bio) according to manufacturer's direction. Two days after transfection, the cells were selected in 0. 5 mg/ml hygromycin B (Wako Chemicals, Japan) containing DMEM with supplements mentioned above. Several colonies with GFP-positive signals were isolated with a penicillin ring and propagated. To check their packaging activity, a VSV-G expression vector (Clontech) was

transfected to the isolated clones and the culture medium was harvested three days post-transfection. The medium was passed through a 0.22 μm filter unit (Millex®, Millipore) and stored at 4° C until use. 2×10^5 BC3 cells or HEK293 were prepared in a 6-well plate and the virus containing medium was contacted with them for 4 hours. The medium was withdrawn and incubated for two days in the DMEM for HEK293 and RPMI1640 for BC3 supplemented with antibiotics and FBS as mentioned above. Then, the medium changed to hygromycin B containing one as above and GFP-positive colonies were counted and the best clone was maintained for further experiment. The established packaging cell lines were termed gfp/GP2.

To generate HBV pseudotype particles (HBVpp), the established packaging cell line, gfp/GP2 was transfected with the pCEP4 LS-S expression vector with TransIT LT1 (Takara Bio) as mentioned. Two days after transfection, the supernatant was harvested and passed through a 0.22 μm filter as mentioned.

Immunoprecipitation and Western blotting analysis.

166 Supernatant from pCEP4 LS-S transfected gfp/293GP2 cells was adjusted at
167 0.3M NaCl and incubated with goat polyclonal anti-HBs antibodies (Austral
168 Biologicals) at 4 ˚C overnight. Then the supernatant was added 20 μl protein G
169 Sepharose® (GE Healthcare) and incubated for 20 min at RT. The complex was
170 pelleted and washed with sodium phosphate buffer (50mM Na-phosphate pH 7.8, 0.3M
171 NaCl) three times. Finally, half of the pellet was suspended in sample buffer (5mM
172 2-mercaptoethanol, 5% SDS, 0.3% bromophenol blue, 10% glycerol) and an aliquot
173 was subjected to SDS-PAGE followed by Western blotting analysis using a mouse
174 monoclonal antibody against HBsAg (HBs mAb #7-2).
175
176 **CsCl density gradient ultracentrifugation and Enzyme-linked immunosorbent**
177 **assay (ELISA) for HBs and preS1.**
178
179 The culture medium of pCEP4 LS-S transfeted gfp/GP2 cells was harvested
180 as above and was added 30% polyethyleneglycol (PEG6000, nakalai tesque)-3M NaCl
181 at 5% PEG and 0.5M NaCl of final concentration. This solution was stood at 4˚C

more than half an hour and then centrifuged at 10,000 × g for 30 min at 4°C. The precipitate was suspended in 500μl 10mM Tris-HCl, pH 7.6-100mM NaCl-1mM EDTA (TNE) solution and clarified insoluble materials. The soluble fraction was loaded at the top of CsCl gradient solution (39F 350 μl, 35F 2500μl, 31F 250μl, 27F 250μl, 23F 250μl, 19F 250μl and 15F 150μl from the bottom, F; CsCl percentage in weight) and centrifuged at 50, 000 rpm at 10°C for 30 hours using a Beckman Ti55 rotor and Optima® TLX ultracentrifuge (Beckman). After the centrifugation, eighteen fractions were taken from the top by the 120 μl. An aliquot (about 10 μl) from each fraction was diluted with 90μl PBS and subjected to ELISA using HBs Antigen (HBsAg) Quantitative ELISA Kit Rapid II® for HBs and HBV PreS1 Antigen Quatitative Kit® (Beakle Inc., Japan) for preS1, respectively, according to the manufacturer's direction. Forty microliter of the aliquot was subjected to RNA extraction followed by RT-PCR targeting an *EGFP* gene (see below).

Detection of a retrovirus gene in the particle by RT-PCR.

198 The supernatant from pCEP4 LS-S transfected gfp/GP2 cells was adjusted at
199 0.3M NaCl was incubated either with goat polyclonal anti-HBs antibodies (1μg/ml)
200 (Austral Biologicals) or rabbit anti-VSV-G antibodies (1μg/ml) (MBL) at 4°C overnight.
201 This mixture then incubated with 20 μl protein-G Sepharose® (GE Healthcare) for 20
202 min at RT. The complex was pelleted and washed with sodium phosphate buffer
203 (50mM Na-phosphate pH 7.8, 0.3M NaCl) three times. The final pellet and 40 μl from
204 each fraction of CsCl density gradient ultracentrifugation as mentioned above was
205 added 200μl or 160 μl TNE, respectively, and 2 μl 10 % SDS and incubated with 20 μg
206 Protainase K (Roche) at 56 °C overnight, respectively. The solution was extracted
207 with phenol-chloroform-isoamylalcohol (24 : 24 : 1) and precipitated with ethanol with
208 10 μg yeast tRNA by centrifugation at 10, 000 × g at 4 °C for 10 min. The precipitate
209 was rinsed with 70 % ethanol and dried and suspended 20 μl TE (10 mM Tris-HCl pH
210 7.6, 1 mM EDTA).
211 5 μl of the preparation was subjected to reverse transcription reaction by using
212 first strand synthesis kit (Transcriptor First Stand cDNA Synthesis Kit®, Roche)
213 according to the manufacturer's direction. This reaction was stopped with

phenol-chloroform-isoamylalcohol (24: 24: 1) extraction followed by ethanol precipitation with 10 μg yeast tRNA as mentioned above. Finally, the dried pellet was suspended in 20 μl TE. Either 5 μl out of 20 μl cDNA sample or 5 μl of RNA preparation sample was constituted in PCR using an EGFP 5' primer; 5'-ACGGCAACATCCTGGGGCACAAGC-3', and an EGFP 1327RV primer; 5'-TGTACAGCTCGTCCATGCCGAGAG-3' and Ex Taq® DNA polymerase (Takara Bio) according to the manufacturer's direction. The PCR condition was 96°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min with 35 cycles. One forth of the reaction (5 μl) was run in the 2 % agarose gel electrophoresis stained with ethidium bromide. A digital image of the DNA was obtained using a fluorescent imager (FX-800®, BioRad).

Electron microscopy.

Cells were cultured on a polystyrene cover slip, Cell Desk (Sumitomo Bakelite Co., Ltd., Japan), fixed with 2% formaldehyde and 2.5% glutaraldehyde in 0.1M sodium-cacodylate buffer (pH7.4) and washed for 5 min three times in the same buffer.

Cells were post-fixed for 1hr with 1% osmium tetroxide and 0.5% potassium ferrocyanide in 0.1 M sodium-cacodylate buffer (pH7.4), dehydrated in graded series of ethanol and embedded in Epon812® (TAAB Co. Ltd., UK). 80 nm ultra thin sections were stained with saturated uranyl acetate and lead citrate solution. Electron micrographs were obtained with a JEM-1011® transmission electron microscope (JEOL, Japan).

Results

Establishment of HBV psuedotype packaging cells.

We utilized a murine leukemia virus (MLV) packaging system since considering *in vitro* use for biological cloning of an HBV receptor in cultured cell lines based on infectivity and for checking infectivity of HBV with ease afterwards, even though mature hepatocytes have a limited growth activity in vitro. Retroviral genomes are integrated into host genome after reverse-transcribed only in growing cells except those of lentiviruses, which were utilized in a previous report [24]. Thus, the packaging design was a commonly used retrovirus packaging system consisting of 5'-LTR-packaging signal (Ψ)-hyg^{R}-CMV IE promoter-GFP-LTR-3' (Fig. 1A). This construct was integrated into a retrovirus gag-pol expressing GP2-293 cell line (Clontech), the cell line of which was termed gfp/GP2 (Fig. 1B). Thus, the transcribed RNA genome was packaged into MLV capsids. We attempted that upon envelope expression and processing, the capsids would be incorporated into enveloped particles

and secreted. If HBV membrane proteins could envelope the retroviral capsid, HBV pseudotype particles (HBVpp) would be formed (Fig. 1C).

In order to confirm whether the established packaging cell lines were available or not, we transfected to the cells with a VSV-G expression vector (Takara Bio Clontech) to form a VSV-G pseudotype (in case, this pseudotype is called a pantropic retrovirus) and its infectivity. If the packaging cell lines were available, so-called pseudotype retroviruses wrapped by VSV-G envelope would be generated and secreted into the culture medium. Thus, the supernatant of the VSV-G expressing packaging cells was contacted with BC3, a primary effusion lymphoma cell line or with the other cell lines. Two days after the contact, the infected BC3 cells were selected with a hygromycin B containing medium and we observed that hygromycin B resistant cells were growing and such cells showed GFP positive signals (Data not shown). Several cell lines including HeG2, HuH7, and HEK293 cells were effectively infected with the VSV-G pseudotype retrovirus. These results showed that the packaging system was functional and termed gfp/GP2.

Expression of HBV envelope genes.

There are three envelope genes termed large S (LS), middle S (MS) and small S (SS, also called simply HBs) (Fig. 2A) [2; 26]. Each gene is independently transcribed but the coding regions are overlapped. We constructed an HBV membrane protein expression vector termed pCEP4 LS-S under CMV immediate early enhancer and promoter (CMV IEp) in the pCEP4 expression vector (Invitrogen) (Fig. 2B). We were afraid that such strong enhancer and promoter just upstream the LS gene preferentially might drive mainly the LS gene expression, because a balance among HBV envelope proteins affected secretion activity of the virus particles [27]. Transfected cells with the pCEP4 LS-S, however, expressed generally all kinds of HBV envelope genes as reported (Fig. 2C) [28].

Formation and secretion of HBV pseudotype particles into medium.

Next question was whether our designed HBVpp would be formed and secreted into medium from the packaging cells transfected with the HBV envelopes expression vector (pCEP4 LS-S). Immunoprecipitates with anti-HBs antibodies from the supernatant of pCEP4 LS-S transfected and non-transfected gfp/GP2 cells were analyzed by Western blotting analysis to detect HBV envelope proteins. Fig. 2C showed that all HBV envelopes were expressed and actually LS, MS and SS protein expression were detected. Thus, expressed HBV envelope proteins were secreted into medium.

Secreted envelope proteins contains the designed retroviral genome.

In order to check whether HBV membrane proteins can envelope retroviral capsids, culture medium of pCEP4 LS-S transfected gfp/GP2 cells was harvested and immunoprecipitated either with anti-HBs antibodies or anti-VSV-G antibodies in case that VSV-G was expressed. Reverse transcription (RT) was performed on the RNA extracted from the immunoprecipitates. VSV-G pseudotype was also tested as a

300 positive control. As shown in Fig. 3, a correct size of amplified band was observed in

301 LS-S expressed samples as in VSV-G. This result (about ~320bp) suggests that HBV

302 envelope proteins wrapped retroviral capsids containing retroviral genomes, in which

303 *GFP* and *hygR* genes were encoded in this case.

304

305 **HBVpp shows near identical density profiles to HBV particles (Dane particles) in**

306 **CsCl density gradient ultracentrifugation.**

307

308 Next, we checked the density profiles of HBVp particles in CsCl density

309 gradient untracentrifugation. Peak fractions for HBs and preS1 were seen in fraction 8

310 whose density was around 1.17 g/ml. Furthermore, amplified *EGFP*-corresponding

311 DNA by RT-PCR was detected around the fractions positive for HBsAg. The main

312 RT-PCR products were detected in fraction 9 with higher density (1.18 g/ml) than those

313 where the most S and preS1 antigens were detected (fraction 8). The produced

314 pseudotyped HBV particles were a little bit smaller than the native HBV, since it was

315 reported that Dane particles had the heavier density (around 1.25 g/ml) than small S

316 particles (1.20~1.22 g/ml) [29]. Taken together, these observations suggest that
317 retroviral capsids surrounded by HBV membrane proteins were generated and secreted
318 into the medium from the packaging cells expressing HBV membranes and thus the
319 HBV pp was produced.
320
321 **Observation of HBVpp by electron microscopy.**
322
323 Finally, we tried to observe HBVpp from the packaging cells transfected
324 HBV membrane protein expression vectors by electron microscopy. Unfortunately,
325 we were not able to observe the complete particles on the secretion process but
326 viral-like-particles in the endoplasmic reticulum of the packaging cells expressing HBV
327 membrane proteins (Fig. 5A). Most of them might be subviral particles ie., HBs
328 particles. We also observed some typical Dane-like particles in the medium (Fig. 5B),
329 though infectious viral particle formation might not be efficient with this method.

330 **Discussion**

331

332 HBV is a small DNA virus with 3.2 kb genome involved in acute and chronic
333 liver diseases, persistence of which is predisposed to in a sense incurable liver cirrhosis
334 and cancer. It is assumed that there are more than a few hundred million infected
335 people and thus this is one of the biggest infectious diseases in the world [2]. Since
336 vaccine was developed, protection from the virus infection has been enabled except
337 some vaccine escape mutants [30; 31]. And it is certain that several nucleotide analogs
338 effectively inhibit the viral replication and then disease progression [32; 33].
339 Nevertheless, the real virus life cycle has remained to be elucidated. The critical
340 reason why there are a lot of mysteries about HBV is mainly due to no convenient *in*
341 *vitro* and/or *in vivo* infection system for HBV, though there are several animal
342 hepadnavirus models such avian (duck and heron) and rodent (ground tree squirrel and
343 woodchuck) hepatitis B viruses. Hatching and raising such animals and preparation of
344 primary cultured hepatocytes from the liver of them are not so easy for daily research

345 activities and animal hepadnaviruses are quite divergent and unlikely to be good models
346 for HBV.
347 Recent progress of tissue culture system from a hepatocellular carcinoma
348 called HepaRG [7; 34] and from primary hepatocytes of Tupaia belangeri [35; 36]
349 enable us to propagate HBV in *in vitro* infection system to some extent. By
350 differentiation of the cells to differentiated parenchymal hepatocytes with 2 % DMSO
351 as well as hydrocortisone, HBV can infect the HepaRG up to 20 % or so at viral
352 genome equivalent (V.G.E.) to a cell of over 200 [7; 34; 37]. The infected HBV,
353 however, did not produce infectious daughter viruses in spite of observation of
354 Dane-like particle formation [34]. Furthermore, it takes more than two weeks to
355 differentiate the HepaRG to susceptible for HBV infection [7]. Thus, HepaRG cells
356 are useful but it would be still less efficient for HBV infection and time-consuming
357 system to analyze the HBV life cycle.
358 It is reasonable to find out an HBV receptor in order to overcome this
359 situation followed by establishing a HBV infection system. Several candidates for
360 HBV receptors have been reported. Starting from polymerized human serum albumin