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Establishment and Characterization of a Woodchuck Hepatocellular Carcinoma Cell Line (WH44KA)

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A continuous cell line was established from a hepatocellular carcinoma obtained from a woodchuck that was sero-positive for woodchuck hepatitis virus (WHV). The cell line, designated WH44KA, grows as an adhering monolayer with a doubling time of 36 hr in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The cells not only showed epithelial origin on light and electron microscopic examination but also possess biosynthetic markers of the latter, such as albumin and α-fetoprotein, which were demonstrated in cultured cells. When they were transplanted into athymic nude mice, tumors developed at the site of inoculation. These tumors were shown to be hepatocellular carcinoma, similar in morphology to the original tumor from which the WH44KA cells were derived. Chromosome analysis revealed a chromosome number ranging from 31 to 126, with a modal number of 35. Integration of WHV DNA was shown by Southern blot analysis. However, WHV surface antigen was not demonstrated in the cultured cells or supernatant medium. The WH44KA cell line appears to be a useful *in vitro* model for the study of virus-induced hepatocellular carcinoma.

Key words: Woodchuck hepatitis virus — Woodchuck hepatocellular carcinoma cell line, WH44KA — Woodchuck hepatitis virus DNA

The woodchuck (*Marmota monax*) is one of the largest and most visible of the north American rodents. This animal has long been investigated intensively on the ecological and biological levels.[1-3] Furthermore, since the discovery of woodchuck hepatitis virus (WHV) by Summers *et al.*[4] in 1978, this animal has been used as a model for viral hepatitis and for subsequent development of primary hepatocellular carcinoma (HCC). The value of the woodchuck as a model for the study of hepatitis and its course of infection has been well established. The natural occurrence of hepatitis in woodchucks with the subsequent development of chronicity and HCC may closely resemble what happens in humans who are infected with hepatitis B virus (HBV) and subsequently develop HCC.[5,6] Thererfore, this animal is now employed as a laboratory animal to study not only obesity, endocrine function, and cardiovascular disease but especially hepatitis B and its associated diseases including HCC. A total of 40 woodchucks were imported from the United States and maintained in our laboratory for a study of virus-induced HCC. This paper reports the establishment and characteristics of a woodchuck HCC cell line, designated WH44KA, and its heterotransplantation into athymic nude mice.

MATERIALS AND METHODS

Source of Tumor Tissues An imported WHV-positive woodchuck (W44), estimated to be 3–4 years old, and trapped in Pennsylvania, was used in this study. The tumor tissue was surgically obtained from the animal. Histologically, the tumor was classified as a moderately differentiated HCC.

Tissue Culture Tumor tissue was washed with Hanks' balanced salt solution (Gibco) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin and then minced with surgical knives into small pieces. The minced tissue was centrifuged (600 rpm, 5 min) and the pellet was resuspended in 5 ml of 0.25% trypsin in PBS (−), and incubated at 37° for 30 min. After exposure to this enzyme solution, the cell suspension was centri-

fuged at 600 rpm for 5 min. The cell pellet was suspended in 5 ml of Dulbecco's modified Eagle's medium (Nissui), supplemented with 10% fetal bovine serum (Gibco) and then centrifuged again in the same manner. The pellet was resuspended in culture medium similar to that just described and then distributed into plastic Petri dishes (35 mm) (Corning/Iwaki Glass, Tokyo), and cultivated at 37° in a humidified atmosphere of 5% CO_2 in air. The growth medium was changed once a week.

Serial Passage and Storage The cell line was passaged by treatment with 0.25% trypsin and 0.02% EDTA in PBS(−), and maintained in growth medium. During serial passages, cells of different passage numbers in growth medium containing 10% dimethylsulfoxide were frozen and stored in liquid nitrogen.

Morphological Examination The cells were removed from a culture flask with 0.25% trypsin and 0.02% EDTA and seeded in a chamber slide. Cells grown on coverslips were fixed by 10% neutral buffered formalin solution, and stained with hematoxylin-eosin. These preparations were examined under a light microscope.

Determination of Cell Growth and Temperature-sensitivity A cell suspension of 1×10^4 cells was seeed into 35 mm plastic Petri dishes with 2 ml of growth medium, which was replaced every day. Twenty-four hours later, the dishes were transferred to the temperature-gradient incubator, in which the temperature and humidity in the chambers could be precisely adjusted. A cell count was taken every 2 days in three dishes. Viable cells were determined by the dye exclusion method after staining with 0.2% trypan blue solution. The doubling time of the cell population incubated at 37° was examined in the logarithmic growth phase.

Chromosome Analysis Cells were treated with 0.1 μg/ml colcemid (Gibco) for 1 hr, then lysed with 0.062*M* KCl for 20 min and finally fixed with methanol-acetic acid (3:1) solution. Giemsa staining was performed, and one hundred metaphases were analyzed for chromosome number.

Detection of Albumin, α-Fetoprotein (AFP) and WHV Surface Antigen (WHsAg) Determination of albumin, AFP and WHsAg localization in cultured cells was performed by indirect immunofluorescence using specific antibodies after fixation of cells on the slide with cold acetone. Rabbit anti-woodchuck albumin serum was kindly provided by Dr. Matsushita (School of Medicine, Kanazawa University, Kanazawa), rabbit anti-woodchuck AFP serum was kindly provided by Dr. Tennant (New York State College of Veterinary Medicine, Cornell University, N.Y.) and antibody specific for WHsAg was made in our laboratory. WHsAg of supernatant media was assayed by enzyme-linked immunoassay with the use of the cross-reacting human hepatitis B virus surface antigen (HBsAg) Auszyme kit (Abbott Laboratories, North Chicago, Ill.).

Xenografts Four-week-old athymic nude mice (BALB/c/*nu/nu*) were used as hosts for tumor cell injection. Inocula of 1×10^7 cells suspended in PBS(−) were injected subcutaneously into the flanks of the nude mice. Passages of the xenografts were also performed. Tumors in nude mice were removed about 1 month after inoculation and then minced into small pieces and passed by retransplantation into nude mice.

Histopathology For light microscopy, the xenograft was fixed in 10% neutral buffered formalin and stained with hematoxylin-eosin (HE). For electron microscopic observation, the tissue was fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in graded ethanols, and embedded in epoxy resin. Ultra-thin sections stained with lead citrate and uranyl acetate were examined under an electron microscope.

Detection of WHV DNA Integration Cells and tissues were quickly frozen and stored at −80° until use. DNA was prepared from minced liver tissues and cultured cells by using lysis buffer consisting of 10m*M* Tris-HCl (pH 8.0), 150m*M* NaCl, 10m*M* Na_2EDTA, 0.2% sodium dodecyl sulfate and 100 μg of proteinase K/ml, at 37° overnight. After chloroform-phenol extraction, the supernatant was treated with RNase and again extracted with chloroform-phenol. The purified DNA (10–20 μg) was digested, according to the suppliers' instructions, with different restriction enzymes, including *Kpn* I, which has no cleavage site on the WHV genome and *Hin*d III, *Bam* HI and *Pst* I, which each have one cleavage site on the WHV DNA. Electrophoresis was done in 1.0% agarose horizontal gels, in 89m*M* Tris, 89m*M* boric acid and 2m*M* Na_2EDTA. The molecular markers were bacteriophage λ DNA digested by *Sty* I. Following electrophoresis, the DNA was transferred to nylon membranes (PALL Ultrafine Filtration Corp.) after alkali and acid treatment. Prehybridization was performed in a prehybridization solution consisting of 5×Denhardt's solution (1×solution: 0.02% each of Ficoll, polyvinyl-pyrrolidone, bovine serum albumin), 5m*M* Na_2EDTA, 50m*M* phosphate buffer (pH 8.0), 0.9*M* NaCl, 0.2% sodium dodecyl sulfate and 1 mg of carrier DNA/ml (salmon sperm DNA) at 65° overnight. Hybridization was done with the ^{32}P-labeled nick-translated probe of a cloned whole WHV gemone inserted into the *Eco* RI site of pBH20, kindly provided by Dr. Summers (Fox Chase Cancer Institute, Pa.) in a hybridization solution at 65° overnight. The filter was washed twice with excess volumes of the washing buffer consisting of 1m*M* Na_2EDTA, 0.2%, sodium dodecyl sulfate and 0.2

Fig. 1. Phase-contrast micrograph of established WH44KA cell line. ×200.

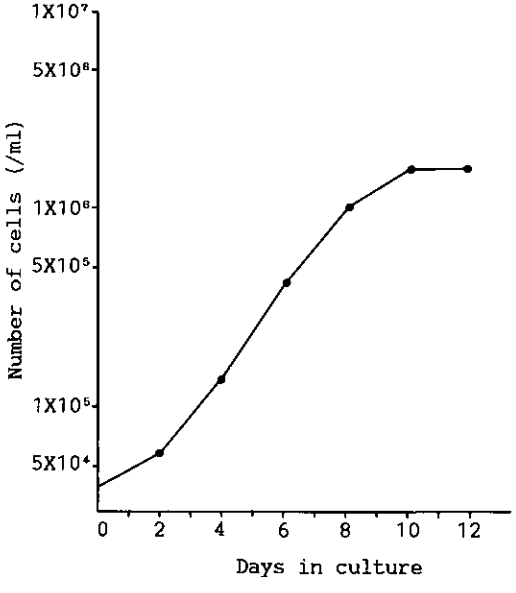

Fig. 2. Growth curve of WH44KA cells at the 28th passage showing a doubling time of 36 hr.

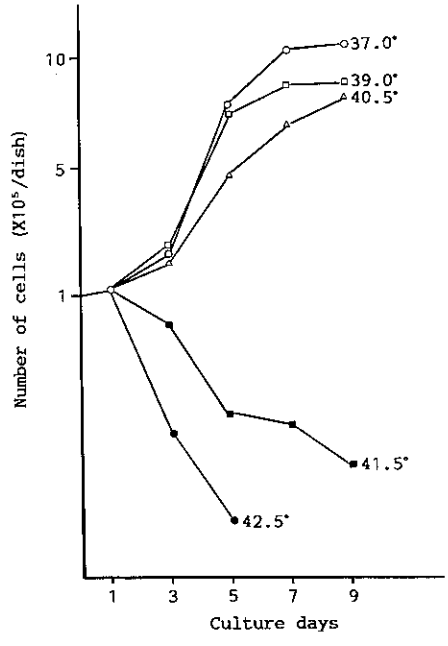

Fig. 3. Effect of temperature on the proliferation of WH44KA cells. Cells were incubated for 24 hr at 37°, and then exposed to each temperature for 5–9 days.

M phosphate buffer (pH 7.0) at room temperature. The dried filter was exposed to X-ray film (XAR, Kodak) in the presence of an intensifying screen at −80° for 2 to 7 days.

RESULTS

Establishment of WH44KA Many colonies of epithelial-like cells were observed surrounded by fibroblasts about one week after the onset of culturing. About one month after the primary culture was completed, epithelial cells became predominant, replacing virtually all the fibroblastic cells. The culture cells exhibited typical epithelial cell morphology characterized by sheets of polygonal cells with trabecular and pavement arrangement (Fig. 1). These epithelial cells seemed to be adapted to the culture conditions after 3 months. This cell line, named WH44KA, thus established has undergone more than 70 passages to date.

Cell Growth and Effect of Temperature The cells grow logarithmically with a doubling time of approximately 36 hr. A typical growth curve is shown in Fig. 2. The cell proliferation was slightly inhibited at temperatures ranging from 39 to 40.5°. At over 41.5°, the cells were apparently damaged, and the number of viable cells gradually decreased with time (Fig. 3).

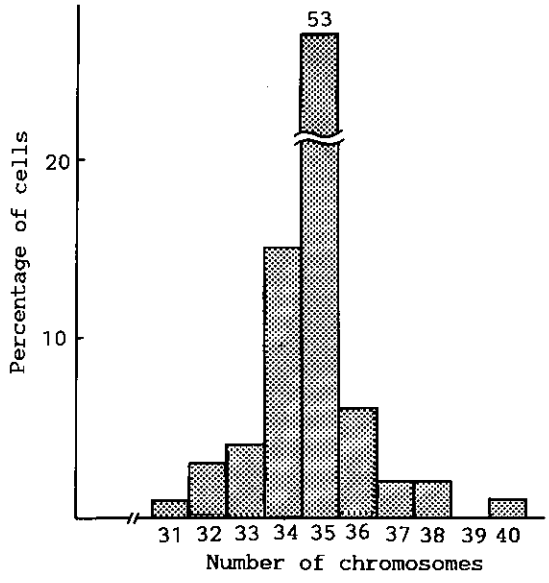

Fig. 4. Distribution of chromosome number of WH44KA cells.

Chromosome Analysis Chromosomal study was done at the 40th passage. The number of chromosomes varied from 31 to 126. The modal number was 35, as indicated in Fig. 4. Metaphase of WH44KA cells is shown in Fig. 5.

Detection of Albumin, AFP and WHsAg Albumin and AFP were detected in the cytoplasm of cultured cells by immunofluorescence (Fig. 6). On the other hand, WHsAg was not detected in cultured cells and supernatant medium.

Growth Characteristics of Xenografts Inoculation of nude mice with 1×10^7 WH44KA cells resulted in a detectable tumor after 16 days. The tumor reached a size of $1.7\times1.5\times1.0$ cm after 28 days. Macroscopically, tumors were well encapsulated, firmly attached to the covering dermis, and exhibited no invasion into underlying tissue. Microscopically, xenografts were surrounded by fibroblastic tissue. The tumor closely resembled the primary tumor, and was classified as moderately differentiated HCC (Fig. 7). Electron microscopic examination revealed tumor cells containing abundant free ribosomes, undeveloped endoplasmic reticulum, dilatations of intercellular space and an irregular distribution of chromatin with large nucleoli (Fig. 8). No viral particles were noted.

Detection of WHV DNA Integration Integration of WHV DNA was demonstrated in WH44KA cells and a xenograft, since distinc-

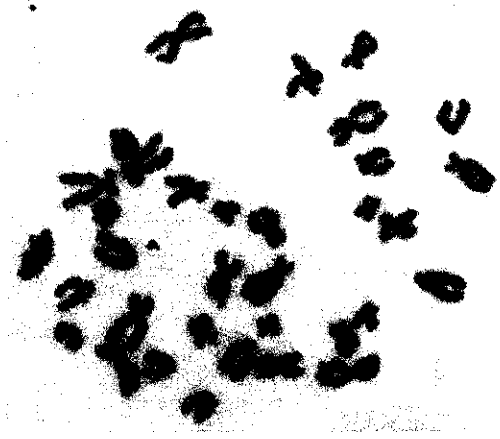

Fig. 5. Metaphase of WH44KA cell showing 35 chromosomes.

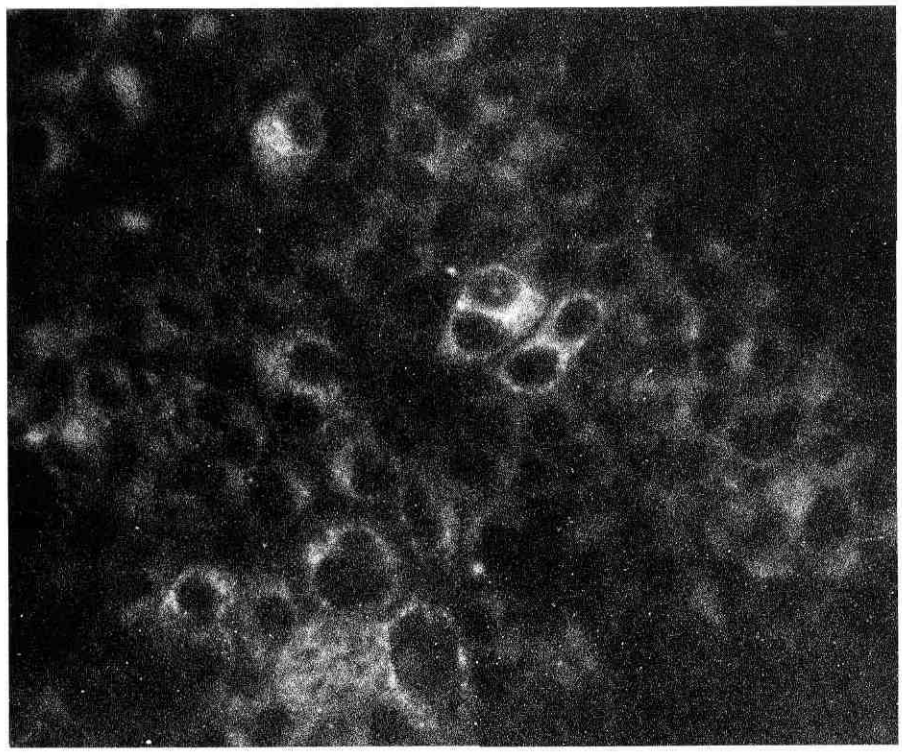

Fig. 6. Immunofluorescence for woodchuck α-fetoprotein in the cytoplasm of WH44KA cells. $\times 200$

tive bands of WHV DNA having a molecular weight larger than the unit length of WHV DNA were observed in digested samples of WH44KA cells and a xenograft (Fig. 9). The number of integration sites of WHV DNA is more than one, because there are more than a single band in the digest of *Kpn* I which has no cleavage site in the WHV genome and more than two bands in the digests of *Hin*d III, *Bam* HI and *Pst* I which have one cleavage site in the genome. In the primary tumor, a large amount of extrachromosomal WHV DNA was present in addition to integrated WHV DNA, although extrachromosomal WHV DNA could not be detected in WH44 KA cell DNA. The pattern of the WHV DNA integration in WH44KA cells and a xenograft was similar to that in the primary HCC.

Discussion

Biological study of HBV has been drastically limited due to its restricted host range and failure to infect tissue culture. Recently three hepatitis viruses infecting, respectively, woodchuck, Beechey ground squirrel, and Pekin duck, were isolated.[4, 7, 8] These viruses have many characteristics in common with human HBV, some being unique to this novel class, and are classified as hepadnavirus. WHV is the most oncogenic virus, and chronic infection with it causes HCC in more than half of infected animals.[5, 9, 10] Due to the close correlation between chronic hepatitis and HCC, studies have been conducted to investigate the role of WHV DNA in the occurrence of HCC. WHV DNA has been found to be integrated in the nuclear DNA of woodchuck HCC.[11] These findings are important in evaluating the behavior of WHV in carcinoma cells and the role of WHV in carcinogenesis. In this study, we have succeeded in establishing an HCC cell line (WH44KA), which was derived from a woodchuck that was sero-positive for WHsAg. WH44KA cells represent the

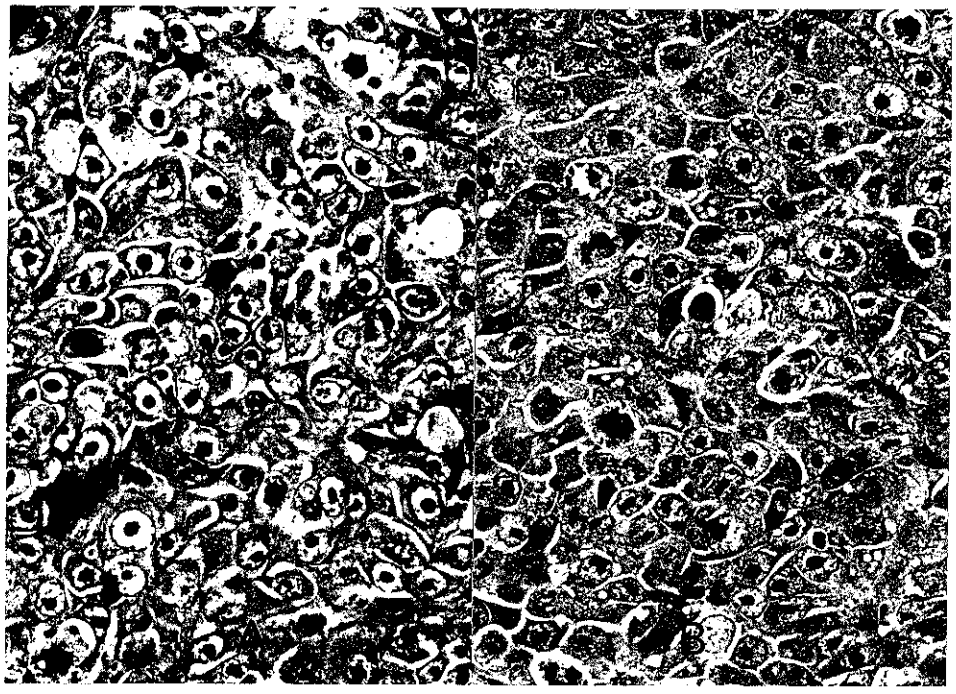

Fig. 7. (A) Histological findings of the original tumor showing moderately differentiated HCC. (B) Histological findings of a xenograft of WH44KA cells in a nude mouse showing a close resemblance to the tumor of origin. HE, ×200.

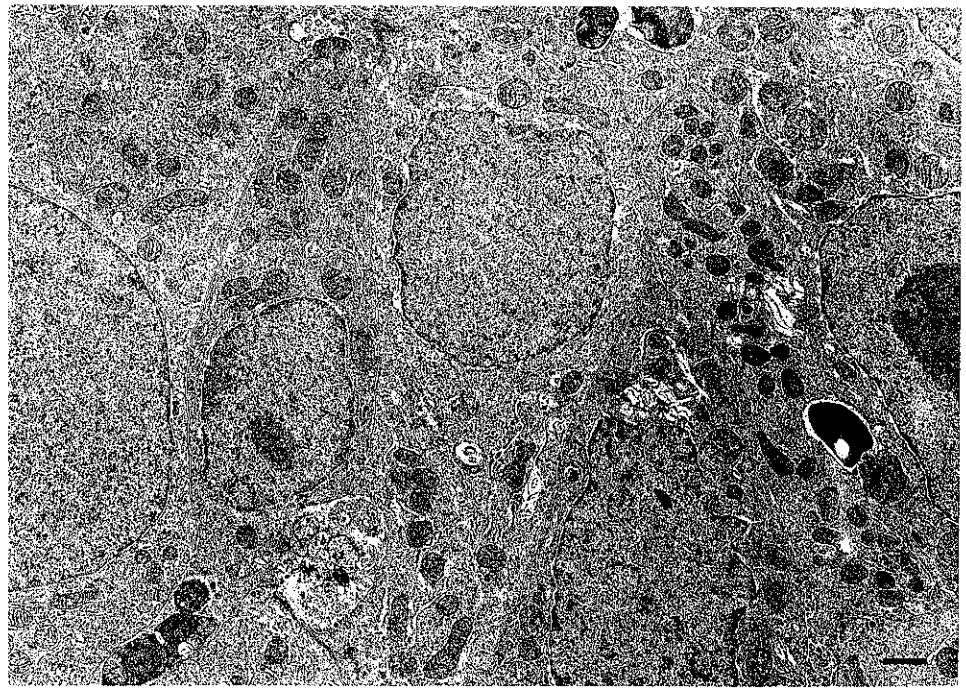

Fig. 8. Electron micrograph of a xenograft of WH44KA cells in a nude mouse showing carcinoma cells containing abundant free ribosomes and undeveloped endoplasmic reticulum. Bar=1 μm. ×6,820.

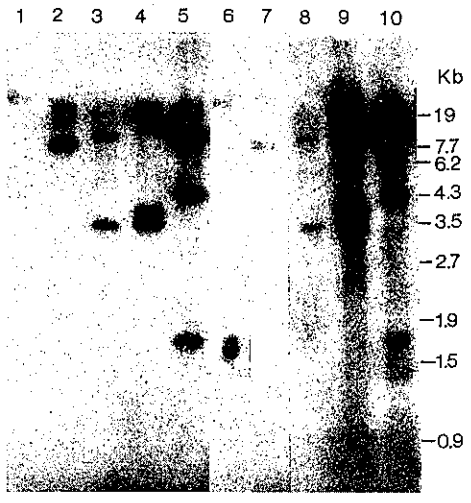

Fig. 9. Autoradiograph showing hybridization of ^{32}P-labeled cloned WHV DNA probes to WHV DNA in Southern blot analysis of the WH44KA cells and a xenograft. DNA samples of WH44KA cells (lanes 1 to 5) and a xenograft (lanes 6 to 10) were applied onto a 1.0% horizontal agarose gel without digestion (lanes 1 and 6) or after digestion with *Kpn* I (lanes 2 and 7), with *Hind* III (lanes 3 and 8), with *Bam* HI (lanes 4 and 9), or with *Pst* I (lanes 5 and 10). Molecular markers are shown on the rigth.

second woodchuck HCC cell line established as a permanent line in tissue culture. The first woodchuck HCC cell line (WH257GE10) was established by Unoura *et al.*[12] in 1985. However, attempts in some laboratories, including our own, to establish cell lines from normal woodchuck hepatocytes have not been successful. The morphologic features of WH44KA cells described above coincide well with what are believed to be those of epithelial cells. In particular, the electron microscopic findings are believed to be typical for carcinoma cells originating from epithelial cells. Another strong indication that this cell line represents carcinoma cells derived from hepatocytes is that albumin and AFP production is detectable. The modal number of chromosomes for the WH44KA cell is 35 as compared with 38 in the woodchuck cells.[13] This chromosomal abnormality in the WH44KA might be related to the pathogenesis of a woodchuck HCC. The WH44KA cells lost their growth potency under high temperature treatment. Concerning the temperature-sensitivity of cultured cells, it is reported that the adaptability of cancerous cells to high temperature is poor in comparison with that of normal cells.[14] Human HCC cell lines which have been reported so far possess the ability to produce HBsAg *in vitro*, which provides evidence that the cell lines have originated from human HCC.[15, 16] In contrast, WH44KA cell line lacks the ability to produce WHsAg in supernatant medium, although WHsAg secretion could be anticipated since the cells were derived from a woodchuck that was sero-positive for WHsAg. However, many reports show that HBsAg was almost always detected in non-cancerous hepatocytes, and rarely in cancerous cells when the localization of HBsAg in liver tissue was studied.[17-19] Similar findings can be seen in the liver of woodchucks infected with WHV.[20] These results indicate that HBsAg and WHsAg may disappear from cancerous tissue and replication of hepatitis virus may cease in cancerous cells. It is interesting that the WHV DNA integration pattern in the established cell line and transplanted tumor cells in nude mice are similar to that of the original woodchuck HCC tissue. The integration of WHV DNA sequence in these transformed cells derived from the primary HCC suggests that the integration of WHV DNA sequences is crucial for maintenance of the transformed state of these cells. Further evidence that WHV induces cell transformation by its presence or integration still awaits a suitable *in vitro* culture system for WHV. In contrast to WH257GE100 cell line, we succeeded in serial heterologous transplantation into athymic nude mice with the WH44KA cell line. Furthermore, the histological findings and pattern of WHV DNA integration of the transplanted tumor were similar to those of the primary tumor. This result suggested that the WHV DNA integration in the host DNA had been maintained without change of the band patterns for more than one year during passages of cultured cells. From these results, the WH44KA cell line is expected to be useful as an *in vitro* model for the study of virus-induced HCC.

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