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Transformation of NIH/3T3 Cells by DNA from a Human Hepatoma Cell Line with Integrated Hepatitis B Virus DNA*

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Abstract—*We have studied by means of DNA-mediated gene transfer the transforming activity of the DNA of the human hepatoma cell line HCC-M, which contains genomes of hepatitis B virus (HBV) in integrated form. DNA from HCC-M induced transformed foci on transfection of NIH/3T3 cells. DNAs from primary transformants were capable of inducing secondary transformants. Most of the DNAs of these transformants were demonstrated to contain both human repetitive sequences and HBV DNA, indicating that the transformants had incorporated exogenous human DNA and HBV DNA as well. These results suggest that transformation occurs as the result of the transfer of oncogene which might be closely associated with HBV genome.*

INTRODUCTION

CLOSE association between hepatocellular carcinoma and hepatitis B virus (HBV) has been noted based on epidemiological evidence [1–3]. HBV DNA has been found integrated in the DNAs of HBsAg-positive human hepatoma cells and human hepatoma derived cell lines [4–7]. HBV DNA sequences were detected even in patients without any serological HBV markers [8]. These data indicate that HBV DNA integration may play a part in tumor generation, although the exact significance of integrated HBV sequences is unclear. Morphological transformation of NIH/3T3 cells by transfection with genomic DNA has been used to detect dominantly-acting transforming genes in human tumors and tumor-derived cell lines [9, 10]. We had established a human hepatoma cell line from a serum hepatitis B surface antigen (HBsAg)- and hepatitis B e antigen (HBeAg)-positive Japanese male patient [11]. In the present study we examined whether DNA from the human hepatoma cell line HCC-M which has HBV DNA in integrated form had an ability to transform NIH/3T3 cells by DNA transfection assay.

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MATERIALS AND METHODS

Cell culture

HCC-M cells were maintained in a medium RPMI-1640 supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 μg/ml streptomycin (Flow Laboratories, Inc., McLean, CA, USA). NIH/3T3 cells were cultured in Dulbecco's modified essential medium (MEM) (Flow Laboratories, Inc.) supplemented with 10% FCS and the same antibiotics. The latter cells were used as recipients of transfection experiments within 2 months after being thawed from liquid nitrogen. Previously it had been demonstrated that HCC-M cells do not produce HBV-associated antigens nor alphafetoprotein [11]. HBV DNA had been demonstrated to be integrated in the genome of HCC-M by Southern blot hybridization [12]. No free viral DNA was detectable in HCC-M.

DNA preparation

DNAs were prepared according to Gross-Bellard *et al.* [13]. Cells cultured in monolayer were trypsinized and suspended in phosphate-buffered saline (PBS, pH 7.4) and washed three times with cold PBS. Cells were resuspended in 0.05 M Tris–HCl (pH 8.0) containing, 10 mM ethylenediaminetetraacetic acid (EDTA) and 0.15 M NaCl in a 50-ml silicon-coated flask and sodium dodecyl sulphate (SDS) was added at a final concentration of 0.5% (W/V) and Proteinase K (Boehringer, Mannheim, FRG) was added at a concentration of 500 μg/ml. They were incubated for 3 hr at 50° C. DNA was extracted three times from the

lysate with phenol saturated with 0.1 M Tris–HCl (pH 8.0) and precipitated with 2 vol. cold ethanol and DNA was collected with a glass rod. It was resuspended in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (TE buffer) and treated with RNase A (100 μg/ml) for 1 hr at 37°C. Proteinase K and SDS were then added to the lysate as above and incubated for 2 hr at 37° C. The DNA was precipitated with ethanol and dissolved in TE buffer (pH 8.0).

Transfection of NIH/3T3 cells with cellular DNAs

DNA transfers used the calcium phosphate precipitation method according to Perucho *et al.* [14]. Two-hundred-and-fifty thousand NIH/3T3 cells were seeded in a 100 mm culture dish. Twenty hours later the medium was changed with 10 ml Dulbecco's medium supplemented with 10% FCS. DNA was sheared by passing through a 23 gauge needle several times and an equal vol. of 0.5 M $CaCl_2$ was added and mixed well. An equal vol. of 50 mM HEPES, 280 mM NaCl, 1.5 mMNa_2PO_4 (pH 7.4) was added dropwise and incubated for 30 min at room temperature. Precipitated DNA (30 μg) in a vol. of 0.9 ml was then added to the above dish. After an additional 12 hr, each plate of treated cells was trypsinized and seeded into five 100 mm dishes in Dulbecco's medium and 5% FCS. The cells were refed with this medium at intervals of 3–4 days.

Approximately 2 weeks later all the cultures were examined for the presence of transformed foci which appeared on a background of semiconfluent normal cells.

DNA probes

The detection of human repetitive DNA sequences used DNA extracted and prepared from HeLa cell according to Murray *et al.* [15] as a probe. The 2300 base pair recombinant HBV DNA, pHBV 114 [16] which was a kind gift from Dr. Murray was used as a probe to detect HBV DNA homologue. These DNA probes were labeled with ^{32}P-dCTP (800 cpm/nmol, Amersham, London, UK) by nick translation procedure of Rigby *et al.* [17] to a specific activity $> 1 \times 10^8$ cpm/μg.

Hybridization

To analyze the DNAs from many transformants the dot hybridization was performed according to Kafatos *et al.* [18]. Nitrocellulose filters (22 mm in dia.) were water washed and placed on a platform consisting, from bottom to top, of dry paper towels, moist 3 MM paper and moist nitrocellulose paper. The filters were washed with drops of 1 M ammonium acetate. Extracted DNA is denatured in 0.4 N NaOH for 10 min and chilled, and diluted with an equal vol. of cold 2 M ammonium acetate to a concentration of 16 μg/ml. The DNA solution was delivered to the filter using a pipette connected to a Clay Adams pipette filler. After each sample was spotted (approx. 1.5 min), the filter was rinsed through with a drop of 1 M ammonium acetate. The filters were washed with approx. 200 ml 4 × SSC (1 × SSC; 0.15 M NaCl/0.015 M sodium citrate) and air dried, shaken in 2 × Denhardt's solution (1 × Denhardt's: 0.02% bovine serum albumin, 0.02% polyvinylpyrollidone, 0.02% Ficoll) for 1.5 hr, drained and air-dried, baked under vacuum at 80° C for 2 hr and stored dry.

The filters were soaked for 1.5 hr in 10 × Denhardt's solution, 4 × SET (1 × SET buffer; 0.15 M NaCl, 0.03 M Tris–HCl, pH 8.0, 1 mM EDTA), transferred to a vinyl bag containing blank hybridization mixture (50% deionized formamide, 2 × Denhardt's solution, 4 × SET, 0.1% SDS, 100 μg/ml yeast tRNA and 125 μg/ml poly(A)), incubated for 1.5 hr at 50° C, and then incubated for 20 hr with gentle shaking in hybridization mixture, containing in addition the radioactive probe (10^6 cpm/ml). The filters were washed at 50° C with 3 changes of blank hybridization mixture and then thoroughly with 50% formamide–2 × SET. They were drained, placed on wax paper on top of a glass plate, wrapped with Saran-wrap, and were exposed at −80° C for 16–48 hr with Kodak XR-5 film with intensifying screens.

RESULTS

Focus formation

Through 6 experiments with 30 Petri dishes totally 19 foci were obtained approx. 2 weeks after the transfection of NIH/3T3 cells with DNA from HCC-M cells. A focus of transformed cells growing piled up was shown in Fig. 1. On the other hand, through 2 experiments with 10 Petri dishes 4 foci were obtained after the transfection with DNA from NIH/3T3 cells. The cells in the foci were picked, transferred to a flask and cultured. The frequency of focus formation per μg DNA from HCC-M cells was 0.106 on average, whereas that from NIH/3T3 cells was 0.067 (Table 1).

Analysis of HCC-M and transformant DNAs

In Fig. 2 an autoradiogram of the dot hybridization of DNAs from transformants, HCC-M cells and pHBV 114 was shown. HCC-M DNA was demonstrated to possess homologue(s) to HBV DNA. DNAs of 14 primary transformants were examined whether they had human repetitive DNA sequences and HBV DNA homologue. Nine of the transformants were demonstrated to have human repetitive DNA sequences and the homologue(s) to HBV DNA (Table 2).

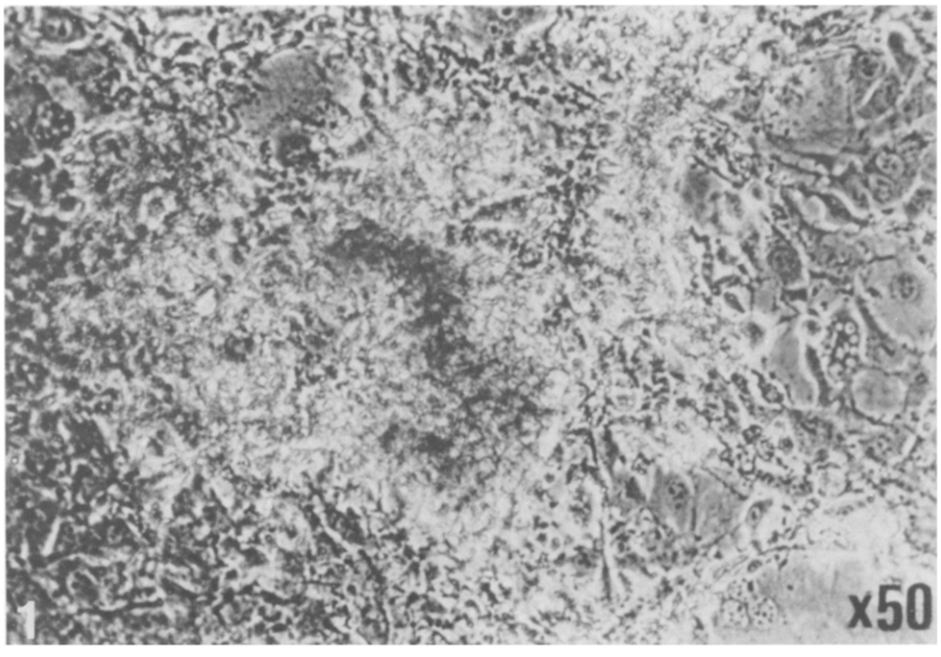

Fig. 1. Morphology of transformed focus induced in NIH/3T3 cells by transfection of DNA from the human hepatoma cell line HCC-M.

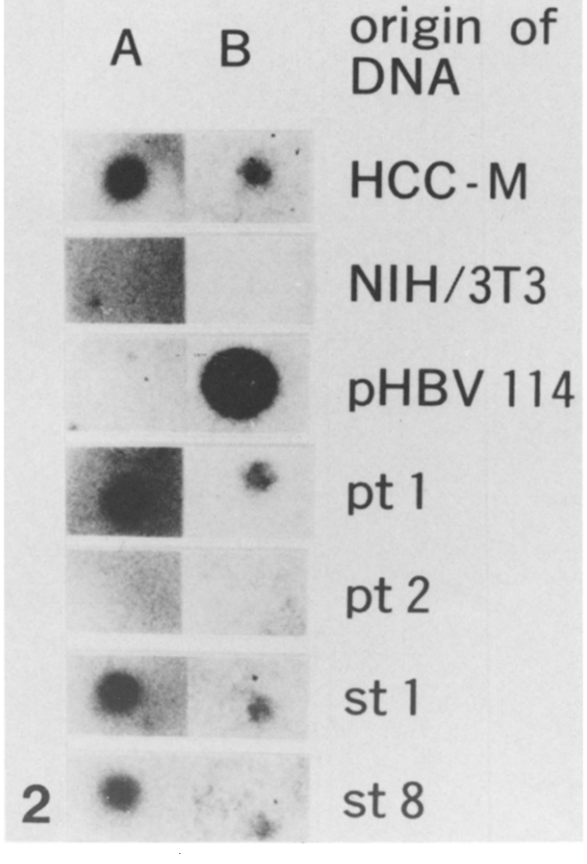

Fig. 2. Detection of human sequences and HBV DNA in NIH/3T3 transformants. Representative cases are shown. DNAs from HCC-M, NIH/3T3 cells, two of the primary transformants, pt1 and pt2 and two secondary transformants, st1 and st8, were hybridized with HeLa cell DNA(A) and with pHBV 114, a recombinant HBV DNA(B).

Table 1. *Focus formation by DNA transfection*

DNA source	No. of experiment	No. of foci obtained from 5 dishes	No. of foci per μg DNA
HCC-M	1	8	0.267
	2	4	0.133
	3	0	0
	4	1	0.033
	5	6	0.2
	6	0	0
	Mean value	3.16	0.106
NIH/3T3	1	2	0.067
	2	2	0.067
	Mean value	2	0.067

Table 2. *Detection of human repetitive sequences and hepatitis B virus DNA homologue in primary transformants*

Transformant	Probe	
	HeLa DNA	HBV DNA
pt1	+	+
pt2	−	−
pt3	+	+
pt4	−	−
pt5	+	+
pt6	+	+
pt7	+	+
pt8	+	+
pt9	+	+
pt10	+	+
pt11	+	−
pt12	+	+
pt13	−	−
pt14	−	−

Table 3. *Focus formation by DNA of primary transformants*

DNA source	No. of foci obtained from 5 dishes	No. of foci per μg DNA
pt1	6	0.2
pt9	4	0.133

Table 4. *Detection of human repetitive sequences and hepatitis B virus DNA homologue in secondary transformants*

Transformant	DNA source for transfection	Probe	
		HeLa DNA	HBV DNA
st1	pt1	+	+
st2	pt1	+	+
st3	pt1	+	+
st4	pt1	+	+
st5	pt1	+	+
st6	pt1	+	+
st7	pt9	+	+
st8	pt9	+	+
st9	pt9	+	+
st10	pt9	+	+

Second cycle of transfection

NIH/3T3 cells were transfected by DNA from two of the primary transformants, pt1 and pt9, which were demonstrated to possess the both human DNA sequences and HBV DNA. Totally, 10 foci were induced by the DNA from these cells (Table 3). All of these secondary transformants were demonstrated to possess the both human DNA sequences and HBV DNA (Table 4).

DISCUSSION

Since Shih *et al.* [19] investigated whether DNA extracted from tumor cells could induce neoplastic transformation of a recipient cell line, NIH/3T3, which has many of the properties of normal cells, it has been shown that DNAs of a variety of neoplasms from several different vertebrate species, including man, induce transformation of this cell line. But, there are few studies concerning hepatocellular carcinoma [20, 21]. To our knowledge, this is the first report about the transforming activity of the DNA from human hepatocellular carcinoma.

The present study suggests that oncogene(s) which are active in HCC-M cells are transferred to NIH/3T3 cells in conjunction with HBV DNA and the both should be closely associated, because in most of the transformants, primary or secondary, HBV DNA was found at a high frequency. And in our previous transformation experiments with HBV DNA alone was unsuccessful [12], suggesting that HBV DNA transfer occurs in conjunction with oncogene(s) that are responsible for the transformation. Analysis of integrated HBV DNA sequences present in hepatoma cells revealed

that integrated viral sequences were often rearranged as well as cellular flanking sequences adjacent to the viral genome [22]. Although it is not known whether such a structural rearrangement is present in HBV DNA sequences integrated in HCC-M, it might be possible that HBV integration provides a potential signal for activation of cellular oncogenes. And also it might be possible that hybrid HBV-host gene products induce transformation, as suggested in the case of other human hepatoma cell line [23].

The great majority identified by transformation of the NIH/3T3 mouse cell line belong to three members of one gene family, called *ras* [24]. The *ras* genes were originally identified as the oncogenes of Harvey and Kirsten murine sarcoma virus. At present it is known that about 15% of all human tumors appear to contain *ras* oncogenes which are active in the transformation assay. Therefore, it is most likely that HCC-M cells possess oncogene(s) of the *ras* family. However, it is also possible that some unknown oncogene(s) would be responsible for the transformation induced by HCC-M DNA, since it is also known that transfection experiments detect oncogenes that are not *ras* and do not seem to be homologous to any of the viral oncogenes.

We are now trying to identify the oncogenes involved by using several oncogene probes. We are also trying to clone HBV DNA-containing sequences from HCC-M DNA genomic libraries for further analysis of the relationship between oncogenes and HBV genome integration.

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