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Structure and expression of integrated hepatitis B virus genes in an HBs antigen producing human cell line (huGK-14)

Noboru Nakamichi, Asao Noda, Tomonori Yonezu, Katsuro Koike¹ & Toshiharu Matsumura
Meiji Cell Technology Center, Meiji Milk Products Co., Odawara 250, Japan; ¹ The Cancer Institute, JGCR, Kami-Ikebukuro, Tokyo 170, Japan

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Abstract

A human continuous cell line (huGK-14) within a lineage of passaged cultures was investigated in the mode of integration and expression of hepatitis B virus (HBV) genes. HBV DNA was integrated in eight different sites of the cellular DNA, in each of which HBV genome was rearranged, fragmented, and/or partly deleted. Complete HBV genome that may lead to production of infectious virus particles was not detected in the cells nor in the culture medium. Clones of cDNA containing a complete coding frame for small HBs antigen protein (type adr) were obtained from mRNA of the cells. The cells were stable over the period of six months of cultivation and more than 60 population doublings in the mode of HBV integration and HBs mRNA expression.

These results provide substantial evidence for the absence of an ability for the integrated DNA to create an infectious product in the cell; for the stable production of HBs mRNA from the cells, and suggest the usefulness of this cell line as a substrate for HBV vaccine production.

Abbreviations: S1, PreS1; S2, PreS2; C, core antigen; X, X protein; P, DNA polymerase; HBV, hepatitis B virus

Introduction

The advantages of using human diploid cell lines in biopharmaceutical production are now widely accepted both scientifically and ethically (Hayflick, 1989), but those of using human continuous cell lines have become recognized only recently (Petricciani, 1992; Pearson, 1992; Petricciani and Horaud, 1995). In the past ten years, progress in purification and detection technologies and viral and oncogenic DNA research has increasingly removed fears of the possibility of transfection of oncogenic principles through purified products from a continuous cell line, and safety concerns are becoming focused more on the qualification of products and validation against possible contamination by adventitious agents in the products, as suggested in recent official documents (CBER, 1993, 1996). Characterization of a substrate cell line is important and informative not only for safety, but also in constructing

a whole system of manufacturing processes using the cell line. To the authors' knowledge, two human continuous cell lines have been approved so far in Japan for biopharmaceutical production, i.e., Namalwa cells of Burkitt lymphoma origin and BALL-1 cells of acute B cell leukemia origin, both for α -interferon production (ABMJ, 1993).

The possibility has long been discussed that a hepatitis B virus (HBV) vaccine may be produced using and HBV surface antigen (HBsAg) protein-producing human continuous cell line, and that such a vaccine may be advantageous in several respects over those obtained from the pooled serum of infected donors (Skelly *et al.*, 1979; McAleer *et al.*, 1979; McAleer *et al.*, 1983). Such possibility has been sought, particularly with the PLC/PRF/5 cell line of hepatocellular carcinoma origin, known as the Alexander cell line (MacNab *et al.*, 1976; Alexander *et al.*, 1978; Barin *et al.*, 1981). The technology of HBsAg

production, and knowledge of HBs genes in the cells and HBsAg obtained have been particularly gained from this cell line (Alexander *et al.*, 1978; Skelly *et al.*, 1979; Marion *et al.*, 1979; Barin *et al.*, 1983; Dejean *et al.*, 1983; Koshy *et al.*, 1983; Zerial *et al.*, 1986; Rivkina *et al.*, 1988). Among other HBsAg protein-producing human hepatocarcinoma cell lines, huH-1 cell line has been suggested to be useful for vaccine production (Huh and Utakoji, 1981). In a series of attempts to select a high HBsAg producer, a sub-line huSP was obtained from huH-1 (Mizusawa *et al.*, 1985), and a colonially selected line huGK-14 was further obtained from huSP (Koike *et al.*, 1985, 1987). It was shown in huH-1, huSP, and huGK-14 cells that HBV DNA was integrated in multiple integration sites of cellular DNA, with rearrangements and deletions, and without any sign of the presence of the whole viral genome (Koike *et al.*, 1983, Koike *et al.*, 1985). An HBV DNA fragment flanked by cellular DNA at both ends was characterized in details showing rearrangements accompanied with an inverted repetition (Mizusawa *et al.*, 1985). HBsAg particles were obtained from a serum-free culture of huGK-14 cells which resembled the small spherical HBsAg particles in the sera of HBV carriers (Koike *et al.*, 1987).

The HBV genome consists of ca. 3.2 kb partly double stranded circular DNA, and transcribes 3.5 kb, 2.1 kb, and a few other poly A⁺ RNAs (For review, ref. to Tiollais *et al.*, 1985; Ganem and Varmus, 1987). The 3.5 kb poly A⁺ RNA which contains the whole genomic sequence is transcribed for the synthesis of viral DNA polymerase and for the replication of viral genome, while other poly A⁺ RNAs are transcribed for coding four protein species. The S, PreS1 and PreS2 regions of the genome contain coding frames for HBsAg proteins of three types, i.e., the major protein (S), middle protein (PreS2 + S) and large protein (PreS1 + PreS2 + S). These genes are located in the order of C, PreS1, PreS2, S and X. The P gene for the HBV DNA polymerase is extended from part of C region to part of X region, overlapping the whole of preS1, preS2 and S regions. There are several subtypes in HBV, which are very similar in terms of their genomic structure.

It is generally accepted, based on numerous studies on HBV DNA-containing hepatocellular carcinoma cells and cultured cell lines thereof, that HBV DNAs are integrated in cellular DNA with frequent structural rearrangements including deletion and inversion, that free HBV genome is rarely detected, and that some of the integrated HBV genes are expressed to produce

Table 1. Cultures of clone huGK-14 subjected to investigation

Cell generation (PDLs) ^a	Culture duration (months)	Designation of cell stages
0		Primary stocks cells
12		Secondary stock cells
18		Tertiary stock cells
18-33		Cells in a reactor
	2	R2M
	4	R4M
	6	R6M
33		Upper limit of inoculation in reactor
62		Excessively grown cells

^a Population doubling levels (PDL)

viral proteins (Brecht *et al.*, 1981, 1982; Shafritz and Kew, 1981; Shafritz *et al.*, 1981; Koike *et al.*, 1983; Yaginuma *et al.*, 1984; Choo *et al.*, 1986; Tokino *et al.*, 1987; Takada *et al.*, 1990; Matsubara and Tokino, 1990). These studies encouraged us to further evaluate the candidate cell line huGK-14 for HBV vaccine production not only for safety, but also in respect to production efficiency and stability.

In this paper, we report the characteristics of huGK-14 cells in a lineage of passaged cultures as outlined above.

Materials and methods

Cultures subjected for analysis

The lineage of passaged cell cultures of huGK-14 studied in this paper is shown in a summarized form in Table 1. Briefly, cells of clone huGK-14 propagated in monolayer cultures in plastic containers were trypsinized and freeze-preserved in ampoules as a primary stock. From a primary stock ampoule, cells were reconstituted, propagated in plastic containers, trypsinized for passage cultures, and frozen again as secondary and tertiary stocks with the number of cell population doublings (PDL) at 12 and 18 respectively when the PDL of the primary was stock regarded as 0.

Cells reconstituted from a stock ampoule were grown in monolayer, trypsinized and then introduced for long term static maintenance culture in a perfusion-type cell reactor of 800 ml volume in which the cells were immobilized on glass fiber cloth of 2800 cm² surface area. The cell reactor system will be described

in detail at a later date. The culture environment was kept at 37 °C with a controlled gas supply of oxygen, nitrogen and carbon dioxide, and the culture medium was continuously supplied and harvested during cultivation. Cells between 18 PDL and 33 PDL were introduced in a reactor and maintained for up to 6 months. Cells obtained from the reactor after 2, 4 and 6 months are referred to as R2M, R4M and R6M respectively. If not otherwise stated cells used in this study were within a few passages from the primary stock.

Growth medium contained DM-160 (Katsuta and Takaoka, 1978) supplemented with 1% or 5% fetal bovine serum (FBS). Maintenance medium used for maintaining the cells in the reactor contained William's E medium (Williams and Gunn, 1974) supplemented with 0.1% bovine serum albumin (BSA) and with 1 μ M dexamethasone.

Since we limited the use of huGK-14 cells within the present cell lineage in this study, huGK-14 cells used in previous studies (Koike *et al.*, 1985, 1987), are referred to below as the original huGK-14 cells.

DNA preparation

Cellular DNAs were prepared from cultured cells using a proteinase K/SDS method (Maniatis *et al.*, 1982). Briefly, approximately 10^7 cultured cells were suspended in one milliliter buffer solution containing 150 mM NaCl; 10 mM Tris-HCl, pH9.5; 10 mM Na₂EDTA. To this cell suspension, SDS was added at 0.1% and incubated for 30 min. at 55 °C to solubilize the cells. To the solution 100 μ g RNase A was added and incubated for 30 min. at room temperature followed by the addition of 250 μ g proteinase K. The solution mixture was incubated for four hours at 37 °C. At the end of the incubation, phenol:chloroform (1:1) extractions and chloroform extractions, twice for each, were carried out to eliminate proteins and other cellular debris. The upper aqueous layer containing purified DNA was dialyzed four times against 100 volumes of a buffer solution containing 10 mM Tris-HCl, pH8.0; 1mM Na₂EDTA.

For the concentration of DNA-containing particulate fractions of the spent medium, a cesium chloride equilibrium density gradient ultracentrifugation was performed before DNA extraction. Here, Pool 1 was obtained containing centrifugal fractions with densities heavier than 1.25 g/ml, which should contain infectious HBV particles (Dane particles) if any. Also obtained was Pool 2 of fractions with densities between 1.16 g/ml and 1.21 g/ml, in which the majority

of HBsAg in the spent culture medium was recovered in a membrane-bound particulate form (Koike *et al.*, 1987). DNAs in each pool were then purified using the proteinase K/SDS methods as described above.

Southern and northern blot methods

Southern and northern blot hybridization analyses were carried out following the methods basically as described (Maniatis *et al.*, 1982). For the restriction analyses, we confirmed that reported HBV DNA sequences of type adr including the present one have only one cleavage site in their sequences for *Bam*HI and no sites for both *Hind*III and *Pst*I (Ono *et al.*, 1983; Fujiyama *et al.*, 1983; Kobayashi and Koike, 1984). Sample DNAs treated either with or without a restriction enzyme, and RNAs were transferred to Zeta probe nylon membrane (Japan Bio-Rad Laboratories, Tokyo, Japan).

The whole 3.2 kbp HBV DNA probe was prepared by *Bam*HI digestion of pBRHBadr125 (type adr) which was kindly supplied by Dr. T. Yoneyama at the National Institute of Health, Tokyo, Japan. HBV gene-specific hybridization probe DNAs were prepared by digesting the whole HBV DNA with appropriate restriction enzymes as follows; S-gene specific 612 bp fragment probe (*Acc*I/*Hind*II), X-gene specific 369 bp fragment probe (*Bam*HI/*Rsa*I), C-gene specific 444 bp fragment probe (*Bg*/II), and preS1/S2-gene specific 395 bp fragment probe containing 364 bp preS1 and 31bp preS2 sequence (*Alu*I).

DNA sequencing

For the purpose of mapping and sequencing analyses, DNA fragments containing HBV DNAs were first cloned either in λ EMBLE 4 or λ ZapII vector and further subcloned after digestion with *Bam*HI and *Eco*RI. Nucleotide sequences were determined using either M13 sequencing kit (TOYOBO Co., Tokyo, Japan) or 7-DEAZA sequencing kit (Takara Co., Shiga, Japan). Template plasmids were purified by a published method (Hattori and Saiki, 1986), M13/M4 universal and M13/RV reversal sequencing primers were purchased from Takara Co. Other primers shown in the figure legends were synthesized using an automated DNA sequencer (Perkin Elmer, Chiba, Japan).

Figure 1. Attempted detection of free HBV DNA in huGK-14 cell and its spent medium by Southern blot analysis. Purified DNAs (10 μ g/lane) from huGK-14 cells (lanes a–d) and spent medium (pool 1 and 2, see materials and methods) were digested with restriction enzymes, electrophoresed on a 0.7% agarose gel and probed with 32 P-labeled 3.2 kbp HBV DNA after transfer to a nylon membrane. a: undigested; b: *hindIII* digested; c: *Bam*HI digested; d: *Pst*I digested; e, g: pool 1 and 2 undigested; f, h: pool 1 and 2 *Pst*I digested. A purified 3.2 kbp HBV DNA was used as a sensitivity standard in lanes i through m; i: 5 fg; j: 50 fg; k: 100 fg; l: 1 pg; m: 10 pg. X-ray film was exposed for 29 hours at -80°C with two intensifying screens. The size marker indicated on the right hand side is Lambda phage DNA digested with *Hind*III.

Results

Attempted detection of free HBV DNA in the cells

In a Southern hybridization analysis using 32 P-labeled HBV DNA as a probe, DNA of the particulate fractions of spent culture medium showed no detectable band in the range of 2.3–3.2 kb, but showed high molecular weight bands presumably of cellular DNA fragments containing integrated HBV genome (Figure 1 lanes e–h). Since infectious HBV particles, if any, should contain 2.3–3.2 kb range DNA in a particulate fraction detected in Pool 1, and its precursor DNA molecule should be found in the undigested cellular DNA preparation as free molecules of corresponding molecular sizes, the present results are all against the presence of infectious HBV in the culture. It was also noted that free HBV DNA in the range of 2.3–3.2 kb was not detected in the undigested total cellular DNA (Figure 1 lane a). The sensitivity of detection is less than 0.2

Table 2. Detection of HBV genes in *Pst*I fragments by Southern blot analysis

Fragment size	Presence of HBV genes			
17 kbp	C	S1/S2	S	X
8 kbp	C	S1/S2	S	X
6 kbp	C	S1/S2	S	^a
4 kbp	^a	^a	S	X
3.6 kbp	C	S1/S2	^a	X
3.2 kbp	^a	S1/S2	S	^a
3.0 kbp	C	S1/S2	^a	X
2.4 kbp	C	^a	S	X

^a genes not detected by partial probes

copy HBV per total DNA equivalent with one copy of cellular DNA (Figure 1 lanes i – m).

Cellular DNA after digestion with either *Hind*III, *Bam*HI or *Pst*I showed a number of bands indicating that HBV genomes are integrated in cellular DNA (Figure 1 lanes b – d). These results extended the previous observation on huH-1 cells and the original huGK-14 cells that no free viral linear HBV DNA (3.2 kb) nor free viral closed circular DNA (2.3 kb) was detected in the cells (Koike *et al.*, 1983). However, since bands of larger than 3.2 kbp size were detected, the possibility was not rejected that one or more of the integrated HBV genome might transcribe a 3.5 kb genomic RNA for the production of Dane particles.

Cellular DNA fragments containing HBV genomes

To investigate the possibility of one or more of the integrants detected in Figure 1 being a template for the genomic RNA transcription, i.e., 3.2 kbp DNA with full set(s) of HBV genes in the right order, cellular DNA was completely digested with *Pst*I and subjected to Southern analyses using the whole HBV DNA as well as the partial HBV DNAs as probes. The *Pst*I was used because the best separation of eight bands, i.e., 17 kb, 8 kb, 6 kb, 4 kb, 3.6 kb, 3.2 kb, 3 kb and 2.4 kb, was obtained (Figure 1 lane d). The 17 kb band and the 8 kb band hybridized with all the four partial probes, suggesting that they might contain full sets of HBV genes. On the other hand the other six bands did not contain a complete set of HBV genes, as indicated by the loss of one or more bands to be detected by the partial probes (Figure 2 and Table 2).

Since there are no *Pst*I sensitive sites in HBV DNA, it is expected that DNA fragments in these six bands are all flanked by cellular DNA fragments at both ends,

Figure 2. Southern blot analysis of huGK-14 genomic DNA. huGK-14 genomic DNA (50 μ g) was completely digested with *Pst*I and loaded 10 μ g DNA per lane. Samples were treated the same way except for the probe hybridization step in which each lane was cut out and hybridized with 32 P-labeled HBV partial probes. A: S-gene specific probe; B: C-gene specific probe; C: preS1/S2-gene specific probe; D: X-gene specific probe; E: 3.2 kbp HBV whole DNA probe. X-ray film was exposed for 18 hours at -80°C with two intensifying screens. The size marker indicated on the right hand side is Lambda phage DNA digested with *Hind*III. ori indicates the electrophoresis start point of each sample.

and therefore that they all lack the complete sequence for infectivity. However, it should be taken into account that by structural alterations, a *Pst*I site could possibly be created in the fragment of integrated HBV DNA, and thus this restriction enzyme could split an integrated HBV DNA into unflanked pieces. If a sequential combination of two or three of the fragments contains C, PreS1, S, and X gene in the right order, then the possibility still remains that an integrated DNA remains in intact form. We investigated the 36 possible sequential pair combinations among the six fragments, and found no such combination. Combinations of three fragments among the six was also investigated with negative results.

These results are in accordance with the previous observation on huH-1, huSP, and the original huGK-14 cells that HBV DNA was integrated in multiple integration sites of cellular DNA, with rearrangements and deletions, and without any sign of the presence of the whole viral genome in them (Koike *et al.*, 1983, 1985, 1987). These results also reject the possibility that an intact HBV DNA sequence remains at these

six integration sites in chromosomal DNA. It is highly probable that the HBV sequences in the present 4 kb fragment and in the hu489 clone previously obtained from huSP cellular DNA fragments (Koike *et al.*, 1985; Mizusawa *et al.*, 1985) are the same, since both could be detected only with the X and S probes and there are no other such HBV DNA fragments noted in huGK-14.

Thus, the only possibility for the presence of complete set(s) of HBV DNA remains in the two large fragments of 17 kb and 8 kb, or sequential combinations including them.

Analyses of 17 kbp and 8 kbp genomic fragments

The 17 kbp and the 8 kbp bands detected in the Southern blot analyses were cloned in λ EMBLE 4 and λ ZAPII vectors, respectively. The results of restriction map analyses showed that the 17 kbp genomic fragment contained HBV genome in two separate locations, and 8 kbp fragment in one. In both cases HBV genome was flanked by cellular DNA at both ends (Figure 3A and 4A). A 3.3 kbp and a 2.7 kbp *Bam*HI-subfragment from the 8.8 kbp *Eco*RI fragment, and a 3.5 kb *Bam*HI/*Eco*RI-subfragment and a 2.7 kb *Bam*HI-subfragment from 7.6 kbp *Eco*RI fragment respectively were subcloned into pUC19 vector for partial nucleotide sequence determination. Nucleotide sequences determined are shown by rightward and leftward arrows under the figures (Figure 3B and Figure 4B).

The results of mapping and sequence analyses are schematically presented in Figures 3B and 4B. In all integrated HBV stretches examined in the 17 kb and 8 kb fragments, deletions, insertions, and inversions of HBV genome were noted, and no complete sequence of HBV genome was recovered from any part of the fragments. Hereafter, an incomplete gene and an inverted gene is assigned with a prime and an underline, respectively. The 7.6 kb fragment contained a stretch of HBV genome in the order of X', C, S1, S2', S, X, C', S2', S1 and C'. The 8.8 kbp fragment contained the same stretch of HBV genome except that its left most terminal sequence including X' and a part of C are deleted. Thus, it contains C', S1, S2', S, X, C', S2', S1 and C'. Details in the point and sizes of deletions, insertions, and inversions are described in the legends of Figures 3 and 4. As shown in Figure 3B, the points of deletion in the left terminal C' and right terminal C' in 8.8 kbp fragments, and that in the right terminal C' in the 7.6 kbp fragment are all the same. Also, the three

Figure 3. Restriction mapping and structure of integrated HBV genomes in the 17 kbp genomic clone. (A) Restriction map of the 17 kbp genomic DNA in λEMBL4 vector was constructed for *Bam*HI and *Eco*RI. E: *Eco*RI recognition site. B: *Bam*HI recognition site. Four subfragments (3.3, 2.7, 3.5 and 2.7 kbp) generated by *Bam*HI and *Bam*HI/*Eco*RI digestions were cloned into pUC19 vector for further analysis. Fragments sizes are in kilo base pair (kbp). (B) Four subclones were partly sequenced as indicated by the rightward and the leftward arrows under the drawings. The structural arrangements of integrated HBV genes in 8.8 kb and 7.6 kb fragments were predicted by combining confirmed (solid line boxes) and presumptive HBV sequences (dashed line boxes) corresponding to W.T. HBV sequences. Gene organization of wild type HBV DNA (W.T. HBV) is shown in the middle as a standard, and gene arrangements in the 8.8 kbp fragments (8.8 kbp fgt.) and the 7.6 kbp fragment (7.6 kbp fgt.) are shown above and below the standard, respectively. ∇: indicates a deletion of 47 bp between the nucleotide number 3096 and 3142, and an insertion of 2 nucleotides (CA). ▼ and ▲: indicate an insertion of 12 bp (the nucleotide numbers 3154 to 3143) and an insertion of 2 nucleotides (TG). Nucleotide numbers are according to Kobayashi and Koike (1984).

Figure 4. Structural analyses of integrated HBV DNA in the 8 kbp genomic clone. (A) Restriction map of the 8 kbp genomic DNA in λZapII vector was constructed for *Bam*HI and *Eco*RI. E: *Eco*RI recognition sites. B: *Bam*HI recognition site. (B) Nucleotide sequences were partly determined as indicated by the rightward and the leftward arrows under the drawings. The structural arrangements of integrated HBV genes in 8 kb fragments were predicted by combining confirmed (solid line boxes) and presumptive HBV sequences (dashed line boxes) corresponding to W.T. HBV sequences. ∇: indicates a deletion of 99 bp between the nucleotide numbers 2014 and 2112. Nucleotide numbers are according to Kobayashi and Koike (1984).

cellular sequences flanking these three C' are all the same when sequenced regions are compared (data not shown). These results suggest that incomplete duplication and inversion of HBV genome occurred during the development of hepatoma and/or cell line establishment in vitro in the locality of chromosomal DNA.

The 8.0 kbp fragment contained a stretch of HBV genome in order of C', S1, S2, S and X' (Figure 4). The 12 bp deletion at the left terminal of X' occurred in the 11-base pair viral direct repeat (DR1) sequence. Integration of HBV DNA through DR1 has been observed frequently in other hepatocellular carcinomas (Dejean *et al.*, 1984).

These results show that none of the three stretches of HBV genome in the 17 kb fragment and the 8 kb fragments has a complete HBV sequence. They may restore the capability of transcribing S gene for HBs major protein from all three stretches, and S1, S2 and S genes for large and middle proteins from the 8.0 kbp fragment, X gene for X protein from 8.8 kbp and 7.6 kbp fragments, and C gene for core protein from 8.0 kbp fragment. But the sequence characteristics do not enable them to transcribe P gene for DNA polymerase, and a complete set of 3.5 kb mRNA for viral replication. The deletion in the S2 region of both 8.8 kb and 7.6 kb fragments is described as 47 base deletion plus two base insertion in the legend to Figure 3. However, since there are variations within the adr subtype, it is also possible that there was only 45 base deletion, and other base differences were due to polymorphism.

Stability of integrated HBV genomes

Preparations of *Pst*I-digested DNA and poly A⁺ RNA were obtained from the cells at various culture stages during the course of cell stock construction, serial passages, and incubation in long terms as shown in Table 1. In southern analyses using the S-gene probe and a whole HBV genome probe no apparent changes were noted in the gel profiles (Figure 5). These results show that the cell line is stable during the serial passages and incubation for up to six months in the static maintenance culture in terms of the mode of HBV genome integration.

Structure and expression of HBs mRNA

Using the HBs-DNA probe, an apparent single 2.1 kb band was detected in a Northern blot of poly A⁺ RNAs which were extracted from three different culture stages (Figure 6).

Figure 5. HBV genome stability in six different cell stocks derived from huGK-14 cell as examined by Southern analysis. Purified DNAs (10 μ g/lane) from different cell stocks were digested with *Pst* I, electrophoresed on a 0.7% agarose gel and probed with ³²P-labeled 3.2 kbp HBV DNA (left panel) or with ³²P-labeled S-gene probe (right panel). 1: Primary stock cells. (PDL = 0); 2: Cells at PDL = 12; 3: Cells at PDL = 18; 4: Excessively grown cells (PDL = 62); 5: Cells cultured for two months in a cell reactor (R2M); 6: Cells cultured for six months in a cell reactor (R6M). X-ray film was exposed for 29 hours at -80 °C with two intensifying screens. The size marker indicated on the right hand side is Lambda phage DNA digested with *Hind*III.

From the Poly A⁺ RNA, two cDNA clones were obtained using HBs DNA as a probe, and one PCR-amplified cDNA preparation using synthetic primers. All the three cDNA preparations were sequenced showing that they all contain the same coding frame for the HBs major protein (type adr). These results show that at least the majority of the mRNA are homogeneous in terms of their HBs nucleotide sequence. The sequence of 226 amino acid residues as deduced from the present cDNA sequence was the same as those of three reported type adr HBs major proteins except for a variation in seven positions of amino acid residues (Table 3). They are considered to be variations within type adr since the seven variation detected here coincide with the reported positions of polymorphism (Fujiyama *et al.*, 1983; Kobayashi and Koike, 1984).

Discussion

Using passaged cultures in a lineage of the continuous human cell strain, huGK-14, it was shown in the results that HBV DNAs were integrated in the cellular DNA, that the integrated HBV DNAs were modified by insertions, deletions, multiplications, and reversions, and no complete sets of HBV genes in the right order were present, and that intact HBV genomes were unde-

Table 3. Amino acid sequence variations of HBsAg between the present and reported adr subtypes

Subtypes	Amino acid position from the N-terminus HBsAg protein						
	3	4	44	114	126	165	184
huGK-14	Asn	Thr	Gly	Thr	Thr	Trp	Val
adr ^a	Ser	Thr	Gly	Ser	Ile	Trp	Ala
adr 4 ^b	Asn	Thr	Gly	Ser	Ile	Gly	Val
adr 27 ^b	Asn	Ser	Glu	Ser	Ile	Trp	Ala

^a: sequence from Kobayashi and Koike (1984)

^b: sequences from Fujiyama *et al.* (1983)

Figure 6. HBs mRNA stability in three different cell stocks derived from huGK-14 cell as examined by Northern analysis. Poly A⁺ RNAs isolated from cells at various stages were processed for a Northern blot which hybridized with ³²P-labeled S-gene probe. The same filter was used to hybridize with ³²P-labeled GAPDH probe as an internal standard. 1: Primary stock cells; 2: Excessively grown cells (PDL = 62); 3: Cells cultured for six months in a cell reactor (R6M). X-ray film was exposed for 29 hours at -80 ° with two intensifying screens. The size markers indicated on the left hand side are RNA markers (BRL, U.S.A.).

tectable either in the chromosome, in the cells, or in the culture medium. In these respects, the present results confirmed and extended the previous observations on

huH-1, huSP and the original huGK-14 cells. In addition, the present results showed that mRNA for HBs major protein in the correct sequence was steadily produced for months over cell generations of more than 30 PDLs. It was also shown that the mode of HBV genome integration and HBs mRNA expression as studied by Southern and Northern analysis was stable during that time and cell generation.

The absence of complete HBV DNA either in a free or integrated form has been reported in a number of hepatocellular carcinoma cell lines, as described in our introduction, and we believe that huGK-14 is not particular in this respect: Previously reported examples include Alexander cells which contain integrated HBV DNA fragments at seven locations (Dejean *et al.*, 1983). One of its seven HBV integrants contains 125% of HBV genome, but does not produce free HBV DNA (Dejean *et al.*, 1983; Chakraborty *et al.*, 1980; Edman *et al.*, 1980; Ziemer *et al.*, 1985). In other studies, HBV DNAs were found to be integrated in all cellular DNAs isolated from 20 human hepatoma patients only in deleted and/or rearranged forms (Brecht *et al.*, 1981), and free HBV genome was not detected when 16 available samples were analyzed (Brecht *et al.*, 1982).

In the present results, we obtained decisive evidence showing that the two large HBV DNA-containing fragments, i.e., the 17 kbp fragment and the 8 kbp fragment, contain only disrupted HBV genomes. The fact that rearrangements of HBV sequence are multiplied in the 17 kb fragment, while that of the 8 kb fragment contained only deletions, supports the classification of integrated HBV DNA into primary integrants and secondary rearrangements (Matsubara and Tokino, 1990). It should be mentioned here that the schematic structures shown in Figures 5 and 6 are rough, since we determined DNA sequences only within limited ranges traversing flanking positions, and

positions of deletions predicted by PCR analyses (data not shown). Therefore, there may be small deletions, insertions and/or rearrangements in other parts of HBV DNA sequences. Also, flanking sequence regions, which remained largely undetermined, may contain substantial information.

It was reported that integrated HBV acts as an insertional mutagen in the process of hepatocellular carcinogenesis (Dejean and de The, 1990). In this study, integrated HBV genomes were shown to be stable over 30 cell generations, as studied by Southern analyses of restriction fragments, and by Northern analyses of HBs mRNA expression. Although duplication and inverted attachment of a part of the integrated HBV DNA fragment were suggested in the 18 kbp fragment, a remarkable preservation of original structure, given some deletions, was observed in the 8.0 kbp fragment. Although we did not directly compare huGK-14 cells with other cells in their gene stability, we believe that the genetic stability of huGK-14 cells is sufficient for their use for production purposes.

In the manufacturer's view of the homogeneity of products, a host cell containing a small number of production genes is preferred. Considering that a number of recombinant cell lines for production have been obtained by gene amplification technology, containing hundreds of copies of genes for production, which are often unstable during cell proliferation, we believe that the present cell line may be appreciated as a production cell line, in view of the copy number and stability of integrated production genes.

To evaluate the present cell line as a candidate substrate for HBV vaccine production, we are aware that the results presented so far do not cover all the aspects to be considered such as analysis for adventitious microorganisms and their genes other than HBV, oncogenes, tumorigenesis, isozymes and karyology. Nevertheless, as far as the molecular genetic properties of this cell line are concerned, we believe that the present results provide substantial evidence to evaluate the cell line for the production of biologicals with respect to its safety, stability and quality of mRNA transcripts. In addition huGK-14 cell line is a candidate for recombinant production. At present, several human continuous cell lines, such as human kidney cell line 293 (Graham, 1987) are being studied as hosts of recombinant production (Garnier *et al.*, 1994). Since huGK-14 cells produce some serum proteins efficiently (unpublished), they may also produce exogenous recombinant proteins efficiently.

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Address for correspondence: Toshiharu Matsumura, Meiji Cell Technology Center, Meiji Milk Products Co., Odawara 250, Japan.