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Loss and Acquisition of Duck Hepatitis B Virus Integrations in Lineages of LMH-D2 Chicken Hepatoma Cells

SHIH S. GONG,* ANNE D. JENSEN, AND CHARLES E. ROGLER

Marion Bessin Liver Research Center, Department of Medicine, The Jack and Pearl Resnick Campus of the Albert Einstein College of Medicine, Bronx, New York 10461

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Hepatocellular carcinoma is the culmination of a series of genetic events which progressively alter the phenotype of a hepatocyte toward malignancy. Hepadnaviral DNA integrations are agents of genetic change which can promote the process of hepatocarcinogenesis. We previously characterized episomally derived duck hepatitis B virus (DHBV) integrations in LMH-D2 cells that replicate wild-type DHBV. In an effort to understand how integrations function as agents of progressive genetic change, we have studied integrations of DHBV DNA in three lineages of LMH-D2 cells through three generations of subclones. Our data have established several features of the integration process. First, single and multiple integrations occur continuously through successive cell generations. Second, the integration frequency can vary dramatically in subclones of the same cell line. Third, integrations can be lost from successive generations of cells and loss of an integration can be accompanied by loss of cellular DNA associated with the integration. Finally, certain subclones which acquire greater plating efficiency have been distinguished by unique new integration patterns. These results provide a basis for DHBV integrations to function as activators of protooncogenes, as well as agents of the loss of tumor suppressor genes during hepatocellular carcinogenesis.

Hepadnaviruses contain a partially double-stranded DNA genome of approximately 3 kbp which contains a terminal redundancy in its minus strand (5, 26). The terminal redundancy mediates circularization of the virus genome during synthesis of the plus strand. Upon infection of host hepatocytes, the open circular (OC) viral DNA is believed to be converted to the covalently closed circular (CCC) form, which serves as the template for synthesis of pregenomic RNA and mRNAs encoding viral structural proteins and polymerase (35). This mechanism for the production of hepadnavirus pregenomic RNA molecules is different from that of retroviruses, which depend on establishment of a provirus for the synthesis of RNA genomes (36).

Although a provirus is not required for replication of hepadnaviruses, integration of hepadnavirus DNA molecules into host chromosomes does occur during persistent infection (1, 21, 24, 27). It is generally believed that integration of hepadnavirus DNA is an infrequent event, although the frequency of hepadnavirus DNA integration has never been directly measured. Hepadnavirus integrations fall into two general categories, including simple integrations (24, 39), which are composed of a single fragment of viral DNA colinear with the viral genome, and complex integrations, which contain highly rearranged viral DNA sequences (20, 21, 25). In both categories, the structure of the integrations does not allow the transcription of a functional pregenomic RNA and the integrations cannot function as proviruses. Open reading frames for viral envelope proteins are often present and may be expressed from integrations which are present in some tumors and cell lines (3, 28, 41).

The structure of "simple" integrations suggests that they are produced by integration of linearized monomeric viral DNAs. Preferred viral integration sites are found adjacent to and between the 5′ ends of the plus and minus strands, which are bounded by the DR1 and DR2 sequences in viral DNA (29). These findings suggest that structural, as well as sequence, features of viral DNA molecules play an important role in the integration mechanism. A cellular enzyme, topoisomerase I, has been found to cleave woodchuck hepatitis virus (WHV) (37) and duck hepatitis B virus (DHBV) (11a) DNAs near DR1, and this leads to linearization of viral DNA molecules. Furthermore, topoisomerase I can mediate integration of WHV DNA into chromosomal DNA in vitro (37). Integration of topoisomerase I-linearized DNA is expected to produce integrations with a simple structure.

The steps involved in producing "complex" integrations are unknown. It is possible that mutant viral DNAs, produced by illegitimate replication of double-stranded linear (DSL) viral DNA molecules (32, 40), are the substrates for these complex integrations. It has also been suggested that novel forms of WHV DNAs which contain highly rearranged WHV genomes and are present in chronically infected woodchuck livers may serve as substrates for complex integrations found in woodchuck hepatocellular carcinomas (14, 23).

The above studies have begun to unravel the molecular mechanisms of hepadnaviral DNA integration. These studies have important relevance to hepatocarcinogenesis because mutagenic agents such as viral DNA integrations can increase cancer risk. One mechanism of hepatocarcinogenesis discovered for WHV integrations is the activation of the N-*myc* or C-*myc* protooncogene (1, 4, 19). Another mechanism suggested for human hepatitis B virus integrations is the expression of the hepatitis B virus X gene from integrated sequences (11, 13, 17, 33). The hepatitis B virus X gene is capable of acting as an oncogene when expressed in the liver of transgenic mice at appropriately high levels (15, 16). Some data have suggested that integrations may also mediate the loss of tumor suppressor genes by functioning as agents of major chromosomal rearrangements (9, 22, 30). Furthermore, expression of human genes involved in cell cycle control (cyclin A) (38),

* Corresponding author. Mailing address: Marion Bessin Liver Research Center, Department of Medicine, The Jack and Pearl Resnick Campus of the Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-2607 or 430-3651. Fax: (718) 430-8975.

maintenance of differentiated cellular phenotypes (retinoic acid receptor β) (3a), and regulation of intermediary metabolism (mevalonate kinase) (7) has been altered by HBV DNA integration in individual tumors.

To understand more fully the mechanism of integration and how the integration process may function in hepatocarcinogenesis, we have utilized a cell culture system to detect and characterize new episomally derived hepadnavirus integrations. By using the LMH-D2 cell line, which replicates DHBV, we have demonstrated the occurrence of new DHBV DNA integrations (6). One integration we characterized completely had a simple structure, with viral integration sites in the preferred region near DR1. In this study, we used a subcloning protocol to trace integrations through three cell lineages of LMH-D2 cells which replicate wild-type DHBV. Analysis of integration patterns of subclones through three generations demonstrated both acquisition and loss of integrations in individual subclones. We found that the integration frequency varied from low to high among the three lineages. In one lineage of subclones, a complex new integration pattern associated with markedly increased plating efficiency was observed. Therefore, new integration patterns may be used to distinguish subclones of cells selectively amplified as a result of altered growth properties.

MATERIALS AND METHODS

Cell culture. The LMH-D2 cell line was the generous gift of William Mason (Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pa.). The cells were maintained in DMEM/F12 medium (GIBCO) containing 10% fetal bovine serum and 200 μg of G418 per ml. The LMH-D2 cell line was derived from LMH chicken hepatoma cells (12) by transfection with a previously reported plasmid (2) which contains the cytomegalovirus immediate-early (CMV-IE) promoter driving transcription of a greater-than-genome-length DHBV DNA genome (18) which produces a functional DHBV pregenome RNA and mRNAs for all of the structural proteins. The plasmid also contains a neomycin resistance (*neo*) gene for selection. Subclones were initially derived from the parental LMH-D2 cell line by plating limiting dilutions of single-cell suspensions in 96-well culture plates or by plating highly diluted single-cell suspensions onto 100-mm-diameter culture dishes. In some cases, to facilitate the early growth of single G418-resistant LMH-D2 cells, G418-sensitive LMH helper cells were plated onto culture dishes that were first seeded with LMH-D2 cells and the mixed cultures were grown in medium without G418 for 2 days; after this time, the medium was changed by addition of G418 to remove the LMH helper cells. LMH-D2 colonies derived from single cells were typically expanded in culture to 5×10^6 to 10×10^6 cells before harvesting for further subcloning, storage, or DNA analysis. This represents approximately 23 generations of cell growth.

DNA analysis. Total nuclear DNA from cultured cells was isolated as previously described (24, 34). Briefly, after the cells were washed in a buffer containing 10 mM Tris-HCl (pH 7.5) and 0.15 M NaCl, they were pelleted, resuspended in a lysis buffer containing 50 mM Tris HCl (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$, and 0.5% Nonidet P-40, and incubated on ice for 10 min. Nuclei were released with several strokes of a Dounce homogenizer, centrifuged for 5 min at 1,500 × *g*, and washed again with the lysis buffer. The nuclei were then lysed with 0.2% sodium dodecyl sulfate and treated with proteinase K (200 μg/ml) for 5 to 16 h at 37°C, and the nucleic acid was extracted once with phenol and once with chloroform and precipitated with ethanol. The resuspended pellet was then digested with RNase A, and nuclear DNA was isolated by phenol-chloroform extraction and ethanol precipitation. Total nuclear DNA was digested with restriction endonucleases, electrophoresed through a 1% agarose gel, and transferred to a Zetabind membrane for Southern analysis as previously described (31).

RESULTS

LMH-D2 chromosomes contain DHBV DNAs that are not linked to the vector plasmid. In a previous study, we utilized the LMH-D2 cell line to identify new integrations derived from episomal DHBV DNAs (6). The LMH-D2 cell line was produced by transfection of LMH chicken hepatoma cells with a DHBV expression plasmid containing the greater-than-unit-length DHBV DNA (18) placed under control of the CMV-IE promoter and the selectable marker *neo*. These cells constitutively express DHBV pregenomic RNAs and replicate wild-type DHBV DNA. Southern blot analysis of LMH-D2 genomic DNA identified two integrated DHBV DNA bands which were associated with plasmid sequences, and these bands are referred to as Tr.1 and Tr.2 (Fig. 1). Band Tr.1 hybridized to both CMV-IE promoter and *neo* sequences, and band Tr.2 hybridized to *neo* but not CMV-IE promoter sequences. In addition, we previously identified two DHBV DNA integrations in LMH-D2 cells which were not linked to the transfected plasmid vector DNA used to establish the line. These integrations are designated a and b (Fig. 1). Cloning and sequence analysis of integration b revealed it to be a complete DHBV genome with the structure of a DSL molecule of virion DNA (6). In addition, there was a 70-bp tandem duplication of cellular sequences flanking integration b. Analysis of LMH-D2 nuclear DNA also revealed episomal forms of DHBV DNA designated OC, DSL, and CCC DNAs (Fig. 1).

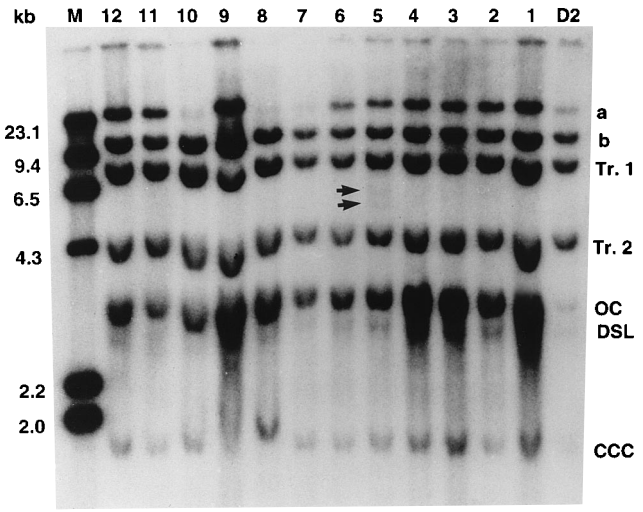

FIG. 1. Southern blot analysis of DHBV DNA integration patterns from parental LMH-D2 cells and representative first-generation single-cell subclones. Nuclear DNAs from parental LMH-D2 cells (lane D2, 7 μg) and 12 single-cell subclones (lanes 1 to 12, 10 μg of each) derived from the LMH-D2 cell line were digested with *Pvu*II and hybridized with a complete DHBV genome probe. Radiolabeled, *Hin*dIII-digested lambda DNA served as the molecular size marker (lane M). Bands a and b did not hybridize to plasmid vector sequences, whereas bands Tr.1 and Tr.2 did hybridize to vector sequences. Bands containing OC, DSL, and CCC DHBV DNAs are indicated. The arrowheads indicate weakly hybridizing bands representing candidate new DHBV DNA integrations.

Single-cell clones derived from the LMH-D2 cell line contain unique new DHBV integrations. We reasoned that if integration of episomal DHBV DNA sequences from newly replicated DHBV occurred frequently in LMH-D2 cells, it would be possible to detect new integrations by isolating subclones of LMH-D2 cells. Therefore, we adopted a single-cell cloning approach to clonally amplify cells which contained integrations. We produced subclones of the original parental LMH-D2 population, and Southern blot analysis of nuclear DNA from 12 of the first-generation subclones (a total of 25 subclones were produced in the first generation) is shown in Fig. 1. DHBV DNA contains no *Pvu*II recognition sites, and therefore *Pvu*II digestion of genomic DNA should produce a new band for each new integration. Two first-generation subclones, designated P1(5) and P1(20), contained additional DHBV-hybridizing bands [subclone P1(5) is shown in Fig. 1, lane 5]. We reasoned that the bands marked with arrowheads in lane 5 might represent new integrations that occurred after the P1(5) colony had partially grown because they were very weakly

hybridizing bands on the Southern blot. Further analysis of this lineage is presented later.

We also observed that the intensity of band a was greatly diminished in five of the first-generation subclones [data for subclones P1(7), P1(8), and P1(10) are shown in Fig. 1]. The consistent presence in these subclones of a weakly hybridizing a band of the same size as the parental band suggests that the a band may be an amplified integration with the flanking cellular DNA containing *Pvu*II sites. Alternatively, the weaker-hybridizing band in these subclones could represent a different integration that comigrated with band a. Coamplification of a hepatitis B virus integration with a flanking cellular transforming gene in a hepatoma DNA has previously been reported (8). The loss of copies of an amplified integration could occur by a homologous-recombination model if the amplified copies were on the same chromosome. Alternatively, if single copies of this integration were present on an amplified chromosome (amplified-chromosome model), the copy number of integration a would depend on the segregation of the amplified chromosomes in each subclone.

The LMH cell line is highly aneuploid and contains over 75 chromosomes, including a large number of morphologically indistinguishable small chromosomes (12). The homologous-recombination model predicts that at least one copy of integration a will always remain, whereas the chromosome amplification model predicts that integration a may be completely lost at a higher frequency as the copy number of the chromosome containing the integration decreases. The data for the second-generation subclones do not, however, allow us to critically distinguish between these possibilities. However, the apparent complete absence of integration a from several third-generation subclones in the P1(21) lineage suggests the amplified-chromosome model.

Analysis of second-generation subclones. The first-generation clones chosen for further subcloning included P1(12), which had no apparent new integrations; P1(21), in which band a had been partially lost; and P1(5), which contained the putative new integrations in submolar quantities, as discussed above. A flow diagram of the subclones we isolated from the parental generation through the third generation in these lineages is illustrated in Fig. 2. Southern blot analysis of genomic DNAs from representative second-generation subclones derived from first-generation subclones P1(12), P1(21), and P1(5) is shown in Fig. 3.

P1(12) lineage analysis. All of the second-generation subclones of P1(12) retained the first-generation pattern (Fig. 3A). Southern blot analysis of DNAs from two of the lineage P1(12) subclones, P1(12)-6 and P1(12)-10, revealed very faint new bands hybridizing to the DHBV probe (Fig. 3A, lanes 6 and 10, arrowheads). These represented 2 colonies of 18 which may have obtained new integrations late during growth of the subclone. Minor variations in the relative intensity of integration a were also apparent; however, all of the subclones retained integration a. Subclones P1(12)-1 to P1(12)-13, which were in the P1(12) lineage, were therefore judged to have a very stable integration pattern and a low frequency of new integrations.

P1(21) lineage analysis. Second-generation subclones in the P1(21) lineage presented a dramatically different result. Several second-generation subclones in this lineage contained new, strongly hybridizing DHBV integrations [Fig. 3B, subclones P1(21)-2, P1(21)-3, P1(21)-4, and P1(21)-7, lanes 2, 3, 4, and 7, respectively], while they retained all of their parental integrations. Rehybridization of the blots with plasmid vector DNA revealed that none of the new integrations contained plasmid DNA, ruling out the possibility that they were derived

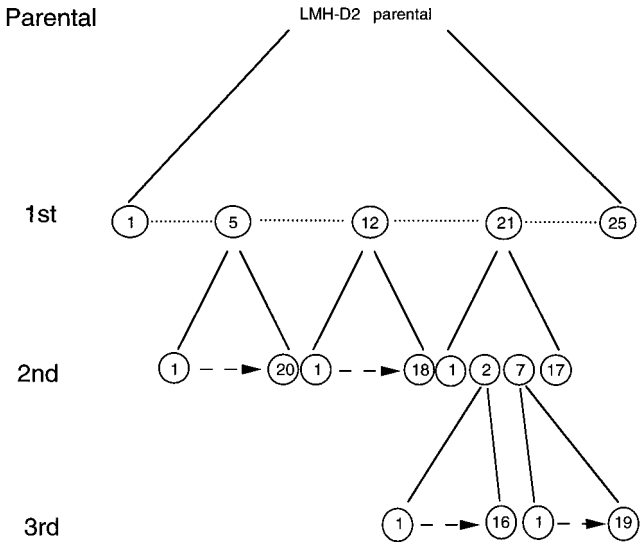

FIG. 2. Flow diagram of the single-cell cloning protocol used for the LMH-D2 cell line. Three successive rounds of single-cell cloning were performed, as indicated. Each circled number is the number of single-cell subclones generated from each cell generation. Cell generations: Parental, LMH-D2 cells; 1st, first-generation subclones designated P1(1) to P1(25); 2nd, second-generation subclones designated P1(5)-1 to P1(5)-20, P1(12)-1 to P1(12)-18, and P1(21)-1 to P1(21)-17; 3rd, third-generation subclones designated P1(21)-2-1 to P1(21)-2-16 and P1(21)-7-1 to P1(21)-7-19.

by amplification of sequences in bands Tr.1 and Tr.2. We concluded that the new bands were the result of new integrations of episomal DHBV DNAs as previously described in this cell line (6). Furthermore, band b was absent in second-generation subclones P1(21)-9 (Fig. 3B, lane 9) and P1(21)-15 (data not shown), while no additional new integrations were present and other preexisting integrations were unchanged. This suggested that integration b was completely lost in those subclones.

The new integrations detected in the second-generation colonies could have been present in cells of the first-generation colony that were in low abundance, or the new integrations could have occurred early during the growth of the second-generation subclones. The first alternative predicts that all of the third-generation subclones should contain the integration, whereas the second alternative predicts that a proportion of the third-generation subclones should not contain the new integration. To distinguish between these possibilities, we produced third-generation subclones from colonies P1(21)-2 and P1(21)-7 (Fig. 3B, lanes 2 and 7). These colonies were chosen because each contained one new DHBV integration.

Southern blot analyses of the third-generation subclones showed that every subclone in the P1(21)-2 and P1(21)-7 lineages contained the new DHBV integrations first detected in their respective second-generation subclones (Fig. 4A and B, respectively, long, thin arrows). In addition, all of the third-generation subclones retained all integrations that were present in the parental second-generation populations, confirming that the new integrations were not derived from rearrangements of the preexisting ones. Thus, the unique new integrations in P1(21)-2 and P1(21)-7 represented new DHBV integrations that were present at the one-cell stage during growth of the second-generation clones.

Finally, some third-generation subclones contained additional new DHBV integrations [subclones P1(21)-2-9 and P1(21)-2-10 in Fig. 4A and P1(21)-7-1 and P1(21)-7-11 in Fig.

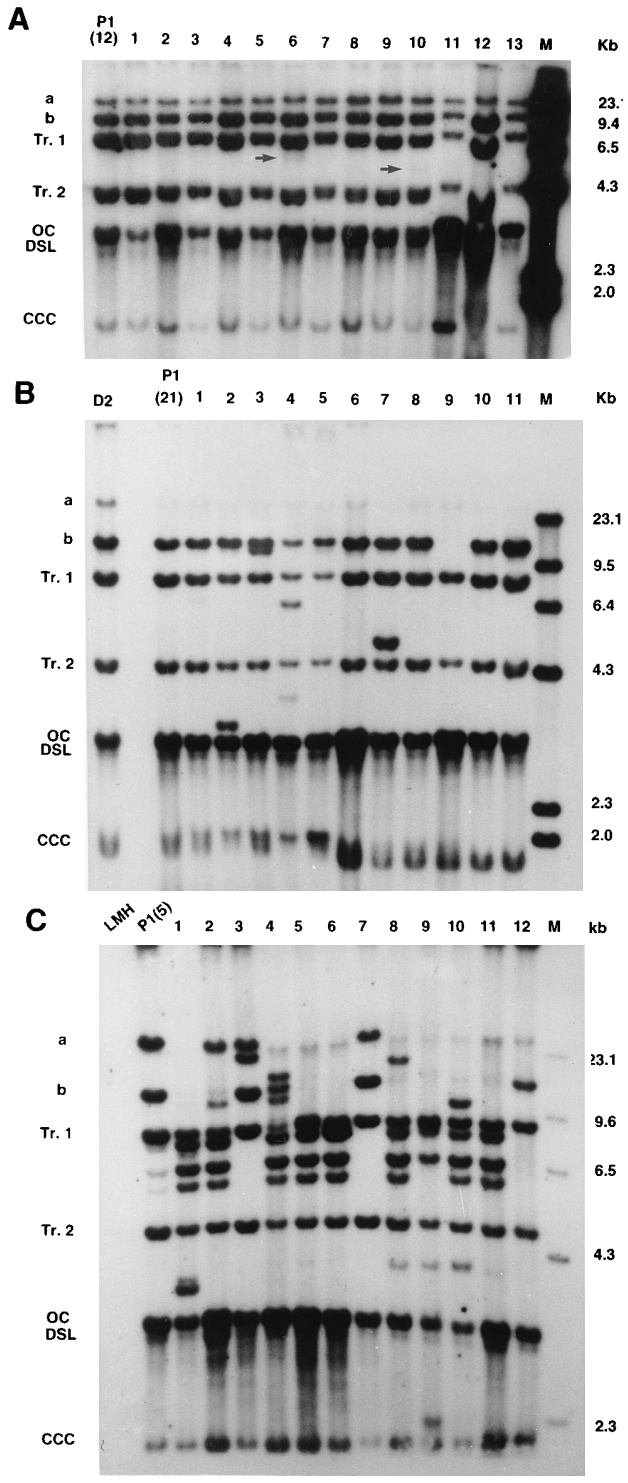

FIG. 3. Southern blot analysis of DHBV DNA integration patterns in second-generation subclones derived from first-generation subclones P1(12) (A), P1(21) (B), and P1(5) (C). DHBV DNA integration patterns of second-generation subclones are in lanes 1 to 13 (A), 1 to 11 (B), or 1 to 12 (C). Lanes M, lambda marker DNA. Lane D2 (B), parental LMH-D2 DNA. Lane LMH (C), untransfected LMH chicken hepatoma cell DNA. DHBV bands representing episomal forms are labeled OC, DSL, and CCC as in Fig. 1. The arrowheads in panel A mark weakly hybridizing bands. Of the DHBV bands in the three panels, Tr.1 and Tr.2 were the only bands which hybridized to plasmid vector sequences, as well as DHBV DNA. Band Tr.1 hybridized to DHBV, CMV-IE promoter (200-bp *Sac*I-*Nco*I fragment probe), plasmid PBR322, and *neo* (1.2-kb *Ava*I fragment probe) sequences. Band Tr.2 hybridized only to *neo* and DHBV sequences.

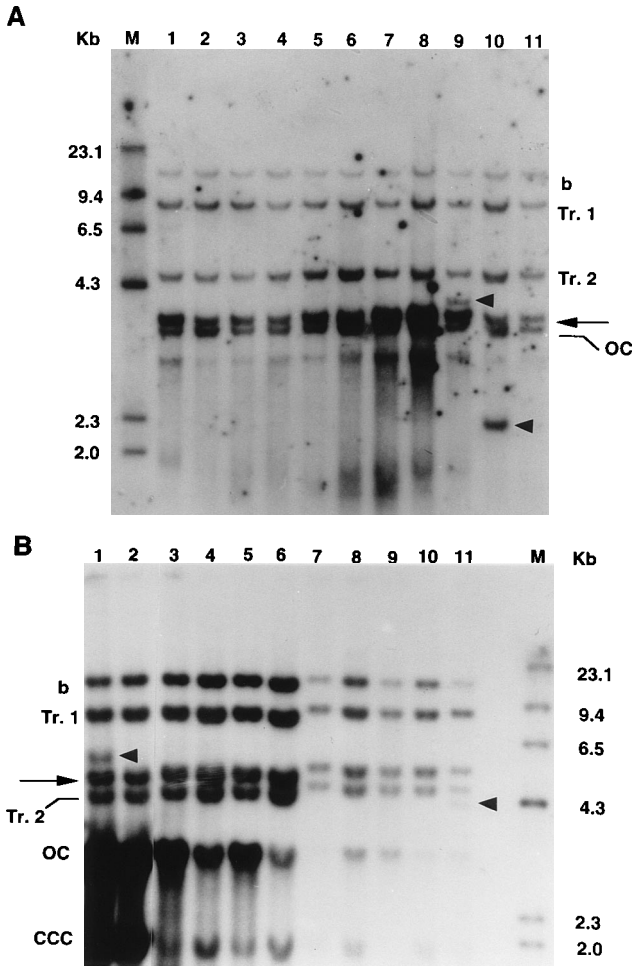

FIG. 4. Southern blot analysis of integrated DHBV DNAs from third-generation subclones derived from second-generation subclones P1(21)-2 (A) and P1(21)-7 (B). The arrows indicate the bands in third-generation subclones that were detected as new integrations in the parental second-generation subclones and were present in 100% of the third-generation subclones. The arrowheads indicate unique new DHBV integrations detected in some third-generation subclones that were not present in second-generation clones. CMV-IE promoter, plasmid PBR322, and *neo* sequences hybridized only to bands Tr.1 and Tr.2 (see the legend to Fig. 3).

4B (arrowheads)], further confirming that integration of DHBV DNA continued to occur in the LMH-D2 cell line. Integration was judged to occur at a moderate frequency in cells of the P1(21) lineage (see Discussion).

P1(5) lineage analysis. Southern blot analysis of first-generation clone P1(5) revealed the presence of two to three weakly hybridizing DHBV integrations which either (i) were present at submolar quantities in the population or (ii) contained subgenomic fragments of DHBV DNA which caused them to hybridize weakly to DHBV DNA probes. To distinguish between these possibilities and also to determine the lineage relationships between cells that may be present at submolar frequencies in the population, we carried out a second generation of subcloning of the P1(5) colony. Southern blot analysis of nuclear DNA from second-generation subclones P1(5)-1 to P1(5)-12 revealed that 8 of the 12 subclones [P1(5)-1, P1(5)-2, P1(5)-4, P1(5)-5, P1(5)-6, P1(5)-8, P1(5)-10, and P1(5)-11 in Fig. 3C] contained the three integrations which had been present in the parental P1(5) DNA (Fig. 1, lane 5). However,

TABLE 1. Plating efficiencies of LMH-D2 subclones P1(5)-9, P1(5)-11, and P1(5)-12

Subclone	No. of cells plated[a]	No. of colonies grown[b]
P1(5)-9	500	10
	1,000	21
	1,500	31
	2,000	58
	2,500	64
	3,000	90
P1(5)-11	500	5
	1,000	17
	1,500	19
	2,000	22
	2,500	37
	3,000	55
P1(5)-12	500	0
	1,000	0
	1,500	0
	2,000	4
	2,500	21
	3,000	22

[a] A single-cell suspension was prepared, the number of viable cells was determined by using a hematocytometer, and cells were plated in 100-mm-diameter dishes.

[b] The culture medium was changed once a week. Three weeks after plating, the colonies were fixed with 10% formalin and stained with crystal violet. Visible colonies were scored.

in the second-generation clones, the intensity of DHBV hybridization was equivalent to that of band b, which we knew from previous work contains one complete DHBV genome (6). Therefore, we concluded that the new integrations in the second-generation subclone most likely contained a nearly unit length DHBV sequence or reiterations of subgenomic fragments. Since transgenes Tr.1 and Tr.2 were stably retained in all of the subclones and the new integrations did not hybridize to vector plasmid or *neo* probes, the new integrations were not the result of rearrangement of the transgene integrations.

The above results suggested that cells containing these new integrations were present at submolar frequencies in the first-generation P1(5) colony. We estimated from the hybridization intensity of first-generation subclone P1(5) that cells containing the three new integrations represented 10 to 15% of the original P1(5) colony. However, after random picking of subclones for the second generation, Southern blot analysis showed that about 70% of the colonies contained the same three new integrations. One simple explanation is that subclones containing the new integration pattern had a higher plating efficiency than the parental cells. In the single-cell cloning experiments for the P1(5) colony, no helper LMH cells were used to facilitate the growth of the colonies.

To test this hypothesis, we conducted a plating efficiency test on subclones which had the three-new-integrations pattern versus subclones which retained the parental pattern. As shown in Table 1, second-generation subclones P1(5)-9 and P1(5)-11, both of which contained the new integration pattern, had plating efficiencies severalfold higher than that of P1(5)-12, which contained the parental integration pattern. To further confirm our hypothesis, we mixed cells of subclones P1(5)-11 and P1(5)-12 in a 1:1 ratio and plated the mixture at low densities to obtain single-cell colonies. Southern blot analysis of 12 clones isolated from the mixed culture revealed that all of the 12 clones contained the new DHBV integration pattern identical to that of P1(5)-11 (data not shown). Thus, we concluded that a lineage of cells containing the new integrations in the P1(5) colony were selectively isolated because of their increased plating efficiency. In the 12 clones isolated from this coculture experiment that we analyzed, the three new DHBV integrations were stably retained, as well as bands Tr.1 and Tr.2, and two clones contained further new single integrations (data not shown).

Flow diagram of proposed lineage relationships between LMH-D2 subclones. Analyses of the specific banding patterns of second-generation subclones P1(5)-1 to P1(5)-12 suggested lineage relationships between them which indicate a high frequency of integration in the P1(5) lineage. The possible lineage relationships deduced from the related Southern blot patterns are presented in Fig. 5. The banding patterns suggest that second-generation clone P1(5)-12 derived from clone P1(5)-7 by partial loss of integration a. Subsequently, clone P1(5)-6 derived from clone P1(5)-12 by loss of band b and acquisition of three new integrations, and clones P1(5)-11, P1(5)-1,

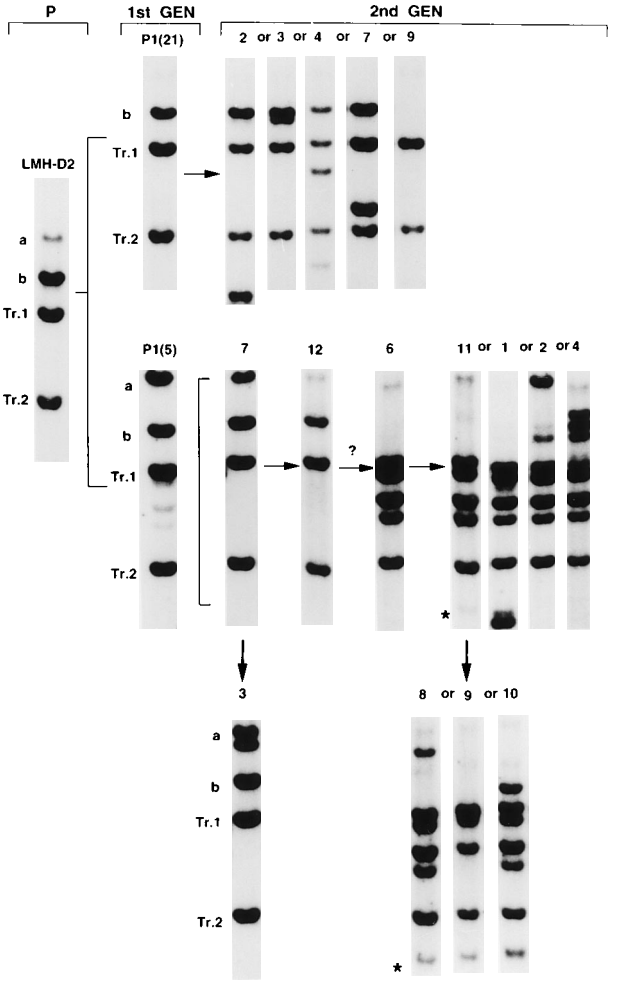

FIG. 5. Proposed lineage relationships between parental and first- and second-generation LMH-D2 subclones based on DHBV DNA integration patterns. The arrows indicate proposed lineage relationships. Second-generation subclones were derived from first-generation subclone P1(5) or P1(21). Single-cell subclone numbers and the DHBV-positive band designations are the same as in Fig. 3. Transgene bands Tr.1 and Tr.2 were stably maintained in all subclones. The asterisks indicate a set of common DHBV integrations unique to second-generation subclones 8, 9, 10, and 11. The question mark above the arrow between second-generation subclones 12 and 6 suggests the possibility of multiple events or a mixture of cell types in the P1(5) lineage.

P1(5)-2, and P1(5)-4 all derived from clone P1(5)-6 by acquisition of additional unique new integrations.

Clones P1(5)-8, P1(5)-9, and P1(5)-10 must have derived from clone P1(5)-11 because they have a unique new integration (asterisks in the lower panel of Fig. 5) in common with clone P1(5)-11, and they also contain additional unique new integrations. Finally, clone P1(5)-3 derived directly from clone P1(5)-7 by acquisition of one new integration. In clone P1(5)-9, one integration was lost and two new integrations are present; one is in common with P1(5)-11 (marked by asterisks in Fig. 5), and the other is unique and migrates at about 2.3 kb (Fig. 3C, lane 9) We conclude that clone P1(5)-9 was derived from P1(5)-11 by loss or rearrangement of one integration.

Clone P1(5)-6 contained a dramatically altered integration pattern compared with P1(5)-12. This included acquisition of three common new integrations and loss of integration b. To determine whether the three new integrations in clone P1(5)-6 were derived from rearrangement of band b, we used oligonucleotide primers and PCR as described previously (6) to test for the presence of any viral-cellular DNA junction sequences unique to integration b. We tested several subclones of the P1(5) lineage that did not contain band b. In no case did we detect any junction sequences unique to integration b (data not shown). Therefore, we concluded that the new integrations in the P1(5)-6 lineage were not derived from rearrangement of band b.

Our proposed lineage relationships in the P1(21) lineage are much simpler since this lineage did not appear to have as high a rate of integration and/or recombination. Thus, second-generation subclones had only one or two unique new integrations without loss of existing integrations or loss of an existing integration without any detectable new integrations (upper panel of Fig. 5).

Admittedly, these proposed lineages are speculative. However, taken as a whole, the data strongly support a mechanism in which some integrations are lost and other new integrations arise on an ongoing basis in LMH cells replicating wild-type DHBV. The apparent higher frequency of loss and gain of integrations in subclones of the P1(5) lineage suggests that genetic and/or epigenetic changes in host cells may affect the rate at which integration occurs.

Rearrangement of cellular DNA near integration b. Since integration b had been lost from subclone P1(21)-9 (Fig. 3B) in our single-cell cloning analysis, we wanted to analyze the cellular DNA at the integration site following the loss of DHBV DNA. We cloned about 3.5 kb of cellular flanking DNA to the left and 2.5 kb of cellular DNA to the right of integration b (Fig. 6B). A unique 1.5-kb *Pst*I-*Bam*HI DNA fragment (Fig. 6B, probe) was used to analyze the integration b site. We detected the normal allele in LMH cells (Fig. 6A, lanes 1 and 5), as well as in LMH-D2 cells (Fig. 6A, lanes 2 and 6). The allele modified by integration b in LMH-D2 cells was detected as a *Pvu*II fragment of approximately 18 kb (Fig. 6A, lane 2, arrow b) or as a *Bam*HI fragment of about 4.6 kb (Fig. 6A, lane 6, arrow).

We showed previously that integration b contained a full 3-kb genome of DHBV DNA. However, the cellular DNA containing the integration b *Pvu*II fragment was about 18 kb long instead of the predicted 6 to 7 kb (3 kb greater than the normal allele of about 3 to 4 kb). Likewise, the total length of the cellular DNA in the two *Bam*HI fragments which each contained part of integration b was at least 6 kb, whereas that of the unintegrated allele was only 5 kb at most (Fig. 6A, lane 5). These data suggested that there was a rearrangement of cellular DNA adjacent to the integration b site in parental LMH-D2 cells.

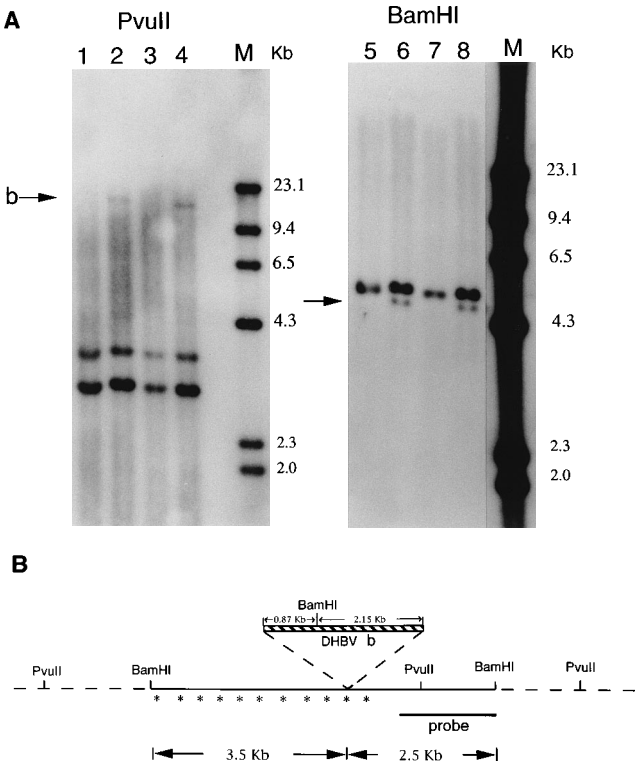

FIG. 6. Southern blot analysis of the normal and integrated genomic DNA loci at the integration b site. (A) Total nuclear DNAs of DHBV-negative LMH cells (lanes 1 and 5), LMH-D2 cells (lanes 2 and 6), P1(21)-9 cells (lanes 3 and 7), and P1(21)-11 cells (lanes 4 and 8) were digested with *Pvu*II (lanes 1 to 4) or *Bam*HI (lanes 5 to 8) and hybridized with a radiolabeled probe of the 1.5-kb *Pst*I-*Bam*HI fragment of cellular flanking DNA to the right of DHBV integration b (dark bar under the restriction map). Arrows point to bands that also hybridized to the DHBV probe. Band b is the same as in Fig. 3. Lanes M contained molecular size markers. (B) Restriction map of the cellular locus containing DHBV integration site b. The dashed lines indicate proposed *Pvu*II sites in cellular DNA (see text). DHBV integration b, containing a genome length DHBV DNA, and the flanking cellular DNAs shown in the map were cloned by PCR protocols which are described elsewhere (6). The asterisks indicate repetitive DNA sequences in genomic DNA.

Loss of flanking cellular sequences along with the DHBV integration from some subclones. Having mapped the cellular sequences at the integration b site, we examined their fate in subclones P1(21)-11, which had retained integration b, and P1-21-9 (Fig. 3B), which had lost integration b. By using the 1.5-kb unique cellular DNA probe, we detected a normal allele and a modified allele in P1(21)-11, as expected (Fig. 6A, lanes 4 and 8). However, we detected only the normal allele in P1(21)-9, which had lost integration b (Fig. 6A, lanes 3 and 7). This suggested that right-hand flanking cellular DNA was lost when integration b was lost from subclone P1(21)-9.

Another model for the deletion event was that very precise excision of the DHBV DNA occurred. To determine whether any modified sequences could be detected at the integration site where integration b was lost, we amplified a DNA fragment from P1(21)-9, which had lost integration b, by using closely spaced primers across the integration site as described previously (6). These primers amplified a 320-bp fragment from both LMH cells and the P1(21)-9 subclone. We did not detect the 390-bp fragment expected if precise excision of the viral sequences from integration site b had occurred in P1(21)-9 (a 70-bp cellular sequence was duplicated at integration site b, increasing the predicted size from 320 to 390 bp

[6]). Nor did we amplify other abnormal-size fragments from P1(21)-9 by using these primers. Sequence analysis of several clones of PCR products amplified from P1(21)-9 showed that they all contained only one copy of the 70-bp cellular sequence adjacent to the integration b site, which was expected of the unmodified allele (data not shown). Thus, we concluded that integration b was probably lost along with a substantial, yet not precisely determined, amount of flanking cellular DNA in subclone P1(21)-9.

DISCUSSION

The hepadnavirus replication cycle does not include integration of viral DNA; however, the presence of integrations in hepatocellular carcinomas, from both mammalian and avian viruses, illustrates that integration does occur. We have utilized LMH-D2 cells, which replicate DHBV efficiently, to study the integration process in vitro. In a previous report, we have demonstrated that this cell line can be utilized to study the occurrence of new viral DNA integrations (6). Cloning and sequencing of one integration, designated b, revealed that it had the structure of a DSL DHBV DNA molecule. The presence of tandemly duplicated cellular sequences flanking the integration suggested that the cellular DNA integration site had been cleaved at staggered positions during the integration process.

In this study, we utilized a single-cell cloning approach to study the occurrence of integrations in three lineages of LMH-D2 cells. Our results established several principles for the integration process in LMH cells. The first and most unexpected result was that integrations occur at a high enough frequency in LMH cells that one can easily detect new integrations by producing as few as 10 subclones. The second result was that the new integrations identified by our subcloning procedure are usually stable upon further subcloning. This was expected, because new integrations which occur and are not stable in the genome are not detected by our subcloning approach.

Because of the limitations of our subcloning and Southern blot approach, it is not possible to determine the absolute rate of integration of DHBV sequences in growing LMH-D2 cells. However, we can make an estimate of the frequency of cells containing stable DHBV integrations in a clone population. Thus, in the second-generation clones of the P1(21) lineage, each of the new integrations was unique and occurred only once in the 17 subclones. Therefore, assuming that these integrations were stable from their initial integration, these integrations most likely occurred after the 16-cell stage of the growth of the first-generation colony. Further single-cell cloning of two second-generation clones, P1(21)-2 and P1(21)-7, detected at least two additional new integrations in 16 third-generation subclones of P1(21)-2 and two new integrations in 19 third-generation subclones of P1(21)-7. Each of the two new integrations detected in the third-generation clones is unique, suggesting that they did not occur earlier than the 16-cell stage of the second-generation colonies. Hence, we estimate that new integrations occur at no greater than 1 in 16 cells in the P1(21) lineage. Since our protocol measures accumulated integration frequencies and the new integrations could have occurred after the one-cell stage of new subclones, the frequencies of cells in clone P1(21) containing a new integration could be lower than our estimate. We believe it provides the first estimate, albeit rough, of the integration frequency in a replicating cell line.

On the other hand, the P1(21) lineage also allows us to estimate the approximate stage during the growth of the first-generation P1(21) colony at which integration b was lost. Of 17 second-generation clones in the P1(21) lineage, 2 lost integration b. Therefore, we estimate that the integration was lost from one cell at approximately the eight-cell stage (i.e., the third to fourth cycle of cell divisions in the colony). Molecular analysis of cellular sequences flanking the integration b site in clones which retained integration b versus those which lost it revealed that a large amount of cellular DNA was lost along with integration b. The loss encompassed the region covered by our hybridization probe so that only one normal allele remained in the deleted clones. Because of the complex aneuploid genotype of the LMH cell line, we have not been able to determine whether the loss of integration b involved an intrachromosomal deletion of sequences or the loss of an entire chromosome containing integration b. Integration b was independently lost in the P1(5) lineage; however, since we have shown that subclones in that lineage had very unequal growth rates, it is not possible to estimate when it was lost.

Loss of integrations, including integration b from the P1(21) and P1(5) lineages and one new integration from clone P1(5)-9, could have important implications. General instability of the chromosomal loci of LMH-D2 cells in culture cannot account for the loss of DHBV integrations. When the first-, second-, and third-generation subclones we generated were probed for plasmid sequences that were not under selection pressure, no loss or rearrangement of the plasmid sequences was detected in any of the 90 subclones analyzed. Plasmid PBR322 sequences hybridized to band Tr.1 and to a band migrating slightly faster than the DHBV DSL DNA in the *Pvu*II digest (data not shown), and both bands were stably retained in every subclone examined. Thus, integrated DHBV DNA sequences may be more unstable than some other foreign sequences in LMH-D2 cells. On the other hand, random loss of the chromosomes that are not lethal may be responsible for the loss of DHBV integrations from the cells.

Genetic instability is expected to be the rule rather than the exception in highly malignant cell lines, and the LMH chicken hepatoma line is not likely to be an exception. Our data suggest that during the growth of the first-generation colony in the p1(5) lineage, a series of three new integrations occurred in one cell, along with loss of integration b and loss of some copies of amplified integration a. The cell in which this occurred was the founder cell for the P1(5)-6 second-generation colony, according to our lineage analysis (Fig. 5). This cell appears to have acquired a selective growth advantage and was genetically unstable, at least initially, because numerous additional new integrations occurred in its subsequent growth to produce a spectrum of related but different integration patterns, as outlined in our lineage analysis (Fig. 5). Although it is formally possible that the P1(5) colony originated from a mixture of two cell types, we feel that this is unlikely since the cells with the selective advantage should predominate in the first generation and this was clearly not the case. Furthermore, our measurements of increased plating efficiency of a lineage of subclones with the new integration pattern suggest the association of the different growth properties of the p1(5) lineage cells with a class of multiple integrations.

We cannot determine whether viral or cellular mechanisms regulate the acquisition and loss of integrations. The structure of OC and DSL DHBV DNA molecules makes them targets for cellular enzymes which may regulate whether they are processed into CCC DNA molecules or integrated. Some evidence suggests that the presence of integrated hepadnavirus DNA increases the recombination frequency of cellular DNA flanking viral integrants (10). This could be due to the presence of preferred cleavage sites for cellular nucleases which may be

present in specific regions of the viral genome (25, 37). On the other hand, the variable frequency of integration in the three lineages we studied suggests that cellular factors are also important regulators of integration. It is possible that integration is much less frequent in normal hepatocytes and that molecular genetic changes associated with increased tumor susceptibility, such as defects in nucleotide excision repair, may promote the occurrence of integrations that may stimulate progression to malignancy. In this scenario, the sustained occurrence of new integrations and the loss of integrations plus flanking cellular DNA sequences would provide a basis for integrations to function as activators of protooncogenes, as well as agents mediating the loss of tumor suppressor genes.

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