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Increase in the Frequency of Hepadnavirus DNA Integrations by Oxidative DNA Damage and Inhibition of DNA Repair

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Persistent hepadnavirus infection leads to oxidative stress and DNA damage through increased production of toxic oxygen radicals. In addition, hepadnaviral DNA integrations into chromosomal DNA can promote the process of hepatocarcinogenesis (M. Feitelson, Clin. Microbiol. Rev. 5:275–301, 1992). While previous studies have identified preferred integration sites in hepadnaviral genomes and suggested integration mechanisms (M. A. Buendia, Adv. Cancer Res. 59:167–226, 1992; C. E. Rogler, Curr. Top. Microbiol. Immunol. 168:103–141, 1991; C. Shih et al., J. Virol. 61:3491–3498, 1987), very little is known about the effects of agents which damage chromosomal DNA on the frequency of hepadnaviral DNA integrations. Using a recently developed subcloning approach to detect stable new integrations of duck hepatitis B virus (DHBV) (S. S. Gong, A. D. Jensen, and C. E. Rogler, J. Virol. 70:2000–2007, 1996), we tested the effects of increased chromosomal DNA damage induced by H_2O_2, or of the disturbance in DNA repair due to the inhibition of poly(ADP-ribose) polymerase (PARP), on the frequency of DHBV DNA integrations. Subclones of LMH-D21-6 cells, which replicate DHBV, were grown in the presence of various H_2O_2 concentrations and exhibited up to a threefold increase in viral DNA integration frequency in a dose-dependent manner. Moreover, inhibition of PARP, which plays a role in cellular responses to DNA breakage, by 3-aminobenzamide (3-AB) resulted in a sevenfold increase in the total number of new DHBV DNA integrations into host chromosomal DNA. Removal of either H_2O_2 or 3-AB from the culture medium in a subsequent cycle of subcloning was accompanied by a reversion back towards the original lower frequency of stable DHBV DNA integrations for LMH-D21-6 cells. These data support the hypothesis that DNA damage sites can serve as sites for hepadnaviral DNA integration, and that increasing the number of DNA damage sites dramatically increases viral integration frequency.

Hepatocellular carcinoma (HCC) is one of the most frequent human cancers in the world, and its occurrence is strongly associated with persistent hepatitis B virus (HBV) infection (1, 2, 14, 34). Pathoanatomical studies have revealed that hepatocarcinogenesis in HBV carriers occurs through a multistage mechanism which includes chronic liver disease, adenomatous hyperplasia, and HCC. The carcinogenic process is driven by genetic alterations which accumulate in hepatocytes during the course of chronic liver disease, which is marked by a cycle of cell death and regeneration (10, 18). Thus, it is very important to identify risk factors which affect genomic stability that are responsible for the occurrence of genetic alterations in carcinogenesis.

Viral DNA integrations can serve as mutagens and can increase the cancer risk in hepadnaviral hosts (33, 47). Proto-oncogene activation by hepadnaviral DNA integration has been confirmed as one major mechanism driving hepatocarcinogenesis in woodchucks which are persistently infected with woodchuck hepatitis virus (WHV) (11). WHV DNA integrations commonly activate one member of the Myc family of proto-oncogenes, the N-*myc*2 gene (19). This gene is activated by the insertion of the liver-specific WHV enhancer either upstream or downstream from the N-*myc*2 allele (11, 53). A second common viral DNA integration site occurs in woodchuck HCCs and is located approximately 200 kb upstream from the most frequently activated N-*myc*2 allele. Since the second site does not encode a gene, it is thought that the WHV DNA insertion functions as a long-range enhancer for the N-*myc*2 gene, which is transcriptionally activated in woodchuck HCCs (12).

In humans, a common HBV DNA integration site has not been identified. Nevertheless, in several HBV-associated HCCs, viral DNA integrations have been observed in genes which may have played a role in hepatocarcinogenesis in those specific tumors, since the HBV DNA integrations occurred in genes which control processes that are generally altered in tumors. Some of these specific examples include viral DNA integration into a cell cycle control gene (cyclin A) (52), a retinoic acid receptor gene β (8), and a mevalonate kinase gene (17). In addition, HBV DNA integrations have been located at sites of genomic DNA deletions and translocations, suggesting that they may function in a broader role to cause gross chromosomal abnormalities which can also deregulate gene expression through many additional mechanisms (20, 34–36, 49).

Pathogenetic mechanisms during hepadnavirus infection which induce or modulate DNA damage might affect the frequency of hepadnaviral integrations, which in turn might have a major impact on the genetically driven process of hepatocarcinogenesis. The necroinflammatory liver disease present in persistent hepadnavirus infection is caused by a limited host immune response to viral antigens, which does not clear the virus from hepatocytes. Activated Kupffer cells present in hepadnavirus-infected livers generate toxic oxygen radicals (37), which induce a cascade of reactions which leads to DNA dam-

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age in the nuclei of hepatocytes (48). One promutagenic DNA lesion caused by H_2O_2 is 8-hydroxydeoxyguanosine, which is a derivate of deoxyguanosine (23, 46, 54). This compound serves as a specific marker of oxidative DNA damage (18) in the liver, and its levels are elevated in HBV-infected livers (48). Increased production of 8-hydroxydeoxyguanosine in human liver is correlated with increased serum alanine aminotransferase levels, a sensitive indicator of liver cell injury (55). Cell proliferation in chronic active hepatitis allows for greater exposure of DNA to reactive oxygen species. Moreover, in rapidly dividing cells there may not be sufficient time to repair DNA damage, which would then be converted into mutations in daughter cells (18).

One of our aims has been to investigate how agents which alter the level of DNA damage in cells replicating hepadnaviruses may affect the frequency of viral DNA integration. Our approach was to identify different means of altering the level of DNA damage and to determine their effects on hepadnaviral DNA integration frequency. First, we increased the exposure of cells to toxic oxygen radicals through treatment with H_2O_2. In a second approach, we interfered with processes of DNA repair by inhibiting the activity of poly(ADP-ribose) polymerase (PARP), an enzyme which plays a role in cellular responses to DNA breakage (45).

PARP is a 116-kDa nuclear enzyme that is present in most eukaryotic cells and binds to DNA strand breaks through the intermediacy of two zinc fingers, which immediately stimulates the catalytic center of this enzyme to covalently modify nuclear proteins with poly(ADP-ribose) by using NAD^+ as a substrate. This posttranslational modification participates in a range of cellular processes which involve DNA strand break formation and rejoining. PARP seems to play a role in maintaining the integrity of the genome and in prevention of accidental recombination events (5, 9, 45). Inhibition of PARP restricts the access of DNA repair enzymes to DNA damage sites and blocks efficient religation of DNA strand breaks. A compound which competitively inhibits PARP is 3-aminobenzamide (3-AB) (13, 27). Cells exposed to DNA damage in the presence of 3-AB fail to demonstrate repair of DNA strand breaks (40). Moreover, as a result of PARP inhibition, increased detection of homologous recombination (51), sister chromatid exchange (28), and carcinogen-induced DNA amplification (3, 4) have been reported.

One of our hypotheses was that H_2O_2 treatment of cells might increase viral DNA integration frequency as a result of an increase in the level of DNA nicking. Another hypothesis was that PARP may normally function to prevent viral DNA integration by promoting efficient DNA repair, and that by blocking PARP with 3-AB, the integration frequency of hepadnaviral DNA might increase. We used a subcloning approach with LMH-D2 cells, which replicate wild-type duck hepatitis B virus (DHBV), to identify and quantitate new viral DNA integrations (15). Our experiments revealed that during two to three generations of subcloning, there was an explicit increase in DHBV DNA integration frequency in response to oxidative stress produced by H_2O_2, and an even more dramatic increase in viral integration frequency when PARP activity was blocked.

MATERIALS AND METHODS

Cell culture. The LMH-D2 cell line was derived from LMH chicken hepatoma cells (24) by transfection with a previously reported plasmid (7), containing the cytomegalovirus immediate-early promoter driving transcription of a greater-than-genome-length DHBV DNA genome (29), which produces a functional DHBV pregenome RNA and mRNAs for all the structural proteins. The plasmid also contains a neomycin resistance (neo) gene for selection. The cells were maintained in Dulbecco modified Eagle medium-F12 medium (Gibco) containing 10% fetal bovine serum (Gemini Bioproducts), antibiotic-antimycotic (Gibco), and 140 μg of Geneticin (G418; Gibco)/ml. H_2O_2 (Sigma) was added to the cell culture medium in concentrations of 0.1 to 25 μM for dose-response experiments and in concentrations of 1.0 and 10.0 μM for subcloning experiments. 3-AB and the noninhibitory analog 3-aminobenzoic acid (3-ABA) (both from Sigma) were added initially for dose-response experiments at concentrations from 10.0 μM to 25 mM. For all subsequent experiments, 3-AB and 3-ABA were added at concentrations of 1.0 mM each. The cell culture medium was changed daily, and cells were treated with H_2O_2 or 3-AB or 3-ABA repetitively until harvesting of the cells. Subclones were initially derived from the parental LMH-D2 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 petri culture dishes. To facilitate the early growth of single G418-resistant cells of the LMH-D2 lineage, 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. The medium was then changed, and G418 (140 μg/ml) was added to remove the LMH helper cells. LMH-D2 subclones 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 25 generations of cell growth.

DNA analysis. Total nuclear DNA from cultured cells was isolated as previously described (15, 35). Briefly, cells were resuspended in a lysis buffer containing 10.0 mM Tris-HCl (pH 7.5), 1.0 mM EDTA, 5.0 mM $MgCl_2$, and 0.5% Nonidet P-40 and were incubated on ice for 10 min. Nuclei were released with several strokes of a Dounce homogenizer, centrifuged for 5 min at $1,000 \times g$, and washed again with the lysis buffer. The nuclei and the supernatants for the detection of cytoplasmic DNA were digested separately with 0.5% sodium dodecyl sulfate and treated with proteinase K (0.5 mg/ml) for 6 to 12 h at 55°C, and nucleic acids were extracted once with phenol and once with chloroform and precipitated with ethanol. The resuspended pellets were then digested with RNase A, and nuclear and cytoplasmic DNA were isolated by phenol-chloroform extraction and ethanol precipitation. Nuclear DNA was digested with restriction endonucleases. Nuclear DNA and cytoplasmic DNA were electrophoresed through 1% agarose gels and transferred to a nylon membrane for Southern analysis as previously described (50).

Statistical analysis. The Fisher exact test was used for statistical analysis determining the probability of observations by using a contingency table (P values of <0.05 demonstrate statistically significant differences).

RESULTS

Analysis of amplified and segregating DHBV DNA integrations in the LMH-D21-6 lineage. The LMH-D2 chicken hepatoma cell line constitutively expresses DHBV pregenomic RNAs and replicates wild-type DHBV DNA (7, 24). Previous studies have demonstrated that DHBV DNA also integrates into chromosomal DNA of LMH-D2 cells and that stable new viral integrations can be detected by Southern blot analysis of subclones (15, 16). A summary of the subclone lineages described in this report is shown in Fig. 1. Southern blot analysis of parental LMH-D2 cells identified two DHBV DNA bands which were associated with integrated plasmid sequences (Fig. 2, bands Tr. 1 and Tr. 2) and two bands (designated a and b) which represented DHBV DNA integrations produced by integration of episomal DHBV DNA during earlier growth of LMH-D2 cells. Episomal forms of DHBV DNA designated open circular, double-stranded linear, and covalently closed circular DNA (Fig. 3) were also detected in nuclear DNA as previously described (16).

All the above integrations serve as stable background integrations in all the subclones in this report. One exception to the general stability is that integration a appears to become highly amplified in some subclones (Fig. 3B, lane 3). This is consistent with previous data for integration a, which also suggested that its copy number appears to vary in sequential generations of cells (16).

Southern blot analysis of nuclear DNA from the LMH-D21-6 subclone, used as the source of cells for the H_2O_2 treatment experiments, revealed two integrations which were not present in the parental LMH-D2 generation of cells. These bands were designated c and d, and they segregated independently in subclones of the LMH-D21-6 cells (Fig. 2). Thus, these two integrations must have occurred independently in

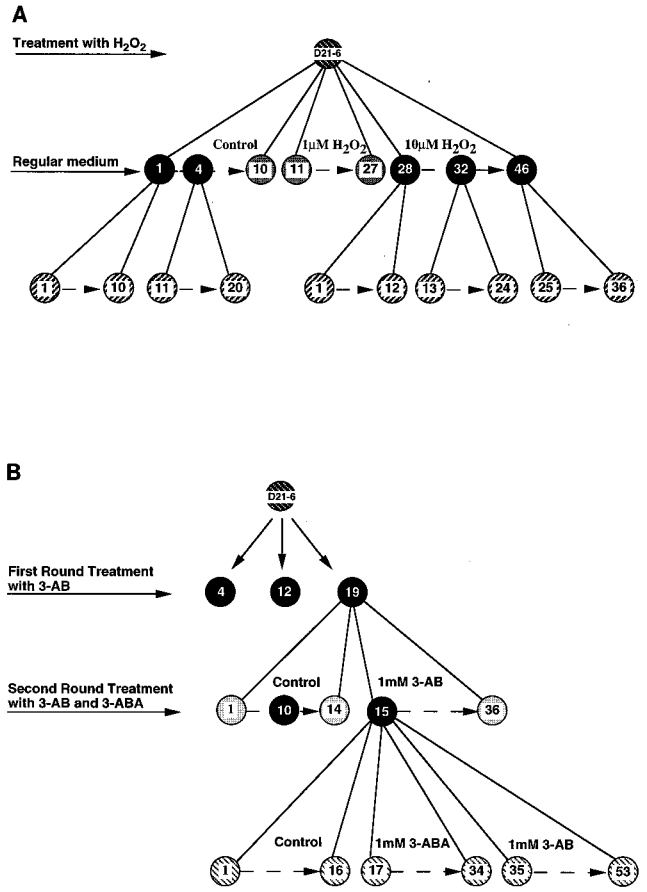

FIG. 1. Flow diagrams of the single-cell cloning protocols for the H_2O_2 (A) and 3-AB (B) experiments with the LMH-D21-6 lineage, a subclone of LMH-D2 cells. Two successive rounds of single-cell cloning were performed for H_2O_2, and three successive rounds were performed for 3-AB, as indicated. Each encircled number represents a single-cell subclone. Subclones from LMH-D21-6 were grown in the presence or absence of 1.0 or 10.0 μM H_2O_2 as indicated. Subclones grown in the first generation in the presence (subclones 28, 32, and 46) and absence (subclones 1 and 4) of H_2O_2 were further subcloned and grown in the absence of H_2O_2. For the 3-AB experiments, the LMH-D21-6 cell line was further subcloned. Three subclones (subclones 4, 12, and 19) were selected and grown in the presence or absence of 1.0 mM 3-AB (shown here in detail only for subclone 19). A subclone that did not show any new viral integration band after initial first generation treatment with 3-AB (subclone 15) was selected for a second generation of subcloning and growth in the presence of 1.0 mM 3-AB or 1.0 mM 3-ABA, or in regular cell culture medium. Another subclone (subclone 10), grown for the first generation in the absence of 3-AB, was subcloned and grown in the presence or absence of 1.0 mM 3-ABA.

two separate progeny cells during the growth of the D21-6 colony. Furthermore, the c integration occurred in 10 of 12 subclones of LMH-D21-6 cells, and the d integration occurred in 3 of 12 (Fig. 2). One subclone, D21-6-12, which contained both the c and d integrations (Fig. 2, lane 12), may have derived from more than one single cell or obtained integrations that were very similar in size to integrations c and d. The frequency of independently assorted integrations c and d is consistent with a model in which both integrations occurred in different cells during the growth of the D21-6 subclone before the colony reached the 16-cell stage.

The above subcloning data, and additional Southern blot data in this and previous reports, have shown that we can detect a DHBV DNA integration as a band on a Southern blot if it is present in only 1/10 of the cells in the colony (15, 16). Thus, our estimates of the numbers of new viral DNA integrations, based on Southern blots, reflect the stable DHBV DNA integrations which occur in the first three to four cell divisions of any given subclone. Second and third rounds of subcloning experiments are needed to determine the frequency of integrations in cells which occurred after the fourth cell division of colony growth. Moreover, in this report, second- and third-generation subcloning experiments were used to estimate the variability of integration frequency once H_2O_2 or 3-AB treatments were terminated.

Increased DHBV DNA integration frequency in LMH-D21-6 subclones grown in the presence of H_2O_2. Subclones of LMH-D21-6 cells were grown for approximately 25 cell generations (to approximately 2×10^7 cells) in media containing 0, 1.0, or 10.0 μM H_2O_2 followed by Southern blot analysis of nuclear DNA digested with *Pvu*II, which does not cut DHBV. Southern blot analysis of nuclear DNA from control subclones revealed only one new DHBV DNA integration in 10 subclones, which is a frequency consistent with previous data from LMH-D2 lineages grown in regular cell culture media (16). However, we identified new viral DNA integrations in 6 of 17 subclones grown in 1.0 μM H_2O_2 and in 10 of 19 subclones grown in 10.0 μM H_2O_2. Both the 1.0 and 10.0 μM H_2O_2 levels of treatment caused an explicit increase in the frequency of DHBV DNA integration in the subclones (Table 1). Interestingly, several subclones grown in the presence of 1.0 or 10.0 μM H_2O_2 also contained more than one new integration. While eight subclones contained one new DHBV DNA band, five contained two, one contained three, and two contained four new DHBV DNA integrations. In 12 subclones treated with 10 μM H_2O_2 we identified 17 new integrations (Fig. 2).

Increase in the DHBV DNA frequency associated with inhibition of PARP. In preparation for the 3-AB treatment experiments, we further subcloned the LMH-D21-6 cells. We used three single-cell subclones, designated LMH-D21-6-4, -12, or -19 (Fig. 1), for the 3-AB experiments. Subclones were grown for 25 generations in media containing 0 or 1.0 mM 3-AB. Repetitive treatment with 1.0 mM 3-AB did not alter the growth rate of LMH-D21-6 cells (data not shown) or the level of DHBV replication in the cells (compare Fig. 3B, lanes 14

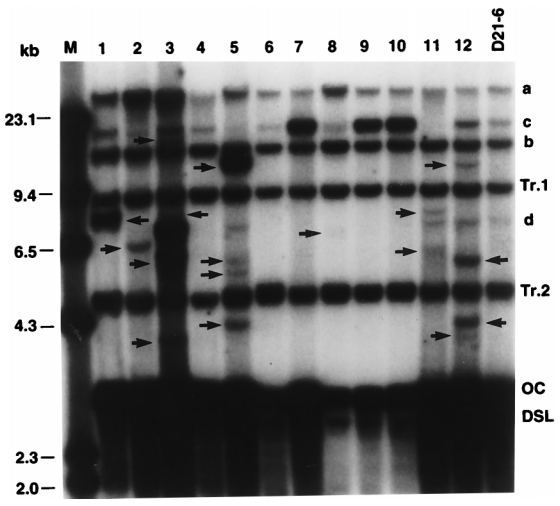

FIG. 2. Southern blot analysis of total nuclear DNAs from subclones of the LMH-D21-6 lineage grown in the presence of 10.0 μM H_2O_2. Arrows, new DHBV DNA integrations; a and b, stable DHBV DNA integrations already present in the parental cell line; c and d, DHBV DNA integrations already present in submolar ratio in LMH-D21-6 cells; Tr. 1 and Tr. 2, sites of stable transgene integration; OC, open circular DNA; DSL, double-stranded linear DNA.

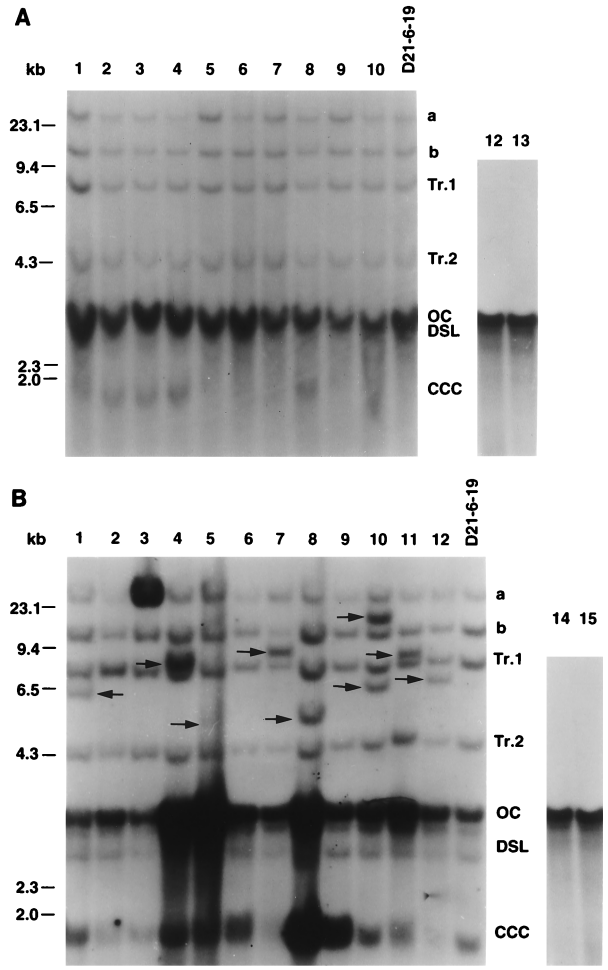

FIG. 3. DHBV DNA integration pattern in first-generation subclones derived from LMH-D21-6-19 and replicative forms from first-generation subclones. Shown are Southern blots of digested total nuclear DNA from subclones of LMH-D21-6-19 cells, grown in regular medium (A) or in medium containing 1.0 mM 3-AB (B). Symbols and abbreviations are as defined in the legend to Fig. 2. CCC, covalently closed circular DNA. Representative cytoplasmic DNAs from subclones of LMH-D21-6-19 cells are shown in panel A, lanes 12 and 13, and in panel B, lanes 14 and 15.

and 15, with Fig. 3A, lanes 12 and 13). For example, Southern blot analysis of 14 control subclones from lineage LMH-D21-6-19 revealed only 2 subclones containing one unique new DHBV DNA integration each (10 representative examples are shown in Fig. 3A). In contrast, Southern blot analysis of nuclear DNA from 3-AB-treated subclones detected 13 new DHBV DNA integrations in 22 subclones (12 representative clones are shown in Fig. 3B) (Table 2).

Including all three lineages that were exposed to 3-AB, we analyzed the nuclear DNA from a total of 62 subclones grown in the presence of 1.0 mM 3-AB and from 42 control subclones grown in regular medium (see Fig. 1 for a summary of lineages LMH-D21-6-4, -12, and -19) (Table 2). Overall integration frequencies were 5 new integrations in 42 control subclones and 55 new integrations in 62 3-AB-grown subclones.

Detection of multiple new DHBV DNA integrations in a single subclone can be an indication of ongoing integration during colony growth. In fact, we detected multiple new DHBV DNA integrations only in the 3-AB-grown subclones. Of 62 3-AB-treated subclones, 18 clones contained one new integration, 14 contained two new DHBV bands, and 3 contained three new DHBV DNA integrations (Table 2). In contrast, the five control cell clones which contained new DHBV DNA integrations contained only a single new DHBV DNA band each (Table 2).

Every new band of the first-generation 3-AB-treated subclones (shown in Fig. 3B) hybridized at least as strongly as the b viral integration band already shown to contain one complete DHBV genome (16). These data suggest that the new viral integrations occurred early during first-generation subclone growth. 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 from amplification of sequences in bands Tr. 1 and Tr. 2. We concluded that the new bands were the result of new integrations

TABLE 1. Summary of the results of subcloning and growth of first- and second-generation subclones from LMH-D21-6 cells in the presence or absence of H_2O_2[a]

Cell clone	H_2O_2 concn (μM)	No. of subclones with new DHBV integrations/total no. of subclones (*P* value)
First-generation		
D21-6-(1–10)	0	1/10
D21-6-(11–27)	1	6/17 (0.20)
D21-6-(28–46)	10	10/19 (0.03)
Second-generation		
D21-6-1-(1–10)	0	1/10
D21-6-4-(11–20)	0	2/10 (1.0)
D21-6-28-(1–12)	0	3/12 (1.0)
D21-6-32-(13–24)	0	3/12 (1.0)
D21-6-46-(25–36)	0	3/12 (1.0)

[a] While 6 of 17 first-generation subclones grown in the presence of 1.0 μM H_2O_2 (not statistically significant, with a *P* value of 0.2) and 10 of 19 subclones grown in the presence of 10.0 μM H_2O_2 (statistically significant, with a *P* value of 0.03) carried new DHBV DNA integrations, only 1 of 10 clones grown in the absence of H_2O_2 carried a new DHBV DNA integration. Second-generation subclones that originated from LMH-D21-6-1 and -4, grown in the absence of H_2O_2, in both the first and the second generation, contained new viral DNA integrations in 1 of 10 (LMH-D21-6-1) or in 2 of 10 (LMH-D21-6-4) subclones. Subclones that originated from LMH-D21-6-28, -32, and -46, grown in the presence of 10.0 μM H_2O_2 in the first generation and in the absence of H_2O_2 in the second generation, each carried new viral DNA integrations in 3 of 12 subclones ($P = 1.0$).

TABLE 2. First-generation cell clones grown in the presence or absence of 1.0 mM 3-AB[a]

Subclone	No. of subclones with new DHBV integrations/total no. of subclones (total no. of new DHBV integrations)		
	Control	3-AB treated[b]	*P*[c]
4	1/13 (1)	9/19 (17)	0.02 (0.0005)
12	2/15 (2)	13/21 (20)	0.006 (0.0005)
19	2/14 (2)	13/22 (18)	0.01 (0.0005)

[a] Among control cell clones, only 1 of 13 subclones from LMH-D21-6-4, 2 of 15 subclones from LMH-D21-6-12, and 2 of 14 subclones from LMH-D21-6-19 contained a single new DHBV DNA integration each. In contrast, among the 3-AB-treated subclones, 9 of 19 subclones from LMH-D21-6-4, 13 of 21 subclones from LMH-D21-6-12, and 13 of 22 subclones from LMH-D21-6-19 showed one or more new DHBV DNA integrations. Of all three lineages from 3-AB-treated subclones, 17 subclones contained a single new viral DNA integration, 16 contained two new viral integrations, and 2 carried three new DHBV DNA integrations (statistically significant, with *P* values of <0.05).

[b] Treated with 1.0 mM 3-AB.

[c] Presented as *P* values for the numbers of subclones with new DHBV integrations (*P* values for the total numbers of new DHBV integrations).

TABLE 3. Summary of results of second-round subcloning and growth of subclones in regular cell culture medium (control), 1.0 mM 3-ABA (specificity control), or 1.0 mM 3-AB

Subclone	No. of subclones with new DHBV integrations/total no. of subclones (total no. of new DHBV integrations)		
	Control	3-ABA treated[a]	3-AB treated[b]
15[c]	3/16 (3)	2/18* (2)*	8/18** (14)***
10[d]	1/14 (1)	1/14 (1)	ND (ND)

[a] Treated with 1.0 mM 3-ABA. *, $P = 1.0$.

[b] Treated with 1.0 mM 3-AB. **, $P = 0.03$; ***, $P < 0.0005$.

[c] In the experiment with subclones from LMH-D21-6-19-15, only 3 of 16 control subclones and only 2 of 18 3-ABA-treated subclones contained one new DHBV DNA integration each (no significant difference; $P = 1.0$). In contrast, 8 of 18 3-AB-treated subclones from LMH-D21-6-19-15 were carrying one or more new DHBV DNA integrations ($P = 0.03$). Three of those subclones contained a single new viral DNA integration, four contained two new viral integrations, and one carried three new DHBV DNA integrations ($P < 0.0005$).

[d] In another, unrelated subcloning experiment, the specificity control experiment again revealed no significant difference in the number of newly detected DHBV integrations in untreated versus 3-ABA-treated cells from LMH-D21-6-19-10 subclones ($P = 1.0$). Only 1 of 14 subclones in both groups contained a single new DHBV DNA integration each.

of episomal DHBV DNAs as previously described for this cell line (16).

Reversion to control integration frequencies—experimental lineages grown in the absence of H_2O_2 and 3-AB. We reasoned that if stable DHBV DNA integration is a continuous unidirectional process, DHBV DNA integrations should accumulate as subclones grow. The first-generation subcloning data for 3-AB-treated cells demonstrating a high overall viral integration frequency (55 integrations in 62 cell clones) suggested that one new viral integration may occur every two cell generations. When we extrapolated this rate, we predicted that virtually all the cells in a subclone grown to approximately 10^7 cells (25 generations) should contain a new stable DHBV DNA integration. In order to test this model, we subcloned cells from first-generation colonies grown in the presence of 3-AB, for a second generation grown in the absence of 3-AB. The rationale for this experiment was that this would measure the percentage of cells in the 3-AB-grown colony which contained a stable DHBV DNA integration. These integrations would not be detectable in the first-generation Southern blotting, because they occurred after the 16-cell stage of colony growth.

This experiment was conducted for two lineages, LMH-D21-6-19-10 and LMH-D21-6-19-15 (Table 3 and Fig. 1). Southern blot analysis of nuclear DNA revealed the unexpected result that only 19% of the second-generation subclones originating from 3-AB-treated cells and then grown in the absence of 3-AB contained new viral integrations (see Fig. 5 and Table 3). This same approach was used for H_2O_2-treated colonies, and we observed that only 9 of 36 (25%) second-generation subclones grown without H_2O_2 carried new viral DNA integrations (12 representative second-generation subclones are shown in Fig. 4), also far below the level we had predicted. Therefore, we concluded that either (i) stable viral DNA integration does not occur continuously during colony growth in the presence of H_2O_2 or 3-AB, (ii) integrations are lost at a relatively high rate, and viral integration is a reversible process (see Discussion), or (iii) this result reflects a balance between the two above alternatives.

Specificity of the 3-AB effects on DHBV DNA integration frequency. To determine the specificity of our data for PARP inhibition, we carried out a specificity control experiment on a separate group of second-generation subclones. This test was conducted by growing the subclones in the presence of either 1.0 mM 3-ABA or 1.0 mM 3-AB. 3-ABA is a structural analog of 3-AB which does not inhibit PARP and which has been widely used as a specificity control for the effects of 3-AB on PARP (5). We analyzed the nuclear DNA of 32 cell clones grown in 1.0 mM 3-ABA, 18 subclones grown in 1.0 mM 3-AB, and 30 control cell clones grown in regular cell culture medium. We detected three new DHBV DNA integrations in 32 DNAs from 3-ABA-grown subclones (12 DNAs of LMH-D21-6-19-15, with new viral integrations in lanes 11 and 12, are shown in Fig. 5B). In contrast, 44% of the second-generation 3-AB-treated subclones from lineage LMH-D21-6-19-15 accumulated new DHBV DNA integrations (Fig. 5C). Of 18 second-generation 3-AB-treated subclones in lineage LMH-D21-6-19-15, 3 subclones carried one new DHBV DNA integration, 4 carried two new viral integrations (Fig. 5C, lanes 3 and 10) and 1 carried three new integrations (Fig. 5C, lane 5) (summarized in Table 3). Multiple integrations were not observed in the 3-ABA-treated subclones (Fig. 5B). Furthermore, in lineage LMH-D21-6-19-10 (Fig. 1), which did not show any apparent new DHBV DNA integration after first-round growth in regular medium, 3-ABA treatment in the second-generation growth did not produce an enhanced integration frequency (Table 3). Therefore, we concluded that the 3-AB effects are specific for PARP inhibition and do not represent a general effect of altered intermediary metabolism.

DISCUSSION

Sequence analysis of hepadnaviral and cellular DNA sequences at the sites of hepadnaviral DNA integrations have demonstrated that integration occurs via a nonhomologous recombination mechanism. A common feature of all mechanisms of genetic recombination is the need for a single- or double-strand break in the DNA strand which will undergo recombination. Unlike retroviruses, hepadnaviruses, which are considered "para-retroviruses," do not encode their own integrase (14, 34). Therefore, nicking of the viral and cellular DNA in preparation for recombination must be mediated by cellular enzymes.

In this study we have investigated the effects of agents that increased DNA damage or inhibited DNA repair on the frequency of DHBV DNA integrations. Our first approach was to

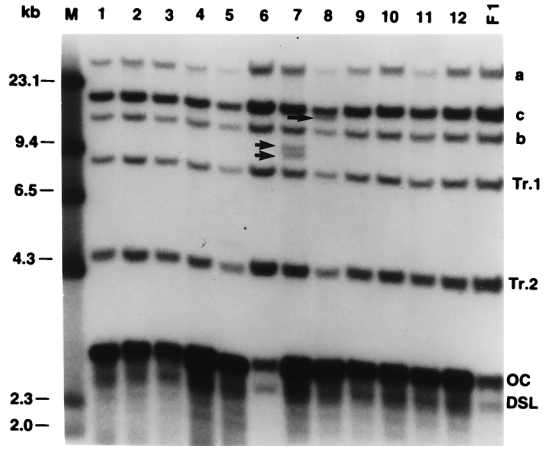

FIG. 4. DHBV DNA integrations in second-generation control subclones grown in the absence of H_2O_2, originating from clone LMH-D21-6-32 (F_1), which was grown in the presence of 10.0 μM H_2O_2 during first-generation subcloning experiments. Symbols and abbreviations are as explained in the legend to Fig. 2.

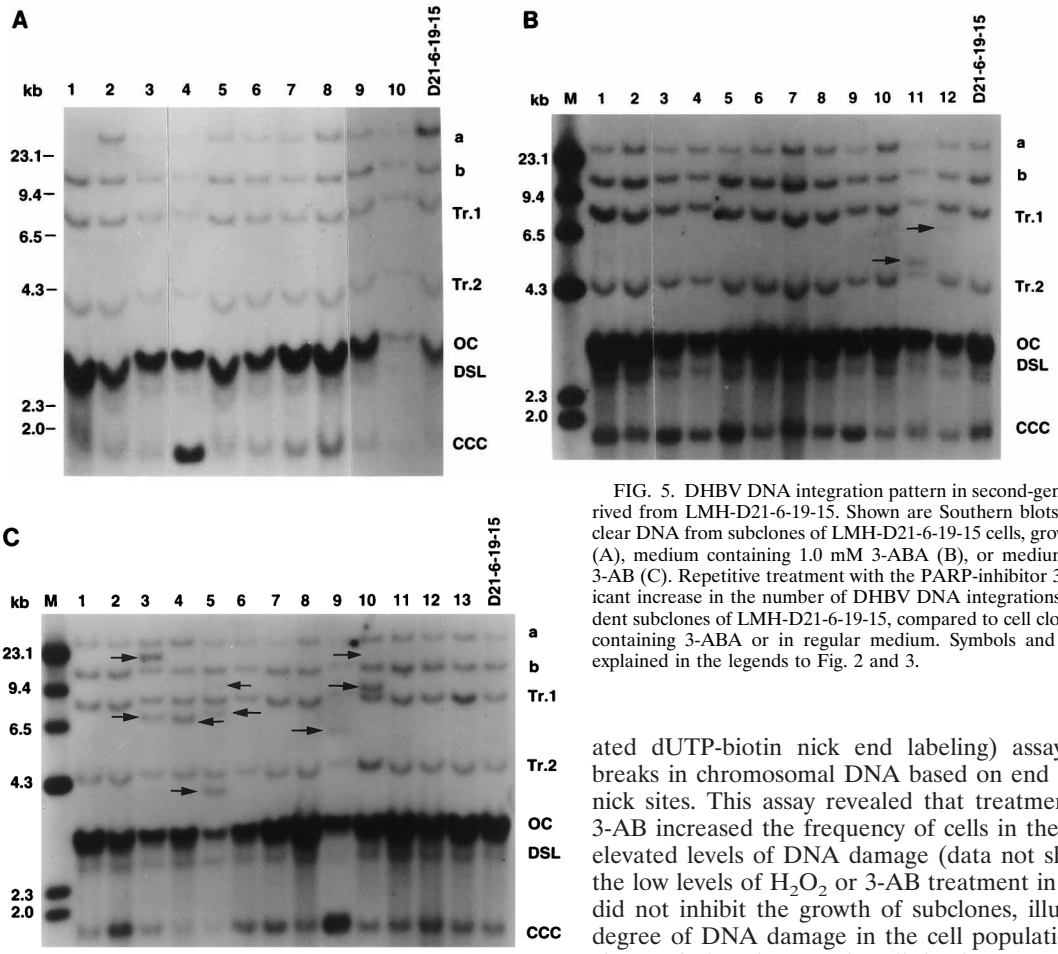

FIG. 5. DHBV DNA integration pattern in second-generation subclones derived from LMH-D21-6-19-15. Shown are Southern blots of digested total nuclear DNA from subclones of LMH-D21-6-19-15 cells, grown in regular medium (A), medium containing 1.0 mM 3-ABA (B), or medium containing 1.0 mM 3-AB (C). Repetitive treatment with the PARP-inhibitor 3-AB leads to a significant increase in the number of DHBV DNA integrations in multiple independent subclones of LMH-D21-6-19-15, compared to cell clones grown in medium containing 3-ABA or in regular medium. Symbols and abbreviations are as explained in the legends to Fig. 2 and 3.

mimic a pathophysiological state of chronic hepadnavirus infection by treating LMH-D2 cells, which replicate DHBV, repetitively with H_2O_2 in cell culture. Second, we investigated how perturbations of the cellular machinery for maintaining the integrity of the genome affect the frequency of hepadnaviral integration. Our results show that DNA damage affects DHBV DNA integration into host chromosomal DNA by increasing the number of new stable DHBV DNA integrations.

Our hypothesis was that DHBV DNA integration might occur preferentially at sites of chromosomal DNA damage. Since the preferred hepadnaviral integration sites are located in the region near DR1 which contains a nick, we assumed that mechanisms which increase the level of nicks in chromosomal DNA could lead to an increase in DHBV DNA integration frequency in LMH-D2 cells. This would indicate that the occurrence of nicks in chromosomal DNA may be a rate-limiting step which normally limits hepadnaviral DNA integration frequency.

Persistent hepadnavirus infections are associated with a wide spectrum of pathophysiological reactions in the liver. One general consequence of persistent infection is the production of toxic oxygen radicals in the liver. Oxidants like H_2O_2 and nitric oxide (NO) are known to induce DNA strand breaks in a dose-dependent manner in various target cells in vitro and in vivo (40, 41). During dose-response experiments we determined treatment levels of H_2O_2 in which the cells could continue to grow and be subcloned. In order to directly confirm the presence of increased DNA damage in treated cells, we used the TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) assay, which detects breaks in chromosomal DNA based on end labeling at DNA nick sites. This assay revealed that treatment with H_2O_2 or 3-AB increased the frequency of cells in the population with elevated levels of DNA damage (data not shown). However, the low levels of H_2O_2 or 3-AB treatment in our experiments did not inhibit the growth of subclones, illustrating that the degree of DNA damage in the cell population was not sufficient to induce large-scale cell death.

In our first set of experiments, we treated LMH-D21-6 cells with H_2O_2 during the growth of subclones in order to place the cells under oxidative stress to mimic the process of toxic oxygen radical formation in a liver during persistent hepadnavirus infection. Southern blot analysis of nuclear DNA from subclones grown in the presence of 10.0 μM H_2O_2 revealed a significant increase in the number of stable new DHBV DNA integrations compared to control subclones (Table 1). The presence of more than one new DHBV DNA integration was detectable only in some of the H_2O_2 treated subclones, which maintained an identical pattern of preexisting parental integrations. This result implied that the same cellular lineage can acquire more than one new DHBV DNA integration, which we confirmed in second-generation subcloning results in this and previous reports (15). New viral DNA integrations detected in first-generation subclones were stably maintained through a second generation of subcloning in the absence of H_2O_2. These data suggest that continuous H_2O_2 treatment also promotes the occurrence of multiple DHBV DNA integrations in specific lineages of LMH-D2 cells.

The molecular mechanisms by which H_2O_2 treatment increases viral integration are unknown. However, previous studies have characterized cellular responses to H_2O_2 which are most likely relevant to our experiments. It has been shown that single-strand breaks of DNA are induced very rapidly after the addition of oxidants, which leads to the activation of DNA repair mechanisms, including PARP activity (41). In HBV transgenic mice, the hepatocellular catalase activity, which is a main cellular defense mechanism that converts H_2O_2 to O_2

and H_2O, is markedly decreased (22). Thus, the cellular antioxidant defense and DNA repair mechanisms may be overwhelmed by the H_2O_2 treatment in LMH-D2 cells. This could cause the amount of DNA damage to exceed the repair capacities and provide the sites for recombination with DHBV DNA, at least transiently.

Our results suggest that viral DNA integration frequency may dramatically increase in HBV-infected patients when hepatocytes are placed under oxidative stress. Such circumstances may commonly occur while chronically HBV-infected patients undergo fluctuations in HBV replication levels and serum aminotransferases during disease exacerbations (30). Hepadnaviral DNA integration frequency may even increase when virus replication resumes after periods of antiviral therapy. The detection of HBV DNA integrations in cirrhotic liver nodules illustrates that integrations occur during persistent infection and that hepatocytes are clonally amplified in certain persistent infection settings (42). Multiple WHV and HBV DNA integrations in nonclonal liver tissue can be demonstrated in a substantial fraction of long-term chronic carriers (34, 43). HBV DNA integrations can also occur within the host genome early after infection, varying dramatically in their frequency (6, 21, 25, 56). The structure of those integrations, which are believed to represent "primary" integration events, closely resembles the DHBV DNA integrations which occur in LMH-D2 cells (16), with their junctions preferentially located in the DR1 region. Our data suggest that toxic oxygen radicals may promote hepadnaviral DNA integration in situations such as those described above.

We next targeted the enzyme PARP, which is involved in specific DNA repair mechanisms, which are highly active in the liver. Our experiments addressed the question of how the alteration of this enzyme's activity might affect stable DHBV DNA integration. Previous studies have demonstrated the involvement of PARP activity in a variety of eukaryotic cellular processes which involve the transient occurrence of DNA strand breaks (9, 32, 45), including the base excision repair mechanism. It has been speculated that a DNA nick protection mechanism, mediated by activated PARP, might prevent DNA recombination and spurious initiation of transcription at DNA strand breaks (38, 39).

3-AB, a nicotinamide analog, is a known inhibitor of PARP. In the presence of this inhibitor, the prolongation of the lifetimes of DNA nicks or gaps has been demonstrated, while PARP was very tightly bound to the DNA ends and could not be released (38, 44). Furthermore, it has been shown that overexpression of the isolated DNA-binding domain of the enzyme PARP can interrupt the base excision repair of damaged DNA (31, 44) and can sensitize cells to genotoxic agents (26). Thus, we hypothesized that blocking of PARP might increase recombination frequencies of hepadnaviral DNA and therefore might increase DHBV DNA integration frequency. In an extensive series of subcloning experiments, using three subcloned lineages for three subsequent rounds of subcloning, we demonstrated that 3-AB treatment of LMH-D2 lineages led to a fourfold increase in the number of subclones carrying new DHBV DNA integrations and a sevenfold increase in the total number of DHBV DNA integrations (see Tables 1 to 3 for *P* values). We further confirmed the specificity of the 3-AB effect for PARP in an experiment which demonstrated that a structural analog, 3-ABA, that does not inhibit PARP (5) did not increase the viral integration frequency in subclones.

At present we can speculate as to the mechanism of the above effects based on previous studies of PARP activity in cells. PARP binds very strongly to DNA nicks and ends, and it synthesizes large ADP-ribose polymers from oxidized NAD^+, which rapidly engenders a very dense concentration of negative charges for a short time period, before this polymer gets hydrolyzed by a specific glycohydrolase. Automodification of PARP decreases the binding affinity of the protein for DNA (38), presumably by electrostatic repulsion between the two negatively charged polymers, which leads to the release of PARP from the damaged DNA to allow repair enzymes, e.g. DNA polymerase and excision enzymes, access to DNA damage sites (38). The polymer may protect free DNA ends from exonuclease action, and it may unravel the chromatin structure (44).

Therefore, by blocking PARP activity, hepadnaviral DNA may have greater access to sites of DNA damage, which also have a longer half-life. If access to the chromosomal DNA is a limiting factor in hepadnaviral integration, inhibition of PARP would be expected to increase recombination opportunities of hepadnaviral DNA and thus might increase DHBV DNA integration frequency. The observed effect of 3-AB on the integration frequency may be explained by (i) a disturbance of repair of endogenous DNA damage that occurred on either the viral or the cellular DNA (or both), with consecutive activation of recombination events induced by persistent damage, or (ii) abrogation of the direct recombination-suppressing function postulated for poly(ADP-ribose), which is thought to be independent of DNA excision repair (39). Perturbation of additional enzymatic mechanisms, such as the nucleotide excision repair pathway, may also play a role in viral DNA integrations in unusual pathobiological circumstances, in which those pathways may be activated in the liver.

Our results contrast with the findings of Gaken and colleagues (13), who reported recently that 3-AB blocks the infection of different cell types with recombinant retroviral vectors. The inhibition appeared to be specific to processes required for the efficient integration of proviral DNA into the host cell genome. This contrast is most likely due to distinct differences in the hepadnaviral and retroviral integration mechanisms used by the two different types of viruses.

We detected a high frequency of integrations in the first-generation subclones treated with H_2O_2 or 3-AB by Southern blot. This led us to hypothesize that virtually all the cells in a given subclone should have at least one new DHBV DNA integration by the time the colony had grown 25 generations (see the rationale in Results). However, Southern blot analysis of our second-generation subclones grown in the absence of H_2O_2 or 3-AB clearly revealed that most of the cells in the first-generation colonies grown for 25 generations did not contain stable new DHBV DNA integrations. The integration frequency was slightly higher than that for controls but clearly not near 100% as expected. Therefore, stable DHBV DNA integrations do not accumulate in a unidirectional mode in LMH-D2 cells.

In previous studies with LMH-D2 cells, we have detected the loss of DHBV DNA integrations in only a small fraction of subclones. Loss of previously stable integrations, as assayed by Southern blot, is a rather infrequent event in LMH-D2 subclones (15, 16). However, our data suggest that the loss of viral DNA integrations occurs at a much higher frequency in growing cells than we expected previously. Another possibility is that integration frequency is reduced greatly as the growth of cell clones proceeds past a certain cell stage. Partially integrated DHBV DNA molecules may be rapidly excised from chromosomes. In contrast, when DNA repair is severely impaired, as in our H_2O_2 and 3-AB treatments, stable integrations may be favored.

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