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Increased Mutant Frequency and Altered Mutation Spectrum of the *lacI* Transgene in Wilson Disease Rats with Hepatitis[1]

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ABSTRACT

The mutant strain Long-Evans Cinnamon (LEC) rat, which accumulates copper in the liver because of a mutation in the *Atp7b* gene, encoding a copper-ATPase, is a model of Wilson disease. It spontaneously develops hepatitis, and subsequently hepatocellular carcinoma and cholangiofibrosis. Excess intracellular copper has been thought to induce DNA damage through reactive oxygen species produced by Cu (II)/Cu (I) redox cycling, and also by direct interaction with DNA. We have developed *lacI* transgenic Wilson disease (WND-B) rats by mating LEC with Big Blue F344 rats carrying a lambda shuttle vector harboring the *lacI* gene. *lacI* mutations of the livers of C-B heterozygous (*Atp7b w/m, lacI*) and WND-B homozygous (*Atp7b m/m, lacI*) rats at 6, 24, and 40 weeks of ages were analyzed. Mutant frequencies in the WND-B rats were $2.0 \pm 0.7 \times 10^{-5}$, $5.3 \pm 0.9 \times 10^{-5}$, and $5.3 \pm 1.0 \times 10^{-5}$, respectively, significantly higher than those of C-B rats. Nucleotide sequence analysis revealed that the frequency of deletion mutations of more than two nucleotides were much higher, 15% in WND-B rats, but only 2% in C-B rats. In addition, the average size of deletion was larger in the former. Loss of oligonucleotide-repeat units was specific and relatively frequent in WND-B rats. This type of mutation might be implicated in the induction of DNA strand scissions by reactive oxygen species. These findings suggest that the increase in mutant frequencies and/or the specific type of mutation according to copper accumulation play a crucial role in hepatocarcinogenesis in LEC rats.

INTRODUCTION

The LEC[3] mutant rat developed at Hokkaido University accumulates copper in the liver because of a mutation in the *Atp7b* gene encoding a copper-ATPase (1–3). This genetic defect is the same as that which exists in Wilson disease patients (4). Under standard breeding conditions, the LEC rat develops hepatitis at around 20 weeks of age, and HCCs at around 18 months of age. Hepatitis development has also been linked to copper accumulation in the studies using F1 backcross rats (5, 6). Further, administration of copper chelating agents has been seen to prevent hepatitis development and HCC development (7, 8). It has been reported by us and others that 8-OHdG (9), 1,N^6-ethenodeoxyadenosine (varepsilon dA), and 3,N^4-ethenodeoxycytidine (varepsilon dC) DNA adducts in the livers are increased (10), whereas levels of antioxidant, such as ascorbate and ubiquinol in plasma, are decreased in LEC rats (11). Etheno-adducts produced from other bases are also known to be produced under oxidative conditions (12, 13). Thus, it is considered that the pro-oxidant status associated with copper accumulation causes cellular damage through ROS produced by Cu (II)/Cu (I) redox cycling. It has been reported that copper itself directly interacts with DNA and results in DNA alterations (14, 15). There is no evidence of infiltration of inflammatory cells in LEC rat livers during hepatitis development, and no induction of nitric oxide synthase (iNOS) has been observed.[4]

In previous studies, levels of oxidative DNA damage, including 8-OHdG and etheno-adducts, were shown to be higher in the acute phase of hepatitis than before onset or during the chronic phase (9, 10). Because the hepatocyte turnover rate also peaks with acute hepatitis (16), DNA modifications caused by oxidative DNA damage could be efficiently fixed as mutations.

Although 8-OHdG and etheno-adducts are known to produce mutations *in vitro* (17–19), their roles *in vivo* with regard to cancer development have not been well elucidated. The LEC rat model has distinct advantages for clarifying the role of oxidative DNA damage in hepatitis-hepatoma development. In particular, analysis of the spectra of mutants induced during hepatitis development might provide information on the types of mutation induced *in vivo* by oxidative DNA damage.

In this study, we analyzed the MF in the livers of WND-B rats harboring homozygous *Atp7b* mutations (*Atp7b m/m*) and the *lacI* gene, with reference to hepatitis development. Further, the mutational spectrum of the *lacI* mutants was analyzed.

MATERIALS AND METHODS

Animals. Female and male Big Blue rats (F344 Tac[LIZd]; homozygous) at 6 weeks of age were purchased from Stratagene (La Jolla, CA). The rats were maintained at 24 ± 1°C with a 12-h light and dark cycle and fed a diet (MF, Oriental Yeast, Japan) and tap water *ad libitum*. Male and female LEC rats purchased from Charles River Japan Inc. were mated with Big Blue rats. The F1 generation was then mated with LEC rats again. A subset of rats that are homozygous for the *Atp7b* mutation (*Atp7b m/m*) and harbor the *lacI* gene, named WND-B rats, were used as the experimental group. Another subset of rats that are heterozygous for the *Atp7b* mutation (*Atp7b w/m*) and harbor the *lacI* gene, named C-B rats, were used as a control group. For detection of the *lacI* gene, which should be heterozygous, dot blot analysis of tail DNA at 4–6 weeks of age was performed according to the method previously reported (20). Genotyping of each rat for *Atp7b* was performed by Southern blot analysis of the tail or liver DNA with a cDNA probe of rWDF41R30. In this analysis, the wild-type allele of the *Atp7b* gene appeared as a single band, and the mutant allele showed no signals.

All animals were cared for and maintained in accordance with the National Institute for Environmental Studies animal care guidelines.

Determination of *lacI* Gene MF. Liver DNA extraction and transgenic lambda phage rescue were carried out according to the manufacturer's instructions (Stratagene, La Jolla, CA). Briefly, liver DNA was packaged by mixing with a phage packaging extracts, Transpack. Rescued phages were then plated on an SCS-8 bacterial cell lawn in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactoside, and blue-colored plaques were counted as *lacI* mutants. MF was obtained as the number of blue-colored plaques over the total number of plaques. Blue plaques were isolated and subjected to mutation analysis.

Analysis and Classification of Mutations. DNA was extracted by SM buffer from the blue plaques subcloned. The *lacI* gene covering the coding and

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[3] The abbreviations used are: LEC, Long-Evans Cinnamon (rat); HCC, hepatocellular carcinoma; MF, mutant frequency; C-B rat, rat heterozygous for the WND gene (*Atp7b w/m*) with *lacI* gene; WND-B rat, rat homozygous for the WND gene (*Atp7b m/m*) with *lacI* gene; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; ROS, reactive oxygen species; GOT, glutamate-oxaroacetate transaminase; GPT, glutamate-pyruvate transaminase.

[4] Unpublished data.

promoter regions was amplified by PCR in a thermal cycler using a primer pair of 5′-GACACCATCGAATGGTGCAA-3′ and 5′-TTCCACACAACATAC-GAGCC-3′. The PCR products were subjected to restriction-single-stranded conformational polymorphism analysis according to the method described by Ushijima *et al.* (21). After locating the mutation in either of the A-I fragments, the PCR products of the fragment containing the mutation were then directly sequenced using ABI 388 or ABI 310 sequencers (Applied Biosystems, Japan). The jackpot mutants were excluded to avoid the influence of clonal growth. When bp deletion or insertion mutations were detected in the repeats of a sequence, the first position in the 5′ upstream site was assigned as the mutation site.

Determination of Plasma GOT and GPT Levels. GOT and GPT were determined using a Hitachi 736 autoanalyzer (Hitachi Tokyo, Japan).

Statistical Analysis of Data. Statistical analyses of MF data and mutation spectra were carried out by the *t* test and χ^2 test using STATVIEW version 4.5 (Abacus Concepts, Inc., Berkeley, CA), respectively.

RESULTS

MF in the *lacI* Gene of the Liver. The results for MF in the *lacI* gene of the livers of C-B (*Atp7b w/m*, *lacI*) and WND-B (*Atp7b m/m*, *lacI*) rats at 6, 24, and 40 weeks of age are summarized in Table 1. MFs in the C-B were $1.3 \pm 0.3 \times 10^{-5}$ at 6 weeks of age, and increased with age, culminating at $2.4 \pm 1.2 \times 10^{-5}$ at 40 weeks, in line with values reported for F344 Big Blue rats (22, 23). The MF in the WND-B rats was $2.0 \pm 0.7 \times 10^{-5}$ at 6 weeks, slightly but significantly higher than that of C-B rats. Plasma levels of GOT and GPT as markers of hepatitis onset, however, were not elevated. At 24 weeks of age, MF in the WND-B rats was $5.3 \pm 0.9 \times 10^{-5}$, 2.4-times the C-B value ($2.2 \pm 0.7 \times 10^{-5}$) and plasma levels of GOT (417 IU/liter) and GPT (317 IU/liter) were much higher. At 40 weeks of age, when the WND-B rats were in the chronic phase of hepatitis, MFs in the livers were almost the same as that at 24 weeks.

Mutational Spectra of the *lacI* DNA Sequence. The DNA sequences of a total of 200 *lacI* mutants isolated from the livers of C-B rats (47 at 24 weeks and 52 at 40 weeks) and WND-B rats (49 at 24 weeks and 52 at 40 weeks) were analyzed, and 186 independent mutations (95 of C-B and 91 of WND) were detected. The mutational types and locations of all mutants are listed in Tables 2–5, and a summary of mutational types is given in Tables 6 and 7. The majority of the recovered mutations in both genotypes were base substitutions (C-B, 81%; WND-B, 78%), giving rise to stop codons or amino acid substitutions (Tables 2, 3, 6, and 7). The others were all simple deletions or insertions of 1–358 bp (Tables 4 and 5). Because mutational types did not principally differ between 24 and 40 weeks in either C-B or WND-B rats (Tables 6 and 7), a comparison between C-B and WND-B was made for the total mutations at 24 and 40 weeks.

The most frequent mutations were G:C to A:T transitions in both strains with frequencies of 41% and 49% in C-B and WND-B, respectively. They were mostly present at CpG sites with frequencies of 62% and 87% of the total G:C to A:T mutations in C-B and WND-B, respectively (Tables 6 and 7), the difference being significant ($P = 0.0164$).

A:T to G:C transitions were observed with a significantly higher frequency in C-B ($P = 0.0121$). Further, it is worthy to note that mutations at the A:T site, including transitions and transversions, were significantly more prevalent in C-B than in WND-B, with frequencies of 24% *versus* 10%, respectively ($P = 0.0184$).

Total frequencies for the frameshifts (one or two bp), deletion (more than two bp), and insertions (more than two bp) were almost the same in C-B and WND-B rats, being 19% (18 of 95) and 22% (20 of 91) of the total, respectively, as shown in Tables 6 and 7. Frameshift mutations were more frequent in C-B (13 of 18; 72%) than in WND-B (6 of 20; 30%). In contrast, only 5 (5%) deletion and insertion mutations ranging from 4 to 358 bp were found in C-B, but 14 (16%) were found in WND-B rats. Of these, one C-B but nine WND-B mutants involved >10 bp deletions. Thus, a tendency toward large deletion mutations was seen in WND-B rats, with the average size of 71 bp in WND-B, in contrast to 16 bp in C-B rats.

Mutational hot spots, defined as more than three mutations, were detected at nucleotide positions 92, 329, and 791 in C-B rats and 92,

Table 1 *MF in the livers of C-B and WND-B rats*[a]

Animal no.	GOT (IU/liter)	GPT (IU/liter)	Mutant/Total plaques	MF ($\times 10^{-3}$)
C-B (*Atp7b w/m, lacI*)				
6 wk				
51	92	50	1/120,432	0.8
57	110	66	1/110,598	0.9
92	106	52	2/117,912	1.7
94	121	44	2/127,824	1.6
97	102	48	2/147,072	1.4
99	94	37	1/102,528	1.0
101	91	37	2/126,592	1.6
Mean	102	48		1.3
SD	12	11		0.3
24 wk				
63	85	49	3/116,004	2.6
71	87	34	12/525,484	2.3
72	99	39	12/540,784	2.2
132	83	31	10/383,479	2.6
136	85	33	1/233,332	0.4
138	100	41	6/203,680	2.9
161	129	42	12/550,175	2.2
Mean	95	38		2.2
SD	15	6		0.8
40 wk				
80	57	27	11/480,883	2.3
91	72	33	7/255,278	2.7
106	66	36	17/402,368	4.2
108	95	56	1/156,038	0.6
109	79	36	3/291,370	1.0
112	64	31	9/260,012	3.5
113	64	31	10/383,024	2.6
Mean	71	36		2.4
SD	12	9		1.2
WND-B (*Atp7b m/m, lacI*)				
6 wk				
52	85	50	2/124,824	1.6
53	94	51	3/132,260	2.3
54	100	58	2/136,680	1.5
55	102	66	3/96,480	3.1
95	125	37	3/132,352	2.3
96	99	37	1/107,776	0.9
100	103	44	3/115,584	2.6
Mean	101	49		2.0[b]
SD	12	11		0.7
24 wk				
8	558	168	5/110,550	4.5
60	501	1088	14/352,752	4.0
61	161	173	18/388,395	4.6
62	170	62	9/137,304	6.6
65	138	119	12/210,776	5.7
67	261	400	13/220,238	5.9
131	1131	212	7/118,419	5.9
Mean	417[b]	317[b]		5.3[c]
SD	357	356		0.9
40 wk				
107	313	849	12/223,337	5.4
111	251	288	8/175,824	4.6
114	189	22	7/186,170	3.8
144	115	98	9/145,180	6.2
145	152	103	10/156,740	6.4
146	174	183	7/163,540	4.3
148	116	46	10/151,300	6.6
Mean	187[c]	227		5.3[b]
SD	67	267		1.0

[a] Rats were sacrificed, and liver tissues and blood were isolated to determine MF in the *lacI* gene and plasma GOT/GPT, respectively.

[b] Significantly different from MF for the corresponding *Atp7bw/m* genotype at $P < 0.05$ using the *t* test.

[c] Significantly different from MF for the corresponding *Atp7bw/m* genotype at $P < 0.001$ using the *t* test.

Table 2 *The type and location of base-substitutive mutations in C-B rats*

Animal no.	No. of plaques	Nucleotide no.[a]	Wild form	Detected form	Base substitution	Codon	Amino acid change
24 wk of age[b]							
63	9,926	92	TCC CGC GTG	TGC	C to T	22	Arg to Cys
63	9,922	329	GAA CGA AGC	TGA	C to T	101	Arg to Stop
63	9,967	791	ATG CGC GCC	AGC	C to A	255	Arg to Arg
71	991	56	GTC GCA GAG	ACA	G to A	10	Ala to Thr
71	992	92	TCC CGC GTG	TGC	C to T	22	Arg to Cys
71	682	150	GCG GCG ATG	GAG	C to A	41	Ala to Glu
71	681	210	CAG TCG TTG	TAG	C to A	61	Ser to Stop
71	703	287	CGC GCC GAT	CCC	G to C	87	Ala to Pro
71	993	400	ATC ATT AAC	ATA	T to A	124	Ile to Ile
71	9,923	707	TTT CAA CAA	TAA	C to T	227	Gln to Stop
71	9,969	896	ATC AAA CAG	TAA	A to T	290	Lys to Stop
72	9,977	77	TCT TAT CAG	AAT	T to A	17	Tyr to Tyr
72	9,940	92	TCC CGC GTG	TGC	C to T	22	Arg to Cys
72	684	178	CCC AAC CGC	AAA	C to A	50	Asn to Lys
72	9,924	329	GAA CGA AGC	TGA	C to T	101	Arg to Stop
72	9,972	468	TTA TTT CTT	TAT	T to A	147	Phe to Tyr
72	9,927	719	ATG CAA ATG	TAA	C to T	231	Gln to Stop
72	9,970	891	ACC ACC ATC	ATC	C to T	288	Thr to Ile
72	9,971	994	GTC TCA CTG	TCC	A to C	321	Ser to Ser
132	9,976	221	ATT GGC GTT	AGC	G to A	65	Gly to Ser
132	9,912	269	GTC GCG GCG	ACG	G to A	81	Ala to Thr
132	9,921	329	GAA CGA AGC	TGA	C to T	101	Arg to Stop
132	9,911	693	AGT GCC ATG	GAC	C to A	222	Ala to Asp
132	9,913	843	GTA GGA TAC	GAA	G to A	272	Gly to Glu
136	662	131	ACG CGG GAA	TGG	C to T	35	Arg to Trp
138	9,950	276	GCG ATT AAA	AAT	T to A	83	Ile to Asn
138	9,916	621	CGT CTG GCT	CCG	T to C	198	Leu to Pro
	9,948						
138	9,957	900	AAA CAG GAT	CCG	A to C	291	Gln to Pro
161	9,973	68	GCC GGT GTC	TGT	G to T	14	Gly to Cys
161	636	83	CAG ACC GTT	GCC	A to G	19	Thr to Ala
161	9,960	92	TCC CGC GTG	TGC	C to T	22	Arg to Cys
161	4,431	178	CCC AAC CGC	AAT	C to T	50	Asn to Asn
161	633	285	TCT CGC GCC	CCC	G to C	86	Arg to Pro
161	9,965	296	CAA CTG GGT	TTG	C to T	90	Leu to Leu
161	683	377	GCG CAA CGC	TAA	C to T	117	Gln to Stop
161	5	383	CGC GTC AGT	GGC	T to G	119	Val to Gly
161	9,974	780	GCG CTG GGC	CCG	T to C	251	Leu to Pro
40 wk of age[c]							
80	806	95	CGC GTG GTG	ATG	G to A	23	Val to Met
80	99,140	96	CGC GTG GTG	GAG	T to C	23	Val to Glu
80	805	178	CCC AAC CGC	AAA	C to A	50	Asn to Lys
80	99,107	232	GCC ACC TCC	ATC	C to T	68	Thr to Ile
80	99,123	346	GAA GCC TGT	GCG	C to G	106	Ala to Ala
80	99,120	525	GAC GGT ACG	GAT	G to A	166	Gly to Asp
80	99,124	537	CTG GGC GTG	GAC	G to A	170	Gly to Asp
80	99,126	896	ATC AAA CAG	TAA	A to T	290	Lys to Stop
91	912	83	CAG ACC GTT	GCC	A to G	19	Thr to Ala
91	916	87	ACC GTT TCC	GCT	T to C	20	Val to Ala
91	915	196	CAA CTG GCG	CTA	G to A	56	Leu to Leu
91	915	197	CTG GCG GGC	TCG	G to T	57	Ala to Ser
91	912	270	GTC GCG GCG	GTG	C to T	81	Ala to Val
106	99,104	54	GAT GTC GCA	GCC	T to C	9	Val to Ala
106	1,063	75	GTC TCT TAT	TTT	C to T	16	Ser to Phe
	1,064						
	1,065						
106	1,066	93	TCC CGC GTG	CAC	G to A	22	Arg to His
106	101	95	CGC GTG GTG	ATG	G to A	23	Val to Met
106	99,103	96	CGC GTG GTG	GAG	T to A	23	Val to Glu
106	99,141	201	GCG GGC AAA	GAC	G to A	58	Gly to Asp
106	102	228	GTT GCC ACC	GAC	C to A	67	Ala to Asp
106	99,145	468	TTA TTT CTT	TCT	T to C	147	Phe to Ser
106	1,062	530	ACG CGA CTG	TGA	C to T	168	Arg to Stop
106	99,142	791	ATG CGC GCC	AGC	C to A	255	Arg to Arg
108	1,081	465	GCG TTA TTT	TAA	T to A	146	Leu to Stop
109	1,097	42	GTA ACG TTA	ATG	C to T	5	Thr to Met
109	1,096	140	AAA GTG GAA	ATG	G to A	38	Val to Met
112	1,125	87	ACC GTT TCC	GCT	T to C	20	Val to Ala
112	1,127	103	GTG AAC CAG	AAA	C to A	25	Asn to Lys
112	1,122	381	CAA CGC GTC	CAC	G to A	118	Arg to His
112	99,102	537	CTG GGC GTG	GAC	G to A	170	Gly to Asp
112	1,126	659	ATT CAG CCG	TAG	C to T	211	Gln to Stop
112	1,123	791	ATG CGC GCC	TGC	C to T	255	Arg to Cys
112	1,121	792	ATG CGC GCC	CAC	G to A	255	Arg to His
	1,124						
113	1,131	30	AAT GTG AAA	GCG	T to C	1	Val to Ala
113	1,138	38	CCA GTA ACG	TTA	G to T	4	Val to Leu
113	1,132	49	TTA TAC GAT	TAA	C to A	7	Tyr to Stop
113	99,131	81	TAT CAG ACC	CGG	A to G	18	Gln to Arg
113	99,133	201	GCG GGC AAA	GAC	G to A	58	Gly to Asp
113	1,137	298	CAA CTG GGT	CTA	G to A	90	Leu to Leu
113	1,136	380	CAA CGC GTC	TGC	C to T	118	Arg to Cys

[a] Location of *lacI* gene.
[b] Total: 37 mutations/38 mutants.
[c] Total: 40 mutations/43 mutants.

Table 3 *The type and location of base-substitutive mutations in WND-B rats*

Animal no.	No. of plaques	Nucleotide no.[a]	Wild form	Detected form	Base substitution	Codon	Amino acid change
24 wk of age[b]							
8	85	92	TCC CGC GTG	TGC	C to T	22	Arg to Cys
8	83	329	GAA CGA AGC	TGA	C to T	101	Arg to Stop
60	1,452	42	GTA ACG TTA	ATG	C to T	5	Thr to Met
60	608	87	ACC GTT TCC	GGT	T to G	20	Val to Gly
60	6,010	150	GCG GCG ATG	GAG	C to A	41	Ala to Glu
60	9,951	269	GTC GCG GCG	ACG	G to A	81	Ala to Thr
60	602	329	GAA CGA AGC	TGA	C to T	101	Arg to Stop
61	9,945	56	GTC GCA GAG	ACA	G to A	10	Ala to Thr
61	9,937	75	GTC TCT TAT	TTT	C to T	16	Ser to Phe
61	9,944	92	TCC CGC GTG	TGC	C to T	22	Arg to Cys
61	612	129	AAA ACG CGG	AAG	C to A	34	Thr to Lys
61	9,936	329	GAA CGA AGC	TGA	C to T	101	Arg to Stop
	9,942						
61	616	783	CTG GGC GCA	GAC	G to A	252	Gly to Asp
62	623	82	TAT CAG ACC	CAC	G to C	18	Gln to His
62	624	95	CGC GTG GTG	ATG	G to A	23	Val to Met
62	6,210	178	CCC AAC CGC	AAA	C to A	50	Asn to Lys
62	621	180	AAC CGC GTG	CAC	G to A	51	Arg to His
62	628	273	GCG GCG ATT	GAG	C to A	82	Ala to Glu
65	652	95	CGC GTG GTG	ATG	G to A	23	Val to Met
65	4,486	131	ACG CGG GAA	TGG	C to T	35	Arg to Ser
65	4,482	269	GTC GCG GCG	ACG	G to A	81	Ala to Thr
65	4,483	332	CGA AGC GGC	AGT	C to T	102	Ser to Ser
65	4,488	375	CTC GCG CAA	GTG	C to T	119	Ala to Val
65	4,484	530	ACG CGA CTG	TGA	C to T	168	Arg to Stop
	4,487						
67	673	79	TCT TAT CAG	TAG	T to G	17	Tyr to Stop
67	995	87	ACC GTT TCC	GGT	T to C	20	Val to Ala
67	671	131	ACG CGG GAA	TGG	C to T	35	Arg to Trp
67	998	180	AAC CGC GTG	CAC	G to A	51	Arg to His
67	9,910	185	GTG GCA CAA	CCA	G to C	53	Ala to Pro
67	9,910	242	CTG GCC CTG	CCC	G to C	72	Ala to Pro
67	676	270	GTC GCG GCG	GTG	C to T	81	Ala to Val
67	672	308	AGC GTG GTG	ATG	G to A	94	Val to Met
67	996	780	GCG CTG GGC	CCG	T to C	251	Leu to Gln
131	1,314	42	GTA ACG TTA	ATG	C to T	5	Thr to Met
131	1,312	329	GAA CGA AGC	TGA	C to T	101	Arg to Stop
131	1,312	346	GAA GCC TGT	GCG	C to G	106	Ala to Ala
40 wk of age[c]							
107	99,125	53	GAT GTC GCA	TTC	G to T	9	Val to Phe
107	99,110	96	CGC GTG GTG	GAG	T to A	23	Val to Glu
107	99,112	103	GTG AAC CAG	AAG	C to G	25	Asn to Lys
107	1,073	104	AAC CAG GCC	TAG	C to T	26	Gln to Stop
107	1,072	162	GAG CTG AAT	CGG	T to G	45	Leu to Arg
107	1,071	329	GAA CGA AGC	TGA	C to T	101	Arg to Stop
111	1,117	51	TAC GAT GTC	GCT	A to C	8	Asp to Ala
111	1,117	66	TAT GCC GGT	GTC	C to T	13	Ala to Val
111	1,113	80	TAT CAG ACC	TAG	C to T	18	Gln to Stop
111	1,113	95	CGC GTG GTG	ATG	G to A	23	Val to Met
111	1,116	131	ACG CGG GAA	TGG	C to T	35	Arg to Trp
111	1,112	179	AAC CGC GTG	TGC	C to T	51	Arg to Cys
111	99,118	222	ATT GGC GTT	GAC	G to A	65	Gly to Asp
114	1,144	188	GCA CAA CAA	AAA	C to A	54	Gln to Lys
114	1,145	702	TCC GGT TTT	GTT	G to T	225	Gly to Val
114	1,147	842	GTG GGA TAC	AGA	G to A	272	Gly to Arg
114	1,146	918	CTG GGG CAA	GAG	G to A	297	Gly to Glu
144	1,441	180	AAC CGC GTG	CAC	G to A	51	Arg to His
144	1,448	579	GCG CTG TTA	CGG	T to G	184	Leu to Arg
144	1,449	944	CTG CAA CTC	TAA	C to T	306	Gln to Stop
145	1,451	188	GCA CAA CAA	GAA	C to G	54	Gln to Glu
145	1,459	270	GTC GCG GCG	GTG	C to T	81	Ala to Val
145	1,453	329	GAA CGA AGC	TGA	C to T	101	Arg to Stop
	1,456						
	1,457						
	1,458						
	14,510						
	14,511						
146	1,461	30	AAT GTG AAA	GCG	T to C	1	Val to Ala
146	1,465	185	GTG GCA CAA	TCA	G to T	53	Ala to Ser
146	1,466	198	CTG GCG GGC	GTG	C to T	57	Ala to Val
146	1,463	381	CAA CGC GTC	CAC	G to A	118	Arg to His
146	1,467	1,005	AAA AGA AAA	AAA	G to A	326	Arg to Lys
148	1,487	84	CAG ACC GTT	ATC	C to T	19	Thr to Ile
148	1,481	92	TCC CGC GTG	AGC	C to A	22	Arg to Ser
	1,483						
148	1,486	93	TCC CGC GTG	CAC	G to A	22	Arg to His
148	1,484	176	CCC AAC CGC	TAC	A to T	50	Asn to Try
148	1,482	542	GTG GAG CAT	TAG	G to T	172	Glu to Stop
148	1,485	569	CAG CAA ATC	TAA	C to T	181	Gln to Stop
148	1,488	681	GAA GGC GAC	GAC	G to A	218	Gly to Asp

[a] Location of *lacI* gene.
[b] Total: 36 mutations/38 mutants.
[c] Total: 35 mutations/41 mutants.

Table 4 *The type and location of mutations other than base substitutions in C-B rats*[a]

Animal no.	No. of plaques	Nucleotide no.	Wild form	Detected form	Deletion or insertion	Mutation type	Remarks
24 wk of age							
63	9,968	670	GCG GAA CGG	GCG AAC GG	G	1-bp deletion	
71	711	220–224	**ATT GGC GTT GCC**	ATT TGC CAC	TGGCG	5-bp deletion	Direct repeat
71	993	401	ATT AAC TAT	ATT AAA CTA T	A	1-bp insertion	
72	9,914	620–623	CGT $(CTGG)_3$ CAT	CGT $(CTGG)_4$CAT	CTGG	4-bp insertion	Gain of repeat
132	665	158–163	ATG GCG GAG	G $(ATG\ GCG)_2$GAG	ATGGCG	6-bp insertion	Gain of repeat
132	9,919	199	GCG GGC AAA	GCG GCA AA	G	1-bp deletion	
138	9,950	40	CCA GTA ACG	CCA GTG AAC G	G	1-bp insertion	
161	9,939	497	ATC AAC AGT	ATC ACA GT	A	1-bp deletion	
161	4,433	620–623	CGT $(CTGG)_3$CAT	CGT $(CTGG)_4$ CAT	CTGG	4-bp insertion	Gain of repeat
Total: 9 mutations/9 mutants							
40 wk of age							
80	99,139	389	AGT GGG CTG	AGT GGC TG	G	1-bp deletion	
80	99,134	519	CAT GAA GAC	CAT GAA AGA C	A	1-bp insertion	
91	99,128	40	CCA GTA ACG	CCA GTG AAC G	G	1-bp insertion	
106	99,146	440	GAA GCT GCC	GAA CTG CC	G	1-bp insertion	
106	99,143	632–693	TGG **CAT...GCC ATG**	TGG CAT GTC	CAT...GC	62-bp deletion	Direct repeat
106	99,105	1,006	AAA AGA AAA	AAA ACG AAA A	C	1-bp deletion	
106	99,144	1,055	TTG GCC GAT	TTG GTC CGA T	T	1-bp deletion	
109	99,127	209	CAG TCG TTG	CAG CGT TG	T	1-bp deletion	
113	99,132	1,013	ACC ACC CTG	ACC CCC TG	A	1-bp deletion	
Total: 9 mutations/9 mutants							

[a] Deleted and inserted bases are underlined in the wild form and in the detected form, respectively. Bold letters indicate direct repeat sequences.

95, 131, 180, and 329 in WND-B rats, with totals of 10 and 18 mutations, respectively. All these sites were CpG, and 8 of 10 and 17 of 18 mutations in C-B rats and WND-B rats, respectively, were G:C to A:T transitions.

Insertion of 5′-CTGG-3′ was observed twice in C-B rats and once in a WND-B rat, at nucleotide positions 620–623 where a three-consecutive repeat of 5′-$(CTGG)_3$-3′ exists. In contrast, three deletion mutations, with loss of one of the three repeats, were detected in WND-B rats but none in C-B. In the two strains of rats, 5 of 19 insertion and deletion mutations were at nucleotide number 620, indicating the 5′-CGT$(CTGG)_3$ CAT-3′ to be a target in both C-B and WND rats.

Other characteristic mutations were also found: A mutation in C-B rats (plaque no. 665) had an ATGCG insertion resulting in a repeat of this sequence. Another mutation in WND-B rats (plaque no. 99113) was implicated with a palindrome structure composed of an inverted 6 bp separated by 45 bp, while 46 bp were deleted.

DISCUSSION

The present study demonstrated the MF in the liver of WND-B rats to be 1.5 times higher than that in the C-B rat, even before the onset of hepatitis at 6 weeks of age. At this age, the copper level in the WND-B rat liver was much higher, 65.3 μg/g wet tissue (n = 8; range, 27–95), than in the C-B case (15.8 μg/g wet tissue; n = 5; range, 5.4–26.[4] Thus, the higher MF in the WND-B rat could have been attributable to accumulation of copper, which is known to induce mutations by direct interaction with DNA or through production of ROS. The BrdUrd labeling index of LEC rats at 6 weeks of age is the same as that of the wild-type rat (24), suggesting that cell proliferation itself played no major role in the difference in MF. The MF ratio of WND-B:C-B was increased to 2.4 at 24 weeks, when hepatitis had developed in the WND-B rats, with high plasma GOT and GPT levels. Some *Atp7b m/m* rats in fact died of fulminant jaundice at around 21 weeks. At 24 weeks of age, the levels of copper in WND-B rats were

Table 5 *The type and location of mutations other than base substitutions in WND-B rats*[a]

Animal no.	No. of plaques	Nucleotide no.	Wild form	Detected form	Deletion or insertion	Mutation type	Remarks
24 wk of age							
8	85	182–204	CGC GTG...AAA CAG	CGC ACA G	GTG...AA	23-bp deletion	
60	642	67–424	**TAT GCC GGT....GAT GCC** ATT	TAT GCG CCA TT	C GGT...GAT	358-bp deletion	Direct repeat
60	934/938	620–623	CGT $(CTGG)_3$ CAT	CGT $(CTGG)_2$ CAT	CTGG	4-bp deletion	Loss of repeat
61	617	350–438	**CC TGT** AAA...GAA **GCT GCC**	CC TGT AGC TGC C	AAA...GA	89-bp deletion	Direct repeat
61	613	620–623	CGT $(CTGG)_3$ CAT	CGT $(CTGG)_4$ CAT	CTGG	4-bp insertion	Gain of repeat
61	943	881–1,023	CCG CCG...CCC AAT	CCG CAA T	CCG...CC	143-bp deletion	Loss of repeat
65	4,481	445–470	GCC TGC...TTT CTT	GCT TGA T	C TGC...TTT C	26-bp deletion	
65	4,485	620–623	CGT $(CTGG)_3$ CAT	CGT $(CTGG)_2$ CAT	CTGG	4-bp deletion	Loss of repeat
67	999	359–384	CG GCG **GTG**...GTC A**GT GG**	GCG CAG T	GTG...GT	26-bp deletion	Direct repeat
131	1,311	901	CAG GAT TTT	CAG ATT TT	G	1-bp deletion	
Total: 10 mutations/11 mutants							
40 wk of age							
107	99,116	635–711	CAT**AAA TCAA ACC**	CAT AAC C	AAA...CA	77-bp deletion	Direct repeat
107	99,111	782–789	CTG **GGC GCA ATG CGC**	CTG GCG C	GGC GCA AT	8-bp deletion	Direct repeat
107	99,113	914–959	CTG *CTG...CAG* GCG	CTG AGG CG	CTG...C	46-bp deletion	Inverted repeat
111	1,111	250	CAC GCG CCG	CAC GCC G	CG	2-bp deletion	Loss of repeat
114	1,148	139–188	AAAGCA CAA CAA	AAA ACA A	A...GCA C	50-bp deletion	
114	99,122	1,113	GCG CAA	GAC GCA A	A	1-bp insertion	
144	1,444	47–190	TTA TACCAA CAA	TTA CAA	TAC...CAA	144-bp deletion	
144	1,443/1,447	860	GAA GAC AGC	GAA ACA GC	G	1-bp deletion	
146	1,464	1,006	AGA AAA ACC	AGA AAA CC	A	1-bp deletion	
148	1,481	731	AAT GAG GGC	AAT AGG GC	G	1-bp deletion	
Total: 10 mutations/11 mutants							

[a] Deleted and inserted bases are underlined in the wild form and in the detected form, respectively. Bold and italic letters indicate direct and inverted repeat sequences, respectively.

highest, with an average of 200 μg/g ($n = 7$; range, 113–275), in line with data for oxidative DNA damage (9, 10) and cell proliferation rate (2). Thus, the DNA lesions would be expected to be efficiently fixed as mutations. Among WND-B rats at 24 weeks of age, there was a positive correlation between the copper levels and MF ($r = 0.398$); however, no correlation between plasma GOT/GPT levels and MF ($r = -0.231$) was observed. At 40 weeks of age, the MF values in C-B and WND-B were the same as those at 24 weeks, and this lack of increase might be partly explained by the lower levels of copper [178 μg/g ($n = 7$; range, 31–301)], DNA adducts (9, 10), and cell proliferation rate (2) at 24 weeks. All LEC rats surviving the acute phase of hepatitis develop HCCs. Thus, the increased MF occurring in the acute phase could play important roles in hepatocarcinogenesis, along with signal transduction induced by ROS, such as through the nuclear factor κB pathway (25).

Ratios of base substitution mutations did not basically differ between C-B and WND-B rats. However, some differences were observed in mutational types: mutations at A:T sites were significantly decreased, and G→A transitions at the CpG site were significantly increased in WND-B rats. Additionally, large deletions were observed at a high frequency in WND-B rats. Recently it was found that 2-OHdA is produced 70–80 times more efficiently in the nucleotide pools than on DNA. 2-OHdA is misinserted opposite dC to give G:C to A:T transitions in subsequent DNA replications (26). Our experimental results, showing a higher tendency for mutation of G:C to A:T in WND-B rats than in C-B rats, suggest 2-OHdA as a possible cause. The predominant base substitutions produced by incubation of single-stranded M13mp2DNA with Cu were C→T and G→T (27). Thus, involvement of direct interaction of copper with DNA in base substitution mutation cannot be ruled out. ROS produced by H_2O_2 or metals, including Fe^{2+}, Cu^{2+}, and Ni^{2+}, in contrast, is reported to induce CC to TT mutations (27, 28); ROS produced by bleomycin is associated with single bp deletions at hot spots of 5′-GTC-3′ or 5′-GCC-3′ in CHO cells (29). However, no such bp substitutions or preferential sites for frameshift were observed here in either C-B or WND-B rats. The oxidative stress in this model system might be attributable to another type of ROS, which resembles spontaneously accumulated mutations during aging.

It has been reported that singlet oxygen contributes to strand breakage by lead acetate ($Pb(CH_3COO)_2$; Ref. 30), and that cobaltous chloride ($CoCl_2$) induces deletion mutations, specifically at direct repeat sequences in *Escherichia coli* (31). There were only two deletions at direct repeat sequences in C-B but four in WND-B rats, and one at an inverted repeat sequence in WND-B rats. Further, some mutation spectra of mutant plaques were not determined because their *lacI* gene fragment could not be amplified by PCR, suggesting that these plaques might include some large deletions.

In conclusion, the present results suggest that the increase of MF and changes in the mutational spectrum in WND-B rats are attributable to DNA damage induced by copper accumulation itself and/or associated oxidative stress. In addition, we hypothesize that the remarkable increase in MF in the LEC rat liver may play a crucial role in its hepatocarcinogenesis.

Table 6 *Types of lacI mutations in C-B rat livers*

Mutational type	24 wk No. of mutations	24 wk CpG (%)	40 wk No. of mutations	40 wk CpG (%)	Total No. of mutations	Total CpG (%)
Transition						
G:C to A:T	18 (39)	10 (56)	21 (43)	14 (67)	39 (41)	24 (62)
A:T to G:C	3 (7)		8 (16)		11 (12)	
Transversion						
G:C to T:A	6 (13)		7 (14)		13 (14)	
G:C to C:G	2 (4)		1 (2)		3 (3)	
A:T to T:A	5 (11)		3 (6)		8 (8)	
A:T to C:G	3 (7)		0 (0)		3 (3)	
Others						
Frameshift[a]	5 (11)		8 (16)		13 (14)	
Deletion	1 (2)		1 (2)		2 (2)	
Insertion	3 (7)		0 (0)		3 (3)	
Total mutations	46 (100)		49 (100)		95 (100)	

[a] Frameshift mutations include one or two bp deletions or insertions.

Table 7 *Types of lacI mutations in WND-B rat livers*[a]

Mutational type	24 wk No. of mutations	24 wk CpG (%)	40 wk No. of mutations	40 wk CpG (%)	Total No. of mutations	Total CpG (%)
Transition						
G:C to A:T	24 (51)	24 (100)	21 (47)	15 (71)	45 (49)	39 (87)
P values		0.0012		>0.9999		0.0164
A:T to G:C	1 (2)		1 (2)		2 (2)	
P values	0.3066		0.0203		0.0121	
Transversion						
G:C to T:A	4 (9)		6 (13)		10 (11)	
G:C to C:G	4 (9)		2 (4)		6 (7)	
A:T to T:A	1 (2)		2 (4)		3 (3)	
A:T to C:G	2 (4)		3 (7)		5 (5)	
Others						
Frameshift[b]	1 (2)		5 (11)		6 (6)	
Deletion	8 (17)		5 (11)		13 (15)	
P values	0.0255		0.0723		0.03	
Insertion	1 (2)		0 (0)		1 (1)	
Total mutations	46 (100)		45 (100)		91 (100)	

[a] Differences of mutations between C-B and WND-B were analyzed by the χ^2 test using Statview version 4.5.

[b] Frameshift mutations include one or two bp deletions or insertions.

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REFERENCES

1. Sasaki, M., Yoshida, M. C., Kagami, K., Takeichi, N., Kobayashi, H., Dempo, K., and Mori, M. Spontaneous hepatitis in an inbred strain of Long-Evans rats. Rat News Lett., *14:* 4–6, 1985.
2. Yoshida, M. C., Masuda, R., Sasaki, M., Takeichi, N., Kobayashi, H., Dempo, K., and Mori, M. New mutation causing hereditary hepatitis in the laboratory rat. J. Hered., *78:* 361–365, 1987.
3. Li, Y., Togashi, Y., Sato, S., Emoto, T., Kang, J. H., Takeichi, N., and Kobayashi, H. Spontaneous hepatic copper accumulation in Long-Evans Cinnamon rats with hereditary hepatitis: a model of Wilson's disease. J. Clin Invest., *87:* 1858–1861, 1991.
4. Wu, J., Forbes, J. R., Chen, H. S., and Cox, D. W. The LEC rat has a deletion in the copper transporting ATPase gene homologous to the Wilson disease gene. Nat. Genet., *7:* 541–545, 1994.
5. Sone, H., Maeda, M., Gotoh, M., Wakabayashi, K., Ono, T., Yoshida, M. C., Takeichi, M., Mori, M., Hirohashi, S., Sugimura, T., and Nagao, M. Genetic linkage between copper accumulation and hepatitis/hepatoma development in LEC rats. Mol. Carcinog., *5:* 199–204, 1992.
6. Ono, T., Takada, S., and Yoshida, M. C. The *WD* gene for Wilson Disease links to the hepatitis of LEC rats. Jpn. J. Cancer Res., *85:* 771–774, 1994.
7. Jong-Hon, K., Togashi, Y., Kasai, H., Hosokawa, M., and Takeichi, N. Prevention of spontaneous hepatocellular carcinoma in Long-Evans Cinnamon rats with hereditary hepatitis by the administration of D-penicillamine. Hepatology, *18:* 614–620, 1993.
8. Sone, H., Maeda, M., Wakabayashi, K., Takeichi, N., Mori, M., Sugimura, T., and Nagao, M. Inhibition of hereditary hepatitis and liver tumor development in Long-Evans Cinnamon rats, by a copper chelating agent, trientine dihydrochloride. Hepatology, *23:* 764–770, 1996.
9. Yamamoto, F., Kasai, H., Togashi, Y., Takeichi, N., Hori, T., and Nishimura, S. Elevated level of 8-hydroxydeoxyguanosine in DNA of liver, kidneys, and brain of Long-Evans Cinnamon rats. Jpn. J. Cancer Res., *84:* 508–511, 1993.
10. Nair, J., Sone, H., Nagao, M., Barbin, A., and Bartsch, H. Copper-dependent formation of miscoding etheno-DNA adducts in the liver of Long-Evans Cinnamon (LEC) rats developing hereditary hepatitis and hepatocellular carcinoma. Cancer Res., *56:* 1267–1271, 1996.

11. Yamamoto, Y., Sone, Hideko., Yamashita, S., Nagata, Y., Niikawa, H., Hara, K., and Nagao, M. Oxidative stress in LEC rats evaluated by plasma antioxidants and free fatty acids. Cancer Res., *10:* 129–134, 1997.
12. El-Ghissassi, F., Barbin, A., Nair, J., and Bartsch, H. 1,N^6-ethenodeoxyadenosine and 3,N^4-ethenodeoxycytidine in liver DNA from humans and untreated rodents detected by lipid peroxidation products and nucleic acid bases. Chem. Res. Toxicol., *8:* 273–283, 1995.
13. Nair, J., Barbin, A., Velic, I., and Bartsch, H. Etheno DNA-base adducts from endogenous reactive species. Mutat. Res., *424:* 59–69, 1999.
14. Tkeshelashvili, L. K., McBride, T., Spence, K., and Loeb, L. A. Mutation spectrum of copper-induced DNA damage. J. Biol. Chem., *266:* 6401–6406, 1991.
15. Nackerdien, Z., Rao, G., Cacciuttolo, M. A., Gajewski, E., and Izdaroglu, M. Chemical nature of DNA-protein cross-links produced in mammalian chromatin by hydrogen peroxide in the presence of iron or copper ions. Biochemistry, *30:* 4873–4879, 1991.
16. Wolf, H. K., and Michalopoulos, G. K. Hepatocyte regeneration in acute fulminant and nonfulminant hepatitis: a study of proliferating cell nuclear antigen expression. Hepatology, *15:* 707–713, 1992.
17. Cheng, K. C., Cahill, D. S., Kasai, H., Nishimura, S., and Loeb, L. A. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G:T and A:C substitutions. J. Biol. Chem., *267:* 166–172, 1992.
18. Pandya, G. A., and Moriya, M. 1,N^6-ethenodeoxyadenosine, a DNA adduct highly mutagenic in mammalian cells. Biochemistry, *35:* 11487–11492, 1996.
19. Palejwala, V. A., Rzepka, R. W., Simha, D., and Humayun, M. Z. Quantitative multiplex sequence analysis of mutational hot spots. Frequency and specificity of mutations induced by a site-specific ethenocytosine in M13 viral DNA. Biochemistry, *32:* 4105–4111, 1993.
20. Kohler, S. W., Provost, G. S., Fieck, A., Kretz, P. L., Bullock, W. O., Sorge, J. A., Putman, D. L., and Short, J. M. Spectra of spontaneous and mutagen-induced mutations in the *lacI* gene in transgenic mice. Proc. Natl. Acad. Sci. USA, *88:* 7958–7962, 1991.
21. Ushijima, T., Hosoya, Y., Suzuki, T., Sofuni, T., and Sugimura, T., Nagao, M. A rapid method for detection of mutations in the *lacI* gene using PCR-single strand conformation polymorphism analysis: demonstration of its high sensitivity. Mutat. Res., *334:* 283–292, 1995.
22. Davies, R., Oreffo, V. I., Martin, E. A., Festing, M. F., White, I. N., Smith, L. L., and Styles, J. A. Tamoxifen causes gene mutations in the livers of lambda/*lacI* transgenic rats. Cancer Res., *57:* 1288–1293, 1997.
23. Dycaico, M. J., Stuart, G. R., Tobal, G. M., de Boer, J. G. Glickman, B. W., and Provost, G. S. Species-specific differences in hepatic mutant frequency and mutational spectrum among lambda/*lacI* transgenic rats and mice following exposure to aflatoxin B1. Carcinogenesis (Lond.), *17:* 2347–2356, 1996.
24. Fujimoto, Y., Oyamada, M., Hattori, A., Takahashi. H., Sawaki, M., Dempo, K., Mori, M., and Nagao, M. Accumulation of abnormally high ploid nuclei in the liver of LEC rats developing spontaneous hepatitis. Jpn. J. Cancer Res., *80:* 45–50, 1989.
25. Hayes, J. D., and Pulford, D. J. The glutathione *S*-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit. Rev. Biochem. Mol. Biol., *30:* 445–600, 1995.
26. Kamiya, H., and Kasai, H. Effect of sequence contexts on misincorporation of nucleotides opposite 2-hydroxyadenine. FEBS Lett., *391:* 113–116, 1996.
27. Reid, T. M., Feig, D. I., Loeb, L. A. Mutagenesis by metal-induced oxygen radicals. Environ. Health Perspect., *102* (Suppl. 3)*:* 57–61, 1994.
28. Newcomb, T. G., Allen, K. J., Tkeshelashvili, L., and Loeb L. A. Detection of tandem CC to TT mutations induced by oxygen radicals using mutation-specific PCR. Mutat. Res., *427:* 21–30, 1999.
29. An, J., Trieff, N. M., and Hsie, A. W. PCR-directed DNA sequencing of "nondeletion" HPRT-mutants induced by bleomycin in CHO K1-BH4 cells. Environ. Mol. Mutagen., *32:* 244–250, 1998.
30. Yang, J. L., Wang, L. C., Chang, C. Y., and Liu, T. Y. Singlet oxygen is the major species participating in the induction of DNA strand breakage and 8-hydroxyguanosine adduct by lead acetate. Environ. Mol. Mutagen., *33:* 194–201, 1999.
31. Iyehara-Ogawa, H, Ohyama, Y., Ohsumi, Y., Kakimoto, K., Kato, Y., Shirai, Y., Nunoshiba, T., and Yamamoto, K. Cobaltous chloride-induced mutagenesis in the supF tRNA gene of *Escherichia coli*. Mutagenesis, *14:* 249–253, 1999.