ABSTRACT

2-Amino-3, 8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) is a mutagenic and carcinogenic heterocyclic amine found in cooked meat. The *in vivo* mutagenicity and hepatocarcinogenicity of MeIQx were examined in mice harboring the *lacZ* mutation reporter gene (Muta^{TM} Mice) and bitransgenic mice over-expressing the c-*myc* oncogene. C57Bl/λ*lacZ* and bitransgenic c-*myc* (albumin promoter)/λ*lacZ* mice were bred and weaned onto an AIN-76 based diet containing 0.06% (w/w) MeIQx or onto control diet. After 30 weeks on diet, only male bitransgenic mice on MeIQx developed hepatocellular carcinoma (100% incidence) indicating that there was synergism between c-*myc* over-expression and MeIQx. By 40 weeks, hepatic tumor incidence was 100 % (17%) and 44% (0%) in male c-*myc*/λ*lacZ* and C57Bl/λ*lacZ* mice given MeIQx (or control) diet, respectively, indicating that either MeIQx or c-*myc* over-expression alone eventually induced hepatic tumors. At either time point, mutant frequency in the *lacZ* gene was at least 40-fold higher in MeIQx-treated mice than in control mice of either strain. These findings suggest that MeIQx-induced hepatocarcinogenesis is associated with MeIQx-induced mutations. Elevated mutant frequency in MeIQx-treated mice also occurred concomitant with the formation of MeIQx-guanine adducts as detected by the ^{32}P-postlabeling assay. Irrespective of strain or diet, sequence analysis of the *lacZ* mutants from male mouse liver showed that the principal sequence alteration was a single guanine-base substitution. Adenine mutations, however, were detected only in animals on control diet. MeIQx-fed mice harboring the c-*myc* oncogene showed a 1.4-2.6-fold higher mutant frequency in the *lacZ* gene than mice not carrying the transgene. Although there was a trend toward higher adduct levels in c-*myc* mice, MeIQx-DNA adduct levels were not significantly different between c-*myc*/λ*lacZ* and

C57Bl/λ*lacZ* mice after 30 weeks on diet. Thus, it appeared that factors in addition to MeIQx-DNA adduct levels, such as the enhance rate of proliferation associated with c-*myc* over-expression, may have accounted for a higher mutant frequency in c-*myc* mice. In the control diet groups, the *lacZ* mutant frequency was significantly higher in c-*myc*/λ*lacZ* mice than in C57Bl/λ*lacZ* mice. The findings are consistent with the notion that c-*myc* over-expression is associated with an increase in mutagenesis. The mechanism for the synergistic effects of c-*myc* over-expression on MeIQx hepatocarcinogenicity appears to involve an enhancement of MeIQx-induced mutations.

Abbreviations: MeIQx, 2-amino-3, 8-dimethylimidazo[4,5-*f*]quinoxaline; HCAs, heterocyclic amines; P-gal, phenyl-D-galactoside.

INTRODUCTION

2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) is a member of a family of mutagenic/carcinogenic heterocyclic amines (HCAs) found in cooked meats including beef, chicken, and fish. MeIQx is currently recognized as the second-most prevalent of the HCAs in the human diet (1). In rodent carcinogenicity bioassays, MeIQx has been shown to be a strong liver carcinogen. In a previous report, the incidence of hepatic cancer in CDF1 mice exposed to 0.06% MeIQx in the diet for 84 weeks was 43 and 91% in males and females, respectively (2).

MeIQx is a procarcinogen that requires metabolic activation to reactive ester derivatives to form DNA adducts, induce mutations, and initiate the process of carcinogenesis. In experimental animals including rats, mice and monkeys fed MeIQx, (3-6), MeIQx has been shown to form adducts at guanine nucleotides. Studies to determine the structures of MeIQx-DNA adducts reveal that the electrophilic metabolite of MeIQx react predominantly at the *C8* and N^2 positions of guanine forming N^2-(deoxyguanosin-8-yl)-MeIQx and 5-(deoxyguanosin-N^2-yl)-MeIQx, respectively (5). N^2-(deoxyguanosin-8-yl)-MeIQx is the major adduct of MeIQx found in liver and other tissues of animals given MeIQx (3, 4).

MeIQx is known to be a potent mutagen in several in vitro assay systems including the Ames Salmonella mutagenicity assay, and in the *lacZ* gene of *Escherichia coli* (7-10). MeIQx has also been shown to be genotoxic in vivo as assessed by an animal-mediated microbial assay (11), and in the *lacZ* gene of the Muta™ mice (12). As with other HCAs (13), *in vitro* mutagenicity studies support that MeIQx-DNA adducts play a role in the induction of specific genetic mutations (10).

In the paradigm of the multi-step model of carcinogenesis, the accumulation of multiple

genetic alterations in critical genes is essential for the development of cancer. Deregulation of the c-*myc* gene by over-expression or amplification is one alteration that has been implicated in the development of hepatocellular carcinoma in both experimental models and humans (14). c-*Myc* is a protooncogene involved in the regulation of DNA synthesis, differentiation, apoptosis and cell cycle progression. Deregulation of c-*myc* can lead to a loss of cell cycle control, improper initiation of DNA synthesis, and genetic instability (14-16). Recent studies in a c-*myc* transgenic mouse model have shown, however, that hepatic over-expression of c-*myc* leads to a low incidence of hepatocellular carcinoma after a long latency period (17, 18). Additional cooperating genetic alterations appear to be essential for hepatocarcinogenesis under conditions of c-*myc* deregulation (19).

The central role of c-*myc* in cell cycle regulation and the requirement for multiple genetic alterations for hepatocarcinogenesis raises the question of whether the sensitivity to a chemical mutagen and hepatocarcinogen such as MeIQx is affected by over-expression of the c-*myc* oncogene. In a previous report, this laboratory described the use of a bitransgenic mouse harboring both the c-*myc* gene and the *lacZ* mutational reporter gene (12). After an acute exposure, the *in vivo* mutagenicity of MeIQx and other HCAs was shown to be higher in c-*myc*/□*lacZ* bitransgenic mice than in control (C57Bl/λ*lacZ*) mice. This study suggested the possibility of an interaction between the specific HCAs and the over-expressed c-*myc* gene. In the current study we examine if hepatic over-expression of the c-*myc* oncogene alters the susceptibility to MeIQx-hepatocarcinogenesis, evaluate the role of MeIQx-induced mutagenesis in hepatocarcinogenesis, and address whether c-*myc* might mediate its effects on MeIQx sensitivity via a mutagenic mechanism.

MATERIALS AND METHODS

Chemicals. 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) was purchased from Toronto Research Chemicals (North York, ON). AIN-76 diet was purchased from Harlan Teklad (Madison, WI). Phenyl-D-galactoside (P-gal) was purchased from Sigma (St. Louis, MO). RNAce-It ribonuclease cocktail and Giga-Pack II were obtained from Stratagene (La Jolla, CA). The *LacZ⁻ galE⁻ E. coli* C strain with overexpressed *galT* and *galK* genes was a generous gift from Dr. Jan Vijg (Harvard Medical School, Boston, MA).

Animals and treatment. Male Muta™ mice (BALB/c x DBA/2 (CD2F1)) carrying the *lacZ* trangene were purchased from Hazleton Research Products (Denver, PA). Female c-*myc* transgenic mice (C57Bl/6J x CBA/J) were a generous gift from Dr. S. S. Thorgeirsson (18). C57Bl/*lacZ* (Muta™) and c-*myc*/*lacZ* (Muta™) mice were generated by breeding male Muta™ mice with female C57Bl/6J and c-*myc* transgenic mice. F1 mice were weaned onto AIN-76 diet containing 0.06% (w/w) MeIQx or AIN-76 (control) diet. Food and water were provided *ad libitum* throughout the study. Animal housing and care were in accordance with NIH guidelines.

Collection of tissue samples and histology. Mice were euthanized after 30 and 40 weeks on diet by cervical dislocation and autopsied. Livers were examined macroscopically for the appearance of tumor. Portions of liver and liver tumors were fixed in 10% formalin or promptly frozen in liquid nitrogen and stored at -80 C until DNA isolation. All fixed tissues were embedded in paraffin and sections stained with hematoxylin/eosin (H&E). Sections of nontumorous liver and grossly visible tumors were examined and the latter diagnosed as hepatocellular adenomas or carcinomas (20).

^{32}P-postlabeling analysis. For ^{32}P-postlabeling analysis, genomic DNA was isolated from frozen tissue essentially by the method previously described (21). The analysis of MeIQx-adducts in genomic DNA was performed by ^{32}P-postlabeling analysis as before (3, 4), using the intensification (ATP deficient) method (22). This method resolves [^{32}P]ATP-labeled bisphosphonucleotide adducts on polyethyleneimine-cellulose thin layer sheets as fingerprints following autoradiography.

Genomic DNA extraction for *lacZ* mutant detection. High molecular weight DNA was isolated from frozen mouse liver and tumor tissues according to the protocol described earlier (23). Briefly, tissues were disaggregated and homogenized in dounce buffer (14 mM sodium phosphate, pH 8.0, 137 mM NaCl, 3 mM KCl and 10 mM NaEDTA) containing 1/50 volume of RNAce-It ribonuclease cocktail. An equal volume of lysis buffer (2 mg/ml proteinase K, 2% SDS and 0.1 M NaEDTA, pH 7.5) was added. After a 3 hour incubation at 50□C, the DNA was sequentially extracted with phenol/chloroform. The DNA was ethanol precipitated, spooled onto a hooked glass Pasteur pipette and then dissolved in 10 mM Tris buffer (pH 8.0) at 37□C to a concentration of 1-3 mg/ml.

Determination of mutant *lacZ* frequency. To determine the frequency of mutations in the *lacZ* transgene in liver of mice, Lambda gt10 DNA was rescued from genomic DNA by packaging into lambda phage using Giga-Pack II packaging extract (23). Each packaging reaction was adsorbed to *E. coli* C at log phase growth. The reactions were then plated with and without P-gal. The number of plaques were counted after overnight incubation at 37C. The *lacZ*$^-$ mutant frequency was determined as the ratio of *lacZ*$^-$ mutant plaques obtained in the presence of P-gal to the number of plaques recovered on the titer plates (23, 24). From each

mouse-liver DNA sample, a minimum of 300,000 total plaques were scored on the titer plates.

Sequencing of mutant *lacZ*. *LacZ* mutants were sequenced as described previously (23). Briefly, mutant plaques were picked randomly from plates and expanded in liquid culture before DNA isolation. Regions of the *lacZ* gene were amplified by polymerase chain reactions and sequenced using the previously reported sequencing primers (23). Sequencing reactions were performed using a DNA sequencing kit for dye terminator cycle reactions (Perkin-Elmer, Foster City, CA). Automated sequencing was carried out on an ABI Prism 377 DNA Sequencer (Perkin-Elmer).

Statistical analysis. The data were analyzed by both two-way ANOVA (mouse strain and carcinogen treatment) and Student's *t*-tests using the computer program Prizm (GraphPad Software Incorporated, San Diego, CA).

RESULTS

DNA adduct analysis

^{32}P-Postlabeling analysis was used to measure MeIQx-DNA adduct levels in hepatic DNA from c-*myc*/*lacZ* and C57Bl/*lacZ* strains of mice that received the MeIQx diet for 30 weeks. Three principle adduct spots, identical to those identified previously as guanine nucleotide adducts of MeIQx (4), were detected in DNA from both strains. The profile of MeIQx-DNA adducts was also identical to that reported previously in c-*myc*/*lacZ* and C57Bl/*lacZ* strains of mice given multiple doses of MeIQx (12). No qualitative differences in MeIQx-DNA adduct formation was noted among the various treatment groups (data not shown). The major adduct comprising at least 50% of total adduct levels coincided chromatographically with the 3', 5' bisphosphate of N^2-(deoxyguanosin-8-yl)-MeIQx, the C8-guanine adduct of MeIQx. For each gender, total MeIQx-DNA adduct levels were not statistically different between c-*myc*/*lacZ* and C57Bl/*lacZ* mice, although there did appear to be a trend for slightly higher adduct levels in mice harboring the c-*myc* transgene (Fig. 1). Irrespective of mouse strain, MeIQx-DNA adduct levels were significantly higher in the females than in the males ($p < 0.05$, two-factor ANOVA). No MeIQx-DNA adducts were detected in animals on control diet.

Mutant frequency

The mutant frequency in the *lacZ* gene of hepatic DNA was measured in c-*myc*/*lacZ* and C57Bl/□*lacZ* mice on MeIQx or control diet for 30 and 40 weeks (Fig. 2). The dietary MeIQx exposure had a striking effect on mutant frequency in the *lacZ* gene in both c-*myc*/*lacZ* and C57Bl/□*lacZ* mice. In both strains and at both time points, MeIQx-fed mice showed at least a 40-fold higher *lacZ* mutant frequency than mice on control diet (Fig. 2, compare panel A to panel

B and panel C to panel D). The presence of the c-*myc* transgene also affected mutant frequency of the *lacZ* transgene in both control and MeIQx treatment groups. In the MeIQx treatment groups, mutant frequency was 1.4 -2.6-fold higher in mice harboring the c-*myc* transgene than that in the control strain (C57Bl/*lacZ*) (Figure 2, B and D). This difference was statistically significant in males after 30 weeks on diet and in both males and females after 40 weeks on MeIQx diet (Figure 2 D, compare c-*myc*/*lacZ* and C57Bl/*lacZ* strains) (Student's *t*-test, $p < 0.05$).

In mice on control diet, the mutant frequency in the *lacZ* gene was 1.4-2.7-fold higher in mice that carried the c-*myc* transgene than in the control strain. The differences between the c-*myc*/□*lacZ* and C57Bl/□*lacZ* strains was statistically higher in female mice after 30 weeks on control diet and in both male and female mice after 40 weeks on control diet (Student's *t*-test, $p < 0.05$). Gender differences in *lacZ* mutant frequencies were also apparent among the animal groups. Within a treatment group, females showed statistically higher mutant frequencies than the respective males (Student's *t*-test, $p < 0.05$).

Hepatic tumor incidence

Hepatic tumor incidence was recorded after 30 and 40 weeks of feeding MeIQx or control diet (Figure 3A,B). After 30 weeks on diet, tumors were detected only in the c-*myc*/*lacZ* male mice that were fed MeIQx (Fig. 3A). All of the four c-*myc*/*lacZ* mice on MeIQx diet examined at this time point had developed several visible tumor masses that were classified histologically as hepatocellular carcinomas. Visible hepatic tumors were not detected in the other groups of male or female mice after 30 weeks. Microscopic changes, including the development of hepatic foci, nodules and adenomas, were detected in 2 of 4 female c-*myc*/*lacZ* mice fed MeIQx.

Thus at the 30 week time point, whereas neither MeIQx nor c-*myc* overexpression alone was

sufficient to induce hepatocellular carcinomas, the combination of MeIQx treatment and c-*myc* overexpression was highly effective in inducing hepatocellular carcinomas in male mice.

After 40 weeks on diet, all of the groups of mice receiving MeIQx developed visible hepatic tumors; however, strain and sex differences in tumor incidence were still observed (Fig. 3B). The highest incidence of tumors at 40 weeks was observed in the male c-*myc*/*lacZ* mice given MeIQx. At this time point, there was extensive involvement of the liver in hepatocellular carcinoma development. Visible isolated hepatic tumors, largely hepatocellular carcinomas, were also seen in 80% of female c-*myc*/*lacZ* mice on MeIQx diet. Hepatic tumors were also observed in 44% and 17% of male and female C57Bl/*lacZ* mice on MeIQx diet for 40 weeks, respectively. The histological changes in these livers, however, were benign, consisting primarily of foci, nodules and adenomas. Approximately 17% of male c-*myc*/*lacZ* mice on control diet developed benign tumors after 40 weeks. The *lacZ* mutant frequency was also analyzed in tumors from MeIQx-treated mice after 40 weeks. There were no significant differences between the mutant frequencies (mean, S.D., n) in tumors and normal tissue from C57Bl/*lacZ* males (1911, 337, 3 versus 2433, 297, 3), c-*myc*/*lacZ* males (5192, 907, 3 versus 4954, 335, 3), or c-*myc*/*lacZ* females (8358, 1505, 3 versus 7136, 659, 3). Tumors from the other groups did not provide sufficient tissue for analysis.

LacZ sequence analysis

Sequence analysis of the *lacZ* gene mutants from liver of male mice was carried out to assess the types of mutations induced in the four different groups of mice: C57Bl/*lacZ* on control or MeIQx diet and c-*myc*/*lacZ* mice on control or MeIQx diet (Table 1). Irrespective of the animal strain or MeIQx treatment, the principal sequence alteration observed in the *lacZ* gene

was a single base substitution. In all four groups, the majority of all single-base mutations were at G:C base pairs which consisted of G:C to T:A transversions, G:C to A:T transitions, and G:C to C:G transversions. In either strain, adenine mutations were observed only when mice were on the control diet.

DISCUSSION

To address the role of multiple factors in MeIQx hepatocarcinogenesis, MeIQx-DNA adduct formation, in vivo mutagenicity, and carcinogenicity were examined in mice carrying the *lacZ* mutational reporter gene and an over-expressed c-*myc* oncogene. It is generally recognized that HCA-DNA adduct formation plays a role in HCA carcinogenesis. In the current study, MeIQx-DNA adduct formation was associated with elevated mutant frequency in the liver of mice. Irrespective of mouse strain, the mutant frequency in mice on MeIQx diet was over 40-fold higher than in control mice, and accordingly MeIQx-DNA adducts were detected only in MeIQx-treated mice. Comparison between male and female mice also illustrate an association between MeIQx-DNA adduct levels and in vivo mutagenicity. In either strain of mice, MeIQx-DNA adduct levels were 2-fold higher in female mice than in male mice (Fig. 1). In accordance with higher MeIQx-DNA adduct levels, the mutant frequency in *lacZ* reporter gene was 1.4-2.6 -fold higher in female mice than in male mice (Fig. 2). The difference in adduct levels between male and female mice is likely to be associated with a higher capacity for metabolic activation in the females (24).

In a previous study, we measured MeIQx-DNA adduct levels and mutant frequency in the *lacZ* gene in liver of mice given ten oral doses of MeIQx (12). Comparison with the current data shows that the level of MeIQx-DNA adducts detected in liver after a 30-week dietary exposure to MeIQx are on average 2-fold higher than the levels found after the 10-dose regimen. Interestingly, mutant frequency in the *lacZ* gene was at least 15-20-fold higher after a 30-week exposure than after a 10-day exposure despite the mere doubling in MeIQx-DNA adduct levels. These findings suggest that the duration of exposure to MeIQx or the persistence of MeIQx-DNA

adducts is a more important determinant of in vivo mutagenesis than is the total adduct level. It appears likely that mutagenesis by MeIQx-DNA adducts occurred as an extended period of time was allowed for cellular replication and the fixation of mutations. The data also suggest that DNA damaging mutations accumulate with chronic exposure to MeIQx.

The mutation spectra found in the *lacZ* gene of MeIQx-treated mice is also consistent with the involvement of MeIQx-DNA adducts in mutagenesis *in vivo*. In accordance with the formation of guanine adducts, all of the base substitution mutations observed in MeIQx-treated mice, irrespective of strain, occurred at the guanine base. The contribution of the different MeIQx-DNA adducts, such as the *C8*- guanine adduct, N^2-guanine adduct, or the role of oxidative base damage, including 8-oxo-dG formation by MeIQx (4) in the mutation spectra of MeIQx is not yet known. In a previous study, Solomon et al. (10) showed that base substitution mutations associated with MeIQx-DNA adducts in the *lacZ* gene of *E. coli in vitro* include primarily G to T transversions followed by G to A transition mutations. Both of these types of mutations were also observed in MeIQx-treated mice, although in C57Bl/*lacZ* mice, G to A transition mutations were more common than G to T transversions. It is noteworthy, however, that the percentage of guanine base substitution mutations in mice on control diet was similar to that observed in mice on the MeIQx diet. Although the number of mutants sequenced in this study was limited, the spectra of guanine base substitution mutations in the *lacZ* gene did not appear to be overtly different between control and MeIQx-treated mice or between mice of different strains. Adenine mutations, however, were observed only in mice (of either strain) on control diet, and these mutations have been shown to occur spontaneously in the *lacZ* gene of the Muta™ mice (26).

The results from this study are consistent with the notion that the carcinogenicity of MeIQx is related to the accumulation of MeIQx-induced genetic mutations. Other studies have supported the general hypothesis that accumulation of mutations is pertinent to the development of cancer (27, 28). The high mutant frequency of the *lacZ* gene in MeIQx-treated mice was paralleled with the development of preneoplastic alterations and tumors in these mice. However, factors in addition to mutagenesis play a role in MeIQx-induced hepatocarcinogenesis. For example, in C57Bl/*lacZ* mice on MeIQx diet for 40 weeks, *lacZ* mutant frequency was higher in females than in males mice whereas tumor incidence was lower in females than males. Sex differences in susceptibility to chemically-induced hepatocarcinogenesis and sex differences in the incidence of hepatocarcinogenesis in humans (29, 30) have been noted previously. Androgen levels in the male have been shown to contribute to a higher susceptibility of males to liver cancer (30).

The use of the c-*myc*/*lacZ* bitransgenic mouse strain for studies with MeIQx indicated that c-*myc* over-expression was associated with a dramatic acceleration of MeIQx-induced hepatocarcinogenesis. In fact after 30 weeks on either MeIQx or control diet, only male c-*myc* mice fed MeIQx developed hepatocellular carcinoma. Neither c-*myc* over-expression alone, or MeIQx treatment alone induced hepatocellular carcinoma at this time point. These *in vivo* findings are consistent with a previous study showing that rodent embryo fibroblasts were more susceptible to *in vitro* transformation by chemical carcinogens when the cells expressed a high level of c-*myc* (31).

The results from the current study in bitransgenic mice further suggest that the mechanism of the synergism between c-*myc* and MeIQx in hepatocarcinogenesis involves an

enhancement of MeIQx-induced mutagenesis. In mice on MeIQx diet, mutant frequency was approximately 2-fold higher in the c-*myc* mice than in the control strain. Therefore, the relatively modest increase in mutations in the *lacZ* gene was associated with a 100% increase in the incidence of hepatocarcinogenesis. This finding raises the possibility that other types of genetic alterations, such as large deletion mutations or amplifications which are not detectable by the *lacZ* gene (23), also contribute to the enhancement of mutations and carcinogenesis. An additional explanation for this finding may be that as a mutational reporter gene, *lacZ* provides an estimate of the mutations occurring in the genome whereas mutations in specific critical genes that cooperate with c-*myc* are likely to lead to the enhancement of carcinogenesis.

Previous studies have shown that the rate of hepatic proliferation is significantly higher in the c-*myc* transgenic mice than in wild-type mice. For example, between 4 and 8 months of age mitotic activity in mouse liver is 50-100-fold higher in male c-*myc* mice than in control mice not carrying the transgene (32). This high rate of hepatic proliferation in c-*myc* mice is likely to facilitate the fixation of MeIQx-induced mutations from MeIQx-DNA adducts. In the multistep model of carcinogenesis, the higher frequency of MeIQx mutations in the genome would be expected to contribute to the accumulation of critical genetic alterations that give rise to neoplasia. A previous study showed that MeIQx-DNA adduct levels were statistically higher in c-*myc*/*lacZ* mice than in C57Bl/*lacZ* mice after 10-doses of MeIQx (12). Although the trend for higher MeIQx-DNA adduct levels was observed with chronic feeding with MeIQx, the difference was not statistically significant. The results therefore suggest that the high rate of hepatic proliferation associated with c-*myc* over-expression rather than higher adduct levels in this strain is likely to be the major factor in determining higher mutant frequency in c-*myc* mice.

Recent studies have indicated that c-*myc* over-expression is associated with genomic instability (33, 34). For example, over-expression of c-*myc* in a variety of cell lines is followed by the amplification and rearrangement of the dihydrofolate reductase gene (34). The results shown here indicate that c-*myc* over-expression is associated with an increase in the frequency of spontaneous base substitution mutations as well as the frequency of carcinogen/mutagen-induced base substitution mutations. In both male and female mice on control diet for 40 weeks, *lacZ* mutant frequency was significantly higher in mice that harbored the c-*myc* gene than in the control strain. The findings raise the possibility that in addition to amplification and genomic rearrangments, the genomic instability associated with c-*myc* over-expression also includes point mutations. To our knowledge, this is the first report showing that c-*myc* increases the frequency of base substitution mutations *in vivo*. Whereas enhanced replication rate and disruption of the G1-S stage of the cell cycle with c-*myc* over-expression may partly account for the increase in mutagenesis (34), the mechanisms by which c-*myc* over-expression enhances the frequency of spontaneous base substitution mutations still requires further study.

Studies in transgenic mouse models have indicated that c-*myc* cooperates with a variety of other genes to enhance carcinogenesis. For example, the combination of c-*myc* with an over-expressed *TGF* gene or with mutated H-*ras* in bitransgenic mouse models causes a striking acceleration of hepatocarcinogenesis (32, 35). The results from this study also indicate that the synergistic effect of MeIQx and c-*myc* is likely to involve MeIQx-induced mutations in a critical gene or series of genes that cooperate with c-*myc* to accelerate malignant conversion. Additional studies are required to elucidate the genes mutated by MeIQx that cooperate with the over-expressed c-*myc* gene to facilitate hepatocellular carcinogenesis.

References

1. Layton, D. W., Bogen, K. T., Knize, M. G., Hatch, F. T., Johnson, V. M., and Felton, J. S. Cancer risk of heterocyclic amines in cooked foods: and analysis and implications for research. Carcinogenesis, *16:* 39-52, 1995.

2. Ohgaki, H., Hasegawa, H., Suenaga, M., Sato, S., Takayama, S., and Sugimura, T. Carcinogenicity in mice of a mutagenic compound 2-amino-3,8dimethylimidazo[4,5-*f*]quinoxaline MeIQx) from cooked foods. Carcinogenesis, *8:* 665-668, 1987.

3. Davis, C. D., Schut, H. A. J., Adamson, R. H., Thorgeirsson, U. P., Thorgeirsson, S. S., and Snyderwine, E. G. Mutagenic activation of IQ, PhIP and MeIQx by hepatic microsomes from rat, monkey and man: low mutagenic activation of MeIQx in cynomolgus monkeys *in vitro* reflects low DNA adduct levels *in vivo.* Carcinogenesis, *14:* 61-65, 1993.

4. Snyderwine, E. G., Davis, C. D., Nouso, K., Roller, P. P., and Schut, H. A. J. ^{32}P-Postlabeling analysis of IQ, MeIQx and PhIP adducts formed in vitro in DNA and polynuceotides and found *in vivo* in hepatic DNA from IQ-, MeIQx and PhIP-treated monkeys. Carcinogenesis, *14:* 1389-1395, 1993.

5. Turesky, R. J., Rossi, S. C., Welti, D. H., Lay, Jr., J. O., and Kadlubar, F. F. Characterization of DNA adducts formed in vitro by reaction of N-hydroxy-2-amino-3-methylimidazo[4,5-*f*]quinoxaline at the C-8 and N^2 atomes of guanine. Chem. Res. Toxicol., *5:* 479-490, 1992.

6. Ochiai, M., Nagaoka, H., Wakabayashi, K., Tanaka, Y., Kim, S.-B., Tada, A., Hukaya, H., Sugimura, T., and Nagao, M. Identification of N^2-(deoxyguanosin-8-yl)-2-amino-3,8-dimethyl-imidazo[4,5-*f*]quinoxaline 3,5-diphosphate, a major DNA adduct, detected by nuclease P1 modification of the ^{32}P-postlabeling method, in the liver of rats fed MeIQx. Carcinogenesis, *14:* 2165-2170, 1993.

7. Sugimura, T. Overview of carcinogenic heterocyclic amines. Mutat. Res., *376:* 211-219, 1997

8. Felton, J. S. and Knize, M. G. Occurrence, identification, and bacterial mutagenicity of heterocyclic amines in cooked food. Mutation Res., *259:* 205-217, 1991.

9. Watanabe, M. and Ohta, T. Analysis of mutational specificity induced by heterocyclic amines in the *lacZ* gene of *Escherichia coli.* Carcinogenesis, *14:* 1149-1153, 1993.

10. Solomon, M. S., Morgenthaler, P. L., Turesky, R. J., and Essigmann, J. M. Mutational and DNA binding specificity of the carcinogen 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline. J. Biol Chem., *271:* 18368-18374, 1996.

11. Knasmuller, S., Kienzi, H., Huber, W., Hermann, R. S. Organ-specific distribution of genotoxic effects in mice exposed to cooked food mutagens. Mutagenesis, *7:* 235-241, 1992.
12. Davis, C. D., Dacquel, E. J., Schut, H. A. J., Thorgeirsson, S. S., Snyderwine, E. G. In vivo mutagenicity and DNA adduct levels of heterocyclic amines in Muta mice and c-*myc*/lacZ double transgenic mice. Mutat. Res., *356:* 287-296, 1996.

13. Endo, H., Schut, H. A. J., and Snyderwine, E. G. Mutagenic specificity of 2-amino-3-methylimidazo[4,5-*f*]quinoline and 2-animo-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in the *supF* shuttle vector system. Cancer Res., *54:* 3745-3751, 1994.

14. Grisham, J. W. Interspecies comparison of liver carcinogenesis: implications for cancer risk assessment. Carcinogenesis, *18:* 59-81, 1997.

15. Facchini, L. M. and Penn, L. Z. The molecular role of Myc in growth and transformation: recent discoveries lead to new insights. FASEB J., *12:* 633-651, 1998.

16. Packham, G. and Cleveland, J. L. c-Myc and apoptosis. Biochim. Biophys. Acta., *1242:* 11-28, 1995.

17. Sandgren, E. P., Quaife, C. J., Pinkert, C. A., Palmiter, C. D., and Brinster, R. L. Oncogene-induced liver neoplasia in transgenic mice. Oncogene, *4:* 715-724, 1989.

18. Murakami, H., Sanderson, N. D., Nagy, P., Marino, P. A., Merlino, G., and Thorgeirsson, S. S. Transgenic mouse model for synergistic effects of nuclear oncogenes and growth factors in tumorigenesis: interaction of c-myc and transforming growth factor □ in hepatic oncogenesis. Cancer Res., *53:* 1719-1723, 1993.

19. Leone, G., DeGregori, J., Sears, R., Jakoi, L., and Nevins, J. R. Myc and Ras collaborate in inducing accumulation of active cyclin E/Cdk2 and E2F. Nature, *387:* 422-426, 1997.

20. Frith, C. H., Ward, J. M., and Turosov, V. S. *In:* Turosov, V. & Mohr, U. (eds.), Pathology of tumors in Laboratory Animals, pp223-270. Lyon, France: IARC Scientific, 1994.

21. Gupta, R. C., Earley, K., and Sharma, S. Use of human peripheral blood lymphocytes to measure DNA binding capacity of chemical carcinogens. Proc. Natl. Acad. Sci. USA. *85:* 3513-3517, 1988.

22. Randerath, E., Agarwal, H. P., Weaver, J. A., Bordelon, C. B., and Randerath, K. ^{32}P-postlabeling analysis of DNA adducts persisting for up to 42 weeks in skin, epidermis and dermis of mice treated topically with 7,12-dimethylbenz[□]anthracene. Carcinogenesis, *6:* 1117-1126, 1985.

23. Vijg, J. and Douglas, G. R. Bacteriophage lambda and plasmid *lacZ* transgenic mice for studying mutations *in vivo*. *In:* Pfeiffer, G. P. (eds.), Technologies for detection of DNA damage and mutations, New York: Plenum, 1996.

24. Dean, S. W. and Myhr, B. Measurement of gene mutations *in vivo* using Muta™ Mouse and positive selection for *LacZ* phage. Mutagenesis *9:* 183-185, 1994.

25. Shapiro, B. H., Agarwal, A. K., and Pampori, N. A. Gender differences in drug metabolism regulated by growth hormone. Int. J. Biochem. Cell Biol., *27:* 9-20, 1995.

26. Gossen, J. A., de Leeuw, W. J. F., Verwest, A., Lohman, P. H. M., and Vijg, J. High somatic mutation frequencies in a LacZ transgene integrated on the mouse X-chromosome. Mutat. Res., *250:* 423-429, 1991.

27. Hemminki, K. DNA adducts, mutations and cancer. Carcinogenesis, *14:* 2007-2012, 1993.

28. Loeb, L. A. Many mutations in cancers. Cancer Surv., *28:* 329-42, 1996.

29. Lotze, M. T., Flickinger, J. C., Carr, B. I. Hepatobiliary neoplasms. In: Devita, V. T., Jr., Hellman, S., Bosenberg, S. T., Jr., eds. Cancer: principle and practice of oncology. 4th ed. Philadelphia: Lippincott, 1993: 883-914.

30. Blanck, A., Hansson, T., Eriksson, L. C., and Gustafsson, J. A. On mechanisms of sex differences in chemical carcinogenesis: effects of implantation of ectopic pituitary grafts on the early stages of liver carcinogenesis in the rat. Carcinogenesis, *5:* 1257-1262, 1984.

31. Hsiao, W. L., Castro, M., Giezentanner, J., Fan, H., Hanecak, R. Acquisition of responsiveness to chemical carcinogens by rodent embryo fibroblasts expressing high levels of the c-myc proto-oncogene. Mol. Carcinogenesis, *5*: 140-54, 1992.

32. Santoni-Rugiu, E., Preisegger, K. H., Kiss, A., Audolfsson, T., Shiota, G., Schmidt, E.V., Thorgeirsson, S.S. Inhibition of neoplastic development in the liver by hepatocyte growth factor in a transgenic mouse model. Proc. Natl. Acad. Sci. USA 93:9577-82, 1996.

33. Chernova, O. B., Chernov, M. V., Ishizaka, Y., Agarwal, M. L., and Stark, G. R. MYC abrogates p53-mediated cell cycle arrest in *N*-(phosphonacetyl)-L-aspartate-treated cells, premitting CAD gene amplification. Mol. Cell. Biol., *18:* 536-545, 1998.

34. Mai, S., Hanley-Hyde, J., and Fluri, M. c-Myc overexpression associated DHFR gene amplification in hamster, rat, mouse and human cell lines. Oncogene, *12:* 277-288, 1996.

35. Leone, G., DeGregori, J., Sears, R., Jakoi, L., and Nevins, J. R. Myc and Ras collaborate in inducing accumulation of active cyclin E/Cdk2 and E2F. Nature, *387:* 422-426, 1997.

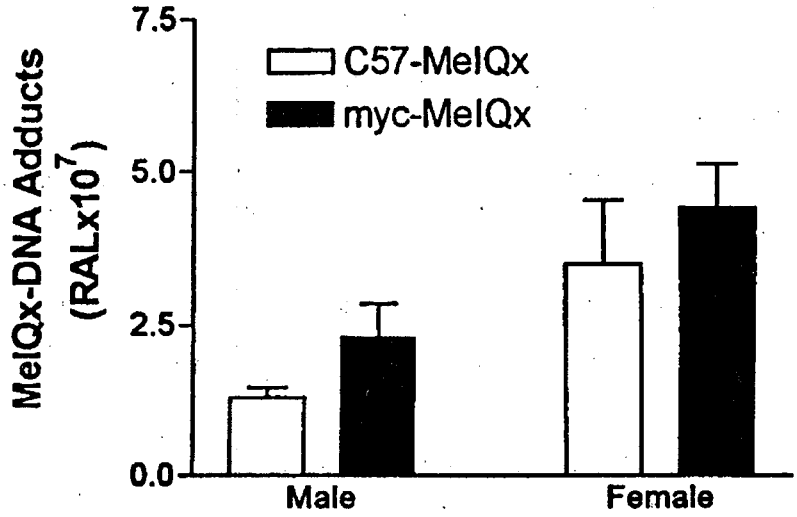

Fig. 1. MeIQx-DNA adduct levels in the liver of C57Bl/λ*lacZ* and c-*myc*/λ*lacZ* mice vho were given 0.06% (w/w) MeIQx containing diet for 30 weeks are shown. DNA dduct levels were determined by the ^{32}P-postlabeling assay, as described in "Materials nd Methods." Values are mean ± SE (n = 3). No adducts were detected in animals on ontrol diet (data not shown). *RAL*, relative adduct labeling.

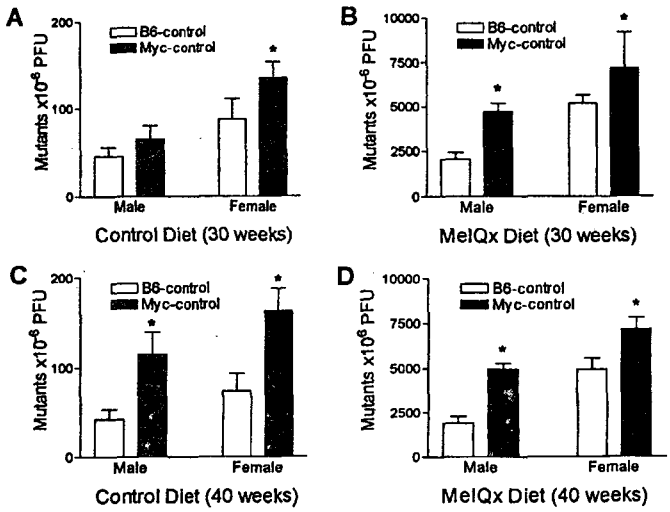

Fig. 2. *LacZ* gene mutant frequency in the liver of C57Bl/λ*lacZ* and c-*myc*/λ*lacZ* mice of both sexes who were given 0.06% MeIQx or control diet for 30 (*A* and *B*) and 40 (*C* and *D*) weeks is shown. Values are mean ± SE ($n = 3$). □, C57Bl/λ*lacZ*; ■, c-*myc*/λ*lacZ*; An *asterisk* represents that *lacZ* gene mutant frequency in C57Bl/λ*lacZ* and c-*myc*/λ*lacZ* mice is significantly different (Student's *t* test, $P < 0.05$). *PFU*, plaque-forming units.

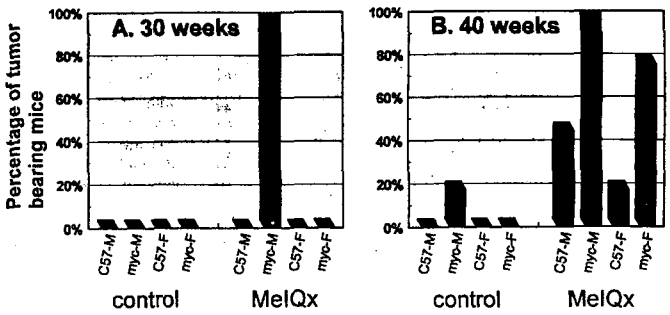

Fig. 3. Incidence of grossly visible liver tumors in C57Bl/λlacZ and c-*myc*/λlacZ mice who were given 0.06% MeIQx containing diet for 30 (*A*) and 40 (*B*) weeks is shown. Values are the percentage of tumor-bearing mice out of $n = 3–4$ and $n = 10–13$ for 30 and 40 weeks, respectively. Tumors were classified as hepatocellular carcinomas. *M*, male; *F*, female.

Table 1 *Representative types of sequence alterations found in the lacZ gene of male transgenic mice*

After 30 weeks on MeLQx or control diet, DNA was isolated from the liver of male mice. DNA was packaged and plated, as described in "Materials and Methods." At least one to two mutant plaques/plate (per mouse, $n = 3–4$) were selected at random. Plaques were expanded in liquid culture, and regions of the *lacZ* gene were amplified by PCR and sequenced, as described in "Materials and Methods." Of the base substitution mutations the frequency of CpG mutations was 6% (1 of 16), 40% (6 of 15), 28% (7 of 25), and 14% (1 of 7) in control-C57Bl/*lacZ*, MelQx-C57Bl/*lacZ*, control-c-*myc*/*lacZ*, and MelQx-c-*myc*/*lacZ* mice, respectively.

		C57Bl/*lacZ*		*myc*/*lacZ*	
		Control	MeLQx	Control	MeLQx
Base substitution					
Single		15	16	25	7
G:C (%)		93%	100%	80%	100%
	G:C to T:A	60%	38%	24%	57%
	G:C to C:G	27%	0%	8%	14%
	G:C to A:T	7%	62%	48%	29%
A:T (%)		7%	0%	20%	0%
	A:T to T:A	7%	0%	4%	0%
	A:T to C:G	0%	0%	8%	0%
	A:T to G:C	0%	0%	8%	0%
Tandem		0	0	0	0
Multiple		0	0	0	0
Insertions		0	0	2	0
Deletions					
Single bp		1	0	0	2
≥2 bp		0	0	0	0
Total number of plaques analyzed		16	16	27	9