must be generated from a minimum of two dose levels and a vehicle control. For laboratories that have demonstrated competence with these assays, concurrent positive control animals are not normally necessary but it is recommended that positive control DNA should be included with each plating step. Tissues can be stored at or below −70 °C for several years but the upper limit for storage is not known. Isolated DNA, stored refrigerated in appropriate buffer, should be used for mutation analysis within 1 year. Standard laboratory or published methods for the detection of mutants are available for the recommended transgenic models [26,27]. Modifications should be justified and properly documented. There is no biological justification for setting a minimum acceptable number of plaque or colony forming units (pfu or cfu) from an individual packaging: all data can be used and aggregated. Tissues should be processed and analyzed using a blocked design, where a sample from the negative control group, the positive control group, and each treatment group are processed together. Reporting a regulatory study should be as defined for all Good Laboratory Practice (GLP) studies. The report should include the total number of pfus or cfus and the MF for each tissue and for each animal. For statistical analysis, pairwise analysis is appropriate for one dose, and a test for a dose–response is appropriate if two or more doses were used. Nonparametric statistics, such as the generalized Cochran–Armitage test, allow for analysis of variable data such as those typically obtained with these assays. The animal should be considered as the experimental unit. Whilst the criteria for positive and negative results were also defined, it is important to consider the biological relevance of any result and that equivocal results may require some form of repetition. For a review of all the items previously agreed upon, see reference [1].

The three critical issues for which no consensus was reached in Washington [1], and which are revisited in this document, were the duration of treatment, the sampling time, and the need for sequencing. Furthermore, little guidance was provided as to which tissues should be analyzed and under what circumstances. A large amount of data has been generated over the past 10 years, with a variety of transgenic models being used for a range of purposes. The aim of this document is to provide recommendations for a generalized protocol that can contribute to risk assessment, for example to complement other in vivo genotoxicity assays. This does not mean that other protocols may not be more appropriate for other specific purposes, if they are scientifically justified.

An important consideration in the definition of a standard protocol is the reason the test is being performed. The purpose of this document is to provide guidelines for conduct of transgenic mutation assays for the purpose of regulatory assessment of safety. The assumption is that studies will be conducted under GLP compliance expressly for submission to a regulatory agency. While these guidelines should be applicable to non-regulatory applications, there are clearly different approaches that may be applied to research or mechanistic investigations, and these guidelines are not intended to stifle or limit the appropriate design of research experiments. A database developed to facilitate the working party discussions [20] indicates that transgenic mutation assays have been used for a wide range of applications, from the mechanistic evaluation of pharmaceuticals to the safety assessment of environmental pollutants. It is clear that there will always be a need for flexibility in a standard protocol so that specific scientific problems may be addressed. The published literature also illustrates the great variety of agents and the large number of different organs that can be investigated. A standard protocol should not only be applicable to those tissues that are examined routinely (such as bone marrow and liver), but also be useful for a wide range of other tissues and applications, including site-of-contact and more exotic tissues in very specific circumstances. The purpose of this document is to describe a general protocol suitable for detecting the effects of most genotoxic compounds in most tissues. It avoids discussing modifications that might allow the detection of non-genotoxic carcinogens.

2. Duration of treatment

To date there are only a limited number of studies in which the influence of multiple treatments has been examined carefully. Recent studies [28] have clearly shown that multiple treatments produce a larger response than single treatments and have generally been in accord with the prediction of Heddle et al. [29] that the results for a neutral gene would be additive.

This has been true for continuous treatments lasting 90 days [30] and seems likely to be true for even longer treatments. It would appear that the longer one treats, the more mutants are induced and the more sensitive the test for mutation becomes, in practice as in theory. However, longer exposures may increase the risk of false-positive results due to non-genotoxic mechanisms caused by chronic toxicity (e.g. tumor induction, inflammatory response, etc.). Since there seems little point in having a "short-term" assay that requires a lifetime exposure, some limit on the duration of treatment must be made whilst still providing sufficient sensitivity. It is also true that there are many mutagens that have been detected by single or five-daily treatments [20]. At this time, there are no adequate data to determine what proportion of weak mutagens would be missed if the duration of exposure was limited.

Based on observations that mutations accumulate with each treatment, a repeat-dose regimen is strongly encouraged, with daily treatments for a period of 4 weeks considered generally adequate both for producing a sufficient accumulation of mutations by weak mutagens and for providing a sampling time adequate for detecting mutations in slowly proliferating organs such as liver. This is based upon a current database of 155 agents evaluated in these assays, assembled from both published and unpublished studies [20]. Although daily treatments are generally recommended, some compounds induce strong positive responses in multiple tissues following single doses or once-weekly dose regimens [31,32]. Alternative treatment regimens such as weekly dose administration may be appropriate for some evaluations (e.g. when evaluating therapeutics that will be administered weekly) and these alternative dosing schedules should be justified in the protocol. We note, however, that treatments should not be shorter than the time required for the complete induction of all of the relevant metabolizing enzymes, and shorter treatments may necessitate the use of multiple sampling times that are suitable for organs with different proliferation rates. Further considerations include tissue exposure, kinetic data and toxicity. Some compounds (e.g. tamoxifen [33]) may require a few weeks of treatment before DNA adducts begin to form. In addition, treatment times of 12 weeks or longer can produce an apparent increase in MF through clonal expansion, or genomic instability in developing pre-neoplastic foci or tumours; therefore, treatment times longer than 8 weeks should be employed with caution [34].

For more complex or investigative applications, there can be no set treatment time that is suitable for all such studies. Consistent with current regulatory guidelines for general toxicity studies, the treatment period could be selected to meet or exceed the maximum anticipated exposure in humans, within the limits defined. For example, if the proposed Phase I/II clinical regimen of a drug is for daily oral administration for up to 3 weeks, a 28-day study in transgenic rodents might be appropriate. If the test agent is intended to be given once weekly by i.v. administration for 4 weeks, a similar schedule of i.v. dosing could be proposed for rodents. While single-dose treatments for transgenic mutagenesis studies are not encouraged, there may be situations where a single administration in humans would be best assessed using a single-dose protocol in transgenic rodents. The specific exposure and manifestation periods should be clearly justified in the protocol and should be based on the known action of the compound, the anticipated human exposure (if appropriate), and other considerations. The rationale for exposure times of less than 4 weeks and more than 8 weeks, should be clearly explained and justified.

3. Sampling time

The time between the last treatment and the time of sampling, which has been variously referred to as manifestation time, fixation time, expression time and sampling time, is a critical variable [35]. It has been demonstrated with model compounds that the induced MF in the liver can increase at least 10-fold over the month following an acute exposure, both in mice [36] and in rats [37]. Eventually, the MF seems to reach a maximum, as predicted for a neutral mutational target, and to remain relatively constant thereafter [38]. There are, however, examples in the literature of a decrease in MF from the maximum at longer sampling times; therefore, sampling longer than 3–28 days (depending upon tissue turnover) after the recommended treatment regimen should be approached with caution (Figs. 1 and 2 [39–42]). The time required to reach the maximum MF is tissue-specific, and seems to be related to the turnover time of the cell population, with bone marrow and intestine being rapid responders and

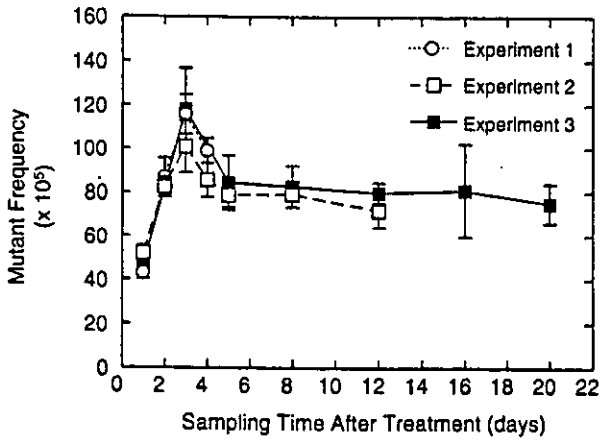

Fig. 1. *lacZ* mutation response in female Muta™mouse bone marrow following a single i.p. dose of 80 mg/kg ENU. Animals received pregnant mare serum gonadotrophin (5 IU per animal), 48 h before sacrifice [40].

the liver being much slower [36,42–44]. It is inferred, but not demonstrated, that there will be little difference in the manifestation time for different mutagens, rates of metabolism aside [45]. Accordingly, following 28 consecutive daily treatments, it should be possible to sample all tissues at a single time point appropriate for both rapidly and slowly proliferating tissues. Sampling 3 days after the final treatment will ensure that mutations in rapidly proliferating tissues induced soon after the final treatments are not lost. Furthermore, the data indicate that the 28-day treatment regimen should allow sufficient accumulation of mutants to detect an increase in slowly proliferating tissues, even 3 days after

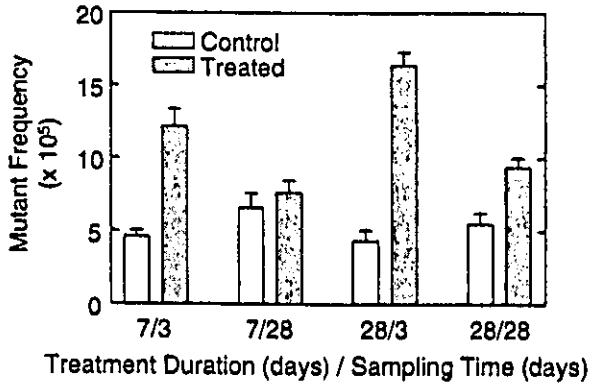

Fig. 2. *lacZ* mutation response in male Muta™mouse bone marrow after exposure to acrylamide. Transgenic mice were exposed to 25 mg/kg, i.p. for durations of 7 or 28 days, and sacrificed at sampling times of 3 or 28 days [41].

the final treatment, although the maximum mutation frequency may not manifest itself under these conditions. Therefore, in tissues for which the sampling time has not been determined, sampling 3 days following 28 consecutive daily treatments should be used [46]. If slowly proliferating tissues, such as the liver, are of particular importance, then a sampling time greater than 3 days following 28 consecutive daily treatments may be more appropriate, e.g. 28 days after the final treatment. In the case of germ cells where the kinetics are well defined the sampling time should be selected according to the stage of interest [47]. Potential effects on cell proliferation (induction or inhibition) should be taken into consideration in defining or modifying the sampling time [48,49]. It should be noted that there are limited data in the literature describing the optimal sampling time following 28 consecutive daily treatments, and the working group may wish to re-evaluate this recommendation should further relevant data become available in the future. There are several tissues for which little or no data exist, and it will be important to evaluate the effectiveness of the 28 + 3 protocol more rigorously through experiments.

4. Selection of gender and species

Since this was not adequately defined previously, the working group confirms that male animals should normally be used, consistent with guideline recommendations for other in vivo genotoxicity tests [50–53]. However, if there are significant differences between the sexes in terms of toxicity or metabolism, then both males and females will be required (e.g. five per sex per group). There may be cases where females alone would be justified, for example where testing human gender-specific drugs, or in case of sex-specific metabolism. These recommendations are applicable to the rat as well as to the mouse, although little mutation data exists for the rat. The choice of rat or mouse will depend upon other toxicological information and the availability of an appropriate rat model.

5. Rationale for tissue selection

The selection of tissues to be sampled will depend upon several criteria and it is almost impossible to

define a list suitable for all applications. The choice must be made on a case-by-case basis, following certain rules. The choice of tissues to be analyzed will be based upon the reason for conducting the study and any existing mutagenicity, carcinogenicity or toxicity data for the compound under investigation. Other important factors include the route of administration and the likely tissue distribution and subsequent exposure (e.g. lung following inhalation, skin following topical application [15]), as well as the possible mechanism of action or even intended use of or likely human exposure to the compound. In the absence of any background information, at least one rapidly dividing (e.g. bone marrow) and one slowly dividing tissue (e.g. liver) should be evaluated. The rationale for tissue selection should be made clear.

6. Criteria for sequencing of mutants

When testing drugs or chemicals for regulatory applications, the sequencing of mutants is not normally required, particularly where a clear positive or negative result is obtained. However, a number of situations were identified where sequencing of mutants may provide additional information to the results of a study.

For equivocal results, sequencing may offer the opportunity to identify outliers or to assign a weak increase in MF to a specific mutation type. There was a consensus that if this rare mutation does not result in a significant elevation in the overall MF, then it is insufficient to conclude a positive response.

Sequencing data may be useful when high inter-individual variation is observed. In these cases, sequencing can be used to rule out the possibility of "jackpots" or clonal events by identifying the proportion of unique mutants from a particular tissue. There are other alternative methods for eliminating outliers using formal statistical procedures such as the Grubbs–Smirnov method [54]. While removing an obvious outlier from a sample provides a simple means for reducing animal-to-animal variation, sequencing and subsequent appropriate correction of MF for clonality allows the "rescue" of a sample value, so that the respective sample MF is not lost. This can be particularly important when the number of animals or tissues is small, and removal of data-points from the sample results in an inadequate sample size. The group felt that sequencing up to 10 mutants per tissue is sufficient for simply identifying clonal mutants, but that sequencing as many as 25 mutants may be necessary for correcting MF mathematically for clonality.

Another alternative to sequencing when identifying outliers is to analyse an additional equivalent tissue sample, or another part of the same tissue (e.g. the second lung or kidney of the same animal, another region of the same liver) that can reveal the existence of clonality and allow for a MF correction [55]. In other test systems, a repetition of the experiment is an option; however, for in vivo experiments, ethical considerations related to animal use, not to mention factors of cost, make this less practical. While true jackpots or clonal events that can be regarded as experimental artifacts have been reported [35,56–58] they are in fact not very common in the literature and the application of sequencing or other strategies for their correction is expected to be rarely necessary.

It also should be remembered that outliers can represent true biological variation, indicating a "responder versus non-responder" phenotype. In such cases, sequencing of mutants could be useful in identifying and obtaining otherwise inaccessible mechanistic insights.

Finally, sequencing may also be useful for providing mechanistic information about the biological mechanisms underlying mutation induction by specific mutagens. In general, this is achieved by comparing mutation spectra of treated and negative control animals, after sequencing of a representative number of mutants. However, the results of this approach may not be always easy to use, and a proper evaluation of mutation spectra may require a significant effort and the sequencing of a large number of mutants, depending upon the molecular mechanism of the mutagen. Thus, the spontaneous mutation spectrum itself is subject to a considerable variability, and it may be difficult to determine a significant change in the mutation spectrum, particularly for mutagens that induce a mutation spectrum that is similar to the background.

When sequencing is to be included as part of the study protocol, special care should be taken in the design of the sequencing component, in particular with respect to the number of mutants sequenced per sample. While recommendations exist to collect and sequence at least 50 mutants per dose group [59], these suggestions are based upon data obtained with potent

mutagens such as PhIP, and may not be applicable to many unknown mutagens. The group was unable to provide a general recommendation for the number of mutations necessary to produce an adequate spectrum but agreed that they may revisit this issue in the future. It therefore might be useful to preserve as many mutant clones as is practical to allow for a later analysis of mutations, should this turn out to be necessary.

For the analysis of mutation spectra, various methods have been described [60–62]. These methods differ in their approach to analysis, such as a dose–response trend analysis versus a pairwise comparison of mutation types, so that it is left to the discretion of the experimenter to choose the method appropriate for their purpose. However, it is advised to use a more popular method such as the Adams–Skopek algorithm [60], which allows an easier comparison of new with published data, unless specific questions recommend otherwise.

7. Conclusion

Using a comprehensive database containing published and unpublished observations, the working group has agreed upon a general approach for the design of mutation studies using transgenic rodents. The proposed treatment/sampling regimen of 28 + 3 days will form the basis of future investigations and its effectiveness will be re-evaluated as the database grows. Sequencing will continue to be a supplementary investigation that may provide useful information in some circumstances.

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Regional mutagenicity of heterocyclic amines in the intestine: mutation analysis of the *cII* gene in lambda/*lacZ* transgenic mice

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Abstract

Transgenic mouse assays have revealed that the mouse intestine, despite its resistance to carcinogenesis, is sensitive to the mutagenicity of some heterocyclic amines (HCAs). Little is known, however, about the level and localization of that sensitivity. We assessed the mutagenicity of four orally administered (20 mg/kg per day for 5 days) HCAs—2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) hydrochloride, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) acetate—in the intestine of male Muta^{TM}Mice. Two weeks after the last administration, we isolated epithelium from the small intestine, cecum, and colon and analyzed *lacZ* and *cII* transgene mutations. PhIP increased the *lacZ* mutant frequency (MF) in all the samples, and in the small intestine, *cII* and *lacZ* MFs were comparable. In the *cII* gene, G:C to T:A and G:C to C:G transversions were characteristic PhIP-induced mutations (which has also been reported for the rat colon, where PhIP is carcinogenic). In the small intestine, PhIP increased the *cII* MF to four-fold that of the control, but IQ, MeIQ, and Trp-P-2 did not have a significant mutagenic effect. In the cecum, *cII* MFs induced by IQ and MeIQ were 1.9 and 2.7 times those in the control, respectively. The MF induced by MeIQ in the colon was 3.1 times the control value. Mutagenic potency was in the order PhIP > MeIQ > IQ; Trp-P-2 did not significantly increase the MF in any tissue. The cecum was the most susceptible organ to HCA mutagenicity.

Subj. Class.: 105650-23-5 (PhIP hydrochloride); 76180-96-6 (IQ); 77094-11-2 (MeIQ); 72254-58-1 (Trp-P-2 acetate)

Keywords: Heterocyclic amine; *lacZ*; Lambda *cII*; Mutant frequency; Intestine

1. Introduction

Spontaneous cancers are common in the human intestine, where there is frequent exposure to dietary mutagens and carcinogens such as the heterocyclic amines (HCAs) found in well-cooked meat. In experimental animals, however, the organ is resistant to the carcinogenic effects of the majority of genotoxic compounds [1]. Although five HCAs—2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-

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3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)—induce intestinal tumors in rats, they have rarely been shown to do so in mice [2,3].

The weak carcinogenicity of HCAs in the mouse intestine is not due to a lack of local mutagenic activity, as has been shown by somatic mutation assays based on endogenous markers and transgenic shuttle vectors [4,5]. Transgenic mouse assays reveal that HCAs increase mutant frequencies (MFs) in the intestines at least as much as they do in the target organs for carcinogenesis. Although PhIP is mutagenic in several organs, it is most potent in the small and large intestine of *lacZ* transgenic Muta™Mice [6] and the colon of *gptΔ* transgenic mice [7]. In female Big Blue® transgenic mice, MeIQ induces *lacI* mutations at a much higher rate in the colon than in the liver and forestomach, which are target organs for carcinogenesis [8]. MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline), a hepatocarcinogen, induces *lacI* mutations in the mouse colon as well [9].

We know that the mouse intestine is sensitive to HCAs, but we know little about the level of sensitivity in specific areas. PhIP is mutagenic in both small and large intestine [4–6], but the mutagenic potencies of other HCAs have been reported for only a part of the intestine or not at all.

We used the male Muta™Mouse intestine to study the genotoxic potential of four HCAs—PhIP hydrochloride, IQ, MeIQ, and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) acetate. After the animals were treated, we isolated epithelium from the small intestine, cecum, and colon and extracted the DNA, which we subjected to an in vitro packaging reaction. We assayed lambda shuttle vectors, recovered as viable phages, for *lacZ* and lambda *cII* mutations. We analyzed the frequency and mutational specificity of *cII* mutants induced by PhIP, and showed that PhIP induced the same mutations in the small intestine of mice and the large intestine of rats, despite differences in their susceptibility to carcinogenicity. We compared *cII* MFs induced by HCAs and showed that the epithelium in each intestinal region was distinct in its susceptibility to HCA mutagenesis. The transgenic mouse intestine served as a useful model for the study of genotoxic potency of intestinal carcinogens.

2. Materials and methods

2.1. Chemicals

PhIP hydrochloride, IQ, MeIQ, and Trp-P-2 acetate were purchased from Wako Pure Chemicals (Tokyo). Just before administration, PhIP hydrochloride and Trp-P-2 acetate were dissolved in physiological saline while IQ and MeIQ were dissolved or suspended in corn oil, at a concentration of 2 mg/ml. Phenyl-β-D-galactopyranoside (P-gal) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were purchased from Sigma Chemical (St. Louis, MO).

2.2. Animals and treatment

Male *lacZ* transgenic mice (Muta™Mouse) with a CDF1 (BALB/c × DBA/2 F1) genetic background were obtained at 4–7 weeks of age from Covance Research Products (Denver, PA). The Muta™Mouse test system carrying integrated lambda gt10 shuttle vectors containing the bacterial *lacZ* gene has been described in detail elsewhere [10]. The mice were housed in plastic cages with hard wood chips for bedding and were fed a standard diet (Oriental MF, Oriental Yeast, Tokyo) and tap water ad libitum. A week before administration of the test compounds, the diet was switched to AIN-76 purified diet (Crea Japan, Tokyo). Administration of the compounds started when the mice were 10 weeks old. The mice in the HCA treatment groups ($n = 5$–7) were fed a HCA daily in a volume of 10 ml/kg body weight for 5 days. The control mice received the same volume of corn oil daily for 5 days. We used the carcinogenic potency database to set the dose of HCA at 20 mg/kg per day [11], which approximates to the TD_{50} values for PhIP, IQ, MeIQ, and Trp-P-2: 28.6, 19.6, 12.3, and 12.6 mg/kg per day, respectively [3].

2.3. Tissue and DNA isolation

Two weeks after the last administration of HCA, the mice were killed by post-caval incision under anesthesia and the intestines were removed. The lumen was flushed with phosphate-buffered saline (PBS), and the intestine was divided into small intestine, cecum, and colon. Each section was cut along the mesenteric line, washed with PBS, quickly frozen

with liquid nitrogen, and stored at −80 °C. Before use, each frozen segment was placed in a plastic dish containing 30 mM EDTA-PBS solution at <4 °C and left to defrost for 30 min. After removing the luminal contents and mesenteric nodes, we placed the segments lumen-side-up in a plastic dish and, with the aid of a microscope, stroked them gently with the back of mosquito forceps to isolate crypt-villus units (sheets) from the mucosal layer. We collected the units in a tube and immediately incubated them with proteinase K solution at 50 °C for 3 h. We extracted DNA as described previously [12]. Without bias (distal or proximal), we cut the colon segment into pieces and randomly partitioned them into two portions. We used one portion (consisting of about two thirds of the total amount of cut pieces) for DNA isolation from the mucosal epithelium and the other portion for DNA isolation from the whole colon.

2.4. *lacZ* mutation analysis

A 5–10 μl aliquot of DNA solution was removed and used for lambda prophage rescue with a MutaPlax™ packaging extract (Epicentre Technologies, Madison, WI). Screening for *lacZ* mutant phages was carried out by P-gal-based positive selection with *Escherichia coli* strain C (*lac*$^-$*galE*$^-$), and all mutants were confirmed on X-gal plates as previously described [13].

2.5. Lambda *cII* mutation analysis

We used a MutaPlax *cII*-Select™Kit (Epicentre Technologies) for the lambda *cII* assay. The kit contained lambda packaging extracts and cultures of *hfl*$^+$ *E. coli* strain G1217 for the determination of recovered phage titers and *hfl*$^-$*E. coli* G1225 for the selection of mutant phages. Lambda packaging and positive selection for *cII* mutants was performed according to the manual in the packaging insert, essentially as described by Jakubczak et al. [14] with slight modification. Briefly, 500 μl of the packaged phage solution was incubated with the same volume of strain G1225 cells (OD_{660} = 1.0) at room temperature for 30 min, mixed with 11 ml LB top agar, and plated on four 9 cm dishes containing 6 ml bottom agar. The plates were incubated at 24.5 ± 0.5 °C for 48 h. For titration, a 100 μl aliquot of a 1:100 dilution of the packaged phage was mixed with the same volume of strain G1225 cells and 6 ml LB top agar, plated on two dishes, and incubated at 37 °C for 24 h. Wild-type phage recovered from Muta™Mouse has a *cI*$^-$ phenotype, which permitted plaque formation on the *hfl*$^-$ strain at 37 °C but not at 25 °C. We determined the MF by dividing the number of mutant plaques by the total number of recovered phage titers evaluated from each animal. Generally, we analyzed >2 × 10^5 plaque forming units in each case.

2.6. Sequencing of *cII* mutants

We determined mutations in the lambda *cII* transgene using the DNA cycle sequencing method described elsewhere [15]. The *cII* gene region (294 bp) was PCR-amplified directly from mutant plaques using the primer pair 5′-AAAAAGGGCATCAAATTAAACC-3′ and 5′-CCGAAGTTGAGTATTTTTGCTGT-3′. A 446 bp PCR product was purified and used for the sequencing reaction with a BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Tokyo). PCR amplification and DNA sequencing was performed using a Program Temp Control System PC-800 (Astec, Tokyo) and an ABI Prism™ 310 Genetic Analyzer (Applied Biosystems), respectively.

2.7. Statistical analysis

We expressed MF as mean ± S.D. We compared between-group means by the unpaired *t*-test and variance among groups by one-way analysis of variance (ANOVA). We used the Dunnet test for multiple comparisons. Identical mutations from the same mouse were counted as a single event.

3. Results

3.1. Mutagenicity of PhIP

Comparison of *lacZ* MFs in the whole colon and colon epithelium of the same animals exposed to PhIP or corn oil (control) is shown in Fig. 1. The spontaneous MF in the colon epithelium was about twice that in the whole colon. The MF in the PhIP treatment group was significantly higher than in the control group in both the whole colon ($P < 0.05$) and the

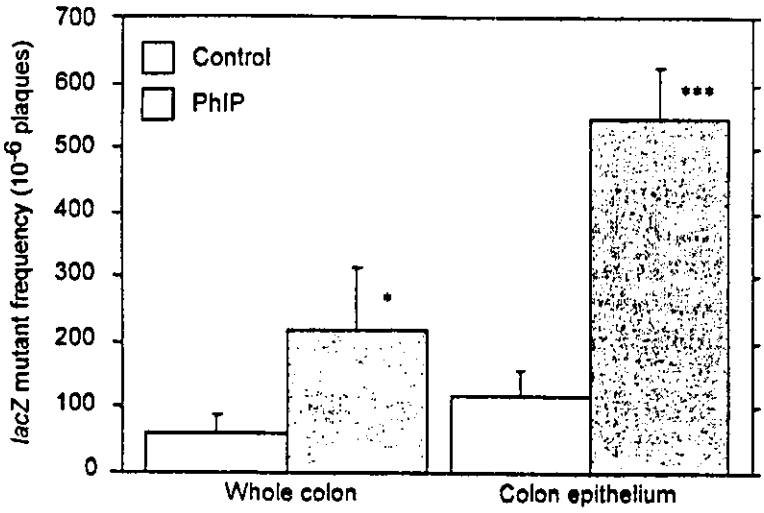

Fig. 1. *lacZ* mutant frequencies induced by PhIP in the whole colon and colon epithelium of male Muta^{TM}Mouse. The animals were orally fed 20 mg/kg PhIP or corn oil (control) daily for 5 days and sacrificed 2 weeks after the last administration. The MFs in the whole colon and colon epithelium of the same animals ($n = 5$) were analyzed. Asterisks indicate significantly different levels of induced MF in the control and PhIP treatment groups as determined by the unpaired *t*-test (* $P < 0.05$, *** $P < 0.0005$). Values are mean ± S.D.

colon epithelium ($P < 0.0005$). Because the degree of increase in mean MF in treated animals was greater in the colon epithelium than in the whole colon (4.7 versus 3.5 times the control value, respectively), we used epithelium for the remainder of the experiments. The MFs in the PhIP treatment group were significantly higher than those in the control group in the epithelium of the small intestine, cecum, and colon (Fig. 2). The induced MFs did not differ significantly among the various sections of the organ (one-way ANOVA).

The effect of PhIP treatment on the MF of the *lacZ* and *cII* genes in the small intestine are shown in Table 1. The MF was lower for the *cII* gene than the *lacZ* gene in both control and PhIP-treated mice. The

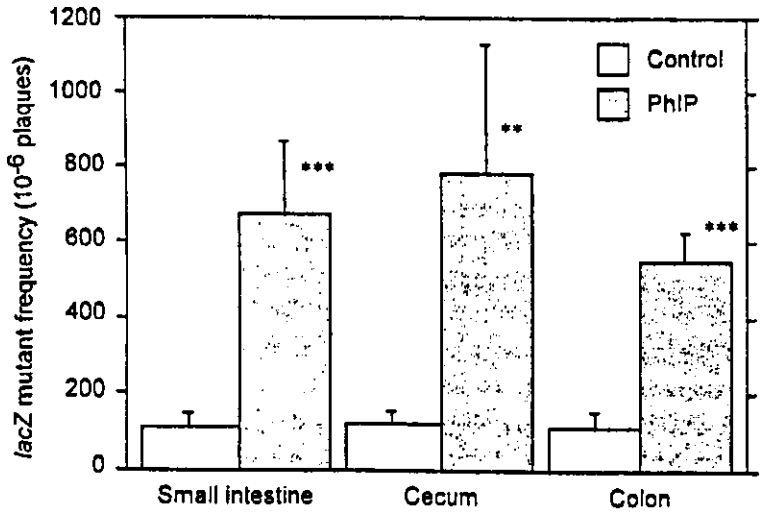

Fig. 2. *lacZ* mutant frequencies induced by PhIP in the intestinal epithelia of the small intestine, cecum, and colon. The animals are treated as described in the legend to Fig. 1. The MFs in the small intestines and ceca of seven animals were determined. Asterisks indicate significantly different levels of MF in the control and PhIP treatment groups as determined by the unpaired *t*-test (** $P < 0.005$, *** $P < 0.0005$). Values are mean ± S.D.

Table 1
Spontaneous and PhIP-induced mutant frequencies of *lacZ* and *cII* loci in small intestine epithelium

Locus	Compound[a]	n[b]	Number of plaques		Mutant frequency[c]
			Total	Mutant	Mean ± S.D.
lacZ	Control	7	1909100	190	112.3 ± 30.6
	PhIP	7	1006992	741	672.0 ± 198.0[d]
cII	Control	6	1602500	54	49.6 ± 41.7
	PhIP	5	736000	165	199.4 ± 87.8[e]

[a] Animals were treated orally with 20 mg/kg per day PhIP for 5 days and killed 2 weeks after the last treatment.
[b] Number of analyzed animals.
[c] Mutant frequencies ($\times 10^{-6}$ plaques) were analyzed in DNA extracted from the small intestine epithelium.
[d] Significantly different from the control value at $P < 0.0005$.
[e] Significantly different from the control value at $P < 0.005$.

PhIP-induced increase in MF was six times the control value in the *lacZ* gene and four times the control value in the *cII* gene.

3.2. *cII* mutant sequences

We subjected 33 PhIP-induced mutants and 31 spontaneous mutants to sequence analysis. The mutations are characterized in Table 2 and summarized in Table 3. Spontaneous mutations from the control mice consisted mainly of base substitutions. G:C → A:T transitions were predominant, with 72% (13/18) occurring at 5′-CpG-3′ dinucleotide sequences. Other base substitutions (21%) and single base pair deletions (16%) were also observed. PhIP-induced mutations also consisted mainly of base substitutions (32/33), but the majority of those (72%) were G:C → T:A transversions compared with 8% of the spontaneous base substitutions. No obvious hot spots were seen, and mutations were distributed over the whole coding region.

3.3. Mutagenicity of IQ, MeIQ, and Trp-P-2

One-way ANOVA revealed a significant variance in the cecum and colon but not in the small intestine (Fig. 3). In the cecum (B), significant differences from the control were found in the IQ ($P < 0.0005$) and MeIQ groups (Dunnet test, $P < 0.0005$). The MFs in the IQ, MeIQ, and Trp-P-2 groups were 1.9, 2.7 and 1.4 times those of the control, respectively. MeIQ also significantly increased the MF in the colon (C) ($P < 0.0005$, Dunnet test). The MFs in the IQ and MeIQ groups were 1.5 and 3.1 times those of the control, respectively. We observed no significant increase in MF in any sample from the Trp-P-2 treatment group. We observed no significant variance in spontaneous MFs of the control groups in the colon, cecum, or small intestine (one-way ANOVA).

4. Discussion

In the transgenic mouse assays, isolation of proliferative tissues is important for sensitive detection of gene mutation, since DNA lesion (adduct formation, etc.) is fixed as a mutation during DNA repair and replication. In the present study, the isolated colon epithelium was more sensitive to PhIP than the whole colon (Fig. 1). The cell turnover in the epithelium is more proliferative than that in the other smooth muscle and connective tissues. The presence of resting or quiescent cells in the crude intestine could level down the apparent MFs, because the mutational target genes are rescued from not only proliferating cells, but also quiescent cells, which are in the inactive state of mutation fixation that arises as a consequence of DNA damage [16].

Almost uniform induction of *lacZ* mutation in the mouse intestine was reproduced in our experiments using the epithelia of the small intestine, cecum, and colon (Fig. 2). It is known that a long-term feeding of HCA in the diet is more effective for inducing mutations [5,9], but, as confirmed in this study, short-term exposure followed by appropriate

Table 2
PhIP-induced and spontaneous *cII* mutations from the small intestine epithelium of Muta™Mouse

Mutant no.	Position	Nucleotide change	CpG[a]	Sequence	Amino acid change
PhIP-induced mutants					
P1	3	G to T		ATG GTT CGT GCA AAC	Met to Ile
P2	25	G to T		CGC AAC GAG GCT CTA	Glu to Stop
P3	28	G to T		AAC GAG GCT CTA CGA	Ala to Ser
P4	34	C to T	*	GCT CTA CGA ATC GAG	Arg to Stop
P5	34	C to G		GCT CTA CGA ATC GAG	Arg to Gly
P6	35	G to C		GCT CTA CGA ATC GAG	Arg to Pro
P7	40	G to T		CGA ATC GAG AGT GCG	Glu to Stop
P8	40	G to A	*	CGA ATC GAG AGT GCG	Glu to Lys
P9	64	G to C		AAA ATC GCA ATG CTT	Ala to Pro
P10	88	G to T		AAG ACA GCG GAA GCT	Ala to Ser
P11	100	G to T		GCT GTG GGC GTT GAT	Gly to Cys
P12	103	ΔG		GTG GGC GTT GAT AAG	Frameshift
P13	111	G to T		GTT GAT AAG TCG CAG	Lys to Asn
P14	115	C to G		AAG TCG CAG ATC AGC	Gln to Glu
P15	115	C to T		AAG TCG CAG ATC AGC	Gln to Stop
P16	123	C to A		CAG ATC AGC AGG TGG	Ser to Arg
P17	125	G to T		ATC AGC AGG TGG AAG	Arg to Met
P18	134	G to T		TGG AAG AGG GAC TGG	Arg to Met
P19	145	C to A		TGG ATT CCA AAG TTC	Pro to Thr
P20	146	C to A		TGG ATT CCA AAG TTC	Pro to Gln
P21[b]	163	C to T		ATG CTG CTT GCT GTT	Lue to Phe
P22	163	C to A		ATC CTG CTT GCT GTT	Lue to Ile
P23	166	G to T		CTG CTT GCT GTT CTT	Ala to Ser
P24	167	C to A		CTG CTT GCT GTT CTT	Ala to Asp
P25	175	G to T		GTT CTT GAA TGG GGG	Glu to Stop
P26	179	G to T		CTT GAA TGG GGG GTC	Trp to Lue
P27	179	G to T		CTT GAA TGG GGG GTC	Trp to Lue
P28	193	G to A	*	GTT GAC GAC GAC ATG	Asp to Asn
P29	196	G to T		GAC GAC GAC ATG GCT	Asp to Thr
P30	210	G to T		GCT CGA TTG GCG CGA	Leu to Phe
P31	212	C to A		CGA TTG GCG CGA CAA	Ala to Glu
P32	220	G to T		CGA CAA GTT GCT GCG	Val to Phe
P33	220	G to T		CGA CAA GTT GCT GCG	Val to Phe
Spontaneous mutants					
C1	25	G to A	*	GCC AAC GAG GCT CTA	Glu to Lys
C2	34	C to T	*	GCT CTA CGA ATC GAG	Arg to Stop
C3	34	C to T	*	GCT CTA CGA ATC GAG	Arg to Stop
C4	40	G to A	*	CGA ATC GAG AGT GCG	Glu to Lys
C5	89	C to T	*	AAG ACA GCG GAA GCT	Ala to Val
C6	89	C to T	*	AAG ACA GCG GAA GCT	Ala to Val
C7	89	C to T	*	AAG ACA GCG GAA GCT	Ala to Val
C8	94	G to T		GCG GAA GCT GTG GGC	Ala to Ser
C9	94	G to A		GCG GAA GCT GTG GGC	Ala to Thr
C10	101	G to A		GCT GTG GGC GTT GAT	Gly to Asp
C11	101	G to C		GCT GTG GGC GTT GAT	Gly to Ala
C12	113	C to T	*	GAT AAG TCG CAG ATC	Ser to Leu
C13	113	C to T	*	GAT AAG TCG CAG ATC	Ser to Leu
C14	116	A to G		AAG TCG CAG ATC AGC	Gln to Arg
C15	123	C to A		CAG ATC AGC AGG TGG	Ser to Arg
C16	125	G to A		ATC AGC AGG TGG AAG	Arg to Lys
C17	131	A to C		AGG TGG AAG AGG GAC	Lys to Thr

Table 2 (*Continued*)

Mutant no.	Position	Nucleotide change	CpG[a]	Sequence	Amino acid change
C18	150	ΔG		ATT CCA AAG TTC TCA	Frameshift
C19	163	ΔC		ATC CTG CTT GCT GTT	Frameshift
C20	170	T to G		CTT GCT GTT CTT GAA	Val to Gly
C21	179–184	ΔG		GAA TGG GGG GTC GTT	Frameshift
C22	183–184	GG to T		GAA TGG GGG TC GTT	Frameshift
C23	196	G to A	*	GAC GAC GAC ATG GCT	Asp to Asn
C24	214	C to T	*	TTG GCG CGA CAA GTT	Arg to Stop
C25	214	C to T	*	TTG GCG CGA CAA GTT	Arg to Stop
C26	214	C to T	*	TTG GCG CGA CAA GTT	Arg to Stop
C27	217	C to T		GCG CGA CAA GTT GCT	Gln to Stop
C28	217	C to T		GCG CGA CAA GTT GCT	Gln to Stop
C29	220	G to C		CGA CAA GTT GCT GCG	Val to Leu
C30	241–246	ΔA		ACC AAT AAA AAA CGC	Frameshift
C31	249–251	ΔC		AAA CGC CCG GCG GCA	Frameshift

[a] C to T transition at CpG site (*).
[b] Identical mutation from same mouse.

expression (manifestation) time is sufficient to induce gene mutation in the intestinal epithelia.

In the *cII* gene in the small intestinal epithelium of the male Muta^{TM}Mouse, PhIP was the only HCA that increased MF (Table 1 and Fig. 3). MeIQ induced *cII* mutations in the cecum and colon, but IQ was mutagenic only in the cecum. In both the cecum and colon, the MFs induced by MeIQ were higher than those induced by IQ. PhIP, in contrast, induced MFs uniformly throughout the intestinal tract. These results suggest that PhIP is the most potent mutagen for the mouse intestine, followed by MeIQ and then IQ (Trp-P-2 was not mutagenic), and the epithelium in the cecum is the most susceptible to HCAs.

Table 3
Classification of PhIP-induced and spontaneous *cII* mutations from small intestine epithelium of Muta^{TM}Mouse

Mutation type	PhIP-induced		Spontaneous	
	Number	%	Number	%
Base substitutions				
Transitions				
G:C to A:T (at CpG)	5 (3)	15 (9)	18 (13)	58 (42)
A:T to G:C	0	0	1	3
Transversions				
A:T to T:A	0	0	0	0
A:T to C:G	0	0	2	6
G:C to T:A	23	70	2	6
G:C to C:G	4	12	2	6
Single base pair deletion				
G:C	1	3	5	16
A:T	0	0	0	0
Other mutations	0	0	1	3
Total[a]	33	100	31	100

[a] Identical mutations from the same mouse were counted as single event.

The reasons for regional differences in HCA susceptibility are not clear. Differences of the distribution of DNA adducts may play a role. Metabolic activation leading to the formation of DNA adducts is critical to the genotoxicity of HCAs. These compounds are first metabolized by cytochrome P450 and are converted in the liver to *N*-hydroxy intermediates and released back into the gut [17]. The intermediates are converted into ultimate mutagens, capable of binding to DNA in the intestinal epithelium. The distribution of ^{32}P-post-labeled DNA adducts in the small intestine, cecum, and colon of male CDF1 mice given dietary PhIP are almost the same [18]. The distribution in those given IQ, on the other hand, is higher in the cecum than in the colon, and IQ-DNA adduct formation is lowest in the small intestine [19]. Such differences in the distribution of DNA adducts may help to explain the tissue-specific difference in HCA-induced mutagenesis.

The regional differences in HCA susceptibility were shown in the intestine of the male CDF1 Muta^{TM}Mouse, but mutation alone is not sufficient to explain differences in carcinogenicity. PhIP induces

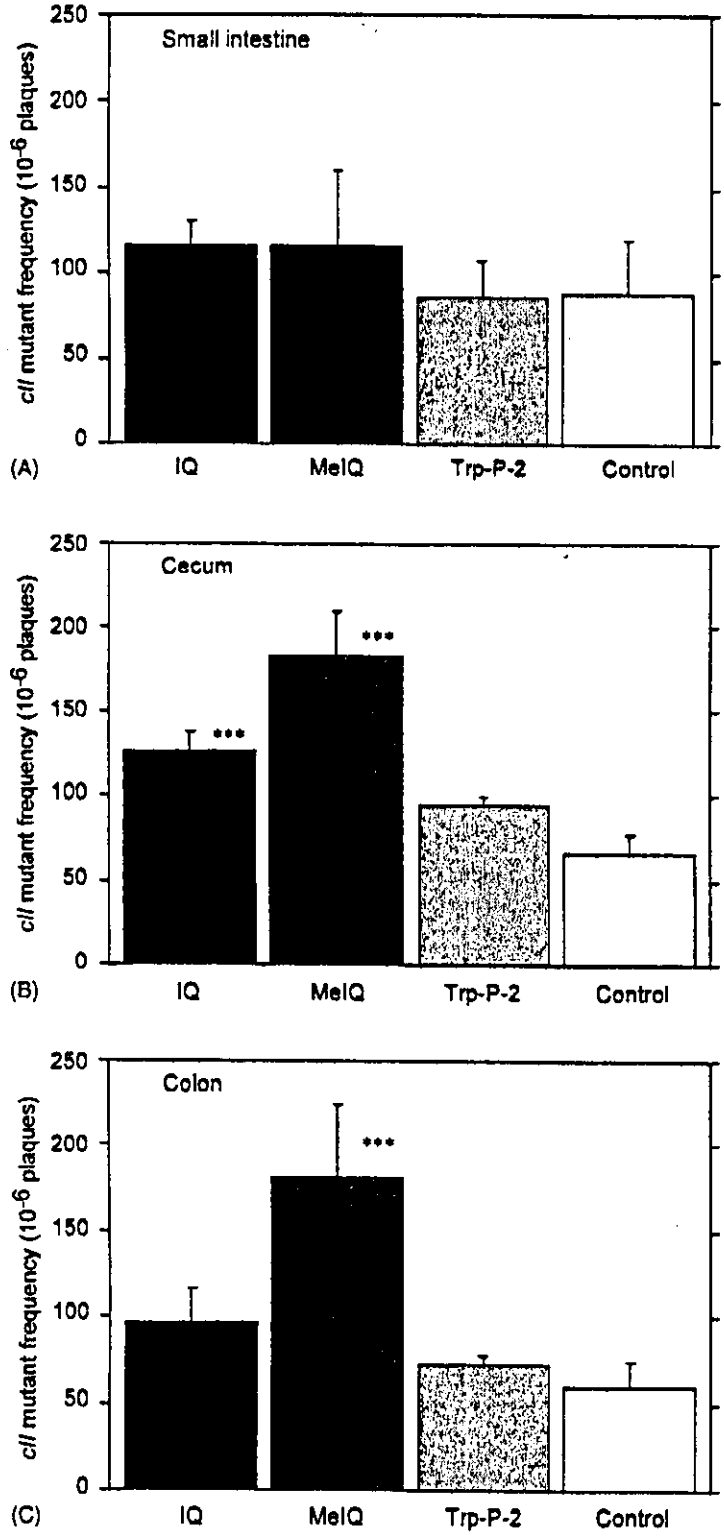

Fig. 3. *cII* mutant frequencies induced by IQ, MeIQ, and Trp-P-2 in the epithelium of small intestine (A), cecum (B) and colon (C). The animals were fed 20 mg/kg HCA or corn oil (control) daily for 5 days and sacrificed 2 weeks after the last dose. The MFs in the same animals ($n = 4$–5) in each treatment group were analyzed. Asterisks indicate significantly different levels of MF in the control and HCA treatment groups as determined by Dunnet's test ($P < 0.0005$). Values are mean ± S.D.

colon cancer in male F344 rats but not in females [20] and not in either sex of CDF1 [21] and C57BL/6 mice [22], yet there is no marked difference in the frequency and spectrum of *lacI* mutations in the colon of both sexes of C57BL/6 Big Blue® mice and F344 Big Blue® rats [23]. In the present study (Tables 2 and 3), analysis of the *cII* mutants recovered from the small intestine of PhIP-treated Muta™Mice showed that the mutations were similar to those induced by PhIP in the colons of Big Blue® rats, where G:C → T:A and G:C → C:G transversions were induced in both the *cII* [24] and *lacI* genes [25]. Thus, these mutational characteristics have been well conserved between the small intestine of mice and the large intestine of rats, although the organs differ in susceptibility to PhIP carcinogenicity.

MeIQ is an intestinal carcinogen in female C57BL/6 mice but not in male or female CDF1 mice [26,27], and it induces *lacI* mutations in the colon of female Big Blue® mice [8]. Although we used the male CDF1 Muta™Mouse in the present study, MeIQ significantly induced *cII* mutations in the cecum and colon. IQ induces tumors in the large and small intestines of F344 rats but not of CDF1 mice [28], and it induces *lacI* mutations in the colon of Big Blue® rats [29]. We believe that the present study is the first to show IQ mutagenicity in the mouse intestine. The difference in the intestinal carcinogenicity of HCAs was not attributable to differences in mutational specificity in the intestinal epithelia but might be due to differences in a post-initiation stage in carcinogenesis.

Since two non-transcribed transgenes such as *lacZ* and lambda *cII* are not expected to differ in rates of DNA damage or repair, it is tempting to attribute the differences in mutagenic responses observed in this study to differences in either target size or sequence context. The spontaneous MF of the *cII* gene was about a half that of *lacZ* gene even though the *cII* was much smaller than *lacZ* (294 bp versus 3096 bp), suggesting that *cII* is more mutable than *lacZ*. In response to PhIP treatment, however, *cII* and *lacZ* were nearly identical. Observations made with other mutagenic chemicals also suggest that the response of the *cII* gene is similar to that of the *lacZ* gene [30–32].

In both *lacI* of Big Blue® mice and *lacZ* of Muta™Mice, PhIP preferentially induces single G:C base pair deletions in the sequence 5′-GGGA-3′ [24,25,33]. The *cII* gene, however, contains only one 5′-GGGA-3′ sequence, and we found no PhIP mutational hot spot. Although all 33 PhIP-induced mutants in this study had a G:C base pair mutation, we found no increase in the frequency of PhIP-induced G:C base pair deletions. This has been also demonstrated in the *cII* and *lacI* genes in the Big Blue® rat colon [24]. Compared with the spontaneous mutational spectra from the colons of untreated rats, the proportion of −1 frameshifts (almost exclusively deletion of a G:C base pair) in PhIP-induced mutants is elevated more than 10-fold in the *lacI* but not the *cII* gene [24]. This suggests that *cII* differs from *lacZ* and *lacI* in its mutagenic response to PhIP.

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DNA Cleavage via Superoxide Anion Formed in Photoinduced Electron Transfer from NADH to γ-Cyclodextrin-Bicapped C_{60} in an Oxygen-Saturated Aqueous Solution

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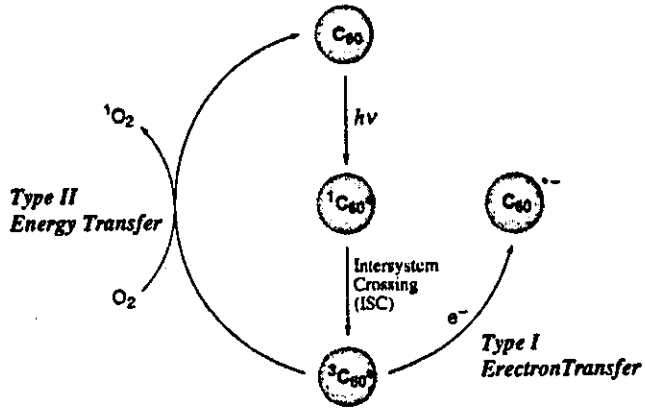
γ-Cyclodextrin-bicapped C_{60} (C_{60}/γ-CyD) shows an efficient DNA cleaving-activity in the presence of NADH (β-nicotinamide adenine dinucleotide, reduced form) in an O_2-saturated aqueous solution under visible-light irradiation. No DNA cleavage has been observed without NADH under experimental conditions that are otherwise the same, although singlet oxygen ($^1O_2$) has been detected by the ESR spin-trapping of the C_{60}/γ-CyD-O_2 system. This indicates that neither triplet excited state of C_{60}/γ-CyD ($^3C_{60}$*/γ-CyD) nor $^1O_2$ produced via an energy transfer from $^3C_{60}$*/γ-CyD to O_2 is an actual reactive species, which is responsible for the DNA damage under the present experimental conditions. In the presence of NADH, photoinduced electron transfer from NADH to $^3C_{60}$*/γ-CyD occurs to yield two equivalents of the radical anion ($C_{60}^{\bullet-}$/γ-CyD), which exhibits its characteristic NIR band at 1080 nm. The dynamics of the photoinduced electron transfer have been examined by monitoring the decay of triplet−triplet absorption band at 740 nm and concomitant rise of the NIR absorption band at 1080 nm due to $C_{60}^{\bullet-}$/γ-CyD with use of the laser flash photolysis for the C_{60}/γ-CyD-NADH system. In the presence of O_2, $C_{60}^{\bullet-}$/γ-CyD disappears via the electron transfer to O_2 and an electron transfer from NADH to $^1O_2$ to produced $O_2^{\bullet-}$. The formation of $O_2^{\bullet-}$ has been confirmed by the spin trap with DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide), which is an efficient $O_2^{\bullet-}$-trapping agent. The reorganization energy for the reduction of O_2 to $O_2^{\bullet-}$ is evaluated as 43.4 kcal mol^{-1}, which agrees with the literature value determined directly for the self-exchange between $^{36}O_2^{\bullet-}$ and $^{32}O_2$. This indicates that the electron transfer from $C_{60}^{\bullet-}$/γ-CyD to O_2 proceeds via an outer-sphere pathway. The $O_2^{\bullet-}$ thus produced gives H_2O_2, ultimately yielding hydroxyl radical, which is shown to be an actual DNA-cleaving reagent.

Introduction

Fullerenes, such as C_{60} and C_{70}, are known to have strong photosensitizing activities[1,2] and the potential utility of fullerenes as pharmaceuticals has been extensively explored in recent years.[3,4] Fullerenes, which are sensitive to light at wavelengths longer than 500 nm, are expected to be an effective photodynamic therapy agent because bodily tissues are most transparent in this region of wavelengths.[5] C_{60} and its derivatives are reported to promote chondrogenesis,[6] exhibiting enzyme-inhibiting activity[7] and radical-quenching activity.[8] In particular, the DNA-cleaving and lipid peroxidation activities of fullerenes have attracted considerable attention.[9−11] At first, it was presumed that the mechanism of DNA cleavage involves photoexcitation of the fullerene group followed by sensitized formation of singlet oxygen ($^1O_2$) which would then cleave DNA.[8,9] Photoirradiation of C_{60} results in the formation of the singlet excited state $^1C_{60}$*, which undergoes efficient intersystem crossing (ISC) to give the triplet excited state $^3C_{60}$* (Scheme 1).[12] The $^3C_{60}$* thus formed efficiently transfers energy to molecular oxygen to give $^1O_2$ (type II energy-transfer pathway).

SCHEME 1



The possible intermediacy of $^1O_2$ was examined in some detail by comparing the reactivity of the fullerene-oligonucleotide

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linked system with a similarly linked eosin-origonucleotide.[10] A singlet oxygen quencher, sodium azide, was found to inhibit the eosin-oligonucleotide cleavage, but not the fullerene-oligonucleotide cleavage.[10] This suggests that the fullerene-oligonucleotide cleavage does not involve the singlet oxygen mechanism, but rather some other mechanism must be involved.[10] The most probable mechanism may be an electron transfer because the reduction potential of $^3C_{60}$* is significantly high (+1.14 V vs SCE in benzonitrile),[13] and an electron transfer from reductants, such as amines and antioxidants, to $^3C_{60}$* occurs to give the radical anion of C_{60} ($C_{60}^{\bullet-}$) (type I electron-transfer pathway). Formation of $C_{60}^{\bullet-}$ in the photoinduced electron-transfer reactions from a variety of electron donors to $^3C_{60}$* has been demonstrated with use of the transient vis-NIR spectroscopy as well as the ESR technique.[14-19] Foote et al. have reported that the $^3C_{60}$* directly oxidizes guanine in a DNA stack via the type I electron-transfer mechanism because the oxidation potential of a guanosine derivative is located at 1.26 V which is close to the reduction potential of $^3C_{60}$* (1.14 V).[20,21] When photoinduced electron transfer occurs from electron donors to $^1C_{60}$*, the back electron transfer to the triplet excited-state rather than to the ground state can occur provided that the triplet excited state is lower in energy than the radical ion pair state.[22] In such a case, formation of $^1O_2$ may occur via photoinduced electron transfer and subsequent energy transfer from $^3C_{60}$* to oxygen.[17] Thus, there are several possible pathways for the DNA-cleaving process involving $^3C_{60}$*: via superoxide anion, singlet oxygen, or direct oxidation of DNA. However, the poor solubility of fullerenes to water has so far precluded the detailed mechanistic studies of C_{60}-photosensitized DNA damage. The preparation of a water-soluble C_{60} complex has been achieved by using γ-cyclodextrin (γ-CyD) as a host in the complex where the fullerene core is embedded between the cavities of two γ-CyD molecules.[23] Although the reactivity of the fullerene in this complex is noticeably lower than that in the free molecule, C_{60}/γ-CyD is still susceptible for the sensitizing and redox processes.[24,25]

We have previously reported photocleavage of DNA in the presence of NADH (β-nicotinamide adenine dinucleotide, reduced form) in water, when C_{60} or C_{70} used as a sensitizer was dissolved with use of poly(vinylpyrrolidone) (PVP).[26] However, the actual intermediate for the photocleavage of DNA has yet to be clarified.[11a]

We report herein that photoinduced DNA cleavage occurs efficiently by C_{60}/γ-CyD in an O_2-saturated aqueous solution containing NADH which is the most important redox coenzyme acting as the source of electrons in the living system. Detailed spectroscopic and kinetic studies using a laser flash photolysis technique, detection of superoxide ($O_2^{\bullet-}$) with use of DEPMPO,[27] and the effects of hydroxyl radical scavengers on DNA cleavage are now performed to provide confirmative mechanistic insight into the C_{60}/γ-CyD-photosensitized DNA damage by oxygen in the presence of NADH.

Experimental Section

Materials. C_{60} (>99.99% pure) was purchased from MER Co., Tucson, AZ. γ-Cyclodextrin was obtained commercially from Wako Pure Chemical Ind. Ltd., Japan and dried under vacuum prior to use. NADH (β-nicotinamide adenine dinucleotide, reduced form) was purchased from Sigma Chemical Co. DNA pBR322 (0.51 $\mu g\ \mu L^{-1}$) was purchased from Wako Pure Chemical Ind. Ltd., Japan. 2,2,6,6-Tetramethyl-4-piperidone (TEMP) used as a trapping agent of singlet oxygen was purchased from Aldrich. 5-Diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) used as an $O_2^{\bullet-}$ -trapping agent was obtained commercially from OXIS International, Inc. and used as received.

Preparation of γ-Cyclodextrin-Bicapped C_{60} (C_{60}/γ-CyD). γ-Cyclodextrin-bicapped C_{60} (C_{60}/γ-CyD) was prepared by ball milling of the mixture containing 100 mg (0.14 mmol) of C_{60} and 900 mg (0.70 mmol) of γ-Cyclodextrin (γ-CyD) for 2 h at room temperature, followed by an addition of 10 mL of water.[23,29] Aqueous solutions containing C_{60}/γ-CyD were filtered with a membrane filter (pore size: 0.22 μm). The concentration of C_{60}/γ-CyD was estimated by using the ϵ value of 51 900 $M^{-1}\ cm^{-1}$ at 330 nm determined for the cyclohexane solution.[28a,29]

DNA Cleavage. The 30 μL of aqueous solution of DNA pBR322 (0.51 $\mu g\ \mu L^{-1}$) was diluted by adding 270 μL of water. Typically, 10 μL of aqueous solution of C_{60}/γ-CyD (1.8×10^{-4} M), 10 μL of NADH (4.0×10^{-2} M), 12 μL of aqueous solution of DNA pBR322 (2.84×10^6 D; 0.051 $\mu g\ \mu L^{-1}$), and 8 μL of phosphate buffer (250 mM, pH 7.4) were mixed in a micro test tube under dark conditions. Samples were incubated under irradiation with a 300-W reflector lamp for 1 h at 273 K, mixed with 10 μL of loading buffer (0.1% bromophenol blue and 30% glycerol in TBE buffer), and loaded onto a 1% agarose gel containing ethidium bromide (1 $\mu g\ mL^{-1}$). The gels were run at a constant voltage of 70 V for 2 h in TBE buffer, washed with distilled water, visualized under a UV transilluminator, and photographed using an instant camera.

ESR Measurements. In a typical experiment of the ESR measurements for the detection of $^1O_2$, 50 μL of aqueous solution of C_{60}/γ-CyD (2.3×10^{-4} M), 50 μL of 250 mM phosphate buffer solution (pH 7.4), 100 μL of water, and 50 μL of aqueous solution of TEMP (0.5 M) were mixed in a LABOTEC LLC-04B ESR sample tube. The ESR samples were then irradiated with a 300-W reflector lamp prior to the measurements. ESR spectra were measured with a JEOL JES-RE1XE and were recorded under nonsaturating microwave power conditions. The magnitude of the modulation was chosen to optimize the resolution and the signal-to-noise ratio (S/N) of the observed spectra. The g values were calibrated using an Mn^{2+} marker. The detection of $O_2^{\bullet-}$ with use of DEPMPO as a $O_2^{\bullet-}$-trapping agent was carried out in a similar manner.

Spectroscopic Measurements. Typically, to a deaerated 50 mM phosphate buffer solution (pH 7.4; 3 mL) of C_{60}/γ-CyD (1.3×10^{-4} M) in a quartz cuvette (10 mm i.d.) under an atmospheric pressure of argon was added NADH (1.5×10^{-4} M), and the solution was irradiated with a Xe lamp (Ushio Model V1-501C) through a UV cut-off filter (Toshiba Y-47) transmitting $\lambda > 470$ nm at 298 K for 2 h. The vis-NIR spectra were measured on a Shimadzu UV-3100PC spectrophotometer.

Laser Flash Photolysis. The nanosecond time-resolved absorption spectra were measured using second-harmonic generation (532 nm) of a Nd:YAG laser (QuantaRay GCR-130, fwhm 6 ns) as an excitation source. For the transient absorption spectra in the NIR region (600-1600 nm), a Ge avalanche photodiode (APD) (Hamamatsu Photonics, B2834) was employed as a detector for monitoring light from a pulsed Xe flash lamp.[30] The long time scale phenomena up to millisecond-region were measured using an InGaAs-PIN photodiode (Hamamatsu Photonics, G5125-10) or a Si-APD (Hamamatsu Photonics, S5343) as a detector for monitoring light from a continuous Xe-lamp (150 W).[31] Because the purple solution of C_{60}/γ-CyD in water disappeared by each laser shot (532 nm; 7 mJ) in the presence of NADH, the transient spectra were recorded using

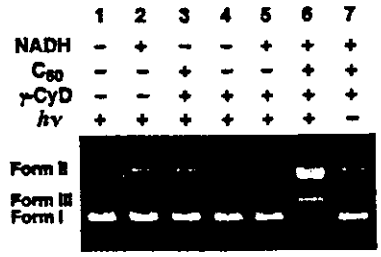

Figure 1. DNA-cleaving activities of C_{60}/γ-CyD in phosphate buffer (50 mM, pH 7.4) after visible-light irradiation with a 300-W reflector lamp for 1 h at 273 K. $[C_{60}] = 4.6 \times 10^{-5}$ M; [γ-CyD] = 9.2×10^{-5} M; [NADH] = 1.0×10^{-2} M; $[O_2] = 1.3 \times 10^{-3}$ M.

fresh solutions in each laser excitation. All experiments were performed at 295 K. The concentration of O_2 in the solution was adjusted by argon, air, or O_2 purging for 15 min prior to the measurements ($[O_2] = 0$, 2.7×10^{-4}, or 1.3×10^{-3} M, respectively). Pseudo-first-order rate constants were determined by a least-squares curve fit using an Apple Macintosh personal computer. The first-order plots of $\ln(A_\infty - A)$ vs time (A_∞ and A are the final absorbance and the absorbance during the reaction, respectively) were linear for three or more half-lives with the correlation coefficient $\rho > 0.999$.

Quantum Yield Determination. A standard actinometer (potassium ferrioxalate)[32] was used for the quantum yield determination of the photochemical reactions of C_{60}/γ-CyD and NADH. A square quartz cuvette (10 mm i.d.) which contained a 50 mM phosphate buffer solution (pH 7.4; 3 mL) of C_{60}/γ-CyD (1.3×10^{-4} M) and NADH ($1.0 \times 10^{-5} - 1.3 \times 10^{-2}$ M) was irradiated with monochromatized light of $\lambda = 532$ nm from a Shimadzu RF-5000 fluorescence spectrophotometer. The solution was first evacuated with ultra sonic irradiation and then argon gas was bubbled for 15 min. Under the conditions of actinometry experiments, both the actinometer and C_{60}/γ-CyD absorbed essentially all the incident light. The light intensity of monochromatized light of $\lambda = 532$ nm was determined as 2.2×10^{-8} einstein s^{-1} with the slit width of 10 nm. The photochemical reaction was monitored by using a Shimadzu UV-3100PC spectrophotometer The quantum yields were determined from the increase in absorbance due to $C_{60}^{\bullet-}$/γ-CyD ($\lambda_{max} = 1080$ nm, $\epsilon = 1.2 \times 10^4$ M^{-1} cm^{-1})[33] and the decrease from the absorbance due to NADH ($\lambda = 380$ nm, $\epsilon = 1.2 \times 10^3$ M^{-1} cm^{-1}).

Results and Discussion

Photoinduced DNA Cleavage in the Aqueous C_{60}/γ-CyD-NADH-O_2 System. The DNA-cleaving activity of C_{60}/γ-CyD was examined using pBR322 supercoiled DNA. In the presence of NADH as an electron donor, pBR322 was efficiently cleaved into form II (nicked DNA) and form III (linear DNA) after 1 h visible-light irradiation of a phosphate buffer solution (50 mM, pH 7.4) containing C_{60}/γ-CyD and molecular oxygen with use of a 300-W reflector lamp (Figure 1, lane 6). The effect of NADH as well as C_{60}/γ-CyD was dose-dependent (see Supporting Information). NADH, C_{60}/γ-CyD, or γ-CyD alone shows no DNA-cleaving activity even in the presence of O_2 under irradiation (Figure 1, lane 2, 3, or 4, respectively). NADH in the presence of γ-CyD also shows no DNA-cleaving activity (Figure 1, lane 5). Under dark conditions, no DNA cleavage occurs even in the presence of all the components, i.e., C_{60}/γ-CyD, NADH, and O_2 (Figure 1, lane 7).

As shown in Scheme 1, photoirradiation of the aqueous C_{60}/γ-CyD solution in the absence of an electron donor under aerobic conditions may result in the formation of singlet oxygen ($^1O_2$) via energy transfer from the triplet excited state of C_{60} to molecular oxygen (type II pathway). In fact, $^1O_2$ was detected by the ESR spin-trapping with use of TEMP (2,2,6,6-tetramethyl-4-piperidone), which is a $^1O_2$-trapping agent.[34] TEMP reacts with $^1O_2$ to give a $^1O_2$-adduct, TEMPO.[34] The characteristic three ESR signals of TEMPO with a_N value of 15.8 G was observed under visible-light irradiation of the aqueous C_{60}/γ-CyD-O_2 system as shown in Figure 2a.

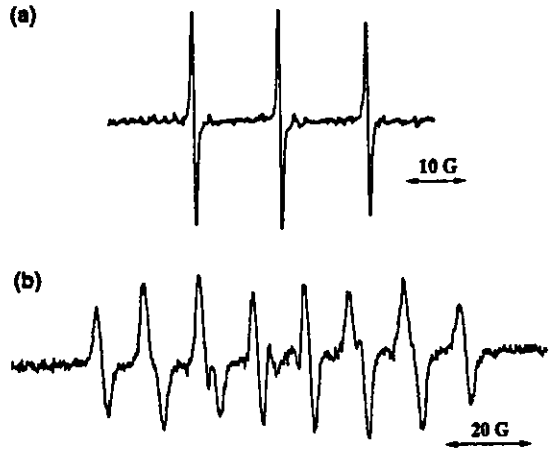

Figure 2. X-band ESR spectra of (a) TEMPO formed after visible-light irradiation of a phosphate buffer solution (50 mM, pH 7.4) of C_{60}/γ-CyD (4.6×10^{-5} M) and TEMP (0.1 M) in the presence of O_2 for 180 s at 298 K and (b) DEPMPO−OOH formed after irradiation of a phosphate buffer solution (50 mM, pH 7.4) of C_{60}/γ-CyD (4.6×10^{-5} M), NADH (1.0×10^{-3} M), and DEPMPO (1.0×10^{-2} M) in the presence of O_2 for 60 s at 298 K.

Although $^1O_2$ was suggested to have a DNA-cleaving activity,[8,9] no photoinduced DNA cleavage occurs in the aqueous C_{60}/γ-CyD-O_2 system under our experimental conditions (Figure 1, lane 3). In the presence of NADH, however, superoxide anion ($O_2^{\bullet-}$) was detected by the ESR spin-trapping with use of DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide) as an $O_2^{\bullet-}$ -trapping agent.[27] DEPMPO reacts with $O_2^{\bullet-}$ to give the $O_2^{\bullet-}$ -adduct, DEPMPO−OOH, which shows a characteristic ESR signal different from that of •OH-adduct, DEPMPO−OH. Figure 2b shows the ESR spectrum of DEPMPO−OOH observed after visible-light irradiation of an O_2-saturated phosphate buffer solution (50 mM, pH 7.4) containing C_{60}/γ-CyD, NADH, and DEPMPO at 298 K. These results indicate that the DNA cleavage occurs via $O_2^{\bullet-}$ formation via type I electron-transfer pathway, where the triplet excited state $^3C_{60}$*/γ-CyD formed by visible-light irradiation undergoes one-electron reduction by NADH to give the radical anion $C_{60}^{\bullet-}$/γ-CyD. Because the oxidation potential of $C_{60}^{\bullet-}$/γ-CyD (−0.56 V vs SCE)[35] is more negative than the reduction potential of O_2 (−0.40 V vs SCE),[36] electron transfer from $C_{60}^{\bullet-}$/γ-CyD to O_2 to give $O_2^{\bullet-}$ is energetically feasible.

Photoinduced One-Electron Reduction of C_{60}/γ-CyD. Visible light irradiation of a deaerated aqueous solution containing C_{60}/γ-CyD and NADH results in the formation of the radical anion of C_{60}/γ-CyD ($C_{60}^{\bullet-}$/γ-CyD). No reaction occurs in the dark. The formation of $C_{60}^{\bullet-}$/γ-CyD is detected by the typical NIR band at 1080 nm as shown in Figure 3. The $C_{60}^{\bullet-}$/γ-CyD generated in the photochemical reaction is stable in deaerated aqueous solution, and from the spectral titration (inset of Figure 3) is established the stoichiometry of the reaction as shown in eq 1, where NADH acts as a two-electron donor to reduce 2 equiv of C_{60}/γ-CyD to $C_{60}^{\bullet-}$/γ-CyD.[37]