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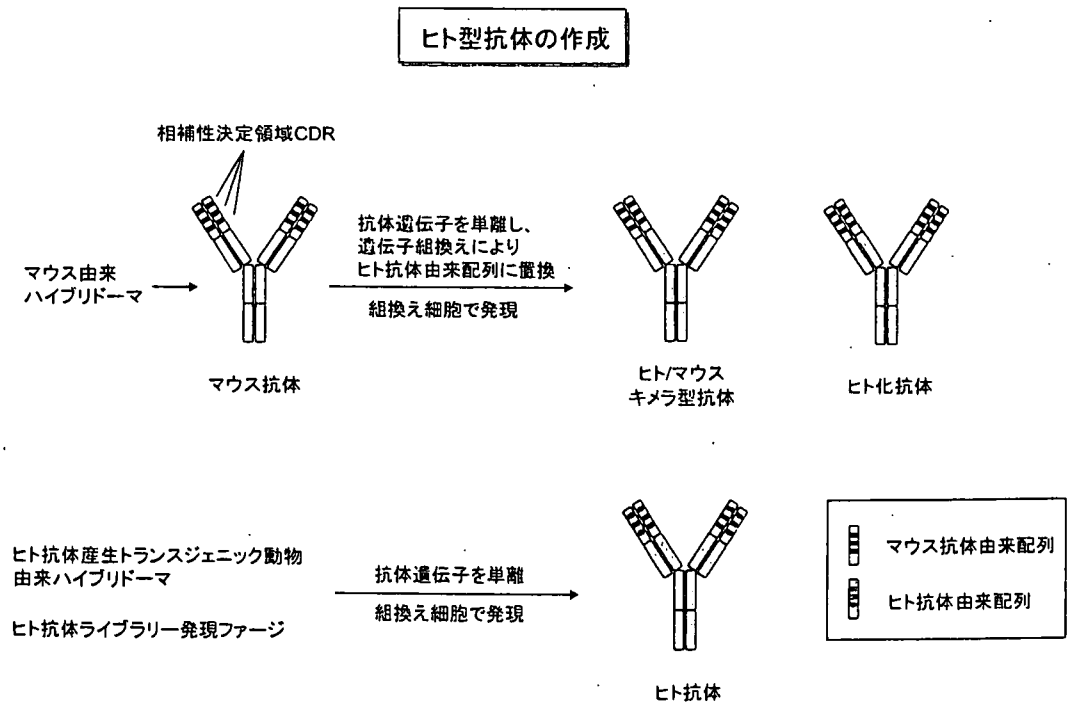
注1：バイオ医薬品

遺伝子組換え技術や細胞培養技術などのバイオテクノロジーを用いて製造される医薬品。組換えタンパク質医薬品、細胞培養医薬品、遺伝子治療薬、細胞治療薬等が含まれる。狭義には、バイオテクノロジーを応用して製造されるタンパク質性医薬品を指し、インスリン、成長ホルモン、エリスロポエチン、インターフェロン類、サイトカイン類、モノクローナル抗体などが代表例。

注2：ヒト化抗体

マウスに抗原を免疫して得た脾細胞とマウスミエローマを融合させたハイブリドーマから産生されるモノクローナル抗体はマウス抗体であるため、ヒトに投与すると、抗原性を示す、血中半減期が短い等の問題があり、医薬品としては適していない場合が多い。これらを解決するために、目的の抗体を産生するハイブリドーマから単離した抗体遺伝子を改変し、抗原決定に関与しない部分をヒト抗体に置き換える“抗体のヒト化技術”が開発された。マウス抗体遺伝子の不変領域をヒト抗体遺伝子に置換して作製した抗体を「キメラ型抗体」、不変領域に加えて、相補性決定領域（complementarity determining region：CDR）のみを残して可変領域もヒト抗体遺伝子に置換して作製した抗体を「ヒト化抗体」という（注2－図）。最



注2－図

近では、ヒト抗体遺伝子を導入したマウスやファージディスプレイ法を用いてヒトモノクローナル抗体を取得する技術が開発、実用化され、既に上市されたヒト抗体もある。

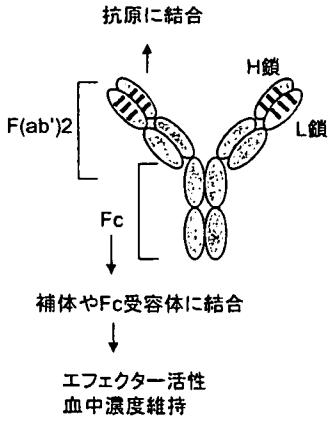
TGN1412の開発過程では、ラットCD28に対するマウスモノクローナル抗体JJ316が作製されて疾患モデルにおけるCD28アゴニスト抗体の有用性が明らかにされ、続いて、ヒトCD28に対するマウスモノクローナル抗体5.11A1が作製された。5.11A1抗体遺伝子の改変によりヒト化を行い、作製されたものがTGN1412、あるいは、そのIgG1バリアントTGN1112である。

注3：抗体の構造と機能

抗体は、相同な2本の重鎖（H鎖）と相同な2本の軽鎖（L鎖）がS-S結合で結ばれた構造を持つ。抗体の機能としては、第一義的には可変領域の抗原結合部位を介して抗原に結合することであり、さらに、Fc部分を介してFc受容体あるいは補体と結合し、抗体依存性細胞障害（Antibody Dependent Cellular Cytotoxicity: ADCC）や補体依存性細胞障害（Complement Dependent Cytotoxicity: CDC）により抗原を発現する細胞を障害するエフェクター機能を有している（注3－図）。

抗体には、IgG、IgA、IgM、IgD、IgEの5つのクラスが存在するが、これまでに医薬品として開発されたモノクローナル抗体は、すべてIgGである。ヒトIgGには、IgG1～4の4つのサブクラスが存在し、サブクラスごとにエフェクター機能等に差があることが知られている（Filpula D. *Biomol. Eng.* 24: 201, 2007）。組換え抗体医薬品では、目的とする医薬品の特性に応じたサブクラスが選択される。これまでに開発されている組換え抗体医薬品では、サブクラスがIgG1のものがほとんどであり、エフェクター機能により標的分子を発現する細胞を障害する効果が期待できる。IgG2はエフェクター機能が低い。IgG3はヒンジ領域が長いため他のサブクラスと比較して分子量が大きく、エフェクター機能は高い。凝集体を形成することがあるとされている。IgG4は補体結合能を持たないことが特徴である。生体内のIgG4では、重鎖＋軽鎖の交換が起こり、バイスペシフィックな分子にもなることが知られている（Aalberse RC et al., *Immunol.* 105: 9, 2002）。

抗体の構造と機能



IgG各サブクラスのFc受容体および補体との結合

		IgG1	IgG2	IgG3	IgG4
単球	FcγRI	++	−	+++	++
	FcγRIIa	+	(+)	++	−
	FcγRIIIa	+	−	+	−
好中球	FcγRIIa	+	−	+	−
	FcγRIIIb	+	−	+	−
補体		++	+	+++	−

＜多田富雄監訳　免疫学イラストレイテッドより改変＞

注3－図

注4：抗体医薬品の品質

抗体医薬品は、作製した抗体遺伝子をCHO細胞、あるいは、Sp2/0細胞、NS0細胞などに導入して細胞の培養上清中に抗体を分泌させ、その培養上清から目的の抗体を精製することにより生産する。(TGN1412はCHO細胞で製造された。) したがって、最終的に得られる抗体医薬品の品質（有効成分の構造・組成、物理化学的性質、免疫化学的性質、生物学的性質などの特性の他、目的物質由来不純物、製造工程由来不純物、混入汚染物質の存在等も含めて評価される）は、生産細胞の特性や培養条件、あるいは精製工程などの製造工程に大きく影響を受ける。そのため、抗体医薬品をはじめとするバイオ医薬品の品質・安全性確保においては、目的産物の特性解析を詳細に行い、望ましい有効性・安全性プロファイルの得られる目的物質の品質を規定する方法（規格および試験方法）を明らかにすると共に、製造ロットごとに品質の差が生じないよう、製造工程を厳密に管理することが重要である。

バイオ医薬品の品質・安全性確保に関しては、日米欧でこれまでに議論された結果が国際調和ガイドラインとしてまとめられている（注4－表（1））。我が国においては、バイオ医薬品の承認申請に際して申請者がどの程度のレベルでどの程度の量のデータを蓄積すればよいかなどを示すため、注4－表（2）のようなガイドラインが定められている。

注4－表（1）

バイオ医薬品の品質・安全性確保に関するICHガイドライン

Q5A ヒト又は動物細胞株を用いて製造されるバイオテクノロジー応用医薬品のウイルス安全性評価
Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin
(2000.2.22) 【厚生省医薬安全局審査管理課長 医薬審第329号】

Q5B 組換えDNAを応用したタンパク質生産に用いる細胞中の遺伝子発現構成体の分析
Quality of Biotechnological Products: Analysis of the Expression Construct in Cells Used for Production of R-DNA Derived Protein Products
(1998.1.6) 【厚生省医薬安全局審査管理課長 医薬審第3号】

Q5C 生物薬品（バイオテクノロジー応用製品／生物起源由来製品）の安定性試験
Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products
(1998.1.6) 【厚生省医薬安全局審査管理課長 医薬審第6号】

Q5D 生物薬品（バイオテクノロジー応用医薬品／生物起源由来医薬品）製造用細胞基剤の由来、調製及び特性解析
Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products
(2000.7.14) 【厚生省医薬安全局審査管理課長 医薬審第873号】

Q5E 生物薬品（バイオテクノロジー応用医薬品／生物起源由来医薬品）の製造工程の変更にともなう同等性／同質性評価
Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process
(2005.4.26) 【厚生労働省医薬食品局審査管理課長 薬食審査発第0426001号】

Q6B 生物薬品（バイオテクノロジー応用医薬品／生物起源由来医薬品）の規格及び試験方法の設定
Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products
(2001.5.1) 【厚生労働省医薬局審査管理課長 医薬審第571号】

S6 バイオテクノロジー応用医薬品の非臨床における安全性評価
Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals
(2000.2.22) 【厚生省医薬安全局審査管理課長 医薬審第326号】

国際合意に達したICHガイドラインの内容は、国内におけるバイオ医薬品の試験や評価を行う際の基本となり、そこに記載されている内容については遵守する必要がある。合意された各ICHガイドラインの内容については国内版が作成され、国内通知として出されている。

注4－表 (2)

我が国におけるバイオ医薬品関連ガイドライン

バイオ医薬品の承認申請に関して、申請者が、どの程度のレベルでどの程度の量のデータを蓄積すればよいかなどを示すために、我が国では以下の3つのガイドラインが公表されている。

(1) 薬審第243号通知(昭和59年3月30日)
「組換えDNA技術を応用して製造される医薬品の承認申請に必要な添付資料の作成について」

(2) 薬審1第10号通知(昭和63年6月6日)
「細胞培養技術を応用して製造される医薬品の承認申請に必要な添付資料の作成について」

(3) 都道府県衛生主管部(局)薬務主管課宛事務連絡(平成元年5月)
「薬審1第10号通知に関する質疑応答について」

注5：サイトカイン放出症候群に関する添付文書上の記載

医薬品添付文書においては、サイトカイン放出症候群は、"薬剤投与中または投与開始後24時間以内に多く現れる有害反応の総称"であるinfusion reactionに含めて記載されている場合が多い。例えばRituximabの場合は、infusion reactionとサイトカイン放出症候群を臨床上、厳密に区別することが困難であること、また、薬剤投与に伴って認められる有害事象に対して2つの異なった用語が用いられることによって対処方法が異なる印象を与え、臨床現場に混乱を来す可能性が懸念されるとして、infusion reaction

注5－表

サイトカイン放出症候群に関連する医薬品添付文書中の記載概要(抜粋)

	Muromonab-CD3 (抗CD3抗体)	Rituximab (抗CD20抗体)	Alemtuzumab (抗CD52抗体)
日本	【重要な基本的注意】 投与初期に発熱等のインフルエンザ様症状があらわれるので、その旨を患者にあらかじめ説明しておくこと。また、その間は患者を厳密に観察すること。	【重要な基本的注意】 本剤の初回投与中又は投与開始後24時間以内に多くあらわれるinfusion reactionが約90%の患者において報告されている。 【警告】 本剤の投与開始後30分～2時間よりあらわれるinfusion reactionのうちアナフィラキシー様症状、肺障害、心障害等の重篤な副作用により、死亡に至った例が報告されている。	未承認
US	【警告】サイトカイン放出症候群 ほとんどの患者で、活性化リンパ球または単球からのサイトカイン放出に起因する急性の臨床症状(サイトカイン放出症候群)が生じる。 【有害事象】サイトカイン放出症候群 腎移植の急性拒絶反応の治験で、最初の2回の投与後に、2%以下の患者で致死的なレベルの肺浮腫が報告されている。	【有害反応】Infusion reactions 大部分の患者で初回投与時に、発熱や悪寒/硬直などの軽度～中程度のinfusion reactionが生じた。 【枠付き警告】Fetal infusion reactions: 投与24時間以内の死亡例が報告されている。	【警告】Infusion-related events: 低血圧、硬直、発熱、気管支痙攣、悪寒などのinfusion-related eventsが生じている。 【枠付き警告】Infusion reactions:重篤な、時に致死的なinfusion reactionが生じ得る。
EU *	未承認	【投与方法】 サイトカイン放出症候群の発生に備え、患者を注意深くモニターすること。 【特別な警告と使用上の注意】 重篤なサイトカイン放出症候群が生じるリスクの高い腫瘍細胞数の多い患者では、他の治療法がない場合にのみ、特別な注意のもとに使用すること。	【副作用】Infusion-related reactions: 非常に一般的に報告された反応(サイトカイン放出に起因する)は、発熱、硬直、悪心、嘔吐等の急性のInfusion-related reactionsであった。 稀に、サイトカイン放出に伴って気管支痙攣、低酸素などの重篤な反応がおこり、致死的な結果となる例があった。

EU * : Summary of product characteristicsより抜粋。
いずれも、患者用添付文書ではinfusion reactionとしての記載のみ。

に統一されている（衛研発第2458号　審査報告書：http://211.132.8.246/shinyaku/g0106/04/38010100_21300AMY00273_110_1.pdf）。注5－表にMuromonab CD3、Rituximab、およびAlemtuzumabの日米欧における添付文書中のサイトカイン放出症候群に関連する表現を記載した。これらの医薬品では、サイトカイン放出症候群の発生を防ぐために、解熱薬や抗炎症薬の前投与が推奨されている。

Infusion reactionの原因としては、アナフィラキシー反応がよく知られているが、アナフィラキシー反応と比較して、サイトカイン放出症候群は抗体医薬品の初回投与時に起こる頻度が高いことが特徴である。これは、生体内の標的分子数が初回投与時に最も多く、2回目以降は初回に投与された抗体の効果により標的分子を発現する細胞が減っていることが原因であると考えられている。

☆コラム「生殖発生毒性の理解に役立つホームページ」コラム☆

最近は生殖発生毒性のみを専門にしている毒性研究者は少なくなりました。一般毒性も生殖発生毒性も兼任されている方や特殊毒性すべてを担当されている方もいます。若い方では実際に動物室でラット新生児の性別を見分けたり、胎児の骨格を染色して観察をした経験がなく、報告書の評価をされている人もいると聞きます。もちろん、教科書等でしっかり勉強されておられるのですが、現実感がわかないのも無理のないことかも知れません。

そんな専門ではないが、生殖発生毒性に関係しなくてはならなくなった方へ、

私がお勧めするホームページは「おくすり１１０番」の中の「妊娠とくすり」のページです。このページは一般の妊婦さんのために書かれたもので、非常にわかりやすい言葉で書かれていますが、内容は包括的かつ専門的なものを含んでいます。また、臨床の立場で書かれていますので、非臨床に身を置く者にとって大局的な目を見開かされるところもあります。また、薬剤ごとの妊婦における危険度がリストされていますので、自分の担当する薬剤の類薬における生殖発生毒性を調べる際にも役に立ちます。生殖発生毒性でなにか疑問に感じた時、ぜひともアクセスしてみて下さい。

http://www.okusuri110.com/kinki/ninpukin/ninpukin_00top.html

（下村　和裕）

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Nitrogen-substitution effect on in vivo mutagenicity of chrysene

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Abstract

We have previously reported the in vivo mutagenicity of aza-polycyclic aromatic hydrocarbons (azaPAHs), such as quinoline, benzo[*f*]quinoline, benzo[*h*]quinoline, 1,7-phenanthroline and 10-azabenzo[*a*]pyrene. The 1,10-diazachrysene (1,10-DAC) and 4,10-DAC, nitrogen-substituted analogs of chrysene, were shown to exhibit mutagenicity in *Salmonella typhimurium* TA100 in the presence of rat liver S9 and human liver microsomes in our previous report, although DACs could not be converted to a bay-region diol epoxide, the ultimate active form of chrysene, because of their nitrogen atoms. In the present study, we tested in vivo mutagenicity of DACs compared with chrysene using the *lacZ* transgenic mouse (Muta™Mouse) to evaluate the effect of the nitrogen substitution. DACs- and chrysene-induced mutation in all of the six organs examined (liver, spleen, lung, kidney, bone marrow and colon). The mutant frequencies obtained with chrysene showed only small differences between the organs examined and ranged from 1.5 to 3 times the spontaneous frequency. The 4,10-DAC was more mutagenic than chrysene in all the organs tested. The highest *lacZ* mutation frequency was observed in the lung of 4,10-DAC-treated mice and it was 19 and 6 times the spontaneous frequency and the frequency induced by chrysene, respectively. The 1,10-DAC induced *lacZ* mutation in the lung with a frequency 4.3- and 1.5-fold higher than in the control and chrysene-treated mice, respectively, although the mutant frequencies in the other organs of 1,10-DAC-treated mice were almost equivalent to those of chrysene-treated mice. Not only chrysene but also DACs depressed the G:C to A:T transition and increased the G:C to T:A transversion in the liver and lung. These results suggest that the two types of nitrogen substitutions in the chrysene structure may enhance mutagenicity in the mouse lung, although they showed no difference in the target-organ specificity and the mutation spectrum.

Keywords: Aza-substitution; In vivo mutagenesis assay; Mutation spectrum

1. Introduction

We have been investigating the mutagenicity of aza-polycyclic aromatic hydrocarbons (azaPAHs) with

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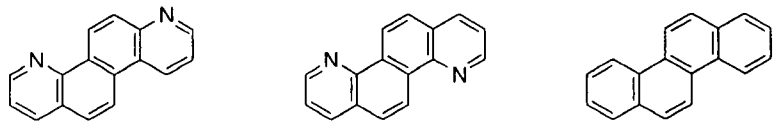

1,10-diazachrysene (1,10-DAC) | 4,10-diazachrysene (4,10-DAC) | chrysene

Fig. 1. Chemical structures of 1,10-DAC, 4,10-DAC and chrysene.

special attention to their metabolic activation mechanism. The 10-azabenzo[*a*]pyrene (10-azaBaP), a 10-aza-analog of benzo[*a*]pyrene (BaP), was reported to be as mutagenic as BaP in *Salmonella typhymurium* TA100 in the presence of PCB-treated rat liver S9 [1,2], although 10-azaBaP could not be converted to a bay-region diol epoxide, the ultimate mutagenic form of BaP [3,4], because of its nitrogen atom. We have previously reported that 10-azaBaP showed a higher mutagenicity than BaP in the Ames test using pooled human liver S9 [5]. However, in the in vivo mutagenesis assay system using the *lacZ* transgenic mouse (Muta^{TM}Mouse), 10-azaBaP was mutagenic only in the liver and colon and showed much less mutagenicity than BaP, which showed high mutagenicity in all of the organs tested [5]. Thus, 10-azaBaP interestingly showed differences in mutagenicity between the in vitro and in vivo assay systems.

We have also reported that quinoline, an aza-analog of naphthalene, one of simplest azaPAHs and a hepatocarcinogen [6,7], showed mutagenicity only in the liver of Muta^{TM}Mouse [8]. We also observed that it caused remarkable induction of G:C to C:G transversion [9] and suggested that it might be metabolically activated in the pyridine moiety to the ultimate mutagenic form [10]. Its active form was supposed to be an enamine epoxide (1,4-hydrated 2,3-epoxide), which would be responsible for the mutagenic modification of DNA [11–14]. Furthermore, three trycyclic azaPAHs, i.e., benzo[*f*]quinoline, benzo[*h*]quinoline and 1,7-phenanthroline, were shown to exhibit mutagenicity in Muta^{TM}Mouse in our previous report [15]. Benzo[*h*]quinoline and 1,7-phenanthroline were suggested to be converted to the ultimate genotoxic form in the pyridine moiety [16].

1,10-Diazachrysene (1,10-DAC) and 4,10-DAC are diaza-analogs of chrysene (Fig. 1), consisting of two quinoline moieties, and have structures similar to 10-azaBaP. We have previously reported that these DACs showed mutagenicity in Ames tests in the presence of rat liver S9 or human liver microsomes [17], although formation of the bay-region diol epoxide from DACs seemed impossible because of their nitrogen atoms. DACs have not been found in our living environments, but these are expected to be useful compounds to investigate the nature of mutagenicity in azaPAHs.

In the present study, we undertook to investigate the in vivo mutagenicity of DACs in comparison with chrysene by the in vivo mutation assay system using the *lacZ* transgenic mouse (Muta^{TM}Mouse) to evaluate the nitrogen-substitution effect in the chrysene skeleton on their mutagenicity.

2. Materials and methods

2.1. Materials

Chrysene (CAS Registry No. 218-01-9) and phenyl-β-D-galactoside (P-gal) were purchased from Sigma Chemical Co. (St. Louis, MO), proteinase K and olive oil from Wako Pure Chemicals (Osaka) and RNase from Boehringer Mannheim. The 1,10-DAC (CAS Registry No. 218-21-3) and 4,10-DAC (CAS Registry No. 218-34-8) were synthesized in this laboratory according to the reported methods [18].

2.2. In vivo mutagenesis assays using Muta^{TM}Mouse

2.2.1. Animals and treatments

Male Muta^{TM}Mice, at 7–8 weeks of age, were supplied by COVANCE Research Products (PA, USA) and acclimatized for 1 week before use. Chrysene and 4,10-DAC dissolved in olive oil (10 mL/kg body weight)

were injected intraperitoneally into four mice each at a single dose of 200 mg/kg once a week for 4 consecutive weeks (800 mg/kg in total). The 1,10-DAC dissolved in olive oil (10 mL/kg body weight) was injected into four mice at a single dose of 100 mg/kg similarly (400 mg/kg in total). Four control mice were given 10 mL olive oil/kg.

2.2.2. Tissues and DNA isolation

All mice were killed by cervical dislocation 7 days after the last administration of test chemicals. The liver, spleen, lung, kidney, bone marrow and colon were immediately extirpated, frozen in liquid nitrogen and stored at −80 °C until DNA extraction. The genomic DNA was extracted from each tissue by the phenol/chloroform method according to the Muta™Mouse/PS Mutation Assay Manual (Corning Hazleton, 1995). The isolated DNA, which was precipitated with ethanol, was air-dried and dissolved in an appropriate volume (20–200 μL) of TE-4 buffer (10 mM Tris–HCl at pH 8.0 containing 4 mM EDTA) at room temperature overnight. The DNA solution thus prepared was stored at 4 °C.

2.2.3. In vitro packaging

The lambda gt10/*lacZ* vector could be efficiently recovered by in vitro packaging reactions [19]. Our homemade packaging extract (HM) consisting of sonic extract (SE) of *Escherichia coli* NM759 and freeze–thaw lysate (FTL) of *E. coli* BHB2688 was prepared according to the method of Gunther et al. [20]. As a general procedure for handling the HM extract, approximately, 5 μg DNA was mixed with 15 μL of FTL and 30 μL of SE and incubated at 37 °C for 90 min. Then SE and FTL were added again and the mixture was incubated for another 90 min. The reaction was terminated by the addition of an appropriate volume of SM buffer (50 mM Tris–HCl at pH 7.5, 10 mM $MgSO_4$, 100 mM NaCl and 0.01% gelatin) and the mixture was stored at 4 °C. By this procedure, the lambda gt10 vector to form an infectious phage was efficiently rescued from genomic DNA.

2.2.4. Mutation assays

2.2.4.1. lacZ mutant frequency determination. The positive selection for *lacZ* mutants was performed as previously reported [21–23]. Briefly, the phage

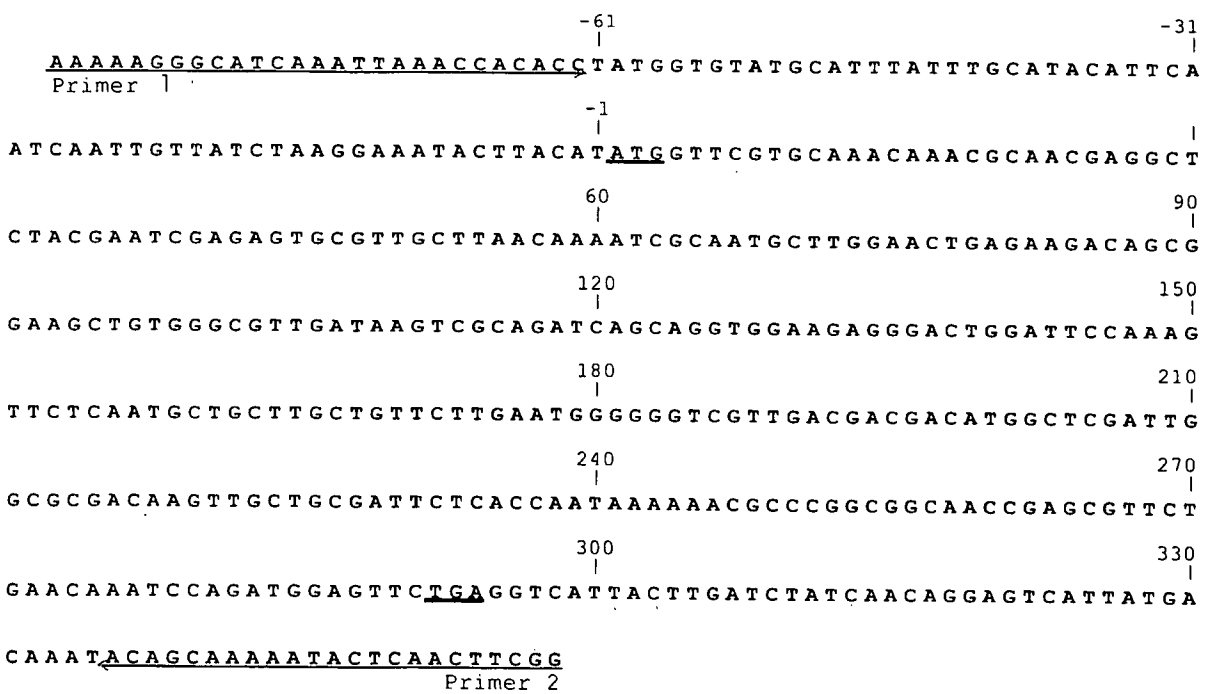

Fig. 2. Sequence map of the *cII* gene; primers used for PCR amplification and sequencing are shown by arrows. The PCR gives 446 bp products that involve the entire (294 bp) *cII* gene. Initiation and stop codons are underlined.

Table 1
Mutant frequencies induced by 1,10-DAC, 4,10-DAC and chrysene in six organs of Muta™Mouse

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ±S.D.
		No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6	No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6
Liver	Control (olive oil)	463000	36	77.8		1547000	31	20.0	
		173000	12	69.4		602000	20	33.2	
		1899000	247	130.1		1618000	47	29.0	
		396000	30	75.8	88.2 ± 24.3	1398000	22	15.7	24.5 ± 7.0
	1,10-DAC	391000	77	196.9		1299000	90	69.3	
		1126000	127	112.8		989000	40	40.4	
		371500	63	169.6		1805000	72	39.9	
		244000	36	147.5	156.7 ± 30.8*	1095000	23	21.0	42.7 ± 17.3
	4,10-DAC	192000	72	375.0		731000	79	108.1	
		355500	193	542.9		1446000	182	125.9	
		506500	225	444.2		2080000	233	112.0	
		503000	308	612.3	493.6 ± 90.9**	1751000	311	177.6	130.9 ± 27.8**
	Chrysene	452500	98	216.6		1025000	51	49.8	
		322500	88	272.9		1220000	81	66.4	
		362500	88	242.8		1343000	68	50.6	
		522000	89	170.5	225.7 ± 37.6**	1799000	60	33.4	50.0 ± 11.7*
Spleen	Control (olive oil)	866000	47	54.3		966000	14	14.5	
		1145000	59	51.5		1180000	25	21.2	
		870000	36	41.4		1024000	34	33.2	
		653500	23	35.2	45.6 ± 7.7	1039000	15	14.4	20.8 ± 7.7
	1,10-DAC	1700500	150	88.2		1550000	70	45.2	
		769000	77	100.1		909000	59	64.9	
		1252000	125	99.8		2119000	129	60.9	
		1316000	131	99.5	96.9 ± 5.0**	1837000	110	59.9	57.7 ± 7.5**
	4,10-DAC	469500	109	232.2		550000	56	101.8	
		1506000	525	348.6		1754000	260	148.2	
		1410000	374	265.2		2071000	268	129.4	
		1115000	271	243.0	272.3 ± 45.7**	1438000	219	152.3	132.9 ± 19.9**
	Chrysene	2225000	339	152.4		470500	12	25.5	
		638000	66	103.4		970000	72	74.2	
		1433500	202	140.9		2313000	102	44.1	
		247500	34	137.4	133.5 ± 18.2**	612000	13	21.2	41.3 ± 20.9
Lung	Control (olive oil)	310000	10	32.3		376000	14	37.2	
		296500	28	94.4		263500	11	41.7	
		646000	63	97.5		2016000	73	36.2	
		1402000	73	52.1	69.1 ± 27.8	1429000	23	16.1	32.8 ± 9.9
	1,10-DAC	1911000	539	282.1		1855000	255	137.5	
		1691000	342	202.2		1921000	151	78.6	
		1242000	430	346.2		1807000	183	101.3	
		1826500	652	357.0	296.9 ± 61.7**	2152000	281	130.6	112.0 ± 23.6**
	4,10-DAC	979500	1402	1431.3		1112000	530	476.6	
		1489000	1659	1114.2		1747000	771	441.3	
		1497000	2343	1565.1		1900000	1093	575.3	

Table 1 (*Continued*)

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ±S.D.
		No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6	No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6
		1673000	1702	1017.3	1282.0 ± 224.0**	1847000	1005	544.1	509.3 ± 53.0**
	Chrysene	1033000	121	117.1		1577000	61	38.7	
		1526000	371	243.1		2067000	231	111.8	
		1101000	241	218.9		1566000	115	73.4	
		871000	177	203.2	195.6 ± 47.5**	1254000	69	55.0	69.7 ± 27.2
Kidney	Control (olive oil)	447000	36	80.5		546000	21	38.5	
		741500	61	82.3		925000	23	24.9	
		1726000	62	35.9		2183000	70	32.1	
		1948000	72	37.0	58.9 ± 22.5	2244000	57	25.4	30.2 ± 5.6
	1,10-DAC	1609000	214	133.0		2570000	109	42.4	
		1510000	138	91.4		2278000	127	55.8	
		1410000	114	80.9		2621000	96	36.6	
		945000	119	125.9	107.8 ± 22.1*	1658000	67	40.4	43.8 ± 7.2*
	4,10-DAC	1378000	175	127.0		1695500	115	67.8	
		2101000	412	196.1		2526000	270	106.9	
		1447000	235	162.4		2217000	153	69.0	
		1355000	402	296.7	195.5 ± 63.3*	2014000	232	115.2	89.7 ± 21.5**
	Chrysene	1177000	161	136.8		2304000	101	43.8	
		1583000	239	151.0		2415000	157	65.0	
		1187500	241	202.9		2224000	115	51.7	
		1785000	257	144.0	158.7 ± 26.0**	2968000	147	49.5	52.5 ± 7.8**
Bone marrow	Control (olive oil)	421000	20	47.5		580000	12	20.7	
		1034000	48	46.4		1094000	25	22.9	
		1080000	61	56.5		1202000	38	31.6	
		1352000	48	35.5	46.5 ± 7.4	906000	17	18.8	23.5 ± 4.9
	1,10-DAC	1131000	78	69.0		1406000	57	40.5	
		764500	86	112.5		916000	39	42.6	
		1209000	108	89.3		1525000	58	38.0	
		732000	72	98.4	92.3 ± 15.8**	858000	48	55.9	44.3 ± 6.9**
	4,10-DAC	503500	147	292.0		779000	90	115.5	
		621000	239	384.9		1074000	191	177.8	
		1010000	200	198.0		1075000	104	96.7	
		829000	196	236.4	277.8 ± 70.2*	945000	124	131.2	130.3 ± 30.0**
	Chrysene	757500	65	85.8		1179000	20	17.0	
		819000	55	67.2		1046000	46	44.0	
		799500	49	61.3		1331000	33	24.8	
		853500	60	70.3	71.1 ± 9.1*	1392000	25	18.0	25.9 ± 10.9
Colon	Control (olive oil)	369500	17	46.0		1058000	43	40.6	
		1064000	77	72.4		1052000	40	38.0	
		660000	63	95.5		840000	36	42.9	
		676000	55	81.4	73.8 ± 18.0	1187000	33	27.8	37.3 ± 5.8
	1,10-DAC	306500	41	133.8		1715000	85	49.6	
		207000	25	120.8		1250000	57	45.6	

Table 1 (*Continued*)

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ±S.D.
		No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6	No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6
		988000	91	92.1		1396000	74	53.0	
		577000	75	130.0	119.2 ± 16.3*	951000	62	65.2	53.3 ± 7.3*
	4,10-DAC	469000	42	89.6		734000	48	65.4	
		369500	211	571.0		1425000	292	204.9	
		1004000	245	244.0		1089000	152	139.6	
		395000	188	475.9	345.1 ± 189.5	954000	187	196.0	151.5 ± 55.7*
	Chrysene	266500	29	108.8		980000	44	44.9	
		379000	52	137.2		1034000	68	65.8	
		1223000	222	181.5		1612000	78	48.4	
		615000	93	151.2	144.7 ± 26.2**	1178000	44	37.4	49.1 ± 10.4

Significantly different from the control group.
* $P<0.05$.
** $P<0.01$.

solution was absorbed to *E. coli* C (*lac*$^-$ *galE*$^-$) at room temperature for 20–30 min. For titration, appropriately diluted phage–*E. coli* solution was mixed with LB top agar (containing 10 mM $MgSO_4$) and plated onto dishes containing bottom agar. The remaining phage–*E. coli* solution was mixed with LB top agar containing phenyl-β-D-galactoside (3 mg/mL) and plated as described above. The mutant frequency (MF) was calculated by the following formula:

mutant frequency = (total number of plaques on selection plates/total number of plaques on titer plates) × dilution factor.

The significance of differences in the mutant frequency between the treated and control groups was analyzed by using Student's *t*-test and Welch's *t*-test in combination with the *F*-test.

2.2.4.2. cII mutant frequency determination. In the present study, we examined the mutagenicity in the lambda *cII* gene, which is also integrated as a lambda vector gene, which serves as another selective marker as reported previously in the *lacI* transgenic BigBlue mouse [24]. The positive selection for *cII* mutants was performed according to the method of Jakubczak et al. [24] with a slight modification as previously reported [9]. Briefly, the phage solution was absorbed to *E. coli* G1225 (*hfl*$^-$) at room temperature for 20–30 min. For titration, appropriately diluted phage–*E. coli* solution was mixed with LB top agar (containing 10 mM $MgSO_4$) and plated onto dishes containing bottom agar and the plates were incubated at 37 °C for 24 h. The remaining phage–*E. coli* solution was mixed with LB top agar and plated onto dishes containing bottom agar. The plates were incubated at 25 °C for 48 h for selection of *cII* mutants. The wild-type phage, recovered from Muta^{TM}Mice, has a *cI*$^-$ phenotype, which permits plaque formation with the *hfl*$^-$ strain at 37 °C but not at 25 °C. The mutant frequency was calculated by the following formula:

mutant frequency = (total number of plaques on selection plates/total number of plaques on titer plates) × dilution factor.

The significance of differences in the mutant frequency between the treated and control groups was analyzed by using Student's *t*-test and Welch's *t*-test in combination with the *F*-test.

2.2.5. Sequencing of mutants

The entire lambda *cII* region was amplified directly from mutant plaques by Taq DNA polymerase (Takara Shuzo, Tokyo, Japan) with primers P1, 5′-AAAAAGGGCATCAAATTAAACC-3′ and P2, 5′-CCGAAGTTGAGTATTTTTGCTGT-3′ as previously reported [9] (Fig. 2). A 446 bp PCR product was puri-

fied with a microspin column (Amersham Pharmacia, Tokyo, Japan) and then used for a sequencing reaction with the Ampli Taq cycle sequencing kit (PE Biosystems, Tokyo, Japan) using the primer P1. The reaction product was isolated by ethanol precipitation and analyzed with the ABI PRISM™ 310 genetic analyzer (PE Biosystems). In this study, about 40 mutants were subjected to sequence analysis in each group both in the liver and lung.

3. Results

3.1. Mutant frequencies by 1,10-DAC, 4,10-DAC and chrysene

Chrysene and its diaza-analogs, 1,10-DAC and 4,10-DAC, were tested for in vivo mutagenicity using *lacZ* transgenic mice (Muta™Mice). Chrysene and 4,10-DAC were injected at the total dose of 800 mg/kg

Table 2
Sequences of *cII* mutations in the liver of 1,10-DAC-treated Muta™Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
C1	117	G to T	TCG	CAG	ATC	Gln to His
C2	42	G to T	ATC	GAG	AGT	Glu to Asp
C3	126	G to T	AGC	AGG	TGG	Arg to Ser
C4	40–43	−GA	GAG	AGT	GCG	Frameshift
C5	166	G to T	CTT	GCT	GTT	Ala to Ser
C6	233	T to A	ATT	CTC	ACC	Leu to His
C7	132	G to T	TGG	AAG	AGG	Lys to Asn
C8	89	C to T	ACA	GCG	GAA	Ala to Val
C9	11	C to TA	CGT	GCA	AAC	Frameshift
C10	178	T to A	GAA	TGG	GGG	Trp to Arg
C11	197	A to G	GAC	GAC	ATG	Asp to Gly
C12	179	G to T	GAA	TGG	GGG	Trp to Leu
C13	29	C to A	GAG	GCT	CTA	Ala to Asp
C14	101	G to T	GTG	GGC	GTT	Gly to Val
C15	89	C to T	ACA	GCG	GAA	Ala to Val
C16	117	G to C	TCG	CAG	ATC	Gln to His
C17	40	G to A	ATC	GAG	AGT	Glu to Lys
C18	294	A to C	TTC	TGA	–	Stop to Cys
C19	150	G to T	CCA	AAG	TTC	Lys to Asn
C20[a]	294	A to C	TTC	TGA	–	Stop to Cys
C21	211	G to A	TTG	GCG	CGA	Ala to Thr
C22	111	G to T	GAT	AAG	TCG	Lys to Asn
C23	123	C to A	ATC	AGC	AGG	Ser to Arg
C24	160	C to A	ATG	CTG	CTT	Leu to Met
C25	173	T to C	GTT	CTT	GAA	Leu to Pro
C26	125	G to C	AGC	AGG	TGG	Arg to Thr
C27	−3	C to G	tta	cat	ATG	Base substitution in the 5′-flanking region
C28	29	C to A	GAG	GCT	CTA	Ala to Asp
C29[a]	111	G to T	GAT	AAG	TCG	Lys to Asn
C30	38	T to A	CGA	ATC	GAG	Ile to Asn
C31	40	G to T	ATC	GAG	AGT	Glu to Stop
C32	163	C to T	CTG	CTT	GCT	Leu to Phe
C33	79	G to T	ACT	GAG	AAG	Glu to Stop
C34	57	C to A	CTT	AAC	AAA	Asn to Lys
C35	89	C to T	ACA	GCG	GAA	Ala to Val
C36	179–184	−G	TGG	GGG	GTC	Frameshift
C37	113	C to A	AAG	TCG	CAG	Ser to Stop
C38	89	C to G	ACA	GCG	GAA	Ala to Gly
C39	123	C to G	ATC	AGC	AGG	Ser to Arg
C40	125	G to A	AGC	AGG	TGG	Arg to Lys

[a] Ascribable to the same mutation obtained in an identical mouse.

intraperitoneally, based on the tolerance dose. The total dose of 1,10-DAC was 400 mg/kg because 1,10-DAC showed more toxicity than 4,10-DAC and chrysene in a preliminary test. The mutant frequencies observed with the DNA preparations extracted from the six organs at 7 days after the last injection are shown in Table 1. More than 10 mutant plaques were analyzed in all organs.

The spontaneous mutant frequencies observed in the control group were similar among the six organs in both *lacZ* and *cII* assays, in the rate ranges of 46×10^{-6} to 88×10^{-6} and 21×10^{-6} to 37×10^{-6}, respectively. These results were comparable to our previous studies [5,8–10,15,25].

All the test compounds significantly increased the mutant frequencies in all the tested organs in the *lacZ*

Table 3
Sequences of *cII* mutations in the liver of 4,10-DAC-treated Muta™Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
D1	113	C to T	AAG	TCG	CAG	Ser to Leu
D2	86	C to A	AAG	ACA	GCG	Thr to Lys
D3	65	C to A	ATC	GCA	ATG	Ala to Glu
D4	106	G to T	GTT	GAT	AAG	Asp to Tyr
D5	101	G to T	GTG	GGC	GTT	Gly to Val
D6	101	G to C	GTG	GGC	GTT	Gly to Ala
D7	29–30	CT to TC	GAG	GCT	CTA	Ala to Val
D8	74	G to T	CTT	GGA	ACT	Gly to Val
D9	115	C to A	TCG	CAG	ATC	Gln to Lys
D10[a]	113	C to T	AAG	TCG	CAG	Ser to Leu
D11	42	G to T	ATC	GAG	AGT	Glu to Asp
D12	119	T to A	CAG	ATC	AGC	Ile to Asn
D13	150	G to T	CCA	AAG	TTC	Lys to Asn
D14	190	G to T	GTT	GAC	GAC	Asp to Tyr
D15	103	G to T	GGC	GTT	GAT	Val to Phe
D16	106	G to T	GTT	GAT	AAG	Asp to Tyr
D17	101	G to A	GTG	GGC	GTT	Gly to Asp
D18	196	G to A	GAC	GAC	ATG	Asp to Asn
D19	226	G to C	GCT	GCG	ATT	Ala to Pro
D20	214	C to T	GCG	CGA	CAA	Arg to Stop
D21	47	C to G	AGT	GCG	TTG	Ala to Gly
D22	91	G to T	GCG	GAA	GCT	Glu to Stop
D23	86	C to G	AAG	ACA	GCG	Thr to Arg
D24	132	G to T	TGG	AAG	AGG	Lys to Asn
D25	123	C to A	ATC	AGC	AGG	Ser to Arg
D26	196	G to C	GAC	GAC	ATG	Asp to His
D27	52	C to T	TTG	CTT	AAC	Leu to Phe
D28	64	G to C	ATC	GCA	ATG	Ala to Pro
D29	163	C to T	CTG	CTT	GCT	Leu to Phe
D30[a]	132	G to T	TGG	AAG	AGG	Lys to Asn
D31	125	G to A	AGC	AGG	TGG	Arg to Lys
D32	196	G to A	GAC	GAC	ATG	Asp to Asn
D33	179–184	−G	TGG	GGG	GTC	Frameshift
D34	148	A to C	CCA	AAG	TTC	Lys to Gln
D35	117	G to T	TCG	CAG	ATC	Gln to His
D36	141	G to T	GAC	TGG	ATT	Trp to Cys
D37	103	G to T	GGC	GTT	GAT	Val to Phe
D38	160	C to A	ATG	CTG	CTT	Leu to Met
D39	125–126	GG to TT	AGC	AGG	TGG	Arg to Ile
D40	166	G to C	CTT	GCT	GTT	Ala to Pro

[a] Ascribable to the same mutation obtained in an identical mouse.

assay and/or *cII* assay. The 4,10-DAC showed the highest mutagenicity among the test compounds, and the highest *lacZ* mutant frequency of 4,10-DAC, observed in the lung, was 19-, 6- and 4-fold over the spontaneously, chrysene- and 1,10-DAC-induced frequencies, respectively. The highest *lacZ* mutant frequency of 1,10-DAC was also observed in the lung. Mutant frequencies obtained with chrysene were not different between the organs examined and ranged from 1.5- to 3-fold over the spontaneous frequency. The mutant frequencies in the *cII* assay showed a tendency similar to those in the *lacZ* assay.

3.2. Mutation spectra of DACs and chrysene in the liver and lung

Thirty-six and 38 control mutants in the liver and lung, respectively, were subjected to sequence analysis, together with 37 and 39 chrysene-induced mutants, 40 and 43 induced mutants of 1,10-DAC and 40

Table 4
Sequences of *cII* mutations in the liver of chrysene-treated Muta™Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
B1	210	G to T	CGA	TTG	GCG	Leu to Phe
B2	214	C to T	GCG	CGA	CAA	Arg to Stop
B3	107	A to G	GTT	GAT	AAG	Asp to Gly
B4	150	G to T	CCA	AAG	TTC	Lys to Asn
B5	113	C to T	AAG	TCG	CAG	Ser to Leu
B6	220	G to T	CAA	GTT	GCT	Val to Phe
B7	132	G to T	TGG	AAG	AGG	Lys to Asn
B8	34	C to T	CTA	CGA	ATC	Arg to Stop
B9	40	G to A	ATC	GAG	AGT	Glu to Lys
B10	272–273	−A	TCT	GAA	CAA	Frameshift
B11	190–198	−GAC	GAC	GAC	GAC	Deletion
B12	107	A to G	GTT	GAT	AAG	Asp to Gly
B13	42	G to T	ATC	GAG	AGT	Glu to Asp
B14	125	G to T	AGC	AGG	TGG	Arg to Met
B15	57	C to A	CTT	AAC	AAA	Asn to Lys
B16	141	G to C	GAC	TGG	ATT	Trp to Cys
B17[a]	190–198	−GAC	GAC	GAC	GAC	Frameshift
B18	74	G to A	CTT	GGA	ACT	Gly to Glu
B19	86	C to G	AAG	ACA	GCG	Thr to Arg
B20	196	G to C	GAC	GAC	ATG	Asp to His
B21	91	G to T	GCG	GAA	GCT	Glu to Stop
B22	178	T to G	GAA	TGG	GGG	Trp to Gly
B23	212	C to A	TTG	GCG	CGA	Ala to Glu
B24	127	T to A	AGG	TGG	AAG	Trp to Arg
B25	88	G to C	ACA	GCG	GAA	Ala to Pro
B26	196	G to A	GAC	GAC	ATG	Asp to Asn
B27	129	G to T	AGG	TGG	AAG	Trp to Cys
B28	88	G to A	ACA	GCG	GAA	Ala to Thr
B29	99–101	−G	GTG	GGC	GTT	Frameshift
B30	212	C to A	TTG	GCG	CGA	Ala to Glu
B31	124	A to T	AGC	AGG	TGG	Arg to Trp
B32	179–184	−G	TGG	GGG	GTC	Frameshift
B33	91	G to T	GCG	GAA	GCT	Glu to Stop
B34	101	G to A	GTG	GGC	GTT	Gly to Asp
B35	89	C to T	ACA	GCG	GAA	Ala to Val
B36	89	C to A	ACA	GCG	GAA	Ala to Glu
B37	163	C to G	CTG	CTT	GCT	Leu to Val

[a] Ascribable to the same mutation obtained in an identical mouse.

Table 5
Sequences of *cII* mutations in the liver of control Muta™Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
A1	40	G to A	ATC	GAG	AGT	Glu to Lys
A2	205–213	−CGATTGGCG				Deletion
A3	34	C to T	CTA	CGA	ATC	Arg to Stop
A4	115	C to A	TCG	CAG	ATC	Gln to Lys
A5	212	C to T	TTG	GCG	CGA	Ala to Val
A6	89	C to A	ACA	GCG	GAA	Ala to Glu
A7	119–208	−ATGGCTCGAT				Deletion
A8	122	G to T	ATC	AGC	AGG	Ser to Ile
A9	233	T to C	ATT	CTC	ACC	Leu to Pro
A10	212	C to T	TTG	GCG	CGA	Ala to Val
A11[a]	89	C to A	ACA	GCG	GAA	Ala to Glu
A12	150	G to T	CCA	AAG	TTC	Lys to Asn
A13	205	C to T	GCT	CGA	TTG	Arg to Stop
A14	95	C to A	GAA	GCT	GTG	Ala to Asp
A15	89	C to T	ACA	GCG	GAA	Ala to Val
A16	212	C to T	TTG	GCG	CGA	Ala to Val
A17	122	G to T	ATC	AGC	AGG	Ser to Ile
A18[a]	150	G to T	CCA	AAG	TTC	Lys to Asn
A19	113	C to T	AAG	TCG	CAG	Ser to Leu
A20	210	G to T	CGA	TTG	GCG	Leu to Phe
A21	212	C to T	TTG	GCG	CGA	Ala to Val
A22	34	C to T	CTA	CGA	ATC	Arg to Stop
A23	110	A to T	GAT	AAG	TCG	Lys to Met
A24	241–246	−A	AAA	AAA	CGC	Frameshift
A25	196	G to A	GAC	GAC	ATG	Asp to Asn
A26[a]	34	C to T	CTA	CGA	ATC	Arg to Stop
A27	107	A to C	GTT	GAT	AAG	Asp to Ala
A28	89	C to T	ACA	GCG	GAA	Ala to Val
A29	196	G to A	GAC	GAC	ATG	Asp to Asn
A30	212	C to T	TTG	GCG	CGA	Ala to Val
A31	3	G to A	–	ATG	GTT	Met to Ile
A32	179–184	−G	TGG	GGG	GTC	Frameshift
A33	95	C to G	GAA	GCT	GTG	Ala to Gly
A34	110	A to C	GAT	AAG	TCG	Lys to Thr
A35[a]	89	C to T	ACA	GCG	GAA	Ala to Val
A36[a]	110	A to C	GAT	AAG	TCG	Lys to Thr

[a] Ascribable to the same mutation obtained in an identical mouse.

and 42 induced mutants of 4,10-DAC in the respective two organs. The mutations are characterized in Tables 2–9 and summarized in Tables 10 and 11. In Tables 10 and 11, the same mutations from an identical mouse were treated as a single event.

In the liver, spontaneous mutations consisted mainly of G:C to A:T transitions (14/30) followed by G:C to T:A transversions (7/30) as shown in the previous report on the *cII* mutant spectrum in the liver of control Muta™Mouse [9,26].

The majority of chrysene-induced mutations were G:C to T:A transversions (13/36), followed by the G:C to A:T transitions (9/36). Both 1,10-DAC- and 4,10-DAC-induced mutations also consisted mainly of G:C to T:A transversions (17/38 and 18/38, respectively). The *cII* mutant spectra by all the test compounds in the lung showed a tendency similar to those in the liver.

4. Discussion

1,10-DAC and 4,10-DAC, the tetracyclic azaPAHs and diaza-analogs of chrysene, could not be converted to the bay-region diol epoxide form because of their nitrogen atoms in the benzene rings of the bay-region

Table 6
Sequences of *cII* mutations in the lung of 1,10-DAC-treated Muta™Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
c1	163–164	CT to AA	CTG	CTT	GCT	Leu to Asn
c2	215	G to C	GCG	CGA	CAA	Arg to Pro
c3	132	G to T	TGG	AAG	AGG	Lys to Asn
c4	79	G to T	ACT	GAG	AAG	Glu to Stop
c5	160–161	CT to AG	ATG	CTG	CTT	Leu to Arg
c6	178	T to A	ATG	CTG	CTT	Trp to Arg
c7	100–101	GG to AT	GTG	GGC	GTT	Gly to Ile
c8	65	C to A	ATC	GCA	ATG	Ala to Glu
c9	150	G to T	CCA	AAG	TTC	Lys to Asn
c10[a]	79	G to T	ACT	GAG	AAG	Glu to Stop
c11	132	G to T	TGG	AAG	AGG	Lys to Asn
c12	34	C to T	CTA	CGA	ATC	Arg to Stop
c13	140	G to C	GAC	TGG	ATT	Trp to Ser
c14	179–184	−G	TGG	GGG	GTC	Frameshift
c15	212	C to T	TTG	GCG	CGA	Ala to Val
c16	141	G to T	GAC	TGG	ATT	Trp to Cys
c17	62	T to C	AAA	ATC	GCA	Ile to Thr
c18	196	G to T	GAC	GAC	ATG	Asp to Tyr
c19	212	C to G	TTG	GCG	CGA	Ala to Gly
c20	42	G to T	ATC	GAG	AGT	Glu to Asp
c21	160	C to A	ATG	CTG	CTT	Leu to Met
c22	215	G to C	GCG	CGA	CAA	Arg to Pro
c23	127	T to G	AGG	TGG	AAG	Trp to Gly
c24	64	G to T	ATC	GCA	ATG	Ala to Ser
c25	100	G to A	GTG	GGC	GTT	Gly to Ser
c26	111	G to T	GAT	AAG	TCG	Lys to Asn
c27	62	T to G	AAA	ATC	GCA	Ile to Ser
c28	196	G to T	GAC	GAC	ATG	Asp to Tyr
c29	179–184	−G	TGG	GGG	GTC	Frameshift
c30	122	G to T	ATC	AGC	AGG	Ser to Ile
c31	126	G to C	AGC	AGG	TGG	Arg to Ser
c32	128	G to T	AGG	TGG	AAG	Trp to Leu
c33	161	T to A	ATG	CTG	CTT	Leu to Gln
c34	129	G to T	AGG	TGG	AAG	Trp to Cys
c35	179–184	−G	TGG	GGG	GTC	Frameshift
c36	164–165	−T	CTG	CTT	GCT	Frameshift
c37	150	G to T	CCA	AAG	TTC	Lys to Asn
c38	178	T to G	GAA	TGG	GGG	Trp to Gly
c39	167	C to G	CTT	GCT	GTT	Ala to Gly
c40	106	G to T	GTT	GAT	AAG	Asp to Tyr
c41	51	G to C	GCG	TTG	CTT	Leu to Phe
c42	133	A to T	AAG	AGG	GAC	Arg to Trp
c43	169	G to C	GCT	GTT	CTT	Val to Leu

[a] Ascribable to the same mutation obtained in an identical mouse.

epoxide or diol moiety. Nevertheless, 1,10-DAC and 4,10-DAC showed in vitro mutagenicity in the Ames tests using rat liver S9 or human liver microsomes in our previous study [17]. Therefore, these DACs might be converted to ultimate mutagenic forms by a mechanism different from that of chrysene. We have investigated the in vivo mutagenicity of some aza-PAHs, such as quinoline and 10-azaBaP [5,8–10,15]. The 1,10-DAC and 4,10-DAC consist of two quinoline moieties and also have a structure similar to 10-azaBaP. In the present study, we attempted to investigate the in vivo mutagenicity of DACs compared with chrysene

Table 7
Sequences of *cII* mutations in the lung of 4,10-DAC-treated Muta™Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
d1	123	C to A	ATC	AGC	AGG	Ser to Arg
	138	C to A	AGG	GAC	TGG	Asp to Glu
d2	29	C to A	GAG	GCT	CTA	Ala to Asp
d3	117	G to T	TCG	CAG	ATC	Gln to His
d4	212	C to T	TTG	GCG	CGA	Ala to Val
d5	125	G to T	AGC	AGG	TGG	Arg to Met
d6	160	C to A	ATG	CTG	CTT	Leu to Met
d7	128	G to T	AGG	TGG	AAG	Trp to Leu
d8	293	G to T	TTC	TGA	–	Stop to Leu
d9	220	G to T	CAA	GTT	GCT	Val to Phe
d10	57	C to A	CTT	AAC	AAA	Asn to Lys
d11	111	G to T	GAT	AAG	TCG	Lys to Asn
d12	115	C to A	TCG	CAG	ATC	Gln to Lys
d13	51	G to T	GCG	TTG	CTT	Leu to Phe
d14	179–184	−G	TGG	GGG	GTC	Frameshift
d15	115	C to T	TCG	CAG	ATC	Gln to Stop
d16	88	G to C	ACA	GCG	GAA	Ala to Pro
d17	132	G to T	TGG	AAG	AGG	Lys to Asn
d18	117	G to T	TCG	CAG	ATC	Gln to His
d19	65	C to A	ATC	GCA	ATG	Ala to Glu
d20[a]	115	C to A	TCG	CAG	ATC	Gln to Lys
d21[a]	88	G to C	ACA	GCG	GAA	Ala to Pro
d22	145	C to G	ATT	CCA	AAG	Pro to Ala
d23	103	G to T	GGC	GTT	GAT	Val to Phe
d24	167	C to G	CTT	GCT	GTT	Ala to Gly
d25	212	C to G	TTG	GCG	CGA	Ala to Gly
d26	163	C to T	CTG	CTT	GCT	Leu to Phe
d27	224	C to A	GTT	GCT	GCG	Ala to Asp
d28	175	G to T	CTT	GAA	TGG	Glu to Stop
d29	40	G to A	ATC	GAG	AGT	Glu to Lys
d30	160	C to A	ATG	CTG	CTT	Leu to Met
d31[a]	103	G to T	GGC	GTT	GAT	Val to Phe
d32	89	C to T	ACA	GCG	GAA	Ala to Val
d33	179–184	−G	TGG	GGG	GTC	Frameshift
d34	95	C to A	GAA	GCT	GTG	Ala to Asp
d35	134	G to T	AAG	AGG	GAC	Arg to Met
d36	101	G to T	GTG	GGC	GTT	Gly to Val
d37	34	C to T	CTA	CGA	ATC	Arg to Stop
d38	129	G to T	AGG	TGG	AAG	Trp to Cys
d39	89	−C	ACA	GCG	GAA	Frameshift
d40	125	G to T	AGC	AGG	TGG	Arg to Met
d41	65	C to A	ATC	GCA	ATG	Ala to Glu
d42	122	G to T	ATC	AGC	AGG	Ser to Ile

[a] Ascribable to the same mutation obtained in an identical mouse.

to understand the nature of the in vivo mutagenicity of azaPAHs.

Table 12 summarizes the in vivo mutagenicity of a series of azaPAHs with the pyridine moiety, investigated in our previous and present studies using Muta™Mouse. One of the authors (T.S.) reported that the median toxic dose (TD_{50}) of chemicals as used for numerical description of their carcinogenic potency is well correlated with in vivo mutagenic potency, which was calculated by division of the chemical-induced mutant frequency by the total injection dose of the chemical in the transgenic mouse [27]. We calculated

Table 8
Sequences of *cII* mutations in the lung of chrysene-treated Muta™Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
b1	112	T to C	AAG	TCG	CAG	Ser to Pro
b2	89	C to T	ACA	GCG	GAA	Ala to Val
b3	34	C to T	CTA	CGA	ATC	Arg to Stop
b4	141	G to A	GAC	TGG	ATT	Trp to Stop
b5	94	G to C	GAA	GCT	GTG	Ala to Pro
b6	126	G to C	AGC	AGG	TGG	Arg to Ser
b7	214	C to T	GCG	CGA	CAA	Arg to Stop
b8[a]	34	C to T	CTA	CGA	ATC	Arg to Stop
b9	190–198	−GAC	GAC	GAC	GAC	Deletion
b10	106	G to T	GTT	GAT	AAG	Asp to Tyr
b11	113	C to A	AAG	TCG	CAG	Ser to Stop
b12	101	G to T	GTG	GGC	GTT	Gly to Val
b13	122	G to T	ATC	AGC	AGG	Ser to Ile
b14	196	G to T	GAC	GAC	ATG	Asp to Tyr
b15[a]	113	C to A	AAG	TCG	CAG	Ser to Stop
b16[a]	190–198	−GAC	GAC	GAC	GAC	Deletion
b17	34	C to T	CTA	CGA	ATC	Arg to Stop
b18	215	G to C	GCG	CGA	CAA	Arg to Pro
b19	113	C to T	AAG	TCG	CAG	Ser to Leu
b20	233	T to A	ATT	CTC	ACC	Leu to His
b21	104	T to G	GGC	GTT	GAT	Val to Gry
b22	42	G to T	ATC	GAG	AGT	Glu to Asp
b23	25	G to T	AAC	GAG	GCT	Glu to Stop
b24	117	G to T	TCG	CAG	ATC	Gln to His
b25	233	−T	ATT	CTC	ACC	Frameshift
b26	123	C to G	ATC	AGC	AGG	Ser to Arg
b27	169	G to C	GCT	GTT	CTT	Val to Leu
b28[a]	215	G to C	GCG	CGA	CAA	Arg to Pro
b29	155	C to T	TTC	TCA	ATG	Ser to Leu
b30	26	A to G	AAC	GAG	GCT	Glu to Gly
b31	34	C to T	CTA	CGA	ATC	Arg to Stop
b32	40	G to A	ATC	GAG	AGT	Glu to Lys
b33	117	G to T	TCG	CAG	ATC	Gln to His
b34	179–184	−G	TGG	GGG	GTC	Frameshift
b35	163	C to A	CTG	CTT	GCT	Leu to Ile
b36	150	G to T	CCA	AAG	TTC	Lys to Asn
b37	94	G to C	GAA	GCT	GTG	Ala to Pro
b38	221	T to C	CAA	GTT	GCT	Val to Ala
b39	73	G to A	CTT	GGA	ACT	Gly to Arg

[a] Ascribable to the same mutation obtained in an identical mouse.

the mutagenic activity by the following formula to compare the mutagenicity between azaPAHs:

fold-increase in *lacZ* MF (%) = *lacZ* MF obtained by test chemical/spontaneous *lacZ* MF × 100.
Mutagenic activity = fold-increase in *lacZ* MF/total dose of test chemical.

The organs given in capital letters in Table 12 indicate those that showed significant mutation and those given in small letters indicate negative organs. The underlined organs are those which showed the highest increase in *lacZ* MF. When the test chemical induced mutation in multiple organs, the mutagenic activity was calculated using the data of the underlined organs.

Although the five azaPAHs tested in our previous reports (quinoline, benzo[*f*]quinoline, benzo[*h*]quinoline, 1,7-phenanthroline and 10-azaBaP) induced mutation only in the specific organ such as the liver, 1,10-DAC and 4,10-DAC showed significant mutant frequencies in all of the six organs examined and the

Table 9
Sequences of *cII* mutations in the lung of control Muta™Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
a1	146	C to T	ATT	CCA	AAG	Pro to Leu
a2	196	G to A	GAC	GAC	ATG	Asp to Asn
a3	113	C to T	AAG	TCG	CAG	Ser to Leu
a4	217	C to T	CGA	CAA	GTT	Gln to Stop
a5	180	G to A	GAA	TGG	GGG	Trp to Stop
a6	73	G to A	CTT	GGA	ACT	Gly to Arg
a7	132	G to T	TGG	AAG	AGG	Lys to Asn
a8	58–61	−A	AAC	AAA	ATC	Frameshift
a9	241–246	−A	AAA	AAA	CGC	Frameshift
a10	149–164	−AGTTCTCAATGCTGCT				Deletion
a11	25	G to T	AAC	GAG	GCT	Glu to Stop
a12	214	C to T	GCG	CGA	CAA	Arg to Stop
a13	197	A to G	GAC	GAC	ATG	Asp to Gly
a14	111	G to T	GAT	AAG	TCG	Lys to Asn
a15	206	G to C	GCT	CGA	TTG	Arg to Pro
a16	212	C to T	TTG	GCG	CGA	Ala to Val
a17	38	T to G	CGA	ATC	GAG	Ile to Ser
a18	40	G to A	ATC	GAG	AGT	Glu to Lys
a19[a]	40	G to A	ATC	GAG	AGT	Glu to Lys
a20	34	C to T	CTA	CGA	ATC	Arg to Stop
a21	90–91	GG to T	GCG	GAA	GCT	Frameshift
a22	113	C to T	AAG	TCG	CAG	Ser to Leu
a23	140	G to A	GAC	TGG	ATT	Trp to Stop
a24	110	A to T	GAT	AAG	TCG	Lys to Met
a25	175	G to A	CTT	GAA	TGG	Glu to Lys
a26	172	C to T	GTT	CTT	GAA	Leu to Phe
a27	89	C to T	ACA	GCG	GAA	Ala to Val
a28[a]	34	C to T	CTA	CGA	ATC	Arg to Stop
a29	221	T to G	CAA	GTT	GCT	Val to Gly
a30	273–275	ACA to CGATGCACG				Insertion
a31	212	C to T	TTG	GCG	CGA	Ala to Val
a32	193	G to A	GAC	GAC	GAC	Asp to Asn
a33	34	C to T	CTA	CGA	ATC	Arg to Stop
a34	31	C to G	GCT	CTA	CGA	Leu to Val
a35	155	C to A	TTC	TCA	ATG	Ser to Stop
a36	128	G to A	AGG	TGG	AAG	Trp to Stop
a37	88	G to T	ACA	GCG	GAA	Ala to Ser
a38[a]	34	C to T	CTA	CGA	ATC	Arg to Stop

[a] Ascribable to the same mutation obtained in an identical mouse.

highest *lacZ* mutant frequency in the lung. Chrysene also significantly induced mutation in all of the six organs examined, although chrysene showed similar mutant frequencies in all the six organs. These results suggest that nitrogen substitution in the chrysene skeleton may enhance the inducibility of mutation in the mouse lung.

4,10-DAC showed the highest mutagenic activity, followed in order by: quinoline > 1,7-phenenthroline ≈ 1,10-DAC > benzo[*h*]quinoline ≈ 10-azaBaP = benzo[*f*]quinoline. The number of mutagenic activity of chrysene is the same as that of benzo[*f*]quinoline, which shows the lowest mutagenic activity among the series of azaPAHs tested. The 4,10-DAC gave mutant frequencies higher than 1,10-DAC in all the test organs and the increase in *lacZ* MF obtained by 4,10-DAC is more than twice that by 1,10-DAC in most of the organs tested. Therefore, these results suggest that the nitrogen-substituted position affects the strength of mutagenicity of the DAC structure.

Table 10
Summary of *cII* mutation spectra in the liver of Muta™Mouse

Mutation class	Control (%)	1,10-DAC (%)	4,10-DAC (%)	Chrysene (%)
Total	30 (100)	38 (100)	38 (100)	36 (100)
Base substitution	26 (87)	35 (92)	35 (92)	32 (89)
Transitions				
GC to AT	14 (47)	7 (18)	8 (21)	9 (25)
AT to GC	1 (3)	2 (5)	0 (0)	2 (6)
Transversions				
AT to TA	1 (3)	3 (8)	1 (3)	2 (6)
AT to CG	2 (7)	1 (3)	1 (3)	1 (3)
GC to TA	7 (23)	17 (45)	18 (47)	13 (36)
GC to CG	1 (3)	5 (13)	7 (18)	5 (14)
−1 Frameshifts	2 (7)	1 (3)	1 (3)	3 (8)
+1 Frameshifts	0 (0)	0 (0)	0 (0)	0 (0)
Deletion	2 (7)	1 (3)	0 (0)	1 (3)
Insertion	0 (0)	0 (0)	0 (0)	0 (0)
Complex	0 (0)	1 (3)	2 (5)	0 (0)

The same mutations from an identical mouse were counted as a single event.

We further attempted *cII* mutant spectrum analysis in the liver and lung, which were shown to give the highest *lacZ* mutant frequencies by chrysene and DACs, respectively. In both organs, the spontaneous mutations consisted mainly of G:C to A:T transitions and the mutant spectrum in the control Muta™Mice showed no difference between the liver and lung. The G:C to A:T transitions in the control mice are likely to be ascribable to the deamination of 5-methylcytosine at the CpG site as previously reported [28]. DACs predominantly induced the G:C to T:A transversion in both organs. It was especially noteworthy that there was no difference in the *cII* mutant spectrum of 4,10-DAC between the lung and liver in spite of the quantitative difference showed by 4,10-DAC with its 3.9-fold higher *cII* mutant frequency in the lung than in the liver. These results suggest that the high mutagenicity in the lung of 4,10-DAC-treated mice might not arise from

Table 11
Summary of *cII* mutation spectra in the lung of Muta™Mouse

Mutation class	Control (%)	1,10-DAC (%)	4,10-DAC (%)	Chrysene (%)
Total	35 (100)	41 (100)	39 (100)	35 (100)
Base substitution	30 (86)	34 (83)	35 (90)	32 (91)
Transitions				
GC to AT	19 (54)	3 (7)	6 (15)	10 (29)
AT to GC	1 (3)	1 (2)	0 (0)	3 (9)
Transversions				
AT to TA	1 (3)	3 (7)	0 (0)	1 (3)
AT to CG	2 (6)	3 (7)	0 (0)	1 (3)
GC to TA	5 (14)	16 (39)	25 (64)	11 (31)
GC to CG	2 (6)	8 (20)	4 (10)	6 (17)
−1 Frameshifts	2 (6)	4 (10)	3 (8)	2 (6)
+1 Frameshifts	0 (0)	0 (0)	0 (0)	0 (0)
Deletion	1 (3)	0 (0)	0 (0)	1 (3)
Insertion	0 (0)	0 (0)	0 (0)	0 (0)
Complex	2 (6)	3 (7)	1 (3)	0 (0)

The same mutations from an identical mouse were counted as a single event.