INTRODUCTION

The most frequently used in vivo mutational model systems are the commercially available Muta™ Mouse and Big Blue® Mouse. Similarities between these systems include the organization of very similar lambda transgenes in so-called head-to-tail arrays, the existence of roughly 40 copies per haploid genome, and their prokaryotic origin. There are some differences structurally and spatially with regard to the transgenes and their genetic background. Mice bearing the lacZ gene on chromosome 3 [Swiger et al., 1994], whereas lacI copies are integrated on chromosome 4 [Dycaico et al., 1994]. Similarities and differences notwithstanding, the systems appear to be equivalent in sensitivity to induced (both acute and chronic) and spontaneous mutational events.

The transgenes, which are used as surrogates for endogenous loci, do not share all of the properties of endogenous loci. For instance, they exist at high copy numbers, are eukaryotic in origin. Nevertheless, the transgenes provide a means of quantifying the processes of mutagenesis occurring in mammalian cells in vivo, in virtually all tissues, a property not imparted by any other mutagenic end point. Moreover, mutational response exhibited by the transgenes (lacI and lacZ) after acute treatment with various mutagens and clastogens is comparable to that displayed by endogenous loci within those tissues that permit such comparisons [Cosentino and Heddle, 1999; Tao et al., 1991; Skopek et al., 1995]. Surprisingly, despite a 10-fold range in target size, transcription status and sequence origin, the observed spontaneous and induced mutant frequencies in the small intestine of transgenic mice are remarkably similar.
mutated, yield the high frequency lysogeny (Hfl) phenotype. It is postulated that the two loci, hflA and hflB, are proteases directed at the CII protein. Although the mechanism is not fully understood, it is generally accepted that the hflA and B gene products inhibit the lytic repression function of CII at low temperatures [Kihara et al., 1997].

The CII locus provides a new transgenic surrogate for quantifying mutations sustained in vivo. Since Jakubczak's report, numerous papers supporting the use of the CII locus in the Big Blue® Mouse transgenic construct have appeared in literature (e.g., Monroe et al., 1998; Harbach et al., 1999; Zimmer et al., 1999). Recently, the CII selection assay has been successfully applied to the BigBlue® Rat2 embryonic cell line [Watson et al., 1998]. The major advantage of this locus for use with the Big Blue is the positive selection of mutants, which reduces the cost associated with the current assay.

Here, we report the first use of the CII locus residing on the λgt10 shuttle vector in MutaMouse. Since it is 5.9 kb away from the lacZ insertion site, the CII locus is independent of the lacZ with respect to mutational events that are captured by the system. This comparison of the mutational response between the CII and lacZ transgenes and that of the endogenous Dlb-1 locus in the small intestine after acute treatments with N-ethyl-N-nitosourea (ENU) suggests that the CII locus is a reliable target for mutagenesis studies.

The practical advantages of using the CII with MutaMouse is twofold; the first is the ease of sequencing this target for mutational spectra. The currently used lacZ is in excess of 3.0 kb, whereas the CII, at 294 bp, can be sequenced cost-effectively. The second advantage is an important and unmentioned application of the CII locus in both the Big Blue and MutaMouse systems, that is, the facile identification of jackpot mutations (cf. Heddle, 1999) without sequencing. By analyzing two independent loci in the same tissue, DNA, or cored plaque(s), jackpots may easily be eliminated in subsequent mutant frequency determinations.

**MATERIALS AND METHODS**

**CII Selection**

The CII analysis was conducted according to Stratagene’s manual, “λ Select-CII® Mutation Detection System for Big Blue® Rodents.” Briefly, high molecular weight DNA containing the λ transgenes was extracted from the small intestine and packaged in vitro (Transpack, Stratagene, La Jolla, CA). The reactions were arrested using 1.0 ml of SM phage buffer, vortexed vigorously, and placed on ice. Titer samples were prepared by diluting the packaged reaction 100-fold and adsorbing 50 μl of the diluted material into 200 μl strain 1250 E. coli (O.D.600 = 0.5). The remaining 990 μl of the packaging reactions were then adsorbed (100–150 μl) into 200 μl of an Hfl strain of E. coli (strain 1250, Stratagene). All adsorptions were plated on TB-1 top-agar plates (4 ml). Agar and casein peptone (#PEP 403) for use in TB-1 media, plates, and top-agar were obtained from BioShop Canada. Vitamin B₆ was obtained from Sigma (St. Louis, MO). Titer plates were placed at 37°C for 24 hr, whereas selection plates were placed at 23.5°C (±0.1°C) for 48 hr. The selection temperature (23.5 ± 0.1°C) was maintained reliably by placing an incubator in a cold room (6°C). The temperature was monitored using an LCD-Thermometer with memory (Cat. No. 63-859, Micronta®). Temperature minima and maxima were checked at the termination of each experiment. In order to reduce intrasample variance, all treated samples from each experiment were packaged on the same day. All control data were packaged simultaneously.

**CII Mutant Sequence Verification**

Two wild-type and 13 mutant CII plaques were randomly selected and cored, resuspended in 400 μl SM buffer and replated to ensure that the samples were wholly mutant or wild type at the CII locus. The cored plaque stocks were used as template for PCR reactions using standard PCR settings, MgCl₂ and buffer conditions and 1 μl cored suspension as template. The upstream primer sequence: 5’ AAT TAA ACC ACA CCT ATG GTG 3’; the downstream primer sequence: 5’ CCT CTG CCG AAG TTG AGT ATT T 3’. The PCR products (400 bp) were confirmed using standard agarose gel electrophoresis. The reactions were subsequently purified using Qiagen QIAquick PCR Purification Kit and were sequenced at the Molecular Core Facility at York University (Toronto, Canada) using the upstream primer.

**Reconstruction Experiment**

Cored wild-type (CII "X lacZ") and mutant CII (CII "X lacZ") plaques were grown in miniliquid lysis preparations (E. coli. 1250), confirmed wholly mutant or wild type at the CII locus by replating, and the spontaneous mutant frequency was determined. An average of 13.6 mutant phage particles were combined with various amounts of excess wild type and adsorbed into strain 1250 (10 mM MgSO₄); 4 ml of molten TB-1 top-agar was added to the adsorption tubes and plated.

**Ex Vivo Comparison With lacI**

Cored wild-type (CII "X lacI") plaques were grown in miniliquid lysis preparations (E. coli. 1250), confirmed wholly wild type at both loci by replating and the spontaneous mutant frequency was determined for each locus. The phage preparation contained 2 × 10⁸ plaque forming units (pfu)/ml. Phages were treated by placing 10.0 ml of the stock preparation in 60 mm² petri plates (5 ml/plate) on ice under standard (30w G30T8 General Electric) germicidal lamp for 23 min (540 JM –2). Titers for treated and nontreated samples were then plated for lacI and CII mutations. LacI mutations were identified using the Big Blue protocol as per manufacturer instructions (Stratagene).

**Cell Culture**

Big Blue mouse cells were seeded and maintained in DMEM supplemented with 10% FBS. Cultures were treated with ENU (200 μg/ml) in DMEM, pH = 7.0 for 30 min at 37°C. The cultures were washed once with serum-free media and then incubated in 10% FBS DMEM. The cells were quick-frozen with liquid nitrogen at time zero and 4 days after treatment.

**lacZ and CII Analysis of DNA From MutaMouse**

DNA from the small intestine of treated and control animals was purified using phenol chloroform extractions. DNA was resuspended in TE buffer and packaged in vitro. Phages were adsorbed into same day “plating bacteria” cultures (CII, 1250; lacZ, C600) and plated on TB-1 or LB plates as appropriate. The lacZ selection consisted of LB-top agar containing 0.5% phenyl-β-galactopyranoside.
Animals

Nontransgenic SWR mice (homozygous for \(Dlb-1\)) were crossed with the \(\text{lacZ}^{\text{Muta™}}\) Mouse (homozygous for \(Dlb-1\)) to obtain an F\(_1\) generation that were hemizygous for the \(\text{lacZ}\) transgene and heterozygous at the \(Dlb-1\) locus (\(Dlb-1/b/Dlb-1/a\)) because the experiments were designed to compare the endogenous \(Dlb-1\) locus and the \(\text{lacZ}\) transgene. F\(_1\) animals used were 3–4 months old at the start of all experiments. An independent Animal Care Committee at York University approved all experimental protocols used in this study.

Treatments

All animals were treated with \(N\)-ethyl-\(N\)-nitrosourea (ENU).

Dose response. All animals except controls were treated acutely with single ip injections of ENU: 50, 150, or 250 mg/kg dissolved in (9:1) PBS:DMSO. There were six animals (three male, three female) per time point. All animals were sacrificed and tissues collected 10 days posttreatment.

Time course. All animals, except controls, were treated with a single ip injection of 250 mg/kg ENU dissolved in (9:1) PBS:DMSO. There were six animals (three male, three female) per time point. All animals were sacrificed and tissues collected at 10 or 70 days posttreatment.

Controls. Animals were injected with solvent only. Control animals were sacrificed and tissues were collected at the appropriate times. To increase the number of control samples, controls from other experiments have been included. Such additional control samples are identified in the results.

RESULTS

Selection Verification

Selection in the \(cII\) system relies on the bacterial host strain 1250 (\(Hfl\)) and low temperature (<24°C). In order to ensure that mutants arising on selection plates were in fact wholly mutant (sustained in vivo), a time course sample (ip, 250 mg/kg ENU) was randomly selected, packaged, and plated for mutants. Sixty plaques were randomly cored from selection plates and were quantitatively replated at low titers. The total number of plaques on selection plates did not differ significantly from the replicas plated at 37°C (lytic permissive temperature; data not shown).

\(cII\) Sequence Analysis

The \(cII\) locus on the Mutamouse transgene construct was sequenced and found to be identical to that reported previously for the Big Blue (Stratagene manual). The results from the sequencing reactions shown in Table I confirmed a high level of selection stringency and verified the positive selection of \(cII\) mutants. All 13 cored plaques arising on selection plates contained mutations at the \(cII\) locus. The mutation site (bp) position was recorded according to the nucleotide numbering convention adopted from Swartz et al. [1978].

Reconstruction Experiment

Reconstructions are used to determine the efficiency and limits to which mutant phage can be recovered while in the presence of vast excesses of wild-type phages. Because the wild-type preparation had a spontaneous mutant frequency equal to \(1.6 \times 10^{-4}\), the predicted mutant contribution from the wild-type preparation was subtracted from the observed total mutant plaques counted on selection plates. As shown in Figure 1, the average recovery of mutants is 82% over the range shown, with no systematic variation as a function of phage density. Recovery was severely reduced at a ten-fold higher phage density.

### TABLE I. \(cII\) Mutant Sequence Verification

<table>
<thead>
<tr>
<th>Mutant*</th>
<th>(cI)</th>
<th>(cII)</th>
<th>Mutant bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type-1</td>
<td>✓</td>
<td>✓</td>
<td>None</td>
</tr>
<tr>
<td>Wild Type-2</td>
<td>✓</td>
<td>✓</td>
<td>None</td>
</tr>
<tr>
<td>ENU-1</td>
<td>✓</td>
<td>✓</td>
<td>366</td>
</tr>
<tr>
<td>ENU-2</td>
<td>✓</td>
<td>✓</td>
<td>482</td>
</tr>
<tr>
<td>ENU-3</td>
<td>✓</td>
<td>✓</td>
<td>389</td>
</tr>
<tr>
<td>ENU-4</td>
<td>✓</td>
<td>✓</td>
<td>478</td>
</tr>
<tr>
<td>ENU-5</td>
<td>✓</td>
<td>✓</td>
<td>549</td>
</tr>
<tr>
<td>ENU-6</td>
<td>✓</td>
<td>✓</td>
<td>548</td>
</tr>
<tr>
<td>ENU-7</td>
<td>✓</td>
<td>✓</td>
<td>431</td>
</tr>
<tr>
<td>ENU-8</td>
<td>✓</td>
<td>✓</td>
<td>501</td>
</tr>
<tr>
<td>ENU-9</td>
<td>✓</td>
<td>✓</td>
<td>411</td>
</tr>
<tr>
<td>ENU-10</td>
<td>✓</td>
<td>✓</td>
<td>478</td>
</tr>
<tr>
<td>ENU-11</td>
<td>✓</td>
<td>✓</td>
<td>365</td>
</tr>
<tr>
<td>ENU-12</td>
<td>✓</td>
<td>✓</td>
<td>549</td>
</tr>
<tr>
<td>ENU-13</td>
<td>✓</td>
<td>✓</td>
<td>367</td>
</tr>
</tbody>
</table>

*Wild type or mutant plaques (ENU series) were sequenced across the \(cII\) locus. One plaque from one selection plate from different animals treated with ENU were randomly cored, PCR amplified, and sequenced. All mutations represent unique events. The \(cII\) in Mutamouse was found to be identical to that reported previously for the Big Blue.
Ex Vivo, Comparison cII vs. lacI

UV-treated samples experienced a 120-fold loss of viability. As shown in Figure 2, a significant increase in mutant frequency (15-fold) was observed at the lacI locus ($\chi^2 = 209; df = 1; P < 0.001$). Upon plating the same phage preparations for cII selection, no significant increase in mutant frequency was observed. The spontaneous mutant frequency at lacI and cII differed by only 0.1%.

Reproducibility Experiments

The cII mutant frequencies were determined in two independent trials (Fig. 3). The combined mutant frequencies were in agreement, with an average correlation of 79%. The reliability per data point had a range of 38–106%. The titers varied from 35–179% (average = 85%). The reproducibility of the cII selection in this study is within the performance range of the lacZ, as reported by the Collaborative Study Group for the Transgenic Mouse Mutation Assay [EMS Japan, 1996].

**SWR X lacZ F₁ Treatments**

Figures 4 and 5 display the induced mutant frequencies at the cII and lacZ loci from the small intestine of MutaMice treated with ENU. As shown in the figures, the mutational response of the cII is comparable to that of the lacZ. Figure 4 displays the results from a time course study where the induced mutant frequency (average = $137.5 \times 10^{-5}$) at the cII locus reached a maximum 10 days after treatment (plateau), with no significant change ($F = 4.75, df = 1, 9, P < 0.05$) with respect to time thereafter. As shown in Figure 5, the observed mutant frequency at the cII locus is linear ($R^2 = 0.93$) with respect to 0, 50, 150, and 250 mg/kg doses of ENU.

**cII vs. lacZ Spontaneous Mutant Frequency in MutaMouse**

Figure 6 shows the spontaneous mutant frequency resulting from animals that were used as controls for the time course and dose response studies. The spontaneous mutant frequency observed at cII and lacZ were similar, 5.5 and $8.2 \times 10^{-5}$, respectively.
A nontreated control animal from a feeding study was identified as having an unusually high spontaneous mutant frequency after *lacZ* analysis on DNA isolated from the mammary gland, small intestine, and colon (average in the three tissues $5.27 \times 10^{-5}$). The DNA from the mammary and small intestine was packaged and plated for *cII* mutations and was found to display a mutant frequency within the spontaneous range (Table II).

**DISCUSSION**

We analyzed the DNA from MutaMouse X SWR F1 after treatment with ENU, a powerful mutagen in vivo. Figures 4, 5, and 6 display the mutant frequencies at the *cII* and *lacZ* loci resulting from animals treated with various doses of ENU. The mutant frequencies at the *cII* and *lacZ* loci obtained from the dose response and time course treatments appear to be equivalent with respect to mutational response and sensitivity.

The issue of genetic neutrality as it relates to transgenic systems is an important one. A neutral locus is defined as one in which a mutation confers neither selective advantage nor disadvantage to the fitness of the host cell. This is an advantageous property of the current transgenes (*lacI* and *lacZ*) and is shared by the *cII*, as evidenced by a plateau in mutant frequency after the mutational maximum has been achieved (Fig. 4). Further evidence supporting the *cII* locus as being neutral in the MutaMouse system is obtained from the dose response data, where acute treatment of ENU (ip) resulted in a linear dose-response relationship (Fig. 5).

The spontaneous *cII* mutant frequency observed in animals within the control groups for the dose response and time course experiments reside at roughly $5.5 \times 10^{-5}$ (Fig. 6). This is roughly two-thirds of the mutant frequency at the

---

**Table II. Jackpot Analysis**

<table>
<thead>
<tr>
<th>Tissue sample</th>
<th>Pfu plated</th>
<th>Mutants</th>
<th>Mutant frequency a (per 100,000 plaques)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammary gland</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>lacZ</em></td>
<td>81,000</td>
<td>237</td>
<td>293.0</td>
</tr>
<tr>
<td><em>cII</em></td>
<td>161,525</td>
<td>2</td>
<td>1.2</td>
</tr>
<tr>
<td>Small intestine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>lacZ</em></td>
<td>41,750</td>
<td>106</td>
<td>254.0</td>
</tr>
<tr>
<td><em>cII</em></td>
<td>554,216</td>
<td>15</td>
<td>2.7</td>
</tr>
<tr>
<td>Colon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>lacZ</em></td>
<td>268,750</td>
<td>720</td>
<td>268.0</td>
</tr>
<tr>
<td><em>cII</em></td>
<td>41,321</td>
<td>5</td>
<td>12.1</td>
</tr>
</tbody>
</table>

*a* DNA from a control MutaMouse animal displayed unusually high mutant frequencies in the mammary gland, small intestine, and colon upon *lacZ* analysis (p-gal selection). The same DNAs were then repackaged and *cII* analysis was applied. The resulting mutant frequencies at the *cII* locus were found to be in the expected spontaneous range, thus confirming the existence of a “jackpot” mutation at the *lacZ* locus.

*b* Low packaging efficiency.
lacZ locus in the same animals and is comparable to the historical data from lacI (Big Blue) and lacZ (MutaMouse) control animals used in our laboratory.

The transgene construct (λgt10) used to create the strain may have sustained a silent mutation at the cII locus during the cloning of the lacZ target, or possibly subsequent to genomic integration. The MutaMouse transgenic strain (40.6) was produced by microinjection of λgt10, which is similar to the λLiz used to create the Big Blue Mouse. The significant advantage of cII analysis in the Big Blue system is the positive selection of mutants. An additional advantage for Big Blue users and possibly most significant advantage of cII analysis in relation to the MutaMouse transgenic system, is the locus target size, which is 294 bp (roughly one-tenth of that of the lacZ), allowing it to be sequenced easily and cost-effectively. Sequence data are sorely needed information in the MutaMouse and have been explored in great detail using the lacI system [de Boer and Glickman, 1998; refer to http://darwin.ceb.uvic.ca/bigblue/bigblue.htm]. Moreover, sequence data regarding the spectrum of cII mutations in the Big Blue have already begun to be reported [Watson et al., 1998; Harbach et al., 1999]. Two wild-type cII cored MutaMouse plaques were sequenced confirming the cII to be identical in both transgenic systems, as originally reported by Schwarz et al. [1978]. Likewise, selection of cII mutants was confirmed using sequence analysis. Although the sequence analysis was limited, all randomly selected putative mutants cored from selection plates were found to be cII mutants after sequencing (Table I). It is possible that non-cII mutants may arise on selection plates as a result of mutational events occurring elsewhere in the lambda construct [Szalewska-Palasz et al., 1996].

Ex vivo events, leading to mosaic plaques, are readily observed using the Big Blue lacI phage and SCS bacteria when plated on top-agar containing the chromogenic substrate X-gal [Skopek et al., 1996; Paashuis–Lew et al., 1997]. Blue plaques resulting from host cells containing a mixture of wild-type and mutant lacI phages appear blue; after replating cored plaques at low densities, roughly half the progeny plaques will be blue (lacI+), whereas the other half will remain colorless (lacI–). Figure 2A shows that ex vivo mutations cannot be recovered as mutant events using cII selection. As exhibited by the figure, although UV exposure (23 min, 540 JM–2) induced lesions (observed on the lacI), such damage does not affect the mutant frequencies obtained during the selection process in the cII system. This may be explained by the nature of the damage sustained and the nature of the selection, for a mixture of wild-type and mutant CII will result in the cII phenotype. In another experiment, shown in Figure 2B, a Big Blue Mouse cell culture was treated with ENU and analyzed for cII mutations both immediately after treatment and 4 days after treatment. A five-fold induction was found in the cultures after 4 days, whereas immediately after treatment there was no induction of mutations, showing that no cII mutations arose in the bacterial growth phase from adducts present in the DNA. We know the DNA from these cells contained adducts, since the lacI mutation frequency was elevated (data not shown).

Another approach to verify the stringency found with cII selection entailed the use of the Big Blue Mouse cell line. By treating a cell culture with ENU, lesions or adducts cannot be fixed in the host cells at time zero and remain stable in a quiescent state, whereas actively dividing cells replicate and “fix” mutations. In dividing cell cultures (10% FBS serum) an induction of 7.5-fold was observed using cII selection, whereas no noticeable increase was observed in nondividing (serum-free) cell cultures (Fig. 2B).

A powerful use of the cII locus in MutaMouse (and Big Blue) is the determination of jackpot mutations. So-called jackpot mutations have been an issue when interpreting data generated from the transgenic models (cf. Heddle, 1999). Briefly, a jackpot mutation is one that most likely occurred during development and early growth. Such an event may result in an exceedingly high spontaneous mutant frequency (as high as 1000 times that normally expected has been observed). Obviously, a large jackpot is easily identified among animals in control groups. Nevertheless, identifying such an occurrence becomes problematic among animals in treatment groups or when small jackpots occur in a control group. Because the cII and lacZ loci are independent and display a spontaneous mutant frequency of 5 × 10–5, we would expect a double mutant to occur with a frequency equal to $P(cII^-) \times P(lacZ^-) = 2.5 \times 10^{-9}$, so rare that finding a double mutant is a practical impossibility. The likelihood of finding a double mutant among the animals contributing to data in our time course study (mutant frequency approximately 130 × 10–5; 250 mg/kg ENU) is the $P(cII^-)^2 \times P(lacZ^-)^2$, which is equal to approximately $1.7 \times 10^{-16}$, still a very rare event. It follows then, that when encountering any high or outlier samples using lacZ or lacI analysis, cII selection may be applied to the same DNA sample. As shown in Table II, such an application can easily confirm the existence of a jackpot. However, if the sample is an outlier with respect to mutant frequency, at both loci, in multiple tissues, then some underlying biological mechanism may explain such results.

The cII on Big Blue and MutaMouse is the first common transgenic “locus” found in the two widely used systems. Although the sequence of the cII in MutaMouse is identical to that in the Big Blue, the different integration site(s) of the two constructs may warrant nomenclature distinguishing this fact. One point worth mentioning, that may go unnoticed, is the fact that the cII gene and gene product are in no way involved in the metabolism of E. coli. Although the use of host strain addresses this problem in the other approaches to identify mutants, it seems logical to avoid the question of metabolism altogether by using a lambda locus.

Our data support the use of cII analysis in combination with the MutaMouse transgenic model system. We con-
clude that this locus is comparable to the lacZ in sensitivity to acute treatments with ENU and is genetically neutral. We have confirmed that the cII in MutaMouse has the identical sequence to the cII in Big Blue. Moreover, the use of this locus in combination with the lacI or lacZ in the Big Blue or MutaMouse, respectively, will allow the easy identification of jackpot mutations.

ACKNOWLEDGMENTS

The authors thank Mr. David Zimmer for helpful discussions. We also acknowledge Hazelton Research Products (now Covance Labs) for allowing us to breed the Muta-Mouse strain for use in these studies as well as Stratagene for providing gratis the bacterial strain 1250.

In addition to the support of grant sponsors, this work was supported by scholarships: Ontario Graduate Scholarship (Roy R. Swiger), NSERC (Lidia Cosentino), Cancer Research Society (Jason H. Bielas); and a fellowship for research abroad from the Japan Society for the Promotion of Science (Naoko Shima) provided support for this work.

REFERENCES


