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Genetics

Analysis of genetic instability during mammary tumor progression using a novel selection-based assay for *in vivo* mutations in a bacteriophage λ transgene target

(middle T antigen/Big Blue/metastasis/transgenic mice/*cII*)

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ABSTRACT **Genetic instability is thought to be responsible for the numerous genotypic changes that occur during neoplastic transformation and metastatic progression. To explore the role of genetic instability at the level of point mutations during mammary tumor development and malignant progression, we combined transgenic mouse models of mutagenesis detection and oncogenesis. Bitransgenic mice were generated that carried both a bacteriophage λ transgene to assay mutagenesis and a polyomavirus middle T oncogene, mammary gland-targeted expression of which led to metastatic mammary adenocarcinomas. We developed a novel assay for the detection of mutations in the λ transgene that selects for phage containing forward mutations only in the λ *cII* gene, using an *hfl*$^-$ bacterial host. In addition to the relative ease of direct selection, the sensitivity of this assay for both spontaneous and chemically induced mutations was comparable to the widely used mutational target gene, λ *lacI*, making the *cII* assay an attractive alternative for mutant phage recovery for any λ-based mouse mutagenesis assay system. The frequencies of λ *cII*$^-$ mutants were not significantly different in normal mammary epithelium, primary mammary adenocarcinomas, and pulmonary metastases. The *cII* mutational spectra in these tissues consisted mostly of G/C → A/T transitions, a large fraction of which occurred at CpG dinucleotides. These data suggest that, in this middle T oncogene model of mammary tumor progression, a significant increase in mutagenesis is not required for tumor development or for metastatic progression.**

A major challenge of basic cancer research is to define the underlying genetic basis of neoplastic transformation and malignant progression. Cancer is a genetic disease that requires multiple mutational events to achieve a fully malignant state (1). Nowell (2) hypothesized that a source for these multiple mutations in tumors may be genetic instability. Support for this has come from the study of diseases such as Bloom syndrome, Fanconi anemia, ataxia telangiectasia, and xeroderma pigmentosum, in which susceptibility to cancer has been linked to genetic instability (3). It has also been found that defects in the human homologs of *Escherichia coli* mismatch repair genes are the basis of hereditary nonpolyposis colon cancer and some sporadic colon cancers (4, 5). Studies of genetic instability at the level of point mutations using *in vitro* experimental systems have collectively failed to show that enhanced mutation rates correlate with the malignant state or with metastatic potential of tumors, mostly because of limitations associated with the use of cell lines (6). To date, no whole animal systems have been used to address the issue of genetic instability during metastatic progression at the level of point mutations.

The recent development of transgenic mice bearing prokaryotic shuttle vectors integrated into the genome has allowed for the analysis of both spontaneous and induced point mutations and small insertions and deletions from a wide variety of tissue and organ systems (7). One such mouse, Big Blue (Stratagene), is transgenic for bacteriophage λ (8). A bacterial *lacI* gene inserted into the λ transgene serves as a mutational target. Mutations in *lacI* can be detected by screening for blue plaques among colorless plaques after rescue of the λ DNA from the mouse genomic DNA by *in vitro* λ packaging and plating on an appropriate *E. coli* host, in the presence of 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-Gal).

We have combined the λ-based mouse mutagenesis system with a transgenic model of oncogenesis to analyze genetic instability at the level of point mutations during neoplastic transformation and metastatic progression *in vivo*. The ability to detect and analyze mutagenesis in bacteriophage λ transgenic mice was greatly improved by employing a genetic selection system for mutations in the λ *cII* gene. In addition to the relative ease of mutant selection, we show that the sensitivity of the *cII* system is comparable to the *lacI* system, making this an attractive alternative for mutant phage recovery for λ-based mouse mutagenesis assay systems. The *cII* selection system was used to analyze mutant frequencies in genomic DNA from bitransgenic mice containing a bacteriophage λ transgene and a murine mammary tumor virus (MMTV)/polyomavirus (PyV) middle T oncogene (9). Our analysis of λ *cII*$^-$ mutant frequency in normal mammary epithelium, primary mammary adenocarcinomas and pulmonary metastases shows that, at least in the MMTV/PyV middle T model of oncogenesis, there is no significant increase in point mutation frequency during tumor development or progression.

MATERIALS AND METHODS

***E. coli* Strains.** XL1-Blue MRA strain is Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 gyrA96 relA1 lac*^{c} (Stratagene). G1217, the nonselecting strain, is XL1-Blue MRA *supF*. The *hfl*$^-$ selecting strains used were G1225 (G1217*hflA::Tn5 hflB29 tn10*) and G1250 [G1225 that was selected as a spontaneous mutant resistant to a T1-like phage found contaminating some lots of the *in vitro* λ packaging extract (Transpack; Stratagene)]. Other than the T1-like phage resistance, G1250 behaves identically to G1225. In the experiments described below, the term G1250 is used to mean either

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Abbreviations: MMTV, murine mammary tumor virus; PyV, polyomavirus.
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strain G1250 or G1225. Strain SCS-8 (Stratagene) was used when measuring the frequency of *lacI*$^-$ mutants in λ. Strains G1217, G1225, and G1250 can now be obtained from Epicentre Technologies (Madison, WI).

Mouse Strains. Male mice hemizygous for the MMTV/PyV middle T oncogene (ref. 9; FVB/N background) were mated with females hemizygous for the λ transgene (Big Blue; Stratagene) (C57BL/6 background). The genotypes of the F1 generation (FVB/N × C57BL/6 background) were determined by PCR analysis of tail DNA (10). Primers specific for the λ transgene were 5′-GTTATGACGAAGAAGAACGG-3′ and 5′-GCTCAAGAGCGATGTTAATT-3′. Primers specific for the MMTV/PyV middle T transgene were 5′-TACACGATGACTACTGGTCAT-3′ and 5′-TTATGTCACACCACAGAAGTA-3′. All animals were cared for and maintained in accordance with National Institutes of Health animal care guidelines.

Tumor Transplantations. When mammary tumors reached 1–2 cm^3, mice were killed, and tumors and any pulmonary metastases were removed and frozen on dry ice. For mammary tumor transplants, a 2-mm^3 piece of the tumor was removed before freezing and implanted subcutaneously into the backs of athymic mice (NCR-NU). After a tumor reached 1 cm^3, it was removed, frozen, and designated primary passage 1. This procedure was repeated to obtain primary passages 2 and 3. In the case of the metastases transplants, the small size of the tumors necessitated the transplantation of the entire tumor to athymic mice; otherwise the procedure was identical to the primary transplantations. Tumors were measured with calipers and volumes calculated according to the formula $(L \times W^2)/2$, where L equaled tumor length and W equaled width. For purposes of this study, a doubling of the tumor volume was considered at least one cell generation.

Chemical Mutagenesis Assay. The urinary bladder mutagen *p*-cresidine (11) was administered continually in the diet to three male Big Blue mice (C57BL/6) each at doses of 0%, 0.25%, and 0.50%. After 180 days, mice were killed, and the genomic DNA was isolated from bladders.

λ *cII* Mutagenesis Assays. Genomic DNA was isolated from mouse tissue as described (12) with minor modifications. The bacteriophage λ transgene was recovered from the mouse genomic DNA by incubation with *in vitro* λ packaging extract (Transpack; Stratagene). After packaging, the extracts were vortexed for 10 s to reduce the viscosity caused by the remaining genomic DNA. To determine the total titer of packaged phage, 3 μl of packaged phage particles was preadsorbed to 0.2 ml of G1217 ($OD_{600} = 0.5$), mixed with top agar, poured onto 85-mm TB1 plates and incubated at 24°C for 48 h. Phage containing mutations in *cII* were identified by preadsorbing 50 μl of packaged phage particles to 0.2 ml of G1250 ($OD_{600} = 0.5$) and plating as above. As discussed below, both λ$^+$ and λ *cII*$^-$ phage will form plaques on G1217, but only phage which are *cII*$^-$ will form plaques on G1250 (Table 1). For a random subset of plaques, the mutant phenotype was tested by replating the phage on G1250; 68 of 74 (92%) phages formed plaques again. The ratio of plaques that form on G1250 versus G1217 represented the observed λ *cII*$^-$ mutant frequency. Differences among mutant frequencies were tested for statistical significance by a Student's t test. The mutant frequency is expressed as the mean ± standard error of the mean (SEM).

Table 1. Positive selection for λ *cII*$^-$ mutants

	Plaques	
E. coli genotype (strain)	λ$^+$	λ*cII*$^-$
Wild type (G1217)	+	+
hfl$^-$ (G1250)	−	+

***lacI* Mutagenesis Assays.** Bladder DNA isolated from the *p*-cresidine-treated mice was incubated with *in vitro* λ packaging extract (Transpack; Stratagene). The packaged phage particles were preabsorbed to *E. coli* strain SCS-8, mixed with top agar containing 1.5 mg of X-Gal per ml and plated on agar medium at 37°C overnight. λ *lacI*$^-$ mutant frequencies were determined by calculating the ratio of blue plaques containing mutant lacI genes to nonmutant colorless plaques.

DNA Sequence Analysis. λ *cII*$^-$ mutant plaques were picked at random and purified on G1250 by single plaque isolation. Phage DNA was isolated and the entire 294-bp *cII* gene was either sequenced directly from the λ DNA or was PCR-amplified with Vent polymerase (New England Biolabs) under conditions designed to minimize mutations introduced by the PCR (13) and then sequenced. Sequencing reactions were performed with the Applied Biosystems Prism dye-terminator cycle sequencing kit (Perkin–Elmer) and analyzed on an Applied Biosystems model 373 automated DNA sequencer. Primers used to PCR amplify the *cII* region from λ DNA were 5′-CTTGCTCAATTGTTATCAGC-3′ and 5′-GTCATAATGACTCCTGTTGA-3′. Sequencing primers used were 5′-ACCACACCTATGGTGTATGCA-3′ and 5′-GTCATAATGACTCCTGTTGA-3′.

RESULTS

Positive Selection for Mutations in the λ *cII* Gene. In addition to harboring the bacterial *lacI* as a target for scoring mutations, the bacteriophage λ transgene of the Big Blue mouse (8) carries λ genes with the potential to serve as selectable targets for mutagenesis. We have employed a positive selection system for forward mutations in one of these genes, *cII*. The level of cII protein plays a central role in the lytic–lysogenic commitment made by λ upon infection of *E. coli* (14). cII activates the transcription of the genes for the cI repressor and for the λ integrase protein, both of which are required for the establishment of lysogeny. The amount of cII is negatively regulated by the *E. coli* Hfl protease. If cII levels are high, λ will lysogenize; if levels are low, the phage will enter the lytic pathway. Upon λ infection of an *hfl*$^-$ host, the levels of cII remain high and all of the phage lysogenize, resulting in the absence of discernible plaques on agar plates containing *hfl*$^-$ bacterial lawns. Only when an infecting phage is *cI*$^-$ or *cII*$^-$ will λ enter the lytic pathway and form a plaque. For purposes of developing a genetic selection system initially for λ *cI*$^-$ or λ *cII*$^-$ mutants, two *E. coli* strains were constructed. The nonselecting strain, designated G1217, is *hfl*$^+$; it allows λ$^+$, λ *cII*$^-$, and λ *cI*$^-$ phage to form plaques. The selecting strain, designated G1250, is *hfl*$^-$; only phage that are *cII*$^-$ or *cI*$^-$ should form plaques. However, we found that while λ *cI*$^-$ phage could form plaques on the *hfl*$^-$ host at 37°C, λ *cI*$^-$ phage including a λ Δ*cI* phage were unable to do so at 24°C (data not shown). We think that cII is further stabilized at 24°C, and its high activity allows activation of a promoter whose transcripts run counter to the λ P_R promoter (15). Thus phage containing mutations specifically in the *cII* gene can be selected by plating on the *hfl*$^-$ strain at 24°C (Table 1). To assess the λ *cII*$^-$ mutant frequency in a Big Blue mouse tissue, genomic DNA is isolated, λ DNA is recovered by *in vitro* packaging into infective phage virions, and the phage is plated on the nonselecting G1217 and selecting G1250 *E. coli* hosts. The λ *cII*$^-$ mutant frequency is then obtained by dividing the number of plaques on G1250 by the number of plaques on G1217.

To test the feasibility of the *cII* assay in detecting mouse-derived *in vivo* mutations, we determined the spontaneous λ *cII*$^-$ mutant frequency in spleen DNA from five 10-month-old F_1 males of a Big Blue female by FVB/N male cross. The λ *cII*$^-$ mutant frequency was $9.0 \pm 1.6 \times 10^{-5}$. This value is similar to the frequency of λ *lacI*$^-$ mutants measured previously in spleen DNA of aged Big Blue mice ($9.2 \pm 1.7 \times 10^{-5}$; ref. 16).

To compare the sensitivity of the *cII* assay for chemically induced mutations to that of the widely used *lacI* assay (7, 17), we isolated urinary bladder DNA from Big Blue mice that had been treated with the bladder carcinogen, *p*-cresidine. The frequency of both λ *lacI*$^-$ and λ *cII*$^-$ mutants were measured in DNA samples isolated from untreated mice and mice treated with different doses of *p*-cresidine. Both λ *lacI*$^-$ and λ *cII*$^-$ mutant frequencies increased in a dose-dependent fashion (Fig. 1). The frequency of λ *lacI*$^-$ mutants was induced 22- and 37-fold at each dose level, and the frequency of λ *cII*$^-$ mutants was induced 23- and 34-fold at each dose level. Thus, the sensitivity of the *cII* assay for *p*-cresidine-induced mutations was as least as good as that of the *lacI*-based system after considering that the 1080-bp *lacI* gene is 3.7 times larger than the 294-bp *cII* gene. These data demonstrate the utility of the *cII* assay for detecting both spontaneous and induced mutations *in vivo*.

Frequency of λ *cII*$^-$ Mutants during Tumorigenesis and Metastatic Progression. It has been proposed that a mutator phenotype is necessary for tumors to progress to malignancy (18). To test this hypothesis in an *in vivo* model of oncogenesis, we generated bitransgenic mice carrying an oncogene to induce tumorigenesis and a λ transgene to follow mutagenesis. We mated hemizygous MMTV/PyV middle T antigen mice (9) to hemizygous λ transgenic Big Blue mice (8). Both single-transgenic and bitransgenic virgin female mice harboring the MMTV/PyV middle T transgene developed multiple palpable mammary adenocarcinomas by 1–2 months of age; most of these mice exhibited pulmonary metastases by 3–4 months. The incidence and kinetics of tumor development in the (MMTV/PyV middle T)/(λ) bitransgenic females were similar to that in the MMTV/PyV middle T single transgenics; transgenic females containing the λ transgene alone did not develop mammary tumors. Because the mammary tumors that arise in the MMTV/PyV middle T model are epithelial in origin (9), lactating mammary glands, which have a much higher proportion of mammary epithelium than virgin glands, were used as the normal tissue control. These glands were isolated from females bearing the λ transgene alone because these glands remain lesion-free.

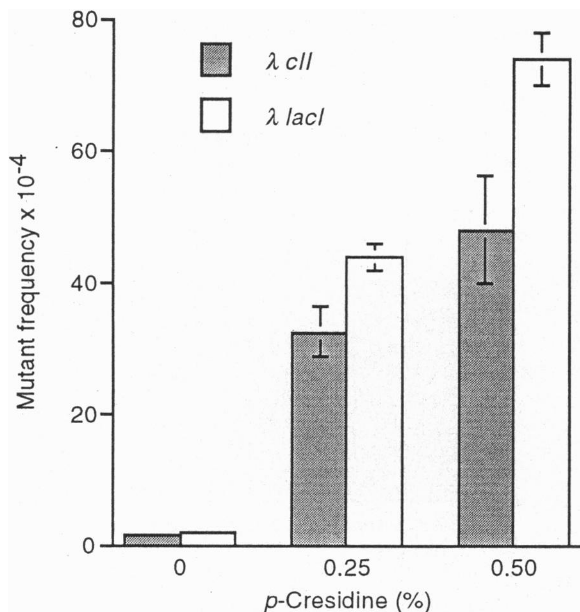

FIG. 1. Comparison of the *cII* and *lacI* genes as mutable targets in λ-based mouse mutagenesis systems. Mutant frequencies were measured for λ *cII*$^-$ (shaded bars) and λ *lacI*$^-$ (open bars) in bladder DNA isolated from *p*-cresidine-treated Big Blue mice. Values are not corrected for the target size difference between *cII* (294 bp) and *lacI* (1080 bp). Error bars represent SEM.

The mutant frequency of λ *cII* was analyzed in the mammary adenocarcinomas from six (MMTV/PyV middle T)/(λ) bitransgenic females and in the lactating mammary glands from four λ single transgenic females (Table 2). After isolation of genomic DNA, the λ transgene was recovered using *in vitro* λ packaging extracts and the packaged phage plated on the selecting and nonselecting *E. coli*. The frequency of λ *cII*$^-$ mutants was then determined by dividing the plaque number on the selecting *E. coli* host by the plaque number on the nonselecting host. The mean frequency of λ *cII*$^-$ mutants in the normal mammary epithelium was $17.1 \pm 2.7 \times 10^{-5}$. The mean frequency of λ *cII*$^-$ mutants in the primary mammary adenocarcinomas was $33.1 \pm 6.0 \times 10^{-5}$ (Fig. 2). The nearly two-fold difference in λ *cII*$^-$ mutant frequency between normal mammary epithelium DNA and mammary adenocarcinoma DNA was not statistically significant ($P > 0.05$).

To determine whether there were changes in λ *cII*$^-$ mutant frequencies associated with further growth of these primary mammary tumors, small viable portions of mammary tumors from three mice were implanted subcutaneously into athymic mice and allowed to grow. For each tumor, three serial transplantations were performed. Based on tumor volume measurements, it was calculated that at least 24 cell generations occurred during the growth of each tumor over the course of the three serial passages. The true number is likely to be considerably higher due to necrosis and apoptosis in the tumors, which results in a substantial loss of cells during tumor growth. Genomic DNA was isolated from an adjacent portion of the tumor saved before each successive transplantation. The λ transgenes were recovered, and mutant frequencies in the *cII* gene were determined as above. As shown in Fig. 3, there was no significant difference in the λ *cII*$^-$ mutant frequency in primary mammary tumor DNA ($33.1 \pm 6.0 \times 10^{-5}$) and in DNA from any of the three passages ($37.8 \pm 12.5 \times 10^{-5}$; $55.7 \pm 18.6 \times 10^{-5}$; and $41.5 \pm 13.0 \times 10^{-5}$; $P > 0.10$).

To determine whether an increase in point mutation frequency, indicative of genetic instability, correlated with the metastatic progression of the mammary adenocarcinomas in this system, we compared the λ *cII*$^-$ mutant frequency in the DNA of primary mammary tumors with that of pulmonary metastases DNA. Because of the multifocal nature of tumor development in these mice (9), it was not possible to know which primary mammary tumor colonized which pulmonary metastasis site. Genomic DNA was isolated from the lung metastases of three mice. Because metastases were small (≤ 1 mm^3), three to five separate tumors were pooled from each animal to obtain genomic DNA. As shown in Fig. 2, the mean frequency of λ *cII*$^-$ mutants in the metastases DNA from these three animals was $22.5 \pm 5.0 \times 10^{-5}$. This value was not significantly different from the mean λ *cII*$^-$ mutant frequencies in normal mammary epithelium DNA ($17.1 \pm 2.7 \times 10^{-5}$) or in the primary mammary tumor DNA ($33.1 \pm 6.0 \times 10^{-5}$; $P > 0.1$).

To determine whether there were changes in λ *cII*$^-$ mutant frequencies during further growth of the metastatic tumors, three serial subcutaneous transplantations into athymic mice were performed with single metastases isolated from three mice, as described for the primary tumor transplantations. As shown in Fig. 4, the metastatic tumors showed no change in the λ *cII*$^-$ mutant frequency when comparing the lung metastases ($22.5 \pm 5.0 \times 10^{-5}$) with any of the three passages ($46.8 \pm 16.2 \times 10^{-5}$; $20.5 \pm 3.5 \times 10^{-5}$; and $21.5 \pm 3.4 \times 10^{-5}$; $P > 0.30$).

***cII* Mutational Spectrum in Tumorigenesis and Metastatic Progression.** To verify that the phage in the G1250-selected mutant λ plaques contained mutations in *cII*, sequence analysis was performed on mutant phage recovered from normal mammary epithelium, primary mammary tumor, and pulmo-

Table 2. λ cII^- mutant frequencies in normal mammary epithelium, mammary tumors, and pulmonary metastases

Tissue type	Mouse	Total pfu, $\times 10^3$	Mutant plaques	Mutant frequency, $\times 10^{-5}$	Mean, $\times 10^{-5} \pm$ SEM
Mammary epithelium	SF4	144	21	14.6	17.1 ± 2.7
	BB1	182	37	20.3	
	BB2	93	11	11.8	
	BB3	87	19	21.8	
Mammary tumors	JF1	550	126	22.9	33.1 ± 6.0
	JF4	330	65	19.7	
	MF2	119	48	40.3	
	MF5	430	140	32.6	
	RF3	74	18	24.3	
	OF1	58	34	58.6	
Pulmonary metastases	JF1	172	40	23.3	22.5 ± 5.0
	JF4	120	18	15.0	
	RF3	65	19	29.2	

Because there are 40 copies of the λ transgene in the Big Blue mouse (17), every 40 pfu represent one mouse genome. pfu, Plaque-forming units.

nary metastasis DNA. After purification of the mutant phage on G1250, λ DNA was isolated from 62 randomly selected plaques, and the entire 294-bp *cII* gene in each isolate was sequenced. In 61 of 62 cases, mutant plaques were confirmed as having missense, nonsense, or frameshift mutations in the *cII* coding region. Table 3 compares the *cII* mutational spectrum in normal mammary epithelium, primary mammary tumor, and pulmonary metastasis DNA. Mutations that occur more than once at the same nucleotide position in the same animal can be the result of the clonal expansion of a cell containing a mutant λ transgene (17). After excluding these from the data, there were 39 independent mutations at 29 different positions, all of which resulted in a change in the amino acid sequence. No *cII* genes were found to harbor more than one mutation. The predominant mutations were G/C → A/T transitions, representing 74% of all mutations in the normal mammary epithelium DNA, 67% of the mutations in mammary tumor DNA, and 27% of the mutations in pulmonary metastasis DNA. Consistent with evidence that smCpG dinucleotides are highly mutagenic in mammalian cells (19), most of these transitions (83–100%) occurred at CpG dinucleotides. A high frequency of G/C → A/T transitions, a large fraction of which occur at CpG dinucleotides, has also been observed in the *lacI* gene of Big Blue mice (7, 17, 20). At present, the small sample size makes it difficult to draw conclusions about any potential change in the mutational spectrum at *cII* during tumorigenesis or metastatic conversion.

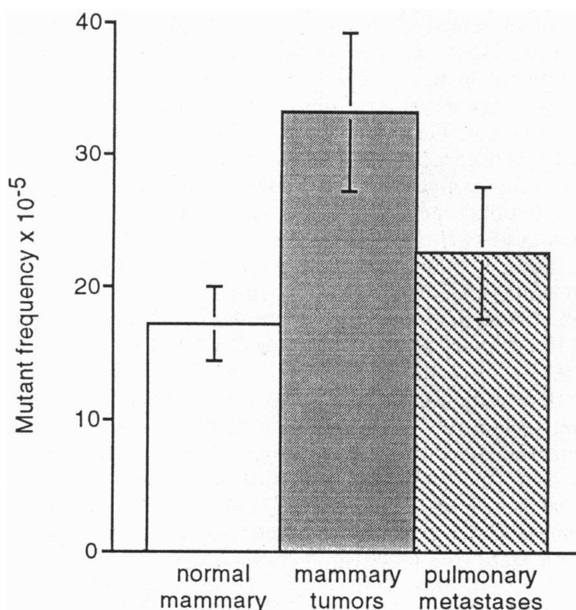

FIG. 2. The frequency of λ cII^- mutants in normal mammary epithelium, mammary adenocarcinomas, and pulmonary metastases. Mean frequencies were calculated in the DNA from four normal, six mammary tumor-bearing, and three metastatic tumor-bearing animals (Table 2). There is no statistically significant difference between the means ($P > 0.05$). Error bars represent SEM.

DISCUSSION

For a tumor to progress to a metastatic state, numerous genetic alterations are required (1). It has been hypothesized that genetic instability is required to fuel these changes (2), but to date this has not been tested at the level of point mutations in

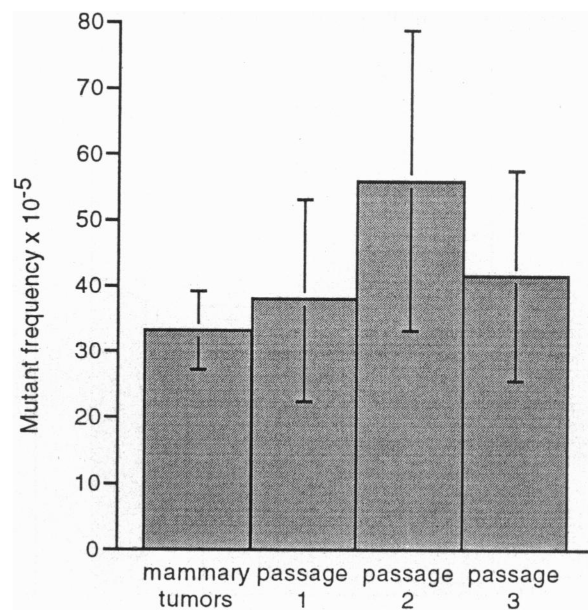

FIG. 3. The frequency of λ cII^- mutants during growth of primary mammary adenocarcinomas. Mean frequencies of λ cII^- mutants were calculated in the DNA from the mammary gland adenocarcinomas of three animals serially transplanted three times in athymic mice. Growth during these passages represented at least 24 additional cellular generations, based on tumor volume measurements. There was no significant difference in the means between the original tumors and any of the passages ($P > 0.10$). Error bars represent SEM.

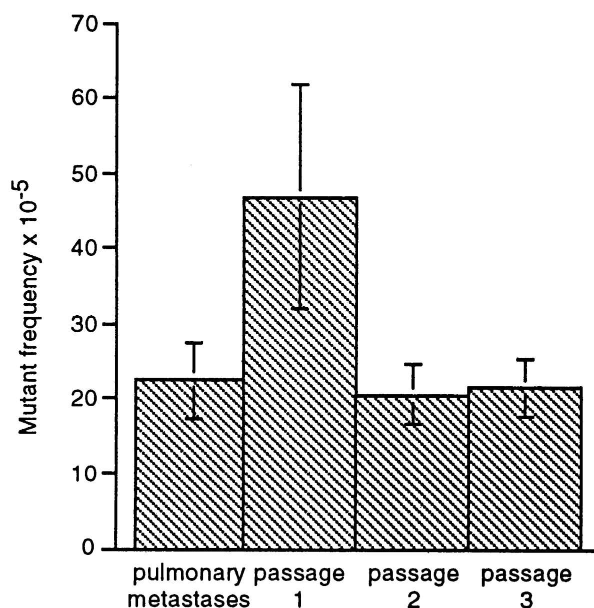

FIG. 4. The frequency of λ cII^- mutants during pulmonary metastasis growth. Mean frequencies of λ cII^- mutants were calculated in the DNA from the pulmonary metastases of four animals serially transplanted three times in athymic mice. Growth during these passages represented at least 24 additional cellular generations, based on tumor volume measurements. There was no statistical difference in the means between the original metastases and any of the transplants ($P > 0.30$). Error bars represent SEM.

an *in vivo* experimental system. In this report, a combined approach employing transgenic mouse models of mutagenesis detection and oncogenesis was used to explore the role of genetic instability in neoplastic transformation and metastatic progression. We generated bitransgenic mice harboring a bacteriophage λ mutational target transgene (8) and the MMTV/PyV middle T oncogene in which mammary gland-targeted expression leads to metastatic mammary adenocarcinomas (9). A novel selection-based assay for mutations, using λ *cII* as the mutable target, demonstrated that there was no significant change in the frequency or pattern of observed base substitutions or frameshift mutations between normal mammary epithelium, primary mammary adenocarcinomas or pulmonary metastases DNA.

Table 3. λ *cII* mutational spectra in normal mammary, mammary tumor, and pulmonary metastasis DNA

	Normal mammary (%)	Mammary tumors (%)	Pulmonary metastases (%)
Transitions			
G/C → A/T	14 (74)	6* (67)	3 (27)
(% at CpG)	(86)	(83)	(100)
A/T → G/C	0 (0)	0 (0)	2 (18)
Transversions			
G/C → T/A	2 (11)	1 (11)	2 (18)
(% at CpG)	(0)	(0)	(50)
G/C → C/G	0 (0)	0 (0)	1 (9)
(% at CpG)	(0)	(0)	(0)
A/T → T/A	2 (11)	0 (0)	0 (0)
A/T → C/G	0 (0)	0 (0)	1 (9)
Other			
+1 frameshift	0 (0)	1 (11)	1 (9)
−1 frameshift	1 (5)	1 (11)	1 (9)
Total	19	9	11

*Two of six mutants isolated from a transplanted tumor.

Genetic Instability in PyV Middle-T Induced Mammary Tumorigenesis. The absence of genetic instability in this system of oncogenesis, as measured by the cII^- mutagenesis assay described here, may reflect the fact that the middle T oncogene is such a potent transforming agent for the mammary epithelium that there is little requirement for secondary genetic changes during initial tumor development or subsequent metastatic progression. In fact, the observation that there is virtually no latency period between the onset of expression of the MMTV/PyV middle T oncogene and the development of mammary tumors suggests that middle T oncogene expression is sufficient for transformation (9). We are currently analyzing the λ cII^- mutant frequency in bitransgenic tumors containing the λ transgene and the *neu* oncogene, which is a model of mammary tumor progression with an increased latency period (21). Alternatively, it is possible there was not sufficient replicative history during the development of these tumors for any increase in genetic instability to be reflected in higher λ *cII* mutant frequencies. However, results from the serial tumor transplantation experiments suggest that this was not the case. It was estimated from tumor volume measurements that the growth of each tumor over the three serial passages represented at least 24 additional cell generations; the true number is likely to be much higher due to tumor cell apoptosis and necrosis, which would lead to underestimation of the number of tumor volume doublings. Despite this additional opportunity for a mutator phenotype to be manifest, there was still no significant increase in λ cII^- mutant frequency. It has been shown that λ-based mouse mutagenesis systems are capable of detecting genetic instability at the level of point mutations. Significant increases in the mutant frequency of a λ transgene containing a *supF* reporter gene (up to 100-fold) have been observed in the skin DNA of the mismatch repair gene *PMS2* null mouse (P. Glazer, personal communication). Although in our middle T model of tumorigenesis no statistically significant increase in λ cII^- mutant frequencies was observed, we cannot rule out enhanced mutagenesis that occurs below the detection limit of the *cII* assay. It also remains possible that other forms of instability such as gene amplification, aneuploidy, or chromosomal rearrangements are present in these tumors. λ-based mouse mutagenesis systems are incapable of detecting large insertions or deletions due to the constraints imposed on the size of the λ DNA during packaging, so neither the *cII* or *lacI* assays would able to measure these types of gross genetic lesions.

The spontaneous λ cII^- mutant frequency in normal mammary epithelium DNA ($17.1 \pm 2.7 \times 10^{-5}$) is almost 2-fold higher than that measured in the spleen ($9.0 \pm 1.6 \times 10^{-5}$). This λ cII^- mutant frequency in mammary tissue is also higher than that seen for the *lacI* gene in other tissues (22), but there is no data available for the frequency of λ $lacI^-$ mutants in the mammary glands for direct comparison. The difference in the spontaneous λ cII^- mutant frequency in the mammary gland and the spleen may simply reflect tissue to tissue variation similar to the tissue specific differences observed in the λ $lacI^-$ mutant frequency (22).

Utility of the λ *cII* Mutagenesis Assay. The Big Blue mouse mutagenesis assay system uses the bacterial *lacI* gene as the mutational target. Spontaneous or chemically induced mutant frequencies in the *lacI* gene are measured after recovery of the λ transgene as infective virions from mouse genomic DNA by *in vitro* λ packaging extracts and blue-white screening for mutant plaques on a Δ*lac E. coli* lawn on agar plates containing X-Gal (8). But the requirement for screening large numbers of plaques at low plating densities using chromogenic reagents to identify mutants makes this an expensive and laborious system (23).

We describe here an improved method for detecting mutations in λ-based mouse mutagenesis systems. The method involves positive selection for forward mutations in the λ *cII*

gene, using an *hfl⁻* *E. coli* host. Like the *lacI* assay, the *cII* assay is capable of detecting only point mutations and small insertions or deletions; gross chromosomal changes cannot be seen. Use of the *cII* assay in *in vivo* mutagenesis studies is advantageous for a number of reasons. First, because *cII* is a λ gene the assay can be used with any λ-based mouse mutagenesis system, including Big Blue (8) and MutaMouse (24). Second, mutant phage can be isolated by selection rather than color-based screening, resulting in a mutagenesis assay that takes less time, uses significantly less reagents, is less expensive, and is less subjective in scoring mutants. Third, the 294-bp *cII* gene is one-third the size of the 1080-bp *lacI* gene, facilitating the analysis of mutational spectra by DNA sequencing. After taking into account the smaller size of the *cII* gene, the sensitivity of the *cII* assay in detecting both spontaneous and chemically induced mutations is as least as good as that of the *lacI* assay (Fig. 1). And fourth, false positives from *E. coli*-derived mutations are unlikely to be seen in the *cII* assay. This is a distinct advantage over the *lacI* system, where *E. coli*-derived mutations can represent a significant fraction of mutant plaques (25). Upon infection, the lytic–lysogenic decision is made before any DNA replication occurs (14). Preexisting DNA adducts in the mouse DNA therefore would not be fixed unless there is already a mutation in *cII* that would allow lytic growth. Mutations occurring in *cII* during lysogen growth would not result in plaque formation, because commitment to lysogeny is already established. Only in the unlikely event of a double mutation in the *cI* repressor gene and in *cII* would lytic growth be possible from a lysogen. Thus, false positives from *E. coli*-derived mutations are unlikely to be seen in the *cII* assay. The wide applicability to any λ-based mouse mutagenesis system, the ability to select for mutants, the comparable sensitivity to the *lacI* system, the small size of the *cII* gene, and the absence of detectable *E. coli*-derived mutations make the *cII* assay a better alternative for studying mutations in λ-based mouse mutagenesis systems.

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