Development of a short-term, *in vivo* mutagenesis assay: the effects of methylation on the recovery of a lambda phage shuttle vector from transgenic mice

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ABSTRACT

Transgenic mice suitable for the *in vivo* assay of suspected mutagens at the chromosome level have been constructed by stable integration of a lambda phage shuttle vector. The shuttle vector, which contains a β-galactosidase (β-gal) target gene, can be rescued from genomic DNA with *in vitro* packaging extracts. Mutations in the target gene are detected by a change in lambda phage plaque color on indicator agar plates. Initial rescue efficiencies of less than 1 plaque forming unit (pfu)/100μg of genomic DNA were too low for mutation analysis. We determined the cause of the low rescue efficiencies by examining primary fibroblast cultures prepared from fetuses of lambda transgenic animals. The rescue efficiency of 5-azacytidine-treated cells increased 50-fold over non-treated controls indicating that methylation was inhibiting rescue. The inhibitory role of methylation was supported by the observation that *mcr* deficient *E. coli* plating strains and *mcr* deficient lambda packaging extracts further improved lambda rescue efficiency. Present rescue efficiencies of greater than 2000 pfu/copy/μg of genomic DNA represent a 100,000-fold improvement over initial rescue efficiencies, permitting quantitative mutational analysis. The background mutagenesis rate was estimated at 1×10^{-5} in two separate lineages. Following treatment with the mutagen *N*-ethyl-*N*-nitrosourea (EtNU), a dose dependent increase in the mutation rate was observed in DNA isolated from mouse spleen, with significant induction also observed in mouse testes DNA.

INTRODUCTION

The development of *in vivo* assays to study the molecular events involved in mutagenesis has been complex, due in part to the difficulty of analyzing these events with mammalian genetics. The use of shuttle vectors, that permit transfer of DNA interchangeably between eukaryote and prokaryote, is one approach to circumventing this difficulty, allowing direct correlation of the specific change in the nucleic acid to a particular mutagenic agent. This is made possible by the ability to rapidly analyze a genetic marker in which the presence of a mutation generated *in vivo* can be easily assayed after transfer to a prokaryotic system.

Previously, cosmid-based shuttle vector systems were proven feasible by stably integrating the vector into the genome of eukaryotic cells and subsequently recovering the DNA by packaging with *in vitro* extracts (1, 2). More recently, a lambda vector was integrated into mouse LTK$^-$ cells via transfection and successfully recovered by *in vitro* packaging, for the purposes of developing a mutagenicity assay (3, 4). Rescue efficiencies from either of these systems did not exceed 5 pfu/integrated copy/μg of genomic DNA (approx. 0.07% of 'theoretical' 7700 pfu/integrated copy/μg of genomic DNA—see Materials and Methods for derivation of this value). These experiments demonstrate that the recovery of lambda phage genomes from eukaryotic cells with a low background mutation rate was possible, with the added advantage of providing a chromosomally integrated mutagenic target.

This work describes the development of transgenic mouse lines that contain an integrated lambda phage shuttle vector that can be rapidly and efficiently recovered from genomic DNA by *in vitro* packaging. The effects of eukaryotic methylation on lambda rescue efficiency is discussed as well as the approaches taken to circumvent this problem. The transgenic mice, lambda packaging extracts, and *E. coli* strains generated in this study provide a sensitive short-term *in vivo* assay for assessing the mutagenicity of suspected genotoxic substances.

MATERIALS AND METHODS

Transgenic mice

B6CBAF1/J mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Transgenic mice were prepared by injecting fertilized single cell embryos from B6CBAF1/J mice with approximately 200 copies of *cos* ligated lambda DNA, essentially as described by Hogan *et al.* (5).

Bacterial strains

E. coli K12 *in vitro* lambda phage packaging extract is a derivative of *E. coli* K12 strains BHB2688 and BHB2690 (*mcr*A$^+$,

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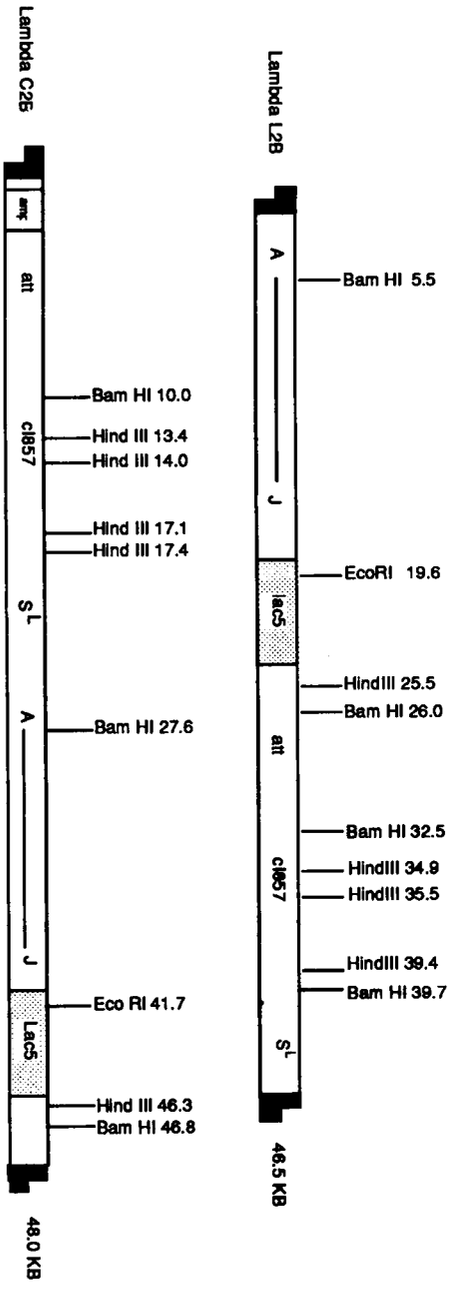

Figure 1. In both Lambda L2B and C2B the β-galactosidase gene is contained within the *lac5* substitution. The S^L gene was obtained by growing a *Sam7* lambda phage on a *SupO* host to obtain a forward mutation which demonstrated improved growth characteristics.

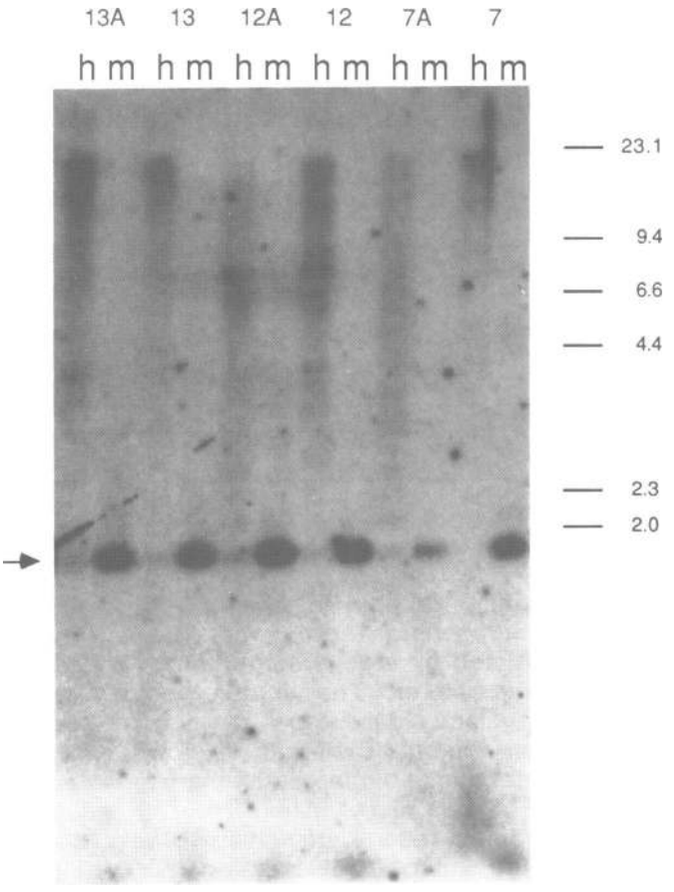

Figure 2A. Southern blot analysis of methylation state of non-treated and 5-azacytidine-treated fibroblast DNA digested with HpaII (h) and MspI (m). Densitometry was performed on band containing lambda P_L promoter region (arrow).

*mcr*B$^-$; *mcr* = methylated cytosine restriction) (6) and was prepared as described by Maniatis *et al.* (7). *E. coli* K12, Δ(*mrr-hsd*RMS-*mcr*B), *mcr*A$^-$ packaging extract strains were generated at Stratagene (8−10) (*hsd* = Eco K restriction system; *mrr* = methylated adenine recognition and restriction). *E. coli* C packaging extract [derived from SMR10 cells (11)] was purchased from Promega Biotech, Madison, WI. DP50 (*hsd*$^-$), Y1088 (*hsd*$^-$, *mcr*A$^-$, *mcr*B$^+$) (12) and K802 (*hsd*$^-$, *mcr*A$^-$, *mcr*B1) (13) were obtained from Stratagene. WB13 and PLK17 (*hsd*$^-$, *mer*A$^-$, *mcr*B1, *lac*$^-$) are derivatives of K802, constructed as described by Bullock *et al.* (14). NM554 (*Rec*A$^-$, *hsd*$^-$, *mcr*A$^-$, *mcr*B$^-$) and NM621 (*Rec*D$^-$, *hsd*$^-$, *mcr*A$^-$, *mcr*B$^-$) were a generous gift of Dr Noreen Murray, University of Edinburgh (15). *E. coli* C strains Cla (16) and C4506, isolated by Dr G.Christie, Medical College of Virginia, were generous gifts of Dr R.Calendar, U.C. Berkeley.

Lambda shuttle vectors

Two lambda shuttle vectors (Stratagene) were used for integration into transgenic mice. Lambda L2B (BK and BL lineages) and Lambda C2B (AL, BA, and LU lineages) were chosen since they both contain an intact β-gal target gene for mutation analysis and an S gene mutation for improved plaque formation (Figure 1). Lambda L2B is 46.5 kb while Lambda C2B is 48.0 kb.

Preparation of primary fibroblast cultures

Fibroblast cultures were prepared from fetuses of a founder transgenic mouse, BK, after 14 days of gestation. The fetuses were homogenized, digested with trypsin, and plated with Dulbecco's Modified Eagle Medium ((DMEM)supplemented with penicillin/streptomycin and 20% fetal calf serum. Cells were cultured at 37°C in 5% CO_2.

Treatment with 5-azacytidine

Confluent plates of fibroblasts at passage number 4−8 were split 1:3, 24 hours before treatment. These cells were than incubated in DMEM supplemented with freshly made 50 μM 5-azacytidine

Table I. Effect of azacytidine treatment and *mcr*$^-$ *E. coli* strains on rescue from fibroblast DNA

E. coli	Fibroblast Line	Rescue Phage (pfu/μg)
Y1088	7	0
Y1088	7-AZA	2.8 ± 2.4
Y1088	9	0
Y1088	9-AZA	0
Y1088	10	0
Y1088	10-AZA	0
Y1088	12	0
Y1088	12-AZA	0.5 ± 0.5
Y1088	13	0
Y1088	13-AZA	0.2 ± 0.1
WB13	7	0.8 ± 0.9
WB13	7-AZA	156.8 ± 61.5
WB13	9	0.1 ± 0.1
WB13	9-AZA	6.0 ± 5.0
WB13	10	0
WB13	10-AZA	0
WB13	12	0.2 ± 0.3
WB13	12-AZA	22.2 ± 9.1
WB13	13	1.9 ± 2.5
WB13	13-AZA	75.5 ± 54.9

The fibroblast DNA used in the methylation analysis experiments (Figures 2A, 2B) was tested for rescue efficiencies in both Y1088 (*mcr*A$^-$, *mcr*B$^+$) and WB13 (*mcr*A$^-$, *mcr*B$^-$). The packaging extract efficiency (3.6×10^8 ± 0.4 $\times10^8$ pfu/μg) was monitored by plating *cI857, ind1, Sam7* lambda DNA with DP50 cells. The plating efficiency of Y1088 (2.7×10^8 ± 0.6×10^8 pfu/μg) and WB13 (2.0×10^8 ± 1.2×10^8 pfu/μg) cells was monitored by plating packaged lambda L2B phage with these cells. Platings were performed in triplicate and the efficiencies are expressed as pfu/μg of genomic DNA. The average efficiency from three experiments is shown with standard deviations.

Table II. Effects of packaging extracts and *E. coli* plating strains on rescue of BK DNA

λ Packaging Extract	*E. coli* Plating Strains Y1088	NM621	NM554	PLK17	C1a	C4506
A. C(SMR10)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Kmcr+	<0.01	0.1	0.2	0.6	102	183
Kmcr−	0.15	0.85	0.45	6.5	−	2,446
B. C(SMR10)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Kmcr+	<0.01	<0.01	<0.01	0.15	47	78
Kmcr−	<0.01	0.15	1.6	1.4	371	1,637

Control. *E. coli* = DP50; Lambda DNA = *cI857, ind1, Sam7*

C(SMR10)	2.1×10^8
Kmcr+	5.3×10^8 ± 1.7×10^8
Kmcr−	3.1×10^8 ± 0.3×10^8

Genomic DNA was prepared from the brains of 2 BK mice (A and B) and packaged using three lambda packaging extracts (*E. coli* K12 (*mcr*A$^+$, *mcr*B$^-$) = K12 mcr$^+$; *E. coli* K12 Δ(*mrr-hsd*RMS-*mcr*B), *mcr*A$^-$ = K12 mcr$^-$; *E. coli* C = C(SMR10). None of the mice were treated with 5-azacytidine. The packaged L2B phage DNA was plated on six different *E. coli* strains. The genotypes of the strains are as follows: Y1088 (hsd$^-$, *mcr*A$^-$, *mcr*B$^+$); NM621 (*Rec*D$^-$, *hsd*$^-$, *mcr*A$^-$, *mcr*B$^-$); NM554 (*Rec*A$^-$, *hsd*$^-$, *mcr*A$^-$, *mcr*B$^-$); PLK17 (*hsd*$^-$, *mcr*A$^-$, *mcr*B1, *lac*$^-$); C4506 [Δ(*mrr-hsd*RMS-*mcr*B), *mcr*A$^-$, lac]; C1a[Δ(*mrr*$^-$*hsd*RMS-*mcr*B), *mcr*A$^-$]. The values listed are expressed as pfu/μg of genomic DNA. Twenty micrograms of DNA were used for each sample with the exception of the more efficient strains C1a and C4506 where five micrograms were used. The efficiency of the lambda packaging extracts was monitored using non-eukaryotic methylated lambda DNA.

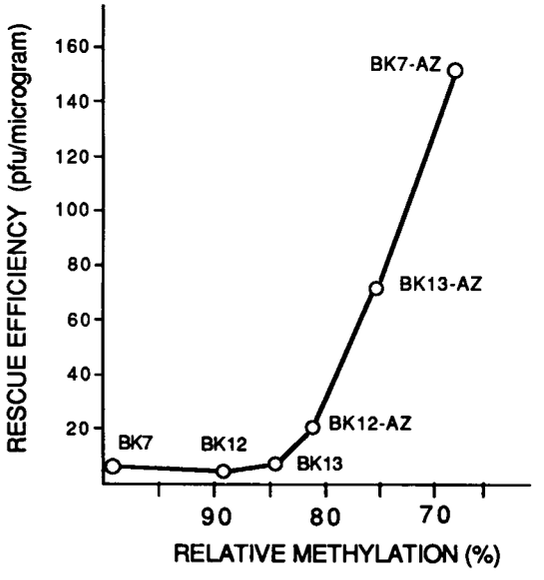

Figure 2B. The rescue efficiencies of lambda DNA from each fibroblast line using WB13 cells listed in Table II (y axis) are plotted against the relative methylation of these cell lines (x axis).

(17) until confluent (3−5 days). DNA was extracted from the fibroblasts as described previously (7).

Rescue of plaques from genomic DNA

Plating bacteria were grown in 1X TB (5 g/L NaCl, 10 g/L tryptone) supplemented with 0.2% maltose and 10 mM $MgSO_4$ overnight at 30°C. Cells were harvested by centrifugation, resuspended in 10 mM $MgSO_4$ in preparation for plating. In a typical experiment, 1−5 μg of genomic DNA was exposed to *in vitro* lambda phage packaging extract and incubated for 2 hr. at room temperature. The packaging reaction was diluted in 500 μl SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris, pH 7.5, and 0.01% gelatin) and incubated with bacteria (2.0 ml of OD_{600} = 0.5), and then plated onto NZY/agar Nunc Bioassay Dishes (245mm×245mm×20mm) with molten top agar containing 1.25 mg/ml X-gal and 2.5 mM IPTG at a density of less than 20,000 pfu per plate. Plaque numbers were determined after overnight incubation at 37°C. Mutations in the β-gal target were detected by white (or faint blue) plaque morphology, while non-mutated plaques remained blue. Mutant plaque color was verified by replating isolated plaques at low density on X-gal and IPTG indicator plates. All rescues and analysis of white plaques were done in 'blind' experiments.

Analysis of methylation state of transgene via restriction digestion

To estimate the extent of methylation, genomic DNA was digested with the isoschizomers HpaIII (inhibited by CpG methylation) and MspI (not inhibited by CpG methylation). Following restriction digestion, the DNA was gel electrophoresed, Southern transferred (18) to nylon membrane, and hybridized to a ^{32}P-labeled pP_L-Lambda DNA probe (19). Densitometry was then used to quantitate the degree of digestion inhibition of HpaII relative to MspI. As a control for complete digestion, 2 μg of phiX174 RFI DNA was added to duplicate samples of genomic DNA and evaluated by ethidium bromide staining of the agarose gel before Southern transfer.

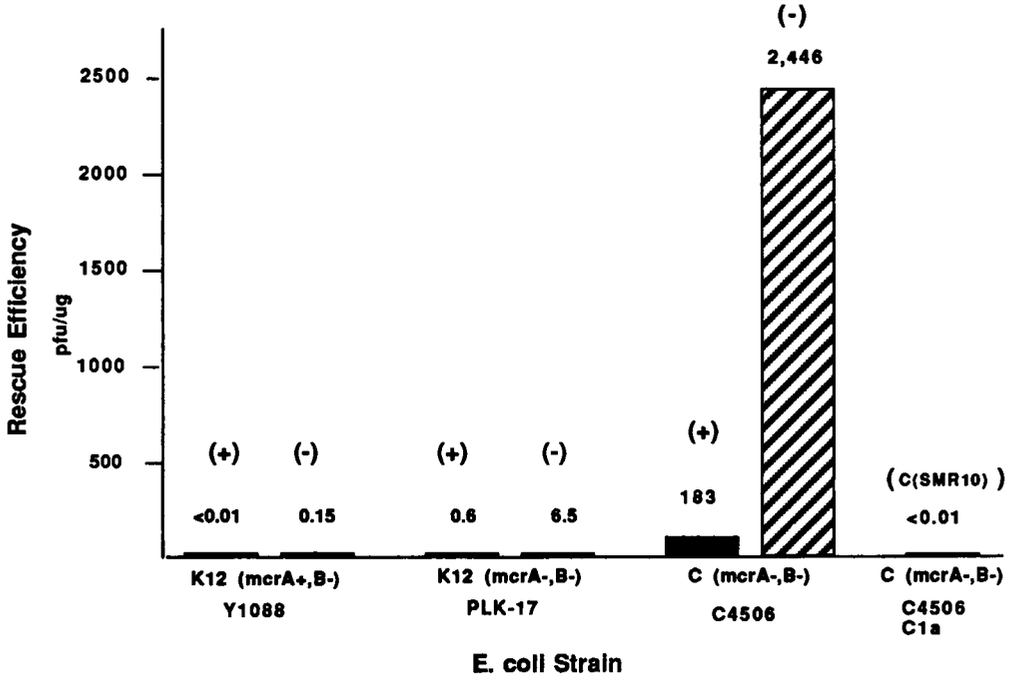

Figure 3. Comparison of the relative efficiency of different *E. coli* plating strains and lambda packaging extracts for the recovery of the lambda shuttle vector from BK mouse brain DNA. The (+) symbol denotes K12 (*mcr*A$^+$, *mcr*B$^-$) packaging extracts and the (−) denotes K12 Δ(*mrr-hsd*RMS-*mcr*B), *mcr*A$^-$ packaging extracts. C(SMR10) is an *E. coli* C derived lambda packaging extract.

Mutagen treatment

Six to eight week old male mice were treated on day 1 and day 4 by intraperitoneal injection of either 125 or 250 mg EtNU per kg body weight. Control animals were injected with phosphate buffer (0.03M, pH 6.0) at 10 ml/kg body weight. Tissues were collected two hours after the final injection.

Calculation of 'theoretical' shuttle vector rescue efficiencies

$$1\ \mu g \text{ of mouse DNA} \times \frac{4.6\times10^4 \text{bp/lambda L2B genome}}{6.0\times10^9 \text{bp/mouse genome}} \times 1\times10^9 \text{ pfu}/\mu g^{**}$$

$$= 7{,}667 \text{ pfu}/\mu g/\text{integrated lambda copy}$$

From this calculation, it is estimated that approximately 7700 pfu/μg can be recovered from 1 μg of transgenic mouse genomic DNA carrying one excisable lambda genome using a lambda packaging extract with a packaging efficiency of 1×10^9 pfu/μg** of lambda DNA (lambda *cI857, ind1, Sam7*) plated on *E. coli* DP50 cells.

RESULTS

Characterization of transgenic mice

Five transgenic lineages, LU, AL, BA, BK, and BL were generated. The BK and BL lineages contain approximately 1 and 2 copies of Lambda L2B, respectively. AL and BA contain approximately 2 copies and LU contains 3 copies of Lambda C2B. Southern blot analysis of the mouse tail genomic DNA indicated that the lambda DNA for all lineages integrated in an intact form with no rearrangement (data not shown).

Initial rescue experiments from spleen DNA

In order to determine if lambda shuttle vector DNA could be recovered from transgenic mouse tissue, genomic DNA was prepared from the spleen of the BL and BK lineages. This DNA was packaged using *in vitro* lambda phage packaging extract (*mcr*A$^+$, *mcr*B$^-$) and plated with *E. coli* Y1088 cells. Although rescue from the BK lineage using Y1088 host cells was successful, the efficiency was less than 0.0001% of the expected value of approximately 7,700 pfu/μg genomic DNA/copy of lambda (see Materials and Methods for calculation). No lambda phage were recovered from the BL lineage under these conditions.

Table III. Rescue efficiency comparisons (pfu/μg) among transgenic lineages

	Spleen	Liver	Testis	Ovary
BK Line	2,541	698	2,893	3,493
LU Line	4,123	1,608	5,896	12,392
AL Line	790	4,267	1,053	257
BA Line	1,668	4,403	1,438	644
BL Line	–	0.2	5.5	2.0

Rescue efficiencies were determined by packaging from 10−170 μg of genomic DNA, depending on tissue type. Greater than 20,000 plaques were rescued from each tissue to determine rate, with the exception of the BL line, which was significantly less efficient.

The effects of 5-azacytidine treatment on rescue from BK derived fibroblasts

To determine if a reduction in eukaryotic methylation would increase lambda phage rescue, six primary fibroblast cultures (#s 7, 9., 10, 12, 13 and 14) were prepared from fetal offspring of a hemizygous BK transgenic female which was mated with a non-transgenic B6CBA/J F1 male. Cell lines 7, 9, 12, and 13 contained approximately 1 copy of lambda with no evidence of rearrangement or deletion, as verified by Southern blot analysis

(data not shown), while lines 10 and 14 did not contain the lambda transgene and served as negative controls. Each cell line was treated with 5-azacytidine. The approximate amount of demethylation caused by this treatment was measured by Southern analysis of HpaII and MspI digested genomic DNA within the lambda P_L promoter (Figure 2A). Quantitation by densitometry indicates that treatment of the BK7 line resulted in the largest decrease in methylation, followed by BK13 and BK12 (BK9 not measured). The high amount of methylation observed in the non-treated cell lines was comparable to that of the BK mouse DNA from spleen and testes (data not shown).

The level of methylation was then correlated with lambda rescue efficiency. Rescue efficiency of the lambda vector from treated and untreated cell lines was measured by packaging 10 μg of genomic DNA and plating with *E. coli* Y1088 cells (Table I). No phage were recovered from untreated cells, which was analogous to the initial rescue data from tissue. However, 5-azacytidine treatment allowed the lambda phage vector to be recovered. BK7 exhibited the highest rescue efficiency followed by BK12 and BK13, while BK9 remained resistant to rescue (Table I).

The effect of *E. coli* restriction systems on rescue of phage genomes from 5-aza-treated and non-treated fibroblasts

There are a number of restriction/methylation systems endogenous to *E. coli*. Two of these systems, *mcr*A and *mcr*B, recognize and cleave DNA at a number of sequences in which cytosine residues have been methylated (13). When introducing eukaryotic genomic DNA into *E. coli* Y1088 (*hsd*$^-$, *mcr*A$^-$, *mcr*B$^+$), degradation of the incoming DNA by this restriction system may be significant since cytosine methylation is the predominant mode of methylation in eukaryotes (20). To examine this effect, the plating efficiency with WB13 (*hsd*$^-$, *mcr*A$^-$, *mcr*B1) was compared to that obtained when using Y1088. The use of the WB13 strain significantly increased rescue efficiencies from both treated and untreated BK cell lines (Table I). Unlike Y1088, WB13 permitted phage to be rescued from the untreated BK7, BK9, BK12 and BK13 fibroblast DNA. As expected, no phage were recovered from the negative control cell line, BK10, regardless of experimental treatment.

There were differences in rescue efficiencies for each cell line, however, increased rescue efficiency correlated well with decreased methylation (Figure 2B). Cell line BK7−5AZ had the highest rescue efficiency upon treatment (156.8 ± 50.2 pfu/μg) and also showed the most significant degree of demethylation by 5-azacytidine (Table I, Figure 2B). This trend of increased rescue efficiency and decreased methylation was repeated with cell lines BK13 and BK12.

Effect of lambda packaging extracts and *E. coli* plating cultures on rescue from mouse tissues

Due to the effects of *mcr* activity in the plating cultures on rescue efficiency from fibroblast DNA, it was reasonable to assume that this activity may also be present in the lambda packaging extract. In order to test this possibility, *E. coli* C packaging extracts were used to rescue phage from the mouse since it was known that some *E. coli* strains lack *mcr* activity (21). However, the *E. coli* C extract was 100-fold less efficient than the *mcr*$^+$ *E. coli* K12 packaging extract (Table II). This result suggested that the SMR10 *E. coli* C extract was inhibitory to lambda phage rescue by a mechanism other than the *mcr* activity observed in *E. coli* K12. However, consistent with the fibroblast data, the *E. coli* K12

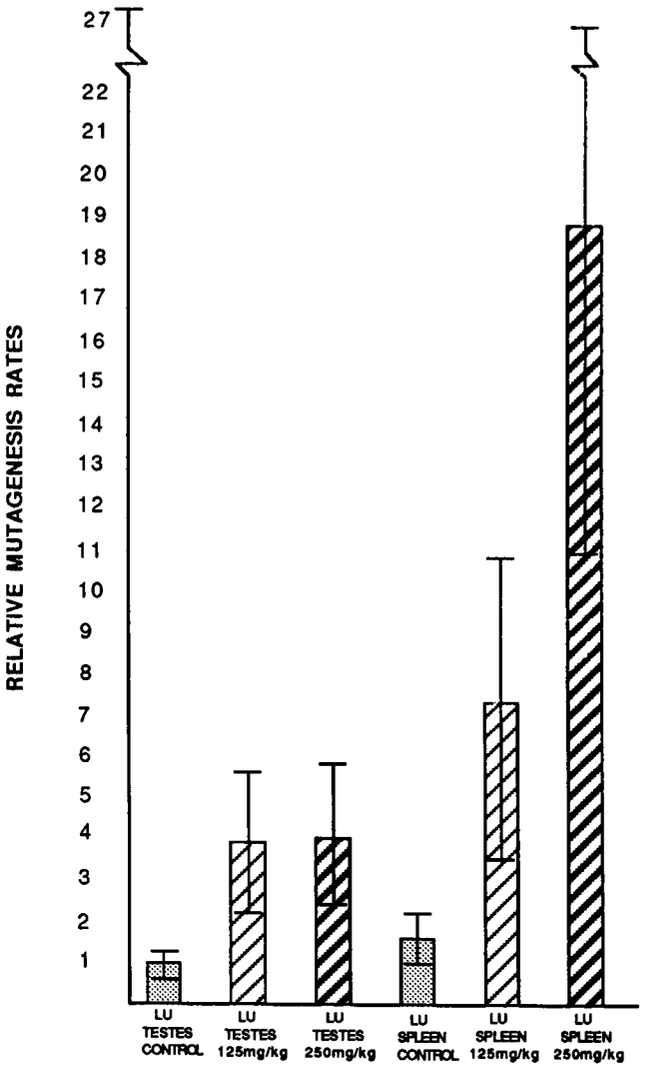

Figure 4. Relative mutation frequencies normalized to LU testes background rate (phosphate buffer control). Standard errors for these values are displayed. Increase in the mutation rate is seen following treatment with either 125 mg/kg or 250 mg/kg EtNU in both testes and spleen.

*mcr*A$^-$, *mcr*B1 strains used for plating improved rescue efficiencies from the mouse when used with the *E. coli* K12 (*mcr*A$^+$, *mcr*B$^-$) packaging extract, confirming that *mcr* activity in the plating culture inhibits rescue.

Based on these experiments, the *mcr* activity in the *E. coli* K12 extract strains was removed by P1 transduction and these strains were used to prepare the modified *E. coli* K12 extract (8−10). Table II shows results of a comparison of *E. coli* K12 *mcr*$^+$, *E. coli* K12 *mcr*$^-$, and *E. coli* C(SMR10) *mcr*$^-$ lambda packaging extract efficiencies using BK mouse brain DNA. These results indicated that the *mcr* activity survived the packaging extract preparation and reduced lambda rescue by as much as 10-fold. Removal of all sources of *mcr* activity increased rescue efficiencies 1000-fold over efficiencies obtained with *mcr*$^+$ packaging extracts and plating strains. These results were consistent with the observation that methylation was the primary block to efficient lambda phage recovery, as predicted from the fibroblast experiments.

In order to investigate the cause for reduced packaging

Table IV. Spontaneous background and mutagenicity testing

CONTROL, PHOSPHATE BUFFER [10 ml/kg body weight]					
A: Spleen			B: Testes		
mouse	plaques rescued	white plaques	mouse	plaques rescued	white plaques
LU03-150	112408	1	LU03-150	154104	1
LU03-73	104216	1	LU03-73	152424	2
LU03-135	141712	2	LU03-135	151552	2
LU03-142	101760	2	LU03-142	173120	1
LU03-141	87672	2	LU03-141	138036	1
	547768	8		796236	7
5 sample avg.:		1 in 76453	5 sample avg.:		1 in 123449
EtNU, [125 mg/kg body weight]					
mouse	plaques rescued	white plaques	mouse	plaques rescued	white plaques
LU03-62	106888	9	LU03-62	161728	3
LU03-137	120992	14	LU03-137	140416	5
LU03-74	112280	6	LU03-74	161004	8
LU03-77	84552	3	LU03-77	155336	6
	424712	32		618484	22
4 sample avg.:		1 in 16853	4 sample avg.:		1 in 32001
EtNU, [250 mg/kg body weight]					
mouse	plaques rescued	white plaques	mouse	plaques rescued	white plaques
LU03-90	92936	34	LU03-90	134596	5
LU03-126	81710	12	LU03-126	153600	8
LU03-145	104536	11	LU03-145	162684	3
LU03-144	120780	14	LU03-144	180408	7
LU03-140	119160	25	LU03-140	156280	5
	519122	96		787568	28
5 sample avg.:		1 in 6487	5 sample avg.:		1 in 31475

Animals were treated by IP injection on days 1 and 4. Tissues were taken two hours after final dose. Mutation rates are expressed as the ratio of white plaques to total plaques rescued with the frequency from each mouse treated as an individual sample. Range of genomic DNA packaged was 10 to 50 μg.

efficiency with *E. coli* C(SMR10) packaging extracts, several *mcr*$^-$ *E. coli* K12 and *E. coli* C plating strains were tested (Table II). Unlike the experiments with the *E. coli* C packaging extract, the *E. coli* C strains allow efficient lambda rescue. It was observed that the rescue efficiency using these *E. coli* C plating strains was at least 10-fold higher than that obtained with the *mcr*$^-$ *E. coli* K12 plating strains. Use of C4506 with *mcr*$^-$ *E. coli* K12 packaging extract demonstrated the highest rescue efficiency using BK mouse brain DNA (Figure 3). In addition, rescue efficiency using *E. coli* C strain C1a was greater than with the *mcr*$^-$ *E. coli* K12 strains, NM621, NM554, and PLK17 (Table II). This latter result is interesting since C1a is the same strain that was reported to be used to produce the less efficient SMR10 *E. coli* C lambda packaging extract. Since this extract was only available commercially, the extract preparation method could not be controlled, nor could the SMR10 genotype be confirmed.

Utilizing *mcr*$^-$ *E. coli* K12 packaging extracts and *E. coli* C plating strains, 4 out of the 5 lineages generated rescued efficiently (Table III). It was noted that rescue efficiencies varied among tissues. However, it is not known if these variations reflect differences in the quality of the isolated DNA or specific DNA modifications affecting rescue. In addition, lambda rescue from the BL mouse line was now successful, although 1000-fold less efficient than from BK mice. Potential explanations for inefficient rescue from the BL line include a) an unfavorable integration site of the shuttle vector and b) a cryptic mutation preventing efficient rescue. However, since BL was the only lineage out of the 5 to rescue inefficiently, these factors will not impede the ability to efficiently recover lambda phage from most transgenic mouse lines.

Mutagenicity testing

To assess the feasibility of this model as a viable mutagenicity assay, two lineages, BK and LU, were chosen to determine spontaneous mutation rates. At least 500,000 lambda phage were rescued from spleen and testes DNA of several BK and LU mice treated with control phosphate buffer. Spontaneous mutation rate from the tissues of both lineages was approximately 1×10^{-5} (LU lineage—Table IV; BK lineage—data not shown), with the spleen exhibiting a slightly higher background rate in the LU lineage (Figure 4). These low background rates were suitable for mutagen testing.

The potent mutagen, EtNU, was administered to nine LU lineage male mice in two doses of either 125 mg/kg or 250 mg/kg. In the spleen the mutation rate was dose dependent, increasing 4.5-fold and 11.8-fold over spleen background mutation rates in response to the 125 mg/kg and 250 mg/kg doses, respectively (Table IV, Figure 4). In the testes, although the higher dose did not increase the mutation rate above that observed with the low dose, a 3.5-fold induction over testes background rate was observed in both cases.

DISCUSSION

We have been able to increase efficiency of recovery of lambda shuttle vectors from BK transgenic mice to approximately 40% of the 'theoretical' limit without the use of 5-azacytidine. The

development of an *mcr*⁻ *E. coli* K12 lambda packaging extract (8–10) permits direct determination of the genetic requirements for rescue of lambda phage shuttle vectors from transgenic mice. We have demonstrated that transgenic mouse DNA packaged with *mcr*⁻ K12 extracts plated less efficiently on *mcr*⁻ K12 strains than on *mcr*⁻ *E. coli* C strains. Gossen *et al.* have recently observed improved rescue efficiencies with the same *E. coli* C plating strains (22, 23) and *mcr*⁻ K12 *E. coli* packaging extract (8–10). However, we have determined that certain *E. coli* K12 plating stains are also capable of rescue efficiency comparable to that of *E. coli* C, indicating that *E. coli* C is not essential for efficient recovery of the lambda phage from transgenic mice (Kretz, P.L., Kohler, S.W. and Short,J.M., personal communication). The difference in efficiency between *E. coli* K12 and *E. coli* C strains can be explained as follows: *mcr*B 'point mutants' (*mcr*B1) allow a certain degree of restriction activity to still occur (24), although not necessarily a direct effect of *mcr*B. However, if this region is deleted Δ(*mrr-hsd*RMS-*mcr*B), *E. coli* K12 is capable of rescue efficiencies equal to that of *E. coli* C. The *mcr*B mutation in our K12 packaging extract is a result of a large deletion, thereby allowing efficient rescue. Characterization of the specific domain within this locus responsible for the inhibitory effect will be described elsewhere.

The varying mutation rates observed between testes and spleen at equivalent doses (Figure 4), as well as the differences with increasing dose, underscore the potential of this *in vivo* assay system for examining tissue specific mutation rates. Tissue specific mutation rates are dependent on a large number of parameters which are impossible to simulate in *in vitro* test systems. Differences in mutagen uptake, restricted blood flow, DNA replication rates, transcriptional activity within the target gene, and/or presence of DNA repair enzymes may explain tissue specific differences in mutation rates, and some of these may also explain the absence of a dose response in the testes. The ability of this assay to account for such differences should improve the quality of data obtained from short term testing and eventually allow correlation of mutation rates with frequency of tumor formation within a single animal (25).

The existing mutagenesis system is currently being modified to utilize the *lac*I gene as the target for mutagenesis. This will simplify detection of mutant plaques (blue plaques on background of white plaques), permit an increase in the density of phage per plate, and provide a target gene with significant historical mutational data for comparison between mutagenesis assays. In addition, the system will allow rapid identification of base mutations by incorporating properties of the lambda ZAP excision vector (26). These features will facilitate the collection of large amounts of data for statistical analysis of the sequence specificity of mutagens in whole animals. Validation of the existing model, through testing of known mutagens and non-mutagens previously identified by the mouse specific locus test (27), is underway.

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