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Spontaneous Mutant Frequency and Mutation Spectrum for Gene *A* of ΦX174 Grown in *E. coli*

Jessica L. Raney,[1] Robert R. Delongchamp,[2] and Carrie R. Valentine[1]*

[1]*Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research, Jefferson, Arkansas*

[2]*Division of Biometry and Risk Assessment, National Center for Toxicological Research, Jefferson, Arkansas*

The use of transgenic targets for measuring mutant frequencies in mammalian tissue requires an estimate of the mutant frequency that results from recovery of the transgene in bacterial recovery systems. In this study, we have determined the spontaneous mutant frequency, estimated the mutation rate, and ascertained the mutation spectrum for gene *A* of ΦX174 grown in *E. coli* strain CQ2 from 156 small independent cultures. The mutant frequency of 12 of the 156 cultures was $17 \pm 1.0 \times 10^{-6}$ and the estimated mutation rate per gene replication was $7.4 \pm 2.3 \times 10^{-6}$. The mutant frequency and spectrum from *E. coli* were not significantly different from that of solvent-treated embryonic mouse cells in culture, $19 \pm 0.5 \times 10^{-6}$ (Valentine CR et al. [2002]: Environ Mol Mutagen 39:55–68), indicating that those spontaneous mutants were primarily derived from *E. coli*. The *E. coli* spectrum was heavily weighted toward two major target sites (hot spots), 4225A→G (56%) and 4218G→A or C (20%). Four new target sites and one new mutational event were recovered by the gene *A* forward assay. A mutant spectrum from an expanded phage stock was also determined to assess the effects of propagating the virus. This mutant frequency was higher (6×10^{-4}), contained more double mutants (15% compared to 0.6%), and had a significantly different spectrum from the spectrum for independent cultures (fewer A:T→G:C and G:C→C:G changes and more G:C→A:T; $P < 0.002$). The *E. coli* mutation spectrum will be useful for determining the origin of gene *A* mutation in tissues of ΦX174 transgenic mice. Environ. Mol. Mutagen. 44:119–127, 2004. Published 2004 Wiley-Liss, Inc.†

Key words: single burst analysis; mutant spectra; spontaneous mutant frequency

INTRODUCTION

Spontaneous mutant spectra obtained from transgenic systems have been compared to spectra of the target gene in *E. coli* in order to identify the origin of spontaneous mutations [Hill et al., 1999; Dollé et al., 2002]. Such a comparison has not been made for the ΦX174 gene *A* system. In this study, the spontaneous mutant frequency and mutation spectrum in gene *A* of ΦX174 is determined in *E. coli* and compared to the frequency and spectrum of gene *A* mutants previously determined for the ΦX174 transgene rescued from a mouse embryonic cell culture line [Valentine et al., 2002].

We would prefer to compare the mutation spectrum of the recovered transgene to its mutation spectrum in the same strain of *E. coli* that is used in recovery of the in vivo transgene. However, it is a unique feature of the ΦX174 recovery assay that the strain used for electroporation, DXHR1, is not a host for ΦX174 phage infection. DXHR1 is a K-12 derivative, being descended from strain DH10B (Bethesda Research Laboratories, now Invitrogen, Carlsbad, CA) [Burkhart et al., 1992; Valentine et al., 2002], which in turn was derived from strain MC1061 [Grant et al., 1990]. *E. coli* strain MC1061 is an *E. coli* K-12 strain [Casadaban and Cohen, 1980], which is not a host for ΦX174 because the wild-type lipopolysaccharide differs from that of the ΦX174 normal host, *E. coli* C [Hayashi et al., 1988].

This property of the recovery strain, that it is not a host for the virus, is the basis for its ability to evaluate the progeny from a single burst of the first cell electroporated. The facts that strain DXHR1 can be productively infected by electroporation of circular double-stranded DNA, but cannot be infected by the progeny phage, mean that progeny of a single recovered transgene molecule from one and only one bacterial cell is scored. This quality is used to distinguish the origin of fixation of mutation based on burst size

*Correspondence to: Dr. Carrie R. Valentine, Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research, 3900 NCTR Road, HFT-120, Jefferson, AR 72079. E-mail: cvalentine@nctr.fda.gov

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[Malling and Delongchamp, 2001; Malling et al., 2003]. The best way to measure the mutant frequency in the recovery strain DHXR1 is to introduce into it DNA produced in a mammal, which would have few inherent mutations. However, this is exactly how the recovery of the ΦX174 transgene is carried out for mutational studies; therefore, the argument becomes circular. We wanted an independent measure of the mutation frequency and spectrum in *E. coli* in order to confirm the origin of in vitro mutations assigned by single burst analysis from animal tissue [Valentine et al., 2004].

The selective strain used for detection of ΦX174 gene *A* mutants is also not a candidate for this analysis because only mutants can grow on it. Therefore, we chose to use the permissive strain, CQ2, which is used to identify the total number of transgenes recovered in the ΦX174 assays. This strain carries the *supF* gene for a suppressor tRNA that inserts tyrosine for the nonsense codon TAG (CQ2Su3$^+$) [Eggertsson and Söll, 1988; Chambers, 1989]. It suppresses the *am3* mutation in gene *E* of the ΦX174 transgene and produces large plaques with ΦX174 infection. Therefore, it is an efficient host for ΦX174 replication and would provide a mutation spectrum in *E. coli*.

The determination of the spontaneous mutant frequency and spectrum in gene *A* grown in *E. coli* should be evaluated from a small number of starting genomes in independent cultures in order to avoid inclusion of preexisting mutants [Schaaper and Dunn, 1991]. Therefore, the *E. coli* spectrum was determined by sequencing one mutant from each of 156 small independent cultures that had each been inoculated with an average of two plaque-forming units (PFUs) of ΦX174. Since expansion of mutant plaques is needed for mutant verification and for PCR for DNA sequencing, we also determined the mutant frequency and spectrum for a single expanded phage stock in order to identify the types of changes occurring from extended propagation of ΦX174.

MATERIALS AND METHODS

Production of Mutant Virus From Independent Lysates

Multiple independent gene *A* mutants were prepared in wells of 96-well microtiter plates containing *E. coli* strain CQ2. A mutant-free phage inoculum was prepared by coring a plaque grown on CQ2 (presumably wild-type with respect to gene *A*) with a sterile 100 μl capillary and storing refrigerated in 50 μl 0.05 M borate, pH 8.0. The plaque eluate was titered by plating on TK bottom agar as described by Valentine et al. [2002] on the same day used to deliver two PFUs per well to 96-well microplates. Each 96-well plated included 8 wells filled with 100 μl/well of TK media [Valentine et al., 2002] supplemented with 1 mM calcium chloride and 30 mM magnesium sulfate (media controls). The remaining wells were filled with 100 μ1 per well of freshly grown CQ2 cells (A_{600} = 0.5) diluted 100-fold in the supplemented TK (eight wells had no further addition for cell growth controls). Two PFUs from the agar core eluate were delivered in a volume between 5 and 10 μl to the remaining 80 wells. Plates were incubated overnight at 33°C. Wells that had poor bacterial growth compared to control wells as judged by eye were selected for plating (3–30 μl) as above on the selective strain, gro89pLS1D. One plaque was cored from each plate for sequencing [Valentine et al., 2002, 2004].

Mutant frequencies were determined from 12 wells in different rows of the 96-well plates in the following manner. After overnight incubation of the microplate, the lysate in each selected well was diluted with serial 10-fold dilutions from 1×10^{-1} to 1×10^{-6} in 0.05 M borate buffer, pH 8.0, by taking 30 μl from each well and adding 270 μl buffer. For plating on selective bacteria, the entire diluted sample (10^{-1} and 10^{-2}, 270 μl after removing 30 μl for next dilution) were plated on TK bottom agar with 150 μl gro89pLS1D cells (A_{600} = 0.5) and 4.0 ml 0.75% top agar. For plating on permissive bacteria for total PFUs, 100 μl of the 10^{-6} dilution was mixed with 200 μl of strain CQ2 and plated similarly in triplicate (total volume 0.3 ml). Mutant frequencies were the ratio of mutant to total PFUs, adjusted for the dilutions.

Production of Mutant Virus Within a Single Lysate

A stock of ΦX174 *am3 cs70* virus was grown in *E. coli* strain CQ2 from a single plaque as follows (based on Fane and Hayashi [1991]). A fresh plaque was cored with a sterile Pasteur pipette tip and stored overnight in 1.0 ml HFB (0.1 M Tris-HCl, pH 7.4, 0.06 M ammonium chloride, 0.09 M NaCl, 0.1 M KCl, 1.0 mM $MgSO_4$, 1.0 mM $CaCl_2$). A culture of CQ2 was grown in 100 ml TK broth (1% tryptone, 0.5% KCl) to a density of 2×10^8 cells (A_{600} = 0.16–0.18) at 37°C with shaking. Magnesium chloride was added to the culture to a final concentration of 10 mM and calcium chloride to a final concentration of 5 mM. Magnesium stabilizes virus at high titer in liquid culture, while calcium promotes adsorption of the virus to cell debris for later collection by centrifugation. The plaque suspended in HFB was added to the culture and shaking continued for an additional 5.5 hr. The lysate was stored overnight at 4°C, since low temperature favors adsorption of the virus to cell debris in the presence of calcium. The lysate was centrifuged in 50 ml conical tubes at 1,500*g* for 10 min. The pellet was resuspended in BE buffer (0.05 M sodium borate, 5.0 mM EDTA) and inverted on a rotating platform at 4°C for 3 days. The centrifugation was repeated and the supernatant collected. The titer of the virus plated on CQ2 was 2×10^{10} per ml and the total volume was 1 ml. Dilutions of this phage preparation were plated on the selective and permissive strains used for the forward assay for gene *A* as above. Mutant plaques were cored for sequencing.

Calculation of Mutation Rate per Genomic Replication

Mutation rates per genomic replication were calculated by the A_0 method of Drake [1991] for geometrical replication. Equations were solved using the program Mathematica [Wolfram, 1999]. The geometric mean of the linear mutation rate (same as mutant frequency) and geometric mutation rate were calculated by averaging the logarithms of both the mutant frequency and geometrical mutation rate; the antilogarithm of this mean was the geometric mean of the mutation rates.

Statistical Analyses

Mutant spectra were compared by the method of Cariello et al. [1994]. The probability that a specific single or double mutation within a spectrum was different from the same mutation within another spectrum was calculated by Pearson's two-tailed chi-square analysis. The significance of the difference of frequencies of double mutants between spectra was also calculated by chi-square analysis. The significance of the difference between means of mutant frequencies with standard deviation was determined by Students' t-test.

TABLE I. Spontaneous Mutant Frequency in Gene *A* of ϕX174 Grown in 12 Independent Cultures of *E. coli* Strain CQ2

Plate number/well	Number of mutant PFUs counted from 27 μl lysate	Number of mutant PFUs calculated for 100 μl well[a]	Total number of PFUs per 100 μl well ($\times 10^6$)	Mutant frequency[b] ($\times 10^{-6}$)
P1/A4	60	222	6.3	35
P1/B7	23	85	5.7	15
P1/C8	70	259	32	8.1
P1/D5	27	90	6.3	14.3
P1/E10	11	41	9	4.6
P1/F3	43	159	14	11.3
P1/G7	151	503	66	8
P1/H12	173	577	33	18
P2/A3	97[c]	1074	36	29
P2/D4	72[c]	2666	80	33
P2/F5	184[c]	6815	489	14
P2/H3	49	181	10	18
Mean				17.4 ± 10
Median				14.6

[a]Previous column × 100/27 or × 100/2.7 if 10^{-2} dilution.
[b]Number of mutant PFUs calculated for 100 μl well/total number of PFUs.
[c]From 2.7 μl of lysate (270 μl of 10^{-2} dilution).

RESULTS

Spontaneous Mutant Frequencies

Over 400 wells were seeded with 2 PFUs/well of ΦX174 and 16% produced good bacterial growth (i.e., no viral infection). Based on this result and the Poisson distribution, the average number of PFUs/well was 1.8, which agreed well with the intended seeding determined by agar plating (2.0). The spontaneous mutant frequency for independent mutants was determined from 12 of the wells that were used to isolate mutants for sequencing (Table I). The average mutant frequency was $17 \pm 10 \times 10^{-6}$ and the median frequency was 15×10^{-6}. The mutation rates per genome replication were also calculated by the A_0 method for geometric growth of Drake [1991] for each independent culture and the average mutation rate was $3.5 \pm 0.8 \times 10^{-6}$ (median, 3.4×10^{-6}).

However, as Drake [1991] points out for M13 replication, rolling circle replication is linear replication, rather than geometric. ΦX174 also proceeds through rolling circle replication after a period of geometric, semiconservative replication. We have used the method of Drake [1991] of calculating a geometric mean between mutant frequency and the geometric mutation rate to achieve an estimate of the mutation rate for ΦX174 replication. This mean for the 12 independent cultures, $7.4 \pm 2.3 \times 10^{-6}$ (median, 7.7×10^{-6}), was about half that of the average mutant frequency.

The spontaneous mutant frequency in gene *A* of ΦX174 for the single expanded culture (single plaque to 2×10^{10} PFUs) was 6×10^{-4}. The mutation rate per genome, replication, μ, was calculated by the A_0 method of Drake [1991] based on $N = 2 \times 10^{10}$ and $f = 6 \times 10^{-4}$ (only one culture evaluated); therefore, $\mu = 4.4 \times 10^{-5}$. The estimated mutation rate was taken as the geometric mean between this geometric rate and the linear rate (6×10^{-4}) and was 1.6×10^{-4}.

Mutation and Mutant Spectra of Single Mutants

For the independent mutation spectrum (frequency of independent mutation at specific target sites), a large portion of each well lysate was plated on selective bacteria. One representative well-isolated plaque was cored from different plates with five or more plaques that were not confluent or overcrowded. Selecting plaques from plates with fewer mutant plaques ensured not only that the mutant was pure, but also that a descendant was picked relatively early in the expansion of the original mutation. Eluates from these agar plugs were spotted on selective bacteria to confirm their mutant status. In all, 156 mutants were sequenced, each originating from a separate well.

The mutation spectrum in gene *A* of ΦX174 for single mutants from the independent cultures and the mutant spectrum (frequency of observed mutation at specific target sites) from the single-expanded *E. coli* culture are shown in Table II. The mutation and mutant patterns (summary of frequency of specific transition or transversion classes) of both types of cultures are shown in Table III. The spectrum of independent *E. coli* mutations was dominated by a single mutation, 4225A→G (56%), followed by mutation at a second site, 4218G→A or C (20%). Although both spectra showed many similar features, they were significantly different from each other ($P < 0.01$). The patterns (Table III) were also significantly different ($P < 0.001$). The expanded stock had the same two primary target sites, but had little 4218G→C and more 4003C→T, 4156C→T, and

TABLE II. Mutation Spectra in Gene *A* of ϕX174 for Single Mutants From Expanded and Independent *E. coli* Cultures

Mutation in ϕX174[a]	Sequence context for new mutations[b]	Amino acid substitution for new mutations	Independent cultures[c]	Expanded stock culture
4003C→T			2	9
4137C→T[d]	CAAG**C**TCTT	A53 leu→phe	0	1
4156C→T			5	9
4218G→A			16	21
4218G→C			15	1
4219C→T			10	14
4224G→A			1	1
4224G→T			1	1
4225A→C			8	2
4225A→G			87	64
4225A→T			5	3
4252C→T[d]	GTT**A**CTGAG	A91 thr→ileu	0	1
4402C→G[d]	GCT**G**CTGAA	A141 ala→gly	0	1
4425C→T[d]	TATT**C**GCGA	A149 arg→cys	0	2
4467G→A			4	1
4468A→G[e]	GAT**G**AGTGT	A163 glu→gly	0	1
Other[f]			2	3
Total number			156	135

[a]Numbering according to Genbank accession number V01128 [Sanger et al., 1978].
[b]Mutated base in bold and mutated codon underlined.
[c]One mutant plaque was sequenced from each well of 96-well plates.
[d]New target site.
[e]New mutation.
[f]No mutation was found from nucleotide 3981 to 4470, the N-terminus of gene *A* [Sanger et al., 1978].

TABLE III. Mutant and Mutation Patterns in Gene *A* of ϕX174 for Single Mutants From Expanded and Independent *E. coli* Cultures

		Mutation patterns	
	Mutant pattern expanded stock culture	Independent cultures	Independent cultures without *E. coli* hot spots[a]
Transitions			
G:C→A:T	59 (45%)	38 (25%)	22 (96%)
at CpG	0 (0%)	0 (0%)	0 (0%)
A:T→G:C	65 (49%)	87 (56%)	0 (0%)
Transversions			
G:C→T:A	1 (0.75%)	1 (0.65%)	1 (4%)
G:C→C:G	2 (1.5%)	15 (9.7%)	0 (0%)
A:T→T:A	3 (2.3%)	5 (3.2%)	0 (0%)
A:T→C:G	2 (1.5%)	8 (5.2%)	0 (0%)
Total	132 (100%)	154 (100%)	23 (100%)
At G:C base pairs	62 (47%)	54 (35%)	23 (100%)
At A:T base pairs	70 (53%)	100 (65%)	0 (0%)

[a]Mutation at nucleotides 4218 and 4225 from Table II not included.

4218G→A. This made the mutation pattern of the expanded culture richer in G:C→A:T mutations and poorer in A:T→G:C and G:C→C:G.

If the two *E. coli* hot spots (4225 and 4128) were removed from the target sites, the preponderance of A:T→G:C mutation disappeared and the predominant transition became G:C→A:T (Table III, shown for independent mutations). The analysis identified four target sites and one mutational event that were not previously reported for gene *A*.

Tables IV and V show a comparison of the gene *A* mutation spectra and mutation patterns from the independent *E. coli* cultures with those of a transgenic mouse embryonic cell line treated with solvent or ENU [Valentine et al., 2002]. The cell culture spectra are also independent with respect to the fact that only one mutant was sequenced from each aliquot, meaning that no two mutations came from the same bacterial cell. There was no significant difference between the mutation spectra ($P > 0.09$) or patterns ($P > 0.08$) from *E. coli* and solvent-treated cells. In contrast, the mutation spectra and patterns were highly different between *E. coli* and either 200 or 400 μg/ml ENU-treated cells ($P < 0.000001$).

TABLE IV. Comparison of ϕX174 Gene *A* Mutation Spectra from *E. coli* and from Mouse Cell Culture

Mutation in ϕX174	Number of occurrences of mutation			
		Mouse embryonic cells in culture[b]		
	E. coli[a]	0 μg/ml ENU	200 μg/ml ENU	400 μg/ml ENU
4003C→T	2	3	5	9
4017G→A	0	1	1	0
4018A→C	0	0	0	2
4019T→A	0	0	2	1
4019T→G	0	0	3	2
4119A→G	0	1	0	2
4126A→G	0	0	1	0
4156C→T	5	3	7	7
4161C→A	0	1	0	0
4162G→A	0	0	4	7
4204A→T	0	0	1	0
4210A→T	0	0	10	10
4218G→A	16	19	18	11
4218G→C	15	11	5	2
4219C→T	10	3	6	7
4224G→A	1	1	0	3
4224G→C	0	1	0	0
4224G→T	1	0	3	1
4225A→C	8	17	9	8
4225A→G	87	65	28	21
4225A→T	5	11	4	4
4239T→A	0	1	2	1
4239T→G	0	0	1	2
4399C→T	0	0	2	0
4452A→G	0	0	0	1
4460T→C	0	0	0	2
4467G→A	4	6	5	9
4468A→C	0	0	1	0
Other	0	0	1[c]	1
Total occurrences	154	144	118	113

[a]Table I, independent cultures.
[b]Valentine et al. [2002].
[c]Three mutants with no mutation from 3981 to 4470 were mentioned in the text in Valentine et al. [2002], but only two were confirmed as having no mutation in this region.

TABLE V. Comparison of ϕX174 Gene *A* Mutation Patterns From *E. Coli* and From Mouse Cell Culture

		Mouse embryonic cells[b]		
	E. coli[a]	0 ENU	200 ENU	400 ENU
Transitions				
G:C→A:T	38 (25%)	36 (25%)	48 (40.7)	53 (47.3)
At CpG	0 (0%)	0 (0%)	4 (3.0%)	7 (6.0)
A:T→G:C	87 (56%)	66 (45.8)	29 (24.6)	24 (21.4)
Transversions				
G:C→T:A	1 (0.65%)	1 (0.7%)	3 (2.5%)	1 (0.9%)
G:C→C:G	15 (9.7%)	12 (8.3%)	5 (4.2%)	2 (1.8%)
A:T→T:A	5 (3.2%)	12 (8.3%)	19 (16.1%)	16 (14.3%)
A:T→C:G	8 (5.2%)	17 (11.8)	14 (11.9%)	16 (14.3%)
Total	154 (100%)	144 (100%)	118 (100%)	112 (100%)
At G:C base pairs	54 (35%)	49 (34%)	56 (47.5%)	56 (50%)
At A:T base pairs	100 (65%)	95 (66%)	62 (52.5%)	56 (50%)

[a]Table II, independent cultures.
[b]Valentine et al. [2002].

TABLE VI. Spectra of Double Gene *A* Mutants From Expanded and Independent *E. coli* Cultures

Relative frequency of each mutation		Number of isolates	
Rarer	Common	Independent cultures	Single expanded culture
4003C→T	4218G→A[c]	0	1
4003C→T	4218G→C	0	1
4003C→T	4225A→G[c]	0	8
4009G→C	4225A→G	0	1
4126A→G	4218G→A	0	1
4217T→C[a]	4218G→A	1	0
4219C→T	4225A→G[c,d]	0	5
4156C→T	4225A→G	0	3
4399C→T	4225A→G[d]	0	1
4156C→T	4218G→A[d]	0	2
4437A→G[b]	4225A→G	0	1
		1	24
Total number of mutants sequenced		157	159
Fraction of total mutants that were double		0.63%	15%

[a]Not previously isolated; silent mutation A79 ala>ala.
[b]Not previously isolated; A153 asn>asp.
[c]Double mutant also isolated from cell culture [Valentine et al., 2002].
[d]Double mutant also isolated from mouse splenic lymphocytes [Valentine et al., 2004].

Double Mutants

The occurrence of double mutants in gene *A* from the single expanded *E. coli* culture and the independent *E. coli* cultures is shown in Table VI. The expanded culture produced a greater proportion of double mutants (15%) than the independent cultures (0.6%), and this difference was highly significant ($P = 0.00001$). The frequency of double mutants from small independent *E. coli* cultures (1/157) was not significantly different from that of spontaneous double mutants from solvent-treated cells (4/141; $P = 0.15$) or 200 μg/ml ENU-treated cells (3/116; $P = 0.19$) [Valentine et al., 2002], although it was significantly different from the 400 μg/ml ENU-treated cells (8/108; $P = 0.004$). However, none of the double mutant frequencies from the cell culture treatments was significantly different from each other ($P > 0.1$).

Three of the double mutants from cells were the same as those found in the independent *E. coli* spectrum (Table VI). All of the double mutants included one of the two hot spots in the single mutation spectra, 4225A→G or 4218G→A or C. However, mutations from both sites were not found together in a double mutant, which would have occurred randomly for 25% of double mutants (2 or 3 mutants out of 11).

DISCUSSION

Mutant and Mutation Frequencies, Mutation Rates

The mutant frequency previously reported for ΦX174 grown on strain gro$^+$ and plated on strain gro89 is 1×10^{-4}

[Ekechukwu et al., 1995]. The mutant frequency for the expanded culture of this study exceeded this frequency, whereas the average mutant frequency for the small independent cultures was lower. The differences probably reflect the different degrees of expansion of a single PFU before mutant frequencies were measured since the mutant frequency of Ekechukwu et al. [1995] was also measured on phage stocks. In addition, the culture conditions may have contributed to different underlying mutation rates.

The accumulated mutant frequency will be higher than the actual mutation rate over a lengthy period of geometric replication because of the multiplication of mutants during expansion [Luria and Delbrück, 1943; Drake, 1991]. The underlying mutation rate is about 1/5 the mutant frequency for geometric replication in the early stages of expansion of mutants. The estimated mutation rate (geometric mean) of the stock culture was 20 times higher than that of the 12 independent cultures, which varied only 2.5-fold between cultures. Therefore, the relatively high genomic mutation rate calculated for the stock culture was not explained solely by the larger size of the population.

Only 1 stock culture was evaluated, rather than 12, so the variance of stock cultures is not known. Also, there was some difference in culture conditions between the stock and independent cultures. The small independent cultures contained only 100 μl medium and were incubated without shaking or agitation; the stock culture contained 100 ml medium and was incubated with vigorous shaking. The higher aerobic conditions may have had an influence on the mutation rate. Although the high mutation rate of the stock culture may not be fully explained, the results emphasize the need for mutant frequencies to be measured in small populations.

There was no significant correlation between the total number of mutants in each of the 12 wells used to determine the spontaneous mutant frequency in *E. coli* and the mutant frequency determined for that well (Table I; $R^2 = 0.0116$). We interpret this to mean that each of these small cultures was analyzed early enough in the propagation of the mutant virus that the accumulation of mutants did not raise mutant frequencies. Most of these wells seem to have stopped propagation after the first or second burst of mutant replication (average burst size, 182.5 [Delongchamp et al., 2001]). We interpret the variation of mutant frequency between wells to reflect the random appearance of the first mutation at different times during the expansion of the several phage particles initiating infection in each well.

The average spontaneous mutant frequency in *E. coli* was not significantly different from that determined from recovery of the transgene from cell culture, 1.9×10^{-5} [Valentine et al., 2002]. This suggests that the spontaneous mutant frequency of gene *A* from cell culture originates from *E. coli*.

It is of interest to compare the mutant frequency of gene *A* of ΦX174 grown in *E. coli* to that of *lacI* in bacteriophage λ (the transgene of Big Blue animals; Stratagene, La Jolla, CA) also grown in *E. coli* because it is claimed that the large majority of spontaneous mutations recovered in the Big Blue assay are fixed in vivo [Paashuis-Lew et al., 1997; Hill et al., 1999]. There exist several reports for the mutant frequency of the *lacI* gene in *E. coli*, each obtained in a different manner. Sui et al. [1999] packaged wild-type phage DNA isolated from lysates derived from a single plaque and obtained a mutant frequency of 6.7×10^{-5} using the same vector and recovery strain as when recovered from animals. However, since the lysates had been expanded before DNA extraction, the mutant frequency might be elevated as shown here for the ΦX174 gene *A* frequency from an expanded lysate. The mutant frequency for *lacI* carried on M13 bacteriophage in a *recA*$^-$ strain of *E. coli*, N9099, has been reported as 1.5×10^{-6} [Yatagai and Glickman, 1990]; the recovery strain for Big Blue mice, SCS-8, is also *recA*$^-$ [Kohler et al., 1991]. The mutant frequency for a portion of the *lacI* gene, without the TGGC hot spot, carried on the F′ plasmid is 1.3×10^{-6} [Schaaper and Dunn, 1991]. This fragment covers approximately 200 base pairs with 58 target sites for base pair substitution. The mutant frequency in *E. coli* for gene *A* without mutations at the two hot spots was here 3.5×10^{-6} for 37 [Valentine et al., 2004] target sites.

We desire a measurement performed in the same vector and with the same *E. coli* strain as for recovery of the λ transgene from animals. A direct analysis has been done by Zhang et al. [1995], who performed a Poisson calculation of the mutant frequency of the *lacI* transgene in *E. coli* from the replating of 18 wild-type plaques recovered from transgenic mice (recalculated by Paashuis-Lew et al. [1997]). This calculation estimates the mutant frequency in *E. coli* to be 16×10^{-6}, which is 4.5 times that for gene *A* of ΦX174 (without hot spots). This compares to about 10 times as many target sites for *lacI* as gene *A*. Therefore, eliminating consideration of mutation at the *E. coli* hot spots for ΦX174, the mutant frequency per target site for gene *A* is about twice that of *lacI*. As more targets are identified for the gene *A* assay, this ratio will decline.

The *E. coli* mutant frequency as measured by Zhang et al. [1995] and Paashuis-Lew et al. [1997] (16×10^{-6}) is slightly higher than the mutant frequencies reported for germ cells, which vary from 6 to 13×10^{-6} and are among the lowest in vivo mutant frequencies measured [Zhang et al., 1995]. Therefore, the calculations of Paashuis-Lew et al. [1997] may be an overestimate, as suggested by the authors. However, the reported mutant frequencies from germ cells are from other laboratories, and interlaboratory variation in the *lacI* assay is greater than twofold for other tissues [Zhang et al., 1995]. Therefore, the difference between germ cell mutant frequencies and the *E. coli* mutant frequency might be the result of interlaboratory variation. Thus, it seems possible that the spontaneous *E. coli* mutant

frequency sets a lower limit for the spontaneous mutant frequency measured from animal tissue.

The practical consequence of these considerations is that the hot-spot mutations generated in *E. coli* for gene *A* of ΦX174 must be identified and discarded by single burst analysis in order to achieve a sensitivity to mutagen treatment comparable to that for the *lacI* transgene.

Mutation Spectra

The independent mutation spectrum of gene A of ΦX174 from *E. coli* was dominated by a single mutation, 4225A→G, followed by a second hot spot at 4218G→A or C. The relative increase of G:C→A:T transitions in the expanded culture may have been the result of the lack of independence of the mutations or the culture conditions (relatively more aeration). The comparison allowed the discovery that secondary mutations (double mutants) are likely on prolonged expansion of mutant plaques.

Spontaneous mutation in *E. coli* is characterized by hot spots that are specific to the DNA context [Maki, 2002]. The sequence context at 4225 (CTTCTG*A*CGTT) has some similarity to a hot-spot sequence in the *lacI* gene (TATAC-G*A*CCGTT), both containing G*A*C separated from GTT by either one or two Cs [Schaaper and Dunn, 1991]. Both sequences mutate predominantly from A→G (base pair 83).

The sequence context at 4218 (AAGGCT*G*CTTCTG) shows some resemblance to another *lacI* hot spot, GGCCT-*G*GTTCAC, both sequences having a CT*G* trinucleotide followed by TTC after either C or G. Two Gs also precede the trinucleotide followed by one or two Cs. Both sequences mutate from G→A (bp 104, opposite strand [Schaaper and Dunn, 1991]). This is also a C→T transition, which, as has been pointed out by Yatagai and Glickman [1990], is more readily achieved spontaneously through cytosine deamination in single-stranded DNA [Lindahl and Nyberg, 1974]. ΦX174 is a single-stranded bacteriophage and replicates through rolling circle production of linear single-stranded genomes, but this idea does not account for the specificity of the hot spot at a particular location. Its similarity to the *lacI* hot spot on a double-stranded episome, F′, suggests that it results from DNA polymerase specificities.

The mutation spectrum from independent *E. coli* cultures is the one that should be compared to spectra derived from animal cells or tissue. When recovering mutant ΦX174 molecules from mammalian cells, only a single round of replication is allowed in the *E. coli* strain for electroporation before plating on the strain for selection. An in vitro mutant must be fixed in one of only two bacterial cells in order to be recovered: the electroporated cell or the first selective bacterium infected. Therefore, the recovery of the transgene from animal cells or tissue limits the expansion of a mutant phage genome before selection to the same extent as the protocol for independent cultures used here.

The fact that the mutation spectrum from independent *E. coli* cultures is not different from that of solvent-treated cells in culture but is highly different from ENU-treated cultures is consistent with the spontaneous mutant spectrum in cell culture being essentially that in *E. coli*. Combined with the fact that the mutant frequencies are also the same for *E. coli* and solvent-treated cells, these results make a strong argument that a large majority of the mutants isolated from solvent-treated cells originate in *E. coli*. These results also argue that the use of *E. coli* CQ2 as a representative *E. coli* strain was reasonable.

The removal of the two hot-spot target sites from the gene *A* mutation spectrum left all of 23 mutations in the G:C→A:T transition category. This is also the most prevalent base substitution class for other transgenes recovered from untreated tissues: the *lacI* gene isolated from spleen and liver [Chen et al., 2001], the *cII* gene and *lacI* gene isolated from liver and colon [Kohara et al., 2001; Chen et al., 2002; Yamada et al. 2002], the *lacZ* plasmid-based transgene isolated from spleen, brain, heart, liver, and small intestine [Dollé et al., 2002; Louro et al., 2002], the *lacZ* gene in bacteriophage λ isolated from spleen, liver, heart, brain, and testis [Ono et al., 1999, 2000], and the *gpt* transgene isolated from bone marrow [Masumura et al., 1999]. However, in most of these studies, a majority of the G:C→A:T transitions occurred at CpG sites, which implicates the deamination of 5-methylcytosine. For gene *A* from cell culture, none of the G:C→A:T transitions were found at either of the two CpG sites known to be recoverable by the assay.

However, similar to our results, the study with the plasmid-based *lacZ* transgene [Dollé et al., 2002] found a minority of G:C→A:T transitions at CpG sites in the brain or spleen of young animals. This suggests that the deamination of cytosine may be a major contributor to spontaneous mutations regardless of whether cytosine is methylated. Although it was noted that the spontaneous mutation patterns from young animal tissue are similar to that of mutants isolated in *E. coli*, the mock-frequency determined by recovery of the plasmid-based target in *E. coli* (background) was lower (0.6×10^{-5} compared to an average of 2.8×10^{-5} for young animal tissues) and 80% of the *E. coli* mutations were not found from mouse tissue, indicating a different mutational spectrum. These results argue for this transgene that most of the spontaneous mutations recovered from animal tissue do not originate in *E. coli*, but does not address ex vivo mutation [Valentine et al., 2004].

Double Mutants

The high incidence of double mutants in the single expanded lysate (15%) sharply contrasted with the lone double mutant found in small cultures (0.6%). Since almost all of the double mutants occurred in the expanded culture, the second mutation in most of these mutants must have been acquired sequentially during prolonged propagation, and

not during a single repair event. The high ultimate frequency of double mutants may reflect a selection for two mutations, replicating more efficiently than mutants with single mutations. Mutant selection is based on the ability of the ΦX174 mutant in gene *A* to compensate for a mutant *rep* gene of *E. coli* [Valentine et al., 2002]. These two proteins interact with each other during stage III DNA synthesis, which is linked to DNA packaging into phage heads. It is possible that a second mutation improves the binding interaction between the two proteins and that a double mutant could replicate more efficiently than single mutants. It is noteworthy that no double mutants contained both of the two most frequently isolated single mutations, 4225A→G and 4218G→A, even though mutation at one of these two target sites was present in all double mutants. This suggests that this particular pair of mutations may produce a nonfunctional protein, which prevents propagation. The fact that all double mutants contained a frequent mutation suggests that the occurrence of double mutations was random, showing the same predominance of the two frequent target sites.

Some of the double mutations found from propagation in *E. coli* were also isolated in other studies from cell culture or splenic lymphocytes (Table VI). This suggests that some common mechanism for their creation operates among these different cell types. This common mechanism could be related to fixation in *E. coli* during recovery of the transgene. Of the three double mutants also isolated from splenic lymphocytes in the accompanying study [Valentine et al., 2004], all had at least one of the two mutations fixed in *E. coli*.

During our sequencing of gene *A* mutants, we frequently observed mixtures with minor components of the most commonly isolated mutations. These were not included in the spectra reported here; only substitutions with sequencing signals equivalent to surrounding bases were reported. We routinely replate isolated plaques on selective bacteria both to confirm their mutant status and to equalize viral progeny between small and large plaques for PCR amplification. The replating is necessary to verify mutant status because a background of wild-type phage is present in all agar plugs and could be amplified during PCR. Even bubbles could be scored as "other" mutation, i.e., no mutation found in the N-terminus of gene *A*. A typical mutant plaque may contain 10^6 phage; after replating, this could expand again several orders of magnitude. Therefore, we were interested to know if we could be introducing further mutations during this expansion process. The accumulation of double mutants in extended culture in *E. coli* suggests that replating of mutants before sequencing may contribute to these secondary mutations.

The mutant frequencies and spectra in gene *A* of ΦX174 grown in *E. coli* determined by this study provide a basis for comparison to spectra generated in transgenic cell culture and from transgenic mouse tissue. The *E. coli* spectrum was dominated by two hot spots, 4225A→G (56%) and 4218G→A or C (20%); the mutant pattern lacking these two target sites was entirely G:C→A:T, which is the most prevalent base substitution for other transgenes. Both the *E. coli* spontaneous frequency and mutation spectrum were not significantly different from those of solvent-treated cells in culture, but were highly different from those of ENU-treated cells. These results lead to the conclusion that almost all of the spontaneous mutants from cell culture for the ΦX174 forward assay [Valentine et al., 2002] originated in *E. coli*. These mutants must be eliminated by single burst analysis [Malling et al., 2003; Valentine et al., 2004] in order to measure in vivo spontaneous mutant frequencies.

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