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Estimation of mutation rates in cultured mammalian cells

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The factors that affect reliable estimations of mutation rates (μ) in cultured mammalian somatic cell populations by fluctuation analysis are studied experimentally and statistically. We analyze the differential effect of the final cell population size in each culture (N_t) and the number of parallel cultures (C) on the variation in the rate estimates ($\hat{\mu}$) inferred from the P_0 method. The analysis can be made after the derivation of the variance of $\hat{\mu}$, which is a measure of variation of $\hat{\mu}$ for a given combination of N_t and C in a number of repeat experiments. The variance of $\hat{\mu}$ is inversely proportional to C and to the square of N_t. N_t determines the probability of occurrence of mutations in a cell culture. By influencing the size of P_0, N_t also determines whether a rate estimate is obtainable from the experiment. Since P_0 is estimated from the fraction of cultures containing no mutation in a set of C cultures, C becomes a determining factor for the accuracy of $\hat{\mu}$. The rate estimated from $\hat{P}_0$ is biased, but the bias is in general 2 orders of magnitude smaller than $\hat{\mu}$. By the selection of an appropriate combination of N_t and C for the experiment, this bias can be reduced even further.

Based on the notion of comparing two proportions, we propose a test statistic and have applied it to experimental results for a test of equality of mutation rates in different cell lines. This development places the comparison of mutation rates on a statistical basis.

Since the publication of Luria and Delbrück's paper [19] on the distribution of the number of mutant colonies in a series of parallel cultures, fluctuation analysis has been widely applied as a method for the determination of mutation rates (μ) in cell populations. The analysis has since been further developed methodologically by Lea and Coulson [15], Newcombe [22], and Armitage [2,3] and modified theoreti-

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cally by Kondo [14]. Although other methods [22–24] are available, fluctuation analysis has reamained the most popular method for the estimation of μ.

In mammalian cell lines grown in vitro, μ values estimated by fluctuation analysis vary considerably. In the data compiled by Morrow [21], for instance, the rate estimates ($\hat{\mu}$) for the same genetic marker in a cell line with the same designation but grown in different laboratories vary by as much as 2000-fold. A variety of factors may be responsible for this discrepancy. First, unidentified differential mutabilities among the sub-lines studied may contribute in part to the variation. Second, the procedures of cell culture such as dissociation of cells by trypsinization, the cell number per culture dish, the growth medium, and the concentration of selective agent may influence the recovery of mutants [10]. Third, a source of variability of $\hat{\mu}$ may come from the phenotypic lag and possible selective disadvantage of the mutant cells, as discussed by Koch [13]. Finally, the number of parallel cultures (C) and the final cell population size (N_t) of any one culture may contribute greatly to the variation in the estimated mutation rate [11]. Taking several published reports as examples, C varies from 6 [26] to 120 [25], and N_t varies from 10^4 to 10^8 cells in different experiments [20,25,26]. It is therefore difficult to know which of the published mutation rates are dependable and whether a rate comparison between cell types, genetic markers or experiments is at all possible.

The factors that most affect the estimation of μ by fluctuation analysis are N_t and C. The purposes of this paper are: (1) to analyze experimentally and statistically the interaction between the two parameters, (2) to design a practical experimental scheme by which reliable estimation of μ may be assured, and (3) to find a test statistic for a comparison of mutation rates in different cell lines. A preliminary account of this work has been published elsewhere [16].

Results

Statistical consideration of the effects of N_t and C on the estimation of μ

According to Luria and Delbrück [19], the probability, P_0, that no mutation has occurred in an experiment can be expressed as:

$$P_o = e^{-\mu N_t} \tag{1a}$$

or

$$\mu = (-\ln P_0)/N_t \tag{1b}$$

Since μ is a constant, P_0 becomes a function of N_t; i.e., N_t determines whether there will be some mutations detectable in any particular culture. In practice, P_0 is derived from the number of cultures (X) containing no mutations in a set of C sib cultures, i.e., $\hat{P}_0 = X/C$. Hence C determines in part whether an appropriate P_0 is obtainable. We have proposed elsewhere [11] that at least $X = 10$ sib cultures containing mutations are needed for a reliable estimation of μ.

The expected value of $\hat{P}_0$ is equal to P_0; $\hat{P}_0$ is therefore an unbiased estimate of P_0.

From Eqn. 1b:

$$\hat{\mu} = \left(-\ln \hat{P}_0\right)/N_t \tag{2}$$

The expectation (E) and variance (var) of $\hat{\mu}$ can be derived by applying a Taylor series expansion to Eqn. 2:

$$E(\hat{\mu}) \doteq \mu + \frac{1 - P_0}{2CN_tP_0} = \mu + \text{bias} \tag{3}$$

and

$$\text{var}(\hat{\mu}) \doteq \frac{1 - P_0}{CN_t^2P_0} \tag{4}$$

N_t is regarded as a constant in the derivation of Eqns. 3 and 4, because in routine practice the cell numbers in each culture are closely monitored. A similar but more cumbersome derivation for a variance of mutant frequency was reported by Furth et al. [12] for a human lymphoblastoid cell line. In that paper, normality was assumed when constructing confidence intervals for the mutant frequency.

The variance of $\hat{\mu}$ should not be confused with the variance of mutant colonies, var(y), in parallel cultures. Var($\hat{\mu}$) is a measure of the variation of $\hat{\mu}$ obtained from a given combination of C and N_t if the experiment is repeated many times.

That μ estimated from $\hat{P}_0$ is biased is somewhat surprising. Armitage [2] warned that some of the methods for the calculation of μ in fluctuation analysis might be biased, but the warning has been generally overlooked and the extend of bias has never been evaluated.

The bias of $\hat{\mu}$ is in general 2 orders of magnitude smaller tham $\hat{\mu}$; its exact size varies depending on the various combinations of N_t and C used in the experiment (see Table 1). Because $P_0 = e^{-\mu N_t}$, however, a larger N_t will increase the bias slightly (see Table 1). The mean squared error (MSE), $E(\hat{\mu} - \mu)^2$, a measure of deviation of $\hat{\mu}$ from μ, is equal to:

$$\text{var}(\hat{\mu}) + (\text{bias})^2$$

The size of MSE can be minimized by maximizing both N_t and C, but N_t has a bigger impact.

Since $\hat{\mu}$ is biased, the 95% confidence interval of μ cannot be constructed directly. However, based on P_0, the approximate upper and lower bounds of μ can be derived by taking the natural logarithm of the 95% confidence interval of P_0, then dividing the interval by N_t. The 95% confidence interval of P_0 can be obtained by using the figure for the confidence limits for proportions from statistics textbooks such as ref. 5.

As a numerical example to illustrate the effect of N_t and C on the recognition of mutations and the reliability of $\hat{\mu}$, consider a hypothetical series of experiments in which C is set to be 25, 50, 100 or 150, and N_t is set to be 10^5, 10^6, or 10^7. The expected results are shown in Table 1. There are 4 combinations of N_t and C in which the ratios (R) of the upper and lower bounds of μ are greater than 220, indicating that $\hat{\mu}$ values are expected to vary greatly. When N_t is 10^6 and C is 50, the

TABLE 1

THE INFLUENCE OF DIFFERENT COMBINATIONS OF N_T AND C ON THE VARIATION OF THE ESTIMATES OF μ WHEN μ IS ASSUMED TO BE 1×10^{-7} MUTATIONS PER CELL PER DIVISION

N_t	C	var($\hat{\mu}$) [a]	Bias of $\hat{\mu}$ [b]	MSE [c]	Bounds of μ [d]	R [e]	CV [f]	$C\mu N_t$ [g]
1×10^5	25	4.02×10^{-14}	20.10×10^{-10}	4.02×10^{-14}	1.10×10^{-8}–23.10×10^{-7}	220.34	2.00	0.25
	50	2.01×10^{-14}	10.00×10^{-10}	2.01×10^{-14}	0.50×10^{-8}–11.00×10^{-7}	219.07	1.42	0.50
	100	1.00×10^{-14}	5.03×10^{-10}	1.00×10^{-14}	0.25×10^{-8}– 5.65×10^{-7}	223.44	100	1.00
	150	6.70×10^{-15}	3.35×10^{-10}	6.70×10^{-15}	0.17×10^{-8}– 3.70×10^{-7}	220.29	0.82	1.50
1×10^6	25	4.21×10^{-15}	21.00×10^{-10}	4.21×10^{-15}	0.15×10^{-7}– 3.01×10^{-7}	19.92	0.65	2.50
	50	2.10×10^{-15}	10.50×10^{-10}	2.10×10^{-15}	0.36×10^{-7}– 2.55×10^{-7}	7.15	0.46	5.00
	100	1.05×10^{-15}	5.26×10^{-10}	1.05×10^{-15}	0.46×10^{-7}– 1.98×10^{-7}	4.31	0.32	10.00
	150	7.01×10^{-16}	3.51×10^{-10}	7.10×10^{-16}	0.56×10^{-7}– 1.80×10^{-7}	3.19	0.26	15.00
1×10^7	25	6.88×10^{-16}	34.40×10^{-10}	6.99×10^{-16}	0.55×10^{-7}– 1.77×10^{-7}	3.20	0.26	25
	50	3.44×10^{-16}	17.20×10^{-10}	3.47×10^{-16}	0.65×10^{-7}– 1.43×10^{-7}	2.18	0.19	50
	100	1.72×10^{-16}	8.59×10^{-10}	1.73×10^{-16}	0.76×10^{-7}– 1.31×10^{-7}	1.73	0.13	100
	150	1.15×10^{-16}	5.73×10^{-10}	1.15×10^{-16}	0.80×10^{-7}– 1.24×10^{-7}	1.55	0.11	150

[a] $\text{var}(\hat{\mu}) = (1-P_0)/(C\cdot N_t^2\cdot P_0)$.

[b] bias of $\hat{\mu} = (1-P_0)/(2C\cdot N_t\cdot P_0)$.

[c] MSE = mean squared error = var($\hat{\mu}$)+(bias of $\hat{\mu}$)^2.

[d] Derived from the 95% confidence interval of P_0.

[e] R = upper bound/lower bound.

[f] Coefficient of variation $(CV) = \sqrt{\text{var}(\hat{\mu})}\,/\hat{\mu}$.

[g] $C\mu N_t$ is the expected total number of mutations in the experiment.

discrepancy of $\hat{\mu}$ from different experiments may reach 7.15-fold. The variation decreases to a range of 3.19-fold when C increases to 150. When N_t is 10^7, the variation of $\hat{\mu}$ becomes less dependent on the size of C, and the expected total number of mutations in the experiment ($C\mu N_t$) is much higher than those at the lower levels of N_t. The results clearly demonstrate the combined influence of N_t and C, with N_t playing the major role, on the reliability of $\hat{\mu}$ in an experiment.

Toward a reliable estimation of μ

One measure of precision in the etimate of μ is the coefficient of variation (CV):

$$CV = \frac{\sqrt{\text{var}(\hat{\mu})}}{\hat{\mu}} \tag{5}$$

When CV is 0.20, the variation of $\hat{\mu}$ from experiment to experiment will be less than 3-fold (Table 1).

At an early stage in designing a fluctuation experiment, the number of sib cultures (C) should be determined. From Eqns. 4 and 5 and a preliminary estimate of $\hat{\mu}_0$,

$$C = \frac{1 - P_0}{(\hat{\mu}_0 N_t)^2 \cdot P_0 \cdot (CV)^2} \tag{6}$$

If CV is set to be 0.20, then $C = 25(1 - P_0)/(\hat{\mu}_0 N_t)^2 P_0$. Thus, if $\hat{\mu}_0 = 1 \times 10^{-7}$ and $N_t = 1 \times 10^7$, then C should be set at 43 to estimate μ within a range of 2- or 3-fold.

For a fluctuation analysis to yield a reliable and reproducible $\hat{\mu}$, the following steps are recommended: (i) predict in advance the order of magnitude of μ to be determined, either from past experience or from the literature, (ii) use Eqn. 1 to choose a value of N_t so as to obtain an appropriate P_0, perhaps N_t equal to 0.5 μ, (iii) choose a CV for an permissible size of variation, and (iv) use Eqn. 6 to compute C. The judicious choice of a combination of N_t and C will also depend on the laboratory facilities and its available resources.

Effects of N_t *and* C *on the estimation of* μ

The results of 5 Expts. on spontaneous mutations from sensitivity to resistance to 6-thioguanine (6-TG) in Chinese hamster V79 cells are summarized in Table 2. The experiments were initiated with several large batches of parallel cell cultures, each being allowed to grow for different lengths of time in order to reach different final cell numbers. Some of the experiments were deliberately made less than ideal, with the intention of demonstrating the differential effect of N_t and C on the reliability of estimation of μ. Mutation rate is expressed as the numbers of mutations per cell per division [19,22]. The ratio of the variance to the mean of the number of resistant colonies, $\text{var}(y)/\bar{y}$, is much greater in the fluctuation experiments than in the control, consistent with the notion that 6-TG resistance occurred randonly during population growth in the absence of 6-TG. When both N_t and C were set small (Expt. 2), no mutation was recovered in any single culture. Mutants became detectable as C was increased (Expt. 1). The large CV of Expt. 1 suggests that a large

TABLE 2

ESTIMATION OF MUTATION RATES FROM 6-THIOGUANINE SENSITIVITY TO RESISTANCE IN CHINESE HAMSTER V79 CELLS IN CULTURE [a]

Parameter	Replicate-sample control	Expt. No.				
		1	2	3	4	5
C	1	61	20	61	20	55
N_0	–	80	56	80	64	80
N_t	1.27×10^6	3.51×10^5	4.6×10^5	1.62×10^6	2.13×10^6	6.62×10^6
Mutant colonies (y)						
Total	18	15	0	575	122	1222
Range	0–3	0–9	–	0–510	0–97	0–1080
Mean ($\bar{y}$)	1.5	0.25	–	9.27	6.1	45.3
Variance, var(y)	0.87	1.86	–	4148.62	442.89	20825.02
var(y)/$\bar{y}$	0.57	7.55	–	447.34	72.60	459.71
$\hat{P}_0$		59/61	20/20	52/61	16/20	27/55
$\hat{\mu}$		0.95×10^{-7}	–	0.99×10^{-7}	1.05×10^{-7}	1.08×10^{-7}
Variance of $\hat{\mu}$		4.51×10^{-15}	–	1.06×10^{-15}	2.76×10^{-15}	4.31×10^{-16}
Bias of $\hat{\mu}$		7.92×10^{-10}	–	8.76×10^{-10}	2.93×10^{-9}	1.43×10^{-9}
MSE		4.51×10^{-15}	–	1.06×10^{-15}	2.76×10^{-15}	4.33×10^{-16}
CV of $\hat{\mu}$		0.71	–	0.33	0.50	0.19
$C\hat{\mu}N_t$		2.03	–	9.78	4.47	39.23

[a] See footnotes of Table 1 for the explanation of the parameters. All the cells in each culture were used. 4×10^5 cells were seeded per culture dish into selective medium which contained 10 μg/ml of 6-thioguanine and 10 ng/ml of TPA (4β-phorbol 12β-myristate 13α-acetate). On day 4 TPA was removed by renewing selective medium. Resistant colonies were stained and counted on day 12.

variation of $\hat{\mu}$ among repeated experiments is expected to occur, including zero estimates. As N_t was increased (Expts. 1, 3 and 5) and C kept at a constant level, the sizes of $C\hat{\mu}N_t$ increased accordingly while $\hat{P}_0$ and CV decreased; more mutations were detected and $\hat{\mu}$ became less variable. In Expts. 3 and 4, the sizes of N_t were similar, but Expt. 4 had a larger CV because its C was smaller. In Expt. 5, the value of CV was 0.19, suggesting that its $\hat{\mu}$ was reliable within a factor of 3.

Applying the same analysis to some of the published data [1,4,9,20,25,26], we find that most of the experimental designs are less than ideal. Values of CV range from 0.81 to 0.22; most of the C values used are less than ideal to ensure a narrow range of $\hat{\mu}$. The stipulation of $CV \leq 0.2$ may seem too stringent a condition for experimental design, but it seems to us that without a reproducible estimate of μ, the whole exercise is useless.

Equality test of μ derived from different cell lines

To assess the biological meaning of the variation of $\hat{\mu}$ obtained from different cell lines, it is necessary to perform tests on the homogeneity of population μ values inferred from the P_0 method. Assuming that the N_t values from 2 Expts. are the same and that $\mu_1 = \mu_2$, then $\mu_1 N_{t1} = \mu_2 N_{t2}$, or $P_{01} = P_{02}$. Therefore, to test the null hypothesis H_0: $\mu_1 = \mu_2$ is the same as to test H_0: $P_{01} = P_{02}$ with $N_{t1} = N_{t2}$. Assuming that the differences of the two proportions are normally distributed, if the statistic

$$Z = \frac{P_{01} - P_{02}}{\sqrt{\left(\frac{C_1 P_{01} + C_2 P_{02}}{C_1 + C_2}\right)\left(1 - \frac{C_1 P_{01} + C_2 P_{02}}{C_1 + C_2}\right)\left(\frac{1}{C_1} + \frac{1}{C_2}\right)}}$$

gives $|Z| \geq 1.96$, then H_0 should be rejected under a 2-tailed test at the 5% level. An alternative test for the equality of two proportions is the exact test at the 0.05 level, based on the hypogeometric distribution. However, the computation is rather tedious for large values of C_1 and C_2.

In Table 3, equality tests on the μ values inferred from $\hat{P}_0$ from the literature and some of our own results are presented. Since the criterion for using the Z statistic is to have the same N_t, only those experiments with similar N_t were analyzed. For this reason and because not all $\hat{P}_0$ values are available, none of the published data on 'mutator' cell lines [20,25,26] can be tested in this fashion. Our Z test is sensitive enough to reject H_0: $\mu_1 = \mu_2$ when $\hat{\mu}_1/\hat{\mu}_2$ is as small as 3.05 (Expt. A). When the μ ratio is as small as 1.35 (Expt. D), however, H_0 is accepted.

Discussion

By the application of simple statistical procedures, we have provided solutions for two long-standing and perplexing problems in quantitative mutagenesis research, viz.: the reliable estimation of mutation rate (μ) and the comparison of μ obtained from different cell lines. The first problem is especially important for mammalian

TABLE 3

EQUALITY TEST OF MUTATION RATE (μ) INFERRED FROM THE P_0 METHOD

Parameter	Marker			
	6-Thioguanine resistance [a]			
	Expt. [ref.]			
	A [8]		B [17,18]	
Cell line [b]	V79 WT	V79, CTPS46	V79 WT	V79, AD^r 4-2
N_0	80	115	1	1
N_t	1.62×10^6	1.70×10^6	3.0×10^6	3.0×10^6
C	61	20	54	26
$\hat{P}_0$	52/61	12/20	49/54	10/26
$\hat{\mu}$	0.99×10^{-7}	3.01×10^{-7}	3.14×10^{-8}	3.08×10^{-7}
$\hat{\mu}_1/\hat{\mu}_2$	3.05		9.80	
Z	3.36		5.00	
$H_0: \mu_1 = \mu_2$	rejected		rejected	

[a] 6-TG at 10 μg/ml in Expts. A and B; ouabain at 0.5 and 3 mM, respectively, in Expts. C and D.

somatic cells in culture, as compared to bacteria, not for cenceptual but for technical and economic reasons.

The usual problem of unreliable estimation of μ stems mainly from the absence of an appropriate set of criteria for experimental design. Through our derivation of var($\hat{\mu}$), the design problem becomes definitive and amenable to analysis. Our study suggests the necessity of using larger N_t and C for a successful fluctuation analysis than are usually employed. There is a large impact of each mutational event on the estimation of μ when only a small fraction of the C cultures exhibited mutations, e.g., 2 out of 120 cultures. The extent of this impact decreases as P_0 decreases because N_t increases. C influences the accuracy of $\hat{\mu}$ by controlling the size of the bias as well as the CV. Once N_t is decided, C can be calculated before performing the experiment by using Eqn. 6 to confine $\hat{\mu}$ within a certain range of variation. $\hat{\mu}$, as inferred from $\hat{P}_0$, is biased. If the experiment is properly designed, however, this bias can be minimized. In that case, the deviation of $\hat{\mu}$ from μ will be too small to be of any practical significance for the accuracy of $\hat{\mu}$. The maximum likelihood method is a good choice for $\hat{\mu}$ calculation, although it does not necessarily yield an unbiased estimate [2,3,13,15].

By proposing a test statistic, we have placed the comparison of μ values on a statistical basis. Lacking a proper test statistic, a mutator effect has traditionally been invoked whenever the $\hat{\mu}$ ratio between two cell lines is larger than either 5 or 10. With the availability of our Z statistic, a possible mutator effect can be detected with greater power. Our results (Table 3) suggest that cell lines CTPS 46 (Expt. A) and

Ouabain resistance [a]			
Expt. [ref.]			
C [8]		D [4]	
V79, CTPS42	V79 CTPS43	Mouse L cells	Mouse L cells
60	90	100	100
4.6×10^6	4.0×10^6	3.0×10^7	3.1×10^7
20	24	28	32
2/20	2/24	6/28	4/32
4.94×10^{-7}	6.21×10^{-7}	5.13×10^{-8}	6.93×10^{-8}
1.26		1.35	
0.19		0.93	
accepted		accepted	

[b] CTPS 42, 43, and 46 are mutant cell lines of Chinese hamster V79 cells with an altered structure of CTP synthetase [7,8]. AD^r 4-2 is a V79 mutant cell line resistant to aphidicolin and has altered DNA polymerase α [6,18].

AD^r 4-2 (Expt. B) may be more mutable than their parental lines.

The derivation of the statistic is based on the notion of comparing two proportions, P_{01} vs. P_{02}, providing that the N_t values from 2 Expts. are equal. This will, however, present some operational difficulties, and it is the principal drawback of our testing procedure. One solution is to monitor the population growth carefully and to stop the growth when N_t reaches a desired cell number. Because the Z statistic is sensitive (Table 3), an equality test should be performed only when the estimation of μ is reliable (a $CV \leq 0.2$). We appreciate the experimental difficulties and economic constraints involved in making CV smaller than 0.2, but if instead one follows the multiple replating technique of Stocker [24], a much larger experimental scale is needed to obtain a rate estimate. Furthermore, the rate estimate so obtained is only an approximation [3], and the labor involved would be prohibitive.

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