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Measuring bidirectional mutation

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Abstract

The estimation of mutation rates is usually based on a model in which mutations are rare independent Poisson events. Back-mutation of mutants, an even rarer event, is ignored. In the hypermutating B cells of the immune system, mutation between phenotypes exhibiting, *vs*. not exhibiting, surface immunoglobulin is common in both directions. We develop three strategies for the estimation of mutation rates under circumstances such as these, where mutation rates in both directions are estimated simultaneously. Our model for the growth of a cell culture departs from the classical assumption of cell division as a memoryless (Poisson) event; we model cell division as giving rise to sequential generations of cells. On this basis, a Monte-Carlo simulation is developed. We develop also a numerical approach to calculating the probability distribution for the proportion of mutants in each culture as a function of forward- and backward-mutation rates. Although both approaches are too computationally intensive for routine laboratory use, they provide the insight necessary to develop and evaluate a third, 'hand-calculator' approach to extracting mutation rate estimators from experiments of this type.

Keywords: Mutation rate; Estimation; Hypermutation; B cells; B lymphocyte; Mutation

1. Introduction

Since the seminal paper by Luria and Delbrück (1943) examining the development in bacteria of resistance to bacteriophage, there have been many proposed estimators of mutation rate. The scenario analysed in the 1943 paper was that of a single cell growing in culture by repeated division. Mutants were identified by their ability to grow on selective medium, and the thrust of the paper was the timing of the mutation rather than the measurement of its frequency. Luria and Delbrück proposed two estimators of mutation rate, both of which assume an ability to identify all mutants existing in the culture at the time of its plating onto selective medium.

Many authors, notably Lea and Coulson (1949), Armitage (1953), Koch (1982), and Ma et al. (1992), contributed to the mathematical development of the field; more efficient estimators were developed, and the experimental assumptions were relaxed. Algorithms are available, although perhaps not widely used, to analyse experiments in which mutants and wild-type grow at different rates, and in which only a (known) fraction of mutants are identified.

Assumptions basic to many formulations of the Luria–Delbrück distribution have been summarized by Angerer (2001). These include that 'mutant cells divide only into cells with mutant properties'. In addition, most approaches have assumed that the mutation rate is small, so that the size of the wild-type pool of dividing cells is independent of the number of mutants. These assumptions give rise to a compound Poisson distribution; the number of new mutations during growth is a simple Poisson distribution, the clone size for each independently follows some probability distribution that depends on relative growth rates, cell death, phenotypic delay, etc., and the final number of mutants is then compound Poisson. In the absence of differential fitness, these models have in common that the proportion of mutants increases monotonically with time. This class of model cannot deal with

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the scenario in which an equilibrium proportion of mutants can be approached from either direction as, for instance, in a hypermutating B-cell line.

Two aspects of experimental design emerging from the literature are central to the focus of this paper. Obviously, bigger cultures yield more information; the last few days of exponential growth yield most information about the mutation rate. Less obviously, for a given number of cells examined for mutants, more information comes from a small fraction of a large culture than from a large fraction of a small culture (Crane et al., 1996; Jones et al., 1999). If, as in the experiments to follow, 36,000 cells are to be examined for mutants, we can grow 15.1 generations from a single cell and examine them all, or grow an extra 2.2 generations and examine just 24% of the resulting 150,000-cell culture. The latter yields more information. This suggests an extension whereby we subculture repeatedly, and examine for mutants after several subcultures.

This paper addresses the design and analysis of such experiments in hypermutating B-cell lines. These express an activation-induced deaminase (AID) that accounts for their high mutation rate (Arakawa et al., 2002; Buerstedde et al., 1990). These rates are still too low to be measured reliably using only a few generations of cell expansion, so analysis of mutation rates in cell lines expressing AID requires weeks of subculturing. We envisage forward-mutation from wild-type to mutant, and also back-mutation from mutant to wild-type. Accordingly the experimental protocol has two arms. In one, cultures from a wild-type inoculum are examined for the development of mutants. In the other, cultures from a mutant inoculum are examined for back-mutation to wild-type.

The theoretical development of the paper is in three parts. The first involves a Monte-Carlo approach to the simulated growing of cultures. The second uses a more numerical approach to develop the probability distribution for the proportion of mutants (or back-mutants) in the aliquot examined. Both approaches are used to derive a confidence region for mutation rates, and the results are compared. Both approaches are computationally intensive, but they set a standard against which a third, 'hand-calculator' approach can be judged. Theoretical issues relating to experimental design are taken up in the Discussion.

2. Materials and methods

2.1. Biological methods

2.1.1. Antibodies and chemicals

All chemicals were supplied by Sigma-Aldrich, unless otherwise stated. Complete culture medium was RPMI supplemented with 10% fœtal bovine serum (Sigma-Aldrich lot 092K2300), 1% chicken serum (IMVS, Adelaide, Australia), benzyl penicillin (0.06 g/l), and streptomycin sulphate (0.1 g/l).

2.1.2. Single cell cloning

Aliquots ($\sim 10^6$ cells) of predominantly surface immunoglobulin positive ($sIgM^+$) DT40-CL18 cells and predominantly $sIgM^-$ DT40-CL18 cells (Buerstedde et al., 1990) were stained with saturating amounts of FITC-conjugated antichicken IgM Ab (clone M-1, Southern Biotechnology Associates, Birmingham, AL) at 4 °C for 30 min in sterile PBS containing 0.5 BSA and 0.02 w/v% sodium azide (PBA). Using a FACSVantage DIVA sorter (Becton Dickinson, Franklin Lakes, NJ), individual $sIgM^+$ or $sIgM^-$ cells were sorted into 384-well tissue culture plates, containing 0.06 ml/well complete culture medium plus Primocin antibiotic (Invivogen, San Diego, CA).

2.1.3. Propagation of clones

Eight days after sorting, random clones were transferred to individual wells in 96-well plates, each well containing 0.15 ml culture medium. Thereafter, when most clones had reached a density of 1 or 2×10^5 cells per well (generally at 2–3 day intervals, but after 8 days in the first instance), one-tenth of each culture (0.02 ml, or 1or 2×10^4 cells) was transferred to a fresh well containing 0.18 ml fresh medium. Subculturing was performed 12 times.

2.1.4. Analysis of surface Ig-loss and -gain (sIg fluctuation)

On day 50 after sorting (cloning), about 80% of each culture was harvested into 1 ml tubes, pelleted (500 g, 5 min) and stained with FITC-conjugated antichicken IgM Ab in a 50 μl volume of ice-cold PBA. After washing with ice-cold PBA, cells were fixed with 2% paraformaldehyde in PBS. Stained samples were subjected to FACS analysis (using a FACScan machine from Becton Dickinson) in an order determined by a random number generator (http://www.random.org/). The frequency of $sIgM^+$ or $sIgM^-$ cells in each sample was determined blind using FlowJo software (Tree Star Inc., Ashland, OR) and the gating strategy of Arakawa et al. (2002). Data on each median of 3.6×10^4 viable cells (defined by forward- and side-light scatters) were collected for each sample.

2.2. Computational methods

Mathematical subroutines were as outlined in Press et al. (1986) and used the subroutine library which accompanies that publication. Source code was changed to use double precision (8 byte) arithmetic.

3. Theoretical development

The process being modelled in the following section is the division of a single cell into 'progeny'. It is assumed that this involves duplication of the genome and that there are constant probabilities (ϕ and β) that the copy differs in genotype in a specific way from the template. We denote by ϕ the probability that a copy from a wild-type is mutant, and by β the probability that a copy from a mutant has 'back-mutated' to wild-type.