

Allele-specific expression patterns of interleukin-2 and Pax-5 revealed by a sensitive single-cell RT-PCR analysis

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Autosomal genes that are subject to random allelic inactivation (RAI), like imprinted genes [1] and genes subject to X-inactivation [2], require mechanisms that dictate the differential transcriptional regulation of two sequence-identical alleles. RAI genes include olfactory receptor genes [3], and the various genes encoding antigen receptors on lymphocytes (immunoglobulin genes, T cell receptor genes and NK receptor genes [4–7]). These observations raise the possibility that other genes might be similarly regulated. Moreover, an interesting possibility is that certain genes might be monoallelically expressed in some cells and biallelically expressed in others. Recently, reports of monoallelic expression of interleukin-2 (IL-2) [8,9] and IL-4 [10,11] have raised the possibility that the cytokine gene family may be subject to monoallelic expression. Another report suggests that the gene encoding the transcription factor Pax-5, which is involved in B-cell (and cerebellar) development [12,13], is also subject to monoallelic expression [14]. Using a novel single-cell reverse transcription-polymerase chain reaction (RT-PCR) approach, we have analyzed the IL-2 and Pax-5 genes in mice. We found that IL-2 is monoallelically transcribed in some T cells and biallelically transcribed in others, raising interesting questions regarding cytokine gene regulation. Additionally, our analyses suggest that Pax-5 is consistently biallelically transcribed. Thus, the IL-2 gene and other cytokine genes may be regulated in a stochastic manner that results in 0, 1 or 2 alleles of a given cytokine gene expressed in each T cell. This type of regulation could account for the wide variety of different combinations of cytokine genes expressed in individual T cells and therefore plays a role in the generation of T cells with a range of different effector functions.

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Results and discussion

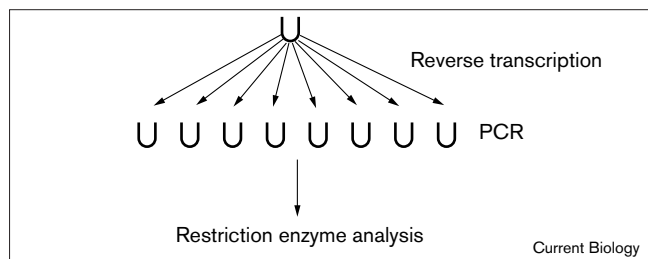
The first important requirement for studying a potentially monoallelically expressed gene is to show unequivocally that the given gene is indeed monoallelically expressed. Towards this goal, we present here a single-cell RT-PCR approach to analyzing cells whose alleles of a given gene can be distinguished based on polymorphisms. One immediate concern in a single-cell RT-PCR analysis is the potential for the assay to incorrectly indicate monoallelic expression. For example, if one were to PCR-amplify from only a single (cDNA) template molecule, it is predetermined that one will amplify only one allele. Thus, if the starting cell in the example were in fact expressing transcripts from both alleles, the low efficiency of conversion of mRNA into cDNA would have caused the RT-PCR experiment to provide the erroneous conclusion that transcription is monoallelic.

To address this concern, our assay has at its heart the following principle: a single molecule (in this case a single cDNA) can only be in one PCR tube. Therefore, by splitting up a RT reaction from a single-cell into multiple tubes (Figure 1), we can be sure that each ‘positive’ PCR reaction represents at least one, if not more than one, individual template(s). Each cDNA template corresponds to an individual mRNA molecule present in the analyzed cell. We suggest that while this assay may or may not be more sensitive at detecting expression of a given gene, it is clearly more sensitive at distinguishing between monoallelic and biallelic expression. Possible outcomes of this approach include the determination that transcription in individual cells is monoallelic or biallelic, a mix of monoallelic and biallelic cells is present, or that the sensitivity of the assay is below the threshold for distinguishing between monoallelic and biallelic expression.

IL-2 expression in individual activated T cells is either monoallelic or biallelic

The IL-2 gene is reasonably highly expressed in each cell, facilitating the analysis of allelic expression. Individual activated CD4⁺ T cells from *Mus spretus* × *Mus musculus* F1 mice were placed in individual tubes using a fluorescence-activated cell sorter (FACS). We then performed

Figure 1



RT-PCR strategy. A key aspect of our RT-PCR protocol is that each single-cell RT reaction is divided into eight aliquots for separate PCR analyses. The parental origins of all PCR products are determined by restriction enzyme analysis.

our RT-PCR assay and were able to detect two or more distinct mRNAs from 54 of 98 cells. In fact, out of the 54 cells revealing two or more of the eight PCRs with products, 31 cells had at least seven PCRs with products. Thus, our ability to detect IL-2 gene expression is well above the threshold for assessing monoallelic vs. biallelic expression. Indeed, our analyses of the IL-2 gene clearly demonstrate that some cells are biallelic and other cells are monoallelic (Figure 2).

The cells that revealed seven or eight PCRs with products are the most informative for analyzing whether some cells express IL-2 from only one allele. Examples of analyses of such cells are shown in Figure 2a. Of these cells, 26% (8/31) showed monoallelic expression. The probability that such a result could arise by chance if expression were truly biallelic and equal is negligible (conservatively estimated at $< 1/10^5$). Thus, the cells with seven or eight products demonstrate that T cells can be monoallelic for IL-2 expression. Of course, one must not ignore the majority

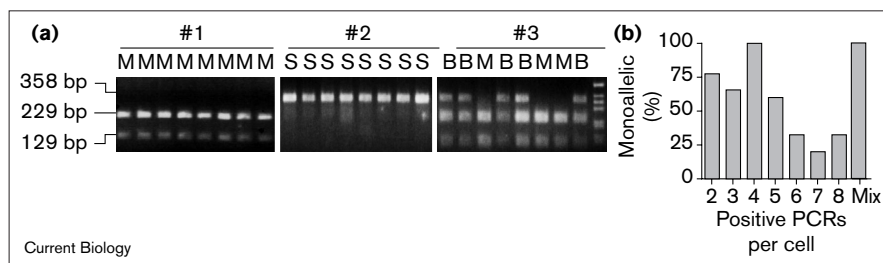
(23/31) of cells that reveal definitive evidence of biallelic expression of IL-2.

We also analyzed cells with between two and six PCR products per cell. The data for all the different cells are presented in Figure 2b. Although it is difficult to determine the exact fraction of cells that are monoallelic, we estimate from an analysis of all the cells shown in Figure 2b that one quarter to one-third of cells are monoallelic. It is important to note that analysis of the mRNA present in a cell at a given time does not necessarily indicate what is going on over the course of time. We can not rule out the possibility that expression switches on and off at either one or both alleles and that, over time, a given cell might switch among three states: monoallelic for the maternal allele, monoallelic for the paternal allele, and biallelic. However, if the regulation of expression of the IL-2 gene is similar to the regulation of the IL-4 gene, then it is likely that in at least some cells, monoallelic expression will be heritable. Naramura *et al.* [9] interpreted their GFP knock-in data as clearly demonstrating biallelic expression of IL-2 and they provided potential explanations for the presence of GFP⁺/IL2⁻ and IL2⁺/GFP⁻ cells that do not invoke monoallelic expression. However, they did not absolutely exclude the possibility that certain cells have monoallelic expression. Thus, our data resolve differences between the claim of pure monoallelic expression by Hollander *et al.* [8] and the suggestion by Naramura *et al.* [9] of the possibility that IL-2 may always be expressed biallelically.

These analyses are approaching the limit of detection of the extremely powerful technique of PCR. Therefore, we performed numerous control experiments including interspersed negative controls, controls to assure that the FACS places only one cell per tube, and various cell

Figure 2

Single-cell RT-PCR analyses of IL-2 gene expression. (a) Examples of CD4⁺ T cells that yielded products in eight out of eight PCR reactions. Digestion with the *Fnu*4HI restriction endonuclease allows one to distinguish the *M. musculus* allele (which has the *Fnu*4HI recognition site) from the *M. spretus* allele. These cells represent clear examples indicating that some T cells express both IL-2 alleles (#3) while others express only one allele (#s 1 and 2). (b) Compiled RT-PCR data on IL-2 expression. The percentage of cells appearing to be monoallelic is plotted as a function of the number of positive PCR reactions observed per cell. The numbers of cells in the seven categories graphed were 9, 3, 3, 5, 3, 10, and 21, respectively. A comparison of this graph with the theoretical graph in Figure 3 allows



the conclusion that some T cells are biallelic and others are monoallelic in their expression of the IL-2 gene. Mix: to ensure that the FACS was indeed sorting single cells into single PCR tubes, we used the FACS to sort a population of T cells consisting of an equal mix of *M. spretus* T cells and *M. musculus* T

cells; in other words, we artificially created 'pure monoallelic expression'. We then analyzed these cells for expression of IL-2 and, as expected, always detected clear evidence for monoallelic expression (20 of 35 cells revealed IL-2 expression and all 20 were 'monoallelic').

mixing and RNA mixing experiments. These controls and data are presented in the Supplementary material.

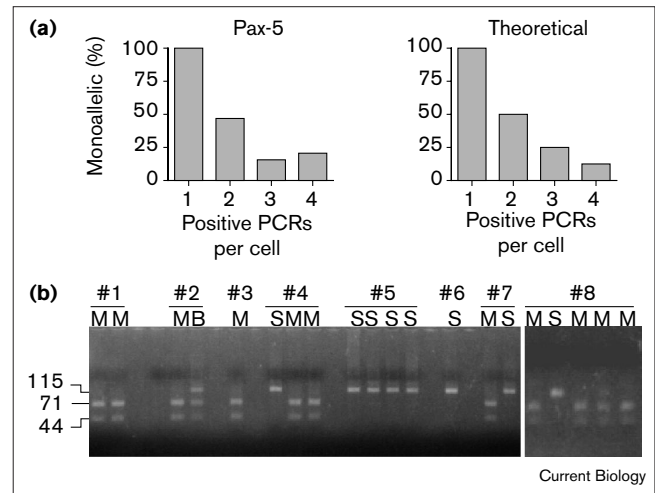
Pax-5 appears to be biallelically expressed in most or all B cells

Pax-5 appears to be expressed at a lower per cell level than IL-2. This is not surprising given that Pax-5 is a transcription factor and IL-2 is a secreted protein. The low level of Pax-5 expression makes analyzing the two alleles in individual cells very challenging and further underscores the importance of the splitting of the RT reaction into multiple tubes, which lies at the heart of our RT-PCR assay. We examined the Pax-5 gene in B lymphocytes from *M. spretus* × *M. musculus* F1 mice. Splenic B cells isolated by FACS or by limiting dilution were subjected to RT-PCR analyses in which PCR was performed on eight aliquots from each RT reaction. We show representative analyses (Figure 3b) and tabulated data (Figure 3a). The analyses of eight PCR reactions from each RT reaction allowed us to distinguish between the threshold for detecting Pax-5 mRNA expression in single cells and the more stringent threshold of detection necessary for determining whether one or both alleles are expressed.

Out of 424 cells analyzed, 199 revealed a Pax-5 PCR product in at least one of eight tubes. Of these 199 Pax-5⁺ cells, 78 revealed only one of eight PCRs with a product and were thus not informative for assessing monoallelic expression. A total of 121 of the 199 cells with products for Pax-5 (out of a total of 424 cells analyzed) revealed products in two or more of the eight PCR reactions performed. Roughly half of the cells (37 of 70) that had exactly two PCR-positive aliquots had both alleles represented, as is predicted by statistical analysis for a biallelically expressed gene (Figure 3a). The cells that revealed a product in more than two of eight PCR samples also had a distribution of the alleles consistent with biallelic expression (Figure 3a). A comparison of the Pax-5 experimental data with the theoretical expectation for a biallelically expressed gene (Figure 3a, right panel) suggests that Pax-5 may indeed be biallelically expressed in all splenic B cells expressing it. Our analyses of lipopolysaccharide (LPS)-activated splenocytes revealed similar results (data not shown). Control experiments are presented and described fully in the Supplementary material.

A previously published RT-PCR analysis (also of splenic B cells of young adult mice) concluded that Pax-5 expression is monoallelic in two-thirds of B cells [14]. The most likely explanation for the difference lies in the difference in experimental design. The Nutt *et al.* [14] assay did not involve splitting the RT into many PCR reactions. One possibility is that Pax-5 expression in the cells they analyzed was biallelic and that the assay lacking splitting could not determine that in fact many of the PCR reactions they performed were ‘single-molecule’ PCRs, all of

Figure 3



Single-cell RT-PCR analyses of Pax-5. **(a)** Graph of Pax-5 expression in 200 B cells. Left panel: the percentage of cells appearing to be monoallelic is plotted as a function of the number of positive PCR reactions observed per cell. These data are averages of three independent experiments. The bar labeled ‘4’ represents cells with 4 or more PCR products per cell. 79 cells revealed one PCR product/cell, 71 cells revealed two, 38 cells revealed three and 14 cells revealed four or more signals per cell. Right panel: the theoretical expectation for an analysis of a biallelically expressed gene. The formula $2/2^n$ (where n = the number of PCR products observed) approximates the expected fraction for low values of n . The percentages presented (100, 50, 25 and 12.5, respectively) leave out a minor correction in the formula for rare instances in which a given signal actually represents more than one cDNA template. A uniformly monoallelically expressed gene would reveal 100% monoallelic expression, independent of the number of PCR products observed. A gene that is sometimes monoallelic and sometimes biallelic in its expression would look like the profile for IL-2 (Figure 2). The observed percentages for Pax-5 (left panel) are within expected statistical variation of the theoretical values (right panel). Note that the appearance of ‘monoallelic cells’ in the theoretical example reflects the potential for a RT-PCR assay to incorrectly suggest monoallelic expression. **(b)** 4% agarose gel analyses of representative single-cell RT-PCRs. Pax-5 RT-PCR products were digested with the *Bsp*1286I restriction endonuclease that recognizes only the *M. musculus* allele. The number of PCR products obtained ranged from one to eight out of eight.

which yield only one allele or ‘two-molecule’ PCRs, half of which theoretically should yield only one product. Indeed, if we group together all eight PCRs for every cell that we analyzed for Pax-5 expression (including cells yielding only 1 of eight PCRs with a product) and reanalyze the data in this incorrect manner, we arrive at 117/199 or 60% ‘monoallelic’.

We also examined the allelic expression pattern of Pax-5 within the nuclei of CD19⁺ B cells using RNA fluorescence *in situ* hybridization (FISH) experiments [15] (see the Supplementary material). Our RNA-FISH experiments correlate with our RT-PCR data in indicating biallelic expression of the Pax-5 gene. The contradictory

conclusions from our FISH experiments and those of Nutt *et al.* [14] (who observed biallelic expression in only half the B cells that they analyzed) could reflect inefficiency in their detection of transcripts. Taken together, our data suggest that Pax-5 is biallelically expressed like most autosomal genes. The observation that Pax-5 is synchronously replicated ([14] and our unpublished observations) is also consistent with biallelic expression of this gene.

We have used a single-cell RT-PCR assay involving splitting the RT reaction into eight PCR reactions to analyze the IL-2 and Pax-5 genes. The assay reveals that around two-thirds of activated T cells contain mRNAs for both alleles of IL-2 and the remaining one-third of T cells contain mRNAs derived from only one allele (or at least over a 10:1 ratio of the two alleles). Our data on the IL-2 gene, taken together with our data on the Pax-5 gene (which is uniformly biallelically expressed), demonstrate the usefulness of the RT-PCR assay we have used in assessing whether or not a given gene is expressed monoallelically. Experiments in which a marker is introduced into a given gene in the mouse germline provide another way to assess whether or not a given gene is expressed monoallelically. However, such genetic manipulations have the potential to alter aspects of gene expression. Variations on the RT-PCR analysis we present here may be generally applicable to expression studies of more than one gene at a time in individual cells.

The observation that the activated CD4⁺ T cell population contains both cells with monoallelic and cells with biallelic patterns of IL-2 expression is reminiscent of the patterns observed for the IL-4 gene and suggests the possibility that other members of the cytokine gene family may also be expressed monoallelically in a fraction of cells [10,11]. Monoallelic expression could reflect a model of gene expression regulation in which each cell contains a limiting number of activating complexes leading to the expression of zero, one or two alleles of a given cytokine gene. While the number of such activating complexes may be influenced by the strength of T cell receptor signaling or alternative signals, the 'decision' to express a given cytokine gene may have a strong stochastic component. This model immediately provides a potential solution to the question of how a number of different effector populations of T cells can be derived from T cells with the same antigen specificity. Perhaps each newly activated T cell stochastically chooses to activate either one or both alleles of a number of different cytokine genes, and the choice of which cytokine genes are activated is then inherited by the progeny of that cell [10]. This would lead different T cell clones to express distinct combinations of cytokines. Stochastic gene expression regulation, leading to the expression of zero, one or two alleles of a given cytokine gene, may therefore play an important role in determining the commitment of precursor T helper cells to distinct differentiated lineages.

Supplementary material

Supplementary material including control experiments supporting the single-cell RT-PCR analyses, RNA FISH experiments analyzing the Pax-5 gene, and additional methodological details is available at <http://current-biology.com/supmat/supmatin.htm>.

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