

Determination of Intrachromosomal Recombination Rates in Cultured Mammalian Cells

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Summary

Recombination is involved in many important biological processes including DNA repair, gene expression, and generation of genetic diversity. Recombination must be carefully regulated so as to prevent the deleterious consequences that may result from rearrangements between dissimilar sequences in a genome. It is of considerable interest to study the mechanisms by which genetic rearrangements in mammalian chromosomes are regulated in order to understand better how genomic integrity is normally maintained and to gain insight into the types of genetic mutations that may destabilize the genome. To explore such issues in mammalian chromosomes, a suitable experimental system must be developed. In this chapter, we describe a model system for studying intrachromosomal recombination in cultured mammalian cells. We discuss two model recombination substrates, a method for stably introducing the substrates into cultured Chinese hamster ovary cells, and a method for determining rates of intrachromosomal recombination between sequences contained within the integrated substrates. The general approach described here should be applicable to the study of a variety of aspects of recombination in virtually any cultured mammalian cell line.

Key Words: homologous recombination, fluctuation analysis, cell culture, DNA transfection

1. Introduction

Homologous recombination is defined as an exchange of genetic information between nearly identical DNA sequences. Homologous recombination can serve as a mechanism to repair double-strand breaks and other forms of DNA damage in mammalian cells. Recombination also plays roles in gene expression and genome evolution. One important aspect of recombination is that it typically occurs only between sequences that display a high degree of sequence identity. In this way, the cell usually manages to avoid the potentially harmful consequences of recombination between dissimilar sequences (*homeologous*

From: *Methods in Molecular Biology*, vol. 262, *Genetic Recombination: Reviews and Protocols*
Edited by: A. S. Waldman © Humana Press Inc., Totowa, NJ

recombination). Consequences of homeologous recombination may include chromosomal translocations, deletions, or inversions. The same proteins that catalyze homologous recombination may function to suppress homeologous recombination.

Homologous recombination in mammalian cells is indeed strongly dependent on sequence identity; as heterology increases, rates of homologous recombination and conversion tract length decrease (1–4). Waldman and Liskay (2) have shown that intrachromosomal recombination between two linked sequences sharing 81% homology was reduced 1000-fold compared with recombination between sequences displaying near-perfect homology. Lukacsovich and Waldman (5) reported that a single nucleotide heterology is sufficient to reduce recombination by about 2.5-fold, and a pair of nucleotide heterologies can act to suppress recombination from 7-fold to as much as 175-fold. It has been learned that mismatch repair (MMR) systems in bacteria, yeast, and mouse embryonic stem cells suppress homeologous recombination, and if any MMR components are lacking, rates of homeologous recombination increase (6–10). Gaining a more complete understanding of how cells normally regulate recombination and prevent unwanted homeologous exchanges is of fundamental importance to an understanding of how genome stability is maintained.

To explore spontaneous homologous and homeologous recombination in mammalian chromosomes, our lab developed a model system utilizing a gain-of-function assay. The system described in this chapter involves a pair of isogenic Chinese hamster ovary (CHO) cell lines designated MT⁺ and Clone B (generously provided by Margherita Bignami). The Clone B cell line is defective for an MMR protein named Msh2. MT⁺ cells are wild-type for Msh2. To study spontaneous intrachromosomal homologous or homeologous recombination, plasmids pLB4 and pBR3 (**Fig. 1**) were constructed to serve as recombination substrates, and MT⁺ as well as Clone B cells were stably transfected with these plasmid substrates. In this chapter we describe the isolation of stably transfected cell lines containing recombination substrates and fluctuation analysis to calculate intrachromosomal recombination rates. Although we describe work done with a specific set of CHO cell lines, the substrates used and the general approach discussed should be applicable to the study of a variety of issues relevant to intrachromosomal homologous and homeologous recombination in virtually any cultured mammalian cell line.

2. Materials

1. Plasmids pLB4 and pBR3 (**Fig. 1**) serving as substrates for homologous and homeologous recombination, respectively.
2. TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
3. Bio-Rad Gene Pulser (or other electroporator).
4. 40-cm Gap cuvetts for electroporator.

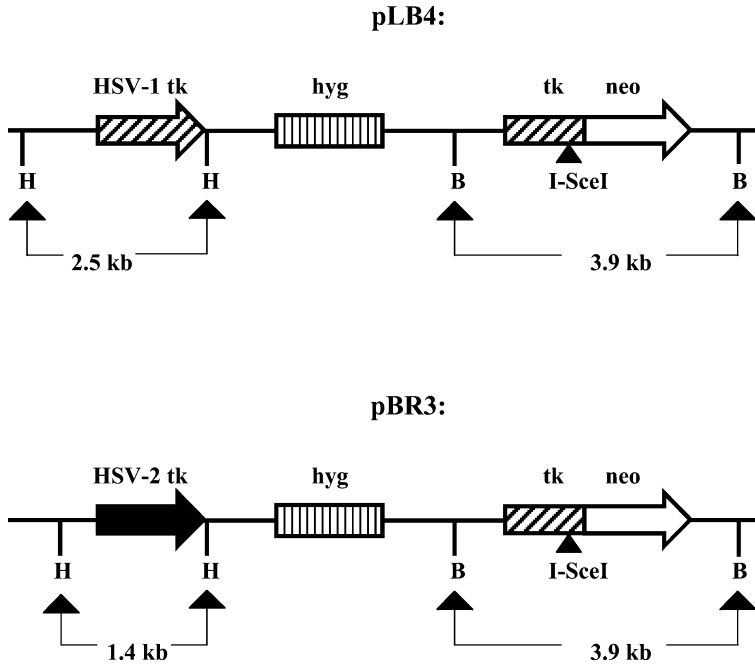


Fig. 1. Recombination substrates pLB4 and pBR3. Substrates pLB4 and pBR3 are suitable for the study of intrachromosomal homologous and homeologous recombination, respectively. Both substrates contain a tk-neo fusion gene that is disrupted by the insertion of an I-SceI recognition site in the tk portion of the fusion gene. Each substrate also contains an additional “donor” tk sequence. The tk portion of the tk-neo fusion gene is from herpes simplex virus type 1 (HSV-1). The donor tk sequence on pLB4 is from HSV-1, and the donor tk sequence on pBR3 is from herpes simplex virus type 2 (HSV-2). Both substrates contain a hygromycin resistance gene (hyg), which allows for the isolation of stable transfectants. For cells containing either substrate, recombination between the tk donor and the disrupted tk-neo fusion gene can eliminate the I-SceI site, restore function to the fusion gene, and produce a G418^r phenotype. Also shown in the figure are the locations of *Bam*HI (B) and *Hind*III (H) restriction sites.

5. G418 and hygromycin.
6. CHO cell lines MT⁺ and Clone B.
7. Alpha-modified minimal essential medium (α MEM), supplemented with 10% fetal bovine serum (heat-inactivated).
8. Trypsin-EDTA solution (GIBCO, cat. no. 15400-054).
9. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.5 mM KH₂PO₄.
10. Cell culture flasks (25 cm², 75 cm², and 150 cm² surface area).

11. 24-Well tissue culture plates.
12. Hemacytometer.
13. Sterile cotton swabs.
14. Fixative/stain solution: 0.04% methylene blue in 20% ethanol.
15. Dimethyl sulfoxide (DMSO).
16. Cryovials, 2 mL (for freezing cell lines).
17. Multiprime labeling kit (Amersham Biosciences cat. no. RPN 1601Z).
18. Restriction enzymes *Bam*HI and *Hind*III
19. Endonuclease I-*Sce*I
20. Agarose

3. Methods

3.1. Plasmid Constructs for Studying Intrachromosomal Recombination

To evaluate spontaneous homologous and homeologous recombination in mammalian cells, the plasmids pLB4 and pBR3 were constructed (**Fig. 1**) (*see Note 1*). A hygromycin resistance gene is included on each plasmid for stably installing the plasmid into mammalian cells. Plasmids pLB4 and pBR3 both contain a herpes thymidine kinase (tk) sequence (flanked by *Hind*II sites) that serves as a potential “donor” sequence for recombination. Each construct also contains a nonfunctional tk/neomycin-resistance fusion gene (flanked by *Bam*HI sites). The “tk-neo” fusion gene is nonfunctional because a 22-bp insertion containing the 18 bp I-*Sce* I endonuclease recognition site has been incorporated into the tk portion of the fusion gene (*see Note 2*). The nonfunctional tk-neo gene in either substrate can be corrected (that is, the I-*Sce*I site can be eliminated) via recombination with the donor tk sequence, and recombinants can be recovered as G418^r segregants. In pLB4, the donor tk sequence shares greater than 97% sequence homology with the tk portion of the tk-neo fusion gene sequence, and this construct is used to study homologous recombination. (The donor and the tk-neo gene on pLB4 do not share *perfect* homology, but the very limited number of scattered nucleotide differences allows for unambiguous identification of conversion tracts upon DNA sequencing.) In pBR3, the donor shares only about 80% sequence homology with the tk-neo gene and this construct is used to study homeologous recombination. As described in **Subheading 3.2.**, cells are stably transfected with pLB4 or pBR3 to study intrachromosomal recombination.

3.2. Establishing Cell Lines for Studying Homologous Recombination and Homeologous Recombination by Stable Transfection With Recombination Substrates

1. Grow MT⁺ and Clone B CHO cells in α MEM to near confluence in 75-cm² flasks, trypsinize, and count cells using a hemacytometer.
2. Resuspend MT⁺ or Clone B CHO cells (5×10^6 cells) in 800 μ L of PBS, mix with 3 μ g of plasmid pLB4 or pBR3 (DNA should be added in a minimal volume of

water, PBS or, TE buffer, not to exceed 50 μ L), and electroporate in a 40-cm gap cuvet using a Bio-Rad Gene Pulser set at 1000 V, 25 μ F (*see Note 3*).

3. Following electroporation, plate cells into a 150-cm² flask and allow cells to grow for 2 d without selection to permit recovery from electroporation.
4. After 2 d, plate 1×10^6 cells per 75-cm² flask containing α MED supplemented with either 500 μ g/mL of hygromycin (Clone B cell lines) or 400 μ g/mL of hygromycin (MT⁺ cell lines) (*see Note 4*).
5. Allow cells to grow until colonies are visible. Typically, CHO colonies are clearly visible after 8–10 d.
6. Draw circles around colonies on the outside of the flasks using a marking pen. Pick hygromycin-resistant colonies with sterile cotton swabs dipped in trypsin-EDTA solution. A swab is aimed at the center of a circle drawn around a colony; by gently brushing the cells in a colony with the cotton swab, the cells are released from the flask and adhere to the cotton. Transfer cells from each colony to a different, single well in a 24-well plate by dipping the cotton swab containing the cells into a well containing 1.5 mL of medium and gently rubbing the swab against the bottom of the well.
7. Incubate the wells at 37°C. When a well becomes full of cells, transfer cells from that well to a 25-cm² flask; when that flask is full, transfer cells to one 25-cm² and one 75-cm² flask.
8. When the 25-cm² and 75-cm² flasks of cells for a particular colony become full, prepare genomic DNA from the cells in the 75-cm² flask and freeze down the cells in the 25-cm² flask. Freeze cells at –80°C in 1 mL of α MED supplemented with 10% DMSO in a 2-mL cryovial.
9. Analyze genomic DNA by Southern blot analysis to determine which cell lines contain a single unrearranged copy of the plasmid construct with the correct restriction fragment sizes (*see Note 5*). Using a tk-specific probe (labeled to greater than 1×10^9 per μ g using a Multiprime labeling kit) and a DNA digestion with *Hind*III plus *Bam*HI, cell lines containing pLB4 should display a 3.9-kb and a 2.5-kb band and cell lines containing pBR3 should display a 3.9-kb and a 1.4-kb band (*see Fig. 1*).
10. After identifying one or more suitable cell lines, remove the vial(s) containing the desired frozen culture(s) from the –80°C freezer and thaw the cells. Propagate the cells and conduct fluctuation analysis as described below.

3.3. Recovery of G418^r Colonies From a Fluctuation Test

To determine spontaneous intrachromosomal homologous and homeologous recombination rates, fluctuation tests are performed. Single-copy cell lines containing pLB4 or pBR3 previously identified by Southern blotting are used. Each cell line is initially sensitive to G418; recombinants from a cell line are recovered as G418^r segregants in a fluctuation test as follows:

1. Separate a given cell line into 10 subclones containing 100 cells per subclone, and plate each subclone into a separate well of a 24-well plate (*see Note 6*).

2. Grow each subclone to confluence in a well, and then transfer to a 25-cm² flask. When the 25-cm² flask is full, transfer cells to a 75-cm² flask. Continue to culture cells until a sufficient number of cells is obtained per subclone. For the experiments described here, 4 million cells per subclone are required (see **Note 7**).
3. For each subclone, plate 1×10^6 per 150-cm² flask in α MEM supplemented with 1000 μ g/mL G418 to select for G418^r segregants arising from homologous or homeologous recombination. We routinely use between four and eight 150-cm² flasks per subclone in our work.
4. Incubate cells for about 10 d, until colonies are visible.
5. As described in **Subheading 3.2., step 6**, pick several colonies per flask using sterile cotton swabs dipped in trypsin/EDTA solution and transfer cells from each individual colony into a separate well of a 24-well plate. Propagate cells and extract genomic DNA from a full 75-cm² flask of cells.
6. Fix and stain any colonies that were not picked with swabs by adding 10 mL of fixative/stain solution per flask and incubating at room temperature for 10 min. Wash out the solution with tap water. Colonies should be stained blue and should be easily visible.
7. Count all stained colonies, and be sure to add to your count the number of colonies that had been picked.

3.4. Calculation of Recombination Rate

Table 1 displays rates of recombination calculated by the “method of the median” for four different cell lines containing pLB4 (see **Notes 8 and 9**). The reader is referred to Lea and Coulson (**II**) for further details and the mathematical theory behind the rate calculation. Here we present a “cookbook” approach to calculating rate:

1. Calculate the median number of colonies per subclone. For example, cell line MT⁺pLB4-22 (**Table 1**) had a median subclone colony number of 7.5. This value is referred to as r_0 .
2. Next, using the value of r_0 , an estimated value of r_0/m is interpolated from Table 3 in Lea and Coulson (**II**). The value of m is the average number of recombination events per subclone. In our example for cell line MT⁺pLB4-22, where $r_0 = 7.5$, the estimated value of r_0/m was found to be 2.38.
3. Using the values of r_0 and r_0/m , calculate the value of m as $[(r_0) \div (r_0/m)]$. In our example, $m = 7.5 \div 2.38 = 3.15$.
4. Calculate the (estimated) rate of recombination by dividing m by the number of cells plated per subclone. For our example using cell line MT⁺pLB4-22, rate = $(3.15) \div (4 \times 10^6) = 7.87 \times 10^{-7}$ recombination events/cell/generation. It is customary to divide this number by the number of copies of integrated recombination substrate to yield recombination rate in terms of recombination events/cell/generation/locus.

Table 1
Intrachromosomal Homologous Recombination Rates

Cell line	Cells plated, total ($\times 10^{-6}$) ^a	Median no. G418 ^r colonies per subclone	Colonies analyzed by <i>AluI</i> digestion	Recombinant colonies ^b	Recombination rate ^c
MT ⁺ pLB4-3	40	4	19	17	5.12 (4.58)
MT ⁺ pLB4-22	40	7.5	20	15	7.87 (5.90)
CBpLB4-9	36	7	18	18	7.5
CBpLB4-20	40	4	16	16	5.12

^aIndependent subclones of 4×10^6 cells each were plated into G418 selection.

^bThe number of G418^r colonies analyzed that displayed the recombinant *AluI* digestion pattern (*see* **Note 10**).

^cCalculated by method of the median (**II**). Presented in parentheses are rates that were corrected by multiplying the initially calculated rate by the percentage of clones determined to actually have arisen by recombination, based on the *AluI* digestion pattern.

3.5. Analysis of Recombinants

It is important to ascertain that G418^r colonies recovered from a fluctuation test were indeed produced by recombination events rather than by some unexpected event that fortuitously produced a G418^r phenotype. This can be accomplished in a number of ways by analyzing samples of genomic DNA isolated from G418^r colonies picked from the fluctuation test. Polymerase chain reaction (PCR) amplification of a portion of the tk-neo fusion gene spanning the I-SceI site is one approach we have used. The first level of analysis should be to confirm that the I-SceI site has indeed been eliminated. This is easily accomplished by digesting PCR products with I-SceI and displaying the products on an agarose gel.

A second level of analysis takes advantage of two *AluI* restriction sites that immediately flank the I-SceI site in pLB4 and pBR3. Both of these *AluI* sites will be eliminated by either homologous recombination (in the case of pLB4) or homeologous recombination (in the case of pBR3). Digestion of PCR products generated from G418^r segregants with *AluI* therefore provides an expedient and reliable screen for recombinants (*see Note 10*).

The ultimate analysis comes from DNA sequence determination, which can be performed directly on PCR products. The donor tk sequences on pLB4 as well as on pBR3 display nucleotide differences when compared with the tk portion of the tk-neo gene. (There are *many* more differences between the donor and the tk-neo sequence on pBR3 than on pLB4 and, hence, pBR3 is useful for studying homeologous recombination). Recombination events can result in the transfer of some of the nucleotide differences from the donor tk sequence to the tk-neo gene, which will be detectable upon DNA sequencing. Detection of the transfer of sequence information between the donor sequence and the tk-neo gene can provide unambiguous confirmation of recombination (*see Note 10*).

It should be noted that there are at least two different types of recombination events that can be recovered from cells containing pLB4 or pBR3. One type of event is a nonreciprocal exchange, also known as a gene conversion. In this case, information is transferred from the donor to the tk-neo gene with no other change. The other type of event is a crossover or “pop-out” in which the donor is essentially “spliced” to the tk-neo gene and the genetic information between the donor and the tk-neo gene (including the hygromycin resistance gene) is “popped-out” or deleted. The two different types of events produce very different restriction patterns on a Southern blot, and it is a good idea to perform Southern blotting to distinguish between these two types of events before attempting to interpret DNA sequence information (*see Note 10*). In our experience, gene conversions comprise at least 80% of events recovered from CHO cells. (Chapter 4 in this volume provides an excellent discussion and further

consideration of a variety of types of recombination events that may occur among mammalian chromosomal sequences.)

After analyzing recombination events, it is useful to correct recombination rate by multiplying the calculated rate by the percentage of recovered clones that were determined to have actually arisen via recombination. This correction has been made to the data presented in **Table 1**. It should be noted that the data in **Table 1** suggest that there is no significant difference between the homologous recombination rate in MT⁺ cells (Msh2 wild-type) vs Clone B (CB) cells (Msh2-deficient).

4. Notes

1. Plasmids pLB4 and pBR3 are available from the authors on request.
2. In this chapter, we describe the use of plasmids pLB4 and pBR3 for the study of spontaneous recombination. The presence of the recognition site for endonuclease I-*Sce*I also makes these plasmids useful for the study of double-strand break-induced recombination. This would be accomplished by an experimental design that includes the introduction of I-*Sce*I into cells to induce a break in a recombination substrate. The reader is referred to Chaps. 4 and 12 in this volume for additional information about strategies for studying break-induced recombination.
3. We routinely use electroporation to transfect mammalian cells with DNA. The optimal conditions for transfection via electroporation will vary depending on cell type and must be determined empirically. However, in our hands, the conditions we describe work reasonably well for a variety of cell types. Other transfection methods, such as liposome-mediated transfection, may be used but, in our experience, electroporation is the method of choice when low-copy-number integrations are desired. We also find that linearization of DNA prior to electroporation somewhat enhances transfection efficiency, but linearization is not necessary.
4. The proper level of hygromycin (or any selective agent) to use will vary by cell type and must be empirically determined.
5. It is not trivial to determine the number of copies of integrated substrate. If a cell line known to contain a single integrated copy of pLB4 or pBR3 is available, restriction digestions of DNA from that cell line can be displayed on a blot along with the DNA samples to be analyzed. Comparison of the hybridization intensity of an experimental sample with that of the established single-copy cell line will allow an estimate of copy number for the sample. Additionally, restriction digestions can be done that are predicted to produce a single junction fragment per integrated copy of substrate. (A junction fragment is a restriction fragment having one terminus within the integrated construct and the other terminus within adjacent genomic DNA). Samples that display only a single junction fragment on a Southern blot would be candidate single-copy lines. Single-copy cell lines sometimes occur at a relatively low frequency among stable transfectants. It is therefore advisable to analyze many (more than 20) transfectants. It is possible to

use cell lines that contain two or three integrated copies of the recombination substrate, but analysis of recombination events is somewhat confounded by the presence of multiple copies of substrate. One can actually only estimate copy number on a Southern blot. Ultimately, copy number is ascertained after recombinants are recovered. In a true single-copy line, the single integrated copy of substrate will be altered by recombination. In a multi-copy line, a single copy of the substrate will be altered by recombination whereas the remaining copies present in a recombinant will remain unaltered. Such a situation is readily revealed during analysis of recombinants.

6. Ideally, one should start with a single cell per subclone to initiate a fluctuation test. Practically speaking, all that is important is that the initial number of cells per subclone is small enough to effectively preclude the presence of a recombinant in the starting population. Starting with 100 cells per subclone satisfies this criterion and helps to expedite the experiment.
7. The appropriate number of cells needed per subclone depends on an approximation of recombination rate, which may not be known in advance. Small pilot experiments involving a couple of subclones may be used to estimate roughly the frequency of occurrence of recombinants. Essentially, for the method of rate determination presented here, one should plate enough cells per subclone to try to ensure the recovery of recombinants in all subclones.
8. There are several other methods for calculating recombination rates other than the method of the median. (See Chap. 1 in this volume for a second rate calculation method.) We find the method of the median to be very easy. Additionally, by virtue of using the *median* number of colonies per subclone, this method avoids potential complications introduced by calculation methods that average in data from “jackpot” subclones, that is, subclones that produce inordinately high numbers of recombinants because of recombination relatively early in the growth of the subclone.
9. Since the recombination substrates are randomly integrated, it is certain that the site of integration is different in each cell line. It is therefore advisable to determine the recombination rate for a given parental cell line using at least two or three stably transfected cell lines for each substrate in order to see if there is any significant position effect on recombination.
10. Detailed sequence information for plasmids pLB4 and pBR3 and further information helpful for analyzing recombinants by *AluI* digestion or other approaches are available from the authors upon request.

Acknowledgments

We are grateful to Margherita Bignami for providing the CHO cell lines, to Laura Bannister and Brady Roth for constructing pLB4 and pBR3, and to Raju Kucheralapati for providing the original tk-neo fusion gene.

This work was supported by Public Health Service grant GM47110 from the National Institute of General Medical Sciences to A.S.W.

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<http://www.springer.com/978-1-58829-236-0>

Genetic Recombination

Reviews and Protocols

Waldman, A.S. (Ed.)

2004, X, 260 p., Hardcover

ISBN: 978-1-58829-236-0

A product of Humana Press