Curry et al., http://www.jem.org/cgi/content/full/jem.20070583/DC1

SUPPLEMENTAL MATERIALS AND METHODS

The LM-TECA assay protocol

Cell preparation. Whole thymuses were extracted from young (1 mo old) mice, and rinsed well with cold PBS to remove blood, if any. Thymic lobes were placed into a cell strainer, and then into a 6-well plate along with 2–4 ml of chilled PBS. The plate was then set on ice for processing. The plunger from a 1-ml syringe was used to gently mush the lobes until they dissociated and the thymic cells passed through the strainer. The strainer and plunger were rinsed well with PBS, and the strained cells were collected into a 15-ml conical tube. They were washed once with PBS and increased to ~1 ml with chilled PBS. Any cell population with many dead or dying cells should be processed over a ficoll gradient to remove those cells from the living population. Dead and dying cells likely contain fragmented DNA, which is not good for this protocol. Cells were counted, and the trypan blue–excluding population was assayed from the others. Cells were increased to 25×10^6 cells/ml in chilled PBS, which is needed to increase the cells to 10^6 cells per 40 μl of PBS.

Genomic DNA in LMP plugs. High molecular weight genomic DNA was isolated by embedding cells (10^6) in agarose plugs (80 μl vol) as previously described (Curry, J.D., J.K. Geier, and M.S. Schlissel. 2005. *Nat. Immunol.* 6:1272–1279). Washed and warmed (37°C) cells in PBS were mixed with molten (56°C) 2.4% low melting point agarose (high-quality NuSieve) in PBS. The mixture held at 42°C and was immediately poured out into arrays of gel plug molds (Bio-Rad Laboratories) and allowed to set at 4°C for 10 min. 20–100 plugs were then extruded into 40 ml lysis buffer (10 mM Tris 8.0, 100 mM EDTA, and 1% sarkosyl) with 400 μg/ml proteinase K, and then slowly rotated in a 56°C incubation oven for >6 h. With the plugs and the buffer, a 50-ml tube should be nearly full, and the rotation should gently mix the plugs in the buffer. The plugs were rinsed with multiple volumes of TE until the soapy nature of the wash was mostly removed. Washed plugs were soaked in TE with DNase-free RNase I at 37°C for 1 h. Plugs were rinsed twice with TE and then placed in 40 ml of a modified lysis buffer (10 mM Tris 8.0, 1 mM CaOAc, and 1% sarkosyl) with 200 μg/ml proteinase K at 56°C for >4 h. Plugs were washed with several volumes of TE to remove the soapy nature of the last lysis buffer, and then soaked with 1 mM PMSF in TE for 2 h for a total of 2 times. Plugs were extensively rinsed in TE with >8 volume changes (with at least 10 min between changes) over an extended time period (ranging from hours to overnight, as needed).

Restriction digest of genomic DNA in LMP gel plugs. Approximately 40 μg of genomic DNA (8×10^6 cells embedded within 8 gel plugs) were completely digested overnight with the restriction enzymes (200 U) MaeIII or RsaI (NEB) at 37°C with their appropriate buffers in a 15-ml conical tube. Completeness of the digestions was monitored by analyzing a small fragment of an agarose plug on an ethidium-stained agarose gel. Digested genomic DNA plugs were rinsed 8 times with 15 ml of TE (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA) over a 2-h period to fully remove the digestion buffer and small DNA fragments. The genomic DNA plugs were spilled out onto a clean piece of tin foil and cleaned using a folded fine paper towel to remove trace amounts of TE buffer. The plugs were transferred with clean flat toothpicks into a 1.8-ml reaction tube.

Primer extension of restriction fragments. Digested genomic DNA was mixed with 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin), 0.2 mM dNTPs, 175 pmol of a 5′-biotynlated oligonucleotide (Table S1), and 20 U of a hot-start Taq polymerase (JumpStart; Sigma-Aldrich) with a final volume of 800 μl. The mixture was heated to 95°C for 10 min. The sample should be vortexed briefly twice at 2 and 4 min, followed by a quick 5-s microcentrifugation (using a personal microcentrifuge to ensure speedy return to the 95°C bath), and the heating should be continued for the remainder of 10 min. The primer extension reaction was incubated at 68°C for 1 h, and then terminated by the addition of 5 μl of 500 mM EDTA, pH 8.0, mixed, briefly centrifuged, and allowed to solidify by incubation on ice for 10 min. The tube-shaped DNA gel plug was removed from the reaction tube by slicing the end of the tube off with a fresh razor blade, cutting it vertically in half, soaking it overnight in 50 ml TE at 4°C, and finally washing it twice with 50 ml TE. The washed DNA plugs were next mixed with an agarose digestion buffer (1× β-agarase buffer; NEB), heated to 68°C for 15 min, and cooled to 42°C for 10 min. 15 U of β-agarase (NEB) was added and allowed to digest the agarose for 4 h at 42°C, and then chilled on ice for 10 min. Undigested agarose was removed by high-speed centrifugation at 4°C for 20 min, and then discarded.

Magnetic fractionation of primer-extension products. Liberated genomic DNA was adjusted to 100 mM NaCl, and 50 μl of μMACs streptavidin-coated paramagnetic beads (Miltenyi Biotech) were added. The mixture was incubated at room temperature for 20 min with mixing every few minutes. During this time, a μMAC column was equilibrated by adding 200 μl of the supplied DNA equilibration buffer to wet the column, followed by two washes with 0.1 M NaCl in TE while being held in the magnetic stand. The DNA bead complexes were loaded onto the equilibrated and magnetized μMAC column. The binding vessel was rinsed twice with 0.1 M NaCl in TE, and the rinses were added to the column. The column was extensively washed with a series of decreasing salt concentration washes; 1-ml washes of decreasing NaCl concentration (0.1, 0.1, and 0.05 M) in TE with 0.01% SDS. The last two wash volumes were at 68°C. The column was then rinsed twice with 2 ml of TE fortified with 0.01% Tween-20. Traces of the final wash buffer were removed by vacuum, with care given to avoid touching or disturbing the iron beads at the bottom of the column. Any drips adhering by surface tension to the bottom of the columns were carefully removed using a clean, dry fine paper towel. The retained volume (~32 μl) of rinse trapped in the iron bead matrix of the column should not be removed or touched. Captured DNA was eluted by removing the column from the magnetic field, placing the column over a receiving 1.8-ml reaction tube, adding 75 μl of the final wash buffer, and centrifuging at 3,000 *g* for 1 min.

Ligation of linker to 3′ A overhang. All of the magnetically fractionated DNA was ligated to a linker (annealed BW-1-T and BW-2; 200 pmol each in TE with 250 mM NaCl heated to no more than 75°C for 5 min and slowly cooled to room temperature, and then to ice temperature) with a 5′ T overhang in a reaction containing 1× ligation buffer (Invitrogen), 20 pmol of the linker, and 15 U of T4 DNA ligase (Invitrogen) in a final volume of 150 μl, which was incubated for 48 h at 14°C. Ligations were terminated by incubation at 68°C for 20 min and brought to 250 μl with the addition of 100 μl of 0.1 M NaCl in TE. This DNA was once again fractionated on an equilibrated and magnetized μMAC column, exactly as before. The μMACs columns used for the previous magnetic fractionation can be reused for the second sample fractionation and then discarded. The DNA was recovered with a 45-μl final wash buffer volume, bringing the total volume to ~73 μl.

Heminested PCR of ligation products. Final ligated DNA fractions were subjected to a heminested PCR. 4 μl of the DNA was mixed with a 1× PCR buffer, 0.2 mM dNTPs, 0.6 pmol BW-1-T oligo, either 0.6 pmol Dβ1-R1 or Jβ1-F2 outer primers, and 1 U of Taq polymerase in a 25-μl reaction volume. The first round of PCR consisted of an initial denaturation step of 5 min at 94°C, followed by 15–17 cycles of 20-s denaturation with a 1-min annealing/ex-

tension temperature of 65°C. 1 μl of the first PCR was carried over to a second PCR reaction identical to the first, but with either the nested Dβ1-R2 or Jβ1-F3 primers for 30 cycles at 68°C. A fraction of the reaction was analyzed on a Gel-Red–stained gel. Reactions that presented discrete bands were either ligated to a PCR ligation vector or particular bands were excised (Zymoclean; Zymo Research) and subjected to automated cycle sequencing. All primer sequences are listed in Table S1.

PCR analysis of genomic insertions sites. Those samples that yielded novel genomic DNA sequences adjacent the Jβ1 or Dβ1 RSS sequences were further analyzed by direct PCR. A set of primers within the target locus adjacent to the transposed signal end were designed, as well as an insertion-specific primer that covers 3–6 bp of the novel N region. Using these genomic DNA primers sets, the other side of the presumed RSS fragment integration event was sought by direct, fully nested PCR on a soluble DNA (12 initial cycles followed by 30 cycles with nested primers). To recover the entire contiguous insertion event, a heminested PCR design was used where the transposition-specific primer with its several 3′ N-region nucleotides was nested with the presumed distal genomic DNA primers sets. An analogous approach was used to detect potential translocations involving each RSS insertion. PCR products were excised from agarose gels, directly ligated to a cloning vector, and subjected to DNA sequence determination.