SUPPLEMENTAL MATERIAL

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Supplemental materials and methods

RNA isolation. Total cellular RNA was isolated from cells by resuspending cell pellets in Trizol reagent (Invitrogen) at a ratio of 1 ml Trizol to 106 cells. The mixture was incubated at room temperature for 5 min to allow the cellular material to fully lyse and dissolve. RNA was extracted by addition of 300 µl of chloroform followed by thorough mixing. The aqueous layer was separated by centrifugation at 14,000 rpm for 30 min followed by careful removal. RNA was then mixed with an equal volume of 70% ethanol, applied to an RNeasy column (QIAGEN), and prepared according to instructions. The eluted RNA was further isolated by ethanol precipitation in the presence of 75 mM sodium acetate and incubated at −20°C for a minimum of 2 h. RNA was collected by centrifugation at 14,000 rpm for 30 min, followed by washing in 100 µl of 70% ethanol and recentrifugation. The final RNA pellet was resuspended in 50 µl of nuclease-free water (Invitrogen) and examined for concentration and purity by optical density (260:280 nm ratio of 1.95 or greater).

Northern analysis. Northern analysis was performed on total cellular RNA isolated from unstimulated (day 0) and stimulated (day 2) B cells as described in the previous section. 10 µg RNA was mixed with loading dye and MOPS buffer, followed by heat denaturation at 65°C for 10 min. Each sample was then analyzed by agarose gel electrophoresis on a 1% denaturing agarose gel (formaldehyde denaturant). RNA was transferred to Hybond N^+ membranes (GE Healthcare) by capillary transfer in the presence on 10× SSC. Samples were immobilized on the membrane by cross-linking under UV light, followed by prehybridization for 3 h at 62°C using 6 ml of ExpressHyb hybridization buffer (Clontech Laboratories, Inc.) and denatured salmon sperm DNA (Applied Biosystems) at a final concentration of 167 µg/ml. DNA probes were amplified using the following oligonucleotides: Iµ forward, 5′-CTTCCCTCTGATTATTGGTCTCC-3′ and Iµ reverse, 5′-CCACCAACC AGCATGTTCAAC-3′, creating a 245-bp probe; Cµ forward, 5′-GTCAGTCCTTCCCAAATGTCTTCC-3′ and Cµ reverse, 5′-CCTCCGTAGTGGATTTTGCATACC-3′, creating a 280-bp probe; and β-actin forward, 5′-TACCACAGGCATTGTGATGGACTC-3′ and β-actin reverse, 5′-GTCAGGCAGCTCATAGCTCTTCTC-3′, creating a 268-bp probe. The probes were radiolabeled using a High Prime DNA labeling kit (Roche) and 50 µCi α-[^{32}P]-dCTP. Hybridization was performed by the addition of 25 ng of labeled probe to the hybridization buffer, followed by incubation at 62°C overnight. The membrane was then washed at 62°C in 2× SSC and 0.1% SDS for 30 min, followed by further washing in 0.1× SSC and 0.1% SDS for an additional 30 min. Products were visualized after exposure to phosphor imaging screens, and analysis was done using a Typhoon TRIO+ (GE Healthcare) imaging instrument using ImageQuant 5.2 software (GE Healthcare).

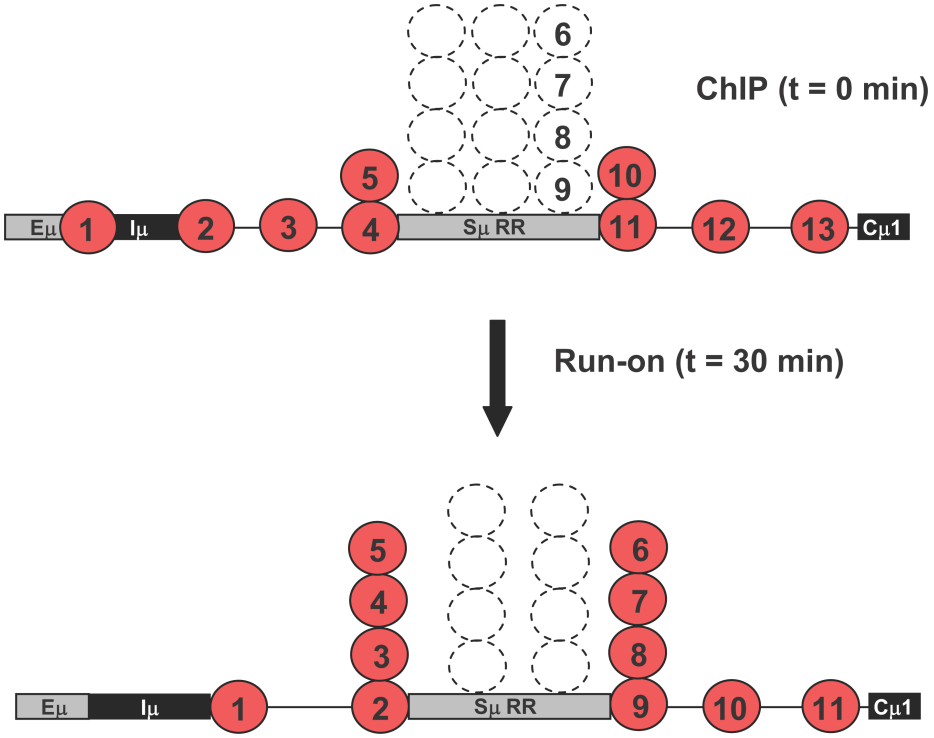

Figure S1. Model to compare the retardation of RNA polymerases in ChIP versus nuclear run-on assays. The ChIP assay depicts the loading of RNA pol II (numbered red circles) at a given instant. Pols 4 and 5 are twice as dense as pols 1–3 because they slow down when reaching R-loops around the repetitive region. Polymerases in the repetitive region (dotted circles) are very dense because of difficulty in unwinding RNA-DNA hybrids formed by the polymerases ahead of them. After the repetitive region, the polymerases move faster. The nuclear run-on assay depicts the activity of these polymerases after initiation of transcription in vitro for 30 min. No new polymerases are loaded, and all existing polymerases move forward. Pols 2–5 pile up at the beginning of the repetitive region, and pols 6–9 come from within the repetitive region. Both these groups have increased labeled transcripts because they are in high density.

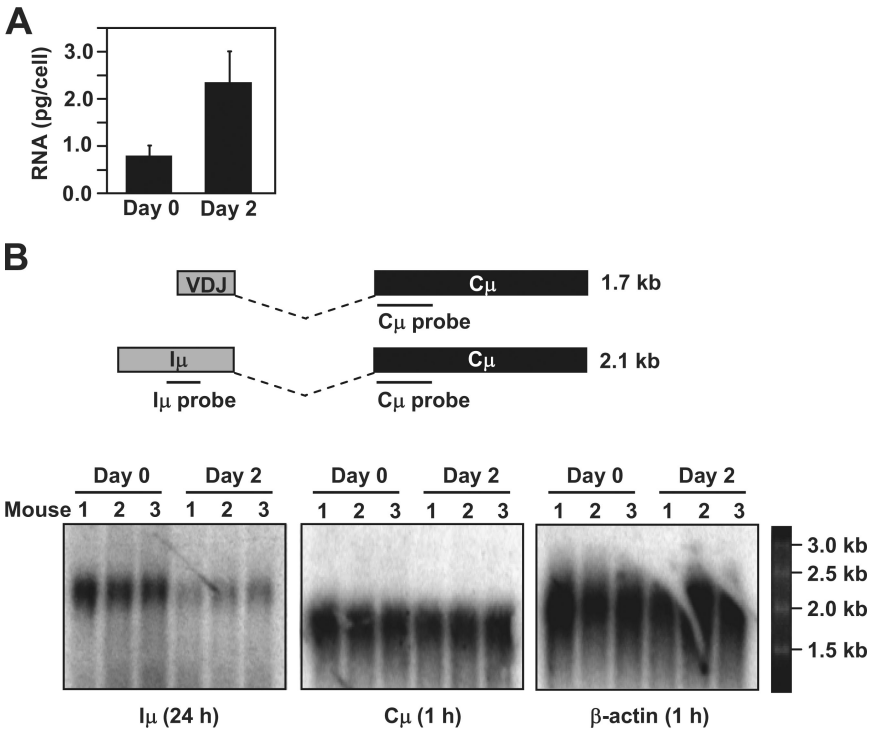

Figure S2. Contribution of VDJ and Iμ promoter activities to Sμ transcription in *Ung*$^{-/-}$ B cells. (A) Total RNA per cell was measured by optical density. Error bars represent the SD of values from two experiments with two mice and three mice. (B) Northern analysis. Labeled probes were hybridized to blots containing 10 μg RNA per lane from three individual mice. Two experiments with three mice each were performed. Specific transcripts were detected by probes for Iμ spliced transcripts (2.1 kb), Cμ spliced transcripts derived from promoters for VDJ (1.7 kb) and Iμ, and β-actin transcripts (2 kb). The Iμ blot was exposed for 24 h, and the Cμ and β-actin blots were exposed for 1 h. Iμ transcripts were not visible on the Cμ blot because Iμ was less abundant and required longer exposure. The decrease in Iμ transcripts on day 2 could be explained by the fact that there is more RNA per cell after activation (A), so that 10 μg RNA on day 2 would derive from fewer cells than 10 μg collected on day 0.

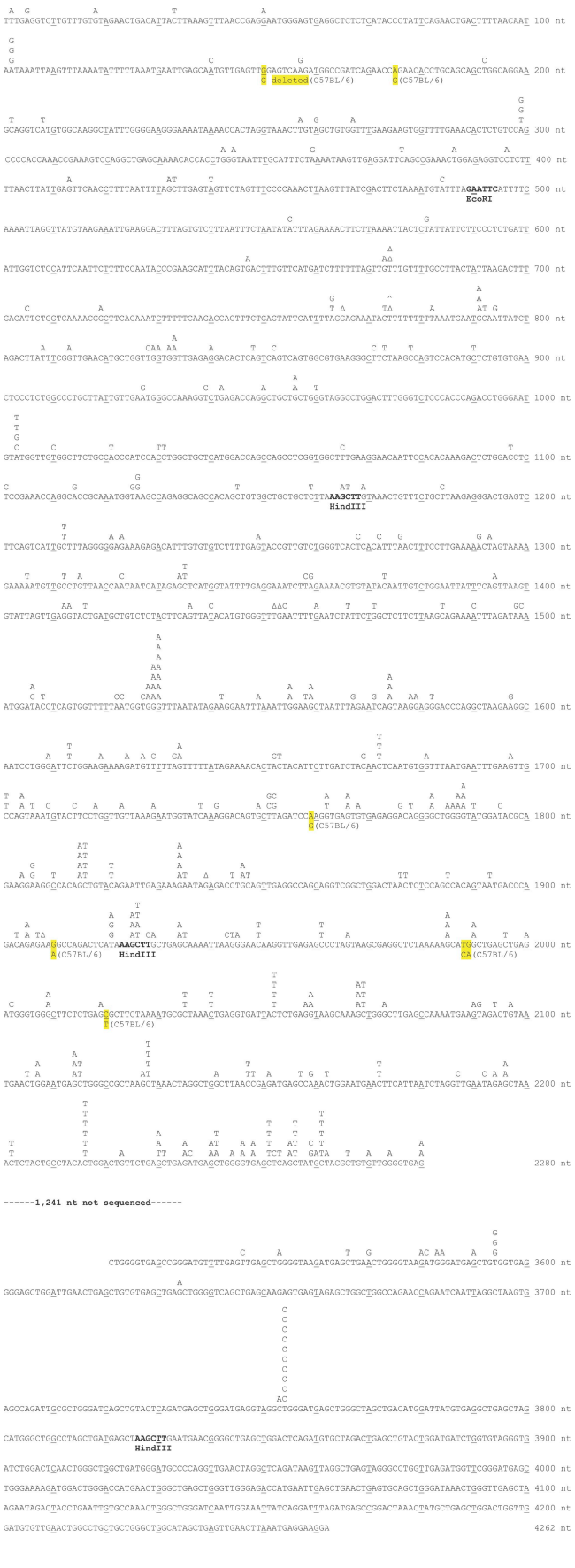

Figure S3. **Location of mutations in the Sμ region.** Every 10th base is underlined. EcoRI and HindIII sites are shown in bold. Allelic differences are noted in yellow, with C57BL/6 shown below the 129SV germline sequence. Mutations are placed above the germline sequence. Δ, deletion; ^, insertion. 1,241 bases in the repetitive region were not sequenced.

S1, 513 bp (143 clones, 29 mutations)

From \ To	A	T	G	C	Total
A	-	8	25	5	38
T	8	-	0	3	11
G	25	0	-	0	25
C	5	21	0	-	26

S2, 669 bp (106 clones, 41 mutations)

From \ To	A	T	G	C	Total
A	-	5	8	7	20
T	3	-	2	8	13
G	46	2	-	0	48
C	0	19	0	-	19

S3, 557 bp (102 clones, 58 mutations)

From \ To	A	T	G	C	Total
A	-	9	10	6	25
T	3	-	1	8	12
G	32	2	-	0	34
C	0	29	0	-	29

S4, 464 bp (111 clones, 122 mutations)

From \ To	A	T	G	C	Total
A	-	4	5	2	11
T	1	-	3	6	10
G	43	2	-	0	45
C	1	32	1	-	34

S5, 385 bp (127 clones, 128 mutations)

From \ To	A	T	G	C	Total
A	-	1	1	1	3
T	1	-	2	5	8
G	35	1	-	0	36
C	1	52	0	-	53

S7, 440 bp (94 clones, 25 mutations)

From \ To	A	T	G	C	Total
A	-	0	5	4	9
T	0	-	13	48	61
G	17	0	-	0	17
C	6	7	0	-	13

S8, 436 bp (91 clones, 0 mutations)

Figure S4. Types of substitutions in the Sμ region from *Ung*$^{-/-}$ mice. Mutations were recorded from the nontranscribed strand and have been corrected for base composition of the nucleotide sequence. Data are expressed as percent of the number of mutations. The nucleotide composition of each fragment is as follows: S1: 32% A, 30% T, 21% G, and 17% C; S2: 25% A, 36% T, 19% G, and 20% C; S3: 28% A, 32% T, 22% G, and 18% C; S4: 34% A, 25% T, 26% G, and 15% C; S5: 29% A, 23% T, 29% G, and 19% C; S7: 22% A, 23% T, 39% G, and 16% C; S8: 25% A, 24% T, 35% G, and 16% C. The increase in mutations of T in S7 is the result of repeated mutations at a hot spot. S8 had no mutations.

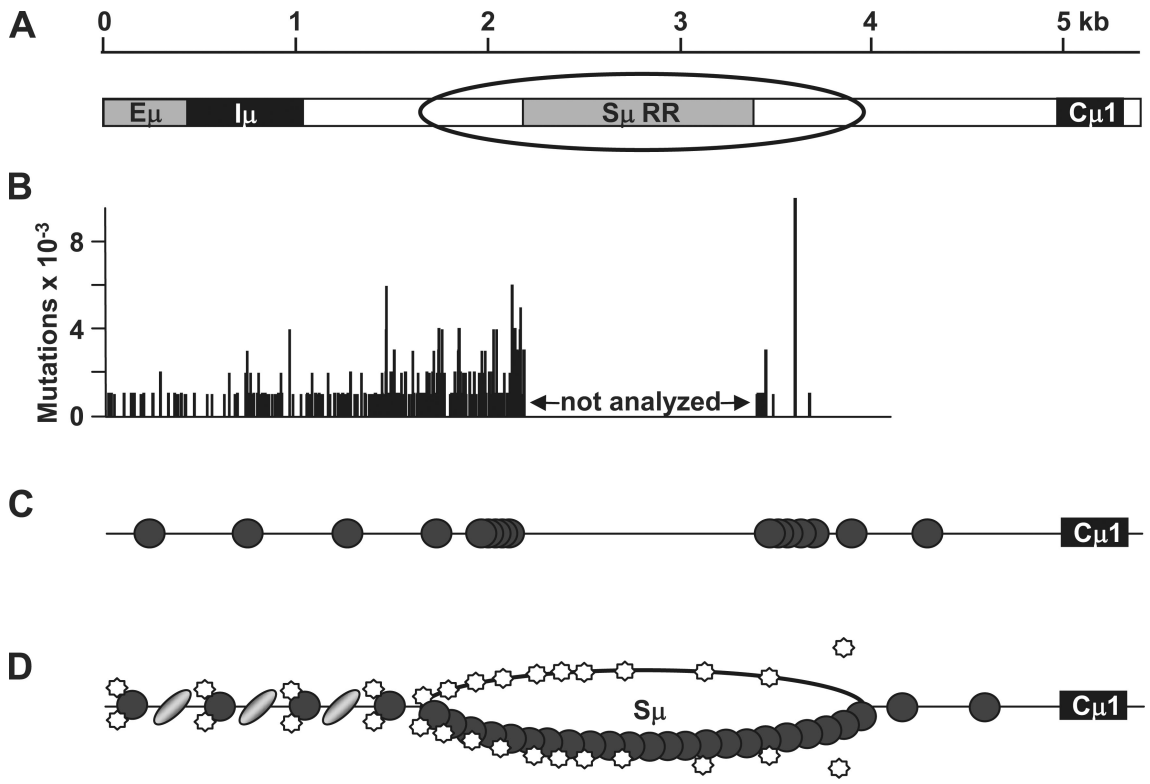

Figure S5. Model of hypermutation and transcription in the Sμ region. (A) The 5-kb region is shown that encodes Eμ, Iμ, the repetitive region (RR), and the first exon of Cμ. The oval depicts the region that forms R-loops (Huang, F.T., K. Yu, B.B. Balter, E. Selsing, Z. Oruc, A.A. Khamlichi, C.L. Hsieh, and M.R. Lieber. 2007. *Mol. Cell. Biol.* 27:5921–5932). (B) The mutational map from Fig. 3 A is shown. (C) The relative frequency of RNA pol II molecules detected in this study is shown as gray circles. (D) Model for distribution of enzymes in the Sμ region. circles, RNA pol II; AID, stars; and ovals, DNA pol η. AID may associate with RNA polymerases as they move through Sμ. RNA pol II molecules accumulate at the beginning of the R-loop, and move slowly though the complexed RNA-DNA hybrids, as AID binds to single-strand DNA and initiates mutation. At the end of the repetitive region, AID dissociates from the transcription complex and mutation stops. RNA pol II then progresses more rapidly to the Cμ gene. DNA pol η is only active in the region before R-loops.

Table S1. Primers for sequencing and run-on assays

Region	Oligonucleotide (5′→3′, 5′ overhang in lower case)
S1 external	Forward, GAGAGGGTTTTCAAGTACTC
S1 external	Reverse, GGAGACCAATAATCAGAGGG
S1 internal	Forward, ccgtcgacCCAAAATTCTTGTCAATCAG
S1 internal	Reverse, cgggatccAAAGTCCTTCAATTTCTTAC
S2 external	Forward, CCACCTGGGTAATTTGCA
S2 external	Reverse, GAGGTCCAGAGTCTTTGTGT
S2 internal	Forward, ccgtcgacGTTGAGGATTCAGCCGAA
S2 internal	Reverse, cgggatccGTTCCTTCAAAGCCACCGAGGC
S3 external	Forward, CTGCTGCTGGGTAGGCCTGGA
S3 external	Reverse, GCCTTCTTAGCCTGGGTCCC
S3 internal	Forward, ggaattcGTCTCCCACCCAGACCTGGG
S3 internal	Reverse, cgggatccCTTACTGATTCTAAATTAGCTTCC
S4 external	Forward, GCTCATGGTACTTTGAGGAA
S4 external	Reverse, CAGCTCAGCTGTGCTTTTTAGAG
S4 internal	Forward, ggaattcGAATCTATTCTGGCTCTTCTTAAGCAG
S4 internal	Reverse, cgggatccGCTTACTAGGGCTCTCAACCT
S5 external	Forward, AAGGCCACAGCTGTACAGA
S5 external	Reverse, CATCTCACCCCATCTCAGCT
S5 internal	Forward, ggaattcCTCTCCAGCCACAGTAATGA
S5 internal	Reverse, cgggatccAGTATCTCATTTCAGATCAG
S7 external	Forward, GTGAGCTGAGCTAGGGTGA
S7 external	Reverse, TTTTCCCAGCTCATCCCGAA
S7 internal	Forward, ggaattcGTGAGCTGAGCTGAGGTGAA
S7 internal	Reverse, cgggatccCCAGGCCCTACTCAGCCTA
S8 external	Forward, CTGGGCTAGCTGACATGGA
S8 external	Reverse, TCAGGCTGGTCCAGCTCA
S8 internal	Forward, ggaattcCTGGCCTAGCTGATGAGCTA
S8 internal	Reverse, cgggatccGCAGGCTAGCCTTGCTCA
S9 external	Forward, GGATAAACTGGGTTGAGCTA
S9 external	Reverse, GGCTTCTGTATCAGCACACA
S9 internal	Forward, ggaattcCTCGGCTGGGATCAATTGGA
S9 internal	Reverse, cgggatccTGGGATTTACACACCAGA
ΔS external	Forward, CTCGTTCTATAGAGGCAGA
ΔS external	Reverse, GCCTTCTTAGCCTGGGTCCC
ΔS internal	Forward, ggaattcCACCTTCCTCCTACCCT
ΔS internal	Reverse, cgggatccCTTACTGATTCTAAATTAGCTTCC
Cμ external	Forward, CTGTCTGTGAAGGCTTCCAA
Cμ external	Reverse, TCTTACCTGGAATGGGCACA
Cμ internal	Forward, ggaattcTGGCTAGAAGGCAGCTCCA
Cμ internal	Reverse, cgggatccGTTTTTGCCTCCGTAGTGGA
β-actin external	Forward, ATCGTACTCCTGCTTGCTGATCC
β-actin external	Reverse, GCATCCTCTTCCTCCCTGGAGA
β-actin internal	Forward, ggaattcATCCACATCTGCTGGAAGGTGGA
β-actin internal	Reverse, gggatccGAGAAGAGCTATGAGCTGCCTGA
γ-actin external	Forward, ACTGAATGAGCACTCATGCCCTTC
γ-actin external	Reverse, GGTCACACACACAGTGCCCATC
γ-actin internal	Forward, ggaattcCACAGGTCCTAAGGCCAGCTCA
γ-actin internal	Reverse, gggatccAGATCCTGACTGAACGGGGCTAC
Cd3δ external	Forward, GTCCATCTTCCCACTGGCTAGA
Cd3δ external	Reverse, GCTGAAGAGGCCTCCCTTAC
Cd3δ internal	Forward, ggtcgacGTGAAGGGTCCTGAGACTGATC
Cd3δ internal	Reverse, gggatccGAGGCAAGCCACAGGATGATCA

All internal forward primers had EcoRI sites, except for S1 and S2 which had SalI sites. All internal reverse primers had BamHI sites.

Table S2. Primers for ChIP analysis

Region	Oligonucleotide (5′→3′ direction)
Sa	Forward, TGAATTGAGCAATGTTGAGTTGGAGTCAAGATG
Sa	Reverse, ACCACTTCTTCAAACCACAGCTACAAGT
Sb	Forward, CCCGAAGCATTTACAGTGACTTTGTTCATGA
Sb	Reverse, GATTTGTGAAGCCGTTTTGACCAGAATGTC
Sc	Forward, GGGAATGTATGGTTGTGGCTTCTGC
Sc	Reverse, GTCCAGAGTCTTTGTGTGGAATTGTTCCT
Sd	Forward, GTTGCCTGTTAACCAATAATCATAGAGCTCATGG
Sd	Reverse, GTATAACTGAAGTAGAGACAGCATCAGTACCTCAAC
Se	Forward, ACACTACTACATTCTTGATCTACAACTCAATGTGGT
Se	Reverse, CGGATCTAAGCACTGTCCTTTGATACCATTC
Sf	Forward, CCAGACAGAGAAAGCCAGACTCATAAAGC
Sf	Reverse, GAAGCACTCAGAGAAGCCCACC
Sg	Forward, CTGCCTACACTGGACTGTTCTGAGC
Sg	Reverse, CAGCTCACCCCATCTCACCCCATC
Sh	Forward, CTGTGGTGAGCGGAGCTGGATTGAAC
Sh	Reverse, CTGATCCCAGCACAATCTGGCTCAC
Si	Forward, GTGTAGGGTGATCTGGACTCAACTGG
Si	Reverse, CTTTTCCCAGCTCATCCCGAACC
Sj	Forward, AGCCTGAGCTGAGTAGGTCTAAACTGAG
Sj	Reverse, GAACGAGGTCCTAGCCTAACTGGTC
SΔ	Forward, ACTGTTTCTGCTTAAGAGGGACTGAGTCT
SΔ	Reverse, CGATGTCGAGTCTAGTTTTTCAAGGAAAGTTAAATGTG
Cμ	Forward, TCTGACAGGAGGCAAGAAGACAGATTCTTA
Cμ	Reverse, GCCACCAGATTCTTATCAGACAGGGG