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Research paper

Quantification of Jκ signal end breaks in developing B cells by blunt-end linker ligation and qPCR

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Abstract

Introduction of a double-strand DNA break at the junction between a rearranging gene segment and its flanking recombination signal sequence (RSS) is the first step of V(D)J recombination. Such DNA breaks can be detected by either Southern blot hybridization or ligation-mediated PCR. While Southern blotting is easily quantifiable, it is often insufficiently sensitive and while LM-PCR is far more sensitive, it is poorly quantifiable. Reported here is a LM-qPCR assay which relies on real-time qPCR to provide an absolute measure of recombinase-mediated, or any other specific, double-strand DNA break in genomic DNA. The efficiency of the initial ligation reaction was found to be relatively low with just 3% of potential targets undergoing linker ligation. Using this assay, approximately 16% of murine bone marrow pre-B cells were determined to contain a dsDNA break adjacent to the Jκ1 gene segment. In addition, the kinetics of Jκ1 dsDNA breaks in a temperature-sensitive cell line induced to recombine its κ locus was determined.

Keywords: qPCR; Blunt-ended ligation; dsDNA breaks; J kappa 1; Murine; Pre-B cells

1. Introduction

Ligation of linkers to DNA molecules is a widely utilized technique in molecular biology. When combined with PCR, the ligation of a linker sequence to fragmented genomic DNA allows for the amplification of DNA sequences adjacent to a DNA break. First developed for genomic sequencing and in vivo DNA foot-printing (Mueller and Wold, 1989), ligation-mediated PCR (LM-PCR) assays have been adapted to detect DNA breaks introduced by the V(D)J recombinase (Schlissel et al., 1993; Roth et al., 1993). V(D)J recombination is a site-specific DNA recombination reaction essential for the generation of immune receptor diversity (reviewed in Jung and Alt, 2004; Gellert, 2002). Germline-encoded V, D, and J gene segments are rearranged to generate exons

Abbreviations: qPCR, quantitative PCR; RSS, recombination signal sequence; LM, ligation-mediated; dsDNA, double-stranded DNA; SE, signal ends.

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encoding the variable domain of Ig or TCR genes. All rearranging gene segments are flanked by a *cis*-acting targeting element known as the recombination signal sequence (RSS). The recombinase, consisting of the RAG1 and RAG2 proteins, recognizes a compatible pair of these RSSs and introduces dsDNA breaks precisely at the junctions between the RSSs and coding segments. The appropriate ends are then joined by DNA repair proteins to create functional genes. Thus, the presence of dsDNA breaks at RSS elements indicates active recombination at that locus. Quantification of these double-strand breaks (termed signal ends or SEs) has relied on semi-quantitative LM-PCR methods that utilize sample dilution to crudely determine relative template amounts. Real-time quantitative PCR (qPCR) technology can now be applied to dramatically improve and simplify these kinds of quantifications. Real-time qPCR assays are sufficiently sensitive that the linker ligation becomes the limiting step in the LM-PCR assay. The efficiency of linker ligation to dsDNA breaks in genomic DNA is unknown. In this report, qPCR was used to detect blunt-end ligation in a LM-qPCR assay and to measure the efficiency of that ligation reaction. A variety of ligation reaction parameters were surveyed and the effects on efficiency determined.

The real-time qPCR assay presented here, along with an accurate measurement of the efficiency of ligation, now permits absolute quantification of specific signal ends. A temperature-sensitive cell line that was induced to recombine its κ locus was examined and the kinetics of Jκ1 breaks determined. In addition, the fractions of Jκ1 gene segments in sorted murine bone marrow pre-B cells containing dsDNA breaks were measured.

2. Methods

2.1. Cell population and lines

The temperature-sensitive Abelson murine leukemia virus-transformed 103 line (Chen et al., 1994) stably transfected with a Bcl-x expression construct (103/BclX-7) was cultured in 5% fetal bovine serum (FBS), RPMI-1640 (100 μM 2-mercaptoethanol), at 33 °C, and then shifted to 39 °C to induce V(D)J recombination. An AMuLV-transformed pro-B cell line (63-12) from a RAG-2 null mouse (Shinkai et al., 1992), was cultured in the same media at 37 °C and served as the source of germline DNA used for background.

Animal experimentation was performed under a protocol approved by the University of California at Berkeley Animal Care and Use Committee (R253-0405). Primary pro- (IgM−, B220+, CD43+, CD25−) and pre- (IgM−, B220+, CD43−, CD25+) B cells were sorted from red blood cell-depleted mouse bone marrow first cleared of IgM+ B cells with rat-anti-mouse IgM microbeads on an AutoMACS column (Miltenyi Biotec, Auburn, CA). IgM− cells were further stained with antibodies (BD PharMingen and CALTAG): PE-anti-B220 (RA3-6B2), -CD25 (PC61); bi-anti-CD43 (S7) followed by SA-conjugated Cychrome (Liang et al., 2002). Cell sorting was performed on an Epics Elite. Sorted population purity, as assessed by flow cytometry, was greater than 95% (Hsu et al., 2003).

High molecular weight genomic DNA was extracted from the various cell populations by proteinase K digestion in the presence of SDS, followed by several phenol chloroform extractions, ethanol precipitation and re-suspension in TE (10 mM Tris–HCl, pH 8.0, 0.2 mM EDTA).

2.2. Construction of model substrate

A plasmid clone containing the murine Jκ1 region (GenBank accession no. M27036) was cut with *Sca*I and *Xho*II (New England Biolabs, Beverly, MA) and the resulting 321-base-pair (bp) fragment was purified by agarose gel electrophoresis. The *Sca*I site is immediately 5′ to the Jκ1 RSS and results in a blunt end while *Xho*II cleavage results in a 5′ overhang (Fig. 1). This substrate was end labeled with γ-^{32}P-ATP in an exchange reaction and then analyzed by polyacrylamide gel electrophoresis to verify its size and purity. The substrate was carefully quantified by spectrophotometry and diluted to 10^6 copies per μl of TE.

To obtain a true Jκ1signal end, a perfect Vκ-Jκ1 signal joint (precise head-to-head fusion of a Vκ and a Jκ1 RSS) was amplified by PCR (primers Jκ-Sub-F1 and Vκ-R1-RSS, Table 1) from bone marrow DNA obtained from a wild-type mouse and then cloned into the pCR2.1-TOPO-TA vector (Invitrogen, Carlsbad

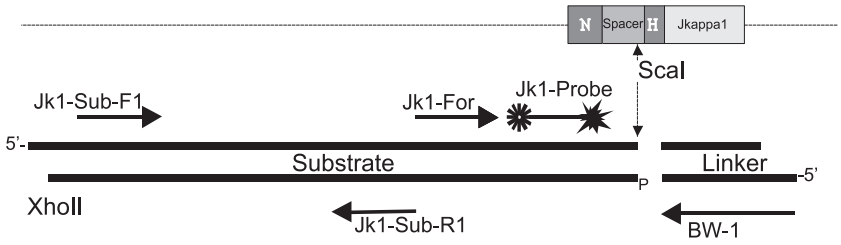

Fig. 1. Diagram of the model substrate for the detection of ligation products. The relative locations of PCR primers and dual-labeled probe are indicated. The nonamer (N), spacer (12 bp) and heptamer (H) are components of the recombination signal sequence that precedes the Jκ1 coding element.

CA). A 428-bp *Bam*HI fragment containing the perfect signal joint was gel-purified and then cut with HypCH4V (New England Biolabs) releasing 303- and 125-bp fragments. The larger fragment containing the complete Jκ1 RSS with a blunt and phosphorylated dsDNA break was purified on a 2% low melting point agarose gel then quantified by spectrophotometry. Purity of this true Jκ1 SE substrate was assayed by end labeling, electrophoresis on an 8% polyacrylamide gel, and phosphorimaging.

2.3. Ligation reactions

Blunt-end ligation reactions were performed using the model substrate described above and a specific linker oligonucleotide duplex (Mueller and Wold, 1989). That linker consisted of two complimentary oligos (BW-1 and BW-2) of different lengths annealed in a salt buffer (TENS; 250 mM NaCl, 10 mM Tris–HCl, pH 8.0, and 0.2 mM EDTA) by heating to 70 °C, cooling slowly to room temperature, then incubating on ice for 30 min. The linker has one blunt end and one 5′ overhanging end and is not phosphorylated. Linker molecules, once annealed, were never permitted to exceed wet ice temperatures during reaction assembly. A 100 pmol/μl stock of linker was used, and then diluted into TENS as necessary.

Ligation reactions (20 μl) assembled on ice consisted of the model substrate at different copy numbers mixed with high molecular weight mouse genomic DNA (63-12) in TE as indicated, 0.8 μl BW linker at differing concentrations, 4 μl 5× ligation buffer (250 mM Tris–HCl, pH 7.6, 50 mM MgCl2, 5 mM ATP, 5 mM DTT, and 25% (w/v) polyethylene glycol-8000), and 2 μl T4 DNA ligase (1 U/μl, Invitrogen). When background genomic DNA was used, that DNA and the substrate DNA were mixed thoroughly first, and then the rest of the reaction components added as a master mix. Reactions were incubated in an electronically controlled cooling block, unless otherwise noted. Reactions were terminated by heating to 68 °C for 20 min. When indicated, completed and terminated ligations reactions were drop dialyzed. A pan was filled with 500 ml of 5% (w/v) polyethylene glycol-8000 and floated on top was a wax laboratory film (Parafilm "M", Pechiney Plastic Packaging, Chicago) with several small rectangular slots. On top of each

Table 1
Sequence of oligonucleotides

Primer name	Sequence
BW-1 (linker)	5′ -GCGGTGACCCGGGAGATCTGAATTC
BW-2 (linker)	5′ -GAATTCAGATC
Jκ1-For	5′ -GCAGCTACCCACTGCTCTGTT
Jκ1-Probe	5′ -TGGCTGTACAAAAACCCTCCTCACTGAAGA
Jκ1-Sub-F1	5′ -AACAAATAATAAGCAGTCCTATGTGACATGCT
Jκ1-Sub-R1	5′ -GCAGTGGGTAGCTGCGAAAGC
Vκ-R1-RSS	5′ -GTTTATTTCTAGGAGGAAGTAGCTAGACCCTAAGG

Sequences are as found in the Jκ germline (GenBank accession no. M27036 (Ponath et al., 1989).

film slot, a single nitrocellulose membrane disc (VSWP02500) (Millipore, Billerica, MA) was placed, shiny side up and allowed to wet completely on the reverse side. The ligation reaction was dropped on to the membrane and allowed to stand for 2 h and then carefully recovered.

2.4. Real-time qPCR reactions

A qPCR primer and hydrolysis probe set was manually designed to detect specific Jκ1 blunt-end ligation products (Table 1). The forward primer (Jκ1-For) and the probe sequence (Jκ1-Probe) are borne on the model substrate, while the BW-1 primer is only found on the linker molecule (Fig. 1).

Real-time qPCR reactions were performed in triplicate on 5 μl of the heat-inactivated ligation reaction. A two-times qPCR buffer was utilized and consisted of 40 mM Tris–HCl, pH 8.4, 100 mM KCl, 6.0 mM $MgCl_2$, 8.0% glycerol, and 400 μM of each dNTP. Reaction volumes were 25 μl and contained 1× reaction buffer, 0.38 units of a hot start *Taq* polymerase (JumpStart™, Sigma, St. Louis, MO), 12.5 pmol of the forward and reverse primers, 6 pmol of a dual-labeled probe (labeled 5′ with the reporter dye 6-carboxy-fluorescein (FAM) and 3′ with the quencher dye 6-carboxytetramethylrhodamine (TAMRA)) (MWG Biotech, High Point, NC). Reactions were thermocycled using an Opticon™ real-time PCR machine (MJ Research, Waltham, MA) as follows: an initial 5 min at 94 °C, followed by 45 cycles of 94 °C for 15 s and 60 °C for 1 min. Data was collected and analyzed with the software supplied by the manufacturer.

To generate an absolute standard curve, a true Jκ1 signal end was captured by ligation using genomic DNA purified from the tsAbl-transformed pro-B cell line 103/BclX-7 grown at the restrictive temperature. That specific product was then cloned into a pCR2.1-TA vector (Invitrogen). The resulting pJκ1/BW1 vector was linearized with *Hin*dIII (NEB), quantified by spectrophotometry, and then used for construction of the standard curve with 20,000, 2000, 200, 20, 2, 0 copies per qPCR reaction, performed in duplicate.

2.5. Southern blot analysis

Genomic DNA obtained from 103/BclX-7 cells cultured at 39 °C for 0–72 h was restricted with *Hin*dIII, *Eco*RI, and *Nco*I (NEB), fractionated by field inversion gel electrophoresis on a 1% agarose gel, and then blotted to a Hybond™-XL (Amersham Biosciences, Piscataway, NJ) membrane. The *Eco*RI to *Bam*HI fragment from a plasmid clone of the germline κ locus was α-P^{32}-CTP labeled by random priming (Invitrogen), purified on a nucleotide removal column (Qiagen, Valencia CA) and used to probe the membrane for 20 h. The membrane was stringently washed at 68 °C down to 0.2× SSC, dried and then exposed to a phosphorimager cassette. The loss of the germline band (3177 bps) was indication of Jκ recombination. Results were corrected for DNA loading inconsistencies using the invariant 2571-bp *Nco*I to *Nco*I fragment between the J segments and the constant region. Quantification was performed using ImageQuant® software (Amersham Biosciences).

3. Results

Our experimental strategy was to first optimize conditions for LM-qPCR detection and quantification of specific dsDNA breaks in genomic DNA, then to apply the optimized assay to DNA purified from cells undergoing V(D)J recombination. A model ligation substrate was generated by restriction digestion of a plasmid clone of the germline κ locus (Fig. 1). The substrate has a blunt end which mimics a broken 3′ RSS end and a 5′ overhanging end and contains the target sequences used for qPCR amplification of authentic Jκ1 RSS breaks. Upon addition of the BW linker and T4 DNA ligase, several ligation products were possible. The model substrate molecules could potentially self-ligate resulting in products that would amplify with either the Jκ1-For or BW-1 primers alone and cause the hydrolysis of two real-time Jκ1 probes per template with each amplification cycle. Although such events were unlikely given the concentrations of the model substrate in our assay system, a control experiment was performed to test this presumption. Ligation reactions containing up to 333333 model substrate molecules were amplified with either the BW-1 primer or the Jκ-F1 forward primer alone and were never observed to generate any detectable qPCR signal (data not shown).

3.1. Optimizing the linker-ligation reaction

Model substrate molecules were mixed with genomic DNA (2000 ng/reaction) from the RAG-deficient pro-B cell line (63-12) at molar ratios of 5% and 10% substrate copies per genome (6 pg) and subjected to linker ligation for various lengths of time. Reactions were terminated, diluted, and assayed by qPCR. Ligation proceeded quickly and products could be detected within minutes of the initiation of incubation (Fig. 2A). Ligation products detected at the zero time point were likely due to the time (<30 s) it takes to mix and then terminate the reactions. Within 60 min, ligation efficiency (molecules detected/input number of substrate molecules ×100%) reached a plateau value of approximately 3% (Fig. 2A). Since incubations longer than 2 h did not significantly increase the efficiency of the ligation reaction, all subsequent ligations had 2 h of incubation.

Variations in incubation temperature only modestly affected the efficiency of the linker-ligation reaction (Fig. 2B). Control substrate and 63-12 genomic DNA mixtures of 5% and 10% were mixed with the ligation reaction cocktail and then incubated at various temperatures ranging from −10 to 68 °C for 2 h.

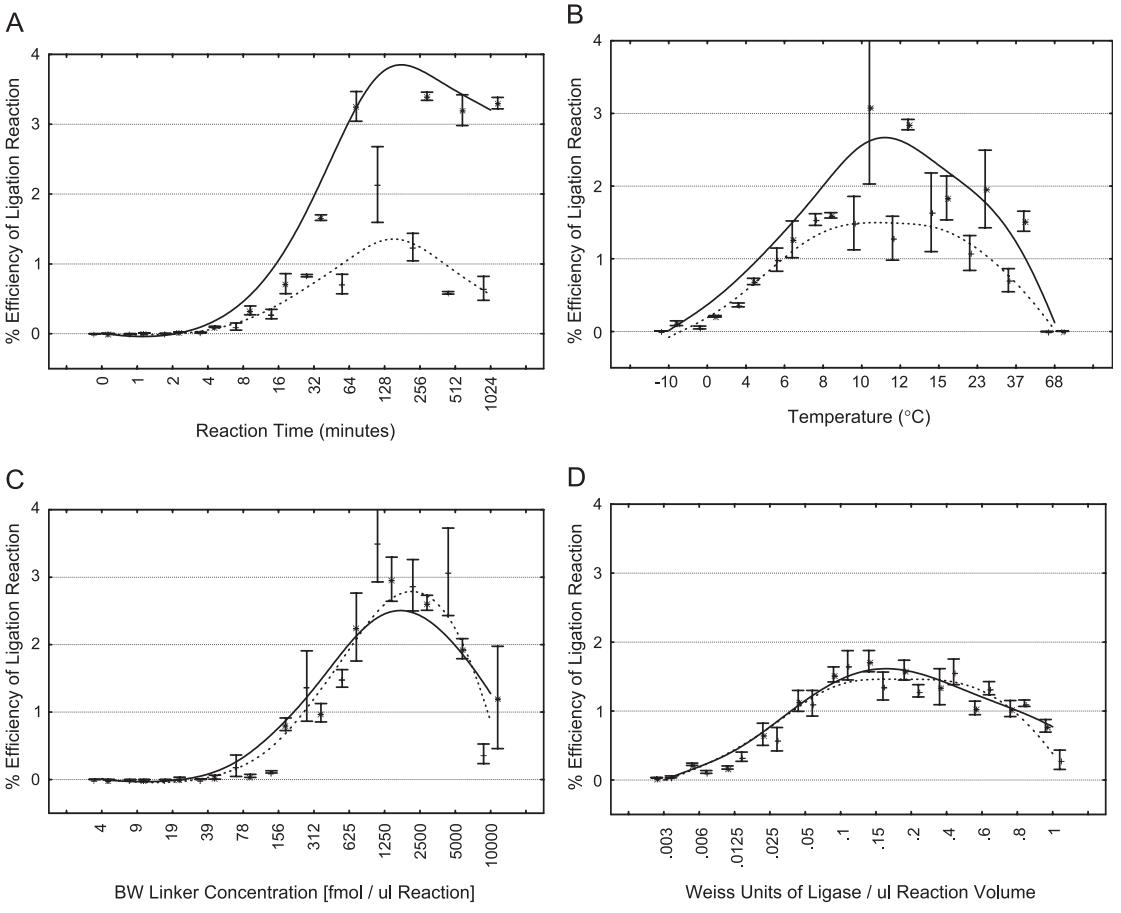

Fig. 2. Effect of (A) reaction time, (B) temperature, (C) BW linker concentration and (D) ligase units (Wiess) on the efficiency (detected output number of ligations/input number of model substrate molecules) of blunt-end ligation reactions. Model substrate molecules were mixed with genomic DNA at ratios of 5% (+) and 10% (*) per genome equivalent (6 pg). Ligation reactions were followed by qPCR and means with standard errors are shown. Best-fit lines (dotted 5% and solid 10%) were calculated using a distance-weighted least squares smoothing procedure (Statistica, Statsoft).

The reactions were then terminated and assayed by qPCR for substrate ligation events. The maximum blunt-end ligation efficiency was found to occur with incubation temperatures in the range from 8 to 15 °C. Ligation efficiency decreased at temperatures higher than 23 °C, possibly due to the dissociation of the two linker strands. All subsequent ligation reactions were incubated at 14–15 °C.

To determine the effects of linker concentration on ligation efficiency, model substrate molecules mixed with genomic DNA (5% and 10%) were subjected to ligation reactions using differing amounts of linker. The efficiency of linker ligation decreased at linker concentrations below 1.25 pmol/μl and above 5 pmol/μl (Fig. 2C). The optimal amount of linker was determined to be approximately 2 pmol/μl and that concentration was used for all subsequent ligation reactions.

Effects of ligase concentration on the efficiency of linker ligation were determined by varying this parameter. Surprisingly, high concentrations (>0.2 unit/μl) of ligase had an inhibitory effect on the efficiency of ligation (Fig. 2D). In addition, at ligase concentrations below 0.1 unit/μl, the efficiency of the

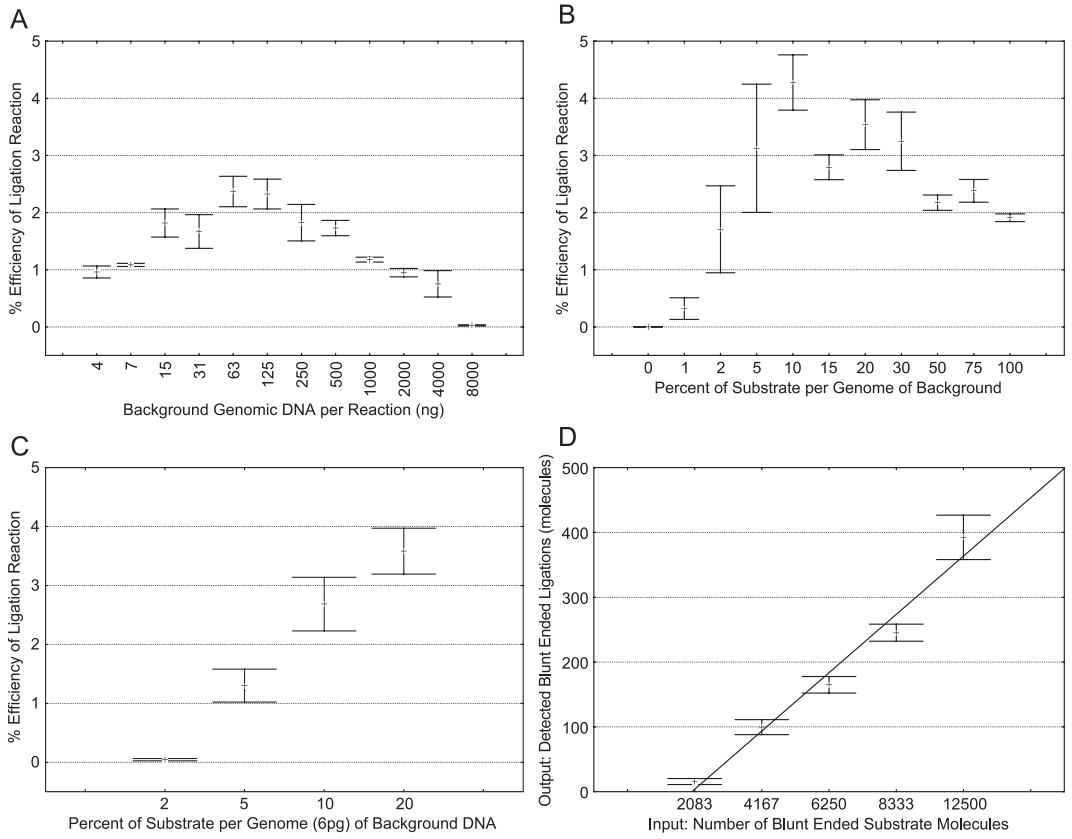

Fig. 3. (A) Effect of increasing genomic DNA background on the efficiency of blunt-end ligation. The number of model substrate molecules per reaction was constant (33333) while the total amount of background genomic DNA was increased by twofold increments from a low of 4 ng to the maximum of 8000 ng. (B) Blunt-ended ligation efficiency varies with the fraction of the model substrate in a fixed background of genomic DNA (2 μg/reaction). (C) The reproducibility of the ligation reaction under the conditions developed is shown for several different substrate percentages. Six separate ligation reactions were performed with each reaction assayed by qPCR three times. Each set of qPCR values was averaged and presented as a mean with standard errors. (D) Input number of model substrate molecules is plotted against the detected output number of successful blunt-ended ligation. This graph generates the linear regression line shown (input=2027+25.27×output, $p<10^{-6}$, R=0.93, df=28). Data from two independent experiments was combined. That equation was then be used to correct the subsequent results for the low efficiency of the blunt-ended ligation reaction.

reaction decreased. Ligase was used at 0.2 unit/μl for the subsequent ligation reactions.

The effect of total genomic DNA concentration on the efficiency of linker ligation was studied by mixing varying amounts of 63-12 genomic DNA with a fixed number of model substrate molecules. As expected, increasing amounts of background DNA had a significantly deleterious effect on the efficiency of the ligation reaction (Fig. 3A). The efficiency with which the assay could detect and quantify dsDNA breaks fell precipitously when the frequency of such breaks was less than 1–2 per 50 genomes (2–4%) (Fig. 3B). Using reaction conditions optimized as described above, reproducibility of the real-time LM-PCR reaction was determined. Ligation standards at 2%, 5%, 10%, and 20% in a background of 2000 ng of genomic DNA were assayed six times each and the mean ligation reaction efficiencies determined (Fig. 3C). The assay was found to be highly reproducible and the standard error around the ligation efficiency was less than ±0.5%. A correction standard curve was generated with which the efficiency of the ligation reaction could be accounted for when quantifying actual Jκ1 breaks using qPCR data (Fig. 3D).

3.2. Adjuvant to the ligation reaction

To determine whether any of several commonly used adjuvants might increase the efficiency of linker-ligation reactions, a minimal ligation buffer consisting only of NaCl, $MgCl_2$, Tris, pH 8.0, and ATP was prepared. Ligation reactions were performed using 63-12 DNA doped with 5%, 10%, or 20% of the model substrate, under the previously optimized conditions. DNA was first mixed with the additive (polyethylene glycol-8000 (PEG) 2.5%, 0.5 mM spermidine, or 1 mM hexaamminecobalt chloride), and then the rest of the ligation reaction components added. Completed ligations were terminated and assayed as before. Spermidine had a clear inhibitory effect compared to the minimal buffer (Fig. 4). The addition of hexaamminecobalt chloride increased ligation efficiency approximately 10-fold, whereas PEG increased efficiency to an even greater extent. Attempts to further

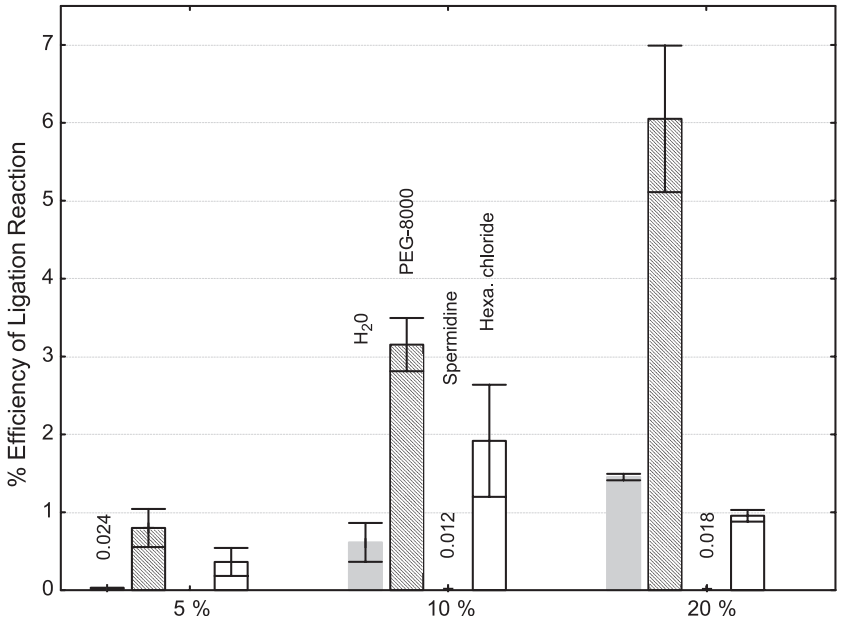

Fig. 4. The effect of potential adjuvants on the efficiency of ligation. Genomic DNA (2 μg) doped with model substrate molecules at ratios of 5%, 10% and 20% per genome equivalent (6 pg) were mixed with either H_2O (solid bars), 5% PEG-8000 (crossed bars), 0.5 mM spermidine (solid), or 1 mM hexaamminecobalt chloride (open bars). A complete ligation master mix utilizing a 10× buffer without PEG-8000 was added and incubated. Completed ligation reactions were assayed by qPCR and the means plotted. Numerical values are indicated for those means that have very low ligation efficiencies.

boost the efficiency of the ligation reaction by adding hexaamminecobalt chloride to ligation reactions containing 5% PEG-8000 were not successful and at levels of 0.5 mM, the genomic DNA began to precipitate from the reaction (data not shown).

3.3. Components of the ligation reaction inhibit the qPCR reaction

Using the optimized conditions, the assay was still unable to detect ligation products in the genomic DNA's doped with 0.5% and 1% model substrate. The ligation conditions did permit the inconsistent detection of the ligation products in the 2% substrate/DNA mixtures and consistently detected the higher (≥5.0%) doped levels. The shapes of the qPCR curves for the 2% ligations (data not shown) led to the hypothesis that some component of the ligation reaction was inhibiting the qPCR reaction and that this inhibition was of consequence only at low target concentrations.

To test whether low molecular weight components of the ligation reaction were inhibitory to the qPCR assay, half of a complete but substrate-free ligation reaction mixture was drop dialyzed against an excess of 5% polyethalene glycol-8000 (PEG) in water. A very small number of a perfect qPCR target molecules was added to a master qPCR mixture to which water, undialyzed or dialyzed ligation reaction mix was added. The qPCR signal in the dialyzed ligation reaction mixture was identical to the H_2O control, while the undialyzed ligation reaction proved inhibitory (8- to 128-fold decrease) to the qPCR reactions (Fig. 5). Further experimentation revealed that unligated BW linker was the principal source of inhibitory activity and that this inhibition was only relevant when the ligation target concentration was below 5% per genome (data not shown).

3.4. Accumulation of signal ends in a transformed pro-B cell line

The 103/BclX7 murine pro-B cell line is transformed with a temperature-sensitive AMuLV mutant (Chen et al., 1994). At the permissive temperature (33 °C), these cells actively divide but express only modest levels of V(D)J recombinase activity. At the restrictive temperature (39 °C), these cells exit the cell cycle and begin to differentiate. Differentiation

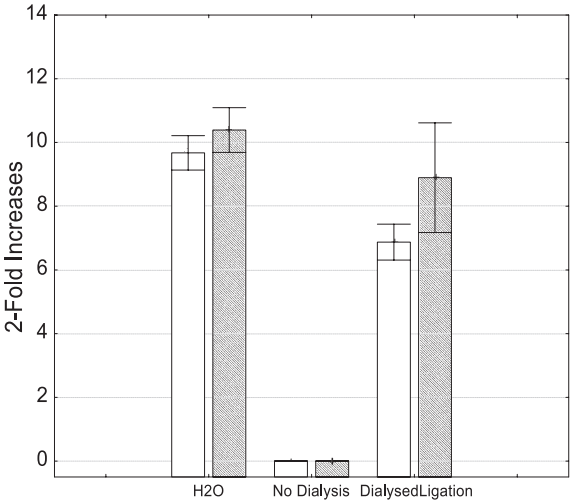

Fig. 5. Ligation reaction components poison qPCR reactions. A single ligation reaction was constructed where the ligase had been heat-inactivated prior to addition. This sham reaction was split into two where one half was drop dialyzed on 5% PEG-8000 for 2 h. Real-time qPCRs containing either 4 (open columns) or 16 (hatched columns) copies of the *Hin*dIII linearized pJκ1/BW1 plasmid were then mixed with 10 μl of water, the sham ligation reaction, or the dialyzed sham ligation reaction. Twofold differences over the lowest values obtained are presented with standard errors.

involves significant increases in expression of RAG proteins and activation of Igκ light chain gene rearrangement. These cells also accumulate high levels of dsDNA breaks at Jκ RSSs (Ramsden and Gellert, 1995). To determine the absolute frequency of these dsDNA breaks, an optimized LM-qPCR assay was performed on DNA purified from 103/BclX7 cells grown at the permissive temperature and then shifted to the restrictive temperature for increasing lengths of time.

As previously detailed, the efficiency of ligation reactions is low and only a fraction of the available dsDNA breaks are ligated with the BW linker molecule. To account for this ligation inefficiency, the standard curve from Fig. 3D was applied to these 103/BclX7 results, permitting the actual number of target dsDNA breaks to be accurately determined. This simply entailed using the linear regression formulae to yield corrected values (actual numbers of Jκ1 dsDNA breaks) from the observed output number of molecules (BW-ligated Jκ1 dsDNA breaks).

An increase in dsDNA breaks at Jκ1 was detectable 32 h after the shift to the restrictive temperature (Fig. 6A) and breaks continued to accumulate

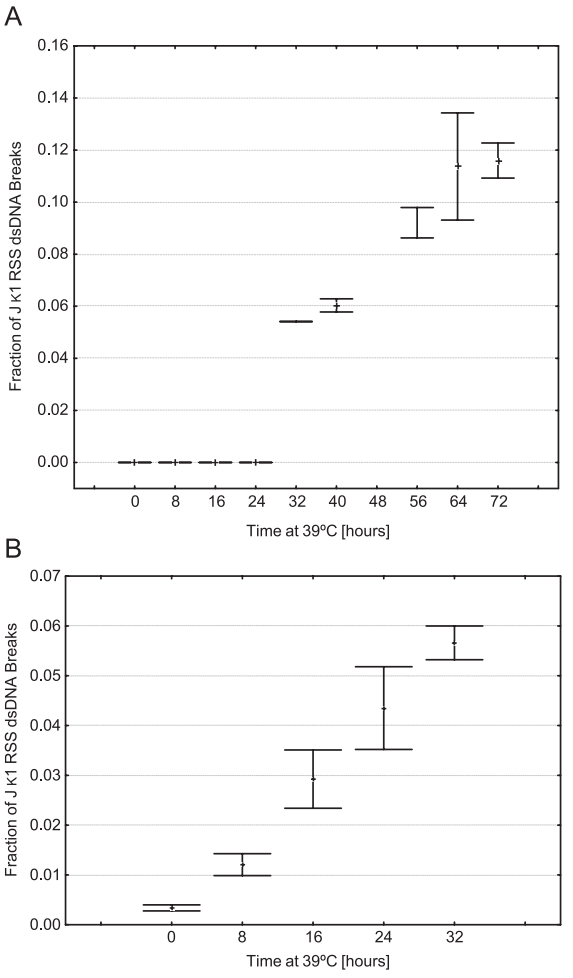

Fig. 6. (A) Fraction of Jκ1 signal ends generated per genome as a function of time in temperature-shifted 103/BclX7 cells. Actual output numbers are corrected against the ligation efficiency standard curve from Fig. 3D. (B) Fraction (%) of Jκ1 RSS breaks detected by LM-PCR on dialyzed ligation reactions. In order to remove the qPCR inhibitory components from the completed ligation reactions, these reactions were dialyzed against 5% PEG-8000 for 2 h and then 10 μl of that dialyzed reaction assayed by the qPCR. A similarly treated standard curve was used to correct the values obtained.

throughout a 72-h culture period. At the 72-h point, 11.6% of the genomic DNA contained a Jκ1 signal end break. For the earlier time points (0–24 h), Jκ1 breaks were below 5% per genome, and no successful ligation products could be detected. At these low levels, the qPCR assay inhibited by components of the ligation reaction. To circumvent this problem, qPCR assays were performed on dialyzed ligation reactions for time points 0–32 h. As the correction standard curve previously used (Fig. 3D) was not accurate in this range, a set of five dialyzed ligation standard curve points ranging from 0.5% to 10% model substrate per 2000 ng genomic DNA was also assayed. This standard curve was used to correct the values obtained for the 0- to 32-h time points and that data presented in Fig. 6B. The dialyzed correction now reveals that there is a measurable level of Jκ1 breaks occurring in the cells at these initial time points. Reassuringly, the 32-h time point value was virtually identical between the two different assays.

To confirm the results obtained by the LM-qPCR assay, a Southern blot hybridization method was used to measure the loss of the germline Jκ locus fragment which occurs with gene rearrangement using the same 103/BclX-7 genomic DNA as used in the LM-PCR assay (Fig. 7). Two different fragments were quantified by phosphorimager; the larger band containing the Jκ segments and a smaller band containing the Cκ exon. Loss of the larger band was taken as evidence of RAG protein cleavage involving one of the four functional Jκ gene segments. The invariant lower band was used to normalize the various samples for DNA recovery and gel loading. The time zero sample was presumed to contain no cleaved or rearranged Jκ segments and thus indicated zero percent cleavage, while subsequent time points were evaluated by comparison. No loss of germline DNA was observed in the time 0 and 16 h samples, though some low level (2.4%) loss was observed at 8 h. These data are within the margin of error associated with this type of assay. Considerable loss of germline Jκ sequence was observed at 24 h and, thereafter, reaching a maximum level of approximately 19% which corresponds closely to the results obtained by real-time LM-PCR.

3.5. Absolute quantification of Jκ1 signal ends in pro- and pre-B cells

To determine the absolute number of Jκ1 signal end breaks in a physiologically relevant cell population, DNA purified from sorted populations of murine bone marrow pro- and pre-B cells was assayed. Pro-B cells do not undergo significant amounts of Vκ-to-Jκ rearrangement and should contain few if any Jκ1 signal end breaks while pre-B cells are actively

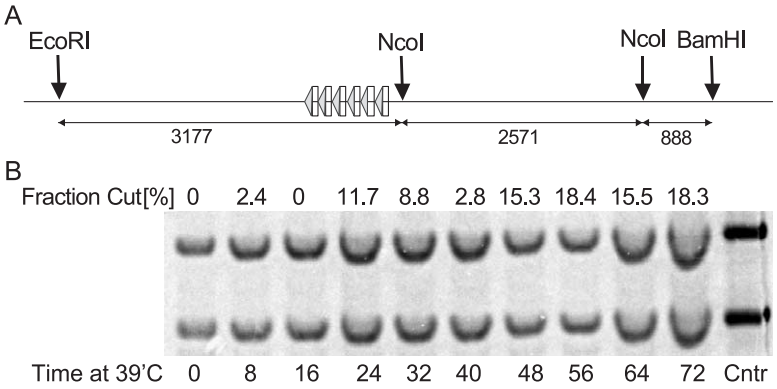

Fig. 7. Map (A) of the murine Jκ cluster with sizes of restriction digestion products. Autoradiograph (B) of *Eco*RI-, *Bam*HI- and *Nco*I-digested 103-Bclx genomic DNA, probed with the *Eco*RI to *Bam*HI fragment of the Jκ cluster. The top band is the 3177 *Eco*RI to *Nco*I fragment containing the Jκ gene cluster. The lower band is the *Nco*I to *Nco*I fragment (2571) just after the Jκ cluster. Fragments were quantified by phosphorimager and used to determine the fraction of the top Jκ band that is lost due to recombinase restriction of the Jκ RSSs. The control band (Cntr) is the *Eco*RI to *Bam*HI interval cut with *Nco*I (50 pg).

rearranging their κ loci and should present a considerable number of Jκ1 signal ends. As expected, Jκ1 signal ends could not be detected by the real-time LM-qPCR assay in the pro-B cell populations even when using the drop dialysis methodology. In the pre-B cell population, assuming 1 genome to be equal to 6 pg of DNA, the frequency of Jκ1 signal ends, after correction for ligation reaction efficiency, was 0.164±S.D. 0.0124 breaks per genome.

4. Discussion

Detection of dsDNA breaks involving RSSs flanking Ig or TCR gene segments has often served as an indirect measure of locus-specific V(D)J recombinase activity (Schlissel et al., 1993). As originally described, LM-PCR is specific and very sensitive for this purpose, but poorly quantifiable. The novel LM-qPCR assay presented here serves two purposes. First, using known amounts of a model substrate doped into a population of high molecular weight DNA, the actual efficiency of the blunt-ended ligation reaction was determined with precision. Factors affecting ligation efficiency were then studied and an optimal set of conditions determined. Second, using these ideal conditions, a correction standard curve was generated which allows LM-qPCR data to be corrected for variations in the efficiency of the ligation reaction. This leads to the ability to generate an absolute measurement of Jκ1 signal end breaks in vivo and was utilized to determine the frequency of such breaks in pro- and pre-B cells as well as a pro-B cell line that was induced to undergo κ locus rearrangements.

Initial experiments used the *Sca*I model substrate since it was technically easy to obtain and fortuitously proximal (12 bp) to the 3′ end of the Jκ1 RSS. This model substrate, however, does not have the same sequence context as the true Jκ1 signal end, most notably the last base pair is a A:T, whereas the true Jκ1 signal end presents a G:C base-pair at its end. Thus, the efficiency of the *Sca*I model substrate ligation might be affected by its divergent sequence context. To address this concern, a true Jκ1 model substrate was subsequently developed. That model employed the HpyCH4V restriction enzyme which generates a blunt end precisely in the middle of the perfect signal joint sequence (Jκ-RSS-G**TG/CA**C-Vκ-RSS). Identical ligation reactions for the *Sca*I model and the HpyCH4V model were performed on between 1 and 10^6 substrates in the absence of competitor DNA. The ligation efficiency was equal for both the model and true substrates leading to the conclusion that the *Sca*I substrate is a valid model for a signal end.

Blunt-ended ligation reaction efficiency was found to be surprisingly low with approximately 3% of the potential target molecules being ligated to the linker under optimal conditions. In order to account for this

low efficiency, a simple correction curve was employed to correct observed values to actual values of initial molecules.

Components of the ligation reaction were found to be poisonous to the qPCR and prevented detection of dsDNA breaks below 5% per genome. A simple drop dialysis methodology was utilized to remove those components and permit a greater volume of the ligation reaction to be assayed. This methodology successfully increased the sensitivity of the LM-qPCR assay but was only necessary for samples that contain rare dsDNA breaks (<5%).

The conditionally transformed 103/Bcl-2 cell line was examined previously for the kinetics and extent of Jκ1 signal ends (Ramsden and Gellert, 1995) and those authors using a Southern blot strategy found a maximum of 30–40% κ rearrangement per κ allele in cells induced to undergo rearrangement. This figure is considerably greater then the 18% figure this study obtained using a similar strategy. The discrepancy between our study and the previous report is likely due to differences between the two cell lines used.

Southern blot analysis of the temperature-shifted 103-Bclx cell line shows that a maximum of 18% cutting within the cluster of Jκ gene segments occurs at the 72-h time point, yet the LM-qPCR assay only shows a 12% cutting. This difference may be due to the fact that Southern blot assay detects dsDNA breaks at any of the five Jκ elements while the LM-qPCR assay detects breaks only at Jκ1. It is not likely that breaks involving any of the other Jκ elements (2–5) are detected by LM-PCR due to the greater distances from the forward and detection primers and resulting decrease in qPCR efficiency. Gel electrophoresis of qPCR products failed to demonstrate the existence of products that would be expected from cleavage of the Jκ2 RSS, thus confirming this assumption.

Signal end breaks at the Jκ1 gene segment RSS are most prevalent in the pre-B cell stage of development (Constantinescu and Schlissel, 1997). In a highly purified population of such cells, it was determined that at least 16% of the cells contained a break at the Jκ1 RSS. Previous reports have demonstrated that Jκ1 and Jκ2 signal end breaks can occur in purified pro-B cells populations as well, although at highly reduced levels compared to the pre-B cell populations (Constantinescu and Schlissel, 1997). Activity of the recombinase on the κ locus in pro-B cells has been confirmed by studies of mutant mice in which the pro-to-pre B cell transition is blocked. These mutants do contain rare V-to-Jκ rearranged alleles. This level of DNA breaks, however, is below the limit of detection of LM-qPCR.

The LM-qPCR assay described here would also be useful for the quantification of other types of sequence specific dsDNA breaks such as those generated by the P-element transposase or the HO endonuclease (Beall and Rio, 1997; Kostriken et al., 1983). For samples containing breaks at frequencies less than 1%, the hemi-nested LM-PCR assay as originally described (Schlissel et al., 1993) might serve to provide relative rather then absolute quantification. Modification of the linker would also permit one to quantify short staggered double-strand breaks presumably with an inherently greater ligation efficiency that might be afforded by a "sticky-ended" ligation reaction as opposed to the low efficiency found with the ligations. Attempts to quantify opened V(D)J recombinase-mediated hairpin loops were refractory to this type of analysis, likely due to the complexity of the ends (Schlissel, 1998) and their rarity.

References

Beall, E.L., Rio, D.C., 1997. *Drosophila* P-element transposase is a novel site-specific endonuclease. Genes Dev. 11, 2137.

Chen, Y.Y., Wang, L.C., Huang, M.S., Rosenberg, N., 1994. An active v-abl protein tyrosine kinase blocks immunoglobulin light-chain gene rearrangement. Genes Dev. 8, 688.

Constantinescu, A., Schlissel, M.S., 1997. Changes in locus-specific V(D)J recombinase activity induced by immunoglobulin gene products during B cell development. J. Exp. Med. 185, 609.

Gellert, M., 2002. V(D)J recombination: RAG proteins, repair factors, and regulation. Annu. Rev. Biochem. 71, 101.

Hsu, L.Y., Lauring, J., Liang, H.E., Greenbaum, S., Cado, D., Zhuang, Y., Schlissel, M.S., 2003. A conserved transcriptional enhancer regulates RAG gene expression in developing B cells. Immunity 19, 105.

Jung, D., Alt, F.W., 2004. Unraveling V(D)J recombination; insights into gene regulation. Cell 116, 299.

Kostriken, R., Strathern, J.N., Klar, A.J., Hicks, J.B., Heffron, F., 1983. A site-specific endonuclease essential for mating-type switching in *Saccharomyces cerevisiae*. Cell 35, 167.

Liang, H.E., Hsu, L.Y., Cado, D., Cowell, L.G., Kelsoe, G., Schlissel, M.S., 2002. The "dispensable" portion of RAG2 is necessary for efficient V-to-DJ rearrangement during B and T cell development. Immunity 17, 639.

Mueller, P.R., Wold, B., 1989. In vivo footprinting of a muscle specific enhancer by ligation mediated PCR. Science 246, 780.

Ponath, P.D., Boyd, R.T., Hillis, D.M., Gottlieb, P.D., 1989. Structural and evolutionary comparisons of four alleles of the mouse Igk-J locus which encodes immunoglobulin kappa light chain joining (J kappa) segments. Immunogenetics 29, 389.

Ramsden, D.A., Gellert, M., 1995. Formation and resolution of double-strand break intermediates in V(D)J rearrangement. Genes Dev. 9, 2409.

Roth, D.B., Zhu, C., Gellert, M., 1993. Characterization of broken DNA molecules associated with V(D)J recombination. Proc. Natl. Acad. Sci. U. S. A. 90, 10788.

Schlissel, M., Constantinescu, A., Morrow, T., Baxter, M., Peng, A., 1993. Double-strand signal sequence breaks in V(D)J recombination are blunt, 5′ -phosphorylated. RAG-dependent, and cell cycle regulated. Genes Dev. 7, 2520.

Schlissel, M.S., 1998. Structure of nonhairpin coding-end DNA breaks in cells undergoing V(D)J recombination. Mol. Cell. Biol. 18, 2029.

Shinkai, Y., Rathbun, G., Lam, K.P., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A.M., et al., 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell 68, 855.