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Matthew L. Brown and Yung Chang

V(D)J recombination cleavage generates two types of dsDNA breaks: blunt signal ends and covalently sealed hairpin coding ends. Although signal ends can be directly ligated to form signal joints, hairpin coding ends need to be opened and subsequently processed before being joined. However, the underlying mechanism of coding end resolution remains undefined. The current study attempts to delineate this process by analyzing various structures of coding ends made in situ from recombination-inducible pre-B cell lines of both normal and scid mice. These cell lines were derived by transformation of B cell precursors with the temperature-sensitive Abelson murine leukemia virus. Our kinetic analysis revealed that under conditions permissive to scid transfectants, hairpin coding ends could be nicked to generate 3' overhangs and then processed into blunt ends. The final joining of these blunt ends followed the same kinetics as signal joint formation. The course of this process is in sharp contrast to coding end resolution in scid heterozygous transformants that express the catalytic subunit of DNA-dependent protein kinase, in which hairpin end opening, processing, and joining proceeded very rapidly and appeared to be closely linked. Furthermore, we demonstrated that the opening of hairpin ends in scid cells could be manipulated by different culture conditions, which ultimately influenced not only the level and integrity of the newly formed coding joints, but also the extent of microhomology at the coding junctions. These results are discussed in the context of scid leaky recombination.

Uniquely, developing lymphocytes, the germline V, D, and J gene segments are somatically recombined to generate a diverse array of Ag receptors (1, 2). V(D)J recombination mechanistically proceeds through two steps, a site-specific cleavage to generate dsDNA breaks, which is then followed by general end joining of these breaks. The proteins encoded by recombination-activating genes (RAG1 and RAG2) recognize recombination signal sequences flanking each gene segment and cleave the DNA at the junction of recombination signal sequences and coding gene segments to produce two types of DNA ends: blunt signal ends and covalently sealed hairpin coding ends (3−5). Resolution of signal ends presumably occurs by direct ligation of blunt ends to form precise signal joints (6). In contrast, resolution of hairpin coding ends is quite complex, but is believed to proceed through nucleolytic processing, end modification, and ultimately religation of the broken coding ends (7−9). This multistep process produces coding joints with considerable junction variability, which significantly contributes to the diversity of the immune repertoire. Signal ends are readily detectable and are persistent in cells undergoing V(D)J recombination, while coding ends are only detectable on limited occasions (7−10). Thus, counterintuitively, the direct ligation of signal ends appears to be much slower than the multistep joining of coding ends.

Genetic analyses of various mutant cells and mice have revealed important roles for many proteins in the V(D)J recombination joining process, such as the DNA-end binding protein, Ku 70/80 heterodimer (11−13), the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) (14−16), XRCC4 (17−19), and ligase IV (20−23). Except for DNA-PKcs, all of these proteins are required for the formation of both signal joints and coding joints (11, 19, 21, 24). Cells with a mutation in the DNA-PKcs gene, incurred in scid mice, retain the ability to form signal joints, but are afflicted with a defect in the formation of coding joints (25, 26). As such, the role of DNA-PKcs may be related to the accelerated resolution of coding ends, even though it remains speculative as to how DNA-PKcs might function in this regard.

Several other proteins have also been implicated in coding end resolution. RAG1 and RAG2 proteins have been shown to possess end-processing activities, including binding of recombination intermediates (27, 28), nicking synthetic hairpin ends (29, 30), and rejoicing cleaved signal ends to coding ends (31). Recently, it has been demonstrated that the Mre11 protein, when complexed with Rad50 and the Nijmegen breakage syndrome gene product, Nbs1, exhibits several nuclease activities in vitro, such as hairpin nicking and processing of opened ends (32). Thus, it is conceivable that the Mre11/Rad50/Nbs1 complex may also participate in coding end resolution in vivo. However, it is not clear how these different protein complexes, DNA-PK, RAG1/2, and Mre11/Rad50/Nbs1, interact and execute their function during the processes of nicking, trimming, and joining.

It is assumed that opened coding ends are the intermediate products during the conversion of hairpin ends into coding joints. Although opened coding ends were detected in developing lymphocytes, direct evidence for their conversion from hairpin coding ends is nonetheless lacking (9). In an effort to elucidate coding end resolution, we have developed recombination-inducible cell lines from both scid homozygous (s/s) and scid heterozygous (s/+ ) mice by transforming B cell precursors with the temperature-sensitive Abelson murine leukemia virus (ts-Ab-MLV). As we reported previously, scid ts-Ab-MLV cell lines exhibit a temperature-dependent ability to resolve recombination coding ends (33). In our current study, we examined the intermediate structures of coding ends
in both scid and s/+ cell lines. We found that the scid cells are capable of converting the newly produced hairpin coding ends into opened ends that possess either a staggered 3’ overhang or a blunt end. The quality and quantity of the end processing in these scid cells can be manipulated by altering the culture conditions. However, this conditional resolution of coding ends is much slower than the coding end resolution in the control s/+ cells. Therefore, by comparing the kinetics of coding end resolution in scid and s/+ cells, we provide evidence for the role of DNA-PKcs in linking the two steps of the recombination process: cleavage and resolution.

Materials and Methods

Cell culture

As described previously, temperature-sensitive pre-B cell lines, A-1 and FL2-1, were derived from bcl-2 transgenic s/s and s/+ mice, respectively, by transformation of fetal B cell precursors with ts-Ab-MLV (33). These ts-Ab-MLV-transformed s/+ and s/+ cells are referred to as s/+ -ts and scid-ts cells, respectively. Cells were maintained at 33°C. To induce V(D)J recombination, cells were incubated at 39°C. To facilitate recombination resolution, the cells cultured at 39°C were returned to 33°C for various times.

DNA preparation

DNA was prepared in agarose plugs, as previously reported (33, 34). Briefly, cells resuspended in the agarose mixture were solidified to form agarose plugs (Bio-Rad, Richmond, CA), and then deproteinized by proteinase-K treatment in 200 μl of lysis buffer (100 mM Tris, pH 8, 25 mM EDTA, 1% Sarkosyl, 400 μg/ml proteinase K) at 50°C overnight. Multiple washes were done over a 24-h period with TE/PMSF (10 mM Tris, pH 8, 1 mM EDTA, 0.5 mM PMSF) and TE only.

RNA preparation and RT-PCR

RNA was prepared from various cell lines using CsCl ultracentrifugation method (35). RNA was reverse transcribed into cDNA with random oligonucleotides. The cDNA was serially diluted and amplified for RAG2 and β2-microglobulin (β2m) genes using the oligonucleotides described previously (35). Amplification of the β2m message served as an internal control for input cDNA. These PCR products were analyzed by Southern blot. A probe for RAG2 was prepared by PCR amplification of RAG2 constructs (kindly provided by M. Gellert, National Institutes of Health). The probe for β2m was a gel-purified PCR product made with primers specific for β2m cDNA.

Assessment of Jk coding end heterogeneity by modified LM-PCR

One-third of the agarose plug was incubated with either 5 U of T4 DNA polymerase (New England Biolabs) or 5 μg of mung bean nuclease (MBN; Boehringer Mannheim, Indianapolis, IN), followed by 5 U of T4 DNA polymerase. The enzymes were either heat inactivated at 75°C for 10 min (to inactivate T4 DNA polymerase) or by treatment with proteinase K (to inactivate MBN). The samples were then subjected to linker ligation with 1/2 coding ends. For better quantitation, only one round of PCR with 28 cycles was applied to amplify the ligated ends (35). For comparison, VJk coding joints and α-actin ends were also amplified by PCR (35). The length of amplified α-actin products served as a control for the amount of input DNA. PCR products were separated by electrophoresis and analyzed by Southern blotting.

PCR analysis of signal joints

The VAIJA1 signal joints were amplified by PCR using oligomers specific to 3’VY (YC24, 5’-CAATGATCTTGTTGGCC-3’) and to 5’JAI (YC23, 5’-GCTGCATACTACACAGAGT-3’). To determine the structure of signal joints as well as signal ends, their corresponding PCR products (one-third of the PCR reaction) were digested with ApoLI (New England Biolabs, Beverly, MA), and analyzed by Southern blotting along with the undigested PCR samples (one-sixth of the PCR reaction).

Probe

Blots were hybridized with 32P-labeled probes of: 1) pJX plasmid (36) to analyze the Jk-related PCR products (signal ends, coding ends, and coding joints); 2) VAI insert to detect VJA1 signal joints; and 3) pActin plasmid to reveal α-actin PCR products (36).

DNA sequence analysis of coding ends and coding joints

The LM-PCR products for Jk coding ends were further amplified by YC25 and YC32 (5’-GAGCATTGTCCTGACCCAG-3’) primers, and the VJk PCR products were amplified with MB46 and the Jc1 primer YC32 for sequence analysis. The amplified products were purified on a 2% Nu-Sieve agarose gel. The isolated PCR fragments were cloned into a TOPO-TA vector (Invitrogen, San Diego, CA) and sequenced on an automated DNA sequencer (ABI 377; Applied Biosystems, Foster City, CA). The sequence from each clone was compared with the germline Vκ and Jκ regions by BLAST similarity from the GenBank database.

Western blot analysis

Cells were swelled on ice in hypotonic buffer (10 mM Tris-HCl, pH 7.9, 10 mM KCl, 1 mM MgCl2, 1 mM DTT) including proteinase inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.5 mM PMSF), lysed by addition of Nonidet-P40 to 0.05%, and microcentrifuged to pellet the nuclei. The nuclear proteins were eluted from the pellet in extraction buffer (50 mM Tris-HCl, pH 7.9, 300 mM KCl, 12.5 mM MgCl2, 1 mM EDTA, 20% glycerol, 1 mM DTT, and proteinase inhibitors). Protein concentration of the extract was determined by Bradford assay (Bio-Rad), and 50 μg of each sample was subjected to 10% SDS-PAGE and transferred to NitroBind membrane (Fisher, Pittsburgh, PA). Membranes were incubated with Abs to RAG2 (PharMingen, San Diego, CA), Mre11 (Novus Biologicals, Littleton, CO), or actin (Santa Cruz Biotechnology, Santa Cruz, CA), followed by HRP-conjugated secondary Abs, and visualized by chemiluminescence detection (Pierce, Rockford, IL).

Results

Opening of hairpin coding ends in scid-ts cells: quantity and quality affected by in vitro culture conditions

Recently, by using recombination-inducible cell lines transformed with the ts-Ab-MLV mutant, we have shown that coding joint formation in scid cells could be manipulated by changing the culture temperature (33). In contrast to the s/+ -ts cells that can form coding joints immediately after recombination induction (at 39°C), scid-ts cells accumulate a substantial population of hairpin coding ends. The resolution of these hairpin coding ends is dependent on returning the cells to 33°C. To pinpoint the detailed steps in end resolution, we have analyzed different structures of coding ends under two culture conditions, specifically a 3-day culture at 39°C (3-0), and a 2-day culture at 39°C, followed by a 1-day culture at 33°C (2-1). The blunt opened ends can be directly amplified by ligation-mediated PCR (LM-PCR), while the staggered ends are revealed by treatment with T4 DNA polymerase, followed by LM-PCR. T4 DNA polymerase fluoresces staggered ends, as it has 3’ to 5’ exonuclease and 5’ to 3’ polymerase activities. The hairpin ends, after being opened by MBN and flushed by T4 DNA polymerase, can also be amplified by LM-PCR. A summary of our detection scheme is presented in Fig. 1A. To minimize any DNA damage caused by the routine extraction procedure, DNA samples were prepared in an agarose plug, as described by Schliessl (9).

Fig. 1B is a representative analysis of several LM-PCR experiments. It is clear that a large amount of blunt opened ends is present in scid-ts cells cultured at 39°C, followed by an incubation at 33°C (Fig. 1B, lane 6). As treatment with T4 DNA polymerase did not significantly increase the amount of LM-PCR products (Fig. 1B, lanes 4 and 6), staggered ends constitute a small fraction of the coding ends. Pretreatment of DNA with MBN and T4 DNA polymerase did not significantly increase the detection of the coding ends (Fig. 1B, lanes 2 and 6), indicating that only a few hairpin ends remained under this culture condition. Although it is possible that the level of hairpin ends is underestimated, the detection of a
were analyzed by LM-PCR at the Jk formation was assayed by PCR using a Vase (T4 Pol) were used to create ligatable blunt ends. Coding joint (CJ) artificially nicked hairpin ends (Fig. 1, B) blunt opened ends seems slightly smaller than the full length of B1) establishes the adequacy of our assay. Interestingly, the size of significant amount of hairpin ends in other samples (Fig. 1B, lane 1) demonstrates the adequacy of our assay. Interestingly, the size of blunt opened ends seems slightly smaller than the full length of artificially nicked hairpin ends (Fig. 1B, lanes 1 and 6), indicating a loss of several nucleotides at the ends during this conversion. Thus, a substantial number of hairpin coding ends can be opened and processed to blunt ends in scid-ts cells once they have been returned to 33°C. Since the appearance of blunt coding ends occurred concurrently with the production of coding joints, it is possible that these blunt ends are readily ligatable and are the immediate precursors of coding joints.

In contrast, very few blunt opened coding ends were detected in the cells cultured at 39°C for 3 days (Fig. 1B, lane 5). A strong LM-PCR band did appear in the sample pretreated with MBN and T4 DNA polymerase (Fig. 1B, lane 1). Thus, the vast majority of the coding ends remained in a hairpin structure. The amount of PCR products that represent opened ends increased when the DNA sample was pretreated with T4 DNA polymerase, indicating the presence of some staggered opened coding ends (Fig. 1B, lanes 1 and 3). The PCR products amplified from these staggered ends are much more heterogeneous than the LM-PCR band without pretreatment of T4 DNA polymerase (Fig. 1B, lanes 3 and 5). The faster moving bands suggest the presence of ends with extensive loss of nucleotides. Hence, even though hairpin nicking may occur

**FIGURE 1.** Coding end resolution in scid ts-Abh-MLV cell line. A, Schematic illustration of the modified LM-PCR for detecting hairpin, staggered, and blunt ends. The DNA fragments and ligation linkers are represented by the thick and thin lines, respectively. B, Detection of coding ends by modified LM-PCR. scid-ts cells were shifted to 39°C for either 3 days (3-0) or 2 days, followed by 1 day at 33°C (2-1). DNA was prepared in agarose plugs, as described in Materials and Methods. Coding ends (CE) were analyzed by LM-PCR at the Jx1 locus. MBN and T4 DNA polymerase (T4 Pol) were used to create ligatable blunt ends. Coding joint (CJ) formation was assayed by PCR using a Vx primer and a Jc2 primer. Amplification of the actin gene was used as a control for the amount of DNA.

at 39°C (3-0), this event is significantly less frequent and considerably more error prone than the hairpin opening in the shift-down culture (Fig. 1B, compare lanes 3 and 4).

To further understand the processing of coding ends at the nonpermissive temperature, we compared the structures of coding ends in cells cultured at 39°C for 2 days (2-0) vs 4 days (4-0). The sample from cells cultured at 39°C for 4 days followed by 1 day at 33°C (4-1) was included as a control, which again showed predominantly blunt opened ends (Fig. 2A, lane 8). The 2-0 sample contained mostly hairpin coding ends, and even though there was a small number of staggered ends, no blunt opened ends were present. The 4-0 sample, on the other hand, showed an accumulation of the staggered ends with a concurrent reduction in the amount of hairpin ends. A small number of blunt opened ends also emerged (Fig. 2A, lane 6), but at a level much lower than that in the 4-1 control (Fig. 2A, lane 8). Thus, at 39°C, both blunt and staggered ends are gradually increased over time (Fig. 2A, lanes 2 and 4). Strikingly, an increase in the level of staggered ends was accompanied by a decrease in the size of these ends over the course of 2, 3, and 4 days (Fig. 2B, lane 3, and Fig. 2A, lanes 2 and 5). These staggered ends are likely to bear a 3' overhang structure since treatment with Klenow exo−/− (Promega), which fills in a 5' overhang, did not increase the detection of the ends (Fig. 2B, lane 3). The size of the blunt ends remained relatively unchanged (Fig. 1B, lane 3, and Fig. 2, A, lane 6, and B, lane 1). This finding indicates that blunt ends are relatively stable, whereas staggered ends are susceptible to successive deletions during an extended culture at 39°C.

A summary of the relative distribution of the possible coding end species is presented in Fig. 3. After quantification of the coding end products (see Fig. 3 legend), it becomes clear that the
hairpin ends are processed differently under the two culture conditions. Only 1 day after returning the cells to 33°C from 39°C, virtually all hairpin ends were converted to blunt opened ends. Even 2 extra days at 39°C (4 days total) only caused a slight reduction in the number of hairpin ends. This limited hairpin opening also resulted in a preponderance of staggered ends over blunt ends.

To gain further insight into the coding end structures, the LM-PCR products were cloned for sequence analysis. As shown in Table I, none of the staggered ends contained palindromic (P) nucleotides that would accompany ends that possessed a 5' overhang. On the other hand, the hairpin end opening made in vitro by MBN

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>DNA Treatment</th>
<th>Deletion at Jκ1 Locus</th>
<th>No. of Isolates</th>
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<td></td>
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<tr>
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<td>T4</td>
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<tr>
<td>4–0</td>
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* See legends to Figs. 1 and 2 for culture conditions.

FIGURE 3. Distribution of the coding end structures is affected by culture temperatures. This graph is tabulated from Figs. 1 and 2A. Each column represents the percentage of each coding end structure in total coding ends under various culture conditions. Note that for samples 2-0 and 4-0, the three types of coding ends do not overlap in size. The amount of relative coding ends, including hairpin (H), staggered (S), and blunt (B), was quantitated using a phosphor imager and ImageQuant software, and adjusted against the actin control (i.e., relative hairpin end level (H) = cpm − CE/cpm − actin). The total level of coding ends (TCE) is the summation of the three types of coding ends (TCE = H + S + B). The relative percentage of each coding end structure was derived by dividing the relative coding ends by the TCE, so that the percentage of hairpin ends = H/TCE × 100%. For the sample 3-0, the TCE is the sum of H and S, and B is not included because S and B overlapped in size (Fig. 1, lanes 3 and 5). For the 2-1 sample (see Fig. 1), S is derived by subtracting the relative activity (cpm − CE/cpm − actin) of lane 4 with B, while H is calculated by subtracting the relative activity of lane 2 with S and B.

Different culture conditions affect the quality of coding joints

Because a longer incubation of scid cells at 39°C leads to an aberrant processing of coding ends, we reasoned that the fidelity of coding joints could be increased by shortening the culture length at this temperature. To that end, we set up two disparate culture conditions. The first condition was a 12-h incubation at 39°C, followed by a 1-day incubation at 33°C (0.5-1), while the second condition was a straight 4-day incubation at 39°C (4-0).

The amount of coding joints is expected to be different because the cells under these two culture conditions have different activities in recombination cleavage (due to different level of RAG expression; unpublished observation) and end joining (attributed to different activity in end opening; Figs. 1 and 3). Instead, our objective was to examine the quality of coding joints. The PCR products were cloned and sequenced, and 24 junctions from the 0.5-1 sample and 20 junctions from the 4-0 sample are presented graphically in Fig. 4. We mainly focused on the Jκ1 region for comparison, as the Vκ primer is only 100 bp upstream of the recombination signal sequence and thus might lead to an underestimation of any excessive Vκ deletions. Consistent with our previous analysis (33), nontemplated nucleotide (N) addition was not detected among all of the recovered joints. P addition with one nucleotide was identified in three clones. Forty-two percent of the coding joints in the 0.5-1 sample contained 0–10 nucleotide deletions. The two perfect joints are independent clones, as their Vκ genes belong to two different Vκ gene families (data not shown). Twelve junctions had deletions ranging from 12 to 56 bp, and only two had lost more than 100 bp. In sharp contrast, 19 of 20 junctions recovered from the 4-0 sample had lost more than 40 nucleotides. Among them, one-half contained deletions ranging from 131 to 228 bp. Thus, scid-ts cells cultured at 39°C for extended time gave rise to more deleted joining products than those cells cultured at 39°C for limited times. In light of the large deletions in the staggered ends from the 4-0 sample (Fig. 2A and Table I), the deletions found at the junction of their coding joints are contributed mainly by the abnormal end processing. The extensive loss of nucleotides at the ends could result from either inappropriate nicking distal from the hairpin termini and/or by successive deletions from the opened ends.

It is striking that 75% of the junctions recovered from the 0.5-1 sample contain 1- to 5-bp homology, whereas only 15% of the junctions from the 4-0 sample have this homology. This could be indicative of two different end joining mechanisms employed by the cells under different culture conditions. As such, the high level of microhomology in the 0.5-1 sample may reflect a preference for homology-directed joining, in which the alignment of two coding ends may be facilitated by the presence of homologous nucleotides. On the other hand, the end joining in the 4-0 sample seems to be relatively independent of microhomology. It is possible that the coding end conformation or the end/protein complex in the 4-0 sample may preclude homology-mediated alignment. Instead, these extensively deleted coding ends might be joined through a random ligation event.

Kinetic analysis of blunt end resolution in both scid-ts and s/+ts cells

We have shown in Figs. 1 and 2 that the majority of the coding ends present at 33°C are blunt opened ends. Is it possible that these blunt opened ends are subjected to further nucleotide deletions, as
FIGURE 4. Quality of coding joints is influenced by the length of incubation at the nonpermissive temperature. *scid-ts* cells were cultured for 12 h at 39°C, followed by 1 day at 33°C (0.5-1) or for 4 days at 39°C (4-0). Coding joints were amplified, purified, cloned, and sequenced, as described in Materials and Methods. Each box in the graphical display represents 20 bp, and the darker boxes represent the coding regions. The number of nucleotides lost during recombination is displayed on the side of each bar. One P nucleotide (C) addition was identified in three clones, designated as +1 (P). The nucleotides with microhomology at the ends of each junction are presented.

<table>
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<tr>
<th>Nucleotide</th>
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<td>0</td>
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seen with the staggered ends at 39°C? Additionally, can these ends be directly ligated similar to the formation of signal joints? To address these questions, we performed a kinetic analysis of end resolution for both signal ends and coding ends in s/+ts and *scid-ts* cells. We set up a 3-day culture at 39°C, followed by a series of incubations at 33°C: 1 day (3-1), 3 days (3-3), and 6 days (3-6).

In the s/+ts cells, coding ends were virtually undetectable by our LM-PCR assay (Fig. 5A, lane 1), and neither could hairpin or staggered coding ends be detected (unpublished observation). Yet, a high level of coding joints appeared upon recombination induction. We subjected the ligated DNA to one round of PCR with 28 cycles of amplification, in which coding ends could be readily detected in *scid-ts* cells, but not in s/+ts cells. The presence of signal ends in the same cell samples indicates that the paucity of coding ends detected is not due to technical limitations in our LM-PCR. Rather, our data demonstrate a rapid conversion of hairpin coding ends to coding joints in s/+ts cells.

In contrast, the blunt opened coding ends in *scid-ts* cells reached a maximum level 1 day after shifting the cells from 39°C to 33°C (similar finding to Fig. 1), indicating temperature-dependent end opening. During the course of their resolution, the size of the coding ends remained relatively intact (Fig. 5A, lanes 6 and 7). Thus, after returning to 33°C, the blunt opened ends are not susceptible to gross nucleotide deletions. Interestingly, even in the presence of an abundant amount of blunt coding ends, the accumulation of coding joints occurred rather slowly over the extended incubation at 33°C. Nonetheless, this was concurrent with the gradual disappearance of blunt coding ends and signal ends (Fig. 5A, lanes 5, 6, 7, and 8). Hence, even though *scid-ts* cells can eventually open all the hairpin ends at 33°C, the joining of these ends is still much slower than the coding joint formation from the uncleaved recombination locus in s/+ts cells (Fig. 5A, lanes 1 and 5–8). This finding suggests that the recombination events, site-specific cleavage, hairpin end nicking, and end joining, are closely linked in s/+ts cells, but dissociated in *scid-ts* cells defective in DNA-PKcs.

It has been known that the *scid* mutation also moderately affects the resolution of signal ends (25). To further test this, we compared the newly generated signal ends and signal joints in s/+ts and *scid-ts* cells. The integrity of signal ends and signal joints can be assessed by *Apa*LI restriction digestion of amplified PCR products, as an *Apa*LI restriction site is created in the perfect signal joints as well as in the intact signal ends that are ligated to the artificial primer. It is clear from Fig. 5B that signal ends made in both s/+ts and *scid-ts* cells are intact without nucleotide modifications, as their LM-PCR products are sensitive to *Apa*LI digestion. Likewise, the integrity of these ends is not altered in the VA1 and JA1 signal ends (unpublished observation).

Due to the simplicity of the *A*-locus, we examined the formation of VA1JA1 signal joints. Although some signal joints were found in the s/+ts cells cultured at 39°C, more signal joints appeared in the cells returning to 33°C (Fig. 5B, lanes 1 and 3). The temperature-dependent resolution of signal ends is even more apparent in *scid-ts* cells (Fig. 5B, lanes 5 and 7). Based on the sensitivity to *Apa*LI digestion, it is clear that *scid* signal joints contain nucleotide modifications (Fig. 5B, lanes 6 and 8), whereas signal joints made in s/+ts cells have perfect junctions (Fig. 5B, lanes 2 and 4). These results are consistent with the previous report that the *scid* mutation alters the efficiency and accuracy of signal end resolution.
Despite the modified signal joints, the structure of the signal ends remains intact (Fig. 5B and unpublished observation). Therefore, the scid mutation seems to interfere with the signal end resolution at the step of alignment and ligation.

**Expression of RAG2 and Mre11**

The biochemical nature of the conditional resolution of coding ends remains to be defined. It has been shown that RAG1/2 proteins can nick artificial hairpin ends in a cell-free system (29, 30). To determine whether these proteins are responsible for processing hairpin ends in vivo, we analyzed RAG2 expression in various cell samples. Similar to the previous report (38), a high level of RAG2 RNA and protein is induced during the culture at 39°C (Fig. 6, A and B). Upon returning the cells to 33°C, both RAG2 RNA and protein levels were substantially reduced (Fig. 6, A and B), during which time coding ends were found to be processed and joined (Fig. 1). Thus, coding end resolution occurs in the cells that express little RAG2.

Mre11, when complexed with Rad50 and Nbs1, was found to possess nuclease activity, nicking fully paired artificial hairpin ends in a cell-free system (32). To determine whether temperature-dependent end resolution could be attributed to the differential expression of Mre11, we examined the amount of Mre11 protein expressed in the cells under our defined culture conditions by Western blot. As shown in Fig. 7, Mre11 protein levels appear comparable in s/1- ts and scid- ts cells, and, importantly, it does not fluctuate during temperature changes. Because the Mre11 nuclease is constitutively available to both s/1- ts cells and scid- ts for possible nicking and trimming, the temperature-dependent resolution of scid coding ends may instead reflect different accessibility of the ends to this enzyme.

**Discussion**

The resolution of hairpin coding ends must proceed through a multitude of steps, which include opening, processing, aligning, and joining. The kinetic comparison on the resolution of newly generated hairpin ends in s/1- ts and scid- ts cells reveals two different pathways for resolving coding ends: DNA-PKcs dependent and DNA-PKcs independent. Upon recombination induction, DNA-PKcs-proficient cells produce a great abundance of coding joints, but very few detectable coding ends (Fig. 5A). This suggests that recombination cleavage is coupled to end processing and joining. This coupled event was also demonstrated in a cell-free assay, in which coding joint formation was much more efficient when intact recombination substrates rather than precleaved coding ends were used.

**Figure 5.** A, Delayed resolution of blunt coding ends in scid-ts cells. scid-ts and s/+ -ts cells were cultured for 3 days (3-0) at 39°C, then shifted back to 33°C for either 1 (3-1), 3 (3-3), or 6 (3-6) days. Both coding ends and signal ends were examined at the Jk1 locus by LM-PCR. Coding joints were examined using a Vκ and a Jκ2 primer. Actin was included as an internal control for DNA in each reaction. B, Signal end resolution. Jκ signal ends and VαιJα1 signal joints were amplified by LM-PCR. Genomic DNA was prepared from both s/+ -ts and scid-ts cells that had been cultured at 39°C for 3 days or 3 days at 39°C, followed by 1 day at 33°C. The PCR products treated with or without ApaLI were analyzed by Southern blot. The slight down-shift of PCR products indicates a digestion by ApaLI, i.e., the presence of the perfect signal joints or intact signal ends.

**Figure 6.** Temperature-dependent regulation of RAG expression. A, RNA was isolated from cells cultured at 33°C (33), 39°C for 2 days (2–0), or 39°C for 2 days, followed by 1 day at 33°C (2–1), and analyzed by RT-PCR for RAG2 and β2m levels. B, Nuclear lysates prepared from the above cell samples were examined for RAG2 protein by Western blot. The same blot was stripped and analyzed for actin.

**Figure 7.** Mre11 expression is not altered by temperature changes. Nuclear lysates were analyzed for Mre11 by Western blot with a polyclonal Ab specific to Mre11. The same blot was stripped and analyzed for actin.
provided (39). In contrast, the resolution of coding ends in DNA-PKcs deficient cells is a very protracted process, as several structures of coding ends could be identified: hairpin ends, staggered opened ends, and blunt opened ends (Fig. 3). Furthermore, the direct ligation of blunt coding ends was much slower than the formation of coding joints from the uncut κ-locus in s/y-ts cells (Fig. 5). Thus, DNA-PKcs may be responsible for coupling those processing events to facilitate coding joint formation. Without functional DNA-PKcs, these events proceed separately and slowly.

The coding end resolution mediated by the DNA-PK-independent pathway can be manipulated in terms of its efficiency and fidelity by altering the culture conditions, as shown in Fig. 1 and Table I. This finding points to two different schemes utilized by scid-ts cells to process coding ends. One, occurring at 33°C, can convert essentially all hairpin ends into blunt opened ends that possess limited nucleotide deletions. Otherwise, at 39°C, only a small fraction of hairpin ends are converted into staggered ends, which are characterized by gross deletions. Our sequence analysis indicates that the staggered ends bear a 3' overhang. These 3' overhang ends could then be further processed into blunt ends by resecting the ssDNA tails. A slight increase in LM-PCR products following the pretreatment of DNA with T4 DNA polymerase implies that the ends with 3' overhangs precede the blunt ends (Fig. 1, lanes 5 and 6). Schlissel (9) has reported that in normal recombination-active cells, the recovered coding ends bear a 3' overhang structure. Thus, the polarity of hairpin end nicking is not altered by the scid mutation. The predominance of blunt opened ends in scid-ts transformants at 33°C may reflect a higher nuclease activity in removing 3' overhangs or, more likely, an increased accessibility of the ends to nucleases at this condition. Experiments are underway to examine these possibilities.

The recent finding of DJ coding joints in DNA-PKcs−/− mutant mice provides direct evidence for the role of a DNA-PKcs-independent pathway in resolving coding ends (16, 37, 40). It is plausible that this mechanism accounts for the rare production of functional recombination joints that promote the development of oligoclonal B and T cells, known as leaky scid cells. In our scid ts-Ab/MLV model, the cells cultured at 39°C possibly resemble the majority of developing scid lymphocytes in vivo, as both scid-ts cells and scid thymocytes contain unresolved hairpin coding ends. On the other hand, the scid-ts cells after returning from 39°C to 33°C may mimic leaky scid lymphocytes, capable of resolving coding ends. Our data further indicate that the scid leaky recombination can be manipulated by external factors.

The unusually high level of coding joints found in our ts-Ab/MLV-transformed scid cells might be attributable to the presence of the bcl-2 transgene and/or the v-abl oncogene in our cell lines. The expression of a bcl-2 transgene renders these recombination-active cells resistant to apoptosis. Owing to their sustained life span, the cells are given the opportunity to eventually resolve their ends and make coding joints. This scenario was observed in bcl-2 transgenic scid mice (41). It is not quite certain how the temperature-dependent reactivation of v-abl might affect scid coding joint formation. It has been speculated that indirect events induced by v-abl reactivation such as cell cycle progression and/or RAG1/2 down-regulation may create conditions that favor the recombination-joining activity (37, 42, 43).

Susceptibility of scid leaky recombination to manipulation is also witnessed in scid thymocytes exposed to ionizing radiation, as these cells increase both the level and the fidelity of their rearranged TCR coding joints (44). Analogous to the temperature-dependent resolution of coding ends in our scid-ts transformants, ionizing radiation may somehow facilitate hairpin end opening and processing in scid thymocytes. This conversion would still be scid-like in that it involves a DNA-PKcs-independent opening, which is slow and uncoupled. This uncoupled recombination process would predict that the unresolved coding ends should be available for nonspecific end association, such as forming hybrid joints and interlocus recombination products. Indeed, we have recently shown that scid-ts cells have a higher frequency of hybrid joints and VκJ λ interlocus joints than their normal counterpart. Thus, the price for a higher level of leaky scid recombination would be an increased risk for chromosomal instability. This correlation was also demonstrated in those scid mice that were exposed to ionizing radiation: a high level of recombination coding joints was accompanied by an elevated level of interlocus recombination products and an accelerated development of thymoma (45). The ability to manipulate our cell lines for coding end resolution will help us to identify the factors responsible for inducing or promoting the development of the scid leaky phenotype, as well as those factors responsible for triggering chromosomal instability.

It has been shown that the recombination intermediates are held in a postcleavage complex that contains RAG1 and RAG2 proteins (27, 28, 46). The purified RAG1/2 proteins were found to mediate the reverse reaction of the cleavage to generate hybrid joints and open-shut products (31). Recently, two laboratories have shown that RAG1/2 proteins can nick hairpin ends (29, 30). Interestingly, however, the resulting opened ends contained 5' overhangs, which differ from the opened coding ends made in vivo (Table I; 9). In our studies, the resolution of hairpin coding ends occurs when the RAG2 protein level is reduced. This finding suggests that hairpin end processing in scid-ts cells is not likely to be mediated by the RAG1/2 proteins. Instead, the reduction of RAG1/2 expression at 33°C (Fig. 6) may help to disassemble the postcleavage complex and to facilitate formation of both coding and signal joints (Fig. 5, A and B). Thus, the most logical explanation for the temperature-dependent resolution of coding ends and signal ends in scid-ts cells is that both types of ends become accessible at 33°C to DNA processing and/or joining machinery, as signal ends do not require end processing.

It has also been demonstrated that Mre11 acts as a 3'-5' dsDNA exonuclease as well as an endonuclease (47). After being complexed with Rad50 and Nbs1, Mre11 can nick fully paired hairpin ends to produce 3' overhang ends in vitro (32). This complex can also cleave 3' overhangs at a double-/single-strand transition, which would generate blunt ends (48). The dual activities of this complex perfectly fit the characteristics of the opened coding ends detected in our scid-ts transformants, in which both blunt opened ends and staggered ends with 3' overhangs are present (Table I). The accumulation of unresolved hairpin ends in scid-ts cells at 39°C may reflect an inaccessibility of these ends to Mre11, presumably due to their binding by the RAG1/2 complex. Then, it should not be unexpected that the level of Mre11 protein does not fluctuate during temperature changes (Fig. 7). Whether or not the Mre11 complex is the nicking enzyme in the DNA-PK-independent resolution of coding ends is currently under investigation. Furthermore, Mre11 protein has been shown to facilitate joining of mismatched ends presumably through its endo- and exonuclease activity to reveal the homologous nucleotides for end aligning and joining (48). In this context, different accessibilities of the ends to Mre11 can also explain the different levels of microhomology observed at the coding junctions made under the two culture conditions (Fig. 3). Collectively, our results support the assumption that

*S. Lew, D. Franco, and Y. Chang. Activation of V(D)J recombination induces the formation of interlocus joints and hybrid joints in scid pre-B cell lines. Submitted for publication.
the Mre11/Rad50/Nbs1 complex is involved in processing recombination intermediates in vivo.

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