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Immunology

Formation of coding joints in V(D)J recombination-inducible severe combined immune deficient pre-B cell lines

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ABSTRACT **Characterization of the severe combined immune deficient (scid) defect in the recombination process has provided many insights into the underlying mechanisms of variable (diversity) joining recombination. By using recombination-inducible scid pre-B cell lines transformed with the temperature-sensitive Abelson-murine leukemia virus, we show that large quantities of recombination intermediates can be generated, and their resolution can be followed during further cell culture. In this study, we demonstrate that the ability of these scid pre-B cell lines to resolve coding ends depends on the cell culture temperature. At the nonpermissive temperature of 39°C, scid pre-B cell lines fail to form coding joints and contain mostly unresolved hairpin-coding ends. Once the cell culture is returned to the permissive temperature of 33°C, these same cells make a significant amount of coding joints concomitant with the disappearance of hairpin-coding ends. Thus, the scid cells are capable of resolving coding ends under certain culture conditions. However, the majority of the recovered coding joints contains extensive deletions, indicating that the temperature-dependent resolution of coding ends is still scid-like. Our results suggest that the inability of scid cells to promptly nick hairpin-coding ends may lead to aberrant joining in these cells.**

Variable (diversity) joining [V(D)J] recombination is a site-specific process unique to developing T and B lymphocytes that involves site-specific cleavage and imprecise end joining (1, 2). Over the last 3 years, much progress has been made in understanding the molecular mechanisms underlying the recombination cleavage. The purified proteins encoded by the recombination activation genes (RAG1 and RAG2) have been demonstrated to be directly responsible for the cleavage step, which gives rise to two types of recombination intermediates: signal ends and hairpin-coding ends (3–5).

Resolution of the two types of ends proceeds differently. Signal joints can be formed from direct ligation of two blunt signal ends, whereas several steps are believed to be involved in resolving hairpin-coding ends, including nicking, modifying, and joining (6, 7). Interestingly, in normal recombinase-active lymphocytes, the signal ends seem to have a much longer half-life than the coding ends, as the former can be readily detected whereas the latter are rarely found (8–10). Thus, although it involves multiple steps, the joining of coding ends appears either much quicker or more efficient than the joining of signal ends in normal cells. This conclusion is further confirmed by results obtained by using a cell-free assay initially developed by Ramsden *et al.* (11) and also reported by other groups (12, 13).

In cells derived from severe combined immune deficient (scid) mice, the joining of coding ends is defective. Whereas scid cells can form signal joints at a frequency comparable to normal cells, their ability to form coding joints is at least 1,000-fold less efficient (14). Furthermore, covalently sealed coding ends can be detected in scid thymocytes but not in those from normal animals, leading to a hypothesis that the scid defect somehow interferes with the hairpin-opening reaction (15). This defect has been attributed to a mutation at the gene coding for the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) (16–21). DNA-PKcs is also believed to be involved in double-strand DNA break repair. Thus, DNA-PKcs may participate in both the hairpin-opening reaction and double-strand break repair. However, much remains to be learned concerning the mechanism by which DNA-PKcs protein carries out these processes. Analysis of the recombination events in scid cells should help to address this question.

The recombination-inducible pre-B cell lines transformed by temperature-sensitive Abelson-murine leukemia virus (*ts*-Ab-MLV) has provided us with a great model to study the ongoing process of V(D)J recombination (22). Using this model, Ramsden and Gellert have shown that the formation of signal joints depends on returning the cell culture to the permissive temperature, whereas the formation of coding joints is independent of culture temperature (9). Thus, this cell line can be a good model to delineate the joining reaction for signal ends. However, its rapid resolution of coding ends presents difficulties when studying the joining process for coding ends.

In an attempt to understand the scid defect in coding joint formation, we developed recombination-inducible, temperature-sensitive *(ts)*-Ab-MLV pre-B cell lines from bcl-2 transgenic scid mice, as the presence of the bcl-2 transgene prevents apoptotic cell death induced at the nonpermissive temperature (23). In this study, we show that large quantities of both signal ends and coding ends can be generated in these cell lines, and that their resolution can be followed during the cell culture. Interestingly, we found that coding joint formation in the scid *ts*-Ab-MLV pre-B cells is temperature dependent. Coding joints were almost undetectable at the nonpermissive temperature; they appeared only on shifting the cell culture to the permissive temperature. Our analyses of the structure of the newly generated scid coding ends suggest that the inability of scid *ts*-Ab-MLV pre-B cells to make coding joints at the nonpermissive temperature can be attributed to their failure to open hairpin-coding ends. In addition, the coding joints made from these scid cells contain extensive deletions, which may result from the inappropriate nicking of hairpin-coding ends.

MATERIALS AND METHODS

Mice. The bcl-2 transgenic scid mice (C.B-17-scid-bcl2–36) were generously made available to us by S. Cory (24) of the

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Abbreviations: *ts*-Ab-MLV, temperature-sensitive Abelson-murine leukemia virus; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, catalytic subunit of DNA-PK; LM-PCR, ligation-mediated PCR; RAG, recombination activation gene; scid, severe combined immune deficiency; V(D)J, variable (diversity) joining; 2-D, two-dimensional; *ts*, temperature sensitive.

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Walter and Eliza Hall Institute of Medical Research, Australia, by way of M. Bosma of the Fox Chase Cancer Center, Philadelphia, PA. Transgenic scid homozygous (s/s) and scid heterozygous (s/+) mice were bred and maintained in a pathogen-free environment at the Animal Research Center facilities at Arizona State University (Tempe, AZ), according to the established protocol (25). Peripheral blood cells were collected from individual mice for DNA-preparation by using the Easy DNA kit (Invitrogen). The purified DNA was screened for the presence of bcl-2 transgene by PCR amplification of transgene vector pEuSV, by using specific primers (YC-7, 5′-GGAACTGATGAATGGGAGCAGTGG-3′ and YC-8, 5′-GCATACACTCTATGCCTGTG-3′) corresponding to the SV40 splice sequence in the vector (24). To obtain fetal liver cells from both bcl-2 transgenic scid heterozygous (s/+-bcl-2) and bcl-2 transgenic scid homozygous (s/s-bcl-2) embryos, both CB-17 wild type (+/+) females and CB17-scid females were mated with bcl-2-transgenic scid males. On their 19th day of pregnancy, the females were sacrificed and fetal livers were dissected from individual embryos. The cell suspensions were made from individual fetal livers, and some were screened for the bcl-2 transgene by PCR assay.

***ts*-Ab-MLV-Transformed Cell Lines.** The fetal liver cells from each embryo, including bcl-2-negative and bcl-2-positive pups, were transformed individually with a *ts*-Ab-MLV (kindly supplied by N. Rosenberg), as described previously (23). The transformed cell lines were maintained at 33°C. The presence of the bcl-2 transgene, as well as the Ab-MLV genome, was further confirmed by PCR amplification with primers YC-7/8 for bcl-2 and primers YC-13/14 for v-*abl* (YC-13, 5′-CTGACCTTTACATCACCC-3′, YC-14, 5′-CTTCTCCCAAGTACTCCAT-3′). Different *ts*-Ab-MLV-transformed cell lines were derived from several transformations of individual fetal livers, including the s/+-A-1 derived from bcl-2 transgenic s/+ fetal liver, and the s/s-2–1cell line derived from bcl-2 transgenic scid fetal liver. This study focused on these two cell lines.

DNA Preparation. Genomic DNA was prepared from treated cells by using the Easy DNA kit (Invitrogen) and dissolved in water at a concentration corresponding to 10^5 cell genome equivalents per microliter. Alternatively, DNA was prepared in an agarose plug according to the procedure developed by Nakajima and Bosma (26) with some modifications. Briefly, cells were resuspended at a concentration of 8×10^7/ml in PBS. Forty microliters of cell suspension (3.2×10^6) were mixed with an equal volume of warmed 1.2% agarose, and 80 μl of agarose mixture was dispensed into plug molds (Bio-Rad), then solidified on ice. The plugs were transferred to Eppendorf tubes and deproteinized by proteinase-K treatment in 100 μl of lysis buffer (100 mM Tris, pH8.0/25 mM EDTA/1% Sarkosyl/400 μg/ml Proteinase K) at 55°C overnight. Several washes were carried out over 24 hr with 10 mM Tris, pH 8/1 mM EDTA solution containing 0.5 mM phenylmethylsulfonyl fluoride to inactivate proteinase K.

Ligation-Mediated PCR (LM-PCR)/Genomic PCR. LM-PCR was used to assay DNA samples for double-strand breaks resulting from the initiation of κ-chain gene rearrangement. As reported before (8), the artificial linker made by annealing the two primers MB216 (5′-CACGAATTCCC-3′) and MB217 (5′-GCTATGTACTACCCGGGAATTCGTG-3′) can be directly ligated to the broken signal ends. The DNA ligation proceeded with T4-DNA ligase according to the manufacturer's protocol (Boehringer Mannheim) at 15°C overnight. After ligation, the DNA was denatured at 95°C for 5 min, and one-fifth of the ligation mixture was tested for signal ends by PCR reaction with primers MB217 and MB224 (5′-AGTGCCACTAACTGCTGAGCCACCT-3′). The PCR reaction for actin (with primers MB76, 5′-ATTAACCCTCACTAAAGGTGTCATGGTAGGTATGGGT-3′, MB77, 5′-CGCACAATCTCACGTTCAG-3′) was also included as a control for DNA input. To examine the rearranged VJκ joints, PCR reaction was carried out on purified DNA with the Vκ degenerate primer (27) and Jκ2 primer (MB47:5′-CCAAGCTTTCCAGCTTGGTCCCCCCTCCGAA-3′).

Two-dimensional (2-D) Gel Electrophoresis. One piece of DNA-agarose plugs (equivalent to 6×10^6 genome content) was digested with the restriction enzyme *Xba*I at 37°C overnight. The digestion buffer was removed the next day. The 2-D gel electrophoresis was carried out according to the protocol developed by Roth *et al.* (15). Briefly, the DNA samples were first separated under the native electrophoresis [1× TBE (90 mM Tris/90 mM boric acid/1 mM EDTA, pH 8.3)] for 6 hr at 80 V. The gel was equilibrated in alkaline solution (50 mM NaOH/0.5 mM EDTA) for 20 min, turned 90° clockwise, and run at 25 V in the same alkaline solution for 14 hr with a glass plate placed on the top of the gel. The separated DNA was transferred to a membrane and analyzed by the Jκ probe.

Probes. The Jκ-probe insert isolated from a pJκ was used to analyze both signal ends and coding joints (28). For detecting coding ends separated by 2-D gel electrophoresis, a PCR product of the Jκ region (from Jκ1 to Jκ4 amplified from Jκ plasmid with primer YC-33, 5′-GTGGACGTTCGGTGGAGGCACC-3′, YC-20, 5′-CGTCAACTGATAATGAGCCCTCT-3′) was used as a probe in Southern blot analysis. The pActin probe was used to detect actin PCR products (28).

Junction Analysis by DNA Sequencing. The PCR coding joints were cloned by the TA cloning kit (Invitrogen) and sequenced by an automated DNA sequencer [ABI 377, Applied Biosystems]. The sequence of each clone was compared with the germline Vκ and Jκ regions from the GenBank database by BLAST similarity. The lack of a corresponding region was characterized as a deletion.

RESULTS

Induction of Vκ-to-Jκ Rearrangement in s/+-*ts* Cells and Scid-*ts*. To study the scid defect in coding joint formation, we developed recombination-inducible cell lines that could be manipulated *in vitro* to give rise to large quantities of recombination intermediates. We obtained such cell lines from both bcl-2 transgenic scid heterozygous (s/+) and bcl-2 transgenic scid homozygous (s/s) mice by transforming B cell precursors with *ts*-Abl-MLV. To simplify the terminology, we refer to these *ts*-Ab-MLVs/+ cell lines and *ts*-Ab-MLVscid cell lines as s/+-*ts* cells and scid-*ts* cells, respectively.

Similar to the findings reported by Chen *et al.* (22), both our s/+-*ts* and scid-*ts* cells showed an up-regulation of RAG1/2 RNA levels over the control cells when cultured at the nonpermissive temperature (unpublished observation). To assess directly recombination initiated at the Ig κ-chain locus, we examined the production of Jκ-signal ends. Concurrent with the up-regulation of RAG expression, Jκ-signal ends appeared in the 39°C cultured cells, but not in the cells cultured at 33°C (shown in Fig. 1). The majority of these ends are made at the junction of signal and coding sequence without significant nucleotide modification (data not shown), as determined by digestion with restriction enzyme *BsiHKA I* (an isoschizomer of *HgiA I,* New England Biolabs) because the primer ligated to perfect signal ends became a *BsiHKA I* restriction site. The signal ends detected by our PCR analysis are made from the primary cleavage, as the Jκ1 primer used in LM-PCR is complementary to the 5′Jκ1 region that is usually removed during the primary rearrangement, and thus this primer would not be able to amplify a secondary V-to-Jκ rearrangement. The majority of signal ends detected in this analysis were made at the Jκ1 and Jκ2 loci.

Delayed-Coding Joint Formation in Scid *ts*-Ab-MLV-Transformed Cell Lines. We next examined coding joint formation in scid-*ts* and s/+-*ts* cells after induction of V(D)J recombination. The rearranged coding joints were amplified

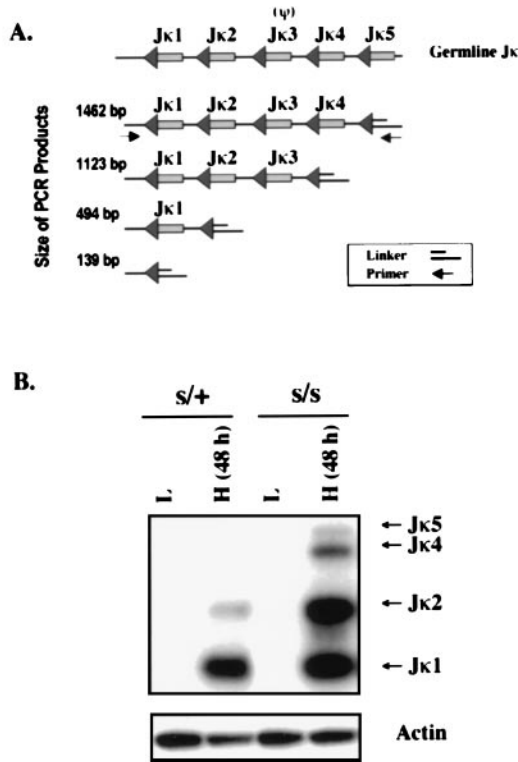

FIG. 1. Initiation of V(D)J recombination as revealed by the presence of signal ends at the nonpermissive temperature. (*A*) Diagrammatic representation of the Jκ-gene loci, along with the predicted sizes of signal ends detected by LM-PCR. Arrows indicate the primers used to amplify the excised signal ends. (*B*) LM-PCR analysis of signal ends. DNA samples isolated from the scid heterozygous (s/+-*ts*) and scid homozygous (s/s-*ts*) cells, cultured either at 33°C (L) or at 39°C (H) were subjected to an LM-PCR assay to assess the level of recombination signal ends from the Jκ gene loci. Amplification of the actin gene is used as a control for the amount of input DNA.

by PCR assay with Vκ and Jκ2 primers (see *Materials and Methods*) and were revealed by Southern blot analysis. For better quantitation of primary rearrangements, only the data on VJκ1-joints are presented. As shown in Fig. 2, a substantial amount of VJκ1 coding joints was detected in the s/+-*ts* cells that were cultured at 39°C for 48 hr. These coding joints appeared as early as 12 hr after incubation at 39°C (data not shown). Extended incubation at 39°C or after return to 33°C did not further increase the amount of VJκ1 coding joints (Fig. 2). Therefore, coding joint formation in s/+-*ts* cells is a very rapid process and does not depend on reincubation at 33°C. In contrast, very low levels of coding joints were found in the scid cells even after 48 hr of incubation at 39°C (Fig. 2), presumably because of the defective DNA-PKcs. Interestingly, however, coding joints did appear in scid-*ts* cells on returning the cells to the permissive temperature for various times (Fig. 2). The increased level of VJκ joints was probably not caused by an expansion at 33°C of the few cells that had succeeded in completing their rearrangements, as the number of cells was comparable to that after 48 hr at 39°C.

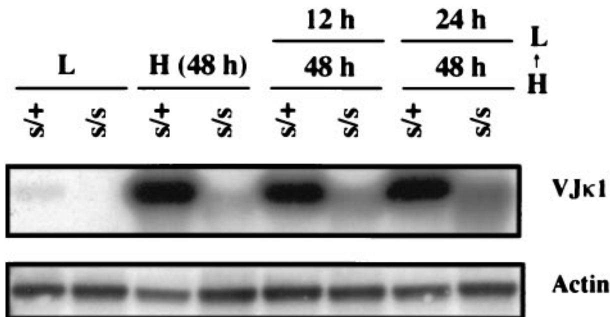

FIG. 2. Analysis of coding joint formation in s/+-*ts* and s/s-*ts* cells. After the s/+-*ts* and s/s-*ts* cells were cultured under their respective conditions [L: 33°C; H: 39°C for 48 hr, or H → L: 39°C (48 hr) followed by 33°C (12 or 24 hr)], DNA samples were isolated and subjected to a PCR assay by using a Vκ primer and a Jκ2 primer, along with actin controls.

The coding joints found in the scid-*ts* cells could result from inefficient scid coding end joining activity and could accumulate only over time. Were this so, an extended period of culture at 39°C (e.g., 3 or 4 days) should increase the amount of rearranged products. Alternatively, scid coding joint formation is temperature dependent, resembling signal joint formation in the *ts*-Ab-MLV transformed pre-B cell line from wild-type mice (9). In these cells, signal joints were found only after the cells incubated at the nonpermissive temperature were returned to the permissive temperature (9). To directly test these two possibilities, we cultured both scid and s/+-*ts* cells at 39°C for 3 and 4 days, or for 2 days at 39°C followed by 1 and 2 days at 33°C. The results are shown in Fig. 3. Again, in s/+-*ts* cells, the amount of VJκ1 coding joints is comparable among the different culture conditions. Similar to the results shown in Fig. 2, very few coding joints (about 5% of the coding joints in s/+-*ts* cells; Fig. 3) were formed in scid-*ts* cells at 39°C even after 3 to 4 days of culture. On the other hand, after the cells were shifted back to 33°C, the amount of VJκ coding joints in scid-*ts* cells increased to 40% of the amount found in the s/+-*ts* cells (Fig. 3). This study clearly demonstrates that an incubation at 33°C after culture at 39°C is essential for scid-*ts* cells to form their coding joints. However, the amount of coding joints in these cells was found to be variable among different experiments, which may reflect variation in the number of cells undergoing rearrangement as well as the quality of these rearrangements, i.e., intact or deleted coding joints.

The amplified coding joint products made in scid-*ts* cells are somewhat smaller than those in the corresponding s/+-*ts* cells (Fig. 3). To examine the sequences of the coding joints made in scid-*ts* cells, the rearranged coding joints were cloned into plasmids for sequence analysis. Table 1 shows the sequences of individual junctions recovered from both s/+-*ts* cells and scid-*ts* cells. As expected, the coding joints made in s/+-*ts* cells

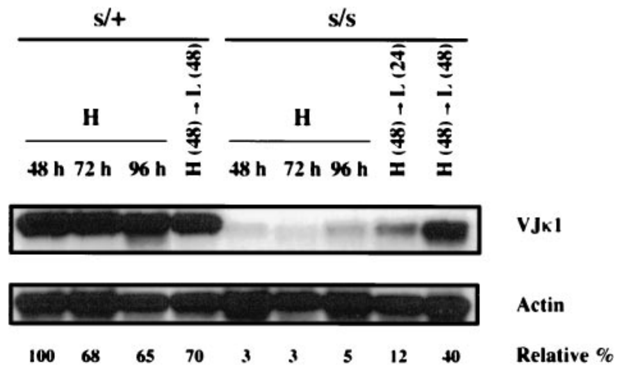

FIG. 3. Temperature-dependent coding joint formation in scid-*ts* cells. DNA samples were prepared from s/+-*ts* and s/s-*ts* cells under various culture conditions. The cells were either cultured at high temperature (H) for 48, 72, or 96 hr, or cultured at high temperature (H: 39°C) for 48 hr followed by an incubation at low temperature (L: 33°C) for 24 or 48 hr. The VJκ coding joints were amplified with a Vκ primer and a Jκ2 primer (see Fig. 2). The amount of VJκ1 PCR products relative to that of actin was analyzed with a PhosphoImager by using IMAGEQUANT software (Molecular Dynamics). The values shown correspond to the relative ratio of the coding joints normalized against the reference control of VJκ1 made in the s/+-*ts* cells at 39°C for 48 hr.

contain essentially intact Vκ and Jκ gene segments with small nucleotide deletion. All of the junctions, except one, are free of nucleotide addition. This lack of nucleotide addition is attributed to a low level of terminal deoxynucleotide transferase in fetal-derived pre-B cell lines (29) as well as a down-regulation of deoxynucleotide transferase during light chain gene rearrangement (30). Among 10 individual coding junctions made by scid-*ts* cells, all but one show deletions ranging from 22 to 158 bps at the Jκ locus and 17 to 53 bps at the Vκ locus (Table 1). This finding indicates that the joining process is still defective in scid-*ts* cells, although their joining activity can be increased on returning the cell culture from the nonpermissive to the permissive temperature.

Unresolved Coding Ends in Scid-*ts* Cells. The inability of scid-*ts* cells to form coding joints at the nonpermissive temperature could result from their dysfunction at any of these stages: hairpin nicking, end filling, or joining. To examine directly the existence of hairpin-structured coding ends, we carried out a 2-D electrophoresis followed by Southern blot analysis (15). The 3.5-kb band was derived from restriction digestion (*Xba-I*) of the germline κ-locus (Fig. 4*A*). Cleavage initiated at the Jκ1 locus should give rise to 1.65-kb Jκ1 and 1.3-kb Jκ2 coding ends (Fig. 4*A*). On the 2-D gel electrophoresis, the open coding ends are expected to run along the diagonal, whereas the hairpin coding ends run off the diagonal.

The DNA sample from s/+-*ts* cells cultured at 39°C contains a 3.5-kb genomic DNA but none of the coding ends, since there is no obvious 1.65-kb band on or off the diagonal. Lack of detectable coding ends in s/+-*ts* cells most likely reflects their rapid resolution of the ends. In contrast, the DNA sample prepared from the scid-*ts* cells incubated at 39°C for 48 hr clearly shows a band at 1.65 kb (Fig. 4*B*). This band resides half on the diagonal and half off the diagonal. We subsequently modified the 2-D gel to give better resolution, as shown in Fig. 4*C Center*. Two bands are running off the diagonal, corresponding to 1.65-kb and 1.3-kb bands in the native dimension and approximately twice these sizes in the denaturing dimension, a pattern indicative of hairpin-structured double-strand DNA. These bands are not present in the cells cultured at 33°C (Fig. 4*C Left*). Thus, a substantial amount of coding ends retained their hairpin structure and could not be resolved in the scid-*ts* cells cultured at the nonpermissive temperature.

Further incubation at 39°C for an additional 24 hr did not seem to increase the opening of the hairpin ends in scid-*ts* cells (data not shown). Interestingly, however, the same cells cultured at 39°C for 48 hr followed by 24 hr at 33°C showed the 1.65-kb species on the diagonal (Fig. 4*C Right*), a pattern that represents open coding ends. Thus, incubation at 39°C followed by 33°C allows the hairpin-coding ends to be opened. These data clearly show that scid-*ts* cells are defective in converting the newly generated hairpin-coding ends to intact open ends at the nonpermissive temperature. The conversion is apparently accomplished after the cell culture is shifted to the permissive temperature, concurrent with the formation of coding joints (see Figs. 2 and 3). In contrast, the resolution of coding ends in s/+-*ts* cells is temperature independent. Thus, as compared with the scid cells, the amount of unresolved hairpin DNA in s/+-*ts* cells is much less and below the detection limit of our experimental system.

DISCUSSION

In this study, we have shown that a significant amount of hairpin-structured coding ends accumulated in scid-*ts* cells 48 hr after initiation of V(D)J recombination, whereas such ends were barely detectable in the wild-type counterpart. Thus, by analyzing the recombination events made *in situ*, we obtained direct evidence to support the previous hypothesis that the scid mutation interferes with the opening of hairpin-coding ends (15). Therefore, the scid-*ts* cells cultured at the nonpermissive temperature resemble the majority of scid lymphocytes in exhibiting a defect in resolving hairpin-coding ends.

These same cells, however, can resolve hairpin-coding ends and make a substantial amount of coding joints after their culture is returned from the nonpermissive to the permissive temperature (Figs. 2, 3, and 4). This finding provides the first indication that the ability of scid cells to resolve hairpin-sealed coding ends is conditional and can be manipulated *in vitro* by changing culture conditions. It is known that shifting the cell culture from the nonpermissive to the permissive temperature results in an activation of v-Abl, which in turn down-regulates RAG and allows the cells to reenter the cell cycle (22). It is possible that these cellular changes allow scid cells to resolve their coding ends. This conditional resolution of coding ends may explain the presence of the few coding joints recovered from scid lymphocytes, especially the leaky scid recombination products. However, the basis of such conditional resolution could never be recognized in developing scid lymphocytes because of their heterogeneity in the expression of RAG and/or the varying stages of the cell cycle. Therefore, the

Table 1. Sequence analysis of coding joints in s/+-*ts* and scid-*ts* cells

Clone	Vκ	N/P	Jκ1
			GTGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAAC (germline sequence)
S/+01	(Vκ4)GGAGTAGTAA-4	—	3-GACGTTCGGTGGAGGCACCAAGCTGGAAATCAAAC
S/+02	(Vκ23)ACAGCTGGCC-1	—	5-CGTTCGGTGGAGGCACCAAGCTGGAAATCAAAC
S/+03	(Vκ12)TGGGGTACTC-1	—	1-TGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAAC
S/+04	(Vκ23)ACAGCTGGCC-1	—	1-TGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAAC
S/+05	(Vκ28)AATTATCC-3	—	2-GGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAAC
S/+06	(Vκ4)-12	—	12-GTGGAGGCACCAAGCTGGAAATCAAAC
ss01	(Vκ1)ACACATCAGCCT	CC (second C underlined)	GTGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAAC
ss02	(Vκ4)GTTACCCACCCA	TGGG (underlined)	26-TGGAAATCAAAC
ss03	(Vκ1)-25*	—	22-AAGCTGGAAATCAAAC
ss04	(Vκ12)-17	—	29-AATCAAAC
ss05	(Vκ10)-32	—	27-GGAAATCAAAC
ss06	(Vκ4)-18	—	−48*
ss07	(Vκ4)-21	—	−53
ss08	(Vκ1)-53	—	−51
ss09	(Vκ8)-52	—	−74
ss10	(Vκ10)-19	—	−158

*The extent of nucleotide loss is indicated either by the number flanking the coding sequences or as −number, without coding sequences. N and P addition are shown in N/P, with P nucleotides underlined.

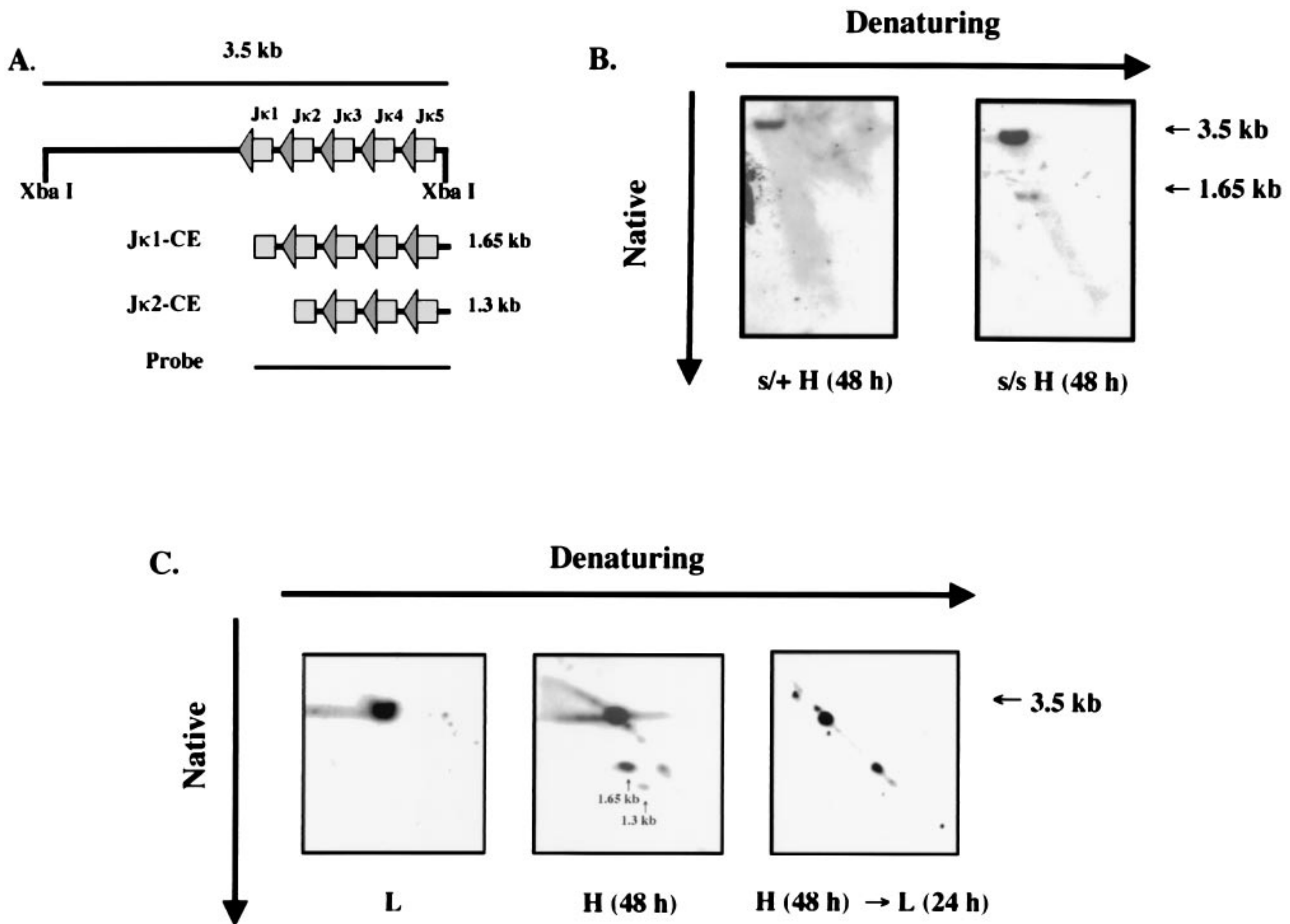

FIG. 4. Hairpin-coding end detection by 2-D electrophoresis. (*A*) Diagrammatic representation of Jκ-gene rearrangement showing the predicted coding ends, the germline fragment after *Xba*I digestion, and the probe used for detection. (*B*) 2-D gel analysis was conducted on the DNA samples prepared from both s/+-*ts* cells and scid-*ts* cells cultured at 39°C for 48 hr. The *Xba*I-digested DNA were electrophoresed in the native condition for the first dimension and in alkaline condition for the second dimension. The germline Jκ fragment as well as the ends are indicated by the arrows. (*C*) 2-D gel analysis was conducted on the DNA samples prepared from the following cells: scid-*ts* cells cultured at 33°C (*Left*), scid-*ts* cultured at 39°C for 48 hr (*Center*), and scid-*ts* cultured at 39°C for 48 hr followed by an incubation at 33°C for 24 hr (*Right*). Expected sizes are 3.5 kb for the germline κ-locus, 1.65 kb for the Jκ-1 coding ends, and 1.3 kb for the Jκ-2 coding ends, as indicated by the arrows.

scid-*ts* cell line with a relatively homogeneous population provides us a valuable model to elucidate the mechanisms of resolving hairpin-coding ends, as well as the regulation of this process.

The conditional formation of coding joints found in scid-*ts* cells is analogous to signal joint formation in the *ts*-Ab-MLV transformed wild-type pre-B cell line. The temperature-dependent joining of signal ends has been postulated to be attributable either to the down-regulation of RAG or to the cells reentering the cell cycle (9). Similarly, we would argue that scid cells with defective DNA-PKcs may still be able to form coding joints once the RAG level is reduced or when the cells can progress into the cell cycle.

Although scid thymocytes accumulated a high level of hairpin-structured coding ends initiated at endogenous gene loci, scid cells were found to be capable of resolving transfected plasmid DNA that contain hairpin structure ends (31). To reconcile these two findings, Zhu and Roth proposed that the scid gene product, DNA-PKcs, may function to modulate the accessibility of coding ends to a nicking nuclease (32). According to this hypothesis, a nuclease is present in scid cells but their chromosomal coding ends may be bound and prevented from nicking by proteins such as RAG, Ku, or poly (ADP ribose) polymerase. Inferred from this, the temperature-dependent resolution of coding ends in scid-*ts* cells may be related to the expression of certain proteins, which ultimately affect the accessibility of the ends. For example, at the nonpermissive temperature, the proteins may bind to the coding ends and make them inaccessible to the nicking nucleases. Incubation at the permissive temperature would then facilitate the removal of these proteins and allow the hairpin ends to be opened. In the case of wild-type cells, functional DNA-PK complexes may promote the removal of blocked proteins, thereby allowing very rapid resolution of coding ends independently of culture temperature. Therefore, the defective DNA-PKcs activity in scid cells would make the newly generated hairpin-coding ends less accessible.

Several studies based on an *in vitro* cell-free recombination system have shown that the RAG proteins can bind to the recombination signal ends and presumably prevent them from degradation as well as from joining (33, 34). Analogous to this argument, we postulate that the RAG proteins may also bind to coding ends and block them from nicking. This postulation is consistent with the recent finding reported by Hiom and Gellert that the RAG proteins can hold all four cleaved ends (35) and mediate the formation of hybrid joints, but not coding or signal joints (36). According to this model, resolution of both coding ends and signal ends may be dependent on dissociation of RAG proteins from these ends. This prediction is contradictory to the observation made by Ramsden *et al.* that the addition of RAG into the *in vitro* cell-free system could increase the formation of coding joints but decrease the

formation of signal joints (11). This cell-free system, however, includes nuclear extracts prepared from Hela cells, which contain high levels of functional DNA-PK complex. Such high levels of DNA-PK may facilitate the removal of RAG specifically from the coding ends but not from the signal ends.

Alternatively, the temperature-dependent resolution of scid coding ends could be explained by their association with cell-cycle progression (37, 38). Analysis of a purified population of cells with defined cell-cycle stages would help to elucidate possible linkage between V(D)J recombination and cell-cycle progression.

Although scid-*ts* cells are capable of making coding joints, this joining activity nevertheless remains scid like. Extensive deletions were found in the coding joints made by scid-*ts* cells, ranging from 22 to 158 bps at the Jκ locus and 17 to 53 bps at the Vκ locus (Table 1). This result indicates that the factors participating in the resolution of coding ends in scid-*ts* cells fail to ensure the fidelity of this reaction. It is not clear how the defective DNA-PKcs leads to aberrant joining. By characterizing nonhairpin open coding ends in normal recombination-active cells, Schlissel has demonstrated that most open ends contain a structure with a 3′ overhang, primarily because of nicking on the 5′ side of the hairpin (10). If this polarity-dependent nicking is used by scid-*ts* cells, nicking at a site away from the hairpin tip would result in a long 3′overhang that could not be filled in before being joined. It is conceivable that such ends would be difficult to align and join, and if they were joined, a deletion would be expected. Hence, the extensive deletions found in the coding joints of scid-*ts* cells may result from inappropriate nicking. The farther the nicking site from the hairpin-tip, the larger the deletion would be at the coding joints.

According to the model proposed above, in which the bound RAG1/2 may prevent coding ends from nicking (e.g., scid-*ts* cells at the nonpermissive temperature), we would argue that if nicking did occur, it might be outside the RAG1/2 binding region, which would lead to a long 3′ overhang and subsequent deletion in joining. On the other hand, once the RAG1/2 dissociate proteins from the coding ends (e.g., at the permissive temperature), the hairpin-coding ends would be subjected to unregulated nicking. The majority of the nicking outside the hairpin tip would still lead to deletion, whereas any nicking near the tip might result in intact joints. Our recent analyses on open coding ends support these speculations (unpublished data). These findings suggest that the scid defect in opening hairpin-coding ends may contribute to both the low level and the aberrant nature of coding joint formation. Therefore, the function of DNA-PKcs may be to promote a rapid and restrictive nicking of hairpin DNA. Such regulated nicking is essential both qualitatively and quantitatively for the formation of functional coding joints.

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