

Coding end resolution in scid recombination-inducible cell lines

Review Article

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Abbreviations: Ab-MLV, Abelson murine leukemia virus; DNA-PK, DNA-dependent protein kinase; DSB, double strand break; LM-PCR, ligation mediated polymerase chain reaction; scid, severe combined immune deficiency; V(D)J, variable (diversity) joining

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Summary

VDJ recombination is the mechanism by which antigen receptor genes are assembled. The site-specific cleavage mediated by recombination activating gene (RAG1 and RAG2) proteins generates two types of broken DNA ends: blunt signal ends and hairpin coding ends. The standard joining of these ends to form signal joints and coding joints employs several proteins involved in double strand break (DSB) repair, including KU70/80, the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), XRCC4 and ligase IV. The cells from severe combined immunodeficient (scid) mice are defective in resolving recombination coding ends due to a point mutation in the DNA-PKcs gene. To study the effect of the scid defect on coding end resolution, we have established recombination-inducible cell lines from scid mice. These cells, at the nonpermissive temperature, actively initiate recombination at the endogenous light chain loci and produce large amounts of hairpin coding ends. After returning to the permissive temperature, scid cells are capable of resolving these coding ends. However, unlike the coding end resolution in normal cells, which is a rapid and regulated process, the resolution of hairpin coding ends in scid cells is slow and error prone. The resulting coding joints contain extensive nucleotide deletions. In addition, the interlocus recombination products are found at much higher frequency in scid cells than in their normal cell counterparts. Our results suggest that functional DNA-PKcs may play an important role in facilitating effective V(D)J recombination and minimizing chromosomal instability.

I. Introduction

V(D)J recombination is a site-specific process that is unique to developing T and B lymphocytes. This process involves a site-specific cleavage and imprecise end joining (Gellert, 1992; Steen et al., 1996). The cleavage, mediated by recombination activation gene (RAG1 and RAG2) proteins, produce two types of recombination intermediates, blunt signal ends and hairpin coding ends (Schatz and Leu, 1996; van Gent et al., 1996). Joining of these ends is dependent on a general double-strand DNA break (DSB) repair system, which includes the Ku-heterodimer, DNA-dependent protein kinase (DNA-PK), XRCC4 and ligase IV (Jackson and Jeggo, 1995; Chu, 1996; Jeggo et al., 1996; Frank et al., 1998). Genetic alteration of any of the proteins that participate in the V(D)J recombination process could

lead to an arrest of lymphocyte development and ultimately to immunodeficiency. For example, the severe combined immunodeficient (scid) mouse bears a point mutation at the gene encoding the catalytic subunit of DNA-PK, and has a defect in resolving recombination coding ends (Danska et al., 1996; Araki et al., 1997).

To understand the molecular mechanisms of the V(D)J recombination joining process, we made recombination-inducible cell lines from both scid and normal mice by transforming pre-B cells with the temperature-sensitive Abelson murine leukemia virus (*ts*-Ab-MLV), which could be manipulated in vitro (Chen et al., 1994; Chang and Brown, 1999). The recombination activity can be induced by incubating cells at the nonpermissive temperature, which leads to site-specific cleavage at L-chain gene loci, both

and (Klug et al., 1994; Liu et al., 1997). Thus, these recombination-inducible scid and normal counterparts provide us with a good model to study the regulation of recombination initiation, and to delineate various steps in the resolution of V(D)J recombination intermediates.

In this study, we have investigated the resolution of coding ends in scid cells. Substantial amounts of recombination hairpin coding ends were found to accumulate in scid but not in scid heterozygous (*s/+*) cells after induction of recombination. By analyzing the kinetics of coding end resolution in both scid and *s/+* cells, we found that such resolution is much delayed in scid cells relative to *s/+* cells. The inability of scid cells to promptly resolve their recombination ends makes these ends vulnerable to genetic mutation. For example, extensive losses of nucleotides were found at the junction of coding joints. In addition, the aberrant end resolution in scid cells was also revealed by an increased level of interlocus recombination products. In contrast, the control *s/+* cells resolve their coding ends rapidly and show very few aberrations in their joined products. These findings lead to the conclusion that functional DNA-PKcs plays an important role in facilitating effective V(D)J recombination and minimizing chromosomal instability.

II. Results and Discussion

A. Recombination initiation

Great effort has been devoted to developing *ts*-Abl-MuLV transformed pre-B cell lines from scid and *s/+* control mice. The scid and *s/+ ts*-Abl-MuLV transformants, referred to as scid-*ts* and *s/+ts* cells, respectively, can be induced to express high levels of RAG1 and RAG2 by incubating the cells at the nonpermissive temperature of 39°C. It has been reported that *ts*-Ab-MLV transformants can be induced to rearrange both of their λ - and κ -chain genes (Chen, et al., 1994; Klug, et al., 1994; Liu, et al., 1997). To directly assess the recombination initiation at these gene loci in scid-*ts* and *s/+ts* cells, we examined the recombination signal ends of J λ , V λ 1 and J λ 1 using a ligation-mediated PCR (LM-PCR) assay as described previously (Roth et al., 1993).

It is clear from **Fig.1** that at 39°C; the level of signal ends generated from three specific gene loci is comparable between scid-*ts* and *s/+ts* cells. This indicates that both cell types attempt similar levels of recombination cleavages. Inferred from this analysis, we expect that a comparable level of coding ends is generated in these two cell types, as well. Therefore, the resolution and various joining products of the newly generated coding ends can be directly compared between scid and *s/+ts* cells.

B. Coding joint formation

We first analyzed and compared coding joint formation in scid-*ts* and *s/+ts* cells by PCR assay. The cells were cultured at 39°C for 48 hours. The *s/+ts*-cells contained a

significant amount of VJ 1 coding joints (**Fig. 2**). Extended incubation at 39°C or after shifting back to 33°C does not further increase the amount of coding joints (**Fig. 2**). Thus, the coding joint formation in *s/+* cells is a very rapid process and is not dependent on incubation at 33°C. In contrast, no detectable coding joints were found in the scid-*ts* cells even after 48 hour-incubation at 39°C (**Fig.1A**). Interestingly, however, coding joints do gradually appear in scid-*ts* cells with an increased level upon shifting cells from the nonpermissive temperature to the permissive temperature for an increased time (**Fig. 2**). This study clearly demonstrates that incubation at 33°C following culture at 39°C is essential for scid-*ts* cells to join their coding ends. Therefore, the ability of scid-*ts* cells to join coding ends can be facilitated by culturing them at the permissive temperature after exposure to the higher levels of RAG at the nonpermissive temperature.

C. Temperature-dependent resolution of recombination hairpin coding ends

The inability of scid-*ts* cells to form coding joints at the nonpermissive temperature could result from a dysfunction at several stages: hairpin nicking, end filling, or joining. To directly examine hairpin-structured coding ends, we carried out a two-dimensional (2-D) gel electrophoresis followed by Southern blot analysis as described by Roth et al (Roth et al., 1992). The 3.4-kb band was derived from restriction digestion (*Xba*-I) of the germline λ -locus. Cleavage initiated at the J λ 1 locus should give rise to 1.7 kb J λ 1 coding ends (shown in **Fig. 3A**). On the 2-D gel electrophoresis, the open coding ends would be expected to run along the diagonal whereas the hairpin coding ends would run off the diagonal.

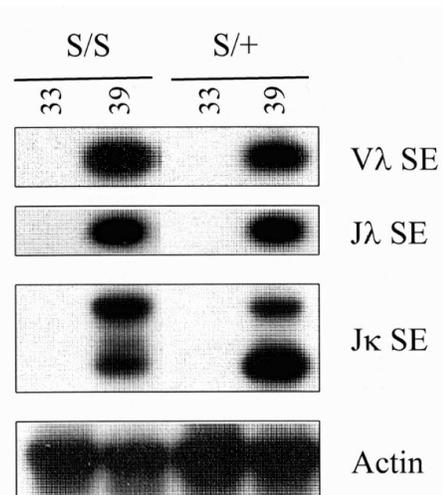


Figure 1. Recombination signal ends (SE) in both λ and κ gene loci detected by Ligation-mediated PCR. The recombination cleavages are initiated upon shifting cells from the nonpermissive temperature (39°C) to the permissive temperature (33°C).

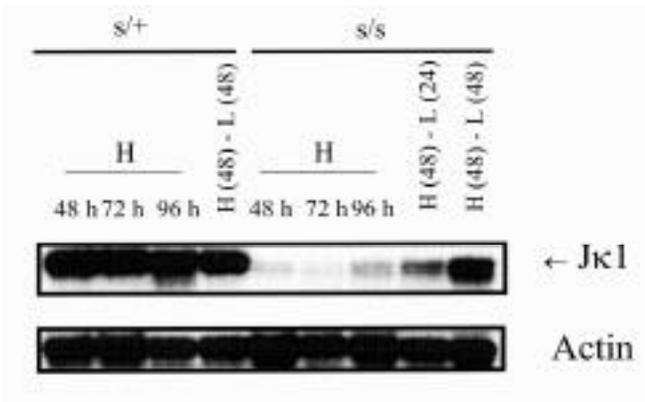


Figure 2: Analysis of coding Joint (CJ) formation in both *scid/+* and *scid/scid* cells. (A) Time course analysis for CJ formation. After the temperature sensitive cells were cultured under their respective conditions (H indicates non-permissive temperature, L indicates permissive temperature), the DNA was isolated and subjected to a PCR assay using a V 1 primer and a J 2 primer, along with actin controls. (B) CJ formation with extended culture. The J 2 primer was used to follow the formation of CJs over a four day period under various conditions as described in the text.

The *scid* sample incubated at 39°C for 48 hours showed a 1.7kb band off the diagonal (**Fig. 3B**, middle panel), a signature of hairpin ends. The same cells cultured at 39°C for 48 hours followed by 24 hours at 33°C showed the 1.7kb band right on the diagonal (**Fig. 2B**, lower right panel), i.e., open coding ends. Thus, incubation at 39°C followed by 33°C seems to allow opening of the *scid* hairpin coding ends. These data clearly show that under the nonpermissive temperature, *scid-ts* cells are defective in converting the newly generated hairpin-coding ends to intact open ends. This conversion might be accomplished after the cell culture is shifted to the permissive temperature, which is concurrent with the formation of coding joints. In contrast, under the same culture conditions, the DNA isolated from the *s/+ts* cells did not show an obvious 1.7 kb band either on or off the diagonal, which may reflect a rapid joining of coding ends (Chang and Brown, 1999). Similar conclusion was also confirmed by a more sensitive assay, a modified LMPCR (described below). These data clearly indicates that the ability of *scid-ts* cells to resolve hairpin-coding ends is conditional

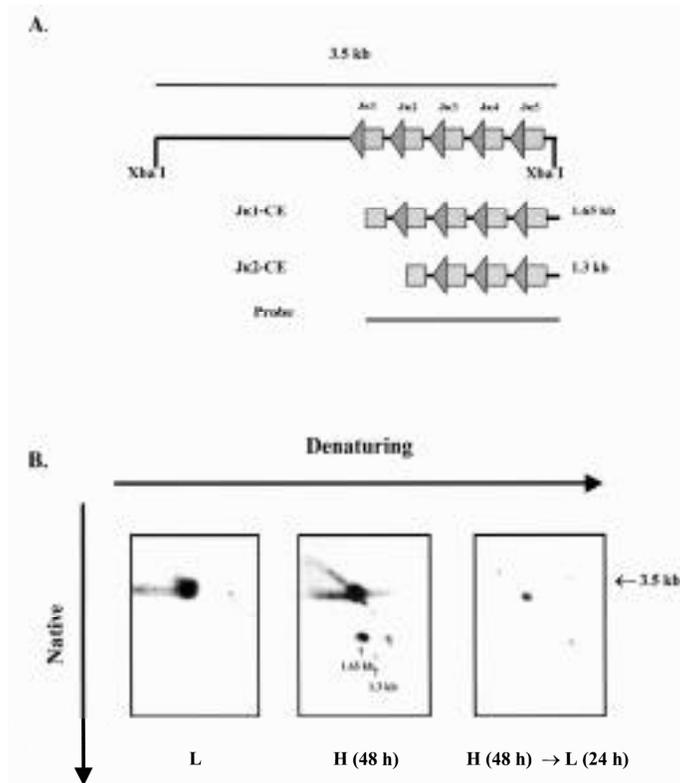


Figure 3: Hairpin coding end detection by two-dimensional electrophoresis. (A) Diagrammatic representation of J-gene rearrangement and predicted sizes after *XbaI* digestion. (B) *scid* DNA samples were digested with *XbaI* and electrophoresed in the native direction, then shifted 90° clockwise and continued under denaturing conditions. Expected sizes are 3.4 kb for the germ-line locus, 1.65 kb for the J 1 CE, and 1.3 for the J 2 CE. The denatured hairpin CE (H, 48 hrs) departed from the diagonal line. In contrast, the opened CE (H, 48 hrs → L, 24 hrs) remained within the diagonal line.

To further delineate the process in end resolution, we analyzed various structures of coding ends: closed hairpin ends, recessive and blunt open ends by a modified LM-PCR. The blunt open ends can be directly amplified by LM-PCR while the staggered open ends are first processed by T4 DNA polymerase before being subjected to the ligation assay. The hairpin ends are pretreated with both Mung Bean nuclease (MBN) and T4 DNA polymerase then followed by LM-PCR. To minimize any DNA damage caused by the routine extraction procedure, DNA samples were prepared in an agarose plug, as described by Schlissel (1998).

Fig. 4 shows that large amounts of blunt open ends are present in cells cultured at 39°C followed by incubation at 33°C (sample 2-1, Lane 6). Pretreatment with T4 DNA polymerase or together with MBN did not significantly increase the detection of these ends (Lane 2 and Lane 4). This indicates that majority of the coding ends are in the form of open blunt ends. However, the size of these ends seems smaller than the full length of artificially nicked hairpin ends (compare lane 6 and lane 2), which may reflect nucleotide loss at the ends.

In contrast, very few blunt open coding ends were detected in cells that were cultured at 39°C for three days (sample 3-0, lane 5). Instead, the majority of the coding ends remained in the hairpin structure (**Fig. 4**, top panel, lane 1). The amount of PCR products was also increased if the DNA sample had been pre-treated with T4 DNA polymerase, indicating the presence of recessive open coding ends (Lane 3). These ends migrate much faster than the other ends, reflecting various deletions of nucleotides. Thus, even though some nicking of hairpin ends may occur at the nonpermissive temperature (3-0), this event is much less frequent and more aberrant than the one observed in the cells returning from 39°C to 33°C (compare lane 3 and lane 4).

Taken together, our data indicate that the ability of scid-ts cells to resolve hairpin coding ends is conditional as well as aberrant. Extensive deletions were found at the blunt open ends and the recovered coding joints (data not shown), which is consistent with our previous finding of scid-like coding joints. Thus, the end resolution in scid-ts cells may be fundamentally different from the control *s/+ts* cells. In normal cells, functional DNA-PKcs may act to protect the ends while recruiting some nucleases and other DSB repair proteins for limited nicking and rapid joining. In scid-ts cells, on the other hand, the temperature-dependent resolution of the ends may reflect different accessibility of the ends to the nucleases and repair proteins under different culture temperatures. For example, the ends may be blocked from nicking at 39°C and exposed to non-regulated nicking after returning the cells from 39°C to 33°C. Therefore, the resolution of coding ends in scid cells proceeds through a DNA-PKcs-independent default pathway, which is inefficient and prone to error.

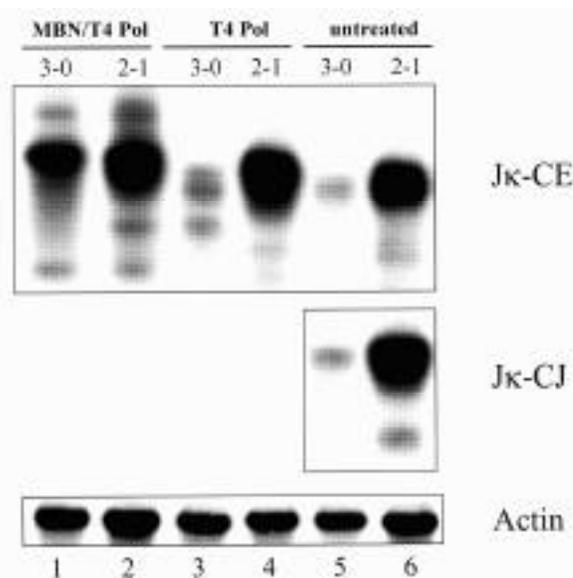


Figure 4. Coding end resolution in scid (*s/s*) temperature sensitive (*ts*) Abl-MLV cell line. Scid-*ts* cells were shifted to 39°C for either 3 days (3-0) or 2 days followed by 1 day at 33°C (2-1). DNA was prepared in agarose plugs as described in materials and methods. Coding ends (CE) were analyzed by ligation mediated-PCR (LM-PCR) at the J 1 locus. Mung Bean nuclease (MBN) and T4 DNA Polymerase (T4 Pol) were used to flush the coding ends. Coding joint (CJ) formation was assayed by PCR using a V primer and a J 2 primer. Amplification of the actin gene was used as a control for the amount of DNA.

D. Chromosomal translocation: presence of interlocus recombination products

It is conceivable that the unresolved recombination coding ends, due to an inefficient pathway for end resolution, could be vulnerable targets for gene mutations such as deletion, insertion and translocation. To directly examine the possibility of chromosomal translocations, we have analyzed the recombination events between two gene segments residing on different chromosomes, e.g., the and gene loci. As shown in **Fig. 1**, both of these gene loci could be induced to undergo recombination cleavages. Thus, it is possible for a rearrangement to occur between these two gene loci. **Fig. 5A** is a schematic diagram that illustrates the detection of an interlocus rearrangement. Specifically, the coding joints between V 1 and J 1 gene segments were analyzed by PCR amplification, and revealed by hybridization with the V probe. **Fig. 5B** is one representative example of the analyses of interlocus recombination. Although substantial amounts of V J coding joints are present in *s/+ts* cells, no V J coding joints were detected. This finding is consistent with the previous report by Bailey and Rosenberg (1997) in which the V J joints were estimated to form at a frequency about 1,000-fold less than that of cis-rearrangement.

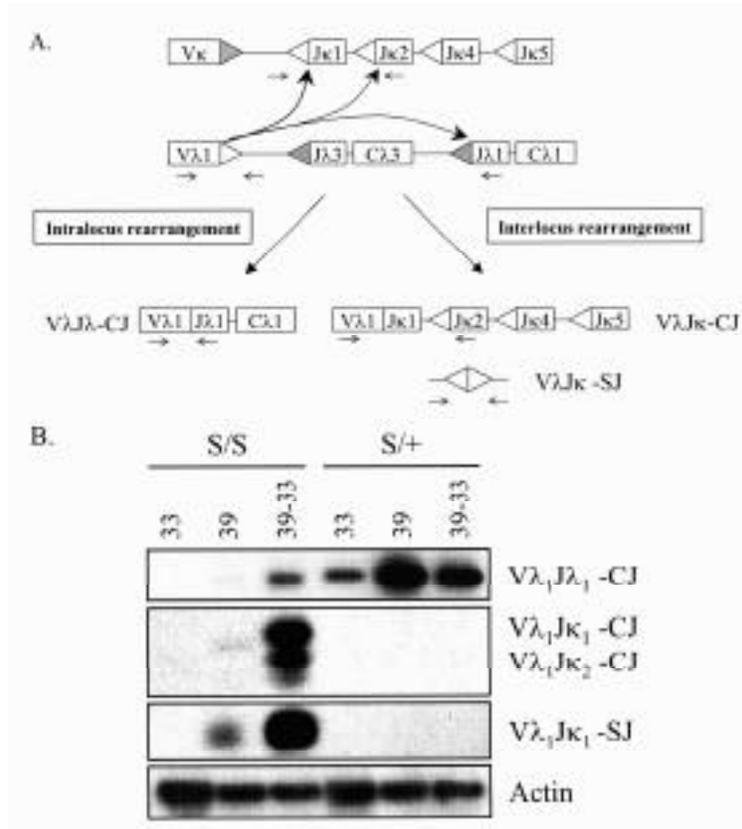


Figure 5. Analysis of light chain interlocus rearrangements in scid and s/+ ts-Abl-MLV transformants. (A). Mechanism of interlocus as compared to intralocus rearrangements. Interlocus rearrangements consisted of V joined to J coding regions and signal joints made up of RSS from both and loci. Primers are represented by small arrows. RSS are represented by triangles and coding regions by rectangles. (B) PCR and Southern blot analysis of standard V J coding joints, V J coding joints, and V J signal joints. Actin served as a control.

Table I. Sequence analysis of interlocus coding joints in scid-ts cells.

Clone	$V\lambda 1/2$ coding sequences'	$J\kappa 1$ coding sequence'
Germline	TACAGCAACCAT	GTGGACGTTTCGGTGGAGGCACC
SL15-a	TACAGCAACCA-1	3-GACGTTTCGGTGGAGGCACC
SL07-a	TACAGCAA-5	-33
SL17-a	TA-11	-87
SL03-a	-13	-87
SL13-a	-25	-58
SL06-a	-49	-54
SL02-a	-75	-90
SL14-a	-35	-105
SL20-a	-50	-95
SL12-a	-75	-134
SL03-b	-94	-260

*The number of nucleotides deleted from the $V\lambda 1/2$ and $J\kappa 1$ germ line sequences. The underlined nucleotides represent the homology between $V\lambda 1$ and $J\kappa 1$ region.

In contrast, V J coding joints were readily detected in the scid-ts cells that were cultured at 39°C followed by incubation at 33°C. The same PCR products detected by a V probe were also revealed by a J-specific probe

(unpublished observation), confirming the rearrangement between and . The junctions of these joining products also contained large deletions (Table I), similar to the abnormality found in the coding joints made by scid-ts cells.

These results indicate that recombination intermediates, including those made from different chromosomes, are in close proximity and accessible for joining to each other. It is possible that these ends are present in a common compartment or that they are free and flexible for engaging in various types of association. The lack of interlocus joining products in *s/+ts* cells suggests that free association of coding ends is prohibited by the functional DNA-PKcs. Therefore, our finding suggests that DNA-PKcs plays an important role in facilitating the resolution of recombination intermediates, one which ultimately prevents the uncontrolled association of the ends.

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