Transient Restoration of Gene Rearrangement at Multiple T Cell Receptor Loci in γ-Irradiated *scid* Mice

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Summary

The developmental arrest of thymocytes from *scid* mice, deficient in variable, (diversity), and joining, or V(D)J recombination, can be overcome by sublethal γ-irradiation. Since previous studies focused on restoration of rearrangement of the T cell receptor (TCR) β locus, productive rearrangement of which is selected for, we sought to examine to what extent locus specificity and cellular selection contributed to the observed effects. We report here that irradiation of newborn *scid* mice induces normal V-D-J rearrangements of the TCR δ locus, which like TCR β, is also actively rearranged in $CD4^-CD8^-$ (double negative) thymocytes. In contrast, no complete V-Jα rearrangements were detected. Instead, we detected substantial levels of hairpin-terminated coding ends at the 5′ end of the Jα locus, demonstrating that TCR α rearrangements manifest the effects of the *scid* mutation. Irradiation, therefore, transiently compensates for the effects of the *scid* mutation in a locus-nonspecific manner in thymocytes, resulting in a burst of normal TCR β and δ rearrangements. Irradiation also allows the development of cells that can initiate but fail to complete V(D)J recombination events at the TCR α locus, which is normally inaccessible in *scid* thymocytes.

T lymphocytes are characterized by the expression of antigen-specific TCR molecules consisting of the clonotypic α and β or γ and δ chains. The V, (D), and J gene segments that encode the variable portion of these polypeptides are joined together during T cell development by a somatic gene rearrangement process termed V(D)J recombination (for a review see reference 1). A number of stages of intrathymic T cell development have been defined by the expression of various surface molecules, with several of the developmental transitions tightly coupled with the ordered assembly of TCR genes (for a review see reference 2). Thymocytes early in the pathway lack expression of CD4 and CD8 (hence referred to as double negative cells) and rearrange their β, γ, and δ TCR loci. Cells with a productive β rearrangement proliferate extensively and develop into $CD4^+/CD8^+$ (double positive) thymocytes, initiate α gene rearrangement, and commit to the α/β T cell lineage, whereas double negative cells with a productive γ and δ rearrangement become γ/δ T cells (3, 4).

Mice genetically deficient in the V(D)J recombination process, either due to the naturally occurring *scid* mutation (5) or to the targeted disruption of one of the two recombination-activating genes, *RAG1*[1] or *RAG2* (6, 7), are unable to generate functional TCR complexes, and their thymocytes are arrested at the double negative stage of development (8, 9). In $RAG^{-/-}$ lymphocytes, V(D)J recombination fails to initiate (10, 11), whereas in *scid* thymocytes, the recombination machinery generates double strand breaks adjacent to TCR gene segments but fails to join the coding segments properly. The unjoined coding ends of the TCR δ locus from *scid* thymocytes have been demonstrated to have a covalently sealed, hairpin structure (12). The rare coding junctions that do form are often characterized by large deletions of coding DNA (13) or the appearance of long palindromic sequences (termed P nucleotides [14]) at the coding junctions (15–17). The defect in *scid* mice has recently been demonstrated to be a deficiency in the catalytic subunit of the DNA-dependent protein kinase (DNAPK; 18, 19). The catalytic subunit is active only when bound to DNA, and this is mediated by the

[1] *Abbreviations used in this paper:* DNAPK, DNA-dependent protein kinase; IRNB, irradiated newborn; RAG1, recombination-activating gene 1.

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Ku nuclear complex, a heterodimer of 70- and 80-kD peptides (20).

It was recently shown that sublethal γ-irradiation of *scid* (21) or $RAG^{-}/^{-}$ (22, 23) mice can overcome the developmental arrest and promote the appearance of large numbers of double positive thymocytes. The mechanism by which γ-irradiation produces this effect in $RAG^{-}/^{-}$ mice is independent of a functional TCR β protein, since these mice are inherently unable to perform gene rearrangement (6, 7). In contrast, in *scid* mice, irradiation induced the development of double positive thymocytes, many of which contained normal TCR β rearrangements and TCR β protein (21). The appearance of large numbers of double positive thymocytes containing normal TCR β rearrangements in irradiated newborn (IRNB) *scid* mice raised two important issues. First, it was unknown whether the effects of irradiation were confined to the β locus or also extended to the TCR γ and δ loci (which are also actively rearranged in double negative thymocytes). Second, it was not known to what extent the double positive *scid* thymocytes induced by irradiation initiated rearrangements of the TCR α locus, and whether the rearrangements displayed the characteristics of wild-type or *scid* recombination. We have therefore analyzed TCR δ and α gene rearrangements in IRNB *scid* mice. We found that normal TCR δ rearrangements occur and that TCR α recombination is initiated after irradiation, but results in broken coding ends terminating in a hairpin structure. Therefore, irradiation compensates for the *scid* defect in V(D)J recombination in a transient and locus-nonspecific fashion, resulting in the development of double positive thymocytes that then initiate defective TCR α rearrangements.

Materials and Methods

Mice. C.B-17 *scid/scid* and wild-type BALB/c mice were bred and housed at the Hospital for Sick Children. Wild-type C57Bl/6 and AKR/J mice used as controls in some experiments were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed at Yale Medical School. Newborn *scid* mice were treated with a single low dose of irradiation (100 cGy) as described previously (21).

Flow Cytometry. Flow cytometric analyses of thymocyte suspensions for CD4, CD8, and CD25 surface markers and for intracellular TCR β chain protein were carried out as previously described (21) on a FACScan® flow cytometer with Lysis II software (Beckton Dickinson & Co., Mountain View, CA).

Nucleic Acid Preparation and Hybridization. High molecular weight DNA was prepared from total homogenized kidney or liver and single cell suspensions of total thymocytes with proteinase K digestion/phenol-chloroform extraction (24). 5–8 μg genomic DNA was restriction digested with the indicated enzymes (Boehringer Mannheim Corp., Indianapolis, IN or New England Biolabs, Beverly, MA) according to the manufacturers' instructions, electrophoresed through 0.7–0.8% agarose gels, and transferred to Gene Screen Plus nylon membrane (NEN DuPont, Boston, MA) on a Posiblot apparatus (Stratagene, La Jolla, CA). Two-dimensional agarose gel electrophoresis was carried out as described previously (12). Briefly, PstI-digested DNA in a wide lane was electrophoresed through a neutral agarose gel in the first dimension; the lane was excised and cut in half lengthwise. One half was saved as first dimension control, whereas the other half was equilibrated with alkaline running buffer (50 mM NaOH, 1 mM EDTA) and inserted into the alkaline agarose gel perpendicular to the direction of the first dimension. After second dimension electrophoresis, the gel was transferred along with the first dimension control as described above. Molecular weight markers were run both in the first and the second dimensions to ensure accurate size determination. Hybridization was carried out in the presence of 50% formamide (24) with random hexamer-primed [α-^{32}P]dCTP-(Amersham Corp., Arlington Heights, IL) labeled probes 4, 8, 9 10, 11, 13, 14, 15, RAG-1 (4), and CaI or CaII (25) as indicated. Autoradiography and quantitative analysis were performed using a PhosphorImager with ImageQuant 3.0 software (Molecular Dynamics, Sunnyvale, CA). The relative hybridization signal for a given probe was calculated as described (4).

DNA Sequencing. TCR V4(D)J1δ and V5(D)J1δ sequences were PCR amplified, restriction enzyme digested at artificially introduced enzyme sites, and directionally subcloned into pBKS+ II vector (Stratagene) as described previously (4). DNA prepared from individually picked clones was double strand sequenced with the Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA), separated on denaturing polyacrylamide gel (National Diagnostics, Atlanta, GA), and analyzed on a Stretch automated sequencer (model 373; Applied Biosystems).

Results

V-D-Jδ Rearrangements in Irradiated scid Thymocytes. To determine the effects of γ-irradiation on the TCR δ locus in *scid* thymocytes, genomic thymus DNA from irradiated and nonirradiated *scid* mice, and from control BALB/c mice, was subjected to Southern blot analysis. EcoRI digestion and hybridization with probe 4 (which lies between Jδ1 and Jδ2; Fig. 1 *B*) allowed detection of any rearrangement to Jδ1. The BALB/c thymus DNA produced a characteristic pattern of bands (Fig. 1 *A*, lane *2*), many of which can be assigned to specific V-to-DJδ or D-to-Jδ rearrangements (4, 26, 27). *Scid* thymus DNA produced a quite different pattern of bands: the germline fragment was retained to a substantial extent, and only three distinct nongermline bands were observed (Fig. 1 *A*, lane *3*). The top-most nongermline band represents D2-J1 rearrangements, whereas the other two (black arrowheads) represent broken DNA molecules that terminate in a hairpin structure at the ends of the Dδ2 and Jδ1 coding regions (hereafter referred to as coding end molecules). These results agree with previous studies that demonstrated that thymocytes from nonirradiated *scid* mice initiate rearrangement of the TCR δ locus generating hairpin-terminated coding end molecules, with most of the events occurring at the Dδ2 and Jδ1 gene segments (12, 28).

We reasoned that if irradiation resulted in a period of normal δ rearrangements in the *scid* thymus, we should see the appearance of diverse nongermline bands similar to those seen in the wild-type thymus, and a decrease in the intensity of the germline-sized band. We might also expect to see the disappearance of the coding end molecules, due either to conversion to coding joint products or to degradation. Analysis of individual IRNB *scid* mice confirmed these predictions. We observed a significant reduction in

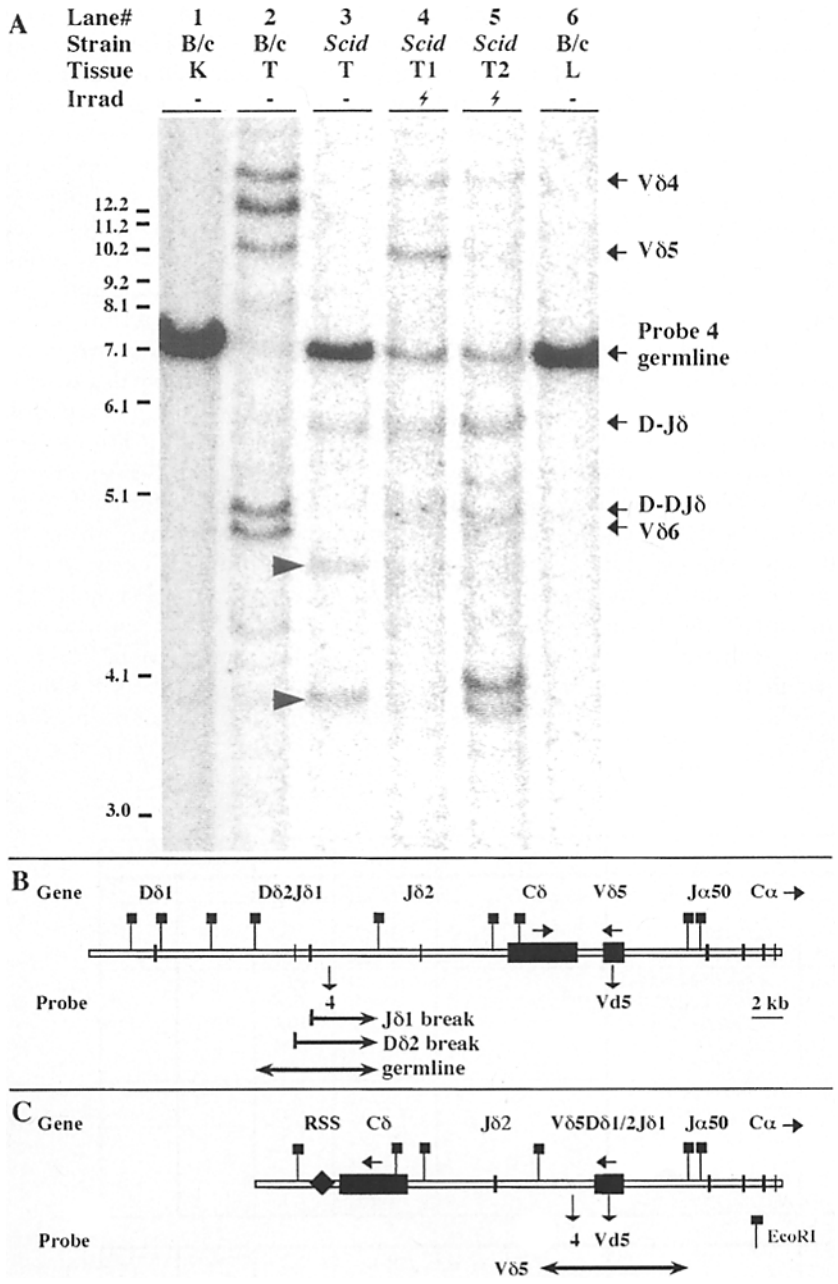

Figure 1. Normalization of Vδ-D-Jδ rearrangements in irradiated *scid* thymocytes. (*A*) Southern blot analysis of EcoRI-digested DNA with probe 4. Samples are DNA from wild-type BALB/c (*B/c*) kidney (*K*), liver (*L*), and thymus (*T*), from nonirradiated 2-wk-old *scid* and from irradiated 1- (*T1*) and 2-wk (*T2*) -old *scid* thymocytes. (*Arrows, right*) Position of germline, partial D-J, and complete V-D-J rearrangements as determined previously (4). (*Arrowheads*) Position of Dδ2 (*upper*) and Jδ1 (*lower*) coding end molecules, as demonstrated previously in non-irradiated *scid* thymocytes (12). (*Dashes, left*) Position of molecular weight standards in kilobases. Note the lack of V-D-Jδ rearrangements and the presence of coding end breaks in nonirradiated *scid* samples (lane *3*), and the appearance of V-D-Jδ rearrangements and the disappearance of coding end breaks in irradiated samples (lanes *4* and *5*). Note also the dramatic reduction of the germline hybridization signal after irradiation. The identity of some of the intense bands present in sample T2 is unknown (lane *5*). (*B*) The location of the probe in the context of the genomic map. (*C*) An example of the generation of a predictable size V-D-Jδ rearranged fragment hybridizing to probe 4. (*Double arrows*) Size of the predicted fragments corresponding to germline, Jδ1, and Dδ2 coding end breaks (*B*) and to V5-D-Jδ rearrangement (*C*). (*Boxes*) Known coding elements, without showing the independent exons of the Cδ and Vδ5 genes. (*C, black diamond labeled RSS*) Fused recombination signal sequence generated during Vδ5 inversion. The probe Vd5 is identical with probe 7 (4).

the intensity of the germline hybridization signal and the disappearance of the two bands corresponding to coding end molecules (Fig. 1 *A*, lanes *4* and *5*, compare with lane *3*). Furthermore, in the 1-wk-old sample (Fig. 1 *A*, lane *4*) there are clearly detectable bands migrating as expected for V4DJ1δ and V5DJ1δ rearrangements that were also seen in independent DNA samples from 9-d-old IRNB *scid* mice (data not shown). The identity of these bands was corroborated by rehybridizing the same blot to a mixture of Vδ4- and Vδ5-specific probes (data not shown). The bands corresponding to complete V-D-Jδ rearrangements became barely detectable at 2 wk after irradiation (Fig. 1 *A*, lane *5*, and data not shown) and were not detectable at 3 wk after irradiation (data not shown). The appearance of V4- and V5-DJ1δ rearrangements 7–9 d after irradiation indicates that a single dose of irradiation resulted in the generation of normal V-D-Jδ recombination products in *scid* mice.

We also wanted to determine whether the disappearance of the TCR δ coding end molecules was due to their conversion into the V-D-Jδ joints or to their quantitative reduction in the large proliferative burst that follows irradiation (21). When *scid* mice are provided with a functional TCR β transgene (29), they develop thymocytes in comparable numbers and with comparable surface phenotypes to those seen in irradiated nontransgenic *scid* mice (data not shown). Southern blotting experiments identical to those

described above revealed that thymus DNA from nonirradiated TCR β transgenic *scid* mice contained substantial quantities of δ coding end molecules but no apparent V-D-Jδ coding joints, similar to that observed in unmanipulated *scid* mice (data not shown). These results suggest that the disappearance of coding end molecules in IRNB *scid* mice is not a dilution effect of the increased number of double positive thymocytes (21) and are consistent with the conversion of coding ends into coding joints after irradiation.

Sequence Analysis of the V-D-Jδ Joints. To confirm that the V-D-Jδ rearrangements seen by Southern blot represented normal coding joints, we cloned and sequenced V4- and V5-DJ1δ rearrangements from the 1-wk and 2-wk-old IRNB *scid* thymus samples (Fig. 1 *A*, lanes *4* and *5*), and from two additional 2-wk old IRNB *scid* samples. We predicted that these sequences would show only small deletions from the coding ends and the absence of long stretches of P nucleotides if they were the product of the normal V(D)J recombination mechanism. Because functional TCR δ rearrangements are not necessary for double positive α/β thymocyte development (30), this experiment also provided the opportunity to analyze rearrangements that occur after irradiation at the molecular level in the absence of cellular selection.

Sequence analysis revealed predominantly normal V-D-Jδ joints (Table 1), with only 8 of the 43 (18.6%) unique, nonprocessed V, D, or J ends having P nucleotide sequences of 3 bp or longer (potentially representative of *scid*-type aberrant rearrangements). Analysis of previously reported (4) V4/V5-DJ1δ rearrangements from normal thymocytes and peripheral T cells found 13 of 162 (8.0%) unique, nonprocessed ends carrying P nucleotides of similar length (data not shown). In contrast, 5 of 13 (38.4%) independent coding ends of D2-J1δ rearrangements amplified from nonirradiated *scid* thymus (the only rearrangements visible by Southern blot; Fig. 1 *A*, lane *3*) exhibited 3 bp or longer P nucleotide sequences (data not shown). We also note that whereas 90% of the previously reported independent TCR β rearrangements were in-frame (21), only 9/20 (45%) of the δ rearrangements were in-frame, which is not statistically significantly different from the 33% expected for random rearrangements ($P > 0.19$). The fact that there is no significant skewing toward in-frame δ rearrangements, yet the majority of the TCR δ sequences are normal (as judged by the absence of extensive P nucleotide addition) demonstrates that normal V(D)J recombination events observed after irradiation do not depend upon cellular selection of the recombination products. This suggests that the previous

Table 1. *V4-D-Jδ and V5-D-Jδ Joints from Irradiated scid Thymocytes*

Source	Vδ4	N	Dδ1	N	Dδ2	N	Jδ1	N°	unique	FRAME
GL	GAGCGC	-	GTGGCATATCA	-	ATCGGAGGGATACGAG	-	CTACCGAC	-	-	-
T 1	GAGC	A			CGGAGGG		ACCGAC	3/5	40%	+
T 1	GAGCGC	**G**	**AT**		ATCGGAGGG		CTACCGAC	2/5		-
T 2.1	GAGCGC	**GA**	GC		CGGAGGGATACGAG		CTACCGAC	1/6		-
T 2.1	GAGCGC	**GCGCT**			ATCGGAGGGATACGAG	**CTCGTA**GCCGTCG	ACCGAC	1/6		-
T 2.1	GAGC	CAC			GGAGGG		ACCGAC	1/6	83%	-
T 2.1	GAGC				CGGAGGG	CTCC	CTACCGAC	2/6		-
T 2.1	GAGC				CGGAGGGA	CCCCCAT	CTACCGAC	1/6		+
T 2.2	GAGCG		TGG	**GAT**	ATCGGAGGGAT	CGC	CCGAC	4/7	29%	+
T 2.2	GAGCG		**AT**		ATCGGAGGGATACGAG		CTACCGAC	3/7		-
T 2.3	GAGCGC	**GAGT**			ATCGGAGGGATACGAG		CTACCGAC	1/5		-
T 2.3	GAGCGC	**GCGCT**	CAT		GAGGGATACGAG	**CT**	CCGAC	1/5	80%	+
T 2.3	GAGCGC	**G**	**AT**		ATCGGAGGGATACGAG		CTACCGAC	2/5		+
T 2.3	GAGCGC	**GCGCCT**			ATCGGAGGG	T	CCGAC	1/5		+

Source	Vδ5	N	Dδ1	N	Dδ2	N	Jδ1	N°	unique	FRAME
GL	GGGTAT	-	GTGGCATATCA'	-	ATCGGAGGGATACGAG	-	CTACCGAC	-	-	-
T 1	GGGTAT	**A**	GG		GGAGGGATACGAG	**CT**	CCGAC	5/5	<20%	-
T 2.1	GG				AGGGATACGAG		CTACCGAC	8/8	<13%	+
T 2.2	GGGTAT	**AT**	**AT**		ATCGGAGGGATACGAG	**CTCGTAT**	GAC	3/6	33%	+
T 2.2	GGGTAT		TC	CCT	ATCGGAGGGATACGAG		CTACCGAC	3/6		-
T 2.3	GGG	ATG	TGG	GT	ATCGGAGGGATACGAG		CCGAC	2/6		-
T 2.3	GGG	CCCGT			ATCGGAGGG		TACCGAC	1/6	50%	+
T 2.3	GGGTAT		TG		GGGATACGAG		CCGAC	3/6		-

DNA sequences of 23 V4-D-Jδ (*top*) and 25 V5-D-Jd (*bottom*) joints PCR amplified from four independent thymus samples obtained from irradiated *scid* mice. T1 and T2.1 represent the samples of 1 wk and 2 wk after irradiation, respectively, shown in Fig. 1 *A*. T2.2 and T2.3 are two more independent thymus samples of 2 wk after irradiation. The entire junction and the ends of the variable and joining genes are shown in a 5'–3' direction. The top lines indicate the germline (*GL*) sequences (28, 41), *N* indicates nongermline nucleotide addition. Potential P nucleotides are highlighted. Note that some AT dinucleotides shown as Dδ1 sequences cannot be assigned unambigously and are also highlighted as potential P nucleotides. (*No*) Number of occurences of each sequence expressed as a ratio of the total number of sequences determined. The next column shows the percent unique sequences in each sample. The rightmost column indicates the reading frame of the sequences, "+" meaning the frame that could generate full-length TCRδ protein.

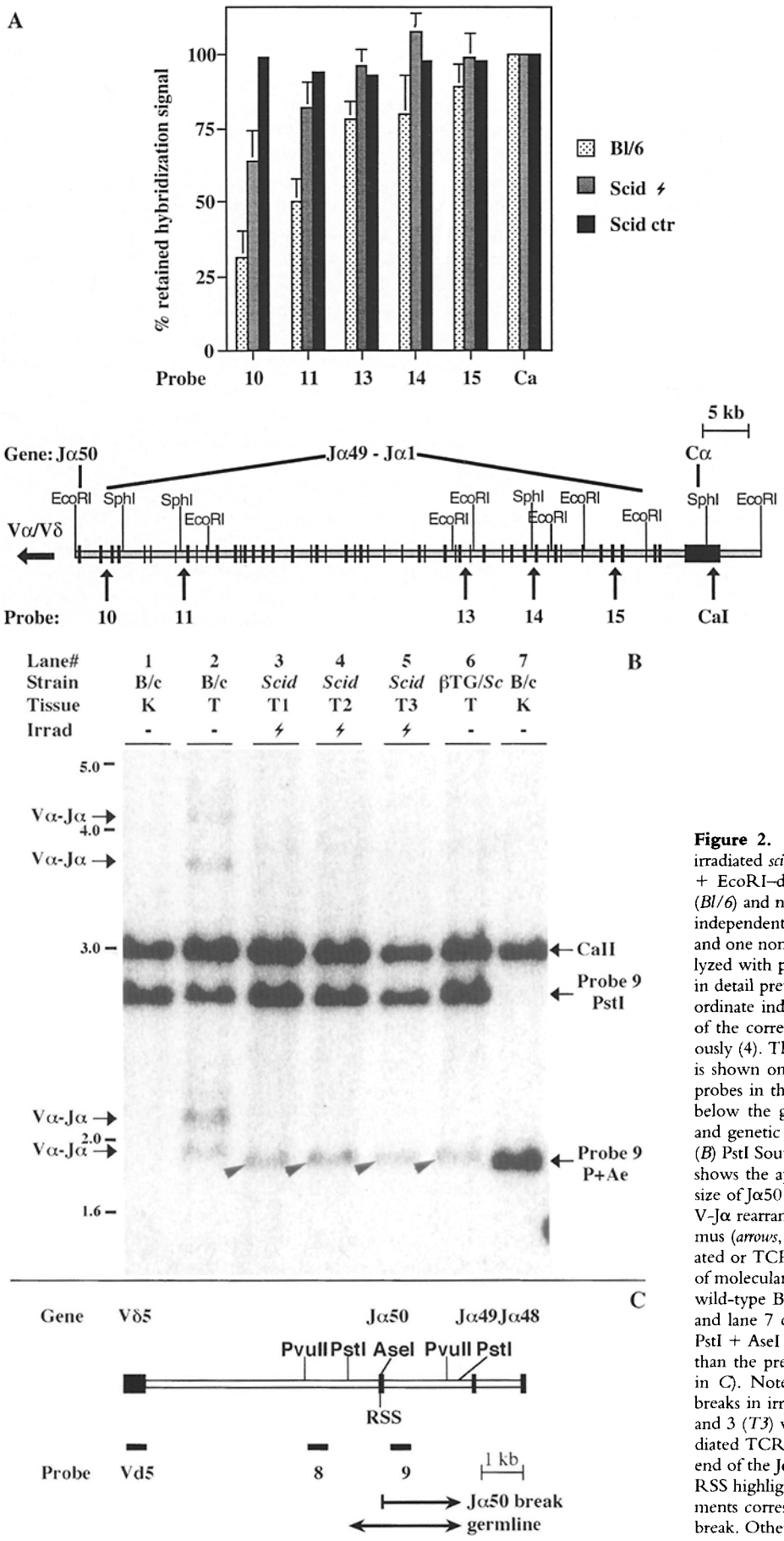

Figure 2. Initiation of TCR α gene rearrangement in irradiated *scid* thymocytes. (*A*) Graphical summary of SphI + EcoRI–digested Southern blots of wild-type C57Bl/6 (*Bl/6*) and nonirradiated or irradiated *scid* thymocytes. Four independent BL/6, seven independent irradiated 1-wk-old and one nonirradiated 2-wk-old thymus samples were analyzed with probes 10, 11, 13, 14, 15, and Ca as explained in detail previously (4). The abscissa shows the probes, the ordinate indicates the average relative hybridization signal of the corresponding probe calculated as explained previously (4). The positive standard deviation from the average is shown on the top of the columns. The location of the probes in the context of the murine J-Cα locus is shown below the graph along with the relevant restriction sites and genetic elements. Symbols are as in Fig. 1, *B* and *C*. (*B*) PstI Southern blot analysis of the 5′ end of the Jα locus shows the appearance of bands migrating at the predicted size of Jα50 coding end breaks (*arrowheads*) and the lack of V-Jα rearrangements present in normal BALB/c (*B/c*) thymus (*arrows*, *left*; compare lanes *2* with lanes *3–6*) in irradiated or TCR β transgenic *scid* mice. (*Dashes*, *left*) Position of molecular weight standards in kilobases. Lane *1* contains wild-type BALB/c kidney DNA (*K*) as germline control, and lane 7 contains the same DNA double digested with PstI + AseI to generate a marker band that is 10 bp smaller than the predicted full-length coding end break (see map in *C*). Note the similar appearance of Jα50 coding end breaks in irradiated *scid* thymus samples of 1 (*T1*), 2 (*T2*), and 3 (*T3*) wk after irradiation (lanes *3–5*) and in nonirradiated TCR β transgenic *scid* thymus (lane *6*). (*C*) The 5′ end of the Jα locus is shown with the position of the Jα50 RSS highlighted. (*Double arrows*) Size of the predicted fragments corresponding to germline and to Jα50 coding end break. Other symbols are as in Fig. 1, *B* and *C*.

observation of polyclonal, structurally normal V-D-Jβ rearrangements in IRNB *scid* mice (21) did not arise merely because of the strong selection for a functional TCR β protein.

In contrast to the diversity of TCR V-D-Jβ junctions identified from IRNB *scid* thymi (81% unique; 21), TCR δ rearrangements were oligoclonal (Table 1). Only 20 of 45 TCR δ sequences (44%) were unique, a somewhat surprising result given the relatively strong hybridization signal seen for V4- and V5-DJδ rearrangements after irradiation (2 and 5% of the total genomic hybridization intensity, respectively; Fig. 1, lane *4*). By contrast, our previous analysis of wild type V-D-Jδ rearrangements (4) found that >95% were unique (data not shown). In IRNB *scid* thymocytes oligoclonal V-D-Jδ rearrangements, representing 2–5% of the genomic loci, indicate that large numbers of double positive thymocytes containing the same V-D-Jδ rearrangement may arise from a limited pool of double negative precursors.

Coding End Molecules of the TCR α Locus in Irradiated scid Thymocytes. TCR α gene rearrangement normally initiates in double positive thymocytes (31) after productive TCR β gene rearrangement (32). Since irradiation of newborn *scid* mice leads to the appearance of normal β rearrangements followed by the emergence of large numbers of double positive thymocytes (21), it was of interest to determine the degree to which TCR α gene rearrangement was initiated in these cells. We first employed a quantitative multiprobe Southern blot analysis we have recently developed (4, 25) to assess the extent of rearrangements throughout the TCR α locus. Southern blots hybridized simultaneously to probes 10, 11, 13, 14, 15, and CaI (for details, see reference 4) were quantitated on a phosphorimager; the retention of hybridization intensity in the germline size band, after normalization for loading differences, was determined for each probe (Fig. 2 *A*). As expected, there was no significant TCR α gene rearrangement in nonirradiated *scid* mice. In contrast, in IRNB *scid* mice, ~30% of the 5′ end of the Jα locus had lost its germline configuration, whereas at the more distal 3′ Jα regions, there was no significant rearrangement and/or deletion.

To investigate the molecular structure of the α rearrangements in IRNB *scid* mice, we focused on the most 5′ Jα gene, Jα50 (33). We have recently demonstrated that this gene segment participates in a large number of diverse V-Jα rearrangements and also exhibits high levels of broken DNA molecules terminating in a recombination signal sequence (signal end molecules) in wild-type thymocytes (25). PstI Southern blots were hybridized to probe 9, which lies just downstream from Jα50 and defines a signal germline hybridization fragment spanning exclusively the Jα50 gene (for map, see Fig. 2 *C*). V-Jα rearrangements were identified by the appearance of novel nongermline fragments present in wild-type thymus but not in kidney DNA (25, and compare Fig. 2 *B*, lanes *1* and *2*). A fragment corresponding to potential Jα50 coding end breaks was also readily identified because double digestion of the germline DNA with PstI + AseI (Fig. 2 *B*, lane *7*) results in a restriction fragment only 10 bp shorter than a putative Jα50 coding end molecule (for map, see Fig. 2 *C*). No band of this size was visible in total wild-type thymus and the closest visible band has been shown to represent complete V-Jα (mostly Vα8 and Vα11) rearrangements (25). In contrast, *scid* thymocytes, 1, 2, or 3 wk after neonatal irradiation, exhibited one clear band migrating exactly at the position expected for a Jα50 coding end molecule (Fig. 2 *B*, lanes *3–5*, compare to the marker band in lane *7*). Thymocytes from nonirradiated TCR β transgenic *scid* mice also showed similar quantities of Jα50 coding end molecules (Fig. 2 *B*, lane *6*). None of these samples, however, contained visible bands corresponding to V-Jα rearrangements, as found in wild-type thymus. In separate experiments, we found Jα50 signal end molecules and reciprocal products in IRNB *scid* thymus samples (data not shown), consistent with previous results demonstrating the capacity of *scid* cells to form signal joints (34).

The lack of detectable V-to-Jα rearrangements and the appearance of a band at the size expected for Jα50 coding end molecules in irradiated *scid* thymocytes suggested that α rearrangements were being performed by a *scid*-type V(D)J recombination machinery. If this were the case, then the Jα50 coding end molecules from IRNB *scid* thymus should terminate in covalently sealed hairpin ends, as has been demonstrated for the Dδ and Jδ genes in nonirradiated *scid* thymus (12). To test this hypothesis, two-dimensional Southern blots were performed as described previously (12). Total thymus DNA from wild-type and from 9-d-old irradiated *scid* mice was digested with PstI and electrophoresed in a neutral agarose gel in the first dimension. Approximately half of each sample was then electrophoresed under alkaline conditions in the second dimension, Southern blotted, and hybridized to probe 9. Most DNA fragments are denatured into two separate strands in the second dimension and distribute along the diagonal of the gel. Fragments with hairpin termini, however, denature to form a single stranded molecule of twice the length of the double stranded molecule, and consequently migrate off the diagonal. No off diagonal bands were detected in wild-type thymus (Fig. 3), as expected (35). In IRNB *scid* thymus, however, the band migrating at the position of the Jα50 coding end molecules in the first dimension was located off the diagonal, migrating in the second dimension at a position corresponding to approximately twice its size in the first dimension (Fig. 3; represented by the gray dot B). As no band is visible at the corresponding position on the diagonal (Fig. 3; represented by open dot A), the majority of the Jα50 coding end molecules in the irradiated *scid* thymus are hairpin-terminated molecules.

Discussion

The exact mechanism of V(D)J recombination remains to be elucidated, but it now appears that the early steps in the process (DNA recognition and cleavage at recombination signal sequences) are catalyzed by the RAG1 and RAG2 proteins (36, 37), whereas later steps (end process-

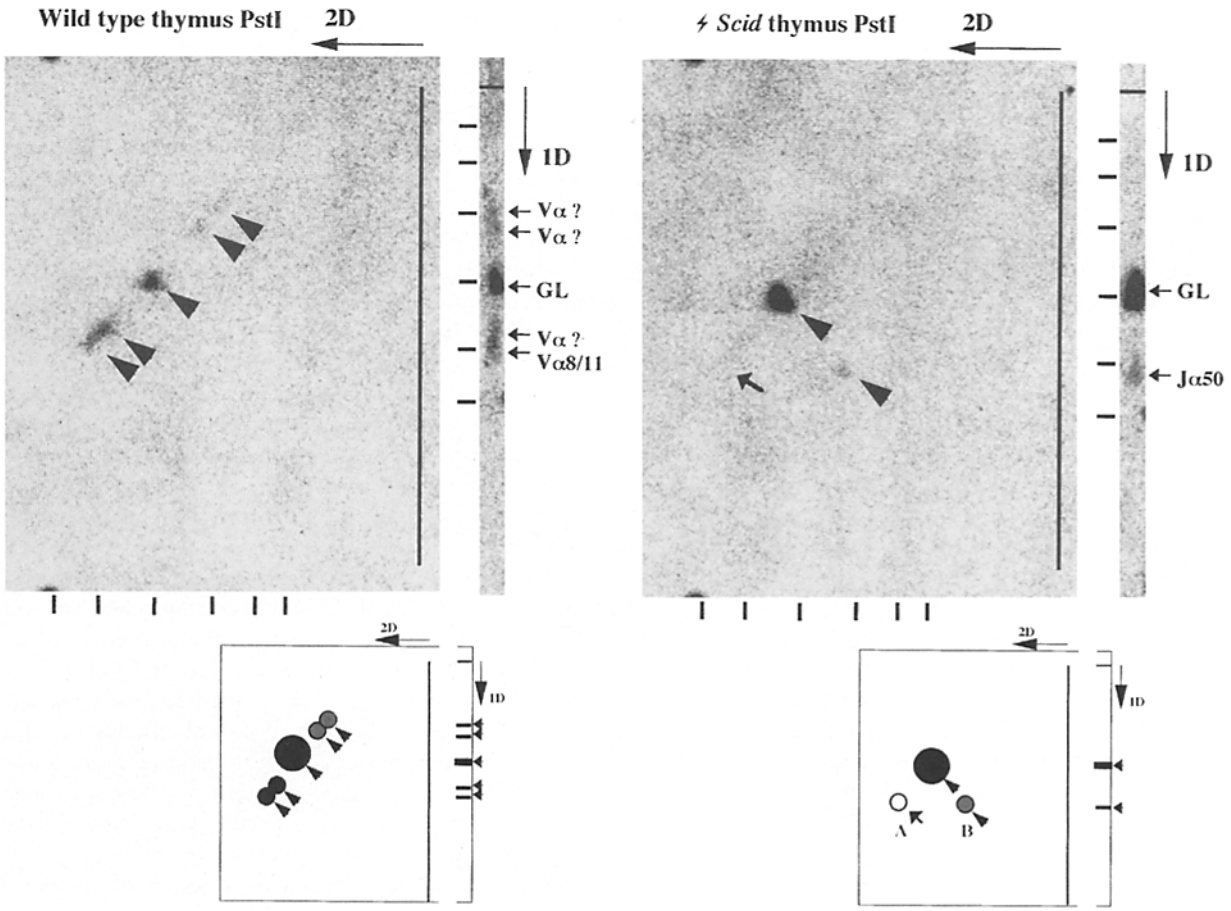

Figure 3. Two-dimensional Southern blot analysis of wild-type and irradiated *scid* thymus DNA. PstI-digested DNA from wild-type and from irradiated 9-d-old *scid* thymus was electrophoresed through a neutral agarose gel in the first dimension (*1D*), half of the samples were then separated in denaturing alkaline agarose gels in the second dimension (*2D*), transferred, and hybridized to probe 9. (*insets*) Schematic representation of the various signals obtained in the hybridization analysis. (*Arrows, sides*) Location of germline (*GL*), rearranged (V-Jα), and potential coding end breaks (*Jα50*) as they migrate in the first dimension. (*Arrowheads*) Position of the same bands after two-dimensional separation. Note the diagonal migration of V-Jα rearrangements in wild-type thymus as opposed to the irradiated *scid* sample, where the single nongermline band corresponding to the Jα50 coding end break migrates off the diagonal (see *arrowhead* and *gray dot B, inset*). No clear signal is visible on the diagonal in this sample (see *small arrow* and *open circle A, inset*), suggesting that the majority of Jα50 coding end breaks terminate in a hairpin (for details see text). Two-dimensional separation of kidney DNA double digested with PstI + AseI confirmed that a restriction fragment of similar size as the Jα50 coding end break would migrate in the diagonal (data not shown). (*Dashes*) At left of the 1D lanes and beneath the 2D blots, molecular weight standards in kilobases, as follows: 1.6, 2.0, 3.0, 4.1, 5.1, and 6.1.

ing and joining) require the direct or indirect involvement of the components of the DNAPK (18, 38). The *scid* mutation, which affects the catalytic subunit of this kinase, interferes both with the formation of proper coding joints of the antigen receptor genes (34) and with DNA double strand break repair, resulting in increased sensitivity to agents causing DNA breaks such as γ-irradiation (39–41). Aberrant V(D)J recombination in *scid* lymphocytes is manifested in three ways: (*a*) extensive deletions of coding sequences of Ig (13) and TCR (17) genes; (*b*) persistence of high levels of hairpin-terminated coding end molecules of the TCR δ locus (12); and (*c*) long P nucleotide additions at the junctions of the rare D2-J1δ (42, and our data) or V-Jγ (17) rearrangements. It has been suggested that the principal defect in *scid* V(D)J recombination is the inability to resolve hairpin coding end intermediates into coding joints (43). Instead, coding end molecules appear to be resolved either through generation of abnormal joints with long P nucleotides or loss of DNA sequences due to nuclease digestion or illegitimate recombination (44).

A principal goal of this study was to determine whether the effects of γ-irradiation on V(D)J recombination in *scid* thymocytes were TCR locus specific. The disappearance of δ coding end molecules and the identification of complete V-D-Jδ rearrangements, which are not selected for in double positive, α/β–lineage thymocytes (4, 30), indicate that irradiation permits productive V(D)J recombination to occur at multiple antigen receptor loci accessible at the time of irradiation. However, this effect is temporally restricted because in the TCR α locus, which is not rearranged to any significant extent before generation of double positive thymocytes (31), no complete V-Jα joints were detectable with our assay, and only rare TCR α VJC transcripts have been observed (21). Instead, we have shown accumulation

of hairpin-terminated Jα50 coding end molecules. The persistence of the Jα50 coding end molecules for several weeks, coincident with the loss of V-D-Jδ alleles (see below), suggests that initiation of TCR α rearrangement continues at substantial levels throughout this time.

It appears that only a limited number of IRNB *scid* thymic precursors perform correct rearrangements at the TCR δ locus. Irradiation results in rearrangement of a substantial fraction of *scid* TCR δ alleles, with the TCR δ germline hybridization signal in the irradiated *scid* thymus approaching the levels seen in normal α/β T cells (Fig. 1 *A*, lanes *4* and *5*). At 1 wk after irradiation, the reduction in germline δ alleles is accompanied by the appearance of readily detectable bands corresponding to V-D-Jδ rearrangements. However, these bands do not accumulate to the extent seen in wild-type thymocytes, and they show a substantial decline in intensity by 2 wk after irradiation (Fig. 1 *A*, lane *5*). In addition, V-D-Jδ rearrangements cannot be detected 3 wks after irradiation, despite the fact that the hybridization intensity of the germline band remains low (data not shown). Importantly, the V4- and V5-D-J1δ rearrangements appear to be oligoclonal, even at 1 wk after irradiation (Table 1), and only 42% of the examined V-D-Jδ rearrangements were unique. This contrasts with the previously reported diversity (81%) of TCR β joints observed after irradiation, (21), and with our previous finding of >95% diversity of TCR δ joints in normal thymocytes (data not shown). Collectively, these data are consistent with the hypothesis that irradiation allows normal V-D-Jδ and V-D-Jδ rearrangements to occur independently in relatively few individual precursors. Since productive TCR β but not TCR δ rearrangements drive expansion of these precursors (32) it is likely that only a fraction of TCR β^+ thymocytes will contain TCR δ gene rearrangements. The limited effect of irradiation on precursor cells might be due to a short period of time during which TCR β or δ alleles can be rearranged normally, and/or to a poor survival of irradiated cells.

A combination of several factors may explain the disappearance of bands corresponding to V-D-Jδ rearrangements at longer time points after irradiation. First, because the effect of irradiation on V(D)J recombination is transient, no new V-D-Jδ rearrangements are generated over time. Second, *scid* double positive thymocytes initiate a substantial amount of TCR α rearrangement (Fig. 2, *A* and *B*), and each such event places the δ locus on an extrachromosomal circle or on a signal end molecule (4, 45, and data not shown). These can be physically lost from the cells, particularly if cellular division occurs. Third, the population of thymocytes in an irradiated *scid* animal becomes oligoclonal over time, and all such mice eventually develop thymic lymphomas (21). Oligoclonality of TCR β rearrangements is clearly manifest on Southern blots by 6–8 wk after irradiation, and the reduction in cellular heterogeneity, presumably caused by proliferation of (pre-) malignant cells, was likely underway some time before this.

The mechanism by which γ-irradiation allows the production of normal V(D)J recombination products in *scid* thymocytes has not been determined. It might directly affect the V(D)J recombination machinery or result in increased production of normal rearrangement products through an independent mechanism that does not involve the *scid* gene product. In the former case, induction of either functional DNAPK or some alternative pathway would allow normal V(D)J recombination to occur. Alternatively, irradiation might significantly extend the G1 phase of cell cycle which in turn could increase the production of complete rearrangements by allowing more time for the resolution of coding end breaks. Whatever the effect of γ-irradiation on V(D)J recombination, our data demonstrate that it is temporally restricted, but not locus specific. It remains to be seen whether the *scid*-type initiation of TCR α rearrangements can similarly be corrected with subsequent exposures to γ irradiation, thereby allowing the generation of significant numbers and diversity of mature α/β T cells in *scid* mice.

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Note added in proof: While this work was under review, similar results on normal V-D-Jδ rearrangements and generation of TCR α coding end molecules after γ irradiation of *scid* mice were reported (Bogue, M.A., C. Zhu, E. Aguilar-Cordova, L.A. Donehower, and D.B. Roth. 1996. p53 is required for both radiation-induced differentiation and rescue of V(D)J rearrangement in *scid* mouse thymocytes, *Genes & Dev.* 10: 553–565).

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