T Cell Receptor γ and δ Gene Junctional Sequences in SCID Mice: Excessive P Nucleotide Insertion
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Summary
The severe combined immunodeficiency (SCID) mutation has been postulated to affect a V(D)J recombinase activity involved in coding joint formation. Analysis of 38 joints from 34 distinct sequences of normally rearranged T cell receptor (TCR) 3' and J genes from adult, SCID thymocytes reveals coding joints with an increased number of P nucleotides. One-third of P sequences are >4 nucleotides in length and P elements of up to 15 bases are observed. This suggests that the SCID defect deregulates P nucleotide addition. Consequently, essential V(D)J recombination intermediates may seldom be generated.

Young adult mice homozygous for the SCID mutation are severely deficient in mature B and T cells (1). Observation of abnormal antigen-receptor gene rearrangements in virally transformed SCID bone marrow cells and spontaneous SCID thymic lymphocytes suggested initially that this might be due to defective V(D)J recombinase activity in these mice (2). Sequence analysis of numerous abnormal SCID Ig (3-7) and TCR (8) rearrangements, as well as studies with recombination substrates (9-11) have subsequently shown that C.B-17 scid/scid mice contain a V(D)J recombinase that recognizes recombination signal sequences adjacent to variable region gene segments (V, D, and J), cleaves the DNA normally at the borders of the variable region gene segments, ligates the signal sequences at a normal frequency, but fails to recombine the V, D, and J gene segments at any frequency comparable to that in normal cells. Further, V(D)J joints frequently result in deletion of one or both of the participating coding elements (2-7).

Recently, while studying natural killer cells and T cell progenitors in the thymus of SCID mice, we obtained evidence for full-length, potentially productive, TCR γ chain transcripts (12). Southern blot analyses revealed normal rearrangements at the TCR γ locus and PCR amplification, cloning and DNA sequencing were performed to capture TCR γ and partner chain, δ, gene junctional sequences with small deletions in the coding segments. Nucleotide sequences reveal that many characteristics of SCID V(D)J recombination are indistinguishable from normal V(D)J recombination (Kienker, L. J., W. A. Kuziel, B. A. Garni-Wagner, V. Kumar, and P. W. Tucker, manuscript submitted for publication), but P nucleotide addition is different. P nucleotides are newly distinguished inserted bases at coding joints that form inverted repeats of the neighboring gene segment termini (13). Presumably, there are up to 2 P nucleotides at the ends of junctional inserts and these occur when the associated gene segment appears in full in the coding joint (13). Here we show that SCID coding joints have an excessive number of P nucleotides in junctional inserts. This suggests that the SCID defect deregulates the addition of P nucleotides leading to the frequent failure to form this essential V(D)J recombination intermediate.

Materials and Methods
Mice. C.B-17/Icr scid/scid mice, originally obtained from Dr. Melvin Bosma, Fox Chase Cancer Center (Philadelphia, PA), were bred and maintained under specific pathogen-free conditions in the barrier facility of the University of Texas Southwestern Medical Center at Dallas. SCID mice were housed in microisolator cages containing sterilized food and water and were 4-6 wk when sacrificed.

DNA Amplification by Polymerase Chain Reaction. Genomic DNA was prepared three times from the pooled thymocytes of five mice each time according to the method of Scott et al. (14). DNA from any one preparation (1 μg) was amplified by the PCR (15) in a reaction buffer consisting of 50 mM KCl, 10 mM Tris (pH 8.4), 2 mM MgCl₂, 100 μg/ml gelatin, 0.05 μM each primer of an appropriate pair, 0.16 mM each deoxynucleotide triphosphate, and 0.5 U of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). Reaction volumes were 100 μl under 60 μl of light mineral oil. The reactions were subjected to 45 cycles of denaturation (1 min 94°C), primer annealing (2 min at the appropriate temperature) and extension (3 min at 72°C). After the 45th cycle, the extension at 72°C was prolonged for 10 min. Temperature cycling was carried out in an automated heating/cooling block (DNA Thermal Cycler; Perkin-Elmer Cetus). One-tenth to one-fifteenth of each reaction was then analyzed in a composite gel of 3% Nusieve agarose and 1% Seakem agarose (FMC BioProducts, Rockland, ME).

Vγ1-Jγ, Vγ2-Jγ, Vγ5-Jγ, and the first sample of Vγ3-Jγ junc-
tional sequences were derived from the first preparation of thymic DNA. The second sample of Vy3-Jy sequences and all of the Vy4-Jy sequences were derived from the second preparation of thymic DNA. The third preparation of DNA was used to derive all of the Vδ junctional sequences.

**Polymerase Chain Reaction Primers.** Jy1: 5'-TATAAGATTCAGGGAGATTAGTCTAGACGCT-3'; Vy1 Subfamily: 5'-CTTGTGATTCCAATGTTAGAGAAGAT-3'; Vy2: 5'-ATTAGATTACCAATGTTAGAGAAGAT-3'; Vy3: 5'-ATATGGAATTCATCAGTTATGACAATATAATC-3'; Vy4: 5'-TAATGGAATTCATCAGTTATGACAATATAATC-3'; and Vy5: 5'-TAAAGATTCATCAGTTATGACAATATAATC-3'. The Jy1 primer hybridizes to all known Jy gene segments. The Vy1 subfamily primer hybridizes to the Vy1.1, Vy1.2 and Vy1.3 gene segments.

| Vγ | Vδ | Jy | Vδ-Jy | Vγ-Jy |
|----|----|----|-------|-------|
| γ1 | δ1 | J1 | 1-4   | 5-8    |
| γ2 | δ2 | J2 | 9-11  | 12-15  |
| γ3 | δ3 | J3 | 16-19 | 20-23  |

**Results**

TCR γ and δ rearrangements were amplified from three thymocyte DNA preparations from 4-6-wk-old SCID mice using the PCR technique. Primers hybridizing to Vγ gene segments 34-54 bp upstream of the Vγ signal heptamer were used individually with a primer that cross-hybridizes with all 4 Jy gene segments 40 bp downstream of the Jy signal sequence to amplify Vγ-Jy junctions. Vδ-Jy junctional sequences were amplified using oligonucleotide primers specific for seven of the eleven Vδ subfamilies and either Jδ1 or Jδ2.

**DNA Sequencing.** After amplification, the overlying oil was removed and PCR reaction mixtures were extracted once each with equal volumes of phenol-chloroform (1:1) and chloroform. A portion of the PCR products were digested with the appropriate restriction enzyme and ligated into either M13mp18 or PUC19. Purified single-stranded or double-stranded DNA's from randomly selected plaques or colonies were then sequenced by the dideoxy chain-termination method (19) using a Sequenase kit (United States Biochemical Corporation, Cleveland, OH) according to the manufacturer's instructions.

![Figure 1](image)

**Figure 1.** Nucleotide sequences of Vγ-Jy junctions from thymocytes of 4-6-wk-old SCID mice. (A) Rearrangements of Vy1 to Jy. (B) Rearrangements of Vy3 to Jy1. (C) Rearrangements of Vy4 to Jy1. (D) Rearrangements of Vy2 to Jy. (E) Rearrangements of Vy5 to Jy. Germline gene segment sequences, with heptamer and nonamer sequences marked, are illustrated at the top of each section (Vy1.2 and Vy1.3 gene segments, (13, 21); Vy2 and Vy3 sequences, (13, 21); Vy4 sequence, (21); Vy5 sequence, (8, 22); Jy1 and Jy2 sequence, (20); and Jy3 sequence, (8, 23)). Bases in the junctional sequences identical to germline are indicated by dashes; nucleotide differences are noted. These mutations are either somatically introduced or due to an artifact of PCR. Junctional sequences are assigned a number given in the first column and the last column lists the number of observations for seven of the eleven Vδ subfamilies and either Jδ1 or Jδ2.
In total, 56 TCR γ and 22 TCR δ rearranged genes were sequenced. The entire collection is presented in another paper (Kienker, L. J., W. A. Kuziel, B. A. Garni-Wagner, V. Kumar, and P. W. Tucker, manuscript submitted for publication). Only those sequences relevant to the issue of P nucleotide addition are presented here. These include sequences which definitely have a coding element in full as well as those which might contain a coding element in full depending upon how the sequence was generated (there are shared nucleotides at the ends of some germline and potential P regions). Fig. 1 shows the γ junctional sequences and δ junctional sequences are shown in Fig. 2. In the figures, assignment of nucleotides that are common to the ends of coding elements or potential P regions were made to maximize the number of P sequences within the joint. However, in analyses of characteristics of P sequences performed below, variations of the TCR γ and δ junctional sequences were considered such that the importance of any feature examined would be minimized. Further in these analyses, although the sample size is dramatically reduced, closely related junctional sequences from the same PCR reaction were grouped together as an identical sequence (e.g., 14, 16, 18, 20, 21, 23, and 25, Fig. 1), and the occurrence of any particular sequence was considered to be one regardless of the observation frequency of that sequence.

Inspection of the SCID TCR γ and δ coding joints in Figs. 1 and 2 shows firstly that the terminals of all Vγ gene segments, Jγ1-3, and the 5' end of Dδ2 have P nucleotides at their junctions. Calculation of the percentage of coding joints with a particular variable region gene segment in its full sequence that have corresponding P nucleotides as junctional inserts (Table 1) shows that P nucleotides are more likely to derive from the 3' recombining element. Furthermore, strengthening the notion that these nucleotides are not really N nucleotides, P nucleotides are not G-C rich. Analysis of the base composition of SCID P sequences shows that A and T are added at a minimum 1.7 times as often as G and C, in accord with previous observations (13, 20, 23).

In contrast to earlier studies on normal mouse thymocytes

Table 1. Percentage of Coding Joints with a Complete TCR Variable Region Gene Segment that Have Corresponding P Nucleotides as Junctional Inserts

| Gene segment | Maximum percentage* | Minimum percentage† | Length of P sequence (bp) |
|--------------|----------------------|---------------------|--------------------------|
| Vγ           | 72.7 (8/11)          | 25.0 (5/20)         | 1–7                      |
| Jγ           | 100.0 (16/16)        | 77.8 (14/18)        | 1–15                     |
| Vδ           | 0.0 (0/4)            | 0.0 (0/4)           | 0                        |
| Dδ (5' end)  | 100.0 (6/6)          | 100.0 (6/6)         | 1–4                      |
| Dδ (3' end)  | NA*                  | NA                  |                          |
| Jδ           | NA                   | NA                  |                          |

* Calculation is based only on the junctional sequences that definitely contain the gene segment in question in full, and the possible P sequences in those rearrangements derived from the gene segment.
† Calculation is based on all the junctional sequences that could possibly contain the gene segment in question in full, and only definite P sequences in those rearrangements derived from the gene segment.

NA: Not applicable. No sequences containing the gene segment in question in full were observed.

Figure 2. Nucleotide sequences of Vδ-Dδ and Vδ-Dδ-Jδ junctions from thymocytes of 4–6-wk-old scid mice. (A) Rearrangements of Vδ1-Dδ2. (B) Rearrangements of Vδ4-Dδ2-Jδ1. Germline gene segment sequences, with heptamer and nonamer sequences marked, are illustrated at the top of each section (Vγ sequence, (25); Vδ sequence, (16); Vδ2 sequence, (25); Jδ1 sequence, (25, 26)). (A) and (C) junctional sequences were each derived from a single PCR. (B) Junctional sequences were derived from 2 PCRs. Asterisks denote sequences obtained from the second PCR (see Materials and Methods). 'X' denotes ambiguous bases. See legend to Fig. 1 for further detail.
the number of P nucleotides in SCID TCR γ and δ junctions can be large. The P nucleotide model of V(J) or V(D)J joining accounts for 2 P nucleotides at the ends of junctional inserts (13). In previously reported sequences of TCR γ and δ genes from normal mice, cases of 4 to 5 P nucleotides are occasionally evident (13, 27). SCID junctions frequently contain long P sequences. Minimally, one-third of the P sequences observed have ≥4 nucleotides. P elements of 7 (Fig. 1, 44) and 15 (Fig. 2, 46) are unprecedented. This suggests that the SCID defect affects the number of P nucleotides added during this intermediate V(D)J joining stage. The possibility also exists that P sequences are altered during inversion to the opposite strand. In 3 sequences (Fig. 1, 9, 10, and 44) the number of P nucleotides in the joint could be increased if a single base mismatch was tolerated in the P sequence. Nucleotide replacement may thus be occurring in SCID mice during P nucleotide addition.

Discussion

Current proposals for the SCID defect focus on steps unique to the process of coding joint formation (3, 9, 10). A recently proposed model of V(D)J joining incorporates a new step unique to coding joint formation - P nucleotide addition (13). According to this model, following cleavage of the recombining gene segments at the signal heptamer borders, the terminal dinucleotides from the 5' strands of either recombinant terminal are obligatorily nicked, inverted, and joined to the 3' ends of the other strands. This creates recombinant termini with protruding single-strands composed of a tetranucleotide palindrome.

The important observation with regard to the TCR variable region junctional sequences in this report is the excessive number of P nucleotides in junctional inserts. One explanation is that the activity responsible for nicking the terminal nucleotides from the 5' strands, before they are inverted and ligated to the 3' strands, is deregulated. Consequently, nucleotide nicking occurs randomly on the 5' strands. It is conceivable that if the 5' ends are nicked too far back, that nucleotides will fail to be inverted and ligated to the 3' strands. Generation of 3' protruding single-stranded tails may be an essential step in the recombination process (13, 28–31). Alternatively, if joining of the terminal bases from the 5' ends to the 3' ends of the recombinant termini precedes the inversion of these bases, and if inversion never occurs, then recombinant ends may simply be blocked for joining. Regardless, this model predicts that the frequency of coding joint formation will be low due to the frequent inability to form a necessary intermediate V(D)J joining structure. Furthermore, among coding joints that are formed, the number of P nucleotides derived from any recombinant terminus will vary within the tolerated number. It is important to note that the SCID defect does not affect the polarity of the nicking activity. This is apparent from the lack of recombination breakpoints 3' of Vγ and Vδ segments, and 5' of intended joining partners (Dδ2 or the corresponding Jγ within a given V-J-Cγ gene cluster) (Kienker, L. J., W. A. Kuziel, B. A. Garni-Wagner, V. Kumar, and P. W. Tucker, manuscript submitted for publication).

We favor the above model over the suggestion that the SCID mutation affects a sequence nonspecific DNA binding protein responsible for juxtaposing and/or ligating recombinant ends in coding joints (3) because P nucleotide addition is obviously faulty in SCID mice. This is evident mainly from our data, but Ferrier et al. (11) also noted longer than normal P nucleotide additions in one of six VH-to-DJH recombinations sequenced following transfection of a recombination substrate into transformed SCID pre-B cells. Finally, while this manuscript was under revision, Schuler et al. (8) reported P nucleotide additions of unusual length at the junctional border in five of eight TCR γ coding joints sequenced from SCID T cell lymphomas. Recently, it has been shown that SCID myeloid cells and fibroblasts have an increased sensitivity to ionizing radiation suggesting that the SCID mutation affects an enzyme involved in DNA repair as well as rearrangement of lymphoid antigen receptor genes (32). P nucleotide addition may therefore be a process utilized not only in V(D)J recombination, but also in repair of radiation damage.
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