Severe Combined Immunodeficiency (SCID) in Man: B Cell–negative (B^-) SCID Patients Exhibit an Irregular Recombination Pattern at the J_H Locus

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Summary

Human severe combined immunodeficiency (SCID) patients were analyzed by a polymerase chain reaction assay for their recombination capability at the D_{HQ52}-J_H region of the immunoglobulin heavy chain locus. Five patients with B cells (B^+ SCID) exhibited a recombination pattern also observed in healthy persons. In contrast, six patients lacking B cells (B^- SCID) showed a grossly altered rearrangement pattern characterized by the (partial) absence of regular D_{HQ52}-J_H recombinations and the presence of abnormal rearrangements. These events were caused by deletions surpassing the boundaries of immunoglobulin coding elements and thus resemble the pattern of deletional recombinations previously described in SCID mice.

Human SCID is a rare, heterogeneous congenital disorder of the lymphatic system (1, 2). Affected infants are characterized by severe and persistent infections due to the impaired function of B and T lymphocytes. Without bone marrow transplantation the patients usually die before the age of two years (1).

In approximately 15–20% of patients, SCID is associated with a deficiency of the enzyme adenosine deaminase (ADA)[1]. Additional immunodeficiencies are characterized by abnormalities of the enzymes purine nucleoside phosphorylase (PNP) and ecto-5′-nucleotidase (3).

Rare cases of SCID can also result from the inappropriate expression of restriction elements of the HLA loci (4–6), defective IL-2 production (7, 8), the failure of T cells to respond to IL-1 (9), impairments in the T cell receptor/CD3 complex (10, 11), abnormalities of the cytoskeleton and from disturbances of signal transduction pathways within T cells (12). Frequently, human SCID is inherited as a X-linked, recessive trait (McKusick no. 30040 SCID X1), characterized by the absence of mature T cells and normal or elevated numbers of B cells (13). The SCID subentities mentioned above all bear detectable numbers of B cells in their peripheral blood. However, occasionally patients have been reported in which B and pre-B cells are completely absent (B^- SCID), suggesting a distinct type of SCID with a profound defect of the lymphocyte development (14).

One of the crucial steps for the development of functional lymphocytes is the correct joining of distinct subgenic elements (V(D)J recombination) to generate coding sequences for Ig or TCR variable regions (15, 16). The joining process is mediated by signal sequences (RSS: recombination signal sequences) flanking each coding element. The juxtaposition of two coding elements is usually associated with modifications at the junctional region, due to the loss of a few bases at the coding ends and de novo insertion of nucleotides (N-nucleotides), most likely mediated by the enzyme terminal desoxynucleotidyl transferase (TdT) (17). The process of N-region insertion is developmentally regulated in B and T cells, with the number of N-nucleotides included in the junctional region increasing with time after birth (18–20). Alternatively, extra bases may represent inverted repeats (pallindromes) at the termini from the adjoining coding segments (P-nucleotides) (18, 19).

The rearrangement of the V, (D) and J elements is tightly controlled, occurs in a preferential order (e.g., D_HJ_H before $V_HD_HJ_H$ recombinations, subsequently V_LJ_L recombination) and is lineage specific (TCR loci are usually not completely rearranged in B cells and vice versa) (21).

The recombination process is thought to be mediated by an enzymatic machinery (V(D)J "recombinase") which is common to B and T cells (22).

A mouse model in which the correct assembly of the subgenic V, (D), J elements is defective, has been described (23).

[1] *Abbreviations used in this paper:* ADA, adenosin deaminase; DTH, delayed type of hypersensitivity; PNP, purine nucleotide phosphorylase; RSS, recombination signal sequence; TdT, terminal desoxyribonucleotidyl transferase.

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This autosomal recessive SCID mutation (24) adversely affects early lymphoid differentiation. Thus young mice, homozygous for SCID, lack functional T and B lymphocytes and phenotypically resemble B^- SCID patients. However, transformed cell lines which have a phenotype characteristic of immature lymphocytes can be obtained from SCID mice. The crucial finding made in those transformed lymphocytes was that rearrangements (on the IgH and TCRβ loci) often deleted the entire J cluster and extended into the flanking regions (25–29). Recent analysis of the TCRγ (30) and Igκ loci (31) confirm these findings. These studies support that the recombinase system in SCID mice is capable of cleaving the DNA at the correct position (between RSS and coding elements) and joining the signal elements, but fails to mediate the formation of normal coding joints (31, 32). It remains to be established whether correct rearrangement events observed in older SCID mice are due to spontaneous revertants or rather reflect a low-frequence "leakiness" of the mutated recombinase (33) and whether the murine SCID mutation is caused by a more general defect in DNA repair (34).

In this report, we have examined the rearrangement capability of B cell negative (B^-) and B cell positive (B^+) human SCID patients. Since in contrast to the murine SCID model, the establishment of transformed cell lines poses a major problem, we used a sensitive PCR assay to characterize endogenous D_{HQ52} to J_H rearrangements. We conclude from these experiments that the B^- SCID patients are characterized by an impaired rearrangement process at the J_H region analogous to the defect in SCID mice.

Materials and Methods

Patients. SCID was diagnosed according to the criteria defined by the World Health Organization classification (2). These included radiological absence of a thymus shadow, severely reduced or undetectable Ig levels, severely impaired cellular immune functions, and negative delayed type of hypersensitivity (DTH) reactions following immunization. ADA and PNP activities were normal. The clinical and immunological characterization of the patients is outlined in Table 1. Antibodies and immunofluorescence techniques for the phenotypic analyses have been described elsewhere (35). On the basis of the expression of the CD20 marker on PBMC cells the patients were subdivided into a B cell positive group (five patients) and a B cell negative group (six patients). All patients except one (patient 7, T.C.) exhibited various amounts of CD3 positive cells, i.e., T lymphocytes. In vitro cultures of PBMC on feeder cells and with rIL-2 showed the presence of CD3 positive cells in five of eight patients. In B^- SCID patients these cells were characterized by serological HLA typing as exclusively of maternal origin (35). Informed consent was obtained from the parents for all diagnostic and therapeutic procedures. Samples of healthy bone marrow or blood donors served as control.

Cell Isolation. Mononuclear cell fractions from bone marrow aspirates and peripheral blood were obtained by density gradient centrifugation over Ficoll-Hypaque (1.077 g/ml; Nycomed, Oslo, Norway).

Southern Blot Analysis. High mol wt DNA was prepared from the isolated cells according to a previously published protocol (36). To investigate IgH gene configuration, genomic DNA (10–15 μg) was digested with Bgl II or Hind III (Pharmacia, Freiburg, Germany), electrophoresed through a 0.8% (w/v) agarose gel, transferred to nylon membranes (Schleicher and Schuell, Dassel, Ger-

Table 1. *Clinical and Immunological Characterization of Severe Combined Immunodeficiency (SCID) Patients*

					Phenotype*					
			Sex	Age‡	CD20	CD3	TCRα/β	CD15	CD16	Maternal T cells§
B^+ SCID	1	D. B.	m	8 mo	18	14	14	25	39	+
patients	2	D. D.	m	1 d	65	7	ND	ND	7	ND
	3	K. K.	m	17 mo	20	0.2	ND	31	1	ND
	4	F. P.	m	1 d	27	5	ND	54	2	–
	5	M. Z.	m	4 mo	26	1	ND	ND	0	–
B^- SCID	6	V. M.	f	1 d	0	45	38	36	12	+
patients	7	T. C.	m	2 mo	0	0	0	34	14	–
	8	P. P.	f	1 mo	0	67	73	5	26	+
	9	J. B.	m	1 mo	0	44	48	32	17	ND
	10	J. J.	m	3 mo	<1	70	64	8	19	+
	11	S. K.	m	6 mo	0	3	2	3	67	+

* Percentage of PBMC which were stained by indirect immunofluorescence.
‡ The age of the patients at diagnosis.
§ Maternal T cells are present (+) or absent (–) in an in vitro culture system of PBMC (35).

many) and fixed by UV crosslinking. Before hybridization, probes were radiolabeled by hexanucleotide priming (37). Washing of the membranes included a high stringency step at 63°C in 0.1 × SSC/0.1% SDS.

Polymerase Chain Reaction. PCR was essentially performed as suggested by Saiki et al. (38). A 100 μl reaction mixture contained 0.5–1 μg of genomic DNA (when indicated, digested with BamH I, Bgl II, or EcoN I), 50 pmol of each 5′ and 3′ oligonucleotide primer, 200 μmol/l dATP, dCTP, dGTP, and dTTP, 10 mmol/l Tris-HCl pH 8.3, 50 mmol/l KCl, 2.5 mmol/l $MgCl_2$ and 0.001% (w/v) gelatine. The reaction mixture was exposed for 10 min to 302 nm UV light on a Vilber Lourmall transilluminator (Bachhofer, Reutlingen, Germany) before the addition of genomic DNA. Oligonucleotide primers are listed below (see: Oligonucleotides) and their position within the J_H region is depicted in Fig. 1. After an initial denaturing step of 10 min at 95°C, 1.5 U Amplitaq polymerase (Cetus Corp., Norwalk, CT) and 1 μl of gp32 (Pharmacia) (39) were added and 30 amplification cycles were run in an automatic PCR processor (Bio-Med, Theres, Germany). Following the initial denaturation, the melting, annealing and extension steps were performed at 94°C for 2 min, 56°C for 90 s and 72°C for 5 min, respectively. The final cycle was followed by an additional extension for 10 min at 72°C.

10% of the amplification products were separated on an agarose gel (0.8% or 1.2%) and studied by Southern blot analysis.

Isolation, Cloning and Sequencing of Amplified DNA. 20–50% of the amplified DNA was cleaved with appropriate restriction enzymes (Pharmacia) to allow for directional cloning, ligated into pBSKSM13+ (Stratagene, Heidelberg, Germany) or pT7T3U18 (Pharmacia) vectors and transformed into RR1lacZΔM15 *Escherichia coli.*

White colonies were picked and rescreened by colony hybridization with oligonucleotides 3, 4, or 5 (see below).

Plasmid DNA for sequence analysis was isolated from each recombinant clone via alkaline lysis. Sequencing followed the dideoxy chain termination method described by Sanger (40) using T7 DNA polymerase according to the instructions of the manufacturers (US Biochemical, Cleveland, Ohio) (Pharmacia).

Evaluation of the sequences was done with version 6.2 of the University of Wisconsin Genetics Computer Group (GCG) sequence analysis package (41).

Probes. For hybridization studies we used two probes, each covering part of the Ig-J_H locus. Probe a (Fig. 1) is a 2.5 kb fragment starting 5′ of J_{H3} and ending at the Bgl II site of the Cμ enhancer (42). A second probe (probe b, Fig. 1) was generated by PCR amplification of undigested genomic DNA using oligonucleotides 2 and 6. A plasmid containing the germline configuration of the D_{HQ52}-J_H region was obtained after cloning and colony hybridization. A BamH I/Nco I digest yielded a 1.1 kb fragment which spans the region from oligonucleotide 2 to base 1103. Nucleotide numbering follows a published sequence of the J_H locus (43).

Oligonucleotides. Synthetic oligonucleotides were prepared according to published sequences (43). The oligonucleotides were used for hybridizations (H), PCR (P), or sequence analyses (S).

Oligonucleotide 1: (P), 5′ ACC CAG CAC TGG TGG ACA C; Oligonucleotide 2: (P), an additional BamHI site (underlined) for cloning was synthesized. 5′ GAC GGA TCC AGT GGG ACG ACG GTG AAC; Oligonucleotide 3; (H, S), 5′ CCT ACC AGC CGC AGG GT; Oligonucleotide 4: (H), 5′ GCG CTA CAA AAA CCA TGC TCC; Oligonucleotide 5: (H, S) 5′ CGT GGT CCC TTG CCC CCA GAC; Oligonucleotide 6: (P), an additional Sal I site is underlined. 5′ GCC GTC GAC GGC AGT AGC AGA AAA CAA AGG.

In addition, we used T_3 and T_7 oligonucleotides (Pharmacia) for sequence analyses.

Results

D_{HQ52}-J_H Polymerase Chain Reaction Assay. We decided to make use of the enormous sensitivity of the PCR technique for the detection of D_{HQ52}-J_H associated rearrangements. The assay was designed such that one primer pair could amplify the germline band and all six possible D_{HQ52}-J_H rearrangements simultaneously (the design of the test system and expected rearrangements are outlined in Fig. 1). In addition, by placing the 5′ PCR oligonucleotide(s) about 500–600 bp upstream of D_{HQ52}, we could also detect possible deletion events. The fragments representing D_{HQ52}-J_H associated recombination events were visualized by Southern blot analysis.

We initially amplified the bone marrow DNA of healthy donors. Fig. 2 *A* is the result of one representative experiment. Amplification products of expected size are visible and represent a "ladder" of D_{HQ52}-J_H rearrangements. Reproducible amplifications especially of the larger products were only achieved after inclusion of gp 32 in our PCR approach (39).

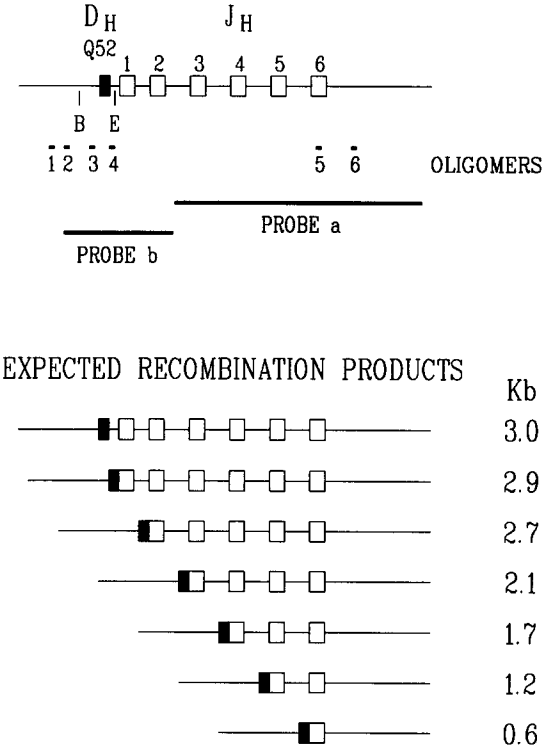

Figure 1. Schematic drawing of the strategy used for the amplification of possible D_{HQ52}-J_H recombination events. A sketch of the D_{HQ52}-J_H locus is shown; pseudo J_H elements are omitted for the sake of clarity. The position of the oligonucleotides used is indicated, as well as the localization of probe a and probe b. Abbreviations of restriction enzymes are: B, Bgl II; E, EcoN I. Rearrangement patterns and expected sizes of the recombination products at the D_{HQ52}-J_H locus are presented. The sizes of the PCR products were calculated for oligonucleotides 2 and 6. When oligonucleotide 1 is used as 5′ primer, the respective sizes will increase by about 100 bp.

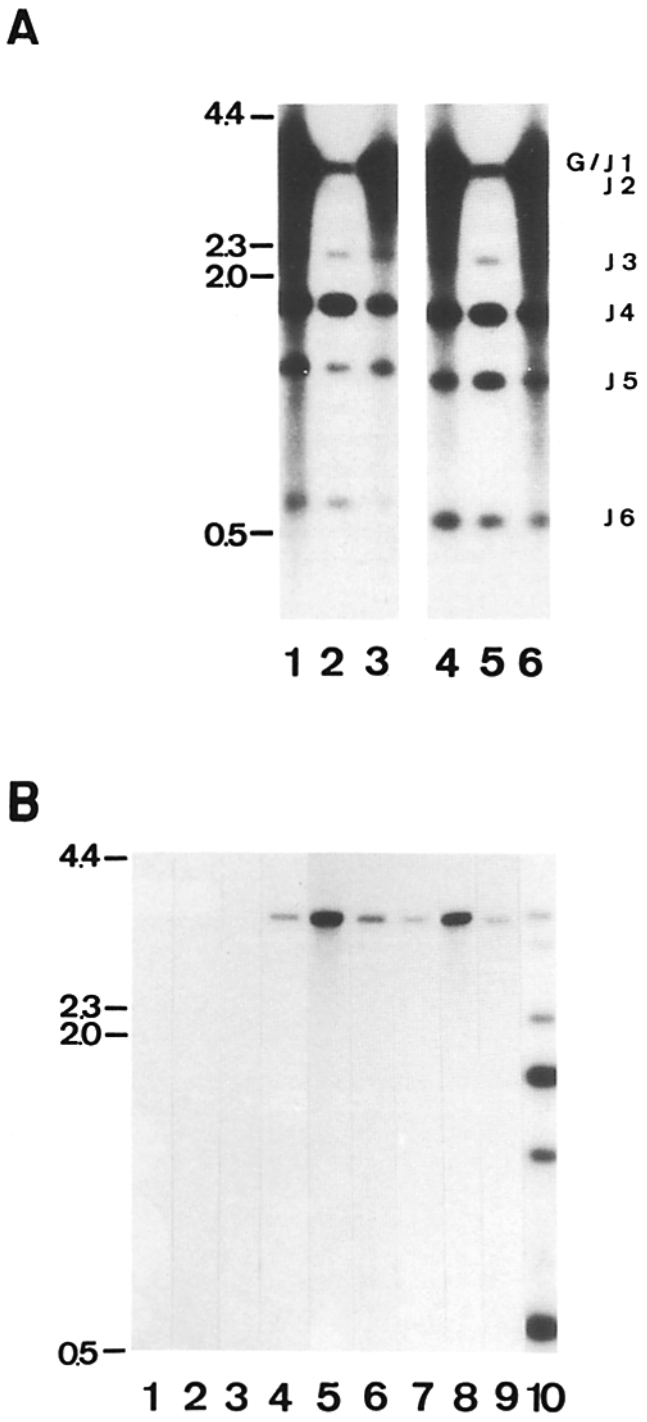

Figure 2. Southern blot analyses of PCR amplification products representing D_{HQ52}-J_H rearrangements. The DNA was separated on a 0.8% agarose gel and hybridized to probe a (Fig. 1 *A*). As size marker, Hind III digested λ DNA was included. (*A*) Genomic DNA of normal bone marrow was either used without restriction enzyme digestion (lane *1* and *4*) or with prior cleavage by EcoN I (lane *2* and *5*) or BamH I (lane *3* and *6*); BamH I does not cut within the D_{HQ52}-J_H region. The DNA was amplified with oligonucleotides 1 and 6 (lane 1–3) or with primers 2 and 6 (lane 4–6). (*B*) Genomic DNA digested with EcoN I was amplified with oligonucleotides 2 and 6. Lane *1*, water; lane *2*, salmon sperm DNA; lane *3*, mouse liver DNA; lane *4*, EG66 DNA (neuroblastoma cell line); lane *5*, HT 29 DNA (colon adenocarcinoma cell line); lane *6*, small cell lung carcinoma cell line DNA; lane 7, lung fibroblast DNA; lane *8*, skin fibroblast DNA; lane *9*, skin fibroblast DNA of a HLA class II deficient SCID patient; lane *10*, bone marrow DNA of a healthy donor.

Undigested DNA or DNA restricted with either EcoN I (cuts between D_{HQ52} and J_{H1}) or BamH I (no restriction site within the amplified region) was tested in the PCR reaction. Amplification of the EcoN I digested DNA reduced the strong signal obtained by the germline fragment in amplifications of undigested or BamH I digested DNA and allowed to differentiate the D_{HQ52}-J_{H2} rearrangement from the germline signal. The D_{HQ52}-J_{H1} rearrangement was probably disclosed by the germline signal in our gel system or, since it occurs very rarely (44), it was too weak to be visualized (Fig. 2 *A*). Even when the DNA was cut with EcoN I we amplified a germline band. This reflects the sensitivity of the PCR reaction to detect rare sequences. The identity of the fragments as D_{HQ52}-J_H rearrangements was further supported by the smaller size of the bands (Fig. 2 *A*, lane *4–6*), when oligonucleotide 2 (Fig. 1) was used as upstream primer instead of oligonucleotide 1. In addition, hybridization with specific oligonucleotides representing J_{H2}-J_{H6} sequences confirmed the conclusion that respective bands represent rearrangement events. Analysis of the amplification products which have been digested with Nco I (cuts between J_{H2} and J_{H3}), ApaL I (cuts between J_{H3} and J_{H4}) or Hinc II (cuts between J_{H4} and J_{H5}) supported this view (data not shown).

The specificity of the assay was proven by the absence of amplification products when nonhuman DNA (salmon sperm or mouse liver DNA) (Fig. 2 *B*) was used. In addition, a panel of human cell lines (Fig. 2 *B*) was analyzed to test the fidelity of the reaction. In fact, only the germline signals were observed upon amplification of DNA obtained from all six cell lines tested.

Sequence Analysis of D_{HQ52}-J_H Associated Rearrangements of Healthy Persons. Amplification products of bone marrow DNA (cut with EcoN I) of two healthy donors were cloned. After selection by lacZ α complementation, rearrangement events were scored by hybridization to oligonucleotide 3 and/or 5 (Fig. 1). Clones hybridizing to oligonucleotide 4 were excluded from sequence analyses since they represented most likely germline amplifications. We sequenced 29 clones (clone 1-29, Fig. 3 *A*) which hybridized to oligonucleotide 3 and 5, and one clone (clone 30, Fig. 3 *A*) which hybridized only to oligonucleotide 5, representing two D_{HQ52}-J_{H4}, five D_{HQ52}-J_{H5} and 23 D_{HQ52}-J_{H6} rearrangements. The distribution of J_H rearrangements probably reflects a double bias in amplification and cloning efficiency where fragments of smaller size are favoured. The J_{H4} and J_{H6} elements that were sequenced were in complete accordance with those published by Yamada et al. (44) and showed some nucleotide substitution compared with another report (43). The J_{H5} sequences derived from our analysis rather represent a composition of J_{H5} nucleotide sequences recently published (43, 44). Six of 30 clones (20%) lacked evidence for N nucleotides insertion (clone 1, 3, 8–11). The GC content of N regions was 65.4%. Both values correspond to published data (45). Twenty of 30 clones (66.7%)

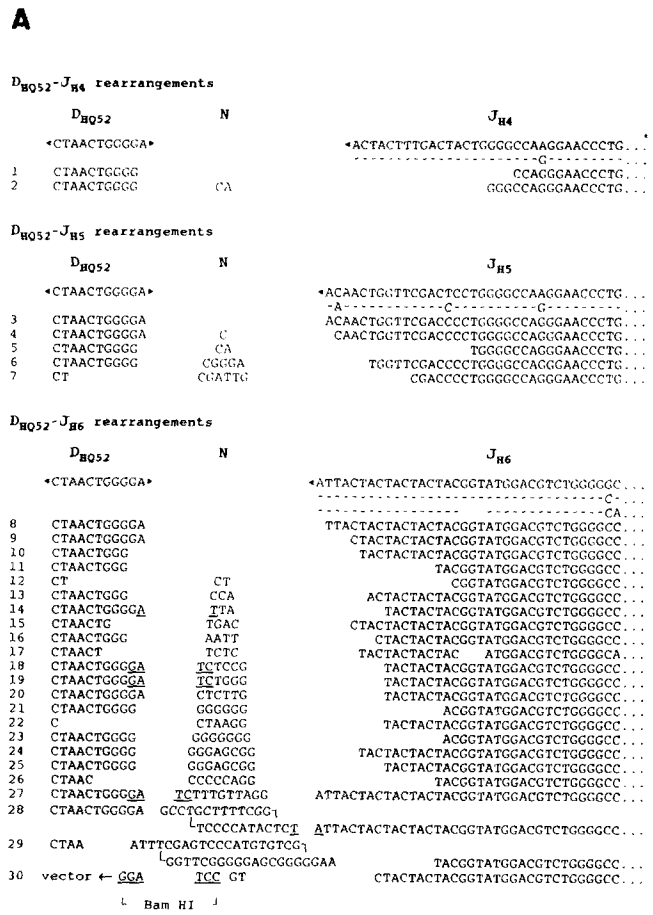

Figure 3. Nucleotide sequences of D_{HQ52}-J_H rearrangements from normal bone marrow of two adult donors. The DNA was cut before amplification with restriction enzyme EcoN I. (*A*) Normal sequences: Dubious nucleotides which could represent D_{HQ52} as well as J_H elements were scored as D_{HQ52} segment. P nucleotides are underlined. Sequence comparison was done according to a published report (asterisk, reference 43). Sequence variations of J_H elements as reported by Yamada et al. (44) are included for comparison. RSS are drawn as triangles. (*B*) An overlapping sequence of D_{HQ52} and J_{H4}, which could be due to homologous recombination in clone 1 (20) is boxed.

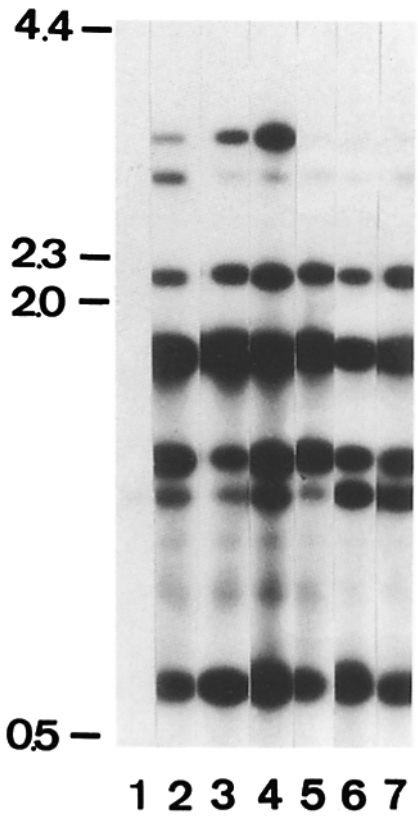

Figure 4. Analysis of D_{HQ52}-J_H rearrangements in PBMC DNA from B^+ SCID patients. Oligonucleotides 2 and 6 were used for amplification, probe a for hybridization. Hind III digested λ DNA served as size marker. As template the following (DNA) samples (cut with EcoN I) were included: Lane *1*, water, treated identically like the samples; lane *2*, PBMC (normal); lane *3*, patient 1 (D. B.); lane *4*, patient 2 (D. D.); lane *5*, patient 3 (K. K.); lane *6*, patient 4 (F. P.); lane 7, patient 5 (M. Z.).

used the last two 3′ nucleotides before the 3′ RSS of D_{HQ52} as junctional nucleotide. The junctional nucleotides at the 5′ site of J_{H6} were spread more evenly, reaching from the first to the seventeenth base with a peak at base nine.

Possible pallindromic sequences (P elements) (18) were detected in 47% of the clones which had not deleted nucleotides at the ends of the coding elements (clone 14, 18, 19, 27, 28, 30). We obtained one clone (clone 30, Fig. 3 *A*) which had vector sequences directly followed by P/N nucleotides and the J_{H6} element. The presence of the vector sequence most likely was due to a 3 nucleotide P element insertion at the 3′ site of D_{HQ52} which creates a BamH I site and juxtaposes P/N elements to vector sequences.

Of six clones without N nucleotides (Fig. 3 *A*), three contained overlapping sequences of D_{HQ52} and J_{H4} or J_{H6} (clone 1, 8, 9). The overlap of one clone (clone 1) (Fig. 3 *B*) encompassed seven nucleotides. Clone 8 and 9 (Fig. 3 *A*) overlapped by one base. One possible mechanistic explanation for such an overlap (as seen in clone 1) could be a homologous recombination event, as previously suggested (20).

D_{HQ52}-J_H Recombinations In Severe Combined Immunodeficiency (SCID) Patients. The amplification of PBMC DNA (digested with EcoN I) from B^+ SCID patients and the hybridization to probe a after blotting revealed that all five B^+ SCID patients show a D_{HQ52}-J_H recombination pattern identical to normal controls (Fig. 4).

The amplification of EcoN I digested bone marrow DNA of six B^- SCID patients showed that all of them exhibit a grossly irregular recombination pattern following hybridization to probe a (Fig. 5 *A*) or probe b (Fig. 5 *B*). Common to all six samples is the disturbance of the normal recombi-

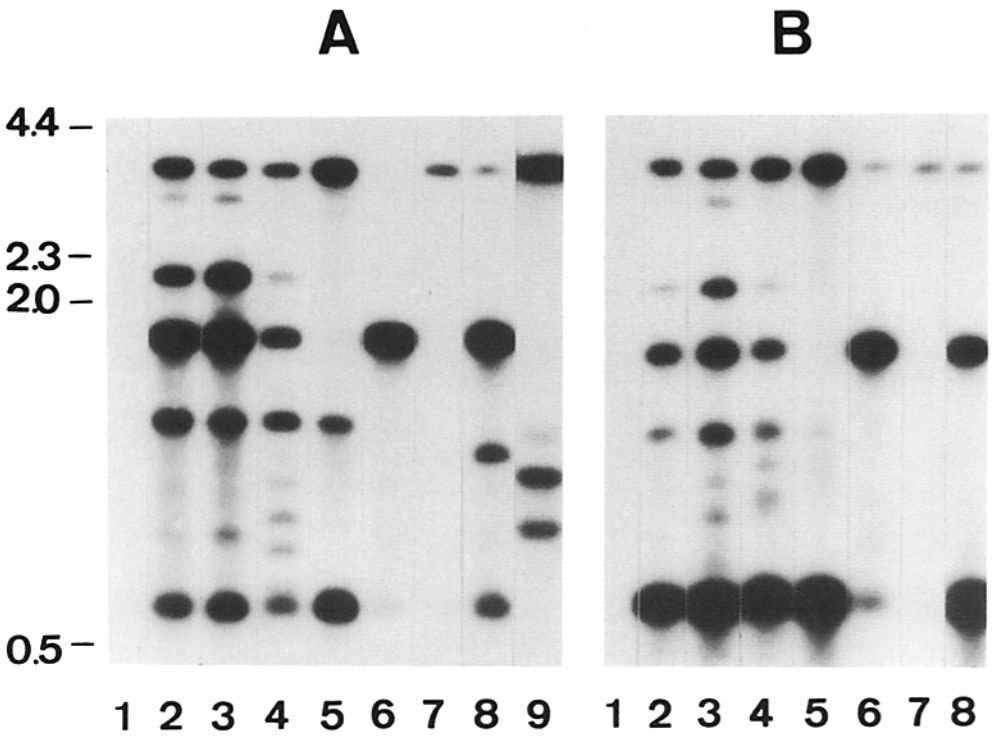

Figure 5. Analysis of D_{HQ52}-J_H associated rearrangements in bone marrow DNA from B⁻ SCID patients. Oligonucleotides 2 and 6 were used for amplification. Probe a (*A*) and probe b (*B*) were hybridized to the identical filter. Mol wt marker, Hind III digested λ DNA. The following samples pretreated with EcoN I were included in the analyses: Lane *1*, water; lane *2*, healthy control 1; lane *3*, healthy control 2; lane *4*, patient 6 (V. M.); lane *5*, patient 7 (T. C.); lane *6*, patient 8 (P. P.), lane *7*, patient 9 (J. B.), lane *8*, patient 10 (J. J.); lane *9*, patient 11 (S. K.).

nation "ladder". However, the amplification products represent a heterogenous pattern: one patient (patient 9, Table 1) did not show any rearranged fragment (Fig. 5, lane 7), all other patients revealed the absence of some bands (Fig. 5, lane *4–9*) and, in addition, several patients (patient 6, 10, and 11) showed amplification products which were of exceptional size (Fig. 5, lane *4*, *8*, and *9*), i.e., not representing regular D_{HQ52}-J_H recombinations.

The latter interpretation was supported by the hybridization of the filter used in Fig. 5 *A* to probe b (Fig. 5 *B*). Patients 6 and 10, who had irregularly hybridizing bands with probe a, lost some of those signals after hybridization to probe b. Thus the upstream regions of these putative D_{HQ52}-J_H rearrangements have obviously lost hybridizable sequences which span from oligonucleotide 2 to sequences between J_{H2} and J_{H3}. Amplification of PBMC DNA of patient 6 and 10 showed similar patterns of D_{HQ52}-J_H recombinations as observed in bone marrow DNA (data not shown). Genotypic analysis of the immunoglobulin gene status was done on PBMC or bone marrow DNA after digestion with Bgl II or Hind III and Southern blot analyses using probe a (Fig. 1). The probe hybridized in all patients to 3.9 kb Bgl II and to 9.5 kb Hind III germline fragments, indicating that no gross DNA abnormalities were present within the analyzed Ig gene loci (data not shown).

Sequence Analysis of B⁻ Severe Combined Immunodeficiency (SCID) Patients. Nucleotide sequences derived from amplification products of bone marrow DNA (EcoN I or Bgl II digests) of two B⁻ SCID patients were in marked contrast to the results obtained in healthy donors. We detected only two clones (S6E2 and S10B3) containing P nucleotide insertions, which resembled regular J_{H4} rearrangements (Fig. 6 *A*). However, the other clones showed deletions of different sizes (Fig. 6 *B*). In all clones the 5′ breakpoint occurred between the upstream amplification primer and D_{HQ52}. The 3′ breakpoint occurred always within a J_H element; clone S10B2 exhibits a deletion of 51 nucleotides of J_{H6} sequences, thus by far exceeding the normal range of J_{H6} nucleotide loss in normal DJ junctions. Three of the deletion clones contained N sequences, supporting the view that this step in V(D)J recombinations is not mutated in B⁻ SCID patients.

Hybridization of a clonospecific sequence derived from one deletion clone (clone S10B1) (Fig. 6 *B*) to the Southern blot analysis of amplification products (Fig. 7 *C*) additionally supported the interpretation that aberrant fragments in the Southern blot analysis actually represent irregular rearrangements.

Abnormal Rearrangements In Normal Bone Marrow Donors. Since we detected abnormal recombinations in B⁻ SCID patients, we asked if recombination failures are unique to B⁻ SCID patients or if these irregular recombinations can also be detected in normal individuals. To enrich for the detection of deletional events, we digested the DNA of two normal bone marrow donors and three B⁻ SCID patients with Bgl II prior to the analyses; Bgl II cuts at position 331, thus between the 5′ amplification ogligonucleotide and D_{HQ52}. Therefore only those events that have deleted position 331 can serve as intact template during the amplification procedure besides a small amount of uncut regular D_{HQ52}-J_H rearrangement events. Fig. 7 *A* shows the result of a Southern blot analysis of amplification products after hybridization to probe a. In fact, healthy persons (Fig. 7, lane *1* and *2*) as

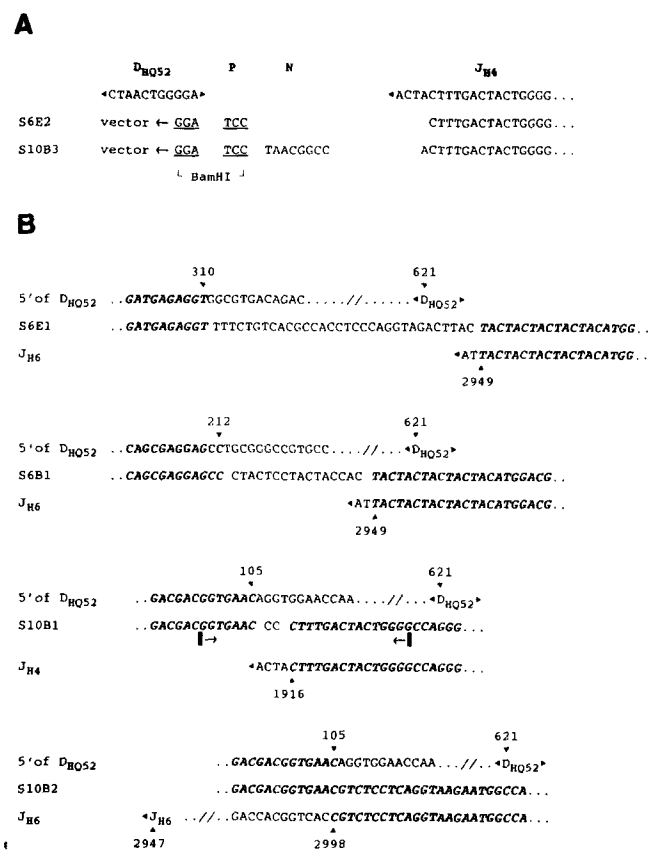

Figure 6. Nucleotide sequences of D_{HQ52}-J_H associated rearrangements from bone marrow of B^- SCID patients. The letter B or E (B = Bgl II, E = EcoN I) in the clone identification number represents the restriction enzyme used prior to the amplification. Sequence comparison was done according to published reports (43, 44). P nucleotides are underlined, RSS are drawn as triangles. The sequence of the clonospecific oligonucleotide (*between arrows*) used for hybridization analysis (Fig. 7 *C*) was derived from clone S10B1.

well as SCID patients (Fig. 5, lane *3–5*) exhibit irregularly sized fragments. Upon rehybridization of the filter to probe b (Fig. 7 *B*), most of the irregular bands either lose their intensity or are no longer visible, indicating that deletions between oligonucleotide 2 and J_{H2}-J_{H3} must have occurred. After cloning Bgl II digested amplified DNA of a healthy person, one clone (clone 31) (Fig. 8) had an irregular recombination pattern, since a deletion of 1584 bp (from base pair 273 to 2857) was detected. The point of recombination did not include any coding elements nor were any cryptic RSS visible in the vicinity of the breakpoints. Thus no principle difference between normal bone marrow donors and B^- SCID patients is observed in this analysis. We conclude that irregular rearrangements are present, albeit rare in normal individuals.

Discussion

We have studied the pattern of Ig gene rearrangements in five B^+ and six B^- human SCID patients by a PCR assay amplifying the D_{HQ52}-J_H region. Either by Southern blot or by sequence analyses the amplification products were characterized in more detail. At the Southern blot level B^+ SCID patients showed a normal D_{HQ52}-J_H recombination pattern, indicating a regular function of the "recombinase" machinery in this disease subentity.

In contrast, all six B^- SCID patients showed a grossly disturbed recombination pattern in the Southern blot analysis, with the lack of or with faulty D_{HQ52}-J_H rearrangements. Sequence analyses of the amplification products of B^- SCID patients showed that a high percentage of the rearrangements included deletions which surpass the boundaries of coding elements. This finding resembles the molecular defect in the SCID mouse (25–29). Since the used PCR approach allowed the detection of either normal rearrangements or of joinings with relatively small deletions on both sides flanking either D_{HQ52} or the J_H cluster, we expected to amplify only a minor proportion of recombinations and especially deletion events. Given the recombination defect in B^- SCID patients, this might explain why each patient exhibits a characteristic lack of usage of particular J_H. Additionally, the clonal expansion (possibly driven by antigenic stimuli) of a few cells bearing a normal D_{HQ52}-J_H rearrangement might explain the oligoclonal pattern of normal recombinations detected in B^- SCID patients. Whether these bands are generated due to the leakiness of the recombination machinery remains to be established.

The enzymatic pathway of Ig gene recombination has been divided into specific steps which are either mediated by the RSS or by nonspecific DNA processing enzymes (46). After RSS are identified by the recombination protein(s) and (probably) brought into physical proximity, cutting occurs at the RSS borders. Upon cleavage, the recombination machinery might continue to hold the coding and signal ends in close proximity to one another. The cut intermediates have to be quite stable since they must be long-lived enough to allow polymerases and exonucleases to modify their termini. Thereafter, the ligation of the open ends will restore the integration of the DNA strand. The two normal clones sequenced in our B^- SCID patients and the presence of N nucleotides in the deletion clones indicate that the B^- SCID recombination machinery is competent for the recognition of RSS, cutting at the RSS sites and the addition of P and N nucleotides. The defect associated with B^- SCID may rather represent a step marking the end of the trimming process of the coding elements. Along this line, one of the two alleles of a transformed cell line derived from a B cell negative (0% B cells, 7% T cells) SCID patient showed a rearrangement of the D_{LR1} element to a region 60 nucleotides downstream from J_{H4}, while the second allele represented a normal D_{XP1}-J_{H5} recombination (14).

Our data also provide evidence for the current view that irregular Ig recombinations are not exclusively observed in B^- SCID patients. The existence of one sequenced deletion clone and several irregular fragments in the Southern blot analysis of healthy individuals points to this fact. Occasionally lymphocyte precursors escape the otherwise tightly controlled recombination process of normal lymphopoiesis. Like-

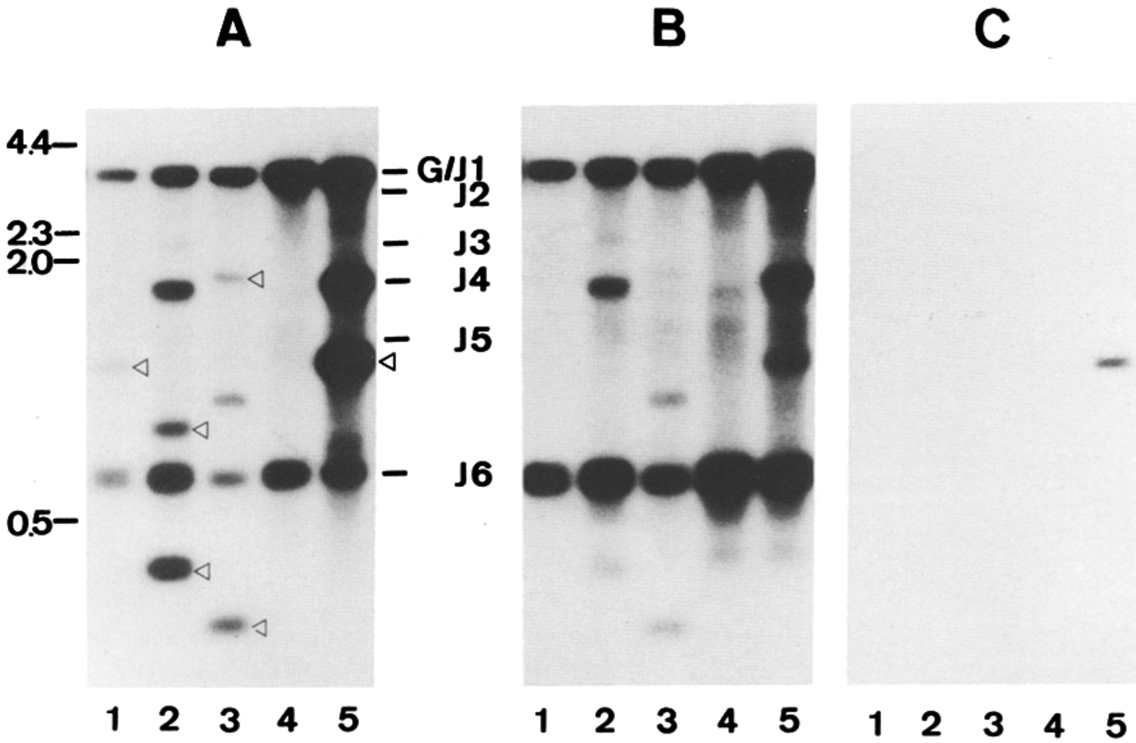

Figure 7. Analysis of D_{HQ52}-J_H associated rearrangements in bone marrow DNA obtained from healthy probands and B^- SCID patients. Oligonucleotides 2 and 6 were used for amplification; probe a (*A*), probe b (*B*) and the clonospecific oligonucleotide 5′ GGT GAA CCC CTT TGA CTA CTG GGG 3′ (*C*) were hybridized to the identical filter. The separation was done on a 1.2% agarose gel. Markers as in Fig. 2. The following samples pretreated with Bgl II were included in the analyses: Lane *1*, healthy individual 1; lane *2*, healthy individual 2; lane *3*, patient 6 (V. M.); lane *4*, patient 7 (T. C.); lane *5*, patient 10 (J. J.). Arrowheads in *A* indicate bands which show a reduced intensity after hybridization to probe b (*B*).

wise, sequencing of several B cell derived clones in the Balb/c mouse (47) has identified only one clone (clone 266 in [47]) where sequences 5′ of D_{HQ52} are rearranged to a region which is downstream of J_{H4}.

One of the critical points concerning our PCR assay is the presence of maternal T cells in B^- SCID patients since it has been realized that T cells can undergo D_HJ_H but no complete $V_HD_HJ_H$ rearrangement (48). These maternal T cells could account for the presence of the normal rearrangements in our B^- SCID panel. Two observations argue against this implication: (*a*) B^- SCID patient 7 (T.C.) who had no maternal T cells on the basis of fluorescence analysis and in vitro culture of PBMC (Table 1) showed a similar recombination pattern as other B^- SCID patients; (*b*) amplification of 99% pure (by cytofluorometry) peripheral T cells of two normal donors yielded the normal recombination ladder as observed with PBMC or bone marrow of healthy persons (K. Schwarz, unpublished data). In vitro culture of PBMC or of lymphocytes from different organ biopsies of patient 6 (V. M.) and patient 10 (J. J.) yielded cell lines with >90% CD3 positive cells which were of maternal origin only. Yet, preliminary PCR analyses of those CD3 positive cell lines revealed only the germline band (K. Schwarz, unpublished data). Thus those maternal cells either have no D_{HQ52}-J_H rearrangement at all or they use other D_H sequences outside of the range of our PCR primers.

Whether pre T cells of the B^- SCID patients recombine TCR genes with faults remains to be established. Patients lacking maternal T cells might allow to approach this problem.

The identification of B^- human SCID patients who may be characterized by a defect within the recombination machinery analogous to SCID mice opens new avenues as to the differential diagnosis of SCID patients and marks a step towards the molecular definition of these heterogeneous entities.

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                                        273                   621
                                         ▼                     ▼
5'of DHQ52   ...CCGCCACCCGGGCCTCTCTGCTGCTGGGAAAACA...//..◂DHQ52▸
Clone 31     ...CCGCCACCCGGGCCTCTCTGCTGGACAGTCAGCTTCCAT.........
5'of JH6               ...GGACAGGGCCACGGACAGTCAGCTTCCAT..//.◂JH6
                                         ▲                      ▲
                                       2857                   2947
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Figure 8. Sequence of a deletional clone obtained after amplification of Bgl II digested DNA of a healthy individual. Sequence comparison was done according to published reports (43, 44). Arrowheads represent RSS.

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