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Analyses of TCR_B Rearrangements Substantiate a Profound Deficit in Recombination Signal Sequence Joining in SCID Foals: Implications for the Role of DNA-Dependent Protein Kinase in V(D)J Recombination

Euy Kyun Shin,* Tonnie Rijkers,† Albert Pastink,† and Katheryn Meek2*‡

We reported previously that the genetic SCID disease observed in Arabian foals is explained by a defect in V(D)J recombination that profoundly affects both coding and signal end joining. As in C.B-17 SCID mice, the molecular defect in SCID foals is in the catalytic subunit of the DNA-dependent protein kinase (DNA-PK CS); however, in SCID mice, signal end resolution remains relatively intact. Moreover, recent reports indicate that mice that completely lack DNA-PK CS also generate signal joints at levels that are indistinguishable from those observed in C.B-17 SCID mice, eliminating the possibility that a partially active version of DNA-PK CS facilitates signal end resolution in SCID mice. We have analyzed TCR_B rearrangements and find that signal joints are reduced by ~4 logs in equine SCID thymocytes as compared with normal horse thymocytes. A potential explanation for the differences between SCID mice and foals is that the mutant DNA-PK CS allele in SCID foals inhibits signal end resolution. We tested this hypothesis using DNA-PK CS expression vectors; in sum, we find no evidence of a dominant-negative effect by the mutant protein. These and other recent data are consistent with an emerging consensus: that in normal cells, DNA-PK CS participates in both coding and signal end resolution, but in the absence of DNA-PK CS an undefined end joining pathway (which is variably expressed in different species and cell types) can facilitate imperfect signal and coding end joining. The Journal of Immunology, 2000, 164: 1416–1424.

V(D)J recombination is the molecular mechanism that generates a diverse repertoire of Ag-specific receptors in B and T lymphocytes (reviewed in Ref. 1). This process is initiated by a lymphocyte-specific endonuclease (the products of the recombination-activating genes I and II; RAG1 and RAG2) that recognizes and cleaves at target sequences adjacent to the immune receptor gene segments (2–4). The centrality of V(D)J recombination to the development of the vertebrate immune system is illustrated in situations in which the process is impaired; defective V(D)J recombination results in a block of B and T cell lymphopoiesis, and the disease SCID (5–11). The first example of this phenomenon was the description of defective V(D)J recombination in C.B-17 mice (12). In 1990, it was demonstrated that the defect in SCID mice not only impairs V(D)J recombination, but also affects the more general process of DNA double-strand break repair (DSBR) (13, 14). This observation was the first to link V(D)J recombination and DSBR.

In eukaryotes, two major mechanisms exist for the repair of double-strand DNA breaks (DSBs): 1) homologous recombination (HR), in which repair of the damaged DNA is directed by gene conversion; and 2) nonhomologous end joining (NHEJ), an error prone end to end joining pathway (reviewed in Ref. 15). In yeast, DSBs are generally repaired via HR, whereas NHEJ plays a very minimal role. Higher eukaryotes are exactly opposite, depending heavily on NHEJ to repair DSBs, while HR plays a less significant role. At least five DNA repair factors are required for V(D)J recombination: XRCC4, Ku70 (XRCC5), Ku 86 (XRCC6), DNA-PK CS (XRCC7), and DNA ligase IV (6, 10, 11, 16–32); three of these are components of the DNA-dependent protein kinase (the Ku heterodimer), and DNA-PK CS (33–37). The RAG endonuclease is targeted specifically to immune receptor gene segments by simple DNA sequence elements (recombination signal sequences, RSS) adjacent to V, D, and J gene segments, and involves two dsDNA cuts and subsequent religations (mediated by the NHEJ pathway). This results in the formation of two new DNA joints, coding joints that are generally modified by nucleotide additions and/or deletions and signal joints that contain the two RSS and are largely unmodified (38). It was initially recognized that the defect in C.B-17 SCID mice affected only coding joint resolution, leaving signal joint formation relatively intact (5), although more recent data demonstrate a modest defect in both the rate and fidelity of signal end resolution (39–42). In 1994, the defect in SCID mice was first shown to be in DNA-PK CS (16). In 1995, we demonstrated that the defective mechanism in SCID foals is V(D)J recombination (9); as with C.B-17 SCID mice, the mutant factor in SCID foals is DNA-PK CS (26). However, our analyses suggested that in SCID foals, both coding and signal end resolution are profoundly diminished. In fact, subsequent reports,
in which two additional rodent cell lines with DNA-PKCS mutations were characterized, also support a role for DNA-PKCS in signal end joining (43, 44), although analyses of a human cell line with defective DNA-PKCS expression suggest that DNA-PKCS is not required for signal joining (45). The murine SCID mutation results in the deletion of only 83 amino acids from the C terminus of DNA-PKCS (46, 47), whereas substantially more of the protein (967 amino acids) is deleted by the equine SCID mutation (26). Thus, we hypothesized that some residual DNA-PKCS activity in SCID mice (possibly independent of kinase activity) might explain the observed difference in signal ligation. Recent reports demonstrating that mice with targeted deletions of DNA-PKCS also have relatively normal signal end resolution negate this hypothesis (39–42). In sum, in recent years, no less than eight reports with considerable diversity have focused attention on the role of DNA-PKCS in signal end resolution. An emerging consensus is that (at least in rodents), in the absence of DNA-PKCS, there is a modest deficiency in both the rate and fidelity of signal end joining coupled with a much more substantial diminution of coding end resolution.

Our initial studies of endogenous Ig light chain rearrangements from SCID foals revealed two differences from SCID mice: 1) an undetectable V-Jk signal joints in equine lymphocytes as compared with murine lymphocytes; and 2) a lack of leakiness when analyzing light chain coding joints from SCID foals as compared with SCID mice (9, 48). Since in our initial studies only Ig light chain rearrangements were analyzed (because of limited available sequence information of horse immune receptor genes), it might be argued that the lack of observed signal joints might actually reflect tighter temporal control of Ig gene rearrangement in equine vs murine lymphocytes. The requirement for successful heavy chain light chain rearrangement before the initiation of light chain rearrangement might be more tightly enforced in equine lymphocytes than in murine lymphocytes. Thus, one possible explanation for the lack of V-Jk signal joints in SCID foals is that light chain rearrangement is not initiated. Although our conclusion (that signal end joining is impaired in SCID foals) was supported by analyses of rearrangements of extrachromosomal substrates in cell lines derived from SCID foals, these experiments were limited by the fact that these plasmid substrates do not replicate in equine cell lines, making quantitation difficult (9).

In this study, we establish that the capacity for both signal and coding end joining at a recombinationally active TCRβ locus in equine SCID lymphocytes is ~4 logs lower than in normal lymphocytes. Additionally, we present data suggesting that the observed differences in end resolution are not because of the specific nature of the mutant DNA-PKCS allele in SCID foals, but more likely reflect differences between species in the ability to bypass the requirement for DNA-PKCS during certain types of end joining.

**Materials and Methods**

**DNA isolation**

DNA was prepared from thymus collected from a 20-day-old SCID foal and from a normal foal ~2 mo old using commercially available DNA extraction buffer (Applied Biosystems, Foster City, CA). Similarly, DNA was isolated from the spleen of both SCID and normal animals.

**Oligonucleotides**

Sequences of oligonucleotides used in this study are as follows: 5′-Dk, CCAACCTCTGGCACTGGTCT; 5′-Dk inner, CTGCCGGTGGCCA GTGGT; 5′-Dk, CCATCCAGAGCAGATTCCCG; 5′-Dk inner, GCTTG TGGGGGGGTITTT; 5′-Jk, TGTCAGCAATGATGTCGCA; 5′-Jk inner, CTAAATTGGGAAATGGGAAG; 3′-Jk, TCTAGAATGAGC CGAGTCCC; 3′-Jk inner, TGGCCGGAAGAGACGCTCT; 5′-Dk, CCCCGAGTCCCAACATTGTA; 3′-Dk, RSS, AAGGCCTCCTCTTA CCTG; 5′-Vγ, TCTGGGCTTTGTTCTGGTCTCT; 5′-Vγ inner, GGTCAATTTGCCATCAG; 5′-Vγ inner-2, AACAATTCTACACCT CAGACGTTG; 5′-DFL6-1, ACCAGAACCATACTGCGCCAGGC; 3′- DFL6-1, CTCAGAAGCTGTCGGACAAATG; 5′-Jγ4, GGTCCT TAAAGGCGGATATTGGAAGAGT; 3′-Jγ4, GTTCGAGAAGATGGG AGAGAGAA.

**Polymerase chain reactions**

PCR reactions were conducted by using the indicated amounts of DNA in 100 μl reactions. For the experiment depicted in Fig. 1, 40 cycles of amplification were performed using Elongase (Life Technologies, Gaithersburg, MD) and the following conditions: 94°C for 30 s, 59°C for 1.5 min, 68°C for 8 min. For the nested PCR experiments depicted in Figs. 2, B–D, and 3, A–C, Taq polymerase was utilized; initial amplification conditions were: 94°C for 1.5 min, 52°C for 2 min, and 72°C for 3 min for 40 cycles. A total of 10 μl of each reaction was subsequently amplified as follows: 94°C for 1.5 min, 52°C for 2 min, and 72°C for 3 min for 40 cycles. In Fig. 2A, amplification conditions were the same, but only 40 cycles were performed. For the experiment depicted in Fig. 5, Taq polymerase was utilized; 40 cycles of amplification were performed as follows: 94°C for 1.5 min, 58°C for 2 min, and 72°C for 3 min. A total of 20 μl of each PCR reaction was analyzed by Southern filter hybridization analysis. For sequence analysis, amplified rearrangements were gel purified and ligated into pCR2.1 (Invitrogen, Carlsbad, CA) and transformed into competent Escherichia coli. Recombinant colonies were identified by hybridization and sequenced on an ABI sequencer (Applied Biosystems, Foster City, CA).

**Cell lines**

The 0176 and 1821 cell lines were established from dermal biopsies from a normal horse (0176) and a SCID foal (1821) and were the generous gift of Dr. Lance Perryman (North Carolina State University, Raleigh, NC). The SF19 fibroblast cell line was established from a C.B-17 SCID mouse and was the generous gift of Dr. Mel Bosma (Fox Chase Cancer Center, Philadelphia, PA). The 100E cell line (a murine SCID fibroblast cell line that harbors a fragment of human chromosome 8, including the DNA-PKCS gene) was the generous gift of Dr. Cordula Kirchgessner (Stanford University, Stanford, CA) and has been described previously (23).

**Immunoblot analysis**

The indicated amounts of whole cell extracts were electrophoresed in an SDS/5% polyacrylamide gel and transferred to polyvinylidene difluoride. The following three fragments spanning the DNA-PKCS coding sequence were used in analyzing light chain coding joints from SCID foals as compared with SCID mice (9, 48). Since in our initial studies only Ig light chain rearrangements were analyzed (because of limited available sequence information of horse immune receptor genes), it might be argued that the lack of observed signal joints might actually reflect tighter temporal control of Ig gene rearrangement in equine vs murine lymphocytes. The requirement for successful heavy chain light chain rearrangement before the initiation of light chain rearrangement might be more tightly enforced in equine lymphocytes than in murine lymphocytes. Thus, one possible explanation for the lack of V-Jk signal joints in SCID foals is that light chain rearrangement is not initiated. Although our conclusion (that signal end joining is impaired in SCID foals) was supported by analyses of rearrangements of extrachromosomal substrates in cell lines derived from SCID foals, these experiments were limited by the fact that these plasmid substrates do not replicate in equine cell lines, making quantitation difficult (9).

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**Construction of DNA-PKCS expression vectors**

The following three fragments spanning the DNA-PKCS coding sequence were amplified from eight shorter RT-PCR fragments isolated from the Ramos human B cell lymphoma cell line: 1–3,860, 3,559–8,173, and 7,911–12,358. The fragments were initially cloned into a low copy number plasmid and using a low copy number plasmid and using E. coli deficient in bacterial recombination systems (STBL cells; Life Technologies). NorF sites were engineered by PCR just 5′ of the translation initiation site and just 3′ of the translation termination site using the following oligonucleotides: 5′-Not, GGCGCCGCCCAGCATGGCC; 3′-Not, GGGCCGGCAGACATCCTGACACATCG. At this point, the sequence of each fragment was confirmed. These three fragments were then cloned into a single plasmid by stepwise cloning of the 7,911–12,358 fragment into the plasmid-containing fragment 1–3,860 fragments via SalI sites at 3,482 and 7,933 in the DNA-PKCS coding sequence and an MluI site in the plasmid backbone. Subsequently, the 3,600–8,050 fragment was inserted into the SalI site. Cloning sites were resequenced at this step. The full-length NorF cDNA was then subcloned into the pcMV6 expression vector that provides CMV promoter elements as well as transcription termination and polyadenylation sequences from the human growth hormone gene.
FIGURE 1. Isolation of the equine D\textsubscript{B}2-J\textsubscript{B}2.1 intervening region. PCR amplification of 1 \mu g spleen DNA isolated from a SCID foal (lane 1) or a normal foal (lane 2) using amplification primers 5'-D\textsubscript{B} and 3'-J\textsubscript{B}. Amplification conditions are described in Materials and Methods.

To generate the truncation mutant, a PCR fragment spanning nucleotides 8057–9480 was generated with the following oligonucleotides: 5'-Fse, GGGCAGGGTGATACAAACAGTT; 3'-mut, GGGGATCTCTAAGGGAATTTGATAAATTGCCTTGTTTGC.

The 5'-oligonucleotide incorporates the frameshift at nucleotide 9453 and adds an EcoRV restriction site. The resulting PCR fragment spans a unique FseI site at position 8159. After subcloning into PCR2.1 and sequencing, this PCR fragment was restricted with FseI and EcoRV and then ligated into the full-length DNA-PK\textsub{cs} cDNA that had been restricted with FseI and Eco721 (unique, blunt site at position 11,145). Cloning sites were resequenced in the resulting plasmid.

Extrachromosomal V(D)J recombination assays

Extrachromosomal substrate assays were performed essentially as described by Hesse et al. (49). Briefly, to assess V(D)J recombination in SF19 cells, RAG1 and RAG2 expression constructs (6 \mu g each; generous gift of Dr. Moshe Sadofsky, Medical College of Georgia, Augusta, GA), DNA-PK\textsub{cs} expression constructs or vector controls (6 \mu g each), and recombination substrates pJH201 or pJH290 (1 \mu g each) were transiently introduced into the SF19 cells via liposome transfection using Fugene (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer’s suggested protocol. Forty-eight hours later, the plasmid substrates were rescued by preparing alkaline lysates and restricting with DpnI. A portion of the recovered substrates was subsequently used to transform competent E. coli (max efficiency D\textsubscript{H} 5 \mu g/ml or both ampicillin and chloramphenicol (22 \mu g/ml)). In each case, plasmid DNA was prepared from at least 20% of the colonies and resequenced in the resulting plasmid.

Assessment of radiation sensitivity

Cells (10^5) were exposed to various amounts of ionizing radiation using a \textsuperscript{137}Cs source calibrated at 558.8 R/min and immediately seeded in complete medium containing 10% FBS. After 8 days, all colonies were fixed with 2% formaldehyde, followed by 100% methanol. Subsequently, the colonies were stained with trypan blue, and colony numbers were assessed.

Results and Discussion

Isolation of equine D\textsubscript{B}2-J\textsubscript{B}2.1 intervening sequence

Like Ig gene rearrangement, TCR rearrangement is temporally regulated. In the thymus, D\textsubscript{B} to J\textsubscript{B} rearrangement precedes V\textsubscript{B} to D\textsubscript{B}J\textsubscript{B} rearrangement, which precedes rearrangement of the TCR\text{\textupsilon} locus. To analyze early TCR rearrangements from SCID thymocytes, the intervening region between TCR DB 2 and TCR JB 2.1 was amplified from spleen DNA using amplification primers derived from published equine TCR\textsubscript{\textupsilon} transcripts (Fig. 1) (50). As can be seen, a single hybridizing band of \sim 800 bp is present in amplifications of SCID spleen DNA (representing the germline configuration of the D\textsubscript{B}2 and J\textsubscript{B}2.1 genes), whereas two bands (\sim 800 and \sim 160 bp, representing unrearranged and rearranged, respectively) are apparent in amplifications from normal spleen DNA. The resulting 800-bp amplification product was sequenced, and amplification primers were generated to analyze TCR DB-JB rearrangements.

Analyses of D\textsubscript{\textupsilon}J\textsubscript{\textupsilon} coding joints

DNA was prepared from the thymus of a 3-wk-old SCID foal and from the thymus of an \sim 2-mo-old normal foal. Although a thymus

FIGURE 2. Analysis of TCR\textsubscript{\textupsilon} rearrangements. A, PCR amplification of the germline D\textsubscript{\textupsilon} gene (using the amount of DNA (\mu g) indicated above) from thymus DNA from a normal foal (lanes 1–4), thymus DNA from a SCID foal (lanes 5–8), or no DNA (lane 9). Amplification of the D\textsubscript{\textupsilon}2 gene segment (as described in Materials and Methods) was done using the following oligonucleotides: 5'-D\textsubscript{\textupsilon} and 3'-D\textsubscript{\textupsilon} B. Nested PCR amplification of D\textsubscript{\textupsilon}B-J\textsubscript{\textupsilon} coding joints (using the amount of DNA (\mu g) indicated above) from thymus DNA from a normal foal (lanes 1–7), no DNA (lanes 8 and 12), or thymus DNA from a SCID foal (lanes 9–11). Amplification of D\textsubscript{\textupsilon}2-J\textsubscript{\textupsilon} mut coding joints (as described in Materials and Methods) was done using the following oligonucleotides: 5'-D\textsubscript{\textupsilon} and 3'-J\textsubscript{\textupsilon} and then 5'-D\textsubscript{\textupsilon} inner and 3'-J\textsubscript{\textupsilon} inner. C, Nested PCR amplification of V\textsubscript{\textupsilon}D\textsubscript{\textupsilon}2-J\textsubscript{\textupsilon} coding joints (using the amount of DNA (\mu g) indicated above) from thymus DNA from a normal foal (lanes 1–7), no DNA (lanes 8 and 12), or thymus DNA from a SCID foal (lanes 9–11). Amplification of V\textsubscript{\textupsilon}D\textsubscript{\textupsilon}2-J\textsubscript{\textupsilon} mut coding joints (as described in Materials and Methods) was done using the following oligonucleotides: 5'-V\textsubscript{\textupsilon} and 3'-J\textsubscript{\textupsilon} and then 5'-V\textsubscript{\textupsilon} inner and 3'-J\textsubscript{\textupsilon} inner. D, Nested PCR amplification of D\textsubscript{\textupsilon}2-J\textsubscript{\textupsilon} signal joints (using the amount of DNA (\mu g) indicated above) from thymus DNA from a normal foal (lanes 1–7), no DNA (lanes 8 and 12), or thymus DNA from a SCID foal (lanes 9–11). Amplification of D\textsubscript{\textupsilon}2-J\textsubscript{\textupsilon} mut signal joints (as described in Materials and Methods) was done using the following oligonucleotides: 5'-J\textsubscript{\textupsilon} and 3'-D\textsubscript{\textupsilon} and then 5'-J\textsubscript{\textupsilon} inner and 3'-D\textsubscript{\textupsilon} inner.
sample was only available from one SCID foal, completely anal-
ogous results (as those presented below) were obtained in exper-
iments using spleen DNA from one normal animal and three ad-
ditional SCID foals. The germline DB 2 gene segment (Fig. 2A) and
J B 2.1 gene segment (data not shown) are amplified equally from
both normal and SCID thymus DNA. In our initial analysis, al-
though both coding and signal joints were readily detectable from
as little as 10 ng of normal thymus DNA, DB J B rearrangements
were not detected from the SCID thymus DNA (data not shown).
Thus, a more sensitive nested PCR strategy was utilized, as shown
in Fig. 2B–D. As can be seen, using this approach, DB J B coding
joints could consistently be detected from 0.5 ng normal thymus
DNA (and in some experiments as little as 0.05 ng). In contrast,
DB J B coding joints could only be detected using 5 mg SCID thy-
mus DNA. Thus, we conclude that the frequency of DB J B coding
joints in SCID thymus is at least 4 logs lower than in normal
thymus. We also assessed complete VB DB J B rearrangements in
both normal and SCID thymus DNA using a nested amplification
strategy and VB primers that should prime only a subset of known
equine VB gene segments. As can be seen in Fig. 2C, VB DB J B rear-
rangements were readily detectable in as little as 5 ng normal
thymus DNA. Complete VB DB J B rearrangements were not consis-
tently detected from SCID thymus; these rearrangements were de-
tected in two of five experiments and only using the highest con-
centration of DNA (5 μg).

To examine the fine structure of the SCID coding joints, ampli-
fications products from several different experiments were cloned
and sequenced. These sequences are presented in Table I. Dupli-
cate sequences isolated from the same PCR product were elimi-
nated. As can be seen, the rare SCID rearrangements are not sub-
stantially different from rearrangements isolated from normal
animals. No excessive P segments were observed, as has been
reported in SCID mice. It has been demonstrated that rare rear-
rangements from Ku-deficient mice lack N segments and that
SCID mice have a less pronounced deficiency in N segment addi-
tion (51). In these sequences, there is a modest decrease in both
the length of N segments (average length: 6 bp vs 2.6 bp in rear-
rangements from normal and SCID thymus, respectively) and the
percentage of N segment-positive rearrangements isolated from
this SCID foal. In the three rearrangements that lack N segments
from SCID thymus, the coding junction occurs at short regions of
sequence homology.

**Analyses of Dp J B signal joints**

The frequency of Dp J B signal joints in both normal and SCID
thymus was similarly assessed (Fig. 2D). As can be seen, signal

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* Nucleotide sequences of each unique Dp J B coding joint isolated from normal and SCID thymus are aligned to germline Dp and J B gene segments
  as indicated. Nucleotides potentially derived as P elements are depicted. Junctional nucleotides which could not be accounted by germline sequences
  are depicted as N segments. Underlined nucleotides represent regions of the sequence that could be derived from either the germline Dp or J B gene segment or from their corresponding P elements.
Hybrid joints represent apparent mistakes of the recombinase and are the result of ligation of the coding end of one gene segment to the signal end of a second gene segment (52). The frequency of hybrid joining, as assessed using extrachromosomal substrates, has been estimated at 10–30% the rate of standard joining (52). The frequency of hybrid rearrangements of endogenous immune receptor gene segments is considerably lower, 100- to 1000-fold less frequently than standard rearrangements (53, 54). Does the mutant equine DNA-PK CS allele inhibit RS joining?

Hybrid joints are less diminished in SCID thymocytes than standard joints

Hybrid joints are readily detected in as little as 0.5 ng normal thymus DNA, whereas signal joints can only be detected in 5 μg of SCID thymus DNA. Detection of signal joints in SCID thymus was inconsistent, being detected in only two of five experiments. Thus, we conclude that the frequency of DB-JB signal joints in SCID thymus is ~4 logs lower than in normal thymus.

FIGURE 3. Analysis of TCRB hybrid rearrangements. A, Nested PCR amplification of VB coding to 5’-D B RSS hybrid joints (using the amount of DNA (μg) indicated above) from thymus DNA from a normal foal (lanes 1–4), no DNA (lane 5), or thymus DNA from a SCID foal (lanes 6–9). Amplification of hybrid joints (as described in Materials and Methods) was done using the following oligonucleotides: 5’-VB inner and 5’-DB inner and then 5’-VB inner and 5’-DB inner. B, Nested PCR amplification of JB coding to 3’-D B RSS hybrid joints (using the amount of DNA (μg) indicated above) from thymus DNA from a normal foal (lanes 1–4), thymus DNA from a SCID foal (lane 5), or no DNA (lane 6). Amplification of hybrid joints (as described in Materials and Methods) was done using the following oligonucleotides: 3’-JB inner and 3’-DB inner and then 3’-JB inner and 3’-DB inner. C, Nested PCR amplification of DB coding to JB hybrid joints (using the amount of DNA (μg) indicated above) from thymus DNA from a normal foal (lanes 1–4), thymus DNA from a SCID foal (lane 5), or no DNA (lane 6). Amplification of hybrid joints (as described in Materials and Methods) was done using the following oligonucleotides: 5’-DB inner and 3’-DB inner and then 5’-J B inner and 3’-J B inner.

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Several conclusions can be derived from these results. First, the fact that some hybrid joints are found at similar frequencies in SCID and normal thymocytes corroborate previous studies suggesting that the RAG proteins alone can mediate hybrid rearrangements in vivo (41, 51, 55, 56). However, the fact that certain hybrid joints are not detected in SCID thymocytes suggests that the RAG proteins cannot mediate all hybrid joints equivalently, and that some hybrid joints are facilitated by the DNA-PK-dependent joining pathway. Finally, the fact that certain hybrid joints can be detected at similar frequencies in wild-type and SCID thymocytes confirms that the TCRB locus is recombinationally active in SCID thymocytes. In sum, these analyses of equine TCRB rearrangements verify the fact that the equine SCID phenotype includes 1) a severe diminution in signal end resolution not observed in SCID mice, and 2) substantially less leakiness in coding end resolution than observed in SCID mice.

Does the mutant equine DNA-PK CS allele inhibit RS joining?

An attractive hypothesis suggested by Gao et al. (39) to explain the discrepancies between the equine and murine SCID phenotypes is that the mutation in the equine DNA-PK CS allele is recessive. In 1998, Melek et al. (57) substantiated this hypothesis, showing in a cell-free system that the RAG proteins alone could mediate hybrid joining.

Three types of TCRB hybrid rearrangements were assessed in normal and SCID thymus DNA (Fig. 3, A–C). When amplifying normal thymus DNA, consistent detection of all three types of hybrid joints required using 500 ng thymus DNA in each amplification. This is in good agreement with previous studies demonstrating that hybrid rearrangements at endogenous loci occur 100- to 1000-fold less frequently than standard rearrangements (53, 54). Relative frequencies of the three different hybrid joints varied considerably in SCID thymocytes. Hybrid joints involving a VB coding segment and the 5’-DB RSS were consistently detected (four of four experiments) at roughly equivalent levels in SCID and normal thymocytes. Several of these rearrangements were cloned and sequenced from both normal and SCID animals (data not shown). In each case, the 5’-DB RSS was completely intact. Because germline VB sequences are not available, it is impossible to determine the extent of nucleotide deletion and/or addition at the VB-D B RSS junction. Still, the modest extent of junctional diversity observed in these rearrangements is consistent with RAG-mediated hybrid joining. Hybrid joints involving the JB coding segment and the 3’-DB RSS could be detected using 5 μg SCID thymus DNA in one of five experiments; thus, these hybrid joints occur at least 2 logs less frequently in SCID thymocytes than in normal thymocytes. Hybrid rearrangements involving the DB coding segment and the JB RSS were not detected in SCID thymus.

Several conclusions can be derived from these results. First, the fact that some hybrid joints are found at similar frequencies in SCID and normal thymocytes corroborate previous studies suggesting that the RAG proteins alone can mediate hybrid rearrangements in vivo (41, 51, 55, 56). However, the fact that certain hybrid joints are not detected in SCID thymocytes suggests that the RAG proteins cannot mediate all hybrid joints equivalently, and that some hybrid joints are facilitated by the DNA-PK-dependent joining pathway. Finally, the fact that certain hybrid joints can be detected at similar frequencies in wild-type and SCID thymocytes confirms that the TCRB locus is recombinationally active in SCID thymocytes. In sum, these analyses of equine TCRB rearrangements verify the fact that the equine SCID phenotype includes 1) a severe diminution in signal end resolution not observed in SCID mice, and 2) substantially less leakiness in coding end resolution than observed in SCID mice.
Thus, we reexamined DNA-PK CS expression in cell lines from not likely present in the mutated protein in equine SCID cells. The mutant DNA-PK CS protein is unstable and not functional. A, Immunoblot analysis of whole cell extracts from the normal equine fibroblast cell line, 0176 (lanes 1–4), using either 100 µg, 50 µg, 25 µg, or 12.5 µg cell extract as indicated, and the SCID fibroblast cell line, 1821 (lanes 5 and 6), using 200 µg or 50 µg extract, as indicated. An anti-DNA-PK CS mAb (18-2, generous gift of Dr. Timothy Carter), which recognizes an epitope within the N-terminal 250 kDa of DNA-PK CS, was utilized. Position of DNA-PK CS and the 200 kDa m.w. marker are indicated with arrows. B, Immunoblot analysis of whole cell extracts from human chromosome 8-transfected cells (lane 1), or SF19 cells transfected with vector alone (lane 2), full-length DNA-PK CS (lane 3), or DNA-PK CS truncated at amino acid 3160. C, Radiation resistance of untransfected murine SCID cells or clonal transfectants transfected with full-length DNA-PK CS expression vector, DNA-PK CS expression vector truncated at amino acid 3160, or harboring a fragment of human chromosome 8. Data are presented as percent survival of unirradiated controls and represent average of triplicate samples. Anti-DNA-PK CS Abs was utilized, two of which specifically recognize epitopes within the C-terminal 150 kDa of DNA-PK CS and not likely present in the mutated protein in equine SCID cells. Thus, we reexamined DNA-PK CS expression in cell lines from both normal and SCID foals using only the Ab that specifically recognizes the N-terminal 250 kDa of DNA-PK CS (Fig. 4A). As can be seen using this Ab, DNA-PK CS is detectable in as little as 12.5 µg whole cell extract from the normal cell line; no unique proteins of ~360 kDa (the predicted size of the mutant DNA-PK CS protein) are detected in as much as 200 µg of whole cell extract from the SCID cell line. Thus, if a mutant form of DNA-PK CS is expressed in equine SCID cells, it appears to be at least 16-fold less abundant than wild-type DNA-PK CS. These data support our initial conclusion that the mutant form of DNA-PK CS in equine SCID cells is unstable.

Although it seems implausible that an unstable form of DNA-PK CS could act as a dominant-negative inhibitor, the possibility exists that a low level of a mutant form of DNA-PK CS inhibits signal end joining in equine SCID cells. Thus, to more formally address this possibility, a human DNA-PK CS expression vector with a mutation analogous to the mutation in SCID horses was constructed. This was done first by assembling full-length human DNA-PK CS from a series of overlapping cDNA fragments. The complete coding sequence of human DNA-PK CS was subcloned into the pCMV6 mammalian expression vector. An internal deletion within the coding region was generated such that a frame shift analogous to the equine mutation occurs after amino acid 3160, adding an additional three amino acids from the altered reading frame before the occurrence of a stop codon. Plasmids encoding both full-length and mutated forms of DNA-PK CS as well as vector only controls were stably transfected into the SF19 murine SCID fibroblast cell line and clonal transfectants derived. In immunoblot analyses of whole cell extracts from the transfectants, full-length DNA-PK CS is easily detected and is expressed at roughly equivalent levels as in mouse SCID cells that harbor a portion of human chromosome 8 that contains the DNA-PK CS gene (Fig. 4B). As with equine DNA-PK CS, truncation of human DNA-PK CS at amino acid 3160 generates an unstable protein that cannot be clearly detected in the transfectants. As expected, full-length DNA-PK CS substantially restores the radiation resistance of the SF19 cell line, demonstrating the efficacy of our DNA-PK CS expression strategy (Fig. 4C). In contrast, transfectants expressing the mutated form have similar radiation sensitivity as the control transfectants. Of note, similar to a recent report (60), the DNA-PK CS transfectants described in this work are more radioresistant than the human chromosome 8 SCID transfectants.

We next assessed both coding and signal joint formation in murine SCID fibroblasts using a transient recombination assay. Three independent transfections are shown; data are presented as number of recombinants/µg substrate transfected. As can be seen, full-length DNA-PK CS substantially reverses the defect in coding joint formation in these murine SCID fibroblasts (Table II). In contrast, coding joint formation was not detected in either control transfectants (including pCMV6 and the RAG expression vectors) or transfections including the expression vector encoding the mutant protein. As expected, the murine SCID fibroblasts are capable of supporting reasonable levels of signal end joining, as assessed with the pJL201 substrate. In this cell line, the fidelity of signal joining is relatively high in the absence of functional DNA-PK CS (85%). The modest improvement in signal fidelity when full-length DNA-PK CS expression vector cotransfected (95%) is not statistically significant. As can be seen, cotransfection of the expression vector encoding DNA-PK CS, which is truncated at amino acid 3160, had no effect on the rate or fidelity of signal end resolution in murine SCID cells. In sum, we find no evidence of a dominant-negative effect by DNA-PK CS truncated at amino acid 3160. These data suggest that the differences in signal end resolution in SCID mice as compared with SCID foals represent actual differences in the absolute requirement for DNA-PK CS during V(D)J recombination.

Do other end joining pathways contribute to coding and signal end resolution in the absence of DNA-PK CS?

There are several discrepancies between the phenotypes of animals (or cell lines) deficient in the different components of the nonhomologous DNA end joining pathway. As discussed above, animals and cell lines vary in their requirements for DNA-PK CS for both coding and signal joint formation. In contrast, deficiencies in either subunit of Ku, XRCC4, or DNA ligase IV result in both defective signal and coding resolution in similar experimental systems.
There are also discrepancies in radiosensitivity of certain cell types, specifically ES cells. Although Ku- and XRCC4-deficient ES cells display extreme radiosensitivity, DNA-PKCS-deficient ES cells have similar radioresistance as normal ES cells. Several different models have been suggested to explain these apparent discrepancies. First, it has been proposed that DNA-PKCS is not involved in signal joining. The data presented in this study demonstrate that DNA-PKCS is involved in signal end joining, at least in certain species.

A second model that has been proposed is that Ku is required to disassemble the RAG postcleavage complex; without Ku, the RAG proteins remain bound to coding and signal ends, thus explaining why Ku-deficient mice have a more severe defect in V(D)J recombination than DNA-PKCS-deficient mice (10). Although there is considerable evidence suggesting that Ku may be involved in re-modeling the recombination complex (10, 61), this still would not account for the lack of signal resolution and more severe coding joint defect observed in SCID foals. Also, this model does not address the discrepancies in radiosensitivity between Ku and DNA-PKCS ES cells.

An attractive theory proposed by several investigators is that additional DNA end joining pathways may function to repair signal ends and some coding ends in DNA-PKCS-deficient mice or in DNA-PKCS-deficient ES cells (39, 41, 45). Bogue et al. have suggested that in normal animals, DNA-PKCS is involved in both coding and signal end resolution, providing for the efficient resolution of hairpinned coding ends and the near perfect ligation of signal ends (41). However, in the absence of DNA-PKCS, an alternative end joining pathway provides for signal end (and certain coding end) joining, but with lower efficiency and fidelity than in the presence of DNA-PKCS. Additionally, Gao et al. (39) have speculated that an alternative end joining pathway that is highly expressed in ES cells repairs DNA strand breaks in DNA-PKCS-deficient ES cells. To explain the marked differences in V(D)J recombination defects in DNA-PKCS-deficient foals and mice, this alternative pathway must not be equivalently active in all species or in different cell types. In fact, there is recent evidence that murine cells have an active DNA-PK-independent end joining pathway not detectable in human cells (62), providing support for the idea that alternative end joining pathways may be more abundantly expressed in certain species than others.

Still, the existence of an alternative pathway does not address the differences in V(D)J recombination defects in Ku vs DNA-PKCS-deficient rodent cells or the differing radiosensitivity of Ku vs DNA-PKCS ES cells. However, if this alternative joining pathway could utilize Ku in targeting DNA ends, this would provide an explanation for the relatively efficient signal joining observed in SCID mice (as opposed to Ku-deficient mice) as well as the radioresistance of DNA-PKCS-deficient ES cells (as opposed to Ku-deficient ES cells). Thus, in murine SCID cells that have an active DNA-PK-independent end joining pathway and adequate levels of Ku, but not DNA-PKCS, Ku can direct the alternative end joining pathway to V(DJ) intermediates, explaining relatively normal signal joining in these cells. Similarly, the possibility that Ku directs this putative alternative pathway to damaged DNA would also explain the different radiosensitivities of Ku- and DNA-PKCS-deficient ES cells. This model could still account for the more severe defect in SCID foals if equine lymphocytes are particularly inefficient in signal joining, at least in certain situations. As discussed above, the two major pathways for repairing DSBs in eukaryotic cells are HR and NHEJ, although there is considerable evidence for other less well-defined mechanisms. An attractive candidate for an alternative pathway that could facilitate the repair of V(D)J recombination intermediates in the absence of DNA-PKCS is single-strand annealing. This pathway depends on regions of sequence homology between the two DNA ends and resection of the DNA ends so that annealing of complementary regions can occur. In yeast, this pathway requires several components of the RAD52 homologous recombination pathway (RAD52, MRE11, Rad50, and XRS2). There is experimental evidence supporting the idea that a mechanism related to single-strand annealing might resolve V(DJ) recombination intermediates in certain situations. First, it is well appreciated that certain coding joints utilize short regions of sequence homology to facilitate joining (63–65), and in the absence of either Ku or XRCC4, the dependence on short sequence homology for efficient joining is accentuated (11, 51). Furthermore, there is precedent for overlapping roles of the nonhomologous DNA end joining factors in other pathways. Yeast that are defective in nonhomologous DNA end joining are not radiosensitive; the importance of this pathway for rejoicing radiation-induced DNA breaks is only observed in yeast.

### Table II. Transient recombination in the murine SCID fibroblast cell line SF19

<table>
<thead>
<tr>
<th>Expression Vectors</th>
<th>Signal (pH201)</th>
<th>Coding (pH290)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amp&lt;sup&gt;B&lt;/sup&gt; Cam&lt;sup&gt;B&lt;/sup&gt;, Rate (%)</td>
<td>Fidelity (%)</td>
</tr>
<tr>
<td>pCMV6</td>
<td>0/20,016, 0</td>
<td>NA</td>
</tr>
<tr>
<td>RAG 1+2</td>
<td>58/28,800, 0.201</td>
<td>85</td>
</tr>
<tr>
<td>Full-length PK&lt;sub&gt;CS&lt;/sub&gt;</td>
<td>26/26,100, 0.099</td>
<td>95</td>
</tr>
<tr>
<td>RAG 1+2</td>
<td>70/27,000, 0.259</td>
<td></td>
</tr>
<tr>
<td></td>
<td>54/45,942, 0.117</td>
<td></td>
</tr>
<tr>
<td>Truncated PK&lt;sub&gt;CS&lt;/sub&gt;</td>
<td>8/5320, 0.150</td>
<td>94</td>
</tr>
<tr>
<td>RAG 1+2</td>
<td>34/15,750, 0.216</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4/3120, 0.128</td>
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</tbody>
</table>

<sup>*Three independent transfections are shown; data are presented as number of recombinants/μg substrate transfected. To eliminate background Cam-resistant colonies, transformants were selected on plates containing 100 μg/ml ampicillin and 22 μg/ml chloramphenicol. In each case, plasmid DNA was prepared from a fraction of the CAM-resistant colonies to ensure authenticity of recombination as assessed by deletion of the sop sequence.</sup>
Amplification of D H J H coding joints (as described in Methods) was done using the following oligonucleotides: 3'-DFL16.1 and 5'-DFL16.1. Amplification of D H J H coding joints (as described in Materials and Methods) was done using the following oligonucleotides: 5'-DFL16.1 and 3'-JH1. Amplification of D H J H signal joints (as described in Materials and Methods) was done using the following oligonucleotides: 3'-DFL16.1 and 5'-JH1.

that are also defective in homologous end joining (66). Direct genetic evidence exists for overlapping roles for factors from these pathways in that the MRE11/RAD50/XRS2 complex is necessary for HR, NHEJ, and repair via single-strand annealing (67, 68). Direct biochemical evidence exists suggesting that Ku may function in other DNA repair pathways, in that Ku has been shown to stimulate DNA ligase activities for all human DNA ligases (69).

In yeast, RAD52 is essential for both homologous recombination and single-strand annealing (68). Mice with a targeted deletion of the murine RAD52 homologue have recently been generated (70). Although homologous recombination is impaired in cells from these animals, the cells are not hypersensitive to agents that induce DSBs, and the animals show no defects in the immune system. The fact that targeted deletion of RAD52 is not terribly detrimental to the mouse perhaps underscores the fact that NHEJ (as opposed to HR) is the major mechanism for repairing DSBs in higher eukaryotes (70). New insight regarding the function of RAD52 in higher eukaryotes has been gained, in that it has recently been demonstrated that RAD52, like Ku, can bind DNA ends in a sequence-independent manner (71). This finding led to the hypothesis that RAD52 may direct other factors involved either in single-strand annealing or homologous recombination to damaged DNA much in the same way that Ku has been proposed to direct components of the NHEJ pathway to damaged DNA (71).

We reasoned that if single-strand annealing contributes to signal end joining in murine lymphocytes in the absence of DNA-PK<sub>CS</sub>, signal joining would be more significantly impaired in lymphocytes lacking both DNA-PK<sub>CS</sub> and RAD52. Thus, we examined Ig D H J H rearrangements in mice deficient in both RAD52 and DNA-PK<sub>CS</sub> (Fig. 5). As can be seen, the level of D H J H coding joints in SCID/RAD52<sup>−/−</sup> animals is depressed ~50- to 100-fold as compared with RAD52<sup>−/−</sup> controls. This level of coding joint diminution is exactly what is observed when comparing D H J H coding joints in DNA-PK<sub>CS</sub>-deficient vs normal mice (41). In SCID/RAD52<sup>−/−</sup>, the level of signal joints is diminished by ~5- to 10-fold as compared with RAD52<sup>−/−</sup> animals. Again, this level of diminution is completely analogous to that observed when comparing D H J H signal joints in DNA-PK<sub>CS</sub>-deficient vs normal mice. In sum, SCID/RAD52<sup>−/−</sup> animals appear to be indistinguishable from SCID animals with respect to both coding and signal joint formation, and we conclude that RAD52 does not participate in resolving V(D)J recombination intermediates in the absence of DNA-PK<sub>CS</sub>. This obviously does not rule out the possibility that Ku directs some other component of this pathway (or others) to unresolved V(D)J recombination intermediates, or that another RAD52 homologue exists (as suggested previously (72)) that may play a more prominent role in DSBR in higher eukaryotes. Finally, this experiment underscores the less severe diminution in signal end resolution in SCID mice as compared with SCID foals and the substantial leakiness in coding end resolution in SCID mice that is not observed in SCID foals (compare Figs. 2 and 5).

**Conclusion**

In sum, these experiments establish that unlike DNA-PK<sub>CS</sub> deficiency in mice, the lack of DNA-PK<sub>CS</sub> in foals results in a profound defect in signal end resolution during V(D)J recombination. This difference cannot be explained by the specific mutation in SCID foals leading to a mutant protein that inhibits signal end resolution. Instead, these data suggest that different species vary in their absolute requirements for DNA-PK<sub>CS</sub> during V(D)J recombination. These data are consistent with the hypothesis that in the absence of DNA-PK<sub>CS</sub>, an undefined end joining pathway joins the nonhomologous DNA ends generated during V(D)J recombination. The observed differences in the absolute requirement for DNA-PK<sub>CS</sub> during V(D)J recombination may reflect the relative abundance of this alternative end joining pathway.

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**References**


