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SCID in Jack Russell Terriers: A New Animal Model of DNA-PKcs Deficiency

Katheryn Meek, Laura Kienker, Clarissa Dallas, Wei Wang, Michael J. Dark, Patrick J. Venta, Maryann L. Huie, Rochelle Hirschhorn, and Tom Bell

We recently described the incidence of a SCID disease in a litter of Jack Russell terriers. In this study, we show that the molecular defect in these animals is faulty V(D)J recombination. Furthermore, we document a complete deficit in DNA-dependent protein kinase activity that can be explained by a marked diminution in the expression of the catalytic subunit DNA-dependent protein kinase catalytic subunit (DNA-PKcs). We conclude that as is the case in C.B-17 SCID mice and in Arabian SCID foals, the defective factor in these SCID puppies is DNA-PKcs. In mice, it has been clearly established that DNA-PKcs deficiency produces an incomplete block in V(D)J recombination, resulting in “leaky” coding joint formation and only a modest defect in signal end ligation. In contrast, DNA-PKcs deficiency in horses profoundly blocks both coding and signal end joining. Here, we show that although DNA-PKcs deficiency in canine lymphocytes results in a block in both coding and signal end joining, the deficit in both is intermediate between that seen in SCID mice and SCID foals. These data demonstrate significant species variation in the absolute necessity for DNA-PKcs during V(D)J recombination. Furthermore, the severity of the V(D)J recombination deficits in these three examples of genetic DNA-PKcs deficiency inversely correlates with the relative DNA-PK enzymatic activity expressed in normal fibroblasts derived from these three species. The Journal of Immunology, 2001, 167: 2142–2150.

The DNA-dependent protein kinase (DNA-PK) is an extremely large multiprotein complex comprised of a regulatory subunit, the heterodimeric Ku protein comprised of 70- and 86-kDa polypeptides, and the catalytic subunit DNA-dependent protein kinase catalytic subunit (DNA-PKcs; a 465-kDa polypeptide) (reviewed in Refs. 1–3). DNA-PK is specifically activated to phosphorylate its target proteins in the presence of DNA ends, suggesting a role for this enzyme in DNA repair (4). Indeed, genetic experiments have demonstrated unequivocally that DNA-PK functions in the repair of DNA double-strand breaks (DSBs) (reviewed in Refs. 5 and 6). In eukaryotes, two major pathways exist to repair DSBs: homologous recombination and nonhomologous DNA end joining (NHEJ). Vertebrates rely most heavily on the NHEJ pathway whereas yeast preferentially use homologous recombination to repair DSBs. DNA-PK functions in the NHEJ pathway (7). Furthermore, recent evidence indicates that at least part of DNA-PK’s role in this pathway is dependent on its kinase activity (8, 9).

In higher eukaryotes, deficiencies in any of DNA-PK’s three component polypeptides effectively disrupt the organism’s capacity to efficiently repair DNA DSBs, resulting in extreme sensitivity to ionizing radiation. This radiosensitivity is not in and of itself lethal. However, in normal environments, DNA-PK deficiencies are generally lethal postnatally because vertebrate immune systems are dependent on the NHEJ pathway to complete V(D)J recombination, the site-specific recombination process that provides for assembly of unique Ag receptor genes (10–12). If V(D)J recombination is disrupted, lymphocyte development is blocked at the prolymphocyte stage, resulting in a profound deficiency of mature B and T lymphocytes and the disease SCID. The lymphocyte-specific endonuclease which initiates V(D)J recombination is comprised of the recombinase-activating gene (RAG) 1 and RAG2 proteins and is targeted to immune receptor gene segments by recombination signal sequences (RSS) which abut the coding sequences of all functional immune receptor gene segments (13, 14). During V(D)J recombination, the RAG proteins introduce two DSBs generating four DNA ends with structurally distinct termini (15, 16). The coding ends have covalently sealed hairpinned ends, whereas signal ends have blunt and 5′-phosphorylated termini. The NHEJ pathway mediates the resolution of both coding and signal ends.

Two spontaneous germline mutations of DNA-PKcs have been characterized previously. The first was discovered in a colony of C.B-17 mice which were shown to have markedly reduced numbers of both B and T lymphocytes (17). Later it was determined that the lack of mature lymphocytes was due to a severe defect in V(D)J recombination (18). This defect in V(D)J recombination produced a dramatic deficit in coding joint formation, but had a negligible effect on signal end joining, and, in 1995, DNA-PKcs was implicated as the defective factor (19). In 1975, McGuire and Poppie (20) described an autosomal recessive form of combined immunodeficiency that occurs at high frequency in Arabian foals (20). Like SCID mice, these animals are profoundly deficient in both B and T cells. In 1995, we characterized the molecular defect...
in these horses as faulty V(D)J recombination that could be explained by a severe deficiency in DNA-PKcs expression (21). In contrast to SCID mice, lymphocytes from SCID foals have dramatically reduced levels of signal joints. We first hypothesized that this difference might be explained by the specific DNA-PKcs mutations in these two animal models of SCID. The DNA-PKcs mutation in SCID foals deletes 976 aa from the C terminus, leaving the entire phosphatidylinositol 3-kinase domain (22). In contrast, the mutation in SCID mice deletes only 83 aa from the C terminus, leaving the phosphatidylinositol 3-kinase domain intact and generating a potentially actively mutant enzyme (23, 24). Thus, we attributed the less severe phenotype in SCID mice to an incomplete mutation. This hypothesis was negated by targeted deletion of DNA-PKcs (25–28). The phenotype of DNA-PKcs null mice is indistinguishable from C.B-17 SCID mice, although these recent studies establish that signal joint formation in SCID mice is also modestly defective—being both slightly reduced (0.1- to 10-fold) and less precise than in normal animals.

Another potential explanation for the phenotypic differences between SCID mice and foals is that the equine mutation might generate a functional protein that blocks other components of the NHEJ pathway in a dominant negative manner. We negated this hypothesis using site-directed mutagenesis of DNA-PKcs (29). Furthermore, we established that signal end joining in SCID foals is reduced by at least 4 logs and that coding end joining is reduced by 5 logs. Thus, both signal and coding joint formation is more dramatically reduced in SCID foals than in SCID mice (where coding joints are reduced by 2–3 logs maximally). We hypothesize that in normal lymphocytes, DNA-PKcs functions in both the efficient joining of coding ends and the efficient and precise joining of signal ends. In the absence of DNA-PKcs, alternative DNA end-joining pathways may facilitate inefficient and imprecise joining of recombination intermediates. Furthermore, this alternative pathway must not be utilized equivalently in all species.

We recently discovered another genetic form of non-B/Non-T SCID in a litter of Jack Russell terriers. Briefly, 12 of 32 siblings from a single breeding pair of Jack Russell terriers succumbed to opportunistic infections within 8–14 wk of age. To investigate this potential genetic immunodeficiency, an additional litter was whelped. From this litter, we determined that four of seven puppies displayed a phenotype of SCID which included extreme lymphopenia, agammaglobulinemia, thymic dysplasia, peripheral lymphoid aplasia, and apparent autosomal recessive inheritance. In this report, we assess cells or tissues from these animals for the following activities: adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP), Ig and TCR gene rearrangement, radiation resistance, and DNA-PK. We have determined that immunodeficiency in these animals is the result of defective V(D)J recombination that can be explained by a deficiency in DNA-PKcs. Our data indicate that as in SCID foals, both coding and signal joint resolution are impaired although the degree of both is intermediate between the murine and equine phenotypes.

Materials and Methods

Detection of ADA and PNP activity

The presence of ADA and PNP was detected by electrophoresis in starch gels and in situ staining essentially as described previously (30).

DNA isolation

DNA was prepared from thymus and bone marrow collected from 6-wk-old SCID and normal Jack Russell terrier littermates and from bone marrow of C.B-17 SCID and BALB/c mice using commercially available DNA extraction buffer (Applied Biosystems, Foster City, CA).

Oligonucleotides

Sequences of oligonucleotides used in this study were as follows: 5’-VH2, GACAGCCGCTTAAGGGGCGC; 5’-VH1, CAGATTGAGCCGCTTAAGGGGCGC; 5’-JH1, CAGATTGAGCCGCTTAAGGGGCGC; and 5’-JH4, GACAGCCGCTTAAGGGGCGC.

PCR

PCR were conducted in a volume of 100 µl using amplification primers described in each figure legend and the indicated amounts of DNA. For the experiment depicted in Fig. 1, 40 cycles of amplification were performed using the following conditions: 94°C for 1.5 min, 55°C for 2 min, and 72°C for 3 min. For nested PCR experiments, initial amplification conditions were: 94°C for 1.5 min, 2°C for 2 min, and 72°C for 3 min for 40 cycles. Ten microilters of each reaction was subsequently amplified as follows: 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min for 40 cycles. Twenty microilters of each PCR was analyzed by Southern blot 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 Applied Biosystems sequencer (Applied Biosystems).

Ligation-mediated PCR (LMPCR)

LMPCR was performed similar to the method described by Roth et al. (15). Briefly, 300 pmol of annealed linker (oligonucleotides 150 and 149) were ligated to 20 µg of normal canine thymus, normal canine thymus, or SCID canine thymus DNA. Ligated DNA was extracted with phenol-chloroform and ethanol precipitated. Subsequently, linker-ligated DNA was used in PCR using primers 150 + cas and 5’Bds for 40 cycles as follows: 94°C for 1 min, 64°C for 45 min, and 72°C for 45 min.

Cell lines

The JRSC and JN005 cell lines were established from dermal biopsies from a SCID puppy and from an unrelated normal dog, respectively. The MSU1.1 is a normal immortalized human fibroblast line, the generous gift of Justin McCormick (Michigan State University, East Lansing, MI). The 0176 and 1821 cell lines were derived from normal and SCID foals, respectively, and have been described previously (21). NS47, a wild-type mouse fibroblast cell line, was generously provided by Dr. K. Ariizumi (University of Texas Southwestern Medical Center, Dallas, TX). The JRSC cell line was generously provided by Dr. M. Bosma (Fox Chase Cancer Center, Philadelphia, PA).

Assessment of radiation sensitivity

Cells (10⁴) were exposed to various amounts of ionizing radiation using a 60Co source and immediately seeded in complete medium containing 20% FBS. After 14 days, cell colonies were fixed with 2% formaldehyde followed by 100% methanol. Subsequently, the colonies were stained with trypan blue and colony numbers were assessed.
DNA-PK microfractionation and measurement of kinase activity

Whole-cell extracts were prepared by a modification of the method of Finnie et al. (31). Briefly, 20 × 10^6 cells were harvested, washed three times in PBS, and cell pellets were frozen at −80°C. Frozen cell pellets were resuspended in 20 μl of extraction buffer (50 mM NaF, 20 mM HEPES (pH 7.8), 450 mM NaCl, 25% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.5 μg/μl leupeptin, 0.5 μg/μl protease inhibitor, and 1.0 μg/ml trypsin inhibitor), subjected to three freeze/thaw cycles (liquid nitrogen/37°C), and centrifuged at 8160 × g for 7 min at 4°C. Supernatants were stored at −80°C before use and concentrations were determined by Bradford analysis using BSA as a standard.

The SignaTECT DNA-PK assay system (Promega, Madison, WI) was used to assay DNA-PK activity with the following modifications. Two hundred micrograms of extract were incubated with 20 μl of preswollen dsDNA-cellulose beads (Amersham Pharmacia Biotech, Piscataway, NJ) for 30 min at 4°C. The dsDNA-cellulose was then washed three times with 1 ml of buffer A (25 mM HEPES (pH 7.9), 50 mM KCl, 10 mM MgCl₂, 10% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT) before it was resuspended in 20 μl of DNA-PK reaction buffer containing 100 μg/ml BSA. Kinase reactions were conducted with 10-μl aliquots of the resuspended DNA-PK-absorbed cellulose beads and were performed in both the presence and absence of a biotinylated DNA-PK p53-derived substrate peptide. Terminated reactions were analyzed by spotting onto SAM² membrane, washing, and counting in a scintillation counter as per the manufacturer’s instructions. All assays were performed in duplicate with at least two different extract preparations.

Immunoblot analysis

The indicated amounts of whole-cell extracts were electrophoresed in a 5% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride. A mouse Ab (18-1, generous gift from Dr. T. Carter, St. Johns University, New York, NY) was used as the primary Ab (1:300). This monoclonal can be used to assay DNA-PKcs from both the presence and absence of a biotinylated DNA-PK p53-derived substrate peptide. Terminated reactions were analyzed by spotting onto SAM² membrane, washing, and counting in a scintillation counter as per the manufacturer’s instructions. All assays were performed in duplicate with at least two different extract preparations.

Genetic marker in the canine DNA-PKcs gene

Primers were designed to conserved regions in exons 5 and 6 of the human and mouse DNA-PKcs gene sequences (33, 34). A 650-bp dog DNA-PKcs fragment was amplified in a 25-μl reaction using 50 mM KCl, 10 mM Tris (pH 8.3 at 21°C), 1.5 mM MgCl₂, 100 μM of each primer, and 50 × 10⁻⁹ g of canine genomic DNA. Cycling conditions were 95°C for 1 min, 55°C for 2 min, and 72°C for 3 min for 35 cycles. The identity of the sequence was confirmed by performing a BLAST search (35). Two additional primers were made to determine the complete sequence of intron 5 (GenBank accession number in process). A single nucleotide polymorphism (SNP) was found in intron 5 using a pool-and-sequence approach (36). Briefly, genomic DNA samples from 10 different dog breeds were pooled, amplified, and sequenced using a Thermosequenase cycle sequencing kit according to the manufacturer’s instructions (United States Biochemical, Cincinnati, OH). The SNP was identified as two bands (A and G) at the identical position in the sequencing ladder. An RsrI restriction site was created for the G allele by altering one base in a diagnostic primer. Amplification conditions were the same as above, except that cycling conditions were 94°C for 1 min, 54°C for 2 min, and 72°C for 3 min for 35 cycles. Three microliters of 50 mM MgCl₂ and 1 μl of RsrI (10 U) were added directly to the PCR product, followed by incubation at 37°C for 1 h. Products were analyzed by electrophoresis on a 2% agarose gel. The A allele was recognized by the presence of a 202-bp band and the G allele was recognized by the presence of 178- and 24-bp bands.

Results

Purine metabolism is normal in SCID puppies

Abnormalities in purine metabolism due to defective ADA or PNP account for a significant percentage of the cases of SCID in humans. To date, no such naturally occurring mutations have been documented in animals (37). We assessed both ADA and PNP activity in normal and SCID dogs by assaying enzymatic activity in hemolysates after electrophoresis in starch gels, as described previously (30). In sum, levels of PNP and ADA activity were similar in hemolysates from normal and SCID dogs (data not shown), and we conclude that ADA and PNP defects cannot explain the immunodeficiency observed in Jack Russell terriers.

Ig and TCR coding joints are severely diminished in SCID puppies

To determine whether V(D)J recombination is intact in SCID puppies, DNA was isolated from thymus and bone marrow samples from affected and unaffected Jack Russell Terrier littermates. Using PCR amplification primers derived from published canine cDNAs, both TCR and Ig coding joints were assessed. As can be seen, unrearranged V₄H (Fig. 1A, top panel) and V₅H (Fig. 1C, top panel) gene segments are equivalently amplified from normal and SCID bone marrow and thymus DNA, respectively. Complete V₄H, D₄H, J₄H (Fig. 1A, bottom panel) or V₅H, D₅H, J₅H (Fig. 1C, bottom panel) coding joints were only consistently detected from DNA derived from normal animals. Furthermore, coding joints in normal dogs were easily detected using as little as 5 ng of thymus or bone marrow DNA. In contrast, only extremely low levels of V₄D₅J₅ coding joints could be detected from SCID thymus and detection of coding joints required 5 μg of SCID thymus DNA. V₅D₅J₅ coding joints were not detected from SCID bone marrow DNA. This level of coding joint depression is representative of experiments when comparing the sensitivity of TCR (representative of six different experiments) and Ig (representative of three different experiments) rearrangements from two normal and two SCID animals (data not shown).

In our attempt to detect coding joints from SCID dogs and previously from SCID foals (29), a more aggressive PCR strategy than that used in studies of SCID mouse Ig and TCR rearrangements was used. Both the quantity of template DNA and the cycle number were increased to detect coding joints in SCID dogs. Therefore, to more directly compare the relative coding joint diminution in SCID dogs to that observed in SCID mice, which was previously reported to be depressed 100- to 1000-fold compared with normal animals (18, 25-27), we assessed Ig V₄H-D₄H-J₄H rearrangements in murine bone marrow DNA using our PCR strategy and a V₄H region primer specific for the J₅58 V₅H gene family (Fig. 1B, bottom panel). As can be seen, the sensitivity of detecting Ig rearrangements from normal mouse and dog bone marrow is similar. Furthermore, Ig V₅H-D₅H-J₅H rearrangements are depressed by ~100-fold in bone marrow from SCID mice, consistent with the reports from other investigators. This level of coding joint depression was also observed using another V₅H segment primer (the X24 gene segment family, data not shown). Thus, we conclude that the block in coding joint resolution is more dramatic in SCID dogs than in SCID mice.

V(D)J recombination is initiated normally in SCID puppies

To date, several different spontaneous germline mutations have been documented which result in defective V(D)J recombination and the disease SCID. In children, mutations in either RAG1 or RAG2 have been shown to account for SCID (38). Similarly in mice, targeted mutation of either RAG1 or RAG2 results in a severe form of SCID (39, 40) which does not include the "leaky" phenotype seen in DNA-PKcs-deficient mice. RAG-deficient mice completely lack either signal or coding rearrangements because, in the absence of the RAG proteins, rearrangement is not initiated and Ig and TCR genes remain in germline configuration. This has been documented using the technique of LMPCR to assess free signal ends that are present in developing lymphocytes as recombination intermediates (16). We reasoned that the RAG proteins could be
implicated (or eliminated) as the defective factor in these SCID puppies by assessing free signal ends by LMPCR.

Using LMPCR, free \( \text{J}_B \) signal ends were assessed from normal canine thymus DNA, canine SCID thymus DNA, and normal canine lung DNA. As can be seen (Fig. 2A), free signal ends are equivalently detected in normal and SCID thymus DNA (compare lanes 3 and 4 to lanes 5 and 6). As expected, free signal ends are not detected in nonlymphoid DNA (lanes 1 and 2). These data suggest that TCR gene rearrangement is initiated normally in SCID dogs and that the RAG proteins are fully functional.

Signal joints are markedly reduced in SCID puppies

Using amplification primers from conserved regions of \( \text{D}_B 2 \) and \( \text{J}_{H2.1} \), the intergenic region between the two gene segments was cloned and sequenced so that \( \text{D}_B \text{J}_B \) signal joints could be examined. Although signal joints were easily detected from normal canine thymus DNA with a standard PCR assay, signal joints were not detected from canine SCID thymus DNA (data not shown). Thus, a more sensitive nested PCR strategy was used which is completely analogous to the strategy used to detect signal joints from SCID foals in our recent study (29). As can be seen (Fig. 2B), signal joints can be detected in as little as 0.5 ng of normal canine thymus DNA; in contrast, signal joints could only be detected in 50 ng of canine SCID thymus DNA. This degree of signal joint diminution was consistent when comparing two SCID and two normal animals and is representative of four independent experiments. Thus, we conclude that the frequency of \( \text{D}_B \text{J}_B \) signal joints in canine SCID thymus is \( \sim 2 \) logs lower than in normal canine thymus.

As discussed above, signal joints are generally precise, lacking either nucleotide addition or deletion. Structurally, signal joints are head to head ligations of the two heptamer sequences which generates a de novo restriction endonuclease site, \( \text{ApaLI} \). It has been demonstrated that the signal joints formed in SCID mice are less precise than in normal animals (27). The fidelity of signal ligation was examined by subjecting the amplified signal joints to \( \text{ApaLI} \) restriction. As can be seen (Fig. 2C), \( \sim 90\% \) of the signal joints amplified from normal dogs are sensitive to \( \text{ApaLI} \), whereas only 10–20\% of the joints amplified from SCID dogs are sensitive to \( \text{ApaLI} \). Thus, we conclude that both the rate and fidelity of signal end resolution in SCID dogs is diminished. Furthermore, the relative diminution in signal end resolution in SCID dogs is intermediate as compared with SCID mice or SCID horses such that the degree of signal joint reduction in these three models of SCID is as follows: equine SCID > canine SCID > murine SCID.

Hybrid joints are modestly reduced in SCID puppies

Hybrid joints are examples of aberrant end resolution and result from ligation of the coding end of one gene segment to the signal end of a second gene segment (41). The frequency of hybrid rearrangements of endogenous immune receptor gene segments has recently been shown to be \( \sim 100\text{-}1000\text{-fold less than standard joining depending on the specific rearrangements analyzed} \) (42, 43). It has recently been shown that hybrid rearrangements can be mediated solely by the RAG proteins in a reversal of the normal cleavage reaction (44, 45). Thus, in the absence of intact nonhomologous DNA end joining, the relative number of hybrid joints should be relatively normal, as compared with either signal or coding joints. We recently examined hybrid joining of TCR gene segments in equine SCID lymphocytes (29). We found that although certain hybrid rearrangements occurred at relatively normal levels, other

![FIGURE 1. Analysis of Ig and TCR coding joints. A, PCR amplification of the germline (top panel) or rearranged (bottom panel) Ig genes from normal dog bone marrow (lanes 1–5), SCID dog bone marrow (lanes 6–10), or no template (lane 11). Amplification of the germline \( \text{V}_H \) gene segment was performed as described in Materials and Methods using the oligonucleotides can 5′-VH-2 and can 3′-JH. B, PCR amplification of germline (top panel) or rearranged (bottom panel) murine \( \text{Ig} \) genes from BALB/c bone marrow (lanes 1–5), from C.B-17 SCID bone marrow (lanes 6–10), or no template (lane 11). Amplification of the germline \( \text{J}_H \) gene segment was done as described in Materials and Methods using the oligonucleotides mur 5′-JH4 and mur 3′-JH4. C, PCR amplification of germline (top panel) or rearranged (bottom panel) canine TCR genes from normal dog thymus (lanes 1–5), from SCID dog thymus (lanes 6–10), or no template (lane 11). Amplification of the germline \( \text{V}_B \) gene segment (as described in Materials and Methods) was performed using the oligonucleotides can 5′-VB1 and can 3′-JB. A–C, The quantities of DNA (ng) used in amplifications are indicated above the figures.]
hybrid joints were severely diminished, suggesting that some hybrid rearrangements are mediated by the NHEJ pathway. Hybrid rearrangements were also assessed in canine thymocytes. As can be seen (Fig. 3A), J_B coding 3’D_B RSS hybrid joints are diminished by 2 logs in canine SCID thymocytes, similar to the reduction observed in signal joints. These data support our previous conclusion that certain hybrid joints are mediated predomi-
nately by the NHEJ pathway.

Structural characteristics of TCR hybrid joints in canine lymphocytes

It has previously been reported that hybrid and coding joints iso-
lated from animals deficient in Ku or DNA-PKcs exhibit less func-
tional diversity than rearrangements isolated from normal animals (46). This is primarily due to diminished numbers and length of N segments (the random nucleotides added at the coding junctions by the enzyme terminal deoxynucleotidyl transferase). We chose to assess junctional diversification of rare rearrangements present in canine lymphocytes by analyzing hybrid joints (as opposed to coding joints) for two reasons. First, hybrid joints are significantly more abundant than coding joints; furthermore, it has been shown that hybrid joints display similar levels of N and P segment addi-
tions as compared with coding joints (46), and thus should be representative with regard to junctional modification. Therefore, hybrid joints were cloned and sequenced from thymus DNA from both normal and SCID dogs (Fig. 3B). As can be seen, excessive P segments (the palindromic nucleotides at the junctions generated by asymmetric opening of the hairpinned coding termini) derived from the Jy coding segment are observed in the SCID rearrange-
ments. Fifty percent of the rearrangements have P elements which range from 1 to 9 nt. None of the hybrid joints isolated from the normal animals have P elements. There is a slight decrease in the number of N segment-positive rearrangements when comparing normal animals to SCID as well as a modest decrease in N segment length (2.4 vs 8.9 nt). Hybrid and coding joints from Ku-deficient mice have virtually no N segments, whereas SCID mice have only a modest deficiency in N segment addition (46). Thus, the struc-
tural characteristics of these hybrid joints are more analogous to those observed from SCID mice than from those observed from Ku-deficient mice.

Radiosensitivity of canine SCID fibroblasts

The data presented thus far are consistent with the NHEJ pathway being defective in these animals. Thus, we next assessed radio-
sensitivity in fibroblasts derived from a SCID puppy and from a normal dog. As can be seen in Fig. 4A, the normal canine fibroblast cell line is more radioreistant than fibroblasts derived from the SCID puppy. These data suggest that the genetic defect in these animals results in impaired NHEJ.

Canine SCID fibroblasts lack DNA-PK activity because of severely diminished DNA-PKcs expression

Of the five known factors known to be requisite for both the V(D)J recombination and NHEJ, three are components of DNA-PK. Thus, to further investigate the NHEJ pathway in these animals, DNA-PK activity was assessed using whole-cell extracts prepared from canine SCID and normal fibroblasts. It is well appreciated that DNA-PK activity is abundantly expressed in human cells but much more minimally expressed in rodent cells. We previously established that equine cells express intermediate levels of DNA-PK activity as compared with human and rodent cells. To compare the relative level of DNA-PK in normal canine cells to the level in human, equine, and murine cells, we assessed DNA-PK activity in normal fibroblast cell lines from these species. As can be seen (Fig. 4B), in normal canine fibroblasts, DNA-PK activity is intermediate as compared with that observed in horse and mouse fibroblasts. Finally, DNA-PK activity was undetectable in the canine, equine, or murine SCID fibroblasts, implicating ei-
ther DNA-PKcs or Ku as the defective factor in SCID dogs.

Next, we performed immunoblot assays to assess levels of DNA-PKcs, Ku70, and Ku86 in whole-cell extracts from canine SCID fibroblasts compared with normal canine cells. Whereas Ku levels did not differ in SCID cells compared with the normal cells (data not shown), canine SCID fibroblasts were found to be de-
fective in DNA-PKcs expression (Fig. 4C). The 465-kDa DNA-
PKcs polypeptide is easily detected in the normal canine cells, but not in the canine SCID fibroblasts, and we conclude that DNA-
PKcs expression is markedly diminished in canine SCID cells.

Identification of a SNP in the DNA-PKcs gene and segregation of one allele with the mutant phenotype

To obtain genetic evidence that DNA-PKcs is in fact the defective factor in these animals, we attempted to identify a polymorphism within the canine DNA-PKcs gene to determine whether within a lineage a single allele segregated with the mutant phenotype. A
polymorphic transition (A/G) was identified at position 95 of intron 5 by using a pool-and-sequence method. A diagnostic PCR-based test was then developed to distinguish the two alleles. When DNA samples were analyzed from members of the pedigree in which the SCID gene was segregating, it was found that segregation of the G allele was consistent with the SCID gene being DNA-PKcs, under the assumption that the G allele is linked with the mutation in the gene (Fig. 4D). The three affected animals are homozygous for the G allele. The three unaffected siblings are either homozygous for the A allele or heterozygotes (Fig. 4D). We have recently tested an additional five animals from a subsequent litter, and homozygosity for the G allele segregates exclusively with the SCID phenotype. Thus, in this limited pedigree analysis, the mutant phenotype segregates with homozygosity of one DNA-PKcs allele. These data are consistent with (although certainly not indicative of) DNA-PKcs being the defective factor in these animals.

**Discussion**

In this study, we have characterized the third example of a genetic DNA-PKcs deficiency. Like mice and horses, DNA-PKcs deficiency in dogs results in SCID because of defective V(D)J recombination. Similarly, cells from SCID dogs are hypersensitive to ionizing radiation. The data presented here demonstrate significant differences between species for the absolute requirement for DNA-PKcs during V(D)J recombination. It is well established that lack of DNA-PKcs in mice results in a "leaky" block in V(D)J recombination such that coding joint resolution is depressed by as much as 100- to 1000-fold, but with considerably less effect on certain "privileged" immune receptor loci, which are apparently unaffected and generate normal levels of coding joints (25–28). Signal joint ligation is even less affected, being virtually normal in some cells and depressed by only as much as 10-fold in some experimental systems. In contrast, DNA-PKcs deficiency in horses results in a much more substantial block of V(D)J recombination, resulting in a 5-log depression in coding joints and a 4-log depression in signal joints (21, 29, 47). The data presented in this report demonstrate that DNA-PKcs deficiency in dogs results in V(D)J recombination defects which are intermediate between those observed in horses and mice. Thus, we conclude that the absolute requirement for DNA-PKcs in V(D)J recombination varies in these species.

**FIGURE 3.** Analysis of TCRβ hybrid rearrangements. A, Nested PCR amplification of JB coding to 3’DB RSS hybrid joints from normal canine thymus (lanes 1–4) or SCID dog thymus (lanes 5–8). Quantities of DNA (μg) used in amplification are indicated above the figure. Amplification of hybrid joints was done as described in Materials and Methods using the oligonucleotides can 3’T/H11032 JB outer and can 3’T/H11032 DB outer and then can 3’T/H11032 JB inner and can 3’T/H11032 DB inner. B, Nucleotide sequences of DβJβ hybrid joints isolated from normal and SCID canine thymus are aligned to germline Dβ and Jβ gene segments. Nucleotides potentially derived from P and N addition are depicted.
three species. This conclusion presents several unanswered questions regarding the biology of DNA-PK.

First, are all cell types affected by species-dependent differences in the absolute requirement for DNA-PKcs? Alternatively, are just lymphocytes from dogs and horses more dependent on DNA-PKcs than murine lymphocytes? In some situations, DNA-PKcs has been implicated as being crucial in preventing apoptosis (48) although in others it has been suggested to promote apoptosis (49). Also, it has been demonstrated previously that unrepaired recombination intermediates in the thymus of SCID mice results in p53 activation (23) which may facilitate apoptosis. Our data could be explained by an increased susceptibility to apoptosis of equine and canine prolymphocytes as compared with mouse prolymphocytes. If canine and equine SCID prolymphocytes undergo rapid apoptosis in response to unrepaired recombination intermediates, any potential leaky rearrangements might not be detected. Thus, the more stringent DNA-PKcs requirement we observe might not actually reflect a more stringent requirement for end joining per se, but instead reflect a lack of rescue from apoptosis or perhaps more rapid apoptosis in prolymphocytes. However, in our previous study, a severe deficit in signal end resolution was demonstrated in SCID horse fibroblasts using a transient recombination assay (21); this assay should be insensitive to apoptotic effects. Using the same assay in our laboratory, most SCID mouse fibroblasts support wild-type levels of signal end joining (29). This suggests that the more stringent requirement for DNA-PKcs in some species is important for DNA end joining, as opposed to rescue from apoptosis, and that this more stringent requirement may be consistent in all cell types. Finally, it has recently been demonstrated that the NHEJ pathway is important for the maintenance of genomic stability (50, 51). Whereas loss of either XRCC4 or DNA ligase IV in mouse embryonic fibroblasts results in a significant increase in various chromosomal aberrations, loss of DNA-PKcs has a clear although less dramatic effect (50). This difference in chromosomal stability in mice with mutations in distinct components of the NHEJ pathway is reminiscent of differences in the rate and fidelity of signal end joining in the same animals (52). It follows that DNA-PKcs deficiency in dogs and horses may result in more significant chromosomal instability than in mice. This question is currently under examination.
A second question is why are there species differences in absolute DNA-PKcs requirements? The severity of the V(D)J recombination defects in these three examples of genetic DNA-PKcs deficiency (this report; Refs, 18, 25–27, and 29) appears to inversely correlate with two factors: life span and normal DNA-PK enzymatic activity. The correlation between severity of V(D)J recombination defects in each of the DNA-PKcs deficiencies and the relative DNA-PK activity expressed in normal cells from the same species is illustrated in Fig. 5. As can be seen, there is a highly significant correlation between relative DNA-PK activity and the log depression in either signal or coding joint formation. Fig. 5 also illustrates that DNA-PKcs deficiency in each of these species affects signal end joining more severely than coding end joining.

It is well appreciated that rodent cells express 50- to 100-fold less DNA-PK activity than human cells (31). This difference in DNA-PK activity can largely be explained by a similar difference in DNA-PKcs and Ku protein levels in human vs rodent cells. Similarly, using mAbs which recognize DNA-PKcs in many species, we have documented that the differences in DNA-PK activity between human cells and either canine or equine fibroblasts are largely the result of protein expression levels (data not shown). Thus, to date, there is no evidence that the specific activity of human DNA-PK differs from that of DNA-PK from other species.

Here, we show that horses (life span ~28 years) and dogs (life span ~14 years) express intermediate levels of DNA-PK activity as compared with rodents and humans. Thus, these data extend this general correlation between relative DNA-PK activity and natural life span. This correlation might predict that loss of DNA-PKcs expression in humans would result in the most severe phenotype. It is interesting that three spontaneous DNA-PKcs germline mutations have now been documented in animals whereas no germline mutations of DNA-PKcs have been implicated in the numerous incidences of genetic combined immunodeficiency disease in humans. This raises the question of whether a germline DNA-PKcs deficiency in humans might be lethal.

It seems reasonable that animals that express very high levels of an enzymatic activity might have a more absolute requirement for that activity in vivo as compared with animals which express minimal levels of the activity. Thus, horse cells that express relatively high levels of DNA-PK have a more stringent requirement for DNA-PKcs than mouse cells. Perhaps expression of high DNA-PK levels reflects an evolutionary preference for NHEJ in some species over other end joining pathways. The question remains: are alternative end joining pathways less active in species which express abundant levels of DNA-PK? If so, perhaps the low expression of this alternative pathway explains the relative lack of “leakiness” in the V(D)J recombination defects in SCID dogs and SCID horses.

In summary, here we describe the third example of genetic DNA-PKcs deficiency. As is the case with SCID mice and SCID foals, DNA-PKcs deficiency in dogs results in a block in V(D)J recombination and extreme radiosensitivity. The severity of the V(D)J recombination defects in these three examples of SCID are as follows: equine SCID > canine SCID > murine SCID. Finally, the severity of the V(D)J recombination defects in these three species inversely correlates with differences in DNA-PK levels expressed normally.

References


