118

Cell, Vol. 68, 869–877, March 6, 1992, Copyright © 1992 by Cell Press

RAG-1-Deficient Mice Have No Mature B and T Lymphocytes

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Summary

The V(D)J recombination activation gene RAG-1 was isolated on the basis of its ability to activate V(D)J recombination on an artificial substrate in fibroblasts. This property and the expression pattern in tissues and cell lines indicate that RAG-1 either activates or catalyzes the V(D)J recombination reaction of immunoglobulin and T cell receptor genes. We here describe the introduction of a mutation in RAG-1 into the germline of mice via gene targeting in embryonic stem cells. RAG-1-deficient mice have small lymphoid organs that do not contain mature B and T lymphocytes. The arrest of B and T cell differentiation occurs at an early stage and correlates with the inability to perform V(D)J recombination. The immune system of the RAG-1 mutant mice can be described as that of nonleaky scid mice. Although RAG-1 expression has been reported in the central nervous system of the mouse, no obvious neuroanatomical or behavioral abnormalities have been found in the RAG-1-deficient mice.

Introduction

The generation of antibody and T cell receptor (TCR) diversity is mediated to a large extent by the somatic DNA rearrangement known as V(D)J recombination, which joins variable (V), diversity (D), and joining (J) gene segments (Tonegawa, 1983). The genes for the antigen receptors are assembled through this process exclusively in lymphocytes. V(D)J recombination occurs at seven different loci: the immunoglobulin (Ig) heavy chain, κ and λ light chain loci in B lymphocytes (Tonegawa, 1983), and the TCR α, β, γ, and δ chain loci in T lymphocytes (Davis and Bjorkman, 1988). The prevailing model is that a common recombinase is active in precursors of both B and T cells and that the sequential recombinations are executed by developmentally controlled targeting of the recombinase to the different loci (Alt et al., 1986).

The cis-acting sequences required for V(D)J recombination have been described in detail (Tonegawa, 1983; Hesse et al., 1989). A gene called the recombination activation gene (RAG-1) was isolated by virtue of its ability to activate V(D)J recombination in NIH 3T3 fibroblasts on an artificial recombination substrate carrying selectable markers (Schatz and Baltimore, 1988; Schatz et al., 1989). A second, structurally unrelated gene called RAG-2 was later identified in the immediate vicinity of RAG-1 (Oettinger et al., 1990). A model has been proposed in which RAG-1 and RAG-2 together are sufficient to induce V(D)J recombination on an artificial substrate in fibroblasts (Oettinger et al., 1990). The expression of both genes is concordant and restricted to cell lines displaying V(D)J recombination activity and to developing lymphoid tissues (Schatz et al., 1989; Oettinger et al., 1990; Boehm et al., 1991; Turka et al., 1991). The only reported discordancies in their spatial patterns of expression are transcription of only RAG-1 in the central nervous system of the mouse (Chun et al., 1991) and of only RAG-2 in the bursa of Fabricius of the chicken (Carlson et al., 1991).

We have introduced a deletion in the RAG-1 gene of pluripotent mouse embryonic stem (ES) cells (Evans and Kaufmann, 1981) by the targeted gene disruption technique (Capecchi, 1989) and produced mice homozygous for the RAG-1 mutation (hereafter called mutant, homozygous mutant, or RAG-1-deficient mice). These mutant mice do not have any mature B and T lymphocytes. Flow cytometric analysis of lymphoid organs reveals a blockade of lymphocyte differentiation at an immature stage, similar to the situation described in the severe combined immunodeficiency (scid) mouse (Bosma et al., 1983; Bosma and Carroll, 1991). Southern blot analysis of DNA from thymus and bone marrow–derived Abelson-transformed cell lines indicates that both TCR and immunoglobulin gene loci remain in the germline configuration. In an accompanying paper (Shinkai et al., 1992 [this issue of *Cell*]), a similar phenotype is described in RAG-2-deficient mice. Taken together, these data suggest that RAG-1 and RAG-2 are both necessary in vivo either to activate or to catalyze the V(D)J recombination reaction.

In view of the reported expression of RAG-1 in the central nervous system (Chun et al., 1991), we have examined the brain of the RAG-1 mutant mice by histological means, but have not detected any overt structural abnormalities.

Results

Generation of the Mutation in RAG-1 in ES Cells and in Mice

The targeting vector pPMKO-31 (Figure 1) was constructed from a RAG-1 genomic clone isolated from a genomic DNA library made from ES cells of a 129/Sv mouse, in order to maximize homology between the targeting plas-

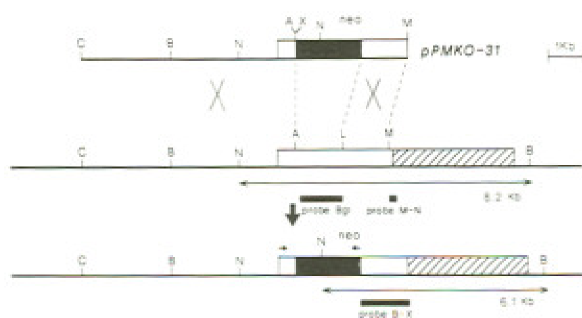

Figure 1. Targeting Scheme

(Top) Targeting vector pPMKO-31. Open box, translated RAG-1 sequences; dark box, *neo*-selectable marker. The location of the small first (untranslated) exon of RAG-1 has not been defined precisely and is therefore not indicated.

(Middle) Genomic structure of the RAG-1 locus. Hatched box, untranslated RAG-1 sequences. Probes are: Bgl, for deleted sequences, see Figure 2B; and M–N, external probe for screening of the ES clones. The size of BamHI–NcoI genomic restriction fragment hybridizing to probe B–X is indicated below the double-arrowed line.

(Bottom) Consequence of homologous integration. Sequences (1356 bp) at the 5′ end of RAG-1 are deleted. The transcription of the *neo* gene is in the opposite orientation of RAG-1 (indicated with arrows). The 8.1 kb BamHI–NcoI genomic restriction fragment is replaced by a 6.2 kb fragment recognized on Southern blot by using probe B–X (see Figure 2A). Abbreviations for restriction sites: C, ClaI; B; BamHI; N, NcoI; A, ApaI; X, XhoI; L, BglI; M, MluI.

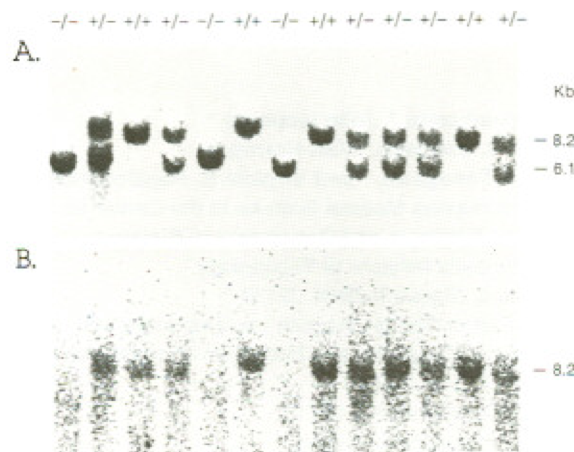

Figure 2. Southern Blot on Tail DNA

(A) Tail DNA was isolated from a litter of 13 mice of a (129/Sv × CD1) F1 heterozygote intercross. DNA was cut with BamHI and NcoI and hybridized to probe B–X. The upper band indicates the wild-type allele, the lower band corresponds to the allele that has undergone homologous recombination. Three mice in the litter are homozygous mutant, as they have only the lower band.

(B) The blot was stripped and hybridized with probe Bgl, which is complementary to sequences within the deletion generated by the targeting event. The three homozgous mutant mice do not contain DNA sequences hybridizing to this probe.

mid and the target sequences. Homologous integration would create a deletion of 1356 bp in the 5′ end of the coding sequence of RAG-1. The resulting mutation is most likely a null mutation, as the deletion encompasses about half of the coding sequence of RAG-1 and includes both the putative nuclear localization signal and the zinc finger–like motif (Schatz et al., 1989). Moreover, as the *neo*-selectable marker is introduced in the opposite transcriptional orientation, a polypeptide correctly initiated at the translation start site would probably be prematurely terminated at the level of the *neo* gene.

The targeting strategy employed was to isolate a number of G418-resistant clones and analyze them individually by Southern blot, since previous experience in our laboratory had shown that the negative selection step utilizing the herpes simplex virus–thymidine kinase gene (Mansour et al., 1988) does not give a significant enrichment and that targeting frequencies among the G418-resistant clones are generally high enough for direct screening (Mombaerts et al., 1991; and data not shown). Using AB1 ES cells (McMahon and Bradley, 1990), we obtained targeted clones in two independent experiments: one (clone A103) out of 130 G418-resistant clones in one experiment and one (clone G113) out of 117 clones in another experiment.

Clone A103 was injected into C57BL/6J blastocysts. From those implanted females that became pregnant (representing 194 implanted blastocysts), 29 male, 1 hermaphrodite, and 12 female chimeras were born, with an average coat color chimerism of about 75%. Of 27 chimeric males that were test mated to CD1 or C57BL/6 females, 17 had live offspring, and 12 were germline chimeras. One out of eight female chimeras had germline offspring. Clone A103 was also injected into eight BALB/c blastocysts, giving rise to two male chimeras and one female chimera. This female proved to be a germline chimera. Clone G113 did not give rise to good chimeras.

(129/Sv × CD1) F1 heterozygotes were intercrossed to produce homozygotes. Offspring were genotyped by Southern blot analysis of tail DNA (data from one litter are shown in Figure 2). Of 20 newborn animals, 6 were homozygous, and 33 out of 112 offspring genotyped after weaning were homozygous.

Macroscopic Analysis

Mice homozygous for the RAG-1 mutation have proven healthy and indistinguishable from their normal littermates by visual inspection up to 21 weeks of age. RAG-1 mutant mice are fertile. Two 12-week-old mutant males could readily fertilize either a homozygous or heterozygous mutant female. In the former case 12 pups were born, and in the latter, 11. Both litters were raised successfully to weaning.

We determined the number of cells in the lymphoid organs of twelve mutant mice of varying ages (newborn and 3- to 9-week-old mice) in comparison with corresponding wild-type or heterozygous littermates. The thymus of the mutant mice contained 15 to 130 times fewer cells than the wild-type or heterozygous littermates. One 4-week-old mutant mouse had the highest number of thymocytes among all mutant mice examined, but that was still 3.4 × 10^6 compared with 230 × 10^6 thymocytes in a wild-type littermate. The numbers of nonerythroid cells in the spleen of mutant mice of 3 weeks or older were between five and nine times smaller than those in corresponding wild-type

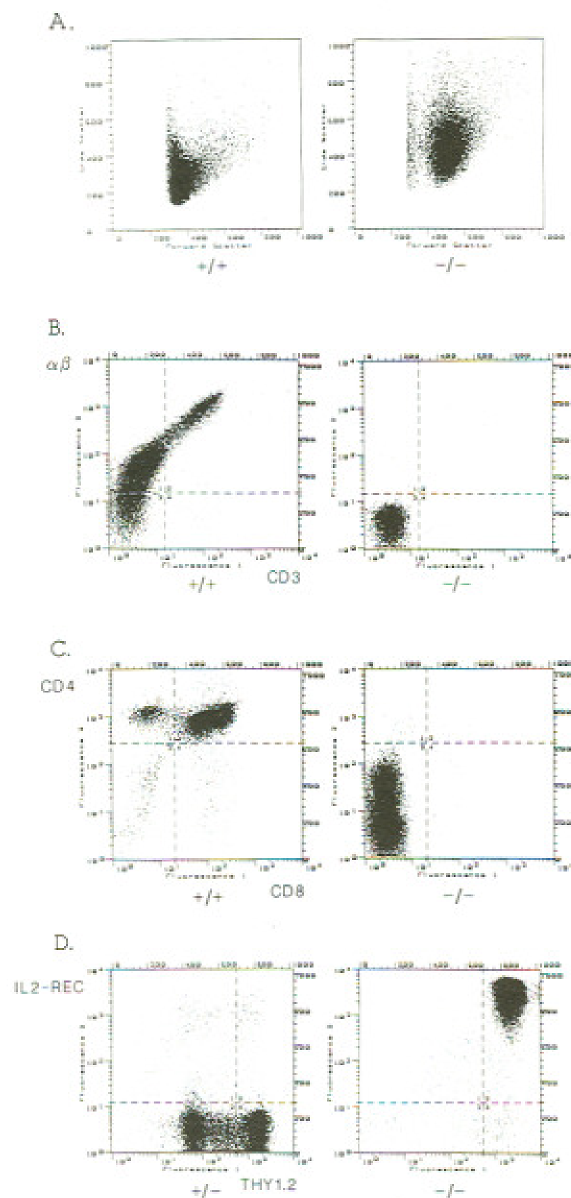

Figure 3. FACS Analysis of the Thymus

Thymus was isolated from either 4-, 5-, or 7-week-old mice. −/− indicates homozygous mutant mouse, +/− indicates RAG-1-heterozygous littermate, and +/+ refers to wild-type mouse.

(A) Forward scatter (FSC) horizontally, reflecting the size of the cells; side scatter (SSC) vertically, corresponding to the granularity of the cells. The thymocytes of the mutant mouse are shifted to higher scatter values.

(B) CD3 (FITC) horizontally and pan-TCR αβ (PE) vertically. No CD3 or TCR αβ thymocytes exist in the mutant thymus.

(C) CD8 (FITC) horizontally and CD4 (biotin–PE) vertically. The dull CD4 staining in the thymus of the homozygous mutant mouse is not reproducible and is further being investigated. No CD8–CD4 double positive cells are observed in the mutant mouse thymus.

(D) Thy-1.2 (FITC) horizontally and IL-2 receptor/CD25 (biotin–PE) vertically. Most thymocytes of the mutant mouse express the IL-2 receptor.

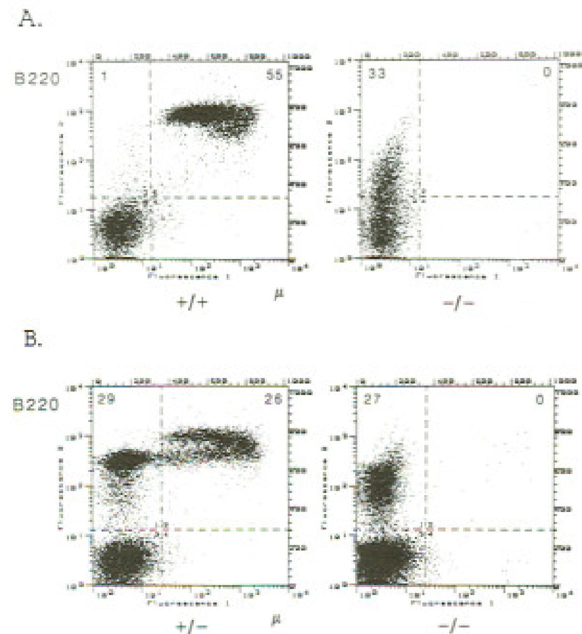

Figure 4. FACS Analysis of the Spleen and Bone Marrow

Samples are from same mice as in Figure 3.

(A) Spleen. A narrow gate was used on the splenocytes to visualize the lymphoid cells only (representing 72% of the nonerythroid cells in the wild-type mouse and 32% in the mutant mouse). IgM (FITC) horizontally and B220 (PE) vertically. In the mutant mouse, only B220 single positive cells exist. Even in the ungated population, no B220–IgM double positive cells are found (data not shown). Percentages for quadrants 1 and 2 are indicated at the corners of the figure.

(B) Bone marrow. Based on the control mouse, a narrow gate on the lymphoid population was used (21% of the total cells in the RAG-1 heterozygous mouse and 13% of the total cells in the mutant mouse). Only dull B220 single positive cells are observed in the mutant mouse.

or heterozygous littermates. Visual inspection revealed a stroma-like structure in the site where the inguinal lymph nodes are located, but only a few nonerythroid cells if any could be recovered from these structures. No other obvious anatomical alterations were observed. As far as a potential neurological deficit is concerned (Chun et al. 1991), we have observed no obvious clinical symptoms to date. The mutant mice are active, have a firm grip with their forelimbs, can hear sounds, can walk on a pencil, can sense heat and feel pain, and can swim as well as their heterozygous or wild-type littermates.

Absence of Mature B and T Lymphocytes

Cells from thymus, spleen, and bone marrow were subjected to flow cytometric analysis. Data are shown for 4-, 5-, or 7-week-old mice (Figure 3 and Figure 4). The thymocytes of the RAG-1-deficient mice are larger than those of wild-type or heterozygous mice, as indicated by the increased forward scatter (Figure 3A). No CD3-positive or TCR αβ–positive cells have been observed (Figure 3B). The thymocytes are CD8–CD4 double negative (Figure 3C), and almost all of them are interleukin-2 (IL-2) receptor–positive (Figure 3D). Finally, they are Thy-1 positive (Figure 3D), bright J11d–positive, bright Sca-1–positive,

and CD5-negative (data not shown). Thus, thymocyte development seems to be arrested at an immature stage.

The spleen does not contain any mature B cells, as judged by the lack of staining by anti-IgM (Figure 4A) or anti-IgD (data not shown) antibody. About one third of the splenocytes (on a narrow gate for the lymphoid population; see legend of Figure 4A) are B220 positive and may represent an early stage of B cell differentiation. A small fraction of the splenocytes is Mac-1 positive (data not shown) and could be macrophages, natural killer cells, or neutrophils. Likewise, the bone marrow contains no mature, IgM- (Figure 4B) or IgD-positive (data not shown) cells. About one fourth of the bone marrow cells (with a narrow gate on the lymphoid population; see Figure 4B legend) are B220-positive and could be early B cell precursors. The intensity of the B220 staining is lower than the majority of the B220-positive cells in the bone marrow of the normal mouse (Figure 4B). The composition of the lymphoid population in the lymphoid organs of the RAG-1-deficient mouse is similar to that of the scid mouse (Bosma and Carroll, 1991). We also measured by ELISA serum IgM levels of seven mutant mice of 5–16 weeks and found that IgM is undetectable (data not shown). It has been shown that the serum IgM level is a sensitive indicator for leakiness in the case of the scid mouse (Bosma and Carroll, 1991).

In summary, no mature B or T lymphocytes have been observed in the lymphoid organs of twelve RAG-1 mutant mice up to the age of 9 weeks, and no serum IgM up to the age of 16 weeks. It remains to be seen whether any leakiness in the phenotype occurs at an older age.

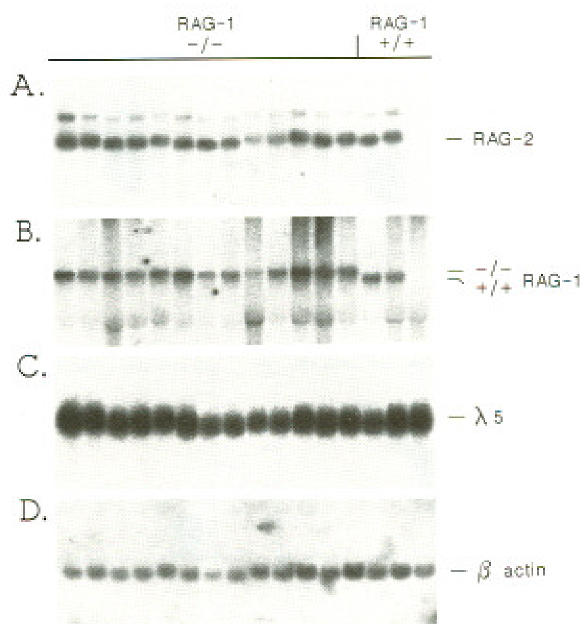

Figure 5. Northern Blot Analysis of Expression of RAG-2, RAG-1, and λ5 in Bone Marrow–Derived Abelson-Transformed Lines

RNA was isolated from individual Abelson lines. The blot was hybridized to: (A) a RAG-2 probe (48 hr exposure); (B) the RAG-1 probe B–X (12 hr exposure); (C) a λ5 cDNA probe (6 hr exposure); (D) a 1.5 kb AvaI–BamHI fragment from the human β-actin gene. Lanes 1–13: Abelson lines from RAG-1-deficient mouse; lanes 14–16, Abelson lines from wild-type littermate. The cell line represented by lane 16 gave neither RAG-1 nor RAG-2 RNA band. This is not unusual because large variations in the levels of RAG-1 and RAG-2 RNA in Abelson-transformed lines from normal mice have recently been observed (L.-C. Wang and N. Rosenberg, personal communication).

Establishment of Abelson-Transformed Lines

The Abelson murine leukemia retrovirus was used to transform bone marrow cells from a 5-week-old female mutant mouse and from a wild-type female littermate. A similar number of cell lines grew up in both mice (transformation efficiency was 1.19×10^{-4} for homozygote and 8.74×10^{-5} for wild-type), as has been reported for the scid mouse (Vulop et al., 1988). All of the Abelson retrovirus-transformed lines are B220 positive (data not shown) and contain RAG-2 and λ5 (Sakaguchi and Melchers, 1986) RNA (Figures 5A and 5C). It is therefore likely that these cell lines originated from immature B cells. Mutant RAG-1 transcripts are present in all of the cell lines established from the mutant mouse (Figure 5B).

Absence of V(D)J Recombination

To confirm that the absence of mature B and T lymphocytes is due to a defect in V(D)J recombination, Southern blot analysis was performed with DNA from the thymus and the Abelson-transformed lines. The $3'J_{\delta1}$ probe (Chien et al., 1987) allows the analysis of rearrangements involving $D_{\delta2}$ or $J_{\delta1}$ and all V_α–J_α rearrangements (in the latter case the TCR δ locus is deleted). Thymus DNA, isolated from 4- or 7-week-old RAG-1-deficient mice, showed no rearrangements (Figure 6A), whereas thymus DNA of a 7-week-old scid mouse showed a low level of rearrangements (Figure 6A) that are presumably aberrant (Carroll and Bosma, 1991). When a $5'D_{\beta1}$ probe was used, no rearrangements were observed at the TCR β locus (Figure 6B), whereas the scid thymus shows a faint additional band that probably represents a D–J rearrangement (Malissen et al., 1984). A TCR C_α probe was used as a control for loading and Southern transfer efficiency of the DNA samples (Figure 6C). Lane 4 (sample of wild-type mouse) is clearly underloaded, but this does not affect the conclusions. We also analyzed DNA from the bone marrow-derived Abelson lines with an immunoglobulin J_H probe. All of the cell lines derived from the RAG-1-deficient mouse revealed a germline configuration, but none of the lines derived from the wild-type mouse retained the IgH locus in a germline configuration (Figure 7).

In conclusion, the inability to perform V(D)J recombination is the most likely explanation fo the absence of mature B and T lymphocytes.

Histological Analysis of the Brain

In light of the reported presence of RAG-1 transcripts in the brain of the mouse (Chun et al., 1991), we assessed the effect of the RAG-1 mutation on the structure of the mouse brain by histological means, although no obvious behavioral deficit was observed (see above). Brains of 7- and 8-week-old male homozygous mutant and wild-type littermates were examined in serial sagittal sections stained with cresyl violet.

No significant defect could be found in the structure of

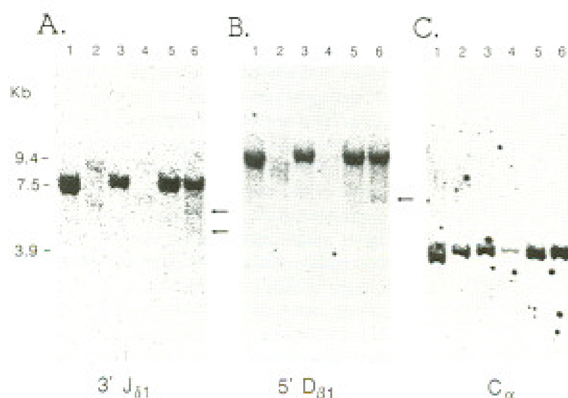

Figure 6. Southern Blot Analysis of TCR Rearrangements in Thymus

(A) DNA from 5 × 10^5 cells was digested with EcoRI. The Southern blot was hybridized to the 3'$J_{\delta 1}$ probe. The size of the germline fragment is 7.5 kb. Lane 1, AB1 ES cells; lane 2, thymocytes from a 4-week-old wild-type mouse; lane 3, thymocytes from a homozygous mutant mouse that is a littermate of the mouse in lane 2; lane 4, thymocytes from a 7-week-old wild-type mouse; lane 5, thymocytes of a homozygous mutant littermate of the mouse in lane 4; lane 6, thymocytes from a 7-week-old scid mouse. There is no evidence for rearrangements in the thymus of the mutant mice. The scid thymus has two faint bands (indicated with arrows) that probably represent D–D or D–J rearrangements.

(B) The blot was stripped and hybridized to the 5'$D_{\beta 1}$ probe. The germline band is at 9.4 kb. No rearrangements are observed in lanes 3 and 5, whereas in lanes 2 and 4, no signal is visible at the level of the germline band as the DNA sequences complementary to this probe are deleted (V–D–J rearrangement) or have an altered size (D–J rearrangement). A faint band (indicated with an arrow) is visible in the scid thymus and probably corresponds to a D–J rearrangement.

(C) The blot was stripped again and hybridized to a TCR C_{α} probe. The intensity of the bands serves to control for variations in amount of DNA loaded and Southern transfer efficiency. As the DNA sample from the wild-type mouse in lane 4 is underloaded, the data in lane 4 of Figures 6A and 6B are not relevant, but this does not affect the conclusions.

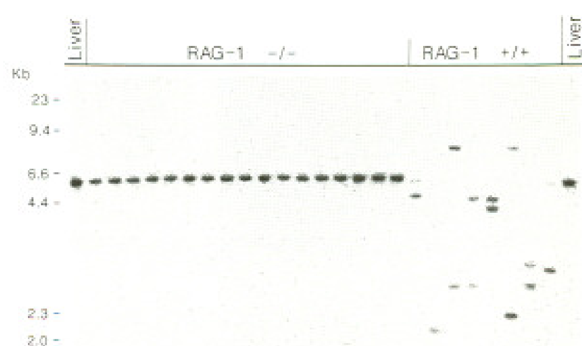

Figure 7. Southern Blot Analysis of Endogenous IgH Rearrangements in Bone Marrow–Derived Abelson-Transformed Lines

DNA was isolated from individual Abelson lines and cut with EcoRI. The blot was hybridized with a 1.9 kb BamHI–EcoRI fragment containing J_{H3} and J_{H4} (Sakano et al., 1980). Lane 1, C57BL/6 liver; lanes 2–18, Abelson lines derived from bone marrow of a 5-week-old RAG-1 mutant mouse; lanes 19–26, Abelson lines derived from bone marrow of a wild-type littermate.

the brain of the mutant mice when compared with control littermates. On gross examination, all major external features of the RAG-1-deficient brain were normal, including its size and the relative placement of the various fissures and major nerve roots. Histological examination revealed that, to a first approximation, most major nuclei found in the wild-type littermate (Figure 8A) were present in the mutant mouse (Figure 8B) and were normal in size and cellular appearance. No structural alterations were observed.

We focused particular attention on the hippocampus and the cerebellum, as these are the two regions that were claimed to have the highest levels of RAG-1 expression by in situ hybridization (Chun et al., 1991). Comparison of Figure 8A (wild-type) with Figure 8B (mutant) shows a nearly identical pattern of foliation of the cerebellar cortex. Similarly, the macroarchitecture of the hippocampus is unaltered in the mutant mouse (Figures 8A and 8B). High magnification views of both cerebellum (Figures 8C and 8D) and hippocampus (Figures 8E and 8F) reveal no distortion of the microarchitecture of the mutant (Figures 8D and 8F) compared with the wild-type (Figures 8C and 8E) brain. As these areas both are sensitive indicators of pattern alterations (Nowakowsky, 1984; Joyner et al., 1991), their normal appearance in the mutant suggest that the absence of RAG-1 function does not interfere in a major way with developmental events such as neurogenesis, migration and differentiation.

Discussion

RAG-1 and RAG-2 Are Both Required for V(D)J Recombination

In this report, we describe the generation and the initial analysis of mice with a mutation of the RAG-1 gene, which has been implicated in the regulation or the catalysis of V(D)J recombination (Schatz et al., 1989). The data show that this gene is necessary in vivo for V(D)J recombination to occur. The presence of RAG-2 transcripts in the Abelson-transformed lines derived from the RAG-1 mutant mouse indicates that the mutation introduced in the RAG-1 gene does not inhibit the expression of the closely linked RAG-2 gene. Formal proof, however, that the lack of V(D)J recombination is due solely to the targeted disruption of RAG-1 awaits complementation of the V(D)J recombination defect either by transfection of the Abelson-transformed lines with a RAG-1 expression plasmid or crossing the RAG-1-deficient mice to RAG-1 transgenic mice. RAG-1 transcripts of a slightly longer size that are observed in the RAG-1-deficient lines are unlikely to give rise to any functional protein (see Results).

Our data, taken together with those of the RAG-2-deficient mice described in the accompanying paper (Shinkai et al., 1992), support the hypothesis that both the RAG-1 and RAG-2 gene products are required in vivo for V(D)J recombination, as in transfected fibroblasts (Oettinger et al., 1990). The low level of rearrangements observed with an artificial substrate upon transfection of either the RAG-1 or RAG-2 gene into 3T3 fibroblasts (Schatz

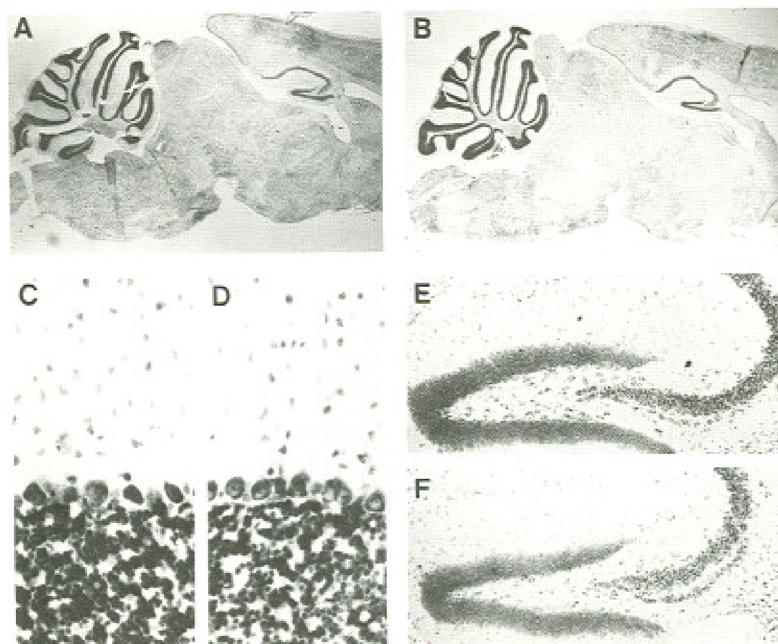

Figure 8. Histological Analysis of the Brain

Histological comparison of the brains of RAG-1 mutant and wild-type littermates. The top panels represent low magnification views of midsagittal sections of wild-type (A) and mutant (B) brain; rostral is to the right, caudal to the left (20.4x). Note the near identity in the foliation pattern of the cerebellar cortex and the close congruence of the structure of the hippocampi. Higher magnification of cerebellar cortex reveals no alterations in the density or Nissl morphology of cells in the molecular, Purkinje, or granule cell layer: (C) wild type (D) mutant (660x). Likewise, the cytoarchitecture of the hippocampus is similar in wild-type (E) and mutant (F) brains (162x).

et al., 1989) can be explained by leaky transcription of, respectively, the endogenous RAG-2 or RAG-1 gene.

Immature B and T Cells

Flow cytometric analysis of cells from lymphoid organs reveals the absence of mature B and T lymphocytes, which is most likely the result of the deficit in V(D)J recombination. B and T cell precursors need to produce functional antigen receptors on their surface in order to survive (Rolink and Melchers, 1991; von Boehmer, 1988). In the case of the T cell lineage, it is well established that the precursors that fail to produce a TCR do not undergo the process of positive selection (von Boehmer, 1988). Thus, like in the scid mouse (Bosma and Carroll, 1991), the thymus of the RAG-1 mutant mouse remains small and contains immature, large, CD8–CD4 double negative thymocytes expressing the IL-2 receptor. Likewise, the bone marrow and the spleen of the RAG-1 mutant mouse contain a population of dull B220-positive cells, a fraction of which may represent pro-B cells. Some of these cells may serve as targets for transformation by the Abelson retrovirus.

No Leakiness Observed

The combination of the flow cytometric data, Southern blot analysis, and serum IgM ELISA measurements has revealed no leakiness in the phenotype to date. It remains to be seen whether any leakiness will be observed when older mice or a larger number of mice are examined. For instance, since serum IgM is a sensitive indicator of B cell development, we intend to follow it by serial bleeding as mice age. We can also follow immunoglobulin and TCR rearrangements by the sensitive polymerase chain reaction technique.

The observations made to date are in contrast to those in the scid mouse. Using the same $3'J_{\delta 1}$ probe (Chien et al., 1987) as the one used in our study, D–J rearrangements at the TCR δ locus have been reported for scid mice (Carroll and Bosma, 1991), as can be seen in Figure 6A. As these mice grow older, some B and T cells express a functional antigen receptor and form an oligoclonal repertoire (Bosma et al., 1988; Carroll and Bosma, 1988; Carroll et al., 1989; Bosma and Carroll, 1991). This leakiness is partly the result of reversion of the mutation in individual lymphocyte progenitors (Petrini et al., 1990). As a result, serum levels of IgM increase in scid mice as they age (Bosma and Carroll, 1991).

If the RAG-1-deficient mice indeed turn out to be nonleaky, as the data obtained to date suggest, these mutant mice may prove to be a useful alternative to the scid mouse for a variety of studies, such as the efforts to produce a human immune system in the mouse—the "SCID-hu mouse" (McCune et al., 1988; Mosier et al., 1988)—as an animal model for AIDS, and reconstitution or infection experiments (McCune, 1991). Indeed, in addition to the leakiness, the *scid* mutation has at least two other drawbacks compared with the RAG-1 mutation: the gene has not been cloned, and the genetic defect is known to be pleiotropic affecting other processes, such as double-strand break-related DNA repair, which results in increased sensitivity to ionizing radiation (Fulop and Phillips, 1990; Biedermann et al., 1991; Hendrickson et al., 1991).

No Overt Structural Alterations in the Brain

The brain is the only nonlymphoid organ in which RAG-1 RNA has been claimed to exist (Chun et al., 1991). Furthermore, unlike lymphoid organs in which RAG-1 and RAG-2 are invariably coexpressed (Oettinger et al., 1990), the brain has been claimed not to express RAG-2 (Chun et al., 1991). On the basis of these observations, it has been speculated that a V(D)J or V(D)J-like recombination may

take place in the brain (Chun et al., 1991; Alt et al., 1991). Our RAG-1 mutant mice offer a direct test of the hypothesis that the RAG-1 gene product plays a physiological role in the brain including promoting somatic DNA rearrangement. However, our initial analysis of such mutant mice revealed no obvious behavioral or neuroanatomical abnormality. In particular, we observed no cytoarchitectural alterations in the cerebellum or the hippocampus, the sites at which the highest levels of RAG-1 transcripts in the central nervous system were reported (Chun et al., 1991). Both regions are sensitive to perturbations in pattern formation, making the absence of a structural defect all the more intriguing.

Several explanations can be offered. First, the observed RAG-1 transcripts may be superfluous and have no physiological function. For instance, they may not be translated. Second, there may be an alternative mechanism for whatever function RAG-1 performs in the brain, i.e., there could be genetic redundancy. Third, the absence of the RAG-1 protein may affect development and functioning of the mouse brain, but the repercussions may be subtle and have escaped our analytical methods.

With respect to the last possibility, it is interesting to note that in the immune system V(D)J recombination is essential for maturation and expansion of lymphocytes and that the mutation in RAG-1 leads to gross diminuition of lymphoid organs. By contrast, there clearly is no dependence of this scale on the RAG-1 product in any of the major subregions of the brain. It is, however, feasible that functional deficits exist in the absence of obvious structural alterations. We will continue to look for such deficits, especially in the areas of olfaction (Buck and Axel, 1991) and learning and memory.

Experimetal Procedures

Construction of Targeting Vector

RAG-1 was cloned by screening a genomic EMBL-3 phage library prepared from D3 ES cells (Gossler et al., 1986) (gift from Alcino Silva) with a probe generated by the polymerase chain reaction technique (gift from Asa Abeliovich). One positive phage out of 1.2 million plaques screened was purified and mapped by restriction enzyme analysis. A 9 kb ClaI and a 6.5 kb BamHI–ClaI fragment were subcloned into pBluescript/SK− (Stratagene) and mapped further. The targeting vector pPMKO-31 was constructed in a quatrimolecular ligation reaction, using a 6 kb ClaI–Apa I fragment upstream of the RAG-1 coding sequence (ending at position 482), a 1.8 kb fragment containing the *pgk–neo* gene (Adra et al., 1987) (gift from Michael A. Rudnicki) excised with ApaI and BamHI, a 1277 bp BglII–MluI fragment containing sequences between positions 1837 and 3113, and the plasmid pGEM7 (Promega) cut with ClaI and MluI. This targeting vector is designed to delete 1356 nucleotides of the RAG-1 coding sequence between positions 482 and 1837 and replace it with the *pgk–neo* gene, transcribed in the opposite orientation. The source of the *pgk–neo* gene is the plasmid pKJ1, described in Tybulewicz et al. (1991).

Targeting Experiment

ES cells were grown on mitomycin C or γ-irradiated primary embryonic fibroblasts, and during the selections on G418-resistant fibroblasts isolated from transgenic embryos carrying a *neo* resistance gene (Gossler et al., 1986). The medium composition was as described in Robertson, 1987. About 5×10^7 AB1 (McMahon and Bradley, 1990) ES cells were electroporated with 75 μg of MluI linearized pPMKO-31 using a Bio-Rad Gene Pulser set at 800 V and 3 μF. Selection was initiated 20 hr later at a concentration of 125 to 150 μg/ml active concentration of G418 (Gibco). Colonies were picked from day 6 to day 9 of selection into 96 or 24 well dishes (Costar) and expanded. Half of a 24 well dish was frozen down and the other half used to isolate DNA for Southern blot analysis. The colonies were screened individually by cutting genomic DNA with BamHI and XhoI and probing the Southern blots with the external probe M–N, a 268 bp fragment containing RAG-1 coding sequences between positions 3113 and 3380 (Schatz et al., 1989).

Generation of Chimeric Mice

Chimeras were generated as described in Papaioannou (1981) and Bradley (1987). Litters of heterozygous intercrosses were housed in autoclaved micro-isolator cages with autoclaved food and water beginning at birth.

Screening of Mice

Mice were screened for homozygosity by Southern blot analysis on genomic tail DNA isolated according to Laird et al. (1991). The DNA samples were cut with BamHI and NcoI (see Figure 2) or with PstI, and the Southern blots were probed with probe B–X, a 1544 bp fragment containing RAG-1 coding sequences between positions 1837 and 3380 (Schatz et al., 1989). Southern blots were exposed using a Fujix Bio-Image Analyzer BAS2000.

Flow Cytometric Analysis

10^5 to 10^6 cells were preincubated in 96-well round bottom dishes (Costar) for 20 min in 12.5 μl containing 5 μl of staining solution (composed of phosphate buffered saline [PBS], 0.1% sodium azide, 1% fetal calf serum) and 2.5 μl each of normal hamster, normal mouse (Jackson ImmunoResearch), and normal rat serum (Caltag). The preincubation with serum was ommitted in the stainings with goat anti-mouse μ. The samples were then stained by adding another 12.5 μl of staining solution containing 0.25 μl of antibodies either biotinylated or conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). The antibodies used were: 2C11 for CD3-ϵ (Pharmingen), H57-597 for TCR $\alpha\beta$ (Pharmingen), 53-6.7 for CD8 (Becton Dickinson), GK1.5 for CD4 (Pharmingen), 53-2.1 for Thy-1.2 (Pharmingen), PC 61 5.3 for IL-2 receptor/CD25 (gift from Kunio Sano), goat anti-mouse immunoglobulin μ (Caltag), RA3-6B2 for B220 (Boehringer Mannheim), M1/70 for Mac-1 (Boehringer Mannheim), 53-7.3 for CD5 (gift from Pablo Pereira), J11d (gift from Pablo Pereira), and anti-Sca-1 (gift from Peggy Goodell). Cells were stained for 30 min, washed twice with staining solution, incubated in 25 μl of staining solution containing streptavidin–PE (Southern Biotechnology) at a 1:200 dilution, washed once in staining solution, once in PBS containing propidium iodide, and finally resuspended in 200 μl of PBS. Cells were kept on ice during the staining procedure and spun in a refrigerated centrifuge. Flow cytometric analysis was carried out with a Becton Dickinson FACScan using FACScan software. Dead cells were gated out by means of propidium iodide staining. Five thousand to seventeen thousand events were acquired using a large gate, and the lymphoid population was analyzed with a narrower gate based upon forward scatter and side scatter.

Southern and Northern Blot Analysis

TCR α and δ rearrangements were analyzed by cutting genomic DNA from thymus with EcoRI and hybridizing with the 3'$J_{\delta 1}$ probe described in Chien et al. (1987) (gift from Yoichi Shinkai). TCR β rearrangements were analyzed by hybridizing with a 5'$D_{\beta 1}$ probe, a 1.2 kb PstI fragment isolated from cosmid 2.3W7 (Malissen et al., 1984). The TCR C_α probe was a 0.6 kb BglII–BamHI fragment (Mombaerts et al., 1991). Immunoglobulin heavy chain rearrangements were analyzed by cutting with EcoRI and hybridizing with a 1.9 kb BamHI–EcoRI JH probe (Sakano et al., 1980).

The RAG-2 probe is a 246 bp fragment clone into pUC12 with the following polymerase chain reaction primers: 5'-ATGTCCCTGCAGATGGTAACA -3' (position 156 to 176 in Oettinger et al., 1990) and 5'-GCCTTTGTATGAGCAAGTAGC-3' (position 401 to 381 in Oettinger et al., 1990). The λ5 (Sakaguchi and Melchers, 1986) cDNA probe was a gift from Eugene Oltz.

Establishment of Abelson-Transformed Cell Lines

The Abelson murine leukemia virus was used to transform bone marrow cells as described in Rosenberg and Baltimore, 1976. Transformation efficiency was determined by counting the number of colonies in soft agar per 2×10^6 infected bone marrow cells.

Histology of the Brain

Mice were deeply anesthetized with avertin, perfused for 2 min with PBS, and then with 4% paraformaldehyde in phosphate buffer (0.1 M, [pH 7.4]) for 15 min. Following perfusion, the brain was removed from the skull and stored in fresh paraformaldehyde overnight at 4°C. The brains were bisected at the midline and embedded in polyester wax (polyethylene glycol distearate 400) according to the protocol of Mullen (1977). Serial ten micron sections were collected, mounted on gelatinized slides, and stained with cresyl violet.

Acknowledgments

P. M. wishes to thank En Li, Yoshi Ishikawa, and Alan Clarke for introducing him to ES cell technology, and Pablo Pereira, Juan Lafaille, and Shige Itohara for advice in the art of the FACScan. The authors thank Rolf Kemler and Rudolf Jaenisch for providing *neo*-resistant transgenic mice, Allan Bradley for AB1 cells and advice, Michael A. Rudnicki or providing *pgk–neo*, Asa Abeliovich for the RAG-1 probe, Alcino Silva for the use of the D3 genomic library, Michele Maxwell, Lili Wang, John Anderson, and James Spencer for technical help, Chris Newcomer and his staff for animal facilities, Bruce Spiegelman for advice and encouragement, Yoichi Shinkai for the $3'J_\delta$ probe and Gene Oltz for the $\lambda5$ probe, Peggy Goodell, Kunio Sano, Juan Lafaille, and Pablo Pereira for antibodies, and Antonio Coutinho and Juan Lafaille for critical review of the mauscript. We thank Fred Alt and Yoichi Shinkai for sharing unpublished information. P. M. is a Howard Hughes Medical Institute Predoctoral Fellow in Biological Sciences; J. I. is supported by an National Insitutes of Health postdoctoral training grant; and R. S. J. is an Albert J. Ryan Predoctoral Fellow. This work was supported by grants from the Howard Hughes Medical Institute and the National Institutes of Health to S. T., by NS18381 to K. H., and by HD27295 to V. E. P.

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Received December 17, 1991; revised January 13, 1992.

References

Adra, C. N., Boer, P. H., and McBurney, M. W. (1987). Cloning and expression of the mouse *pgk-1* gene and the nucleotide sequence of its promoter. Gene *60*, 65–74.

Alt, F. W., Blackwell, T. K., DePinho, R. A., Reth, M. G., and Yancopoulos, G. D. (1986). Regulation of genome rearrangement events during lymphocyte development. Immunol. Rev. *89*, 5–30.

Alt, F. W., Rathbun, G., and Yancopoulos, G. D. (1991). DNA recombination in the brain? Current Biology *1*, 3–5.

Biedermann, K. A., Sun, J., Giaccia, A. J., Tosto, L. M., and Brown, J. M. (1991). *Scid* mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair. Proc. Natl. Acad. Sci. USA *88*, 1394–1397.

Boehm, T., Gonzalez-Sarmientio, R., Kennedy, M., and Rabbitts, T. (1991). A simple technique for generating probes for RNA in situ hybridization: an adjunct to genome mapping exemplified by the *RAG-1/RAG-2* gene cluster. Proc. Natl. Acad. Sci. USA *88*, 3927–3931.

Bosma, M. J., and Carroll, A. M. (1991). The *scid* mouse mutant: definition, characterization and potential uses. Annu. Rev. Immunol. *9*, 323–350.

Bosma, G. C., Custer, R. P., and Bosma, M. J. (1983). A severe combined immunodeficiency mutation in the mouse. Nature *301*, 527–530.

Bosma, G. C., Fried, M., Custer, R. P., Carroll, A., Gibson, D. M., and Bosma, M. J. (1988). Evidence of functional lymphocytes in some (leaky) *scid* mice. J. Exp. Med. *167*, 1016–1033.

Bradley, A. (1987). Production and analysis of chimeric mice. In Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, E. J. Robertson, ed. (Oxford: IRL Press), 113–151.

Buck, L., and Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odorant recognition. Cell *65*, 175–187.

Capecchi, M. R. (1989). Altering the genome by homologous recombination. Science *244*, 1288–1292.

Carlson, L. M., Oettinger, M. A., Schatz, D. G., Masteller, E. L., Hurley, E. A., McCormack, W. T., Baltimore, D., and Thompson, C. B. (1991). Selective expression of RAG-2 in chicken B cells undergong immunoglobulin gene conversion. Cell *64*, 201–208.

Carroll, A. M., and Bosma, M. J. (1991). T-lymphocyte development in *scid* mice is arrested shortly after the initiation of T-cell receptor δ gene recombination. Genes Dev. *5*, 1357–1366.

Carroll, A. M., and Bosma, M. J. (1988). Detection and characterization of functional T cells in mice with severe combined immune deficiency. Eur. J. Immunol. *18*, 1965–1971.

Carroll, A. M., Hardy, R. R., and Bosma, M. J. (1989). Occurrence of mature B (IgM^+,$B220^+$) and T ($CD3^+$) lymphocytes in *scid* mice. J. Immunol. *143*, 1087–1093.

Chien, Y., Iwashima, M., Wettstein, D. A., Kaplan, K. B., Elliott, J. F., Born, W., and Davis, M. M. (1987). T-cell receptor δ gene rearrangements in early thymocytes. Nature *330*, 722–727.

Chun, J. J. M., Schatz, D. G., Oettinger, M. A., Jaenisch, R., and Baltimore, D. (1991). The recombination activation gene-1 (RAG-1) transcript is present in the murine central nervous system. Cell *64*, 189–200.

Davis, M. M., and Bjorkman, P. J. (1988). T-cell antigen receptor genes and T-cell recognition. Nature *334*, 395–402.

Evans, M. J., and Kaufmann, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature *292*, 154–156.

Fulop, G. M., and Phillips, R. A. (1990). The *scid* mutation in mice causes a general defect in DNA repair. Nature *347*, 479–482.

Fulop, G. M., Bosma, G. C., Bosma, M. J., and Phillips, R. A. (1988). Early B cell precursors in *scid* mice: normal numbers of cells transformable with Abelson murine leukemia virus (A-MuLV). Cell. Immunol. *113*, 192–201.

Gossler, A., Doetschman, T., Korn, R., Serfling, E., and Kemler, R. (1986). Transgenesis by means of blastocyst-derived embryonic stem cell lines. Proc. Natl. Acad. Sci. USA *83*, 9065–9069.

Hendrickson, E. A., Qin, S., Bump, E. A., Schatz, D.G., Oettinger, M. A., and Weaver, D. T. (1991). A link between double-strand break-related repair and DNA recombination: the *scid* mutation. Proc. Natl. Acad. Sci. USA *88*, 4061–4065.

Hesse, J. E., Liber, M. R., Mizuuchi, K., and Gellert, M. (1989). V(D)J recombination: a functional definition of the joining signals. Genes Dev. *3*, 1053–1061.

Joyner, A. L., Herrup, K., Auerbach, B. A., Davis, C. A., and Rossant, J. (1991). Subtle cerebellar phenotype in mice homozygous for a targeted deletion of the *en-2* homeobox. Science *251*, 1239–1243.

Laird, P. W., Zijderveld, A., Linders, K., Rudnicki, M. A., Jaenisch, R., and Berns, A. (1991). Simplified mammalian DNA isolation procedure. Nucl. Acids Res. *19*, 293.

Malissen, M., Minard, K., Mjolsness, S., Kronenberg, M., Goverman, J., Hunkapiller, T., Prystowsky, M. B., Yoshikai, Y., Fitch, F., Mak, T. W., and Hood, L. (1984). Mouse T cell antigen receptor: structure and organization of constant and joining gene segments encoding the β polypeptide. Cell *37*, 1101–1110.

Mansour, S. L., Thomas, K. R., and Capecchi, M. R. (1988). Disruption of the proto-oncogene *int-2* in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature *336*, 348–352.

McCune, J. M. (1991). SCID mice as immune system models. Curr. Op. Immunol. *3*, 224–228.

McCune, J. M., Namikawa, R., Kaneshima, H., Shultz, L. D., Lieberman, M., and Weissman, I. L. (1988). The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. Science *241*, 1632–1639.

McMahon, A. P., and Bradley, A. (1990) The *Wnt*-1 (*int*-1) proto-oncogene is required for development of a large region of the mouse brain. Cell *62*, 1073–1085.

Mombaerts, P., Clarke, A. R., Hooper, M. L., and Tonegawa, S. (1991). Creation of a large genomic deletion at the T-cell antigen receptor

β-subunit locus in mouse embryonic stem cells by gene targeting. Proc. Natl. Acad. Sci. USA *88*, 3084–3087.

Mosier, D. E., Gulizia, R. J., Baird, S. M., and Wilson, D. B. (1988). Transfer of a functional human immune system to mice with severe combined immunodeficiency. Nature *335*, 256–259.

Mullen, R. (1977). Site of *pcd* gene action and Purkinje cell mosaicism in cerebella of chimeric mice. Nature *270*, 245–247.

Nowakowsky, R. (1984). The mode of inheritance of a defect in lamination in the hippocampus of BALB/c mice. J. Neurogenet. *1*, 249–258.

Oettinger, M. A., Schatz, D. G., Gorka, C., and Baltimore, D. (1990). RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science *248*, 1517–1523.

Papaioannou, V. E. (1981). Microsurgery and micromanipulation of cells and early embryos. In Techniques in the Life Sciences, vol. P1/1, Techniques in Cellular Physiology Part I, P. F. Baker, ed., *116*, 1–27.

Petrini, J. H. J., Carroll, A. M., and Bosma, M. J. (1990). T-cell receptor gene rearrangements in functional T-cell clones from severe combined immune deficient (*scid*) mice: reversion of the *scid* phenotype in individual lymphocyte progenitors. Proc. Natl. Acad. Sci. USA *87*, 3450–3453.

Robertson, E. J. (1987). Embryo-derived stem cell lines. In Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, E. J. Robertson, ed. (Oxford: IRL Press), 71–112.

Rolink, A., and Melchers, F. (1991). Molecular and cellular origins of B lymphocyte diversity. Cell *66*, 1081–1094.

Rosenberg, N., and Baltimore, D. (1976). A quantitative assay for transformation of bone marrow cells by Abelson murine leukemia virus. J. Exp. Med. *143*, 1453–1460.

Sakaguchi, N., and Melchers, F. (1986). λ5, a new light-chain-related locus selectively expressed in pre-B lymphocytes. Nature *324*, 579–582.

Sakano, H., Maki, R., Kurosawa, Y., Roeder, W., and Tonegawa, S. (1980). Two types of somatic recombination are necessary for the generation of the complete immunoglobulin heavy-chain genes. Nature *286*, 676–683.

Schatz, D. G., and Baltimore, D. (1988). Stable expression of immunoglobulin gene V(D)J recombinase activity by gene transfer into 3T3 fibroblasts. Cell *53*, 107–115.

Schatz, D. G., Oettinger, M. A., and Baltimore, D. (1989). The V(D)J recombination activating gene, RAG-1. Cell *59*, 1035–1048.

Shinkai, Y., Rathbun, G., Lam, K.-P., Oltz, E. M., Stewart, V., Mendelsohn, M., Charon, J., Datta, M., Young, F., Stall, A. M., and Alt, F. W. (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell *68*, this issue.

Tonegawa, S. (1983). Somatic generation of antibody diversity. Nature *302*, 575–581.

Turka, L. A., Schatz, D. G., Oettinger, M. A., Chun, J. J. M., Gorka, C., Lee, K., McCormack, W. T., and Thompson, C. B. (1991). Thymocyte expression of RAG-1 and RAG-2: termination by T cell receptor cross-linking. Science *253*, 778–781.

Tybulewicz, V. L. J., Crawford, C. E., Jackson, P. K., Bronson, R. T., and Mulligan, R. C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the *c-abl* proto-oncogene. Cell *65*, 1153–1163.

von Boehmer, H. (1988). The developmental biology of T lymphocytes. Annu. Rev. Immunol. *6*, 309–326.

Note Added in Proof

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