

- SDS-polyacrylamide gel electrophoresis. After gel separation, proteins were electroblotted onto nitrocellulose membranes and incubated with polyclonal antibodies that recognize PC1/3 and PC2 (provided by I. Lindberg, Louisiana State Medical Center). Membranes were washed and then incubated with goat antiserum to rabbit coupled to horseradish peroxidase (Amersham Pharmacia Biotech, Uppsala, Sweden). The blots were then developed with a chemiluminescence Western blotting detection kit.
23. GTC-1 cells grown to 70 to 80% confluence in 12-well plates were given restricted nutrients for 2 hours in Dulbecco's minimum essential medium (DMEM) with 1.0 mM glucose and 1% fetal calf serum (FCS). Cells were washed and then incubated in 0.5 ml of release media (DMEM plus 1% FCS with either 1.0 or 10.0 mM of glucose) for 2 hours. Insulin levels in media were measured using the human-specific insulin ELISA kit [American Laboratory Products Company (ALPCO), Windham, NH] according to supplier's instructions.
 24. The GIP/Ins fragment (4.2 kb) was excised with Hind III and gel-purified. Transgenic mice were generated by pronuclear microinjection of the purified transgene into fertilized embryos that were then implanted into pseudopregnant females. Transgenic mice were identified by Southern blot analysis. Ear sections were digested, and the purified DNA was cut with Xho I and Pvu II (Fig. 1C), electrophoretically separated, and transferred to nylon membrane. For the detection of the transgene, a 416-bp human insulin gene fragment encompassing intron 2 was amplified by using primers 2 and 4 (Fig. 1C). The PCR product was prepared as a probe by radiolabeling with [α -³²P]dCTP, and bands were detected by autoradiography. Southern analysis results were further confirmed by PCR amplification of the genomic DNA using primers 2 and 4. Positive founders were outbred with wild-type FVB/N mice to establish transgenic lines.
 25. Primers used were human proinsulin-specific, forward 5'-CCAGCCCGCAGCCTTTGTGA-3' and reverse 5'-GGTACAGCATTGTTCCACAATG-3'; mouse proinsulin-specific, forward 5'-ACCACCAGCCCTAAGTGAT-3' and reverse 5'-CTAGTTGCAGTAGTTC-CAGC-3'. PCR conditions were as follows: denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min for 45 cycles. PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining. The human- and mouse-specific primer sets yield 350-bp and 396-bp products, respectively.
 26. Tissues were fixed in Bouin's solution overnight and embedded in paraffin. Tissue sections 5 μ m thick were mounted on glass slides. For immunohistochemistry, the avidin-biotin complex method was used with peroxidase and diaminobenzidine as the chromogen. Sections were incubated with guinea pig antibody to insulin (1:500, Linco Research, St. Charles, MO) or mouse antibody to GIP (1:200, a gift from R. Pederson, University of British Columbia) for 30 min and appropriate secondary antibodies for 20 min at room temperature. Biotinylated secondary antibodies were used for immunohistochemistry, and fluorescein- or Cy3-conjugated secondary antibodies were used for immunofluorescence.
 27. Plasma insulin levels were measured using the ultrasensitive human-specific insulin ELISA kit (ALPCO) according to supplier's instructions. This assay has <0.01% cross-reactivity with human proinsulin and C peptide and does not detect mouse insulin. Plasma C-peptide measurements were made with a rat/mouse C-peptide radioimmunoassay kit (Linco Research). The assay displays no cross-reactivity with human C peptide.
 28. Streptozotocin was administered to 8-week-old transgenic and age-matched control mice via an intraperitoneal injection at a dose of 200 mg/kg body weight in citrate buffer. At this high dose of streptozotocin, mice typically display glucosuria within 3 days after injection. For oral glucose tolerance tests, glucose was administered orally by feeding tube (1.5 g/kg body weight) as a 50% solution (w/v) to mice that had been without food for 14 hours. Blood samples (40 μ l) were collected from the tail vein of

- conscious mice at 0, 10, 20, 30, 60, 90, and 120 min after the glucose load. Plasma glucose levels were determined by enzymatic, colorimetric assay (Sigma), and plasma insulin levels were measured using the ultrasensitive human-specific insulin ELISA kit (27).
29. Pancreata were homogenized and then sonicated at 4°C in 2 mM acetic acid containing 0.25% bovine serum albumin. After incubation for 2 hours on ice, tissue homogenates were resonicated and centrifuged (8000g, 20 min), and supernatants were assayed for insulin by radioimmunoassay.
 30. P. B. Simon, A. G. Renehan, C. S. Potten, *Carcinogenesis* 21, 469 (2000).
 31. O. Sandström, M. El-Salhy, *Mech. Ageing Dev.* 108, 39 (1999).

32. M. A. Croyle, M. Stone, G. L. Amidon, B. J. Roessler, *Gene Ther.* 5, 645 (1998).
33. S. J. Henning, *Adv. Drug Deliv. Rev.* 17, 341 (1997).
34. We thank P. Dickie for performing embryo injections; M. Tourand and J. Lyon for technical assistance; D. Drucker and M. Prentki for STC-1 and INS-1 cells, respectively; M. Magnuson, I. Lindberg, and R. Pederson for providing antibodies to GK, PCs, and GIP, respectively. This project was funded by the Juvenile Diabetes Research Foundation International and the Alberta Heritage Foundation for Medical Research. A.T.C., G.S.K., and T.J.K. also acknowledge salary support from the Canadian Diabetes Association.

11 August 2000; 3 November 2000

Response to RAG-Mediated V(D)J Cleavage by NBS1 and γ -H2AX

Hua Tang Chen,^{1*} Avinash Bhandoola,^{1*} Michael J. Difilippantonio,^{2*} Jie Zhu,¹ Martin J. Brown,¹ Xuguang Tai,¹ Emmy P. Rogakou,³ Tilmann M. Brotz,¹ William M. Bonner,³ Thomas Ried,² André Nussenzweig^{1†}

Genetic disorders affecting cellular responses to DNA damage are characterized by high rates of translocations involving antigen receptor loci and increased susceptibility to lymphoid malignancies. We report that the Nijmegen breakage syndrome protein (NBS1) and histone γ -H2AX, which associate with irradiation-induced DNA double-strand breaks (DSBs), are also found at sites of V(D)J (variable, diversity, joining) recombination-induced DSBs. In developing thymocytes, NBS1 and γ -H2AX form nuclear foci that colocalize with the T cell receptor α locus in response to recombination activating gene (RAG) protein-mediated V(D)J cleavage. Our results suggest that surveillance of T cell receptor recombination intermediates by NBS1 and γ -H2AX may be important for preventing oncogenic translocations.

V(D)J recombination is initiated by lymphoid-specific recombination activating gene 1 (RAG1) and RAG2 proteins, which introduce DSBs precisely between immunoglobulin and T cell receptor (TCR) coding gene segments and flanking recombination signal sequences. RAG-mediated cleavage generates four broken-end intermediates: two blunt signal ends and two covalently closed coding hairpin ends (1). The subsequent resolution of V(D)J ends into coding and signal joints requires ubiquitously expressed factors that function in general DSB repair (2, 3). Although V(D)J recombination generates DNA damage, it has been presumed that broken DNA intermediates, which associate with RAG proteins within a postcleavage synaptic complex (4, 5), are sequestered from the DNA damage

surveillance machinery. Primary DNA damage sensors include histone H2AX, which becomes rapidly phosphorylated (γ -H2AX) in response to external damage (6, 7), and the MRE11/RAD50/NBS1 complex, which forms ionizing irradiation-induced foci at DSBs (8, 9). Although γ -H2AX and MRE11/RAD50/NBS1 appear to play an important role in monitoring chromosome integrity, the physiological conditions that activate these DNA damage surveillance/signaling factors have not been described.

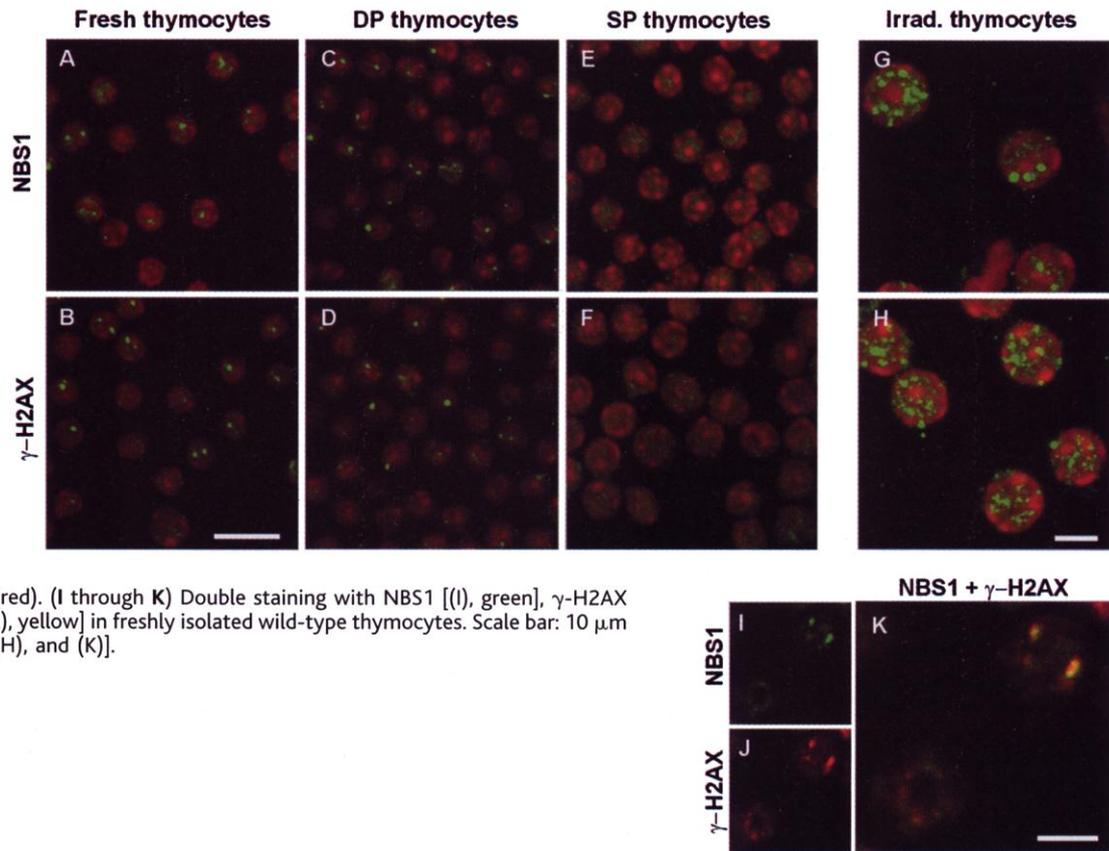
To determine whether NBS1 and γ -H2AX are present at antigen receptor gene-specific breaks introduced during V(D)J recombination, we examined wild-type thymocytes by immunofluorescence analysis (10). We found that approximately 20% of freshly isolated thymocytes showed intense NBS1 and γ -H2AX foci (Fig. 1, A and B). Dual immunostaining revealed that H2AX was generally phosphorylated in the same nuclear domains where NBS1 foci were found (Fig. 1, I through K). The majority of thymocytes with intense NBS1/ γ -H2AX staining contained one distinct spot, although cells were occasionally found to contain two, and less frequently, three or more foci. In contrast, multiple foci were distributed throughout

¹Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA. ²Genetics Department, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA. ³Laboratory of Molecular Pharmacology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA.

*These authors contributed equally to this work.
[†]To whom correspondence should be addressed. E-mail: andre_nussenzweig@nih.gov

REPORTS

Fig. 1. NBS1 and γ -H2AX form foci in immature thymocytes. (A through F) Freshly isolated wild-type thymocytes were stained for NBS1 [(A), (C), (E); green] or γ -H2AX [(B), (D), (F); green], and counterstained with Topro-3 (red). Whole, double positive (DP), and single positive (SP) thymocytes were optically sectioned at 0.5- μ m intervals and recombined into a maximum projection. Cells were imaged under identical conditions. (G and H) Bcl-2 transgenic thymocytes (which are resistant to irradiation-induced apoptosis) were exposed to 5 Gy, incubated at 37°C for 24 hours before fixation, stained for NBS1 [(G), green] or γ -H2AX [(H), green], and counterstained with Topro-3 (red). (I through K) Double staining with NBS1 [(I), green], γ -H2AX [(J), red], and colocalization [(K), yellow] in freshly isolated wild-type thymocytes. Scale bar: 10 μ m [(A) through (F)]; 3 μ m [(G), (H), and (K)].



the nucleus of irradiated thymocytes (Fig. 1, G and H). NBS1/ γ -H2AX foci were present in immature CD4⁺CD8⁺ double-positive (DP) thymocytes (Fig. 1, C and D), whereas mature CD4⁺ single-positive (SP) populations exhibited a diffuse nuclear staining pattern for both proteins (Fig. 1, E and F) (11). Because NBS1/ γ -H2AX foci are specific to DSBs (7, 9), these results indicate that mature T cells contain intact genomes, whereas approximately 20% of immature DP thymocytes have at least one DSB at any given time that is recognizable by NBS1/ γ -H2AX.

In contrast to mature lymphocyte populations, which have completed V(D)J recombination, a fraction of immature DP thymocytes is actively undergoing rearrangement of TCR α genes. Recombination at the TCR α locus ceases during positive selection when successful TCR-major histocompatibility complex (MHC) ligand interactions down-regulate *RAG1* and *RAG2* gene expression (12, 13). To determine whether NBS1 foci formation requires V(D)J recombination in DP cells, we analyzed thymocytes from mice expressing a productively rearranged TCR transgene, termed AND, which is positively selected by MHC class II I-A^b (14). *AND*⁺*MHC*^{-/-} thymocytes, which do not encounter their intrathymic selecting ligands, cannot be positively selected and therefore continue

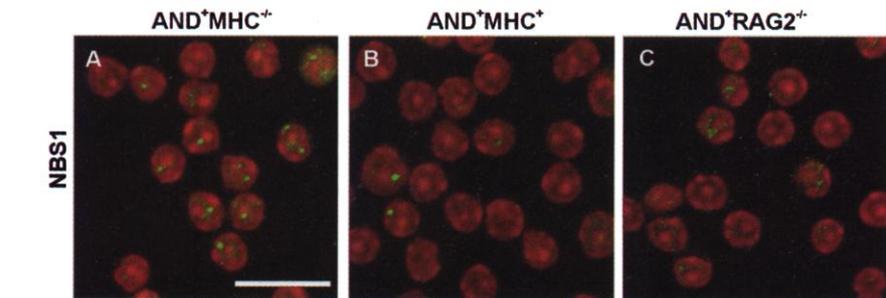


Fig. 2. Foci formation in thymocytes requires V(D)J recombination. Distribution of NBS1 is compared in TCR transgenic thymocytes from *AND*⁺*MHC*^{-/-} (A), *AND*⁺*MHC*⁺ (B), and *AND*⁺*RAG2*^{-/-} (C) mice. Data were obtained as in Fig. 1. Scale bar, 10 μ m.

to rearrange their endogenous TCR α genes. NBS1 foci were detected in 20% of *AND*⁺*MHC*^{-/-} thymocytes, similar to the percentage observed in wild-type thymocytes (Fig. 2A). In *AND*⁺*MHC*⁺ thymocytes, where intrathymic TCR ligation terminates *RAG1/RAG2* expression, NBS1 foci were detectable, but at a lower frequency (approximately 5%) than in the nonselecting *MHC*^{-/-} background (Fig. 2B). By contrast, in *AND*⁺*RAG2*^{-/-} thymocytes, NBS1 foci were undetectable (Fig. 2C), consistent with the absence of V(D)J rearrangement in these mice. Thus, foci formation in immature thymocytes requires RAG cleavage and is suppressed by intrathymic TCR-ligand interactions that terminate *RAG* expression.

To determine whether NBS1/ γ -H2AX foci were present at sites of active V(D)J recombination, we used an immunofluorescence in situ hybridization approach that allowed simultaneous visualization of DNA and protein within intact thymocytes (15, 16). The TCR α locus was identified using a combination of V α and C α probes, and NBS1 and γ -H2AX were detected by immunofluorescence. Coincidence of signals with one or both TCR α alleles was detected in 76% of the cells that contained NBS1 foci ($N > 100$), and in 77% of the cells that contained γ -H2AX foci ($N > 100$) (Fig. 3). In the remaining 23 to 24% of cells, approximately half exhibited only one TCR α hybridizing signal, leaving the possibility that NBS1 or γ -H2AX were localized to the

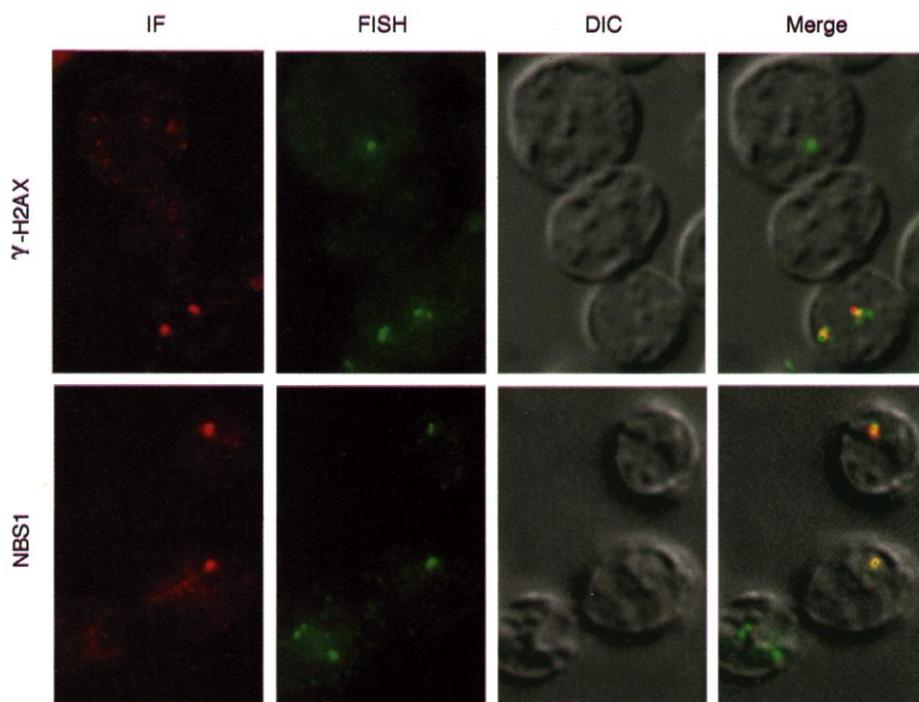


Fig. 3. Colocalization of NBS1 and γ -H2AX foci with the TCR α gene in freshly isolated thymocytes. Freshly isolated wild-type thymocytes were stained with anti- γ -H2AX or anti-NBS1 antibodies [(IF) red] followed by FISH (green) detection of the TCR α locus with probes specific for the variable and constant regions. The cells were visualized by differential interference contrast (DIC) microscopy and the three images merged to determine colocalization (yellow). Fluorescence images represent a single optical section.

undetected TCR α locus. In the remaining half (approximately 2.5% of thymocytes), NBS1 (or γ -H2AX) did not show colocalization with either detectable TCR α locus. Although these foci might be associated with other recombinationally active loci (e.g., TCR β or TCR γ), the vast majority of NBS1 and γ -H2AX foci were coincident with the TCR α locus.

The observation that NBS1 and γ -H2AX are highly enriched in overlapping nuclear foci in immature thymocytes near RAG-induced DSBs was unexpected, because specific breaks introduced during V(D)J recombination do not appear to activate ATM- or p53-dependent DNA damage checkpoints, nor are these DNA damage sensors required for normal V(D)J recombination (17–19). Furthermore, V(D)J recombination coding-end intermediates have an extremely short half-life and are rare in wild-type mice (20–23). By contrast, 20% of thymocytes have broken signal ends at the TCR α locus (24). Thus, there are two possible, though not mutually exclusive, explanations for foci formation. First, NBS1/ γ -H2AX foci may form within the postcleavage synaptic complex (4, 5) and then remain associated with unresolved signal ends, which are stable for several hours before being joined (5, 20). Alternatively, NBS1/ γ -H2AX foci may persist on resolved coding junctions as residual footprints of already completed V(D)J recombi-

nation. In either case, RAG-induced NBS1/ γ -H2AX foci provide a direct visualization of the ongoing process of antigen receptor rearrangement, and constitute the first evidence that DNA damage-sensing pathways are activated during normal V(D)J recombination. Further understanding of how NBS1 and γ -H2AX coordinate repair, signaling, and surveillance functions in response to intrinsic DNA damage during V(D)J recombination will provide insight into the mechanisms underlying chromosomal translocations and malignant transformation in chromosomal breakage disorders.

References and Notes

1. S. D. Fugmann, A. I. Lee, P. E. Shockett, I. J. Vitell, D. G. Schatz, *Annu. Rev. Immunol.* **18**, 495 (2000).
2. G. E. Taccioli *et al.*, *Science* **260**, 207 (1993).
3. M. R. Lieber, *Genes Cells* **4**, 77 (1999).
4. K. Hiom, M. Gellert, *Mol. Cell* **1**, 1011 (1998).
5. A. Agrawal, D. G. Schatz, *Cell* **89**, 43 (1997).
6. E. P. Rogakou, C. Boon, C. Redon, W. M. Bonner, *J. Cell Biol.* **146**, 905 (1999).
7. E. P. Rogakou, D. R. Pilch, A. H. Orr, V. S. Ivanova, W. M. Bonner, *J. Biol. Chem.* **273**, 5858 (1998).
8. R. S. Maser, K. J. Monsen, B. E. Nelms, J. H. Petrini, *Mol. Cell Biol.* **17**, 6087 (1997).
9. B. E. Nelms, R. S. Maser, J. F. MacKay, M. G. Lagally, J. H. Petrini, *Science* **280**, 590 (1998).
10. Rabbit polyclonal antibody was made against a peptide at the extreme COOH-terminus of mouse NBS1, amino acids 733 to 751 (C)KEESLADDL-FRYNPNVKKR (25). Rabbit polyclonal antibody against phosphoserine residue 139 in histone H2AX was described previously (6). We transferred 3×10^6 thymocytes onto 24-well plates containing cover slips coated with 150 μ g/ml poly-L-lysine (Sigma). Cells were fixed with methanol at -20°C

for 20 min, washed $3 \times$ with phosphate-buffered saline (PBS), blocked with 5% goat serum (Jackson ImmunoResearch) + 1% bovine serum albumin for 1 hour at room temperature, incubated with NBS1 (1:1000) or γ -H2AX (1:1000) antibody for 1 hour, washed, and then stained with an Alexa 488- or Alexa 568-conjugated goat anti-rabbit secondary antibody (1:250; Molecular Probes). Double staining for γ -H2AX and NBS1 was performed by staining with anti- γ -H2AX antibody followed by detection with Alexa 568-conjugated goat anti-rabbit secondary antibody, blocking secondary antibody with rabbit immunoglobulin G (100 μ g/ml; Sigma), and finally staining with directly conjugated NBS1-Alexa 488 antibody. Nuclear counterstaining was performed by incubating coverslips with Topro-3 (Molecular Probes) at 1:500 dilution in PBS for 25 min prior to mounting slides with Mowiol (Calbiochem). Images were acquired using a Zeiss LSM 410 confocal microscope with a 63 \times objective.

11. DP (CD4 $^+$ CD8 $^+$) thymocytes were isolated by panning on anti-CD8-coated plates as described (26). CD4 $^+$ SP thymocytes were isolated by negative selection using anti-CD8 (2.43) antibodies to deplete CD4 $^+$ CD8 $^+$ and CD8 $^+$ cells, followed by positive selection of CD4 $^+$ cells using L3T4-microbeads (Miltenyi Biotec, Auburn, CA).
12. L. A. Turka *et al.*, *Science* **253**, 778 (1991).
13. P. Borgulya, H. Kishi, Y. Uematsu, H. von Boehmer, *Cell* **69**, 529 (1992).
14. TcrAND mice were crossed to Rag2 $^{-/-}$ mice (27) and MHC $^{-/-}$ mice. MHC class II knockout mice (28) and $\beta 2$ microglobulin-deficient mice (29) were intercrossed to generate MHC $^{-/-}$ mice.
15. K. E. Brown *et al.*, *Cell* **91**, 845 (1997).
16. In situ hybridization was modified from protocols developed by Fisher and colleagues (15). After protein detection, the antibodies were cross-linked using 50 mM ethylene glycol bis (succinimidyl succinate) for 30 min at 37 $^\circ\text{C}$. Samples were incubated with ribonuclease (100 μ g/ml in $2 \times$ standard saline citrate) for 60 min at 37 $^\circ\text{C}$. The chromosomal DNA was denatured in 0.1 M NaOH for 2 min followed by immersion in cold PBS. The biotin-labeled DNA probe was hybridized at 37 $^\circ\text{C}$ overnight, followed by standard fluorescence in situ hybridization (FISH) washes. Detection and amplification of the probe were with mouse anti-biotin-fluorescein isothiocyanate (FITC) (Sigma F4024) and goat anti-mouse-FITC (BMB #605240). The bacterial artificial chromosome probes (TcrV $\delta 3$ /V $\alpha 6$ -46G9 and TcrC α -232F19) were isolated by polymerase chain reaction library screening (30).
17. C. L. Hsieh, C. F. Arlett, M. R. Lieber, *J. Biol. Chem.* **268**, 20105 (1993).
18. L. A. Donehower *et al.*, *Nature* **356**, 215 (1992).
19. J. S. Danks, C. J. Guidos, *Semin. Immunol.* **9**, 199 (1997).
20. D. A. Ramsden, M. Gellert, *Genes Dev.* **9**, 2409 (1995).
21. F. Livak, D. G. Schatz, *J. Mol. Biol.* **267**, 1 (1997).
22. M. S. Schlissel, *Mol. Cell Biol.* **18**, 2029 (1998).
23. C. Zhu, D. B. Roth, *Immunity* **2**, 101 (1995).
24. F. Livak, D. G. Schatz, *Mol. Cell Biol.* **16**, 609 (1996).
25. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; K, Lys; L, Leu; N, Asn; P, Pro; R, Arg; S, Ser; V, Val; and Y, Tyr.
26. T. Nakayama *et al.*, *Science* **249**, 1558 (1990).
27. Y. Shinkai *et al.*, *Cell* **68**, 855 (1992).
28. M. J. Grusby, R. S. Johnson, V. E. Papaioannou, L. H. Glimcher, *Science* **253**, 1417 (1991).
29. B. H. Koller, P. Marrack, J. W. Kappler, O. Smithies, *Science* **248**, 1227 (1990).
30. M. Liyanage *et al.*, *Blood* **96**, 1940 (2000).
31. We thank A. Singer, R. Hodes, E. E. Max, M. C. Nussenzweig, D. G. Schatz, F. W. Alt, M. Jankovic, D. Allman, and A. G. Fisher for helpful discussions.

16 August 2000; accepted 8 November 2000