Requirement for CD8 β Chain in Positive Selection of CD8-Lineage T Cells

Kei-ichi Nakayama,* Keiko Nakayama,* Izumi Negishi, Keisuke Kuida, Marjorie C. Louie, Osami Kanagawa, Hiromitsu Nakauchi, Dennis Y. Loh†

CD8 is either an αα homodimer or an αβ heterodimer, although most peripheral CD8-lineage T cells express only the CD8αβ heterodimer. The physiological function of CD8β was elucidated with mice that were chimeric for the homozygous disruption of the *CD8β* gene. The *CD8β*$^{-/-}$ T cells developed normally to CD4$^+$CD8$^+$ stage, but did not efficiently differentiate further, which resulted in few peripheral CD8$^+$ T cells. The number of peripheral CD8$^+$ T cells was restored by transfer of an exogenous *CD8β* gene into CD8β-deficient T cells. Thus, CD8β is necessary for the maturation of CD8$^+$ T cells.

CD8 and CD4 are glycoproteins, expressed on both thymocytes and mature T cells, that act as coreceptors for the T cell antigen receptor (TCR) (*1*). CD8 binds to the nonpolymorphic regions of class I major histocompatibility complex (MHC) molecules, whereas the TCR recognizes peptide antigens in conjunction with the polymorphic regions of MHC molecules (*2*, *3*). CD8 can be expressed as an αα homodimer or an αβ heterodimer; surface expression of the CD8 β chain is dependent on expression of the CD8 α chain (*1*). Most peripheral T cells exclusively express the CD8αβ heterodimer (*4*, *5*). Functional and developmental studies of the CD8 molecule have focused primarily on the α chain. The CD8αα homodimer is sufficient for the binding to the α3 domain of class I MHC and for reconstitution of T cell response when hybridoma systems are used (*6*). Transfection experiments with T cell hybridomas suggested that CD8β may increase the avidity between T cells and antigen-presenting cells, or broaden the specificity for the binding to MHC molecules under certain limiting conditions (*7*). However, little is known about the physiological function of CD8β and its role in the development of T cells.

We evaluated the function of CD8β in lymphocyte development. We made homozygous mutations (*8*) in exon 2 of the *CD8β* gene that eliminated most of the immunoglobulin-like domain that is important for the binding to MHC molecules (*9*). In rodents and humans, aberrant splicing connecting exons 1 and 3 has never been detected (*3*, *10*). These constructs were introduced into embryonic stem (ES) cells by sequential homologous recombinations (Fig. 1, A to C) (*11*). Homologous recombination events were screened by polymerase chain reaction (PCR) and verified by Southern (DNA) blot analysis (Fig. 1D). Independent ES cell lines (from mouse strain 129) bearing homozygous mutations at the *CD8β* locus (*CD8β*$^{-/-}$) from two distinct parental ES cell lines, E14 and D3, were obtained. We injected the mutant ES cells (*CD8β*$^{-/-}$) or parental ES cells (*CD8β*$^{+/+}$) into mouse strain C57BL/6 blastocysts (*CD8β*$^{+/+}$) and generated chimeric mice (designated as knockout chimera or control chimera, respectively). Blastocyst-derived cells provide an experimental internal control from the same animal as long as they are phenotypically distinguishable from the ES-derived cells. The ES cell– and blastocyst-derived T lymphocytes in the chimeric mice could be distinguished with a monoclonal antibody (mAb) to Ly-9.1, a cell surface marker that is found on 129, but not on C57BL/6, strain cells (*8*).

Cells from lymphoid organs of the chimeric mice were stained for three-color flow cytometric analysis with antibodies to CD4, CD8α, CD8β, and Ly-9.1 (*12*). We tested for the surface expression of CD8β on Ly-9.1$^+$ (ES-derived) and Ly-9.1$^-$ (blastocyst-derived) cells from control or knockout chimeras (Fig. 2A). No expression of CD8β was detectable on Ly-9.1$^+$ cells from knockout chimeras, whereas CD8β was expressed normally on either Ly-9.1$^-$ cells or Ly-9.1$^+$ cells from control chimeras which were generated by the injection of *CD8β*$^{+/+}$ ES cells. This indicates that the deletion introduced in exon 2 abrogated the expression of CD8β.

The Ly-9.1$^+$ thymocytes from control and knockout chimeras were examined for CD4 and CD8α expression (Fig. 2B). The

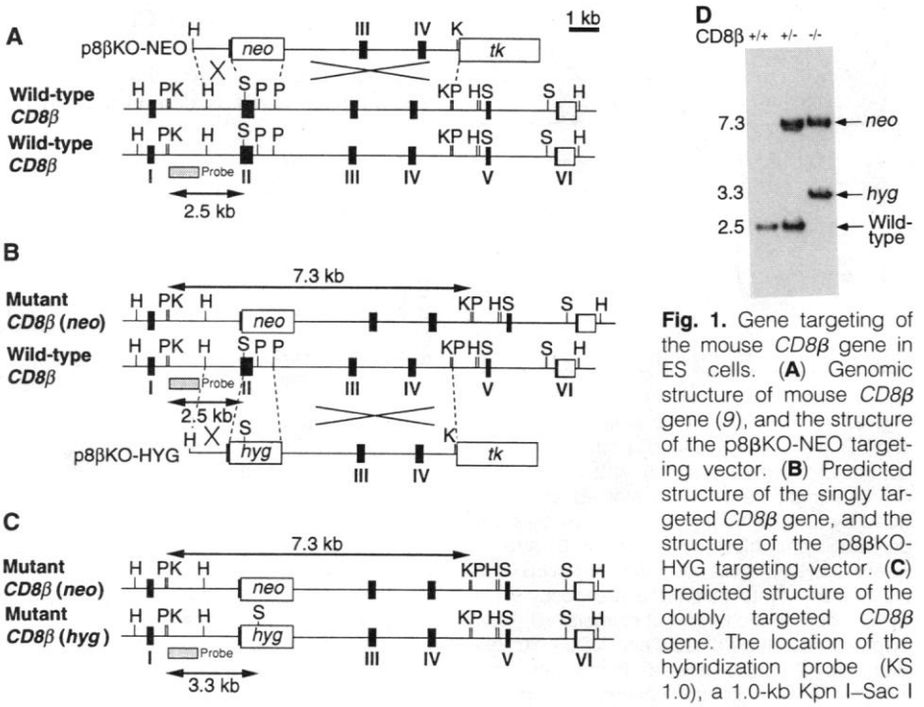

Fig. 1. Gene targeting of the mouse *CD8β* gene in ES cells. (**A**) Genomic structure of mouse *CD8β* gene (*9*), and the structure of the p8βKO-NEO targeting vector. (**B**) Predicted structure of the singly targeted *CD8β* gene, and the structure of the p8βKO-HYG targeting vector. (**C**) Predicted structure of the doubly targeted *CD8β* gene. The location of the hybridization probe (KS 1.0), a 1.0-kb Kpn I–Sac I fragment, is shown as a shaded box. Expected sizes of the Kpn I–Sca I fragments that hybridize with the probe are indicated. H, Hind III; P, Pst I; K, Kpn I; S, Sca I. (**D**) Southern blot analysis of ES cells with the KS1.0 probe. Genomic DNA was digested by Kpn I and Sca I. Lane 1, E14 (*CD8β*$^{+/+}$); lane 2, EE1726 (*CD8β*$^{+/-}$); and lane 3, EEH603 (*CD8β*$^{-/-}$). Expected sizes (in kilobases) for wild-type *CD8β*, mutant *CD8β* by p8βKO-NEO, and mutant *CD8β* by p8βKO-HYG are shown at left.

K.-i. Nakayama, K. Nakayama, I. Negishi, K. Kuida, M. C. Louie, D. Y. Loh, Howard Hughes Medical Institute and Departments of Medicine, Genetics, Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110, USA.
O. Kanagawa, Department of Pathology, Washington University School of Medicine, St. Louis, MO 63110, USA.
H. Nakauchi, Laboratory of Cell Growth and Differentiation, Institute of Physical and Chemical Research (RIKEN), Tsukuba, Ibaraki 305, Japan.

*These authors contributed equally to this report.
†To whom correspondence should be addressed.

$CD4^+CD8\alpha^+$ thymocytes seemed to develop normally in the absence of CD8β, suggesting that CD8β is dispensable for early development of thymocytes and that CD8α expression is independent of the presence of CD8β, consistent with previous transfection studies (*13*). The $CD4^+CD8\alpha^-$ thymocytes also appeared normal. In contrast, however, the percentage of $CD4^-CD8\alpha^+\beta^-$ thymocytes in the knockout chimeras were decreased compared with $CD4^-CD8\alpha^+\beta^+$ thymocytes in the control chimeras. In addition, Ly-9.1$^+$ thymocytes from chimeric mice generated by injection of $CD8\beta^{+/-}$ ES cells showed normal development (*14*), suggesting that it is unlikely that targeted allele of *CD8β* produced any significant amount of "dominant negative" molecules.

The reduction of $CD4^-CD8\alpha^+$ T cells in the absence of CD8β was even more evident in the periphery. Few $CD8\alpha^+$ cells were detected in the absence of CD8β, which increased the CD4:CD8 ratio (normally about 3:1 in 129 strain) to more than 40:1 (Fig. 3). The $CD8\alpha^+\beta^-$ phenotype is prominent among $CD8^+$ T cells from athymic mice and rats, whereas $CD8^+$ T cells from euthymic animals almost exclusively express $CD8\alpha^+\beta^+$ (*5*, *15*). This suggests that the generation of $CD8\alpha^+\beta^+$ T cells is thymus-dependent, whereas $CD8\alpha^+\beta^-$ T cells can be generated by an extrathymic maturation pathway that may not require CD8β expression. The residual (≤1%) $CD8\alpha^+\beta^-$ T cells in the CD8β knockout chimeric mice were possibly from such an extrathymic pathway (*5*, *15*).

To test that the lack of $CD8^+$ T cells was due to the absence of CD8β and not to possible mutations occurring during the manipulation of ES cells, we did gene transfer experiments to *CD8β*$^{-/-}$ ES cells (*16*). These *CD8β*$^{-/-}$ ES cells carrying a single copy of the transfected *CD8β* gene were injected into C57BL/6 blastocysts, resulting in the generation of chimeric mice. The expression of CD8β on Ly-9.1$^+$ T cells was restored (Fig. 3, A and B). The $CD8^+$ T cells appeared in the periphery, so that the CD4:CD8 ratio became about 5:1 (Fig. 3C). The reason why the degree of CD4:CD8 ratio was not fully restored to normal (3:1) is probably because the expression of the transfected *CD8β* in the thymus was lower than in normal cells (Fig. 3A). In contrast, the amount of CD8β expressed on the peripheral T cells was equivalent to normal cells (Fig. 3B). These findings suggested that only thymocytes expressing CD8β over a certain threshold were positively selected. On the basis of these data, we conclude that CD8β is not only necessary, but the amount of CD8β affects the maturation of $CD8^+$ T cells. The CD8αα homodimer cannot efficiently mediate differentiation from $CD4^+CD8^+$ to $CD4^-CD8^+$ thymocytes.

It has been suggested that the fate of developing T cells is determined on the basis of the affinity of the TCR for the antigen-MHC complex (the affinity model) (*17*). According to this model, immature thymocytes expressing high-affinity TCRs for self ligands presented by self-MHC molecules are deleted (negative selection), whereas those with low-affinity TCRs are allowed to mature (positive selection) (*17*). In TCR transgenic mice, the amount of CD8 expressed on peripheral CD8 T cells varies with different MHC molecules for a fixed TCR, which suggests that CD8 contributes to the affinity between TCR and peptide-MHC complexes (*18*). TCR transgenic mice that lack CD8 molecules need CD8 for positive selection but differentially require it for negative selection of T cells during thymic development (*19*). This is consistent with the hypothesis that CD8 contributes to the total avidity between T cells and antigen-presenting cells during thymic selection (*20*). Other evidence was obtained by the overexpression of CD8 in transgenic mice. In double transgenic mice (TCR transgenic × CD8 transgenic mice), the overexpression of CD8 alters the fate of thymocytes from positive selection to negative selection (*21*). The threshold for the alteration from positive to negative selection is different between CD8α transgenic and CD8αβ transgenic mice. An increase of the amount of CD8αβ to two times that of normal mice is sufficient for the switching to negative selection, whereas CD8α requires a 6- to 10-fold increase for a similar effect. These findings support the hypothesis that CD8αβ has a potentially stronger affinity for class I MHC molecules than CD8αα.

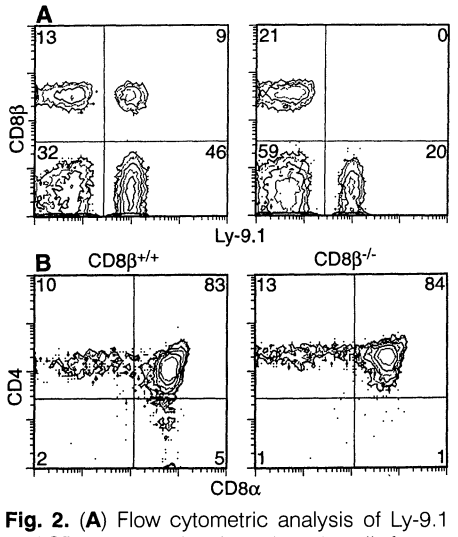

Fig. 2. (**A**) Flow cytometric analysis of Ly-9.1 and CD8β expression. Lymph node cells from a control chimera (left panel) and a knockout chimera (right panel) were stained and analyzed by flow cytometry. The Ly-9.1$^-$ cells are derived from blastocyst (C57BL/6 strain), whereas Ly-9.1$^+$ cells are derived from ES cells (129 strain). Note that there are no Ly-9.1$^+$/$CD8\beta^+$ cells in the knockout chimera. (**B**) Flow cytometric analysis of CD4 and CD8α expression on Ly-9.1$^+$ (ES-derived) thymocytes. Thymocytes from a control chimera (left panel, *CD8β*$^{+/+}$) and a knockout chimera (right panel, *CD8β*$^{-/-}$) were stained and analyzed by flow cytometry. The data were gated on Ly-9.1$^+$ cells. Note that the $CD4^+CD8\alpha^-$:$CD4^-CD8\alpha^+$ ratio is 2:1 in the control chimera and 13:1 in the knockout chimera.

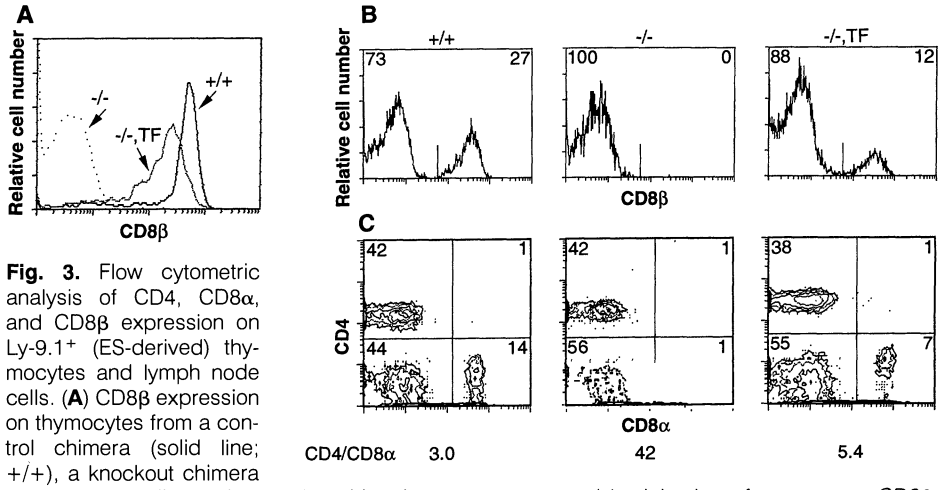

Fig. 3. Flow cytometric analysis of CD4, CD8α, and CD8β expression on Ly-9.1$^+$ (ES-derived) thymocytes and lymph node cells. (**A**) CD8β expression on thymocytes from a control chimera (solid line; +/+), a knockout chimera (coarse dotted line; −/−), and a chimeric mouse generated by injection of exogenous *CD8β*-transfected EEH603 (fine dotted line; −/−, TF) is shown. (**B**) CD8β expression on lymph node T cells from a control chimera (left panels; +/+), a knockout chimera (center panels; −/−), and a chimeric mouse generated by injection of exogenous *CD8β*-transfected EEH603 (right panels; −/−, TF) is shown. The data are gated on Ly-9.1$^+$, $TCR\alpha\beta^+$ cells. (**C**) The pattern of CD4/CD8α expression on Ly-9.1$^+$ lymph node cells (including T and non–T cells) is shown. Note the marked decrease of $CD4^-CD8\alpha^+$ cells in the knockout chimera and the recovery of the $CD4^-CD8\alpha^+$ phenotype by the gene transfer of exogenous *CD8β*. The CD4:CD8α ratios were indicated below the panels. Experiments were done three times with similar results.

Our data suggest that the amount of CD8β expressed is also critical for positive selection. One possible explanation for the role of CD8β is that CD8αβ may bind to class I MHC molecules with higher affinity than that of CD8αα, and only CD8αβ-MHC interaction is sufficient for positive selection. Because positive selection probably requires a relatively weak interaction between the TCR and the peptide-MHC complex (stronger interactions result in clonal deletion), it may be far more sensitive to the relative contribution of the CD8 coreceptor than when studied in in vitro functional assay systems.

In independent studies, we showed that the lack of the CD8α cytoplasmic domain resulted in a dramatically decreased efficiency in positive selection, arguing that not only binding to class I MHC α3 domain but also signaling through the CD8α cytoplasmic region are required for positive selection (*22*). Surprisingly, $p56^{lck}$, which binds to the cytoplasmic domain of CD4 or CD8α, is not necessary for positive and negative selection (*23*). As for the role of the cytoplasmic region of CD8β, it is short and there is no motif for the binding of any known signaling molecules (*24*). In addition, experiments in hybridoma systems have suggested that only extracellular but not the cytoplasmic portion of CD8β is critical for the augmentation of interleukin-2 release (*7*). Taken together, it is unlikely that CD8β contributes to positive selection by signaling through its cytoplasmic region. We thus favor the hypothesis that the significant difference between CD8αβ and CD8αα molecules lies in their extracellular domains. The mice generated in this study should be useful in elucidating the molecular requirements for positive selection in the CD8 lineage.

REFERENCES AND NOTES

1. S. L. Swain, *Immunol. Rev.* **74**, 129 (1983); D. R. Littman, *Annu. Rev. Immunol.* **5**, 561 (1987).
2. M.-L. Blue, K. A. Craig, P. Anderson, K. Branton, S. F. Schlossman, *Cell* **54**, 413 (1988); A. Norment, R. D. Salter, P. Parham, V. H. Englehard, D. R. Littman, *Nature* **336**, 79 (1988); J. M. Connolly, T. A. Potter, E.-M. Wormstall, T. H. Hansen, *J. Exp. Med.* **168**, 325 (1988); T. A. Potter, T. V. Rajan, R. F. Dick II, J. A. Bluestone, *Nature* **337**, 73 (1989); R. D. Salter *et al.*, *ibid.* **338**, 345 (1989); R. D. Salter *et al.*, *ibid.* **345**, 41 (1990).
3. A. Norment and D. R. Littman, *EMBO J.* **7**, 3433 (1988).
4. J. R. Parnes, *Adv. Immunol.* **44**, 265 (1989).
5. N. Torres-Nagel *et al.*, *Eur. J. Immunol.* **22**, 2841 (1992).
6. J. Gabert *et al.*, *Cell* **50**, 545 (1987); Z. Dembic *et al.*, *Nature* **326**, 510 (1987).
7. C. J. Wheeler, P. von Hoegen, J. R. Parnes, *Nature* **357**, 247 (1992); F. Letourneur *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2339 (1990); S. Karaki, M. Tanabe, H. Nakauchi, M. Takiguchi, *J. Immunol.* **149**, 1613 (1992).
8. K.-i. Nakayama *et al.*, *Science* **261**, 1584 (1993).
9. K.-i. Nakayama, Y. Shinkai, K. Okumura, H. Nakauchi, *J. Immunol.* **142**, 2540 (1989).
10. K.-i. Nakayama *et al.*, *ibid.* **148**, 1919 (1992); K.-i. Nakayama and H. Nakauchi, *Int. Immunol.* **5**, 419 (1993).
11. Cloned genomic DNA corresponding to the *CD8β* locus was isolated from a library of strain B10.A mouse DNA as described (*9*). The targeting vectors, p8βKO-NEO and p8βKO-HYG, were constructed by a replacement of a 0.9-kb Sca I–Pst I fragment containing most of the CD8β immunoglobulin-like domain (*9*) with either a PGK-neo-polyadenylate [poly(A)] cassette derived from pKJ-1 (*25*) or a PGK-hyg-poly(A) cassette [H. te Riele, E. R. Maandag, A. Clarke, M. Hooper, A. Berns, *Nature* **348**, 649 (1990)]. The targeting vectors contained 1.3 kb of homology 5′ and 6.0 kb 3′ of the drug resistance marker. The PGK-tk-poly(A) cassette (*25*) was ligated into a restriction site in a vector polylinker at the 3′ end of the insert. Maintenance, transfection, selection, and injection of ES cells were carried out as described elsewhere (*8*). Frequency of the homologous recombinations was 25.5% with p8βKO-NEO after first-round transfection and 18.8% with p8βKO-HYG after second-round transfection.
12. Lymph node cells and thymocytes (5×10^5) were prepared from control and knockout chimeras and stained with fluorescein isothiocyanate (FITC)–conjugated antibody to Ly-9.1, phycoerythrin (PE)–conjugated antibody to CD8β or PE-conjugated antibody to CD4, and biotinylated antibody to CD8α (PharMingen). Biotin conjugates were revealed by Red 613–streptavidin (GibcoBRL). The expression of CD8β (Fig. 3B) was analyzed with Ly-9.1–FITC, CD8β-PE, and TCRαβ-biotin (PharMingen) by gating on Ly-9.1$^+$TCRαβ$^+$ cells. Dead cells were excluded by staining with propidium iodide (Boehringer Mannheim). Flow cytometric analysis was done by FACScan (Becton Dickinson).
13. K.-i. Nakayama, A. Sarai, H. Nakauchi, *Immunogenetics* **33**, 206 (1991); D. Blanc *et al.*, *Eur. J. Immunol.* **18**, 613 (1988); S. D. Gorman, Y. H. Sun, R. Zamoyska, J. R. Parnes, *J. Immunol.* **140**, 3646 (1988).
14. K.-i. Nakayama and D. Y. Loh, unpublished observations.
15. T. Lin, G. Matsuzaki, H. Kenai, T. Nakamura, K. Nomoto, *Eur. J. Immunol.* **23**, 1968 (1993).
16. A 16.5-kb genomic DNA fragment, containing the whole *CD8β* gene (*9*) ligated to a puromycin-resistant gene driven by the PGK promoter, was transfected by electroporation into CD8β$^{-/-}$ ES cells (EEH603) and selected by puromycin-containing medium (1.0 μg/ml). We injected clones carrying the transfected *CD8β* gene into C57BL/6 blastocysts, generating chimeric mice.
17. D. Y. Loh, *New Biol.* **3**, 924 (1991).
18. W. C. Sha *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6186 (1990).
19. W.-P. Fung-Leung *et al.*, *Eur. J. Immunol.* **23**, 212 (1993).
20. L. A. Sherman, S. V. Hesse, M. J. Irwin, D. La Face, P. Peterson, *Science* **258**, 815 (1992).
21. E. A. Robey *et al.*, *Cell* **69**, 1089 (1992); N. A. Lee, D. Y. Loh, E. Lacy, *J. Exp. Med.* **175**, 1013 (1992).
22. W.-P. Fung-Leung *et al.*, *Eur. J. Immunol.* **23**, 2834 (1993).
23. K.-i. Nakayama and D. Y. Loh, *Science* **257**, 94 (1992); I. T. Chan *et al.*, *ibid.* **261**, 1581 (1993).
24. H. Nakauchi, Y. Shinkai, K. Okumura, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4210 (1987).
25. V. L. Tybulewicz, C. E. Crawford, P. K. Jackson, R. T. Bronson, R. C. Mulligan, *Cell* **65**, 1153 (1991).

26. We thank P. Mombaerts, E. George, and R. Hynes for ES cell lines; A. Berns for the PGK-hyg-poly(A) cassette; P. Laird and R. Jaenisch for the PGK-puro-poly(A) cassette; Y. Shinkai for helpful discussions; and S. Kim and Q. Zhang for technical assistance. Supported by the Howard Hughes Medical Institute (D.Y.L.) and NIH grant AI 34580 (D.Y.L.).

29 October 1993; accepted 6 January 1994

Persistence of Transients in Spatially Structured Ecological Models

Alan Hastings* and Kevin Higgins

Simple discrete-time ecological models for a species with alternating reproduction and dispersal are shown to have complex transient dynamics. If the density dependence (nonlinearity) is strong enough, then the time required to reach the final dynamics is usually very long, approaching thousands of generations, and there are typically very sudden changes in the form of the dynamics. Apparent chaos can change to cycles or vice versa. These results are consistent with observed sudden changes in the form of the dynamics of a single species and imply that transient dynamics of ecological models may be more relevant than long-term behavior.

Ecological theory has typically been based on analysis of the long-term behavior of ecological models, with stability analysis as the primary tool (*1*, *2*). Even studies of nonequilibrium behavior, such as limit cycles or chaos, have focused on long-term behavior (*3*). We show that the long-term behavior of a simple ecological model for a species distributed along a one-dimensional habitat can be essentially irrelevant to the understanding of natural ecological systems because the form of the dynamics changes over long time scales. The model we use, in which juveniles (pelagic larvae) are redistributed along the coast each generation, has transient dynamics that are much longer than the time scale of significant environmental perturbations. Moreover, the transient dynamics can appear to be the final behavior, either chaotic or cyclic, and then the system can quickly change its dynamics without any underlying change in parameters.

These considerations have particular relevance when one is trying to understand

Division of Environmental Studies, Institute of Theoretical Dynamics, and Center for Population Biology, University of California, Davis, CA 95616, USA.

*To whom correspondence should be addressed.