15. O. Nureki *et al.*, unpublished data.
16. N. D. Rawlings and A. J. Barrett, *Methods Enzymol.* **244**, 19 (1994).
17. N. C. Strynadka *et al.*, *Nature* **359**, 700 (1992).
18. M. Miller, M. Rao, A. Wlodawer, M. R. Gribskov, *FEBS Lett.* **328**, 275 (1993).
19. Charging assays: Mutants were examined for their ability to catalyze the formation of Ile-tRNA^{Ile} under standard charging assay conditions. Final concentrations in each reaction mixture were 1 nM enzyme, 1.8 μM tRNA^{Ile}, 20 mM Hepes (pH 7.5), 100 μM EDTA, 150 mM NH_4Cl, bovine serum albumin (10 μg/ml), 2 mM ATP, 4 mM $MgCl_2$, and 20 μM L-isoleucine. Editing assays: Each mutant was evaluated in an adenosine triphosphatase assay that measured tRNA-dependent editing by following the hydrolysis of ATP in the presence of L-valine and tRNA^{Ile}. This type of assay does not distinguish between the hydrolysis of Val-AMP or the hydrolysis of Val-tRNA^{Ile}. Final concentrations in each reaction mixture were 3 μM enzyme, 4.6 μM tRNA^{Ile}, 140 mM tris (pH 7.5), 3 mM ATP (labeled with P^{32} at the gamma position, 12.5 μCi/ml), 0.5 mM $CaCl_2$, 10 mM L-valine, 9.3 mM $MgCl_2$, and pyrophosphatase (5 μg/ml). Each time point was quenched in 1.25 ml of a solution of 2 mM sodium pyrophosphate, 8% activated charcoal, 1.4% perchloric acid, and 0.4% hydrochloric acid. After 10 min at room temperature, the charcoal was pelleted by centrifugation, and the phosphate content of 500 μl of each supernatant was quantitated by scintillation.
20. S. P. Hale, D. S. Auld, E. Schmidt, P. Schimmel, *Science* **276**, 1250 (1997).
21. E. Schmidt and P. Schimmel, unpublished data.
22. S. F. Sneddon and D. J. Tobias, *Biochemistry* **31**, 2842 (1992); S. E. Jackson, M. Moracci, N. Masry, C. M. Johnson, A. R. Fersht, *ibid.* **32**, 11259 (1993); K. Takano *et al.*, *J. Mol. Biol.* **254**, 62 (1995).
23. Z. Y. Zhang, M. J. Fasco, L. Huang, F. P. Guengerich, L. S. Kaminsky, *Cancer Res.* **56**, 3926 (1996).
24. M. A. Farrash *et al.*, *J. Virol.* **68**, 233 (1994).
25. Z. Otwinowski and W. Minor, *Methods Enzymol.* **276**, 307 (1997).
26. CCP4: Collaborative Computational Project No 4, *Acta Crystallogr. D* **50**, 760 (1994).
27. J. P. Abrahams and A. G. W. Leslie, *ibid.* **52**, 30 (1996).
28. T. A. Jones, J.-Y. Zhou, S. W. Cowan, M. Kjeldgaard, *Acta. Crystallogr. A* **47**, 110 (1991).
29. A. T. Brünger, *X-PLOR: A System for X-Ray Crystallography and NMR* (Yale Univ. Press, New Haven, CT, 1992).
30. R. A. Laskowski, M. W. MacArthur, A. L. Morris, J. M. Thornton, *J. Appl. Crystallogr.* **26**, 283 (1993).
31. J. J. Perona *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2903 (1991).
32. We thank M. Nakasako, N. Kamiya, M. Yamamoto, H. Iwasaki, T. Ueki, and Y. Maeda for data collection and helpful discussions; N. Watanabe and N. Sakabe for help with synchrotron data collection at the Tsukuba Photon Factory; and K. Nagai and A. Murzin for helpful comments. Supported in part by Grants-in-Aid for Scientific Research on Priority Areas (04272103 and 09278102) to S.Y. from the Ministry of Education, Science, Culture, and Sports of Japan and by grant GM15539 to P.S. and T.H. from NIH. T.H. is an NIH Postdoctoral Fellow. The coordinates for *T. thermophilus* IleRS have been deposited in the Brookhaven Protein Data Base (1ILE).

15 December 1997; accepted 24 March 1998

Dependence of Germinal Center B Cells on Expression of CD21/CD35 for Survival

Michael B. Fischer,* Siegfried Goerg,* LiMing Shen, Andrey P. Prodeus, Christopher C. Goodnow, Garnett Kelsoe, Michael C. Carroll†

Affinity-driven selection of B lymphocytes within germinal centers is critical for the development of high-affinity memory cells and host protection. To investigate the role of the CD21/CD35 coreceptor in B cell competition for follicular retention and survival within the germinal center, either Cr2^{+} or Cr2^{null} lysozyme-specific transgenic B cells were adoptively transferred into normal mice immunized with duck (DEL) or turkey (TEL) lysozyme, which bind with different affinities. In mice injected with high-affinity turkey lysozyme, Cr2^{null} B cells responded by follicular retention; however, they could not survive within germinal centers. This suggests that CD21 provides a signal independent of antigen that is required for survival of B cells in the germinal center.

The murine Cr2 locus encodes complement receptors CD21 (CR2; 150 kD) and CD35 (CR1; 190 kD) that are expressed primarily on B cells and follicular dendritic cells (FDCs) (*1*). Cr2^{null} mice have impaired immune responses to T-dependent antigens (*2*, *3*); the defect is in the B cell compartment (*2*, *4*). The natural ligands for CD21 and CD35 are activation products of complement C3 (C3d and C3b, respectively) (*5*) that are covalently coupled to antigen (*6*); mice deficient in C3 have impaired humoral responses similar to those observed in Cr2^{null} animals (*7*). In studies in which fusion proteins of C3d and hen egg lysozyme (HEL) were used, B cell activation in vitro and in vivo was enhanced and dependent on the presence and density of C3d (*8*). Thus, CD21/CD35 may be a potent coreceptor for complement-decorated antigens that can raise the intensity of suboptimal activating signals.

To examine directly the fate of B cells deficient in CD21/CD35 in an immune recipient, we bred Cr2^{null} mice with mice expressing a transgenic (tg) immunoglobulin (Ig) consisting of both heavy and light chains, which bind avian egg lysozymes with very high affinity (*9*). The importance of CD21/CD35 as a coreceptor in B cell activation by antigen ligands that bind with increasingly substantial affinities was examined by comparing the response of Cr2^{+} and Cr2^{null} lysozyme-specific Ig tg B cells to DEL and TEL, respectively (*10*, *11*). As expected, 10- to 100-fold less TEL than DEL was required for the induction of proliferative responses and up-regulation of the CD86 costimulator molecule in both groups of tg B cells (*12*). Thus, activation through the B cell receptor (BCR) in the absence of complement is comparable in Cr2^{+} and Cr2^{null} tg B cells, and B cell activation and proliferation is proportional to antigen affinity.

To examine the importance of B cell expression of CD21/CD35 in combination with low-affinity antigen (DEL) in vivo, we adoptively transferred splenic B cells isolated from either Cr2^{+} or Cr2^{null} lysozyme-specific Ig tg mice with DEL into wild-type (WT) recipients that had been primed 7 days earlier with DEL (*13*, *14*). Cr2^{null} tg B cells from the spleen declined over the 5-day period in the DEL-primed recipients (Fig. 1A); by day 5 after transfer, there were 75% fewer Cr2^{null} than Cr2^{+} tg B cells (0.26 ± 0.04% versus 1.14 ± 0.26%, respectively; $P < 0.01$) in the recipient spleens (*15*). An increase in Cr2^{null} tg B cells in the blood was observed by day 5 (Fig. 1B); however, this did not account for the loss of cells from the spleen given the relatively low number of B cells in blood compared with spleen. Loss of Cr2^{null} tg cells from the spleen was confirmed by immunohistochemical analysis (Fig. 2, A to D, and Table 1) and correlated with a low frequency of expression of CD86 (B7-2) at day 1 after transfer (*12*). Although similar numbers of Cr2^{+} and Cr2^{null} tg B cells were present on day 1 in the primary splenic follicles of immune recipients (Fig. 2, A and C), by day 5 less than 2% of follicles held Cr2^{null} tg B cells compared with 85% of follicles positive for Cr2^{+} tg B cells (Fig. 2, B and D, and Table 1) (*16*). The loss of the Cr2^{null} tg B cells in DEL-immune recipients was greater than that in nonimmune con-

M. B. Fischer, S. Goerg, L. Shen, A. P. Prodeus, M. C. Carroll, Department of Pathology, Harvard Medical School, Boston, MA 02115, USA.
C. C. Goodnow, Medical Genome Centre, John Curtin School of Medical Research, Australian National University, Canberra, ACT 2601, Australia.
G. Kelsoe, Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD 21201–1509, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: mcarroll@warren.med.harvard.edu

trols in which over 50% of the follicles were positive for tg B cells 5 days after transfer (Table 1). Thus, binding of intermediate affinity antigen without coligation of CD21/CD19 coreceptor might deliver a subthreshold signal leading to B cell elimination. Cook *et al.* (*17*) have reported that subthreshold BCR stimulus of tg B cells in the splenic outer periarteriolar lymphoid sheath (PALS) can result in an abortive response in the follicle in the presence of T cell help. Whether specific elimination or migration out of the white pulp accounts for the loss of $Cr2^{null}$ tg B cells cannot be determined from these results.

To rule out down-regulation of the Ig receptor, we labeled splenocytes with a fluorescent dye (BCECF AM-2′7′-*bis*-2-carboxyethyl-5 and 6-carboxy-fluorescein) before transfer (*15*). On day 5 after transfer, spleen cells were harvested and fluorescent cells analyzed for expression of the B cell marker CD45R (B220), and HEL binding. Similar numbers of $BCECF^{+}B220^{+}$ and $HEL^{+}B220^{+}$ tg cells were observed, confirming that the majority of the HEL^{+} tg cells were accounted for (*12*). Thus, most $Cr2^{null}$ tg B cells in vivo remained inactivated by DEL antigen despite its substantial affinity and activating potential in vitro. This finding supports the threshold model (*18*) that CD21/CD35 (with CD19 and CD81) is a potent coreceptor for antigen and its coligation with the BCR lowers the affinity threshold for B cell activation (*19*).

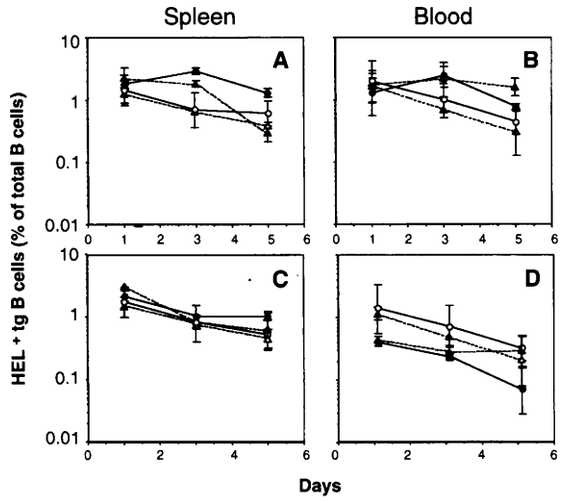

Fig. 1. Flow cytometric analysis of splenic (MNCs) and peripheral blood mononuclear cells (PBMCs) of recipients after adoptive transfer of $Cr2^{+}$ or $Cr2^{null}$ lysozyme-specific Ig tg B cells. (**A** to **D**) Frequency of $Cr2^{null}$ or $Cr2^{+}$ HEL-specific tg B cells in the spleen or blood 1, 3, and 5 days after transfer into nonirradiated MHC class II–matched WT recipients that had been immunized 7 days previously with 50 μg of soluble DEL (A and B) or TEL (C and D) antigen. Values represent the mean ± SD of HEL-binding B cells within the total B cell population ($B220^{+}$) of three to four experiments with pooled cells from two mice for each group in each experiment. Closed and open circles represent $Cr2^{+}$ HEL^{+} tg B cells with and without antigen; closed and open triangles represent $Cr2^{null}$ HEL^{+} tg B cells with and without antigen.

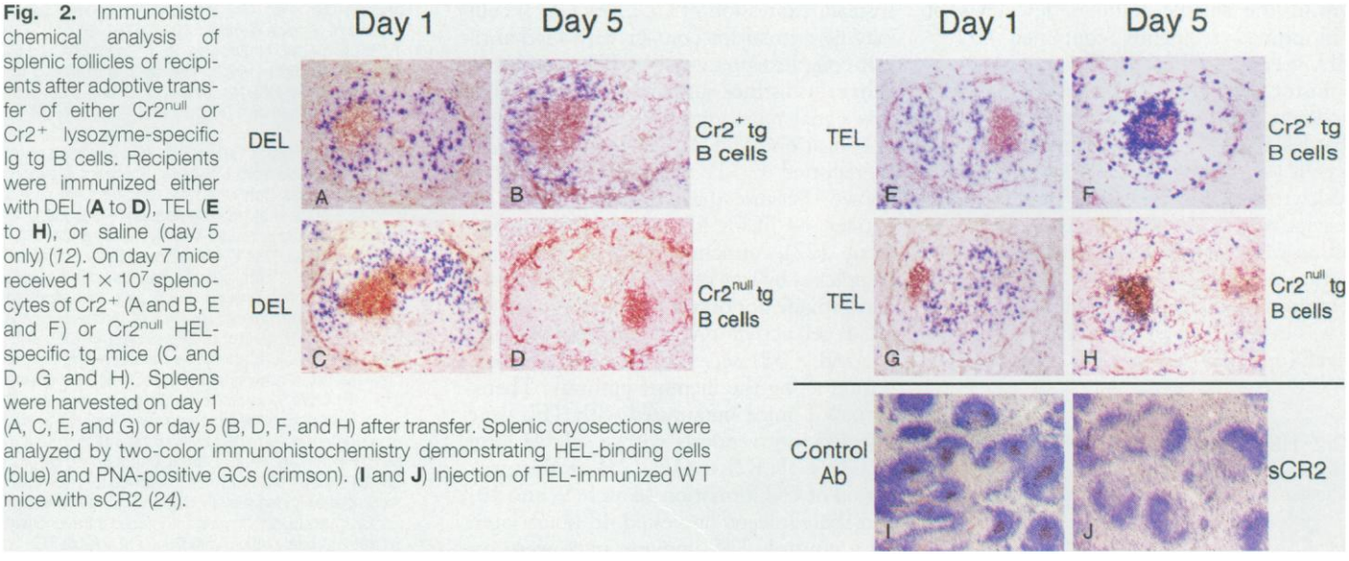

Fig. 2. Immunohistochemical analysis of splenic follicles of recipients after adoptive transfer of either $Cr2^{null}$ or $Cr2^{+}$ lysozyme-specific Ig tg B cells. Recipients were immunized either with DEL (**A** to **D**), TEL (**E** to **H**), or saline (day 5 only) (*12*). On day 7 mice received 1×10^{7} splenocytes of $Cr2^{+}$ (A and B, E and F) or $Cr2^{null}$ HEL-specific tg mice (C and D, G and H). Spleens were harvested on day 1 (A, C, E, and G) or day 5 (B, D, F, and H) after transfer. Splenic cryosections were analyzed by two-color immunohistochemistry demonstrating HEL-binding cells (blue) and PNA-positive GCs (crimson). (**I** and **J**) Injection of TEL-immunized WT mice with sCR2 (*24*).

Table 1. Morphometric analysis of splenic sections of recipients on day 5 after transfer of tg B cells. HEL-binding B cells and PNA-positive GC B cells were identified by immunostaining as described (*16*). Total numbers of follicles or follicles and GCs with more than 10 tg B cells were counted for six recipients each for $Cr2^{null}$ and $Cr2^{+}$. Values represent the mean ± SEM.

Recipients	TEL-primed		DEL-primed		Nonimmune	
	$Cr2^{+}$	$Cr2^{null}$	$Cr2^{+}$	$Cr2^{null}$	$Cr2^{+}$	$Cr2^{null}$
No. of follicles	35.7 ± 2.1	32.2 ± 2.9	33 ± 1.9	32.2 ± 2.4	35 ± 1.3	35.8 ± 1.4
No. of GCs	28.7 ± 2.3	24 ± 3.6	30.5 ± 2.7	28.7 ± 3.1	8.8 ± 1.6	7.2 ± 2.2
No. of follicles with >10 HEL-specific B cells	32.3 ± 3.1*	17.7 ± 4.4	28.3 ± 3.7†	0.5 ± 0.5	27 ± 2.1	19 ± 5.3
No. of GCs with >10 HEL-specific B cells	15.5 ± 2.0‡	1.7 ± 0.7	1.2 ± 0.7	0.2 ± 0.2	0	0
GCs with >10 HEL-specific B cells/GCs (%)	53.3 ± 3.6	6.3 ± 2.2	4.1 ± 2.4	0.5 ± 0.5		

*Comparison of $Cr2^{+}$ versus $Cr2^{null}$ tg B cells in follicles of TEL-immune mice ($P < 0.01$). †Comparison of $Cr2^{+}$ versus $Cr2^{null}$ tg B cells in follicles of DEL-immune mice ($P < 3 \times 10^{-6}$). ‡Comparison of $Cr2^{+}$ versus $Cr2^{null}$ tg B cells in GCs of TEL-immune mice ($P < 3 \times 10^{-5}$).

To determine if the binding of high-affinity antigens by $Cr2^{null}$ tg B cells could drive in vivo activation, follicular retention, and participation in the germinal center (GC) response, we transferred tg cells into TEL-primed recipients. Analysis of the frequency of $CD86^+$ $Cr2^{null}$ tg B cells in TEL-immune versus nonimmune recipients (26% versus 17%, respectively; $P < 0.05$) indicated a significant increase in expression of this activation marker. By day 5 after transfer, an ~30-fold difference was observed in the number of follicles in TEL-immune versus DEL-immune recipients that held $Cr2^{null}$ tg B cells (60% versus 1.5%, respectively) (Fig. 2, H and D, respectively, and Table 1). This difference is not likely due to the differential availability of T cell help because (i) similar numbers of GCs were observed in spleens of both DEL- and TEL-primed recipients (Table 1); (ii) mice immunized with TEL or DEL showed similar antibody responses (*12*); and (iii) T cells isolated from both groups of immune recipients on day 3 after transfer expressed similar amounts of the CD25 activation marker and proliferated comparably in vitro when cultured with the relevant antigen (*12*).

Despite their activation and persistence within the splenic follicles, few GCs of TEL-primed recipients contained $Cr2^{null}$ HEL-specific tg B cells. Two-color immunohistochemical analyses of splenic sections on day 5 after transfer demonstrated that less than 7% of GCs included $Cr2^{null}$ tg B cells as compared with greater than 53% positive for $Cr2^+$ tg B cells (Fig. 2, H and F, respectively, and Table 1). Although interaction with a very high affinity antigen was sufficient for the activation and follicular retention of coreceptor-deficient tg B cells, it was insufficient for their entrance or survival (or both) within GCs. The relative absence of GCs that include $Cr2^{null}$ tg B cells is not explained by the migration of tg B cells out of the spleen because less than a twofold difference was observed in the number of follicles (85% versus 60%) occupied by $Cr2^+$ and $Cr2^{null}$ tg cells, respectively (Table 1). To examine if the $Cr2^+$ and $Cr2^{null}$ tg B cells were actively dividing in GCs, we pulsed both groups of TEL-primed recipients with bromodeoxyuridine (BrdU) on day 5 after transfer and harvested their spleens 2 hours later. Similar proportions of BrdU-labeled endogenous B cells and $Cr2^+$ tg B cells were present in the GCs of mice immunized with TEL (Fig. 3A) (*20*). However, few $Cr2^{null}$ tg B cells incorporated BrdU (Fig. 3B). Thus, high-affinity B cells that lack CD21 are activated by antigen and enter GCs but do not proliferate there.

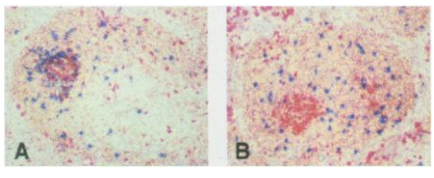

Fig. 3. $Cr2^+$ lysozyme-specific Ig tg B cells localized within the GCs of TEL-primed recipients are actively dividing. BrdU uptake of dividing (**A**) $Cr2^+$ ($n = 4$) or (**B**) $Cr2^{null}$ ($n = 5$) HEL-specific tg B cells in splenic follicles 5 days after transfer into nonirradiated MHC class II–matched WT recipients that had been immunized 7 days previously with TEL antigen. B cells are stained with B220-HRP (crimson), tg cells are stained with HEL-biotin-strepavidin-AP (blue), and proliferating cells that incorporated BrdU were revealed by sequential incubation with BU20a, goat anti-mouse IgG-biotin, and strepavidin-AP (pink).

The reduction in the number of $Cr2^{null}$ tg B cells within the GCs of TEL-primed mice suggests that CD21/CD35 expression is required for survival in GCs. Given the extraordinary affinity of TEL binding to the BCR of $Cr2^{null}$ tg cells, it seems unlikely that coreceptor amplification of BCR signals alone could account for the failure of tg B cells to participate in the GC reaction. Instead, expression of CD21 by GC B cells may be critical for contact with C3d-antigen complexes retained by FDCs and may deliver a distinct survival signal. Whether this signal represents the synergistic effect of coligation of CD21/CD19 with the BCR as reported by Tooze *et al.* (*21*) is not known. Because the efficient retention of antigen by FDCs is mediated by complement (*22*), attachment of C3d-antigen complexes by way of CD21/CD35 receptors may provide not only a source of antigen for GC B cell activation and selection, but also a ligand (C3d) necessary for B cell differentiation along the memory pathway. Therefore, WT mice immunized with TEL were injected intravenously with a soluble form of CD21 ($[CR2]_2$-IgG1) (*23*) at the peak period of GC formation (days 8, 9, and 10) and their spleens harvested 48 hours later. As a control, TEL-immune mice were injected with a similar concentration of IgG1. Soluble CD21 treatment resulted in an 87% reduction in total GC area within the splenic white pulp (Fig. 2, I and J) (*24*). $[CR2]_2$-IgG1 has been well characterized and is specific for iC3b and C3d (*23*); unlike human CD21, mouse CD21 does not bind CD23. This reduction in GC area is most likely explained by the blockade of C3d on FDCs within the GC, which would disrupt contact between activated B cells and FDCs as well as coligation of the CD21/CD19 coreceptor. Although $[CR2]_2$-IgG1 treatment would also inhibit activation of naïve B cells at other sites such as the PALS, this would not explain the elimination of preexisting GCs at day 8. Coculture of FDCs with primed B and T cells promoted B cell survival, and the effect was dependent on expression of CD21 and CD21L (C3d) by B cells and FDCs, respectively (*25*). Thus, expression of the CD21 and CD19 coreceptor provides not only an enhancing signal for lowering the threshold of B cell activation, but also mediates an unanticipated survival signal for B cells within the GC.

REFERENCES AND NOTES:

1. J. J. Weis, T. F. Tedder, D. T. Fearon, *Proc. Natl. Acad Sci. U.S.A.* **81**, 881 (1984); H. Molina, T. Kinoshita, K. Inoue, J. C. Carel, V. M. Holers, *J. Immunol.* **145**, 2974 (1990).
2. J. M. Ahearn *et al.*, *Immunity* **4**, 251 (1996).
3. H. Molina *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3357 (1996).
4. D. A. Croix *et al.*, *J. Exp. Med.* **183**, 1857 (1996).
5. D. T. Fearon and W. W. Wong, *Annu. Rev. Immunol.* **1**, 243 (1983).
6. S. K. Law, N. A. Lichtenberg, R. P. Levine, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7194 (1980).
7. M. B. Fischer *et al.*, *J. Immunol.* **157**, 549 (1996).
8. P. W. Dempsey *et al.*, *Science* **271**, 348 (1996).
9. C. C. Goodnow *et al.*, *Nature* **334**, 676 (1988). HEL-specific Ig tg mice (MD4 line; >90% of the B cells express the rearranged transgene, which encodes heavy and light chain isolated from the HEL-specific hybridoma, HyHEL-10) were crossed with mice bearing disrupted Cr2 loci ($Cr2^{null}$; genetic background of all mice is mixed 129/C57BL/6).
10. T. B. Lavoie, W. N. Drohan, S. J. Smith-Gill, *J. Immunol.* **148**, 503 (1992). The source of the Ig tg, hybridoma Hy-HEL10, binds DEL and TEL with relative affinities of 1×10^7 and 2×10^{10} M^{-1}, respectively.
11. DEL was purified as described (*26*) from fresh duck eggs purchased from Dewhurst Butchers, Newton, MA. Purity was confirmed by NH_2-terminal amino acid sequence analysis. The sequence was identical to duck III lysozyme (Lys-Val-Tyr-Ser-Arg-Cys-Glu-Leu-Ala-Ala-Ala-Met-Lys-Arg-Leu). Splenocytes of $Cr2^{null}$ and $Cr2^+$ HEL-specific transgenic mice were depleted of macrophages and T cells as described (*2*) and incubated in the presence of various concentrations (1 pg to 10 μg) of TEL (Sigma) or DEL antigens or in medium alone. To measure lymphocyte proliferation, ^{3}H-thymidine (0.2 mCi/ml) was added after 48 hours, and labeled cells were harvested 6 hours later onto glass filter strips and their ^{3}H-thymidine content determined by liquid scintillation (*2*).
12. M. B. Fischer and M. C. Carroll, unpublished results.
13. Six- to 8-week-old WT mice [matched for major histocompatibility complex (MHC) and gender with donor $Cr2^+$ and $Cr2^{null}$] were immunized intravenously (iv) (retro-orbital plexus) with 50 μg of soluble TEL or DEL, and 7 days later they received 1×10^7 $Cr2^{null}$ or $Cr2^+$ Ig tg B cells in 100 μl of Hanks' balanced salt solution along with 50 μg of soluble TEL or DEL. Control nonimmune recipients received similar numbers of $Cr2^+$ and $Cr2^{null}$ tg B cells. Mice were killed 1, 3, and 5 days after transfer. One-third of the spleen was used for two-color flow cytometry and the remainder for immunohistochemistry.
14. K. M. Shokat and C. C. Goodnow, *Nature* **375**, 334 (1995).
15. Peripheral blood (PBMCs) and splenic mononuclear cells (MNCs) were prepared on a buoyant density gradient as described (*2*). PBMCs were further treated with lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) to remove the remaining erythrocytes. To detect lysozyme-specific Ig tg B cells, PBMCs and splenic MNCs were stained with HEL-biotin, followed by streptavidin-phycoerythrin (PE) and counterstained with fluorescein isothiocyanate (FITC)–conjugated monoclonal antibody (mAb) to

CD45R (B220; PharMingen, San Diego, CA) [HEL-biotin was prepared with biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma) by standard protocols]. To detect activated lysozyme-specific Ig tg B cells, splenic MNCs were stained with HEL-biotin, followed by streptavidin-FITC and counterstained with PE-labeled anti-CD86 (B7-2) mAbs (all from PharMingen). The HEL-biotin probe stained only the adoptively transferred tg B cells, because no HEL staining was detected when B cells from DEL- or TEL-immune mice (where no adoptive transfer was performed) were stained with HEL-biotin. To confirm that the majority of the transferred tg B cells were detected with the HEL-biotin label over the 5-day period, a second labeling method was used. Splenic B cells from lysozyme-specific Ig tg mice were labeled with 2′,7′-*bis*-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein (BCECF AM) (Molecular Probes, Eugene, OR) as described (*27*) and transferred according to the adoptive transfer protocol. PBMCs and splenic MNCs of immune recipients were harvested as described above, stained with either HEL-biotin, followed by streptavidin-PE, or with biotinylated anti-CD45R (B220), followed by steptavidin-PE, and analyzed by flow cytometry. Results from three to four experiments with pooled cells from two mice demonstrated similar numbers of cells positive for both HEL binding and BCECF or B220 and BCEF.

16. Spleens were prepared for cryosections as described (*2*). For immunohistochemical staining of lysozyme-specific Ig tg B cells, a two-layer sandwich of sequential incubation with biotinylated HEL was used followed by streptavidin–alkaline phosphatase (AP), and GC B cells were detected with peanut agglutinin (PNA)–horseradish peroxidase (HRP) (EY Laboratories, San Mateo, CA). Bound HRP and AP activities were visualized by reacting with 3-aminoethyl carbazole and naphthol AS-MX phosphate–Fast Blue BB (Sigma), respectively.
17. M. C. Cook, A. Basten, B. Fazekas de St.Groth, *J. Exp. Med.* **186**, 631 (1997).
18. R. H. Carter and D. T. Fearon, *Science* **256**, 105 (1992); C. J. M. van Noesel, A. C. Lankester, R. A. W. van Lier, *Immunol. Today* **9**, 8 (1993).
19. R. C. Rickert, K. Rajewsky, J. Roes, *Nature* **376**, 352 (1995); P. Engel *et al.*, *Immunity* **3**, 39 (1995).
20. On day 5 after cell transfer (as described above), 2 mg of BrdU (Sigma) was administered intraperitoneally; mice were killed 2 hours later, and spleens were analyzed by three-color immunohistochemistry (*28*). In brief, sections were blocked with Fcγ-block (1 mg/ml) (CD16/CD32, 2.4G2) and then incubated with FITC-conjugated anti-CD45R (B220, PharMingen) and HEL-biotin. After incubation with streptavidin-AP (Sigma) and anti-FITC coupled to HRP, bound HRP and AP activities were visualized as described above. Labeled sections were then treated in 1 M HCl for 20 min at 70°C to expose and partially degrade the DNA and to terminate the enzymatic reactions that had taken place previously without displacing the precipitates. Slides were then stained with mAb specific for BrdU (BU20a, Dako) and incubated with biotinylated goat anti-mouse IgG $F(ab')_2$ fragments (Boehringer) and streptavidin-AP. BrdU-positive cells were visualized with Fast Red TR/naphthol AS-MX (AP substrate; pink color).
21. R. M. Tooze, G. M. Doody, D. T. Fearon, *Immunity* **7**, 59 (1997).
22. G. G. Klaus, J. H. Humphrey, A. Kunkl, D. W. Dongworth, *Immunol. Rev.* **53**, 3 (1980); M. B. Fischer, M. Ma, N. C. Hsu, M. C. Carroll, *J. Immunol.*, in press.
23. T. Hebell, J. M. Ahearn, D. T. Fearon, *Science* **254**, 102 (1991). $[CR2]_2$-IgG1 is a fusion protein that represents the two NH_2-terminal short consensus repeats of CD21 (which includes the binding site for iC3b and C3d) coupled to the NH_2-terminus of the heavy chains of a NIP[(4-hydroxy-3 nitrophenyl)acetyl]-specific IgG1 antibody.
24. WT mice were immunized on days 1 and 7 with 50 μg of TEL as described (*12*), then received three injections (0.2 mg each, intravenously) of either sCR2 ($[CR2]_2$-IgG_1) (n = 4) (*23*) or control IgG_1 (n = 5) on days 10, 11, and 12. After 48 hours, spleens were harvested, quick-frozen in OCT, and examined by immunohistological staining (B220, blue; PNA, crimson) as described (*16*). Results show significant reduction in both the mean number of PNA^+ GCs and the GC area of sCR2-treated animals. Total GC area per spleen (mean GCs per spleen × mean area per GC = mean GC area per spleen) was determined by morphometric analysis of at least three sections per spleen of animals injected with either sCR2 (n = 4) or control IgG1 (n = 5). sCR2 versus control mAb (mean ± SEM, Student's t test): GCs per spleen = 9.5 ± 1.6 versus 34.0 ± 2.8 ($P < 0.4 \times 10^{-6}$); area per GC = 11.20 ± 1.29 versus 24.67 ± 1.75 μm^2 ($P < 0.2 \times 10^{-6}$); total GC area per spleen = 106.40 versus 837.78 mm^2.
25. J. Wu *et al.*, *J. Immunol.* **157**, 3404 (1996); D. Qin, J. Wu, M. C. Carroll, G. F. Burton, A. K. Szakal, J. G. Tew, in preparation.
26. E. M. Prager and A. C. Wilson, *J. Biol. Chem.* **246**, 5978 (1971).
27. T. G. Diacovo, K. D. Puri, R. A. Warnock, T. A. Springer, U. H. von Andrian, *Science* **273**, 252 (1996).
28. B. Zheng, S. Han, G. Kelsoe, *J. Exp. Med.* **184**, 1 (1996).
29. We thank M. Gendelman for preparation of the antibody to sCR2 and control antibody and E. Alicot for assistance with the analyses of sCR2-treated mice; J. Cohen (Harvard Medical School) for NH_2-terminal amino acid sequence analysis; and D. T. Fearon (Cambridge, UK) and T-Cell Sciences (Boston, MA) for the $[Cr2]_2$-IgG1 hybridoma. Supported by the Austrian Science Foundation (M.B.F.), DAAD Sonderprogramm Rheumatologie, Germany (S.G.), the National Institutes of Health (M.C.C. and G.K.), and the New York Chapter of the Lupus Foundation (M.C.C.).

17 November 1997; accepted 18 February 1998

Visualization of Single RNA Transcripts in Situ

Andrea M. Femino, Fredric S. Fay,† Kevin Fogarty, Robert H. Singer*

Fluorescence in situ hybridization (FISH) and digital imaging microscopy were modified to allow detection of single RNA molecules. Oligodeoxynucleotide probes were synthesized with five fluorochromes per molecule, and the light emitted by a single probe was calibrated. Points of light in exhaustively deconvolved images of hybridized cells gave fluorescent intensities and distances between probes consistent with single messenger RNA molecules. Analysis of β-actin transcription sites after serum induction revealed synchronous and cyclical transcription from single genes. The rates of transcription initiation and termination and messenger RNA processing could be determined by positioning probes along the transcription unit. This approach extends the power of FISH to yield quantitative molecular information on a single cell.

The identification of specific nucleic acid sequences by FISH has revealed sites of RNA processing, transport, and cytoplasmic localization (*1*). Recognition of these sites of hybridization is possible only when sufficient concentrations of the target sequence provide contrast with regions of lesser or no signal. Here we describe a quantitative approach to identify single molecules in these regions of low concentration. The methodology also facilitates accurate quantitation of the regions containing multiple copies of RNA, such as is found at transcription sites. Analysis of individual transcription sites with single molecule accuracy generated precise information on nascent chain initiation, elongation, and termination.

FISH images are composed of points of light with variable intensities resulting either from hybridization or from background fluorescent noise. We used multiple probes targeted specifically to β-actin mRNA to generate high-intensity point sources that result from hybridization to individual RNAs. We then quantitated the light intensity from each point source to distinguish hybridization events from spurious fluorescence.

The strategy involves (i) synthesizing several oligonucleotide probes to adjacent sequences on an RNA target such that their collective fluorescence will be emitted as a point source after hybridization; (ii) conjugating fluorochromes to specific sites on each oligonucleotide probe so that the fluorescent output per molecule of probe can be calibrated (Fig. 1, A to C); (iii) acquiring digital images from a series of focal planes through a hybridized cell; and (iv) processing these images with a constrained deconvolution algorithm such that out-of-focus light is quantitatively restored to its original points of origin.

To identify single β-actin mRNA molecules, we hybridized multiple probes to the isoform-specific 3′-untranslated region (UTR) of the mRNA in normal rat kidney (NRK) cells. The acquired fluorescence image was made up of numerous bright points

A. M. Femino and R. H. Singer, Department of Anatomy and Structural Biology and Cell Biology, Albert Einstein College of Medicine, Bronx, NY 10461, USA.
F. S. Fay and K. Fogarty, Biomedical Imaging Facility and Department of Physiology, University of Massachusetts Medical School, Worcester, MA 01655, USA.

*To whom correspondence should be addressed.
†Deceased.