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Simultaneous expression of mouse immunoglobulins M and D is determined by the same homolog of chromosome 12

(membrane proteins/Syrian hamster cell hybrid/acid phosphatase 1 isoelectric focusing)

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ABSTRACT **A hamster–mouse hybrid cell line expressing both murine IgM and murine IgD on the membrane was shown to have only one copy of mouse chromosome 12. This chromosome is known to carry the structural genes for the immunoglobulin heavy chains. Cloning of populations selected for loss of mouse membrane IgM yielded cells that had also lost expression of membrane IgD, but not the expression of hamster immunoglobulin heavy chain. Karyotype analysis of these subclones demonstrated the concurrent loss of the chromosome 12 present in the parental hybrid. Absence of this chromosome was confirmed by use of the isozyme acid phosphatase 1. The results of the genetic analysis prove that the coexpression of μ and δ immunoglobulin heavy chains is not due to long-lived immunoglobulin mRNA nor to the transcription of genes on homologous chromosomes. We conclude that the genetic information for IgM and IgD expressed by a single cell lies on the same chromosome.**

In all species of mammals that have been carefully examined the majority of the normal B lymphocytes carry two classes of membrane immunoglobulins, IgM and IgD (1–5). The evidence suggests that both immunoglobulin heavy (H) chains are synthesized by the same cell (1, 6), although neither simultaneous transcription nor translation has been demonstrated. Several studies have attempted to reconcile the production of two heavy chains by a single cell with the monospecificity postulated by the clonal selection theory of antibody formation. These studies have tried to show serologically and by antigen binding that membrane IgM and IgD have identical variable region sequences. Thus μ and δ chains on the membrane of individual rabbit spleen cells carry the same variable region allotype (5). Similarly, IgM and IgD chains on cells from human lymphomas (7) and bone marrow from patients with Waldenström macroglobulinemia (8) have been shown to carry related idiotypes and specificity, respectively. Furthermore, Herzenberg *et al.* (9) have studied spleen cells from F_1 mice doubly heterozygous at the μ (*Ig-6*) and δ (*Ig-5*) loci by using antiallotypic sera in immunofluorescence. They observed that anti-Ig-6b and anti-Ig-5b antisera used individually or combined did not stain more than 50% of B cells in a heterozygous animal. This implies that each H chain locus is allelically excluded, and that both loci are expressed by one or the other parental chromosome (haplotype exclusion). The availability of a hybrid cell line, GCL2, that produces both murine μ and δ chains, offered us the possibility of studying the genetic basis of this phenomenon.

Subclone GCL28 was found to have only one copy of mouse chromosome 12, which carries the structural genes for the immunoglobulin H chains (10, 11). Biochemical and cytogenetic analysis of GCL28 subclones demonstrated that the simultaneous expression of μ and δ is controlled by the same copy of chromosome 12.

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MATERIALS AND METHODS

Cell Lines. Cell line GCL2 was established and kindly provided by W. C. Raschke, San Diego, CA (12). This cell line is derived from a fusion between a simian virus 40-transformed hamster lymphoma, GD36A, and lipopolysaccharide-stimulated spleen cells from a BALB/c mouse. GD36A was established by G. Diamandopoulos, Boston (13), and made azaguanine resistant by R. Hyman, La Jolla, CA. The hybrid line was subcloned by limiting dilution, and the GCL28 was selected for further studies.

Antisera. Cell lines 10.4.22 and 11.6.3, established by Oi *et al.* (14), were provided by the Salk Institute, Cell Distribution Center. They produce anti-Ig-5a and anti-Ig-5b allotype antibodies, respectively. The antibodies were used either as present in the culture supernatant or as prepared by ammonium sulfate precipitation of ascitic fluids.

Goat anti-mouse μ, anti-mouse γ_2, and anti-mouse γ_1 were prepared and coupled with fluorochrome by using standard methods (15). The fluorescent reagents were absorbed with insolubilized hamster immunoglobulin and with the GD36A cells. They then were tested for specificity in double staining experiments for μ and δ or μ and γ on both membrane and cytoplasmic immunoglobulins on the following cells: Sp1 ($\mu\kappa$), Sp6 ($\mu\kappa$), Sp2 (γ_{2b}), and Sp3 ($\gamma_1\kappa$) of G. Köhler, Basel, Switzerland; 10.4.22 ($\gamma_{2a}\kappa$), GD36A, and spleen cells from C57BL/6 and BALB/c mice. Rabbit antiserum to hamster immunoglobulin, which had been coupled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC), was purchased from Cappel Laboratories, Cochranville, PA, and absorbed with insolubilized mouse immunoglobulin and mouse spleen cells.

Immunofluorescence. Cells, 2×10^6, were incubated with antibodies produced by the hybridoma cell line 10.4.22. This cell line secretes γ_{2a} antibody with specificity for murine δ chain of Ig-5a allotype. After washing, the cells were allowed to react with a mixture of TRITC-labeled anti-mouse μ and FITC-labeled anti-mouse IgG_2 previously absorbed with insolubilized hamster immunoglobulin and with the GD36A cells.

Selection for Subclones with H Chain Loss. GCL28 cells, 2×10^7, were incubated with 300 μl of FITC-labeled anti-μ

Abbreviations: IgM, immunoglobulin M; IgD, immunoglobulin D; H, immunoglobulin heavy chain; L, immunoglobulin light chain; μ, H chain of IgM; δ, H chain of IgD; γ_1, H chain of IgG_1; γ_2, H chain of IgG_2; κ, immunoglobulin L chain; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; ACP-1, acid phosphatase 1.

antiserum (0.5 mg of protein per ml) and washed in phosphate-buffered saline containing 10% fetal calf serum and 10 mM sodium azide. Cells devoid of fluorescence were selected in the fluorescence-activated cell sorter and immediately cloned in microtiter plates at a dilution of 0.15 cell per well in the presence of 10^5 peritoneal cells per ml.

Radiolabeling of Membrane and Cytoplasmic Immunoglobulin. Membrane labeling was done by lactoperoxidase-(Sigma) catalyzed iodination with carrier-free [^{125}I]iodide (Amersham) as described by Haustein (16). Biosynthetic labeling was performed by culturing cells (3×10^6 per ml) overnight in methionine-free RPMI 1640 medium supplemented with [^{35}S]methionine (1069 Ci/mmol, 500 μCi/ml of culture; 1 Ci = 3.7×10^{10} becquerels) and 5% undialyzed fetal calf serum. Cell lysates (0.5% Triton X-100 at 4°C) were incubated with specific antisera and antigen–antibody complexes precipitated by the addition of *Staphylococcus aureus* (17). The immunoprecipitates were analyzed by polyacrylamide gel electrophoresis, as described by Takács (18). Labeled material was detected by autoradiography.

Cytogenetic Analysis. Metaphases and Giemsa bands were prepared by using standard methods (19, 20). After photography of the banded metaphases, mouse chromosomes were recognized by the differential C-banding method, which does not stain the centromere of hamster chromosomes (21).

Assay for Acid Phosphatase 1 (ACP-1). Mouse and hamster isozymes were separated by isoelectric focusing on polyacrylamide gels. Cells were lysed in 2% Triton X-100 at 10^8 per ml. Samples (50 μl) were applied to filter papers (0.6 × 1 cm) 2 cm from the anode. Gels were prepared with final concentrations of 5.3% acrylamide, 0.14% *N,N'*-methylenebisacrylamide (Bio-Rad), 0.025% *N,N,N',N'*-tetramethylethylenediamine (Fluka), 12.5% D-sorbitol (Merck no. 7759), 1% Triton X-100, 0.0002% riboflavin, 1.5% Ampholine pH 4–6 and 3.5% Ampholine pH 5–8 (LKB). Focusing was performed at constant voltage at 4°C; isoelectric equilibrium was judged by the migration of hemoglobins, and the resulting gradient was measured. The enzyme activity of ACP-1 bands was detected by overlaying the gel with a filter paper impregnated with 4-methylumbelliferyl dihydrogen phosphate (Serva, Heidelberg, Federal Republic of Germany), 0.5 mg/ml in 0.1 M acetate buffer, pH 5.5, as described (22). After incubation for 30 min at 37°C the bands were visualized under long-wave ultraviolet light (365 nm).

RESULTS AND DISCUSSION

Clone GCL2 has been reported to synthesize three different immunoglobulin heavy chains: mouse μ and δ, and hamster γ_2 (12, 23). We established that subclone GCL28 produces δ and μ chains and that those chains were indeed of mouse origin, and not due to activation of hamster genes after fusion.

This was done by using immunofluorescence and polyacrylamide gel electrophoresis of radiolabeled immunoprecipitates. Cells treated with antibody to Ig-5a δ chain and subsequently stained with both TRITC-anti-mouse μ and FITC-anti-mouse γ_2 showed that both μ and δ chain were present on the same cells. Whereas μ chains were abundant, the amount of δ chain was much less than that found on BALB/c spleen cells. Controls were performed by omitting the anti-δ Ig-5a antiserum and by using instead anti-Ig-5b antiserum and subsequent staining with anti-mouse γ_1. Additional controls, in which UPC10 myeloma proteins of the γ_{2a} class were incubated in combination with the anti-γ_2 antiserum with the GCL28 cells, were negative. This excludes that binding of the 10.4.22 monoclonal antibodies, which are of the γ_{2a} class, occurs via Fc receptors specific for γ_{2a}. After staining for hamster immunoglobulin, GCL28 cells showed strong fluorescence. Note that hamster light (L) and H chains were not discriminated in this assay. The above immunofluorescence results were confirmed by the use of cell surface radioiodination (Fig. 1) as well as of biosynthetic radiolabeling (not shown) of the immunoglobulin chains. Anti-mouse μ antiserum precipitated mouse μ chain and κ chain. The μ chain had the same molecular weight as the μ on the membrane of BALB/c spleen cells. The antiserum also precipitated some material from the GD36A cells with the same molecular weight as mouse μ chain. Membrane μ of hamster spleen cells, however, ran at a different position (data not shown). Monoclonal antibodies to the Ig-5a δ chain precipitated murine δ chain of identical molecular weight on GCL28 and BALB/c spleen cells, but no material on the GD36A line. On hamster spleen cells some material was precipitated with the monoclonal antibodies to δ of Ig-5a allotype and *S. aureus*, though banding at different positions from mouse δ chain in gel electrophoresis. Presumptive hamster spleen cell δ chain precipitated by polyspecific anti-hamster Ig migrated at a position different from δ chain of mouse spleen cells or clone GCL28 (not shown). These results conclusively show that GCL28 cells synthesize δ and μ chains of murine origin.

Clone GCL28 has a modal complement of 92 chromosomes, determined on 100 metaphases. As evidenced by nine karyotyped metaphases (Table 1), only one copy of chromosome 12, which carries the genes for the immunoglobulin H chains (10, 11), is present. Most of the hamster chromosomes are metacentric, and are thus easily distinguished from the acrocentric mouse chromosomes. Furthermore, centromere staining allows a direct identification of mouse chromosomes, because the few hamster acrocentrics fail to stain by this method (21). Only one copy of chromosome 17 and one sex chromosome are present. Except for a short translocation carried by one copy of chromosome 1, all mouse chromosomes are structurally indistinguishable from the normal mouse karyotype.

The finding of production of both murine μ and δ by a cell

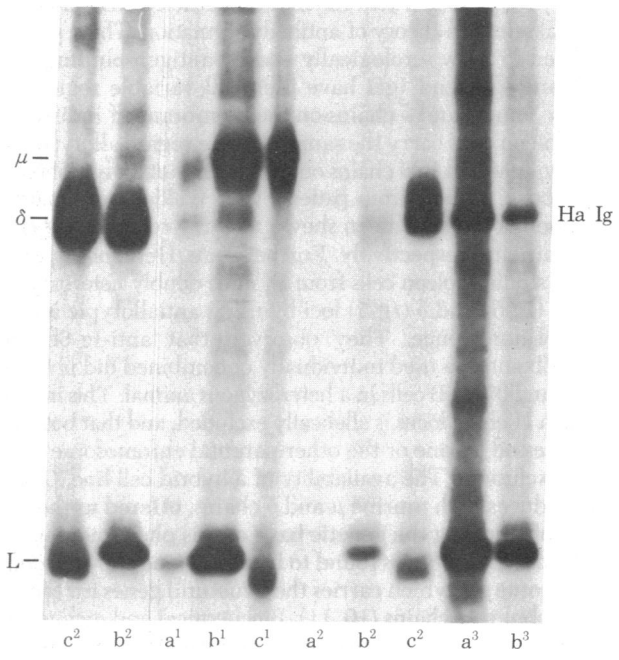

FIG. 1. Polyacrylamide gel electrophoresis of immunoprecipitated surface immunoglobulin on GD36A (lanes a), GCL28 (lanes b), and BALB/c spleen cells (lanes c), labeled with ^{125}I. The different classes were precipitated with anti-μ (lanes ^{1}), anti-δ (lanes ^{2}), and anti-hamster immunoglobulin (Ha Ig) (lanes ^{3}).

Table 1. Distribution of mouse chromosomes in clone GCL28 and four subclones that lack μ and δ chains

Clone	Metaphases karyotyped	Chromosomes																			
		1	2	3	4	5	6	7	8	9	10	11	12*	13	14	15	16	17*	18	19	X*
GCL28	9	0.9	0.9	1.0	1.0	0.9	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	0.9	1.0	0.9	1.0	0.9	1.0	1.0
21M3	10	0.5	0.4	0.9	0.8	0.9	0.9	0.8	0.3	0.8	0.4	0.3	—	0.7	1.0	0.3	0.9	1.0	0.3	0.2	0.6
7M6	14	—	—	0.4	0.7	0.6	0.4	0.6	—	0.5	0.8	0.1	—	0.5	0.7	0.9	0.9	0.8	0.6	0.5	0.9
1M10	6	—	0.8	0.2	0.5	—	0.2	0.7	—	0.3	1.0	0.3	—	0.3	0.7	0.5	0.8	0.7	0.3	0.5	1.0
3M6	10	0.1	—	—	0.7	0.5	0.6	0.8	0.2	0.4	0.7	—	—	0.6	0.7	0.9	0.7	0.9	0.5	0.8	0.6

Numbers indicate the fraction of metaphases with both copies of a given chromosome. Dashes indicate absence of one or both copies of a given chromosome.

* Chromosomes 12, 17, and X are present in a single copy in the GCL28 parent clone.

containing a single copy of chromosome 12 indicated that the genes coding for those heavy chains are transcriptionally active on the same chromosome. This was verified by segregation analysis of μ and δ chains on the GCL28 subclones. Enrichment for cells that had lost membrane μ by the fluorescence-activated cell sorter yielded clones (5 out of 168) that upon subsequent testing with immunofluorescence proved to be devoid of membrane μ chains. Absence of μ chain synthesis was confirmed by biosynthetic protein labeling. These clones were further analyzed for the presence of surface immunoglobulins by polyacrylamide gel electrophoresis of immunoprecipitated membrane proteins. As illustrated in Fig. 2, all of the four clones examined had lost the mouse δ chains concomitantly with μ. Retention of the hamster membrane Ig indicates that the cells have not undergone a mutation with broad regulatory effects on Ig expression. A fifth clone had also lost δ together with μ, as determined by immunofluorescence. Ten other subclones that had retained μ also expressed δ, as demonstrated by immunofluorescence. That the loss of μ and δ chains coincides with the loss of the single copy of mouse chromosome 12 was shown by the chromosome analysis of the isolated subclones (Table 1). The results show that the only karyotypic change that is correlated with lack of expression of μ and δ is the loss of chromosome 12. Besides the immunoglobulin H chain gene cluster another gene marker assigned to this chromosome is the enzyme ACP-1 (24). The Syrian hamster and mouse ACP-1 isozymes were found to be distinguishable by isoelectric focusing, and this difference was exploited to confirm the loss of chromosome 12 in the μ- and δ-negative hybrid subclones (Fig. 3). Isoenzyme analysis would still detect expression of the mouse enzyme even if less than 1% of the cells carry the chromosome with the gene marker for ACP-1. Therefore we conclude that in these subclones loss of mouse μ and δ chains is caused by the loss of the single chromosome 12 present in the parent clone GCL28.

The results of our genetic analysis formally demonstrate that the simultaneous expression of two H chain classes by a lymphocyte can be due to the expression of two H chain loci on the same chromosome homolog. This evidence renders plausible the current hypothesis that multiple immunoglobulin H chains expressed by a single lymphocyte have identical variable regions. This finding also suggests that the gene deletion model for the recommitment of a μ-producing lymphocyte to the expression of another H chain gene (H chain switch) cannot be applied to describe the μ–δ situation. This model was proposed by Kabat (25) and later was proposed by Honjo and Kataoka (26) and corroborated by evidence from several laboratories (27–31). According to the model, H chain switch involves a site-specific recombination that results in the deletion of DNA sequences. This lost DNA segment will contain the gene for the constant region of the μ chain ($C\mu$) and all other sequences located between $C\mu$ and the constant region gene that is expressed by a particular lymphocyte. Thus a cell producing δ should lack the constant region sequences for μ on the homolog from which δ is transcribed. Our data clearly show that this does not occur in the cells of GCL28.

We have shown that μ and δ chains are stably and concomitantly produced in cloned cell hybrids. Lack of production is seen in those clones that have lost the chromosome carrying their structural loci. It follows, therefore, that production of the two H chains is not due to the stability of immunoglobulin mRNAs. It remains to be determined whether these genes are transcribed individually or in tandem on a single precursor mRNA. The finding that the two co-expressed heavy chains of GCL28 share the same variable region segment (R. Maki and S. Tonegawa, personal communication) and the evidence that the genes coding for μ and δ are adjacent on the chromosome (32) render the latter a more plausible hypothesis.

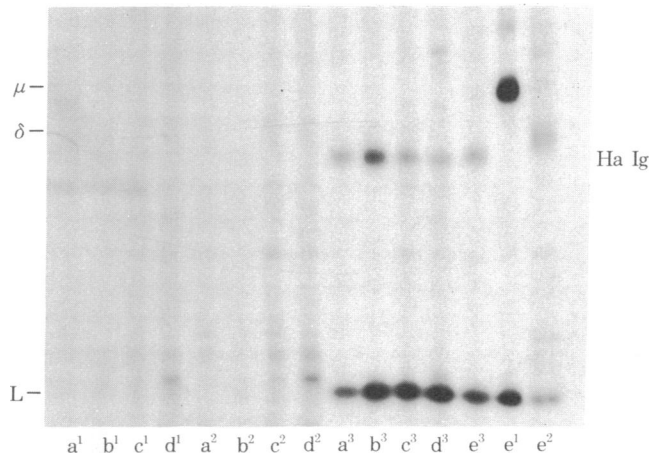

FIG. 2. Polyacrylamide gel electrophoresis of immunoprecipitated surface immunoglobulin on clones 21M3 (lanes a), 4M3 (lanes b), 1M10 (lanes c), 7M6 (lanes d), and GCL28 (lanes e). Immunoprecipitations were with anti-μ (lanes 1), anti-δ (lanes 2), and anti-hamster Ig (Ha Ig) (lanes 3).

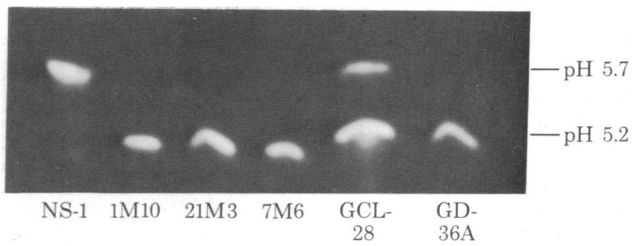

FIG. 3. Isoelectric focusing analysis of mouse and hamster ACP-1 isozymes expressed by the different hybrid clones. The mouse isozyme focuses at pH 5.7, the hamster isozyme at pH 5.2. From left to right: mouse myeloma line NS-1; subclones 1M10, 21M3, and 7M6; parent clone GCL28; and hamster parent line GD36A.

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1. Rowe, D. S., Hug, K., Forni, L. & Pernis, B. (1973) *J. Exp. Med.* **138,** 965–972.
2. Knapp, W., Bolhuis, R. L. H., Radl, J. & Hijmans, W. (1973) *J. Immunol.* **111,** 1295–1298.
3. Melcher, U., Vitetta, E. S., McWilliams, M., Lamm, J. M., Phillips-Quagliata, J. M. & Uhr, J. W. (1974) *J. Exp. Med.* **140,** 1427–1431.
4. Abney, E. R. & Parkhouse, M. E. (1974) *Nature (London)* **252,** 600–602.
5. Pernis, B., Forni, L. & Knight, K. L. (1975) *Membrane Receptors of Lymphocytes,* eds. Seligman, M., Preud'homme, J. L. & Kourilsky, F. M. (North Holland, Amsterdam), pp. 57–64.
6. Ferrarini, M., Corte, G., Viale, G., Durante, M. L. & Bargellesi, T. (1976) *Eur. J. Immunol.* **6,** 372–378.
7. Fu, S. M., Winchester, R. J., Feizi, T., Walzer, P. D. & Kunkel, H. G. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 4487–4490.
8. Pernis, B., Brouet, J. C. & Seligman, M. (1974) *Eur. J. Immunol.* **4,** 776–778.
9. Herzenberg, L. A., Herzenberg, L. A., Black, S. J., Loken, M. R., Okumura, K., van der Loo, W., Osborne, B. A., Hewgill, P., Goding, J. W., Gutman, G. & Warner, N. L. (1976) *Cold Spring Harbor Symp. Quant. Biol.* **41,** 33–45.
10. Hengartner, H., Müller, E. & Meo, T. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4494–4498.
11. Meo, T., Johnson, J. P., Beechey, C. V., Andrews, S. J., Peters, J. & Searle, A. G. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 550–553.
12. Raschke, W. C. (1978) in *Current Topics in Microbiology and Immunology: Lymphocyte Hybridomas,* eds. Melchers, F., Potter, M. & Warner, N. (Springer, Berlin), Vol. 81, pp. 70–76.
13. Diamandopoulos, G. (1973) *J. Natl. Cancer Inst.* **50,** 1347–1359.
14. Oi, V. T., Jones, P. P., Goding, J. W., Herzenberg, L. A. & Herzenberg, L. A. (1978) in *Current Topics in Microbiology and Immunology: Lymphocyte Hybridomas,* eds. Melchers, F., Potter, M. & Warner, N. (Springer, Berlin), Vol. 81, pp. 115–129.
15. Kearney, J. F. & Lawton, A. R. (1975) *J. Immunol.* **115,** 671–676.
16. Haustein, D. (1975) *J. Immunol. Methods* **7,** 25–38.
17. Kessler, S. W. (1975) *J. Immunol.* **115,** 1617–1624.
18. Takács, B. (1979) in *Immunological Methods,* eds. Lefkovits, I. & Pernis, B. (Academic, New York), pp. 81–105.
19. Buckland, R. A., Fletcher, J. M. & Chandey, A. C. (1976) *Experientia* **32,** 1146–1159.
20. Nesbitt, M. N. & Francke, U. (1973) *Chromosoma* **41,** 145–158.
21. Marshall, C. J. (1975) *Exp. Cell Res.* **91,** 464–469.
22. Swollow, D. M., Porey, S. & Harris, H. (1973) *Ann. Hum. Genet.* **37,** 31–38.
23. Coe, J. E. & Green, I. (1975) *J. Natl. Cancer Inst.* **54,** 269–270.
24. Francke, U., Lalley, P. A., Moss, W., Ivy, J. & Minna, J. D. (1977) *Cytogenet. Cell Genet.* **19,** 57–84.
25. Kabat, D. (1972) *Science* **175,** 134–140.
26. Honjo, T. & Kataoka, T. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2140–2144.
27. Rabitts, T. H., Forester, A., Dunnick, W. & Bentley, D. L. (1980) *Nature (London)* **283,** 351–356.
28. Maki, R., Traunecker, A., Sakano, H., Roeder, W. & Tonegawa, S. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2138–2142.
29. Davis, M. M., Calame, K., Early, P. W., Livant, D. L., Joho, R., Weiss, I. L. & Hood, L. (1980) *Nature (London)* **283,** 733–739.
30. Cory, S. & Adams, J. M. (1980) *Cell* **19,** 37–51.
31. Coleclough, C., Cooper, D. & Perry, R. P. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1422–1426.
32. Liu, C.-P., Tucker, P. W., Mushinski, J. F. & Blattner, F. R. (1980) *Science* **209,** 1348–1353.