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Control of lymphokine expression in T helper 2 cells

(interleukin 2 gene regulation/lymphokine production/nuclear run-on/repressor mechanism)

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ABSTRACT **In this report we present evidence that Con A-activated T helper 2 cells transcribe the interleukin 2 (IL-2) gene when cycloheximide is added 3 hr after stimulation. This IL-2 mRNA can be translated into functional protein. However, no steady-state mRNA for γ-interferon can be detected under the same experimental conditions. Furthermore, when cycloheximide is administered at time 0 of activation, no IL-2 mRNA is seen. Nuclear run-on experiments indicate that IL-2 transcription in T helper 2 cells depends on the presence of cycloheximide. It is likely, therefore, that activation of T helper 2 cells leads to the production of a labile regulatory protein that represses the expression of the IL-2 gene. These findings provide insight into the molecular mechanism that governs the differentiation of interleukin 4-producing T helper 2 cells from IL-2-producing precursor cells.**

T-cell receptor (TCR)-mediated stimulation leads to T-cell activation, measured in part by lymphokine production and lymphokine receptor expression (for review, see ref. 1). Some lymphokines are autocrine growth factors, allowing T-cell proliferation. In addition, lymphokines can act in a paracrine manner on other cells, thereby serving as regulators of the immune system (2)—e.g., interleukin 4 (IL-4) has only a weak autocrine effect but exerts an important paracrine effect on B cells where it regulates immunoglobulin isotype switching (3). Two types of murine $CD4^+$ T helper (Th) cells have been described that differ in their lymphokine production; namely, Th-1 cells that generate interleukin 2 (IL-2) and γ-interferon (IFN-γ), and Th-2 cells that secrete IL-4 and interleukin 5 (IL-5) (4).

To the best of our knowledge, nothing is known about the mechanism that controls the various patterns of lymphokine production in the two types of $CD4^+$ Th cells and at what stage in T-cell differentiation this distinction is imposed. We report here that the IL-2 gene is activated in Th-2 cells upon TCR-mediated stimulation. IL-2 transcription, however, is controlled by a cycloheximide (CHX)-sensitive element. These results provide insight into the mechanism that regulates the differentiation of $CD4^+$ cells into Th-1 and Th-2 cells.

MATERIALS AND METHODS

Mice. BALB/c (*H-2*^{d}) and AKR/J (*H-2*^{k}) were purchased from The Jackson Laboratories.

Cell Lines and Monoclonal Antibodies (mAbs). The generation and maintenance of the cloned Th-2 cell lines D10.G4.1 (D10) (obtained from the American Type Culture Collection) and CDC25 (kindly provided by D. Parker, Univ. of Massachusetts Medical School) have been described (5, 6). Briefly, cell lines D10 (specific for conalbumin in the context of I-A^k) and CDC25 (specific for rabbit IgG in the context of I-A^d) were stimulated every 2 weeks with antigen and irradiated syngeneic splenocytes as antigen-presenting cells. Supernatant from rat spleen cells that had been stimulated with Con A for 48 hr was added to the culture medium as source of lymphokines. Each experiment was carried out with resting cells obtained 10–14 days after antigen stimulation. For IL-2/IL-4 bioassays the HT-2 indicator cell line was used, which synthesizes DNA in response to either of these lymphokines. The mAb anti-murine IL-4, 11B11 (kindly provided by W. Paul, National Institutes of Health), was produced as ascites fluid and used at a 1:1000 dilution. The mAb anti-murine IL-2, S4B6.1 (kindly provided by T. Mosmann, DNAX), was prepared by ammonium sulfate precipitation of tissue culture supernatant and used at a 1:320 dilution.

RNA Analyses. The T-cell clones (10^6 cells per ml) were stimulated with Con A (5 μg/ml) for the indicated time, in the presence or absence of CHX (10 μg/ml) (Sigma). The cells were then lysed in Nonidet P-40 buffer (50 mM Tris Cl, pH 8/100 mM NaCl/5 mM $MgCl_2$/0.5% Nonidet P-40) at 4°C and the nuclei were removed. Cytoplasmic RNA was purified by extracting the lysate twice with phenol/chloroform/isoamyl alcohol (7). RNA (20 μg) was electrophoresed on a "Northern gel" [1% agarose/2% (vol/vol) formaldehyde/1× Mops] (1× Mops = 40 mM Mops, pH 7.0/10 mM sodium acetate/1 mM EDTA, pH 8.0) and the gel was blotted onto a nylon membrane (Biotrans Nylon Membrane; ICN). The RNA was UV-crosslinked as described by Church and Gilbert (8). The blot was prehybridized in 5% (wt/vol) SDS/100 mM NaCl/50 mM Pipes, pH 6.8/50 mM sodium phosphate, pH 6.8/1 mM EDTA for 2 hr at 65°C, and then hybridized for 12 hr in a fresh aliquot of the same solution containing an α-^{32}P-labeled random-primed probe (Boehringer Mannheim). The hybridized membrane was washed for three 5-min periods at 65°C in 5% SDS/1× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) and for three 30-min periods at 65°C in 0.1% SDS/0.5× SSC. Membranes were exposed to Kodak XAR film at −70°C with enhancer screens.

Nuclear Run-On Analyses. Nuclei from D10 cells were prepared as described (9). The transcription assays were carried out immediately after isolation of nuclei according to the protocol of Groudine and coworkers (10). Generally 1–2 × 10^7 cpm per 5 × 10^7 cells was obtained. The same number of cpm was hybridized to filters containing 5 μg of plasmid DNA per slot with fragments of the following genes: IL-2 [8.7 kilobases (kb), 5′ untranslated region and exons 1–3], IL-4 (4 kb, exons 3 and 4) (kindly provided by L. Glimcher, Harvard School of Public Health, Boston), or the ribosomal gene pRA (1.3 kb) (11). Hybridization and washing conditions were carried out as described (10).

RESULTS AND DISCUSSION

To study the regulation of lymphokine production in murine T cells, we have reexamined the Th-2 cell line D10. This

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Abbreviations: CHX, cycloheximide; mAb, monoclonal antibody; TCR, T-cell receptor; Th, T helper; IL-2, -4, and -5, interleukin 2, 4, and 5, respectively; IFN-γ, γ-interferon.

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T-cell clone produces IL-4 and IL-5 upon TCR-mediated activation. Fig. 1 shows the kinetics of steady-state lymphokine mRNA expression after stimulation with Con A. This mitogen has been shown to exert its function through the TCR (12). IL-4 mRNA was readily detectable 3 hr after activation and peaked at 6 hr. Thereafter, it decreased progressively and was undetectable 48 hr after stimulation. Similar kinetics were observed for IL-5 mRNA. However, neither IL-2 nor IFN-γ mRNA was detected at any point of this kinetic curve, confirming results obtained by others (13).

It has been suggested that IL-2 gene expression is regulated at two levels: namely, pretranscriptionally by regulatory elements acting on specific 5′ upstream sequences (14) and posttranscriptionally by factors that influence mRNA stability acting over the 3′ untranslated region (15). To test whether the IL-2 gene in D10 cells is under negative regulation by transacting elements, the cells were activated with Con A for 3 hr and then treated with CHX, a known inhibitor of protein synthesis. Cytoplasmic RNA was extracted at various times thereafter. We show in Fig. 2 that IL-2 mRNA was detected under these conditions and that IL-2 mRNA accumulated over time in the presence of CHX. In addition to the full-length 1-kb IL-2 mRNA, a shorter 0.7-kb transcript was seen at an equal molar ratio in cytoplasmic RNA in the presence of CHX. This could be due to usage of a different start site for transcription or differential mRNA splicing.

Identical results were obtained with another Th-2 cell line, CDC.25. Steady-state IL-2 mRNA was seen in these cells only when CHX was added 3 hr after activation with Con A (Fig. 3). Again, IL-4 mRNA did not accumulate in the presence of CHX. In fact, IL-4 mRNA was undetectable 24 hr after Con A activation in the presence of CHX (Fig. 3).

It has been suggested that the transcription of the IL-2 gene is dependent on an inducer protein that presumably acts on the gene promoter (16, 17). Therefore, we tested whether such positive regulation is also required for lymphokine transcription in D10 cells. For this purpose, CHX was added

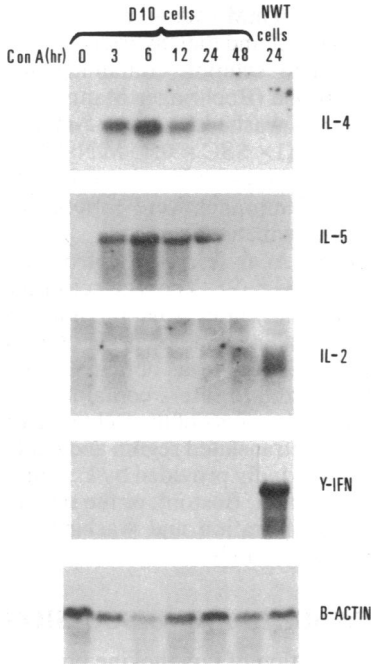

FIG. 1. Kinetics of lymphokine mRNA expression in Con A-activated D10 cells. Cytoplasmic RNA was extracted at 0, 3, 6, 12, 24, or 48 hr after Con A activation. As a positive control for IL-2 and IFN-γ, nylon wool-purified splenic T cells (NWT) were activated with Con A for 24 hr. The Northern blot was hybridized with the indicated random-primed probes.

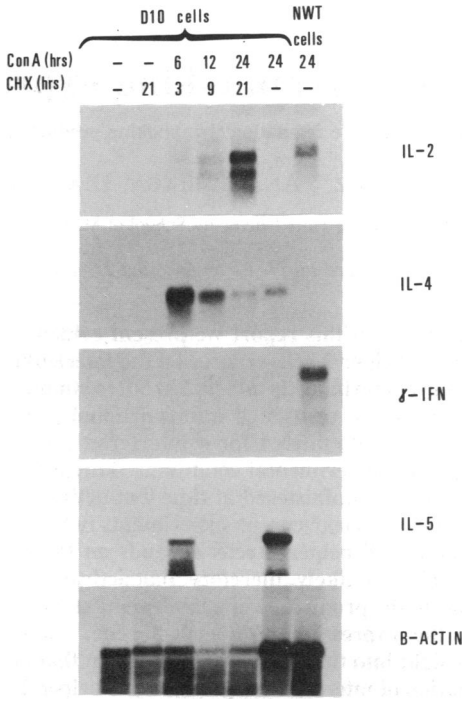

FIG. 2. Lymphokine mRNA expression in CHX-treated Con A-activated D10 cells. Resting D10 cells were cultured in the presence (as indicated) or absence (–) of Con A in complete medium for various times (as indicated), and 3 hr later CHX was added to some cultures (–, cultures to which no CHX was added). Cytoplasmic RNA was extracted at various times thereafter (as indicated).

with Con A at time 0 of culture, and cytoplasmic RNA was extracted 24 hr later. As shown in Fig. 4, neither IL-2 nor IL-5 mRNA was detected, although a normal level of IL-4 mRNA was seen under these experimental conditions. These results suggest that the IL-4 gene is transcribed as a result of second mediators that are generated upon TCR-mediated stimulation and act upon proteins already present in the cell. On the other hand, the IL-2 and IL-5 genes require the presence of a newly synthesized inducer protein for the initiation of transcription. It is likely, therefore, that in Th-2 cells the initial activation of the IL-2 gene follows the same pathway as in other T cells (18).

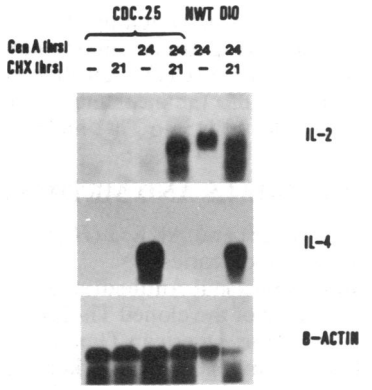

FIG. 3. Lymphokine mRNA expression in CHX-treated Con A-activated CDC.25 cells. Resting CDC.25 and D10 cells were cultured in the presence (as indicated) or absence (–) of Con A in complete medium (as indicated), and 3 hr later CHX was added to some cultures (–, cultures to which no CHX was added). Cytoplasmic RNA was extracted after 24 hr of culture. As a positive control for IL-2 expression, cytoplasmic RNA was extracted from nylon wool-purified splenic T (NWT) cells that had been stimulated with Con A for 24 hr.

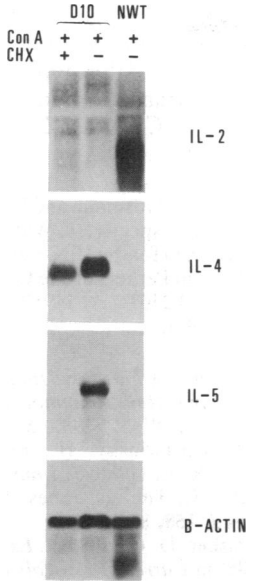

FIG. 4. Lymphokine mRNA expression in D10 cells after simultaneous treatment with Con A and CHX for 24 hr. +, Con A or CHX present; −, CHX absent.

At least three mechanisms can account for the CHX effect seen in activated Th-2 cells. (*i*) CHX could inhibit the translation of proteins that lead to rapid degradation of IL-2 mRNA, thereby stabilizing the level of steady-state IL-2 mRNA (19). This can take place as IL-2 mRNA accumulates in D10 cells in the presence of CHX. On the other hand, IL-4 and IL-5 mRNA degradation was not sensitive to CHX (Fig. 2), since their steady-state levels decreased even in the presence of CHX. (*ii*) CHX could inhibit the formation of a labile repressor protein that binds to a regulatory region of the IL-2 gene. Such a region controlling constitutive expression of IL-2 has been mapped proximal to the "TATA box" near an inducible DNase I hypersensitive region (20). The existence of a labile repressor protein regulating IL-2 expression has been suggested by other authors (21, 22). (*iii*) CHX could posttranscriptionally activate an inducer protein. A candidate for this is NFκB, a nuclear factor that was originally described to be specific for the immunoglobulin κ enhancer (23), but has recently been shown to upregulate transcription of many other genes, including IL-2 (24–26).

To test whether CHX exerts an effect directly on IL-2 transcription in Th-2 cells, in addition to mRNA stabilization, we performed nuclear run-on experiments. As can be seen in Fig. 5, IL-2 transcription occurred only in Con A-activated D10 cells when CHX was added 3 hr later. On the other hand, IL-4 transcription solely depended on the activation of the cells with mitogen and was not influenced by CHX. These results clearly indicate that IL-2 transcription is regulated in Th-2 cells by a CHX-sensitive element. This conclusion is strengthened by our finding that chloramphenicol acetyltransferase expression driven by IL-2 5′ upstream sequences occurred in Th-2 cells only when the cells were activated with Con A, followed by CHX treatment 3 hr later (A.Z., E.M., and B.T.H., unpublished data). It should be mentioned that fusion between IL-2-producing and IL-4-producing cells leads to hybrids that secrete both IL-2 and IL-4 (27). These findings suggest that the element that represses IL-2 transcription in Th-2 cells is not codominantly expressed.

To examine whether a similar mechanism regulates IL-2 gene expression in other cell lineages that do not secrete IL-2 upon activation, we screened the RNA extracted from a B lymphoma, A20, and a macrophage cell line, P388D1, that had been stimulated with the mitogen lipopolysaccharide,

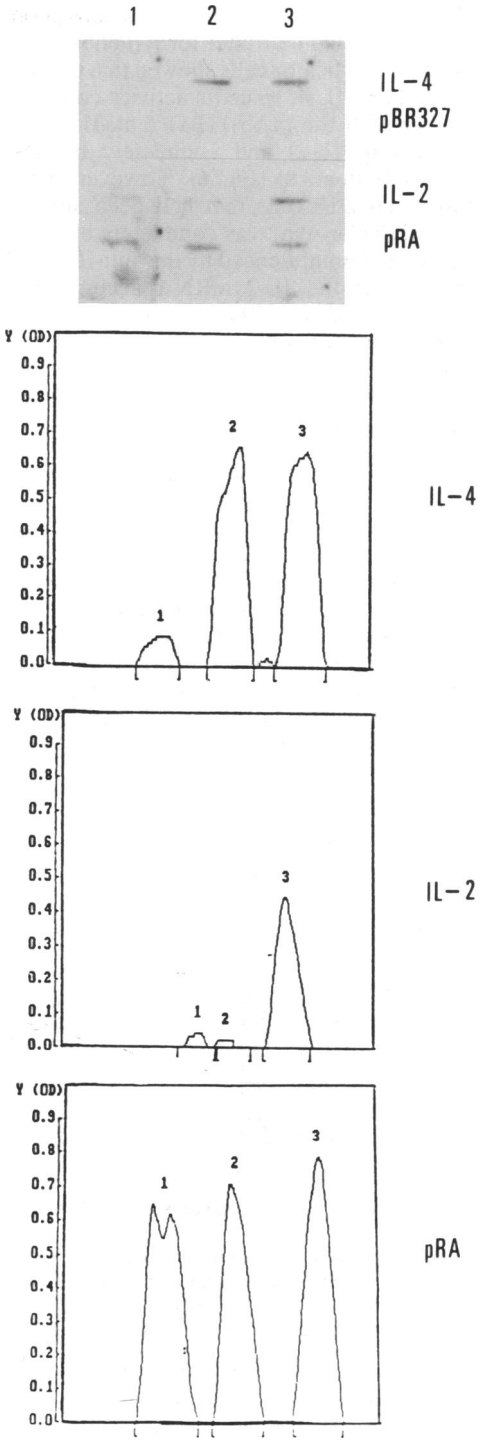

FIG. 5. Effect of CHX on IL-2 and IL-4 gene transcription in Con A-activated D10 cells. (*Upper*) Resting D10 cells (5×10^7 cells) were cultured in the absence (lane 1) or presence (lanes 2 and 3) of Con A for 3 hr. In lane 3, CHX was then added and the cells were cultured for an additional 3 hr. Newly transcribed RNA was hybridized to filters containing excess IL-4, pBR327, IL-2, and pRA (28S rRNA) DNA. (*Lower*) Quantitative measurements were carried out on a Bio-Rad model M-620 densitometer.

followed by CHX treatment 3 hr later. No IL-2 mRNA accumulated under these conditions (results not shown). This implies that activation of the IL-2 gene is specific for the T-cell lineage.

Finally, we tested whether the IL-2 mRNA in CHX-treated Con A-activated D10 cells is functional. Therefore, CHX was

removed from the cells 24 hr after initial activation, and the supernatant was tested 4 hr later for lymphokine content. A bioassay on HT-2 indicator cells showed that this supernatant contained IL-2 and IL-4, since its activity could be partially blocked either with the mAb 11B11 (anti-IL-4) or with the mAb S4B6.1 (anti-IL-2) and completely blocked with a combination of both mAbs (Fig. 6). However, the activity of supernatant from D10 cells that had been stimulated only with Con A (D10 ConAsn) was completely blocked with the anti-IL-4 mAb and uninfluenced by the anti-IL-2 mAb. These results prove that the IL-2 mRNA accumulated in Con A-activated Th-2 cells in the presence of CHX can be translated into functional protein.

In conclusion, the data presented here provide evidence that stimulation of Th-2 cells by Con A activates the positive regulatory elements that are necessary for IL-2 transcription. In addition, the data suggest that a negative regulatory element(s) represses IL-2 transcription. We hypothesize that the differentiation of precursor T cells into Th-2 cells is controlled by the development of a specific regulatory system for lymphokine gene expression and/or repression. The question remains whether this distinction is imposed before or after antigen contact. Hayakawa and Hardy (28) have elegantly demonstrated that IL-2-producing cells are the precursors of IL-4 secretors. In this regard, it is of interest that we were unable to detect IFN-γ mRNA under any experimental conditions tested in Th-2 cells. It is likely, therefore, that Th-2 cells are derived from precursors that do not produce IFN-γ. According to this model, the IL-2/IFN-γ-producing Th-1 cells would be derived from separate precursors. In support of this hypothesis we have found that the IL-4 gene is not transcribed in Con A-activated Th-1 cells in the presence of CHX (A.Z., E.M., and B.T.H., unpublished observation).

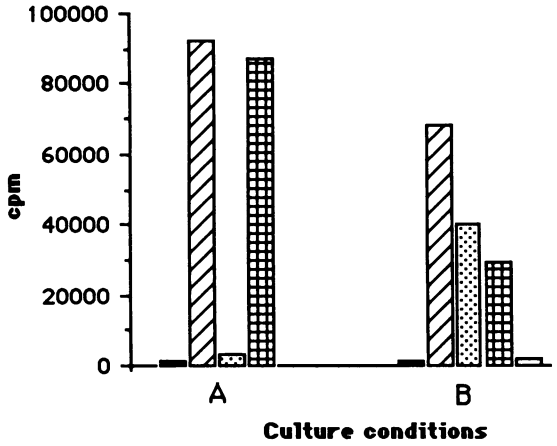

FIG. 6. Bioassay of supernatant obtained from D10 cells activated with Con A in the presence or absence of CHX. D10 cells (10^6 cells per ml) were activated as described in Fig. 2. CHX was removed 24 hr after initial mitogen activation by washing the D10 cells three times in complete serum-free RPMI 1640. The cells (2×10^6 cells per ml) were then cultured in complete medium for an additional 4 hr at 37°C. The supernatants were harvested and tested on HT-2 indicator cells for lymphokine content in the presence or absence of the mAbs anti-IL-4, anti-IL-2, or both. Bars: A, supernatant from Con A-activated D10 cells in the absence of CHX; B, supernatant from Con A-activated D10 cells in the presence of CHX. Culture conditions are shown by the various types of bars. Solid, medium; hatched, 50% (vol/vol) D10 ConAsn; stippled, D10 ConAsn + 11B11; cross-hatched, D10 ConAsn + S4B6.1; open, D10 ConAsn + 11B11 + S4B6.1.

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1. Schwartz, R. H. (1985) *Annu. Rev. Immunol.* **3,** 237–261.
2. Smith, A. (1984) *Annu. Rev. Immunol.* **2,** 319–333.
3. Snappe, C. M. & Paul, W. P. (1987) *Science* **236,** 944–947.
4. Mosmann, T. R., Cherwinsky, H., Bond, M. W., Gieldin, M. H. & Coffman, R. L. (1986) *J. Immunol.* **136,** 2348–2355.
5. Kaye, J., Porcelli, S., Tite, J., Jones, B. & Janeway, C. A. (1983) *J. Exp. Med.* **158,** 836–856.
6. Tony, H.-P. & Parker, D. C. (1985) *J. Exp. Med.* **161,** 223–245.
7. Gilman, M. (1988) in *Current Protocols in Molecular Biology*, eds. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (Greene and Wiley, New York), Vol. 1, pp. 412–416.
8. Church, G. M. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1991–1995.
9. Greenberg, M. E. & Ziff, E. B. (1984) *Nature (London)* **311,** 433–440.
10. Linial, M., Gunderson, N. & Groudine, M. (1985) *Science* **230,** 1126–1132.
11. Erickson, J. M., Rushford, C. L., Dorney, D. J., Wilson, G. N. & Schmickel, R. D. (1981) *Gene* **16,** 1–9.
12. Valge, V. E., Wong, J. G. P., Datlof, B. M., Sinskey, A. J. & Rao, A. (1988) *Cell* **55,** 101–112.
13. Kupper, T., Horowitz, M., Lee, F., Robb, R. & Flood, P. (1987) *J. Immunol.* **136,** 4280–4287.
14. Durand, D., Shaw, J. P., Bush, M. R., Replogle, R. E., Belagaje, R. & Crabtree, G. R. (1988) *Mol. Cell. Biol.* **8,** 1715–1724.
15. Shaw, G. & Kamen, R. (1986) *Cell* **46,** 659–667.
16. Shaw, J. P., Utz, P. J., Durand, D. B., Toole, J. J., Emmel, E. A. & Crabtree, G. R. (1988) *Science* **241,** 202–205.
17. Swoboda, R., Wecker, E. & Schimpl, A. (1987) *Immunobiology* **174,** 300–312.
18. Weiss, A., Shields, R., Newton, M., Manger, B. & Imboden, J. (1987) *J. Immunol.* **138,** 2169–2176.
19. Shaw, J. P., Meerovitch, K., Bleackley, R. C. & Paetkau, V. (1988) *J. Immunol.* **140,** 2243–2248.
20. Nabel, J. G., Gorka, C. & Baltimore, D. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2934–2938.
21. Efrat, S. & Kaempfer, R. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2601–2605.
22. Heckford, S. E., Gelmann, E. P., Agnor, C. L., Jacobson, S., Zinn, S. & Matis, L. A. (1986) *J. Immunol.* **137,** 3652–3663.
23. Sen, R. & Baltimore, D. (1986) *Cell* **47,** 921–928.
24. Hoyos, B., Ballard, D. W., Bohnlein, E., Siekevitz, M. & Greene W. C. (1989) *Science* **244,** 457–460.
25. Osborn, L., Kunkel, S. & Nabel, G. J. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2336–2340.
26. Lenardo, M. J., Fan, C. M., Maniatis, T. & Baltimore, D. (1989) *Cell* **57,** 287–294.
27. Hagiwara, H., Yokota, T., Luh, J., Lee, F., Arai, K.-I., Arai, N. & Zlotnik, A. (1988) *J. Immunol.* **140,** 1561–1565.
28. Hayakawa, K. & Hardy, R. R. (1989) *J. Exp. Med.* **169,** 2245–2250.