1986

Lymphokine and nonlymphokine mRNA levels in stimulated human T cells: Kinetics, mitogen requirements, and effects of cyclosporin a

Angela Granelli-Piperno

Linda Andrus

Ralph M. Steinman

LYMPHOKINE AND NONLYMPHOKINE mRNA LEVELS IN STIMULATED HUMAN T CELLS

Kinetics, Mitogen Requirements, and Effects of Cyclosporin A

BY A. GRANELLI-PIPERNO, L. ANDRUS, AND R. M. STEINMAN

The Rockefeller University and Irvington House Institute, New York 10021

The production of a number of polypeptide mediators or lymphokines underlies many of the differentiated functions of T lymphocytes including their capacity to influence B cells, macrophages, killer cells, as well as a variety of nonleukocytes. Lymphokine release begins when T cells are stimulated with antigen or mitogen. It quickly ceases when the stimulant is removed. A primary control is at the level of gene transcription. The stimulation of resting T cells, or the restimulation of isolated lymphoblasts and T cell lines, induces the formation of active lymphokine mRNA (1–5).

Evidence that lymphokine mRNA levels are subject to distinct controls has come from studies of the immunosuppressive drug, cyclosporin A (CsA).[1] This cyclic peptide blocks the production of several lymphokines but has minimal effects on the expression and function of the T cell's receptor for growth factor or IL-2 (IL-2-R) (1, 3). Several recent studies (1–4) indicate that the mechanism of action of CsA is to inhibit the synthesis of lymphokine mRNA that is initiated upon addition of mitogens. CsA blocks the production of IL-2 mRNA as assessed by nuclear transcription and northern blotting assays, as well as the mRNAs for several other lymphokines (IFN-γ, B cell–, and cytolytic T cell–stimulating factors) as shown with oocyte translation systems (1–4). Another control of lymphokine mRNA appears to be an endogenous protein, which in some way downregulates the level of IL-2 message (6, 7).

We have used DNA-RNA blotting to further analyze the control of lymphokine mRNA levels. The initial goal was to use a large panel of DNA probes to compare lymphokine and nonlymphokine gene expression in terms of kinetics, mitogen requirements, and sensitivity to CsA. The probes identified the mRNAs for the protooncogenes c-*fos*, c-*myb*, and c-*myc*, the IL-2-R, the lymphokines IL-2 and IFN, and the 70 kD heat shock protein (HSP). The results show that lymphokine and nonlymphokine mRNAs are induced by distinct exogenous stimuli. However, IL-2 and IFN mRNAs share with c-*myc* certain intracellular controls, including kinetics of mRNA accumulation and sensitivity to CsA.

This work was supported by grant CA 30198 from the National Institutes of Health, Bethesda, MD.

[1] *Abbreviations used in this paper:* CsA, cyclosporine A; CX, cycloheximide; HSP, heat shock protein.

Materials and Methods

Materials. We purchased leukocyte-rich buffy coats from the New York Blood Center; con A, (crystallized three times) from Miles Laboratories Inc., Elkart, IN; PMA and cycloheximide (CX) from Sigma Chemical Co., St. Louis, MO; PHA from Gibco, Grand Island, NY; OKT3 mAb from Ortho Diagnostics, Raritan, NJ. We were generously supplied with: CsA and CsH by Drs. J. F. Borel and B. Ryffel, Sandoz Pharmaceutical, Basel, Switzerland; purified rIL-2 (1.2×10^6 U/mg) by Dr. S. Rudnick, Biogen, Cambridge, MA; and several DNA probes. Dr. P. Barr (Chiron Corp., Emeryville, CA) provided oligonucleotide probes to IL-2 and IFN-γ. The IL-2 probe was complementary to the 5′ region of the IL-2 leader sequence 5′-ATG TAC AGG ATG CAA CTC CTG TCT TGC-3′, and the IFN probe complementary to the 3′-untranslated region of sequence 5′-CTG TAC CCA AAT GGA AAG TAA CTC ATT TGT TAA AAT TATC-3′. Alternatively, an IL-2 cDNA, described by Clark et al. (8) was used. cDNA probes for c-*fos* (9), c-*myc* (10), c-*myb* (11), and IL-2-R (12) were provided by Drs. H. Hanafusa, The Rockefeller University, K. B. Marcu, State University of New York, Stony Brook, NY, Dr. J. M. Bishop, University of California, San Francisco, CA, and Dr. W. Greene, National Institutes of Health, Bethesda, MD, respectively. The origin and specificity of the cDNAs for β-actin and β_2-microglobulin, have been described (13, 14), and a description of the 70 kD HSP (HSP-70) is in preparation (L. Andrus).

Cell Cultures. Blood mononuclear cells were obtained by applying 10 ml of buffy coat preparations to 10 ml Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). For studies of unprimed T cells, mononuclear populations were cultured at 5×10^6 cells/ml in complete medium consisting of RPMI-1640 (Gibco) supplemented with 2% heat-inactivated FCS (Sterile Systems, Logan, UT), 20 μg/ml gentamicin sulfate, 5×10^{-5} M 2-ME, and different mitogens: PMA at 10 ng/ml, and PHA at 1 μg/ml. For studies of lymphoblasts, we cultured cells at 10^6 cells/ml for 4–5 d in PHA (1 μg/ml). The cells were collected, washed, counted and plated at 2×10^6 cells/ml in RPMI supplemented with 2% serum to which different stimulants or inhibitors (CsA; CX) were added (see Results). To separate CD4 and CD8 subsets, stimulated nylon wool–nonadherent T cells were coated with OKT4 or OKT8 hybridoma culture supernatant (American Type Culture Collection, Rockville, MD) and centrifuged onto Petri dishes coated with anti–mouse Ig (Jackson Immunochemicals, Avondale, PA). The nonadherent cells were examined by indirect immunofluorescence and found to be depleted of the T cells that were reactive with the panning mAb, and to be highly (>95%) enriched in the reciprocal $T4^+$ or $T8^+$ subset. In most experiments, the mitogens and CsA were applied continuously, but in experiments in which lymphokine release was measured, these agents were applied as a 2-h pulse in serum-free medium. Most cells attached to the dishes in the absence of serum. The mitogens and CsA were removed by washing and the attached cells recultured in medium (above).

RNA Blot (Northern) Analysis. Total cellular RNA was isolated by a guanidine isothiocyanate method (15) with cesium chloride modification (16), and quantified by absorbance at 260 nm. Samples were denatured at 60°C for 10 min in electrophoresis buffer (20 mM morholinopropane sulfonic acid, 5 mM sodium acetate, 1 mM EDTA, 3% formaldehyde, pH 7.0) containing 50% formamide. RNA was then size-fractionated by electrophoresing through 1% agarose gel containing 6% formaldehyde, then transferred to gene screen filters (New England Nuclear, Boston, MA). Filters were dried, baked at 80°C in vacuo for 2 h, treated at 42°C for 16–20 h with prehybridization solution (50% formamide, Denhardt's solution [0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA], 1% SDS, 1 M NaCl, 50 mM Tris HCl, pH 7.4, 250 μg/ml denatured salmon sperm DNA, and hybridized at 42°C for 24 h in the same solution containing [^{32}P]cDNA radiolabeled by nick translation, or [^{32}P]IL-2 and [^{32}P]IFN oligonucleotides labeled with [^{32}P]ATP and polynucleotide kinase (17). Filters were washed twice in 2× NaCl/citrate (300 mM NaCl, 30 mM sodium citrate) for 5 min, twice in 2× NaCl/citrate with 1% SDS at 60°C, and twice at room temperature for 30 min. Filters were exposed 1–4 d at −70°C to Fuji film using intensifying screens.

Lymphokine Assays. IL-2 activity in conditioned medium was assayed on mouse lym-

phoblasts prepared by stimulating spleen cells 3–4 d with Con A (18). Dilutions of medium (50 μl) were cultured with 50 μl of lymphoblasts (4×10^5 cell/ml). After 18–20 h, the cultures were pulsed with 1.7 μCi [^{3}H]TdR for 4 h. The uptakes we report are from the linear portion of the dose-response curve. IFN was determined by RIA (Centocor, Malvern, PA).

In Vitro Nuclear Transcription Assay. In vitro transcription was performed according to the method of McKnight and Palmiter (19). Briefly, nuclei (10^8) were isolated and used immediately to prepare nascent RNA transcripts labeled with [^{32}P]UTP (3,000 Ci/mM; New England Nuclear) for 30 min at 36°C. The nascent RNA transcripts were purified by DNase I (RNase free; 50 μg/ml; Boehringer Mannheim, Houston, TX) treatment, proteinase K (25 μg/ml; Boehringer Mannheim) digestion, phenol extraction, and ethanol precipitation. The labeled nuclear RNA was then hybridized to excess specific cDNA (10 μg) immobilized on nitrocellulose filters. Autoradiographic exposures were 2–4 d.

Results

Stimulus Requirements for Optimal Release of Lymphokines. Lectin-primed blood mononuclear cells proved to be a useful source of T cells for studies of mRNA levels in stimulated T cells. We first confirmed that the requirements for lymphokine release from primed cells were similar to those previously reported for unprimed populations.

Human mononuclear cells were enriched in IL-2-responsive T lymphoblasts by culturing for 4–5 d in PHA. The cells were washed, restimulated for 16 h with a number of stimuli singly or in concert, and assayed for IFN and IL-2 release. Little IL-2 release occurred after challenge with PMA, PHA, or the OKT3 mAb, which reacts with the T3 component of the T cell receptor for antigen/MHC. Low levels of IFN were induced by PMA or PHA. However, the mixture of lectin with PMA (Table I) or OKT3 with PMA (data not shown), were synergistic and induced high levels of lymphokine release. A similar synergism has been reported in studies of lymphokine production by unprimed guinea pig and human T cells, and the human Jurkat T cell line (2, 20, 21). The immunosuppressive drug CsA (Table I), but not the inactive analog CsH (not shown), blocked the release of both IL-2 and IFN from T blasts.

IL-2 also induced low levels of IFN release from T blasts (Table I). This response could not be attributed to NK cells, as reported for unprimed populations (22, 23), since IFN production was not altered after treatment with anti-NK mAb (Leu-7 and Leu-11) and complement (not shown).

The effect of exogenous accessory cells was evaluated. Lectin-primed populations had few monocytes and dendritic cells, as assessed by cytology and by surface markers; i.e., there were <1% cells stained with the 3C10 antimonocyte mAb, and <0.1% Ia-rich dendritic cells (24). The primed T cells did not respond to lectin (Table I), but addition of plastic adherent cells at a ratio of 1:3 restored the lectin response (not shown). With this background information, we proceeded to analyze the regulation of lymphokine release at the level of specific mRNAs.

Kinetics of Induction of Several mRNAs During Optimal Stimulation of Lymphokine Release. The level of several mRNAs was monitored in primed and unprimed T cells that were stimulated with lectin and PMA (Figs. 1 and 2). Northern blotting showed that three mRNAs: actin, β_2-microglobulin, and c-*myb*, were present in the nonstimulated cells and were not increased by lectin/PMA. However, six other mRNAs were induced. c-*fos* mRNA peaked at 0.5 h and

TABLE I
Production of IL-2 and IFN-γ upon Restimulation of Human T Lymphoblasts

Stimulus	IL-2 (cpm [^{3}H]TdR)	IFN-γ (U/ml)
None	937	0
PHA (1 μg/ml)	985	26
PHA + CsA (1 μg/ml)	781	0
PMA (10 ng/ml)	2,428	31
PMA + CsA	857	4
PHA + PMA	122,889	144
PHA + PMA + CsA	1,307	3
rIL-2 (300 U/ml)	ND	28
IL-2 + CsA	ND	20

Conditioned medium (15%) was derived from lymphoblasts (10^6 cells/ml) that had been cultured for 2 h with different stimuli. After 2 h, all stimuli except IL-2 were removed, and the incubation was continued for 18 h. IL-2 activity was measured in a bioassay using Con A blasts as responders, while IFN-γ was measured in an RIA.

quickly disappeared; IL-2, IFN, and c-*myc* peaked at 3 h in T blasts and at 12 h in unprimed T cells; and IL-2-R and HSP-70 peaked at 20 h or later. In all experiments, HSP-70 mRNA was detected in unstimulated cells, but during mitogenesis, mRNA levels increased several fold. In comparing the responses of T blasts to unprimed T cells, we noted that: (*a*) the levels of mRNA per cell were about fivefold higher in the blasts, (*b*) the IL-2 and IFN responses were more rapid, and (*c*) unprimed T cells had baseline c-*fos* mRNA. We elected in the next experiments to use lymphoblasts, and we monitored c-*fos* mRNA levels at 30 min, and the other mRNAs at 4–6 h or at 20 h.

Stimuli for Lymphokine and Nonlymphokine mRNAs, and Effects of CsA. The highest levels of IL-2 and IFN mRNAs were induced when lectin and PMA were added in concert (Fig. 3), just like the release of these lymphokines (Table I). Lectin or PMA alone induced little or no response in either IL-2 or IFN mRNA. In contrast, the induction of other genes (c-*fos*, c-*myc*, IL-2-R) required only lectin or PMA, and no synergism was observed (Fig. 3).

The mitogenic OKT3 mAb had no independent effect on any of the mRNAs we studied, but was markedly synergistic with PMA in inducing IL-2 and IFN mRNAs (Fig. 4). c-*fos*, c-*myc*, and IL-2-R again responded to PMA alone, and there was no synergism with OKT3.

CsA is known (1–4) to inhibit IL-2 gene transcription, and indeed blocked the induction of both IL-2 and IFN mRNAs (Figs. 2–4). CsH was not inhibitory (Fig. 5). CsA also blocked, although not completely, the induction of c-*myc* (Figs. 2–4) as was also noted by Reed et al. (25). The addition of CsA for 1 h before stimulation with lectin or PMA did not lead to a complete block of c-*myc* induction. In contrast, CsA did not alter the mRNA levels of other nonlymphokine genes (c-*fos*, and HSP) in response to either lectin, PMA, OKT3, or combinations thereof (Figs. 2–4). CsA reduced IL-2-R mRNA levels little (Figs. 2 and 4) or not at all (Fig. 3).

The northern blotting results were pursued with nuclear transcription assays.

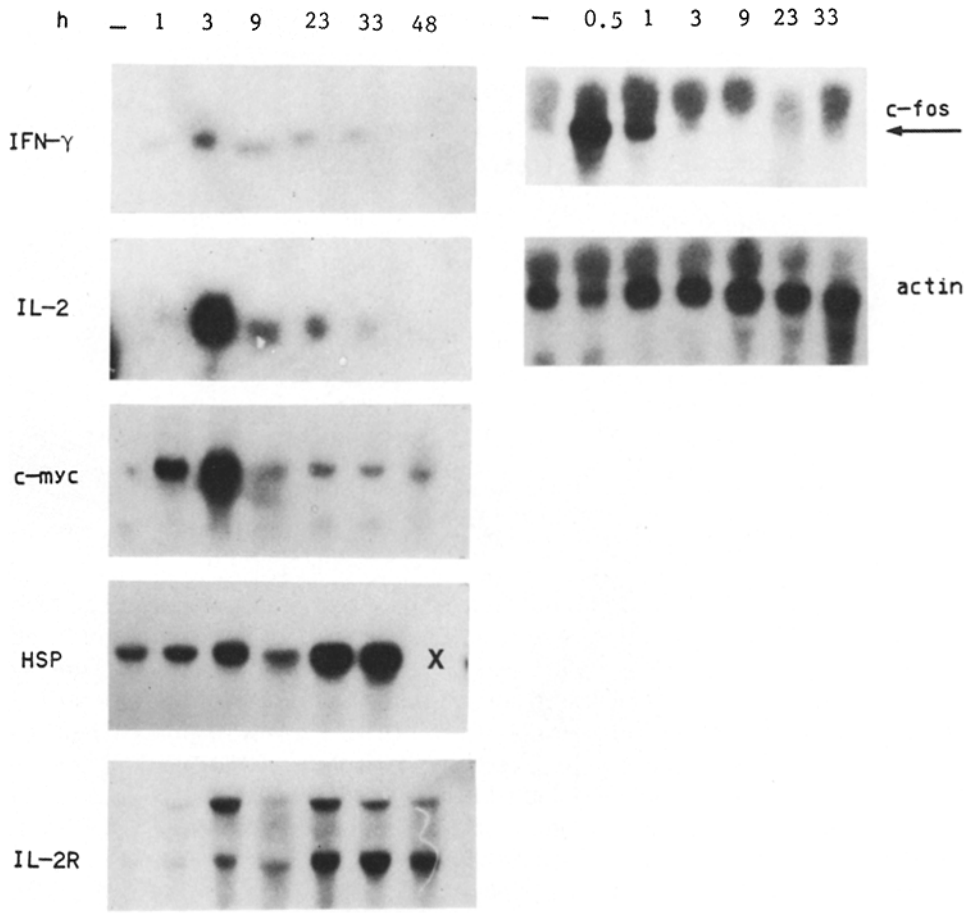

FIGURE 1. Time course of mRNA accumulation in T lymphoblasts. Human T blasts were restimulated with 1 μg/ml PHA and 10 ng/ml PMA for the indicated times (h). Total RNA was isolated, size fractionated (20 μg for probing with IL-2 and IFN, and 10 μg for the other genes), and hybridized to ^{32}P-labeled probes. Note that all mRNAs except actin are induced, although the kinetics for each varies. The c-*fos* panel has a background band, just above c-*fos* mRNA that was not seen in other blots (see other figures). ×, not tested.

As expected from the prior work of Kronke et al. (26), the induction of IL-2 mRNA by PMA and PHA was at the level of transcription, and was CsA sensitive (Fig. 6). Increased transcription of c-*myc* was also apparent and was blocked by CsA (Fig. 6). We conclude that the induction of lymphokine mRNAs differs from nonlymphokine mRNAs in the requirement for the concerted action of PMA and either lectin or OKT3, and in sensitivity to CsA, with the exception that c-*myc* is also CsA sensitive.

mRNA Levels in T4 and T8 Subsets. Highly enriched populations of $T4^+,T8^-$ and $T4^-,T8^+$ cells were prepared by panning, and were stimulated with PHA and PMA in the presence or absence of CsA. IL-2, IFN, and c-*myc* mRNAs were induced in both subsets, and the induction was CsA sensitive (Fig. 7).

Effects of IL-2. IL-2 was also evaluated as a stimulus and was found to induce c-*myc*, HSP and IL-2-R mRNAs (Fig. 8, *left*). Peak levels were reached in 5 h,

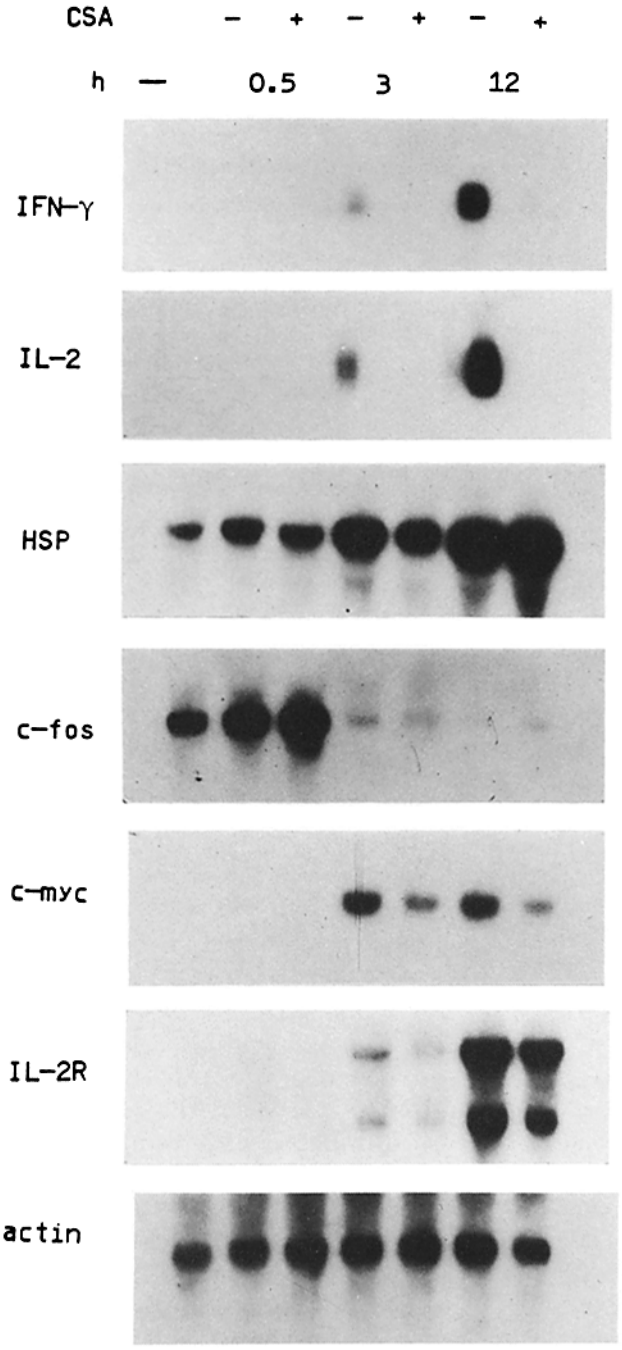

FIGURE 2. Time course of mRNA accumulation in freshly isolated blood mononuclear cells. The cells were stimulated with 1 μg/ml PHA and 10 ng/ml PMA in the presence or absence of CsA (1 μg/ml) for the indicated times (h). Total RNA was isolated, size fractionated (70 μg for probing with IL-2 and IFN, and 10 μg for the others) and hybridized to ^{32}P-labeled probes. Note that the kinetics of expression of lymphokine and c-*myc* genes are slower than those observed in T blasts (Fig. 1), but again are similar to one another and blocked by CsA.

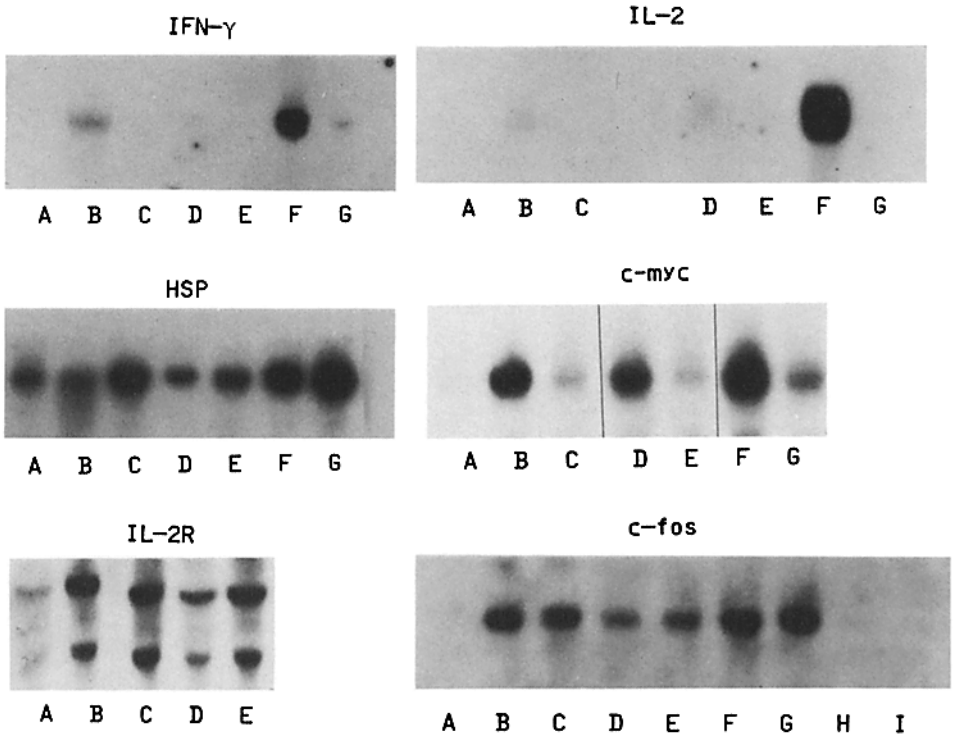

FIGURE 3. Expression of mRNAs for lymphokine and nonlymphokine genes after PHA and PMA stimulation; effects of CsA. T blasts were cultured in the presence of PHA (1 μg/ml), PMA (10 ng/ml), or IL-2 (300 U/ml), with or without CsA (1 μg/ml). After 30 min (c-*fos*) or 5 h (all other probes), total RNA was extracted, electrophoresed in 1% agarose gel (20 μg RNA for probing with IL-2 and IFN; 10 μg for the others), and hybridized with ^{32}P-labeled probes. Note that IL-2 and IFN mRNAs require the concerted action of PMA and PHA, whereas the other mRNAs respond to either stimulus; and CsA blocks the induction of lymphokine and c-*myc* mRNAs. *A*, control; *B*, PHA; *C*, PHA + CsA; *D*, PMA; *E*, PMA + CsA; *F*, PHA + PMA; *G*, PHA + PMA + CsA; *H*, IL-2; *I*, IL-2 + CsA.

and remained elevated for >24 h (not shown). IL-2 was a less effective inducer than PHA/PMA, but only a fraction of our mononuclear populations (30–50% of the cells) stained clearly with the anti-TAC mAb against IL-2-R (not shown). IL-2 induced a small IFN mRNA response, and this induction was not blocked by CX (Fig. 8, *right*). IL-2 failed to induce its own mRNA (Fig. 8, *right*). CsA did not inhibit any of the responses to IL-2 (Fig. 8). Therefore, IL-2 primarily stimulates nonlymphokine mRNAs in T cells, and all the responses are CsA insensitive, including that of c-*myc*.

Effects of CX. Prior studies had indicated that the protein synthesis inhibitor CX did not alter lymphokine mRNA levels if applied with the mitogen, but did block the downregulation of IL-2 mRNA that occurs at later time points (6, 7). We tested whether CsA interfered with the CX effect. CX did not alter mRNA levels when given during the first 4 h of mitogen restimulation (Fig. 8, *right*). If CX was applied at 4–8 h, both IL-2 and c-*myc* mRNAs were increased relative to control, or more precisely, the endogenous downregulation of these mRNAs did not occur (Fig. 9). HSP and IL-2-R mRNAs were not influenced by CX at

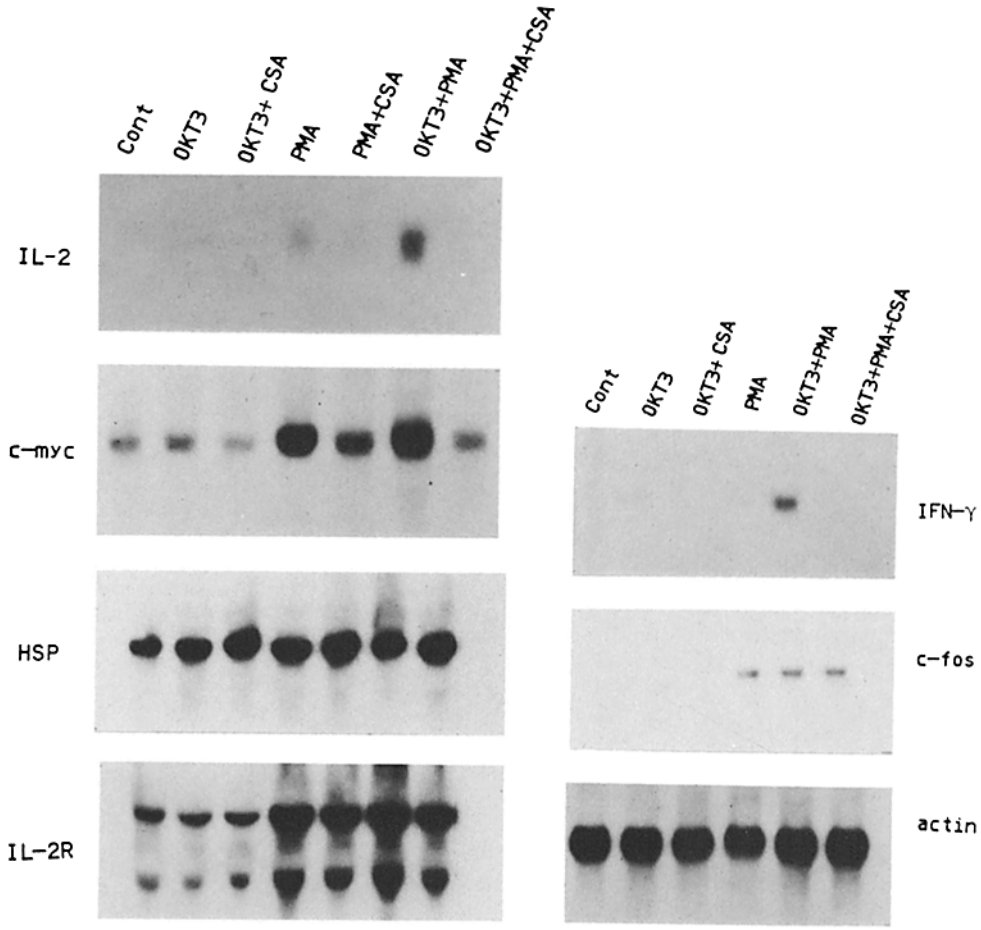

FIGURE 4. Increase in mRNAs after stimulation with OKT3 mAb and PMA; effects of CsA. Human T lymphoblasts were cultured in the presence of OKT3 (25 ng/ml), PMA (10 ng/ml), and CsA (1 μg/ml) added singly or in concert. After 30 min (c-*fos*) or 5 h (all others), total RNA was extracted (20 μg of RNA for probing with IL-2 and IFN; 10 μg for the others) and hybridized with ^{32}P-labeled probes. Note that OKT3 had no effect by itself on mRNA levels, and that CsA primary altered the induction of lymphokine and c-*myc* mRNAs.

this time (Fig. 9). If CsA was applied from 4–8 h, IL-2 and c-*myc* mRNAs were again markedly reduced, but when CX was also present, higher levels of mRNA were found (Fig. 9, right lane). Therefore, there appears to be a CX-sensitive step that develops several hours after T cell stimulation and leads to a lowering of IL-2 and c-*myc* mRNAs. CsA does not seem to inhibit this pathway for increased lymphokine mRNA.

Discussion

The availability of a large number of DNA probes has made it possible to monitor the polyclonal stimulation of T cells at the level of several specific mRNAs. The panel we used detected the mRNAs for two lymphokines, IL-2 and IFN, which are major differentiated products of T cells; three protoonco-

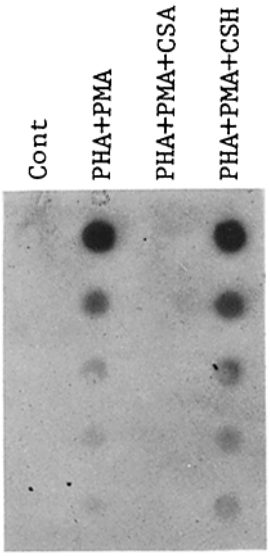

FIGURE 5. Lack of an effect of the cyclosporin analog CsH. Human T blasts were cultured in the presence of PHA (1 μg/ml) and PMA (10 ng/ml), with or without CsA or CsH (1 μg/ml) for 5 h. Serial dilutions of total RNA were applied to nitrocellulose using a BioRad dot blot apparatus and hybridized with ^{32}P-labeled IL-2 cDNA probe.

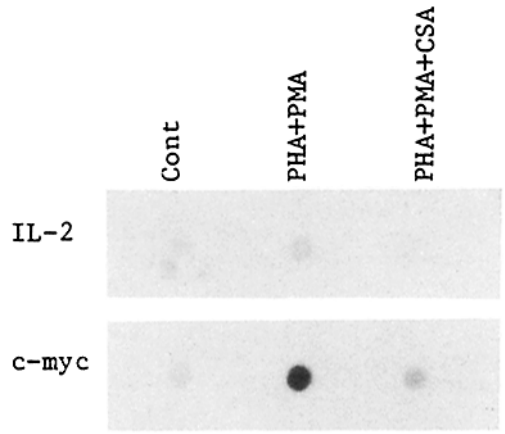

FIGURE 6. CsA inhibits c-*myc* and IL-2 gene transcription. T blasts were cultured in the presence of PHA (1 μg/ml) and PMA (10 ng/ml) with or without CsA (1 μg/ml) for 3 h. Nuclei were isolated and nascent RNA chains labeled with [^{32}P]UTP. Labeled RNA was hybridized with 10 μg of denatured c-*myc* and IL-2 cDNA immobilized on nitrocellulose. Autoradiograms were developed after 2–4 d.

genes, c-*myb*, c-*myc*, and c-*fos*, which encode proteins that localize to the nucleus after stimulation of many cell types (10, 32, 34); HSP-70, previously detected in cells infected with adenovirus or exposed to heat shock (35); and IL-2-R, which is expressed in T cells (26, 36) and other leukocytes. Three parameters were monitored:

Stimuli. The expression of lymphokine mRNAs required distinct exogenous stimuli. For IL-2 and IFN, optimal mRNA induction required the concerted

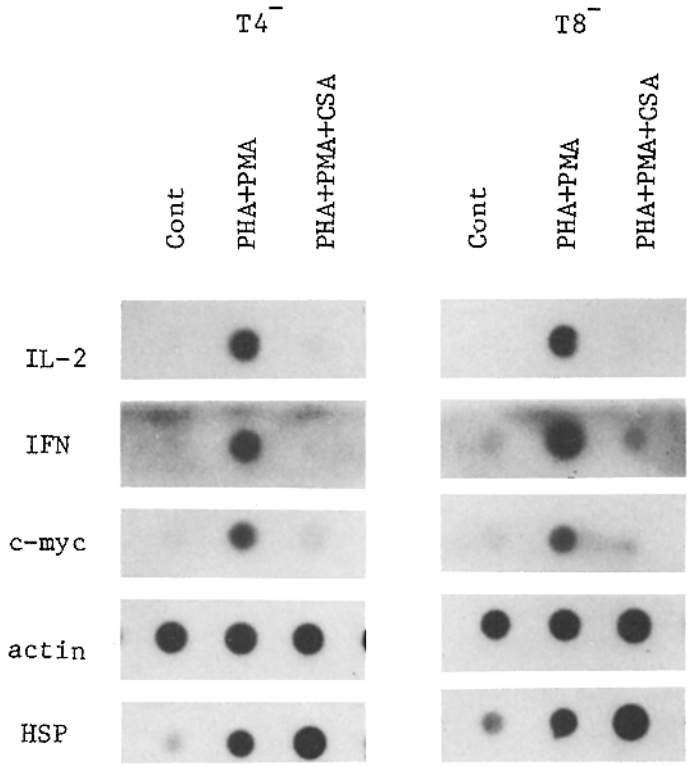

FIGURE 7. Induction of mRNAs in T cell subsets. Human T blasts (taken after 5 d of culture in lectin) were coated with supernatants from the OKT4 or OKT8 hybridoma lines. The cells were washed and spun onto Petri dishes coated with affinity-purified Fab_2 fragment of goat anti-mouse Ig. The nonbound cells were harvested and shown to be highly enriched in T4- or T8-bearing cells (>95% pure). The subsets were stimulated with PHA and PMA with or without CsA for 5 h. Cytoplasmic RNA was extracted with phenol/chloroform (17), and 10 μg was blotted to nitrocellulose. The filters were hybridized with ^{32}P-labeled probes and exposed for autoradiography for 3 d. Note that the T cell subsets show similar induction of IL-2, IFN, c-*myc*, and HSP, and similar sensitivity to CsA.

action of PMA and either lectin or OKT3 mAb (Figs. 3–4). This finding on primary T cells was similar to that reported for lymphokine mRNA in T cell lines (2), and for lymphokine secretion (20, 21). There was little and sometimes no increase in lymphokine mRNA when the T cells were triggered independently by lectin, PMA, OKT3, or IL-2. In contrast, several nonlymphokine mRNAs: IL-2-R, c-*myc*, and c-*fos*, were induced to high levels by either lectin, PMA, or IL-2. All these findings seemed to represent direct effects on T cells, since our populations had few accessory cells by the criteria of direct examination and an absence of lectin responsiveness (Table I). Furthermore, the responses were similar in T4 and T8 subsets (Fig. 7).

The requirement for both OKT3 and PMA during polyclonal activation must have a counterpart in the physiologic or antigenic stimulation of T cells. The T3 complex is associated with the T cell receptor for antigen and MHC (37), and binding of OKT3 mediates a calcium influx (38, 39). One could reason that this influx, and possibly antigen binding, do not independently lead to lymphokine

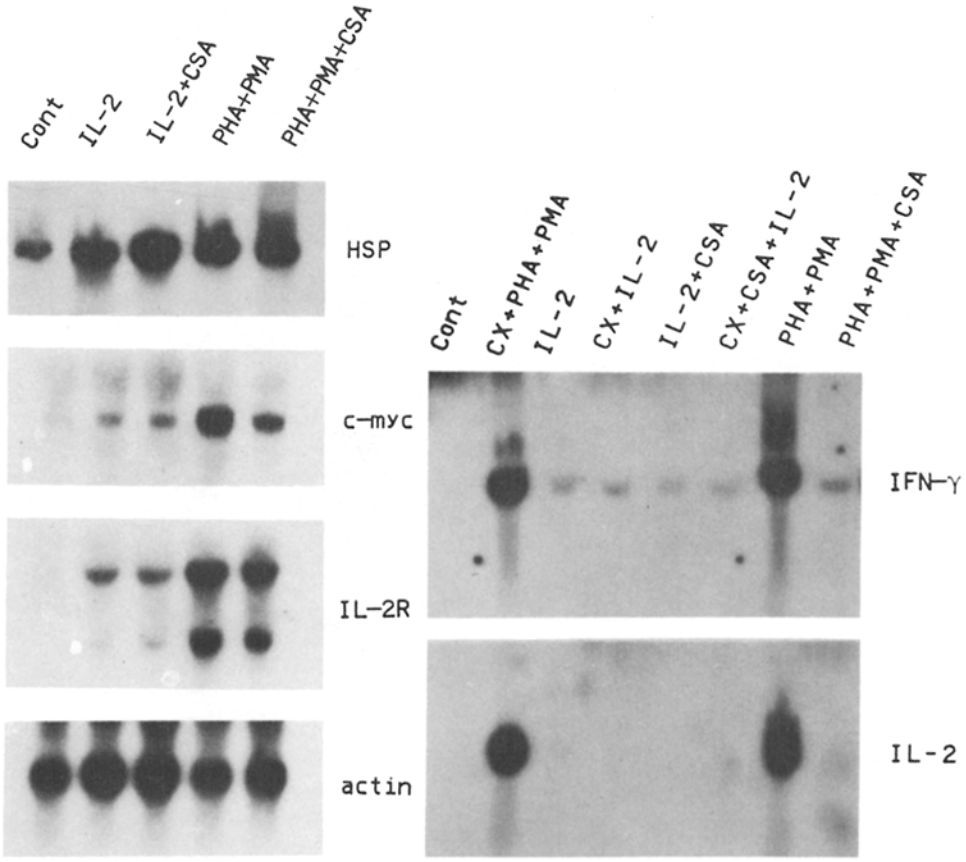

FIGURE 8. Effect of rIL-2 on the expression of inducible mRNAs. T blasts were cultured 6 h with 300 U/ml rIL-2 or with PHA (1 μg/ml) and PMA (10 ng/ml) in the presence or absence of 1 μg/ml CsA. CX was added at 5 μg/ml at the onset of some of the cultures (*right*). Note that IL-2 induces a significant, but CsA-insensitive increase in c-*myc*, HSP, and IL-2-R mRNAs (relative to PHA/PMA; *left*), whereas IL-2 has little (IFN) or no (IL-2) effect on lymphokine mRNAs (*right*).

gene expression or to the induction of several genes that are presumably required for cell division, i.e., c-*myc*, c-*fos*, and IL-2-R. These findings are reminiscent of the failure of antigen to independently trigger primary antigen-specific responses like the mixed leukocyte reaction and T-dependent antibody formation. Instead, primary stimulation requires that antigen be presented in association with dendritic cells, and the response is accompanied by the initiation of lymphokine production (40). We are therefore testing whether dendritic cells trigger lymphokine mRNAs in antigen-specific systems to a comparable extent to that seen with mitogens (OKT3 or PHA) plus PMA.

Kinetics. The application of PMA led to the induction of several genes, but the kinetics were very different. c-*fos* appeared early and briefly in stimulated T cells, as has been observed in other cell types (32, 33). c-*myc* was expressed shortly after c-*fos*, and IL-2-R mRNA appeared much later (Figs. 1 and 2). Whereas the onset of the PMA response likely involves the activation of protein kinase C, the

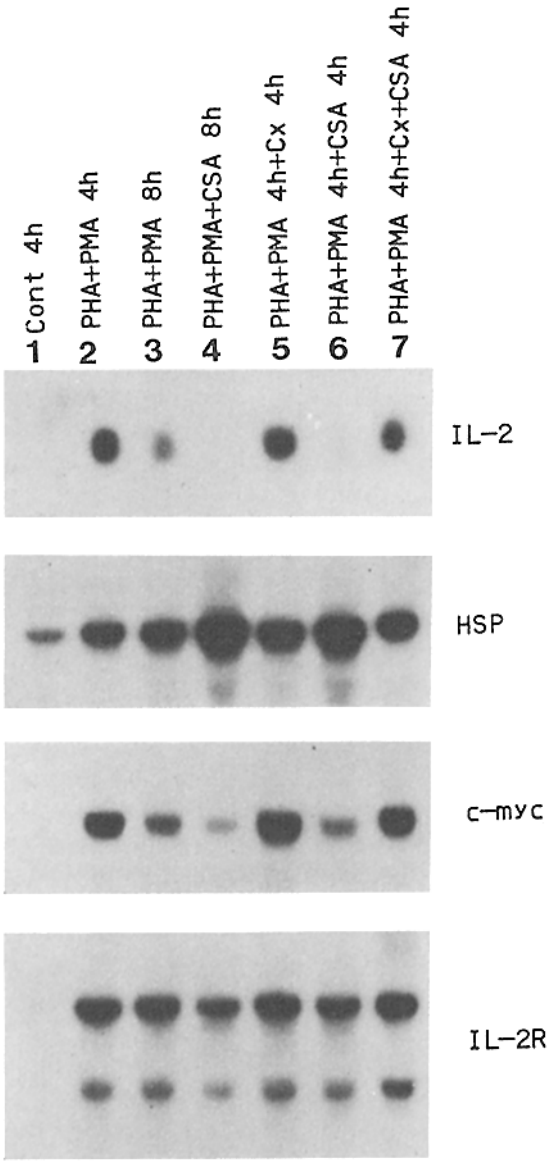

FIGURE 9. Effect of CX on the levels of IL-2 and nonlymphokine mRNA. T blasts were cultured with PHA (1 μg/ml) and PMA (10 ng/ml). CX (10 μg/ml) and/or CsA (1 μg/ml) were added at the indicated times. Cells were harvested after 4 or 8 h of culture. Total RNA was extracted, separated in agarose gel, and hybridized with ^{32}P-labeled probes. Note that *c-myc* and IL-2 mRNA levels fall between 4 and 8 h (lanes *2* and *3*). This decrease is blocked by CX even in the presence of CsA (lanes *5* and *7*).

subsequent steps leading to *c-fos*, *c-myc*, and IL-2-R expression must differ to explain such different kinetics. Expression of IL-2 and IFN mRNAs seems more rapid than expression of IL-2-R (Fig. 2). This suggests that T cells can begin producing their differentiated products before becoming fully competent for cell growth. Therefore, immunosuppressive regimes that are directed to the IL-

2-R, such as application of anti–IL-2-R antibodies, may not efficiently ablate T cell function, since lymphokines could still be formed.

CsA. The profound immunosuppressive effects of CsA on T cells are well known, but it is only recently that a selective effect on lymphokine mRNA has been stressed (1–4). An exception appears to be the c-*myc* gene, which is believed to encode a nuclear binding protein, and which can be induced in many cell types (41). We are testing whether CsA will block c-*myc* expression in non-T cells, since this could lead to nonspecificity in its clinical action.

The sensitivity of c-*myc* to CsA is most useful in analyzing the mechanism of action of this drug. It is difficult to argue that CsA blocks signalling at the cell surface, since it is apparent that CsA blocks the c-*myc* response to PMA or lectin, but not the response of other genes (c-*fos*, IL-2-R) to PMA or to lectin. CsA also does not block the IL-2-induced increase in c-*myc* mRNA, or the smaller increase in IFN mRNA. The fact that c-*myc* and lymphokine mRNAs also exhibited similar kinetics prompts our working hypothesis that CsA acts on a transcriptional control site that is shared by lymphokine and c-*myc* genes, either by binding directly to this region or by inhibiting the function of a control factor acting thereon.

Summary

Northern and dot blotting with a panel of DNA probes were used to monitor the levels of specific mRNAs in mitogen-stimulated human T cells. The induction of IL-2 and IFN mRNAs required the synergistic action of PMA and either PHA or OKT3 mAb. In contrast, several nonlymphokine genes, the protooncogenes c-*fos* and c-*myc*, and the IL-2-R gene, were induced by either PHA or PMA alone. PHA increased the background levels of a 70 kD heat shock protein mRNA, but did not affect the observed background of c-*myb* mRNA. For all mRNAs that were induced, isolated CD4 and CD8 T cell subsets behaved similarly. Exogenous IL-2 had little (IFN) or no (IL-2) effect on lymphokine mRNAs, but significantly increased c-*myc*, IL-2-R and heat shock protein mRNAs. Therefore, the stimuli for lymphokine mRNAs differed from those required for several inducible nonlymphokine genes.

IL-2 and IFN mRNAs exhibited some important similarities with c-*myc*, however. The levels of IL-2, IFN, and c-*myc* mRNA followed similar kinetics, peaking at 3 h in restimulated blasts and at 12 h in unstimulated T cells. The subsequent downregulation of lymphokine and c-*myc* mRNAs was retarded by cycloheximide. The induction of IL-2, IFN, and c-*myc* mRNAs was blocked by the immunosuppressive drug CsA, but not by the inactive analog CsH, and this block occurred at the level of nuclear transcription. Since the exogenous stimuli for lymphokine and c-*myc* gene expression differ, we suggest that intracellular controls must be shared to account for the similarities in their kinetics of expression and CsA sensitivity.

We thank Heidi Gemperle for her excellent technical assistance; Dr. P. Barr for the IL-2 and IFN-γ oligonucleotide probes; Drs. J. F. Borel, B. Ryffel, V. Quesniaux for the gift of CSA and CSH; Dr. D. Foster and H. Hanafusa for providing c-*fos* and c-*myb* cDNA;

Dr. W. Greene for IL-2-R cDNA; Dr. S. Rudnick for rIL-2, and Drs. Z. Cohn and E. Pure for reviewing the manuscript.

Received for publication 17 December 1985.

References

1. Granelli-Piperno, A., K. Inaba, and R. M. Steinman. 1984. Stimulation of lymphokine release from T lymphoblasts. Requirement for mRNA synthesis and inhibition by cyclosporin A. *J. Exp. Med.* 160:1792.
2. Wiskocil, R., A. Weiss, J. Imboden, R. Kamin-Lewis, and J. Stobo. 1985. Activation of a human T cell line: A two-stimulus requirement in the pretranslational events involved in the coordinate expression of interleukin 2 and γ-interferon genes. *J. Immunol.* 134:1599.
3. Kronke, M., W. J. Leonard, J. M. Depper, S. K. Arya, F. Wong-Staal, R. C. Gallo, T. A. Waldman, and W. C. Greene. 1984. Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription. *Proc. Natl. Acad. Sci. USA.* 81:5214.
4. Elliott, J. F., Y. Lin, S. B. Mizel, R. C. Bleackley, D. G. Harnish, and V. Paetkau. 1984. Induction of interleukin 2 messenger RNA inhibited by Cyclosporin A. *Science (Wash. DC).* 226:1439.
5. Hirano, T., K. Fujimoto, T. Teranishi, N. Nishino, K. Onoue, S. Maeda, and K. Shimada. 1984. Phorbol ester increases the level of interleukin 2 mRNA in mitogen-stimulated human lymphocytes. *J. Immunol.* 132:2165.
6. Efrat, S., and R. Kaempfer. 1984. Control of biologically active interleukin 2 messenger RNA formation in induced human lymphocytes. *Proc. Natl. Acad. Sci. USA.* 81:2601.
7. Efrat, S., and R. Kaempfer. 1985. Regulation of human interleukin-2 gene expression. *J. Leukocyte Biol.* 38:49*a (Abstr.).*
8. Clark, S. C., S. K. Arya, F. Wong-Staal, M. Matsumoto-Kobayashi, R. M. Kay, R. J. Kaufman, E. L. Brown, C. Shoemaker, T. Copeland, S. Oroszlan, K. Smith, M. G. Sarngadharan, S. G. Linder, and R. C. Gallo. 1984. Human T-cell growth factor: partial amino acid sequence, cDNA cloning, and organization and expression in normal and leukemic cells. *Proc. Natl. Acad. Sci. USA.* 81:2543.
9. Curran, T., G. Peters, C. Van Beveren, N. M. Teich, and I. M. Verma. 1982. FBJ Murine osteosarcoma virus: identification and molecular cloning of biologically active proviral DNA. *J. Virol.* 44:674.
10. Stanton, L. W., R. Watt, and K. B. Marcu. 1983. Translocation, breakage on truncated transcripts of c-*myc* oncogene in murine plasmacytomas. *Nature (Lond.).* 303:401.
11. Klempnauer, K. H., T. J. Gonda, and J. M. Bishop. 1982. Nucleotide sequence of the retroviral leukemia gene v-*myb* and its cellular progenitor c-*myb*: The architecture of a transduced oncogene. *Cell.* 31:453.
12. Leonard, W. J., J. M. Depper, G. R. Crabtree, S. Rudikoff, J. Pumphrey, R. J. Robb, M. Kronke, P. B. Svetlik, N. J. Peffer, T. A. Waldmann, and W. C. Greene. 1984. Molecular cloning and expression of cDNAs for the human interleukin 2 receptor. *Nature (Lond.)* 311:626.
13. Cleveland, D. W., M. A. Lopata, R. J. MacDonald, N. J. Cowan, W. J. Rutter, and M. W. Kirschner. 1980. Number and evolutionary conservation of α- and β-tubulin and cytoplasmic β- and γ-actin genes using specific cloned cDNA probes. *Cell.* 20:95.
14. Parnes, J. R., B. Velan, A. Felsenfeld, L. Ramanathan, U. Ferrini, E. Appella, and J. C. Seidman. 1981. Mouse β_2-microglobulin cDNA clones: a screening procedure for cDNA clones corresponding to rare mRNAs. *Proc. Natl. Acad. Sci. USA.* 78:2253.

15. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294.
16. Glisin, V., R. Crvenjakav, and C. Byus. 1974. Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry.* 13:2633.
17. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY
18. Granelli-Piperno, A., J. D. Vassalli, and E. Reich. 1981. Purification of murine T-cell growth factor. A lymphocyte mitogen with helper activity. *J. Exp. Med.* 154:422.
19. McKnight, G. S., and R. Palmiter. 1979. Transcriptional regulation of the ovalbumin and conalbumin genes by steroid hormones in chick oviduct. *J. Biol. Chem.* 254:9050.
20. Rosenstreich, D. L., and S. B. Mizel. 1973. Signal requirements for T lymphocyte activation. I. Replacement of macrophage function with phorbol myristic acetate. *J. Immunol.* 123:1749.
21. Hara, T., and S. M. Fu. 1985. Human T cell activation. I. Monocyte-independent activation and proliferation induced by anti-T3 monoclonal antibodies in the presence of tumor promotor 12-*o*-tetradecanoyl phorbol-13-acetate. *J. Exp. Med.* 161:641.
22. Trinchieri, G., M. Matsumoto-Kobayashi, S. C. Clark, J. Seehra, L. London, and B. Perussia. 1984. Response of resting human peripheral blood natural killer cells to interleukin 2. *J. Exp. Med.* 160:1147.
23. Handa, K., R. Suzuki, H. Matsui, Y. Shimizu, and K. Kumagai. 1983. Natural killer (NK) cells as responder to interleukin (IL-2) II. IL-2 induced interferon gamma production. *J. Immunol.* 130:988.
24. Van Voorhis, W. C., R. M. Steinman, L. S. Hair, J. Luban, M. D. Witmer, S. Koide, and Z. A. Cohn. 1983. Specific antimononuclear phagocyte monoclonal antibodies. *J. Exp. Med.* 158:126.
25. Reed, J. C., P. C. Nowell, and R. G. Hoover. 1985. Regulation of c-*myc* mRNA levels in normal human lymphocytes by modulators of cell proliferation. *Proc. Natl. Acad. Sci. USA.* 82:4221.
26. Kronke, M., W. J. Leonard, J. M. Depper, and W. C. Greene. 1985. Sequential expression of genes involved in human T lymphocyte growth and differentiation. *J. Exp. Med.* 161:1593.
27. Bernard, O., S. Cory, S. Gerondakis, E. Webb, and J. M. Adams. 1983. Sequence of the murine and human cellular c-*myc* oncogenes and two modes of *myc* transcription resulting from chromosome translocation in B lymphoid tumors. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:2375.
28. Holbrook, N. J., K. A. Smith, A. J. Fornace, Jr., C. M. Comeau, R. L. Wiskocil, and G. R. Crabtree. 1984. T-cell growth factor complete nucleotide sequence and organization of the gene in normal and malignant cells. *Proc. Natl. Acad. Sci. USA.* 81:1634.
29. Gray, P. W., and D. W. Goeddel. 1982. Structure of the immune interferon gene. *Nature (Lond.).* 298:859.
30. Fujita, T., C. Takoaka, H. Matsui, and T. Taniguchi. 1983. Structure of the human interleukin 2 gene. *Proc. Natl. Acad. Sci. USA.* 80:7437.
31. Mita, S., S. Maeda, K. Obaru, N. Nishino, K. Shimada, T. Hirano, K. Onoue, T. Ogawa, and H. Ogawa. 1983. Isolation and characterization of a human interleukin 2 gene. *Biochem. Biophys. Res. Commun.* 117:114.
32. Muller, R., R. Bravo, J. Burckhardt, and T. Curran. 1984. Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. *Nature (Lond.).* 312:716.
33. Greenberg, M. E., and E. B. Ziff. 1984. Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. *Nature (Lond.).* 311:433.
34. Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder. 1983. Cell-specific regulation of

the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. *Cell.* 35:603.

35. Kao, H. T., and J. R. Nevins. 1983. Transcriptional activation and subsequent control of the human heat shock gene during adenovirus infection. *Mol. Cell. Biol.* 3:2058.
36. Depper, J. M., W. J. Leonard, C. Drogule, M. Kronke, T. A. Waldmann, and W. C. Greene. 1985. Interleukin 2 (IL-2) augments transcription of the IL-2 receptor gene. *Proc. Natl. Acad. Sci. USA.* 82:4230.
37. Meuer, S., O. Acuto, R. E. Hussey, J. Hodgdon, K. A. Fitzgerald, S. F. Schlossman, and E. L. Reinherz. 1983. Evidence for the T3-associated 90 kD heterodimer as the T cell antigen receptor. *Nature (Lond.).* 303:808.
38. Weiss, A., J. Imboden, D. Shobach, and J. Stobo. 1984. Role of T3 surface molecules in human T-cell activation: T3-dependent activation results in an increase in cytoplasmic free calcium. *Proc. Natl. Acad. Sci. USA.* 81:4169.
39. Oettgen, H. C., C. Terhorst, L. C. Cantley, and P. M. Rosoff. 1985. Stimulation of the T3-T cell receptor complex induces a membrane-potential sensitive calcium influx. *Cell.* 40:583.
40. Inaba, K., and R. M. Steinman. 1984. Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen presenting cell) requirements for growth and lymphokine release. *J. Exp. Med.* 160:1717.
41. Ramsay, G., G. I. Evans, and J. M. Bishop. 1984. The protein encoded by the human proto-oncogene c-*myc*. *Proc. Natl. Acad. Sci. USA.* 81:7742.