MOLECULAR AND CELLULAR BIOLOGY, Jan. 1987, p. 342–348
0270-7306/87/010342-07$02.00/0

Vol. 7, No. 1

Regulation of Human T-Lymphocyte Gene Expression by Interleukin 2: Immediate-Response Genes Include the Proto-Oncogene c-*myb*

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Received 16 June 1986/Accepted 6 October 1986

Antigen-stimulated human T lymphocytes must bind the immunoregulatory hormone interleukin 2 (IL-2) if they are to transit from the G1 to the S phase of the cell cycle. Indirect methods, such as the measurement of thymidine uptake rates, were previously the only means available for exploring the mechanism of action of IL-2. Several cDNA clones have been isolated which are expressed subsequent to IL-2 binding, and the expression of two of these genes, Tact52 and Tact75, is regulated directly at the level of transcription; expression of the proto-oncogene c-*myb* is also regulated directly by IL-2 binding. These genes thus constitute a set which is coordinately regulated in the course of the transition from G1 to S phase of human T lymphocytes, and their expression depends on IL-2 binding.

The molecular mechanisms regulating cellular proliferation increasingly appear to be dependent on the binding of growth factors to cells (14). The consequences of growth factor binding include rapid changes in the expression of a small number of genes (13, 18), and the mechanisms regulating coordinate expression of these induced genes clearly are critical in the control of cellular proliferation. Particular attention has been paid to the role of proto-oncogene expression in response to growth factor stimulation (14, 18) because of the examples in which altered expression of these genes, or their homologs, has been correlated with unregulated cellular proliferation (2, 26).

The control of human T lymphocyte proliferation provides a unique model system for the analysis of growth regulation by extracellular factors. Quiescent T cells can be activated by antigenic or mitogenic stimulation and, in the presence of appropriate growth factors, will then proliferate in vitro or in vivo (4, 35). The entity of critical importance in this system is the glycopeptide hormone interleukin-2 (IL-2), which is required by stimulated T cells to effect transit from the G1 to S phase of the cell cycle (4, 20). The IL-2 requirement is absolute in the initial and in all subsequent rounds of T-cell division (4, 11, 23).

Our previous work (submitted for publication) represents the initial work on the isolation of genes whose expression is regulated directly by IL-2 binding. Two of those genes, Tact52 and Tact75, are now shown to be directly responsive to IL-2 binding; indeed, the growth factor induces both transcription and mRNA accumulation from these genes. To these two genes is now added the proto-oncogene c-*myb*, which is also regulated at the transcriptional level. This stands in contrast to the recent report that c-*myb* transcription is not regulated in avian thymocytes, even though cell cycle-dependent changes in c-*myb* expression in both the avian transformed T-lymphoid line MSB-1 and chicken embryo fibroblasts were observed (39).

The present findings attest that the mechanism of IL-2 control of T-cell entry into the S phase involves the trancriptional regulation of a small number of genes. We again focus attention on the regulation of a proto-oncogene as a critical element in the control of normal cell growth. This is an especially significant finding, as the factors which control T-cell proliferation in vitro are the very ones observed in vivo (5, 8, 11, 35, 41). Knowledge of the components of the T-cell response to IL-2 will newly define possible mechanisms for the generation of autoimmunity (24, 25) and lymphomas (10, 22, 38).

MATERIALS AND METHODS

cDNA clones and probes. The Tact50, -52, -53, and -75 clones were isolated from cDNA libraries constructed from RNA extracted from human tonsillar lymphocytes 32 h after phytohemagglutinin (PHA) stimulation. The construction and isolation have been described (submitted for publication). The IL-2 receptor cDNA clone was obtained from T. Honjo (28), and the transferrin receptor clone pTR36 was a gift of J. Miller (37). The c-*myc* clone was obtained from T. Rabbitts (30), and c-*myb* is the 1049 clone obtained from S. Tronick (9).

Radiolabeled probes for RNA blot hybridization analysis were prepared as follows. The human c-*myc* cDNA clone (30) contains a 1.0-kilobase (kb) insert composed of exons 2 and 3 of the c-*myc* gene. The fragment was released by *Pst*I digestion and recovered by pulse elution from a 1% agarose gel. Tact52 is a 1.25-kb cDNA clone in the vector pUC8 (21). A 1.0-kb *Taq*I fragment of the insert sequence was purified as described above and used as a probe. The Tact75 clone is a 0.65-kb cDNA insert also in the vector pUC8. The entire insert was released by *Eco*RI-*Bam*HI digestion and purified by pulse elution. The human c-*myb* plasmid contains a 2.6-kb *Eco*RI fragment of the genomic locus. Within this fragment are two exons, and the insert lacks repetitive DNA elements (9).

Lymphocyte culture. Peripheral blood lymphocyte (PBL) preparations, obtained by leukophoresis of normal donors, were purchased from HemaCare, Inc. (Van Nuys, Calif.). The lymphocytes were purified by Ficoll-Hypaque (Pharmacia) gradient separation and cultured at 4×10^6 cells per ml in RPMI 1640 medium with 5% heat-inactivated fetal bovine serum. PHA (Pharmacia) was added to a final concentration of 5 μg/ml.

Adherent cells were removed by two passages over nylon wool (40). Recombinant IL-2 (Cetus Corp.) was reconsti-

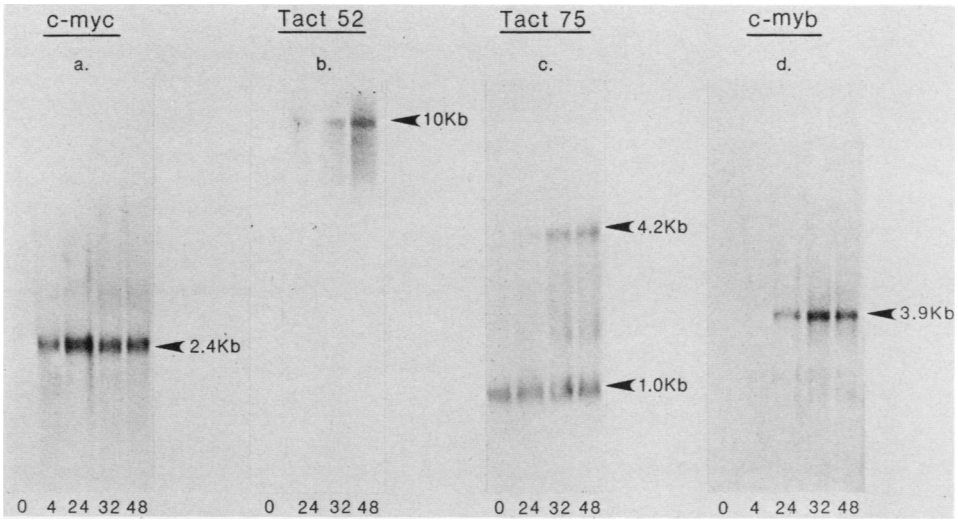

FIG. 1. Time course RNA blot analysis of the expression of the c-*myc*, Tact52, Tact75, and c-*myb* genes. The size of the RNA is marked in each case. Molecular weights were determined from the position of DNA markers run in adjacent lanes. At the bottom of each lane the time (in hours) after PHA addition that the sample was taken is noted. See text for details.

tuted in water and added to appropriate lymphocyte cultures at 150 U/ml.

RNA purification and blot analysis. Cytoplasmic RNA was prepared as described previously (7) with vanadyl ribonucleoside complex. The polyadenylated [poly(A)$^+$] RNA fraction was obtained by one cycle of chromatography on oligo(dT)-cellulose (1). Poly(A)-enriched RNA fractions were quantitated by optical density and then fractionated by electrophoresis on agarose-formaldehyde gels as described elsewhere (submitted for publication). The RNA was transferred to N-bond membrane (Amersham Corp.), except in one case in which the gel was dried under vacuum at 60°C.

Prehybridizaton solutions contained 50% formamide, 0.4 mg of denatured salmon sperm DNA per ml, and 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Hybridization solutions omitted the carrier DNA and contained 10% dextran sulfate. Probes were prepared by nick translation of double-stranded fragments (34) and were added to 5 to 10 ng/ml. The filters were washed twice for a total of 1 h in 2× SSC–0.2% sodium dodecyl sulfate at room temperature, followed by two washes for a total of 1 h in 0.5× SSC–0.1% sodium dodecyl sulfate at 65°C.

Nuclear transcript analysis. Lymphocyte nuclei were prepared by Dounce homogenization of cell suspensions in 0.25% Nonidet P-40. The nuclei were suspended in a buffer containing 20% glycerol and flash-frozen in liquid nitrogen. Nuclei preparation and in vitro transcription were performed by the method of Greenberg and Ziff (13). Plasmid DNA, 5 μg per slot, was denatured by 0.1 N NaOH treatment followed by heating to 90°C for 10 min. The solutions were neutralized and then filtered through a nitrocellulose membrane. Each filter strip was hybridized in 1 ml of solution containing 10^6 cpm of trichloroacetic acid-precipitable material purified from the nuclear transcription reactions. Hybridization and washing were done as described for RNA blot analysis.

RESULTS

Time course of gene expression in heterogeneous lymphocyte cultures. PBL are a functionally heterogeneous cell population. In normal individuals T cells make up 60 to 70% of the PBL, with B cells (approximately 20 to 25%) and monocytes accounting for the remainder of these cell populations. The T cells in a heterogeneous lymphocyte preparation proliferate in response to stimulation by PHA alone. Following the addition of PHA, the T cells increase rapidly in volume, with a concomitant increase in RNA synthesis (16, 27); these events occur rapidly, i.e., within hours. During the initial 24 h the total RNA content of lymphocytes increases approximately fourfold (data not shown). Approximately 48 h after PHA addition, an increase in DNA synthesis can be observed which leads to the initial round of cell division beginning 72 h after mitogenic stimulation. The monocyte population also responds to PHA stimulation and provides a source of interleukin 1, which then acts on antigen- (or mitogen-) stimulated T-helper cells to induce IL-2 secretion (11, 35, 36). In this respect monocytes play a key role in supporting the T-cell proliferation that occurs in response to PHA stimulation. Genes that are expressed after the production of endogenous IL-2, at approximately 22 to 24 h after PHA addition, are candidates for genes that are directly regulated by IL-2 binding. The isolation of Tact52 and Tact75 cDNA clones was sought because they demonstrate a pattern of expression consistent with what was proposed to represent IL-2-regulated genes. Whether expression of the c-*myb* gene was also regulated by IL-2 was examined because of its known role in lymphoid cell differentiation (12) and because expression of this gene is especially high in T-lymphoid tumors representing late blast cell transformation events (42).

The time course of lymphocyte poly(A)$^+$ RNA accumulation resulting from PHA stimulation of PBL cultures is shown in Fig. 1. The time course of accumulation of the 2.4-kb c-*myc* mRNA is shown in Fig. 1a. The c-*myc* gene was expressed at very high levels within 4 h after PHA addition to these cultures, and the level of this mRNA decreased only slightly over time. These data are consistent with the known responsiveness of c-*myc* expression to mitogenic stimulation (18) but are inconsistent with a gene whose expression is uniquely regulated by IL-2 binding.

The time course of Tact52 RNA accumulation was consistent with the pattern expected of a gene regulated by IL-2 binding (Fig. 1b). No specific RNA was detected in samples

before 24 h after PHA addition. Maximum levels of this RNA were observed in either 32- or 48-h samples. The Tact52 cDNA probe hybridized to a large (10-kb) RNA species. This RNA was not detectable by filter hybridization, after the RNA was transferred to the filter by blotting, even though its presence could be confirmed by RNA dot-blotting (not shown). For this reason, the hybridization experiment shown in Fig. 1b was performed by drying the agarose gel and incubating the dehydrated gel in the hybridization solution.

The Tact75 cDNA probe hybridized to two poly(A)$^+$ RNA species observed in lymphocytes. The larger RNA was 4.2 kb and was clearly induced during T-cell activation (Fig. 1c). The Tact75 RNA accumulated with a time course consistent with its being an IL-2-regulated gene. The 4.2-kb RNA was not present in time-zero or 4-h samples. The RNA was observed initially at 24 h and reached a maximum level by 32 h after PHA addition. The probe also hybridized to a 1.0-kb RNA which was present in both unstimulated (time zero) and stimulated lymphocyte samples. The hybridization samples were washed at high stringency (0.2× SSC, 65°C for 1 h), and hybridization to this 1.0-kb RNA was not observed with any other probes.

The time course of accumulation of the 3.9-kb c-*myb* mRNA was very similar to that observed for the 4.2-kb Tact75 RNA. There was no detectable c-*myb* RNA in the samples taken at time zero or at 4 h. The 3.9-kb c-*myb* RNA was detected initially in 24-h samples and was at its maximum level in 32-h samples.

Samples of the same lymphocyte culture used for the RNA analysis (Fig. 1) were also used for the preparation of nuclei. In vitro transcription with these nuclei preparations generated the radiolabeled RNA samples used as probes for the expression of a number of specific genes (Fig. 2). The pattern of hybridization indicated the time course of active transcription of these genes.

The Tact52, Tact75, and c-*myb* genes were expressed coordinately at approximately 32 h after mitogen addition. The presence of the 1.0-kb RNA species recognized by the Tact75 cDNA probe in zero-time samples is interesting because the gene was not being actively transcribed at that time.

Coordinate expression of the Tact52, Tact75, and c-*myb* genes was distinct from that of a second group of coordinately expressed genes. The second group included the transferrin receptor and Tact50 genes, both of which were actively transcribed some 12 h after the c-*myb*-containing group. Accumulation of the 5.0-kb transferrin receptor mRNA species was observed initially at 36 h after mitogen addition and reached a maximum level at 48 h (data not shown). This is in good agreement with the observed time course of transcription for the transferrin receptor gene and supports our previous observations that the increase in transferrin receptor in stimulated T cells was due to increased levels of translatable mRNA (29).

The data shown in Fig. 1 and 2 are from a single experiment. Time course analyses were repeated with samples from three different donors, and the results were similar to the results shown. The fact that Tact52, Tact75, and c-*myb* RNAs could not be detected earlier than 24 h after mitogen addition (with the exception of the 1.0-kb Tact75 RNA species) was quite consistent among the samples. Because of the limited material available for each time point from the lymphocyte culture, it was not possible to determine the precise temporal relationship between the Tact52-Tact75-c-*myb* group of genes and the transferrin receptor-Tact50 group. When the results from four independent experiments were analyzed (by both RNA blotting and in vitro transcription), it was possible to estimate that the time lag separating expression of these two groups of genes was on the order of 6 to 10 h. Alone, these data are insufficient to make precise conclusions about the time course of expression because of the variability between samples from individual donors. Analysis of independent samples allowed generalizations to be made about the time course of T-cell activation events yet did not obscure the potentially important fact that individual responses were unique. For example, the time course of the β-actin gene was not consistent between donors. In one case, actin gene transcription was observed at all time points during the experiment; in two cases, the pattern appeared as shown in Fig. 2.

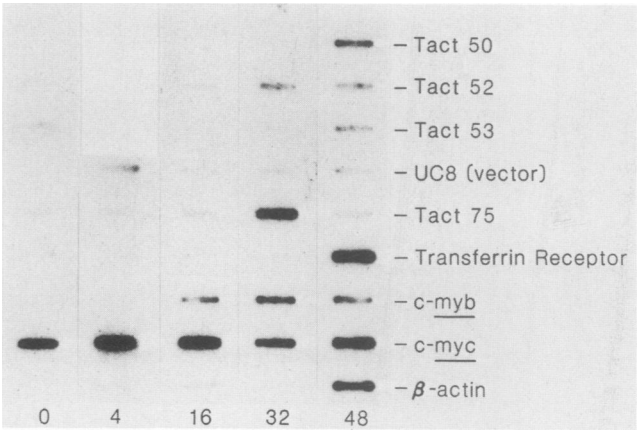

FIG. 2. Time course analysis of nuclear transcription. Time points from the same lymphocyte preparations used in Fig. 1 were used to prepare nuclei and perform in vitro nuclear transcription. The various genes which were analyzed are listed at the right. The UC8 (vector) lanes contained 5 μg of pUC8 DNA (21) as a control for nonspecific hybridization of the probe.

Regulation of transcription by exogenous IL-2. PBL preparations were exhaustively depleted of adherent cells by passage over nylon-wool columns. Such depletion removes most of the endogenous source of interleukin 1 (36). In the absence of this lymphokine, there is essentially no synthesis or secretion of endogenous IL-2 in the stimulated lymphocyte culture. The mitogen-stimulated T cells in the adherent cell-depleted preparation undergo the increase in cell volume characteristic of blast transformation, but no DNA synthesis or cell division occurs in the absence of an exogenous source of IL-2 (4, 20). Figure 3 demonstrates the effect on DNA synthesis of varying the dose of IL-2 in adherent cell-depleted cultures which had been stimulated by PHA. Clearly, exogenous IL-2 was essential for initiating DNA synthesis (as measured by thymidine uptake); the concentration of 100 to 250 U of IL-2 (derived by expression of a recombinant IL-2 gene in *Escherichia coli*) per ml suffices for maximal response (4).

An experimental pattern was developed which demonstrated the effects of IL-2 administration on gene expression. The experimental protocol required that adherent cell-depleted lymphocyte preparations be stimulated with PHA for 24 to 32 h. During this time the T cells underwent morphological transformation and expressed surface receptors for IL-2 (15, 20). The cells were then washed and suspended in fresh medium (without PHA) for 6 h. IL-2 (150 U/ml) was then added, and nuclei were prepared from

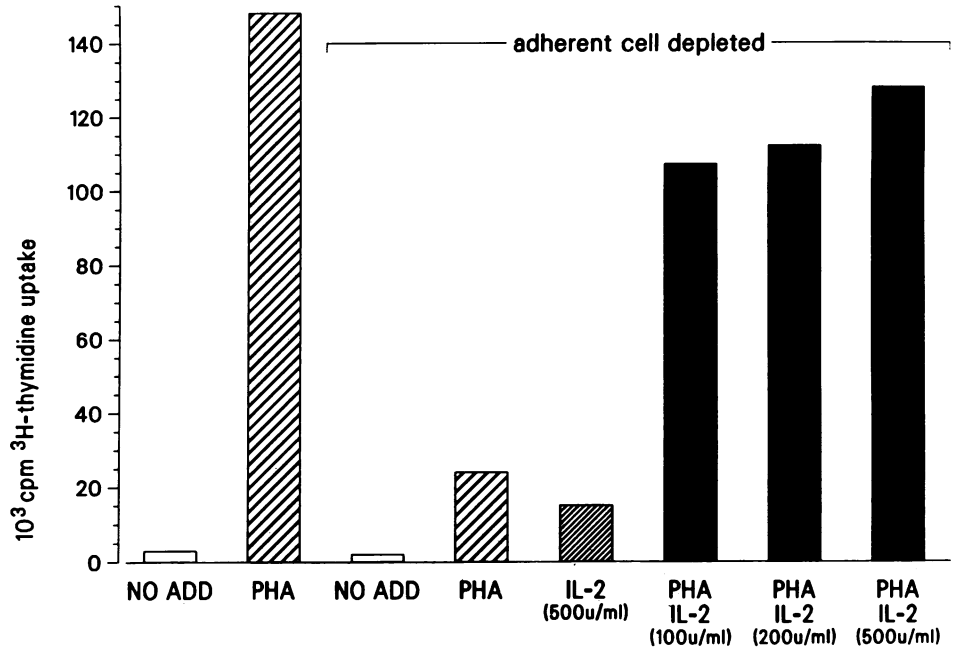

FIG. 3. Thymidine uptake in heterogenous and adherent cell-depleted lymphocyte cultures. In each case, cells were cultured at 4×10^6/ml in 96-well microtiter dishes, with 200 μl of sample per well. Each point represents triplicate determinations. The effects of adding PHA to either heterogeneous (bar 2) or adherent cell-depleted (bar 4) cultures are shown. Adherent cell depletion reduced the rate of thymidine uptake, determined in a 12-h pulse with 1 μCi of [^{3}H]thymidine per well between 40 and 52 h, approximately sevenfold. Although IL-2 alone could not restore thymidine uptake, the addition of both PHA and IL-2 returned the cultures to near normal levels of thymidine uptake. There was little difference between adding 100 and 500 U of IL-2 per ml. [^{3}H]thymidine uptake is shown as 10^3 cpm per 12 h per 10^5 cells.

samples of the cells taken at various intervals after IL-2 addition. Control cultures were stimulated with an equal volume of excipient control solution, also provided by Cetus Corp., which contains the same buffers and stabilizers as the IL-2 preparation but lacks IL-2.

The nuclei preparations were used for in vitro transcription assays to ascertain the effect of exogenous IL-2 on the expression of genes from either the c-*myb* or transferrin receptor group. Typical data from one such assay are shown in Fig. 4. A low level of gene expression was observed in the time-zero sample; this was probably due to trace amounts of IL-2, contributed indirectly from contaminating adherent cells. Note that in Fig. 3 the level of thymidine uptake was not zero in any sample, indicating that traces of residual IL-2 must have been present in the cultures. There was no detectable hybridization of nuclear transcripts to plasmid vector sequences in any of the experiments.

Autoradiograms from four independent IL-2 stimulation experiments were analyzed densitometrically. The intensities were normalized to the IL-2 receptor transcription levels observed at 2 h after IL-2 addition, because the IL-2 receptor gene was found consistently to have the highest level of expression. Normalized values are displayed diagramatically in Fig. 5.

The c-*myb*, Tact52, and Tact75 genes were expressed within 30 min after IL-2 stimulation. The maximum level of expression of these genes was observed at 1 to 2 h following lymphokine addition; these genes are categorized as immediate-response genes. Expression of the late genes, including transferrin receptor and Tact50, occurred 6 to 10 h subsequent to IL-2 addition and was thus separate and distinct from the time course of expression for the immediate-response genes. Addition of exogenous IL-2 recreated the

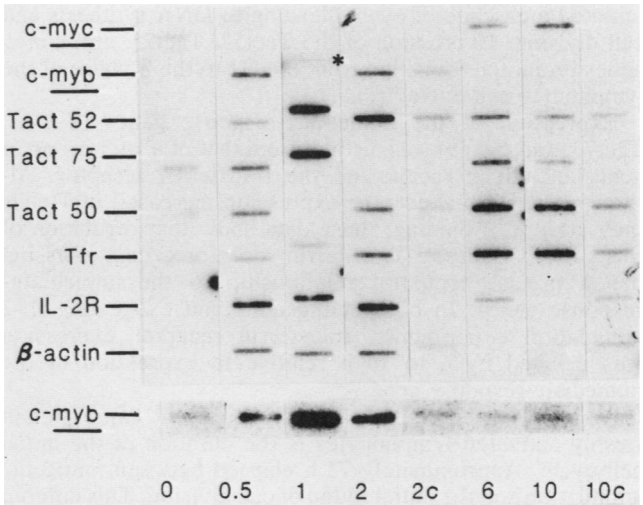

FIG. 4. Effects of exogenous IL-2 on gene transcription rates. Adherent cell-depleted cultures were stimulated for 32 h with PHA. The cells were washed and suspended in fresh medium for 4 h, and then 150 U of IL-2 per ml was added and nuclei were prepared from samples taken at various times as indicated (hours postaddition). The control samples (lanes 2c and 10c) received buffer solutions without IL-2. Nuclear transcription and hybridization are described in the text. The genes used in this analysis are marked on the left: TfR, transferrin receptor; IL-2R, IL2 receptor. The first eight lines (c-*myc* through β-actin) are from one experiment, and the last line (c-*myb*) is from a separate experiment. The asterisk indicates that no plasmid DNA was bound to the filter at this point, and this accounts for the apparent anomaly in the pattern of c-*myb* transcription.

temporal relationships between these two sets of genes that were observed in experiments involving heterogeneous lymphocyte cultures (Fig. 2). Irrespective of whether the source of IL-2 was endogenous (in the heterogeneous cultures) or exogenous (in adherent cell-depleted cultures), the relative time course of gene expression proved to be the same.

A sharp increase in the transcription rate of the IL-2 receptor gene after IL-2 addition was observed consistently. This is similar to results which have been obtained with other systems (6, 17, 31). The effect of IL-2 on the receptor gene is indicative of positive feedback regulation during T-cell activation, but, because the IL-2 receptor gene must be expressed prior to IL-2 binding, does not constitute an example of a gene uniquely regulated by lymphokine binding. Indeed, the IL-2 receptor gene is initially regulated by signals generated several hours before IL-2 secretion is initiated (6; C. D. Pauza and R. C. Holmes, unpublished data). A sharp increase in c-*myc* gene expression after IL-2 addition was not observed, even though a positive effect of IL-2 addition on c-*myc* gene expression was reported previously with 10- to 12-day PHA-treated blasts (32) and T-cell clones (33). The experiments reported here thus deal with an analysis of T-cell response during the initial exposure to IL-2. Should IL-2 exert a more general effect on subsequent rounds of cell division, this may well reflect the responsiveness of cycling cells, as opposed to freshly stimulated T cells.

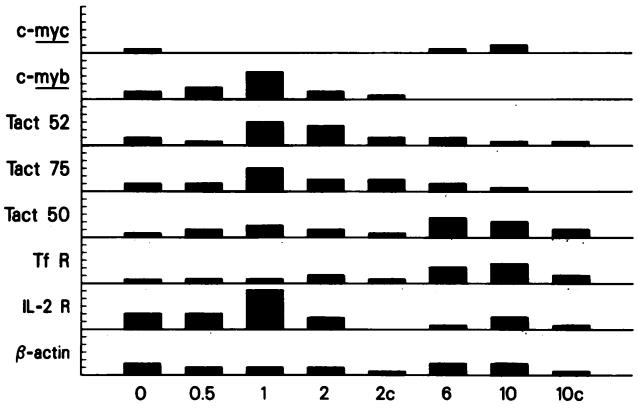

FIG. 5. Normalized average values for the effect of exogenous IL-2 on gene transcription. Four independent experiments with lymphocyte samples from three donors were analyzed densitometrically. The values were normalized to the intensity of IL-2 receptor gene transcription at 1 h after IL-2 addition. The bar heights represent the relative transcription rate of each gene at each time after IL-2 addition (in hours, shown below the bars). The control samples (2c and 10c) received buffer solutions without IL-2. The genes used are shown at the left; TfR and IL-2, transferrin receptor and IL-2 receptor, respectively. The anomalous 1-h time point for c-*myb* expression (Fig. 4) was not included in the analysis.

DISCUSSION

Transcriptional regulation of the Tact52, Tact75, and c-*myb* genes is an essential component of the molecular mechanisms which regulate T-cell proliferation. The addition of IL-2 to stimulated lymphocyte cultures caused an immediate increase in the expression of these genes and initiated a cascade of events leading to DNA synthesis and cell division. Expression of the Tact52, Tact75, and c-*myb* genes promoted transition from the G1 to the S phase of the lymphocyte cell cycle.

Expression of the immediate-response genes (Tact52, Tact75, and c-*myb*) was distinct from that of a later group of genes including Tact50 and the transferrin receptor. Although transferrin receptor expression increased in T-cells only after IL-2 binding, these data show that regulation of this gene is indirect. Transferrin receptor gene expression has a precise temporal relationship to the immediate-response genes. In both endogenous and exogenous IL-2 stimulation experiments, transferrin receptor expression was delayed by 6 to 10 h relative to expression of the immediate-response genes.

An important feature of the analysis of gene expression in freshly activated lymphocytes is the duration of the initial cell cycle. Approximately 72 h elapsed between mitogenic stimulation and the initial round of cell division. This differed markedly from the succeeding rounds of cell division, in which the entire cell cycle can be less than 20 h (11). Transcription of the c-*myc* gene was reported to be positively regulated by IL-2 binding when rapidly dividing cells from a murine T-cell clone (33) or cells derived by 10 to 12 days of PHA stimulation in vitro (32) were analyzed. In the experiments reported here, no positive effect of IL-2 binding on c-*myc* expression was observed. The c-*myc* gene was transcribed throughout the initial G1 to S transition (Fig. 2) but was transcriptionally inactive in adherent cell-depleted populations (Fig. 4). This would suggest that c-*myc* expression, in the initial T-lymphocyte cell cycle, is regulated by an adherent cell-derived factor. This factor would most likely be interleukin 1. In subsequent rounds of cell division, c-*myc* probably comes under the general control of IL-2, which in combination with antigen is sufficient to sustain lymphocyte proliferation (4, 11).

Positive regulation of IL-2 receptor gene transcription occurred in both freshly stimulated (Fig. 4) and rapidly dividing T cells (6, 31). The biological requirement for this feedback regulation is not clear. It may be a cellular adaptation to counteract the short half-life of IL-2 in serum (19), since it is known that the proliferative response to IL-2 depends on both the concentration of the hormone and the number of receptor molecules per cell (4).

It has been reported that T cells require a minimum of 3 h of exposure to IL-2 to initiate the proliferative response (4). However, a small but significant increase in specific gene expression was demonstrable within 30 min after IL-2 addition, which reached high transcriptional rates within 2 h. These findings appear to be contradictory, but we are mindful that prior exposure to very low levels of IL-2 cannot be ruled out; this may have occurred indirectly via contaminating monocytes and may have facilitated the responses observed here. It is also conceivable that sustained gene transcription, resulting from prolonged exposure to IL-2, is required to accumulate a threshold level of immediate-response gene products; the threshold level would then be the signal required to initiate the cascade of gene expression events.

Knowledge of the mechanisms of IL-2 action on T cells is especially important to understanding the normal regulation of immune responses and its aberrations. IL-2 serves as an important regulatory molecule which renders cytotoxic T-cell proliferation dependent on the simultaneous stimulation of T-helper cells. Should this regulatory mechanism be abrogated, either by a continuous source of IL-2 or through a mutation which causes constitutive expression of IL-2-dependent genes, then the potential emerges for development of lymphoproliferative disorders. The human lympho-

proliferative disease angioimmunoblastic lymphadenopathy may be an example of the latter case, as a high constitutive level of c-*myb* expression is evident in lymphocytes from patients with this disease (24, 25) even though the capacity to produce IL-2 in vitro is much lower than normal (17).

ACKNOWLEDGMENTS

I thank Maria Salvato and Pamela Mellon for careful reading of the manuscript and Maurice Landy for his valuable editorial advice. Thanks also to Mel Cohn, Rod Langman, Ed Lennox, and Sydney Brenner for encouragement and advice and to Meg Swingle for help in preparing the manuscript.

Supported by Public Health Service grant AI-05875 and Cancer Training grant CA-09254 from the National Institutes of Health.

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