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Recombinant Interleukin 2 Regulates Levels of c-*myc* mRNA in a Cloned Murine T Lymphocyte

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The cellular oncogene c-*myc* has been implicated in the regulation of growth of normal and neoplastic cells. Recently, it was suggested that c-*myc* gene expression may control the $G_0 \rightarrow G_1$-phase transition in normal lymphocytes that were stimulated to enter the cell cycle by the lectin concanavalin A (ConA). Here we describe the effects of purified recombinant interleukin 2 (rIL2) and of ConA on levels of c-*myc* mRNA in the noncytolytic murine T-cell clone L2. In contrast to resting (G_0) primary cultures of lymphocytes, quiescent L2 cells have a higher RNA content than resting splenocytes and express receptors for interleukin 2 (IL2). Resting L2 cells are therefore best regarded as early G_1-phase cells. Purified rIL2 was found to stimulate the rapid accumulation of c-*myc* mRNA in L2 cells. Levels of c-*myc* mRNA became maximal within 1 h and declined gradually thereafter. In contrast, ConA induced slower accumulation of c-*myc* mRNA in L2 cells, with increased levels of c-*myc* mRNA becoming detectable 4 to 8 h after stimulation. Experiments with the protein synthesis inhibitor cycloheximide demonstrated that the increase in levels of c-*myc* mRNA that were induced by ConA was a direct effect of this lectin and not secondary to IL2 production. Cyclosporin A, an immunosuppressive agent, markedly reduced the accumulation of c-*myc* mRNA that was induced by ConA but only slightly diminished the accumulation of c-*myc* mRNA that was induced by rIL2. Taken together, these data provide evidence that (i) c-*myc* gene expression can be regulated by at least two distinct pathways in T lymphocytes, only one of which is sensitive to cyclosporin A, and (ii) the accumulation of c-*myc* mRNA can be induced in T cells by IL2 during the G_1 phase of the cell cycle.

Expression of the cellular oncogene c-*myc* has been implicated in the regulation of growth of normal and neoplastic cells (2, 7, 10, 19, 41, 47). Unlike malignant cells or continuously dividing cells that maintain high levels of c-*myc* mRNA, expression of the c-*myc* gene in normal cells is transient and correlates with entry of quiescent cells into the cell cycle (3, 24). For example, stimulation of T lymphocytes with concanavalin A (ConA) of B lymphocytes with lipopolysaccharide, or of fibroblasts with platelet-derived growth factor results in rapid accumulation of c-*myc* mRNA (within 1 to 2 h) to levels 10 to 40 times above background. Levels of c-*myc* mRNA remain elevated for 24 to 40 h, then gradually decline as cells begin to synthesize DNA. This rapid expression of the c-*myc* proto-oncogene in lymphocytes and fibroblasts suggests that c-*myc* may be required for the initiation of growth in non-neoplastic cells (26).

Whether expression of the c-*myc* proto-oncogene regulates the growth of normal lymphocytes has not been established definitively. Nevertheless, previous investigations by us and by others have demonstrated that at least two events are required to induce proliferation of resting (G_0) T lymphocytes. (i) Stimulation with specific antigen or with lectin mitogen induces resting T cells to enter the G_1 phase of the cell cycle, where they synthesize and secrete interleukin 2 (IL2) and express receptors for this growth factor, and (ii) interaction of IL2 with its cellular receptor is then necessary for activated T cells to undergo the $G_1 \rightarrow S$-phase transition (4, 33, 36, 43). When lectin-stimulated human lymphocytes stop dividing, they enter a postmitotic phase before returning to G_0 in which they have an increased RNA content, bear IL2 receptors, and proliferate in response to IL2 alone (4, 42). In contrast, unstimulated or naive (G_0) lymphocytes have low levels of RNA and do not bear IL2 receptors.

In this report, we used the cloned murine T-cell line L2 as a model to investigate the regulation of c-*myc* expression in non-neoplastic T cells. T lymphocytes are maintained in culture by weekly stimulation with antigen and IL2. In their resting, nondividing state, cloned T cells have higher levels of RNA than normal splenocytes, they express IL2 receptors, and like postmitotic lectin-stimulated lymphocytes, they proliferate vigorously in response to IL2 alone (13). Thus, cloned T lymphocytes are different from primary cultured (G_0) murine splenocytes or from (G_0) human peripheral blood lymphocytes; they are probably best regarded as cells in a noncycling G_1-transition state (8).

The ability of the cloned murine T-cell line L2 to produce lymphokines after stimulation with antigen or ConA and to proliferate in response to antigen or IL2 has been well established (11, 12, 17, 38, 39). When L2 cells are stimulated with ConA alone in the absence of spleen cells, they produce lymphokines but do not proliferate (11). Similarly, when mouse thymocytes are stimulated with ConA alone or when purified human peripheral blood T lymphocytes are stimulated with phytohemagglutinin, they do not proliferate (27, 45). If accessory cells are added to cloned T cells, thymocytes, or human T cells, lectin stimulation will drive proliferation (11, 27, 45).

Because L2 cells proliferate in response to IL2, but not in response to ConA alone, these cells provide an excellent system for contrasting the effects of a growth factor, IL2, with those of a lectin mitogen, ConA. We therefore compared the ability of IL2 and of ConA to stimulate c-*myc* mRNA accumulation and cell cycle progression in L2 cells. In addition, we investigated the effect of the immunosuppressive agent cyclosporin A (CsA) on L2 cells stimulated by

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IL2 or by ConA. The data provide evidence that c-*myc* gene expression is regulated by at least two distinct pathways in L2 cells. Comparison of these findings with previous reports by us (40) and by others (26) suggests that accumulation of c-*myc* mRNA occurs in both G_0- and G_1-phase lymphocytes that have been stimulated to proliferate.

MATERIALS AND METHODS

Culture conditions. The derivation and maintenance of the murine Mls-reactive T-lymphocyte clone L2 have been described previously (17). At weekly intervals, L2 cells (5×10^4) were stimulated with irradiated allogeneic spleen cells (6×10^6) and secondary mixed lymphocyte culture supernatant (33%, vol/vol). For all experiments, L2 cells at day 7 of the maintenance culture cycle were separated by Ficoll-Hypaque density gradient centrifugation (9) and were washed and suspended in Dulbecco modified Eagle medium (H-21; GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal calf serum (KC Biologicals, Lenexa, Kas.), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and additional amino acids. On day 7 of the maintenance culture cycle, the T cells were resting in IL2-depleted medium and receptive to stimulation with antigen, ConA or IL2 (38, 39). Cloned T cells suspended at 10^6 to 10^7 cells per ml were cultured in a humidified atmosphere with 5% CO_2 at 37°C with 10^2 to 10^3 U of human recombinant IL2 (rIL2; Cetus Corp., Emeryville, Calif.) per ml, excipient control, 10 μg of ConA (Pharmacia Fine Chemicals, Piscataway, N.J.) per ml, 1 μg of CsA per ml (Sandoz), 15 μg of cycloheximide (CHX; Sigma Chemical Co., St. Louis, Mo.) per ml, or various combinations of these reagents. Cells were removed from cultures at various times by centrifugation, and RNA was isolated from cells as described below.

Cell cycle analysis. Cultures of L2 cells, which were initiated at the same time as the cultures for studies of relative levels of c-*myc* mRNA, were stimulated with rIL2 (100 U/ml) in the presence or absence of colchicine (10^{-7} M). Cells were collected at various times and separated by Ficoll-Hypaque density gradient centrifugation. Cells at the interface were washed and pelleted. The pellets were suspended in 0.1% sodium citrate (wt/vol; pH 7.0) containing 50 μg of propidium iodide (Calbiochem-Behring, La Jolla, Calif.) per ml. DNA content was determined with a fluorescence-activated cell sorter (FACS IV; Becton Dickinson and Co., Paramus, N.J.), and analysis of the histograms was performed by a modification of the method of Fried (15).

Protein gels. L2 cells (10^6/ml) were stimulated with rIL2 (100 U/ml), ConA (10 μg/ml), CsA (1 μg/ml), CHX (15 μg/ml), or various combinations of these reagents. At various times, the cells were harvested and pelleted, and 10^6 cells were suspended in 1 ml of methionine-free Dulbecco modified Eagle medium containing 50 μCi of [^{35}S]methionine (specific activity, >800 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and incubated at 37°C. At the end of 1 h, the cells were harvested, washed twice in cold phosphate-buffered saline, and lysed with 100 μl of extraction buffer (0.01 M Tris [pH 7.4], 0.15 M NaCl, 0.5% Nonidet P-40). The lysates were incubated on ice for 20 min, and 40 μl of the cell lysates was combined with 10 μl of 5× sample buffer (0.25 M Tris [pH 6.8], 10% sodium dodecyl sulfate [SDS], 25% sucrose, 0.005% bromophenol blue, 25% 2-mercaptoethanol). Proteins were separated on a 12% polyacrylamide gel containing 0.1% SDS. The gels were treated with Autofluor (National Diagnostics, Somerville, N.J.), dried, and exposed to X-ray film (Cronex; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) for 1 to 7 days at −70°C to determine de novo protein synthesis. A 5-μl sample of the cell lysates was assayed for radioactivity to determine the level of total protein synthesis.

RNA (Northern) blot analysis of c-*myc* mRNA levels. After culture of L2 cells for various times as described above, total cellular RNA was isolated from L2 cells by a guanidine isothiocyanate method (5) with cesium chloride modification (18). Either equal amounts of RNA, determined by A_{260}, or RNA from equal numbers of L2 cells was denatured at 68°C for 15 min in electrophoresis buffer (20 mM morpholinepropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, 3% formaldehyde [pH 7.0]) containing 50% deionized formamide and then size fractionated by electrophoresis through 1% agarose gels containing 6% formaldehyde and 2 μg of ethidium bromide per dl. Agarose gels were visualized by UV illumination to determine the position of 28S and 18S rRNA bands, to assess the integrity of RNA, and, where appropriate, to verify that equal amounts of RNA had been loaded in all wells before transferring RNA to Gene Screen Plus membranes (New England Nuclear). Transfer of RNA to the membranes was accomplished by capillary blotting for 24 h with 2× SSPE (6 M NaCl, 0.5 M NaH_2PO_4, 50 mM EDTA [pH 7.4]). After blotting, gels were restained with ethidium bromide to verify that transfer of RNA to membranes was uniform. Membranes were dried, baked at 80°C in vacuo, prehybridized at 42°C for 16 to 24 h in prehybridization solution (50% formamide, 1× Denhardt solution [0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin], 1% SDS, 1 M sodium chloride, 5 mM Tris [pH 7.4], 0.5 mg of heparin per ml, 250 μg of denatured sheared salmon sperm DNA per ml, 10% dextran sulfate), and hybridized at 42°C for 24 h in the same solution containing 10^6 cpm of heat-denatured, ^{32}P-labeled plasmid DNA per lane. After washing two times in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 25°C for 30 min and twice in 0.1× SSC–0.1% SDS at 50°C for 30 min, membranes were exposed to Kodak XAR film with Cronex tungstate intensifying screens (E. I. duPont de Nemours) at −70°C for 1 to 5 days.

Radiolabeling of recombinant plasmid DNA. A pBR322 plasmid containing a 1,048-base-pair *Pst*I-*Pst*I cDNA encoding exons 2 and 3 of the human c-*myc* gene, pRYC7.4 (1), was a generous gift of C. Croce (Wistar Institute, Philadelphia, Pa.). Plasmid DNA was radiolabeled to high specific activity (0.5×10^9 to 1.5×10^9 cpm/μg) with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) by a random primer method (14) and purified by spun-column chromatography with Sephadex G-50 (34).

Source of purified rIL2. Purified human IL2 (lot LP23Ob) was a generous gift of Cetus Corp. This substance, produced by recombinant DNA technology in *Escherichia coli*, is 98% pure by SDS-polyacrylamide gel electrophoretic analysis, with 0.02 ng of contaminating endotoxin per 2×10^5 U of IL2, as determined by the *Limulus* assay (51). An excipient control was supplied by the manufacturer. Specific activity of rIL2 was assessed by the manufacturer with a standard bioassay for IL2 (16) and is nearly identical to native human IL2 purified to homogeneity from culture supernatants of mitogen-activated T cells (44).

RESULTS

Measurement of DNA content in L2 cells with propidium iodide. To correlate the progression of L2 cells through the cell cycle with changes in levels of accumulated c-*myc* mRNA we quantified relative amounts of DNA content in L2

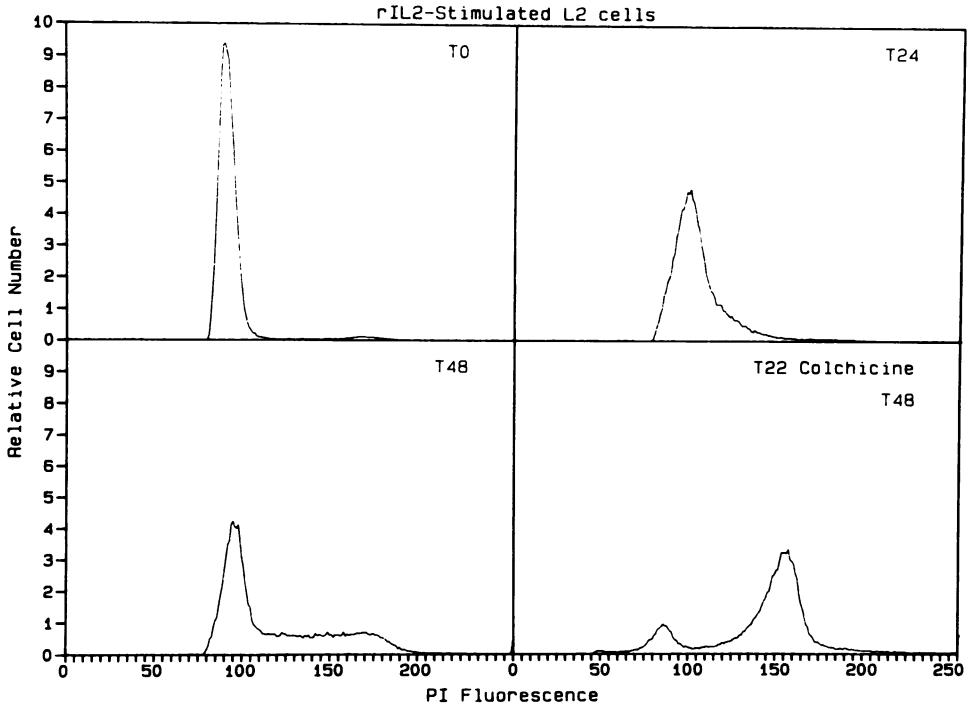

FIG. 1. Kinetics of the cell cycle progression of L2 cells stimulated with purified rIL2. L2 cells (10^6/ml) were quiescent before the addition of 100 U/ml of rIL2 for 0 h (T_0), 24 h (T_{24}), or 48 h (T_{48}). In one set of cultures (T_{22} colchicine, T_{48}), colchicine (10^{-7} M) was added to rIL2-stimulated L2 cells after 22 h, and the cells were recovered at 48 h. Relative DNA content of individual cells was quantified by staining with propidium iodide and by measuring red fluorescence with a FACS IV (15). G_0–G_1-phase cells contain 2n amount of DNA, *S*-phase cells contain 2n to 4n, and G_2–*M*-phase cells contain 4n DNA content. Data are representative of several experiments.

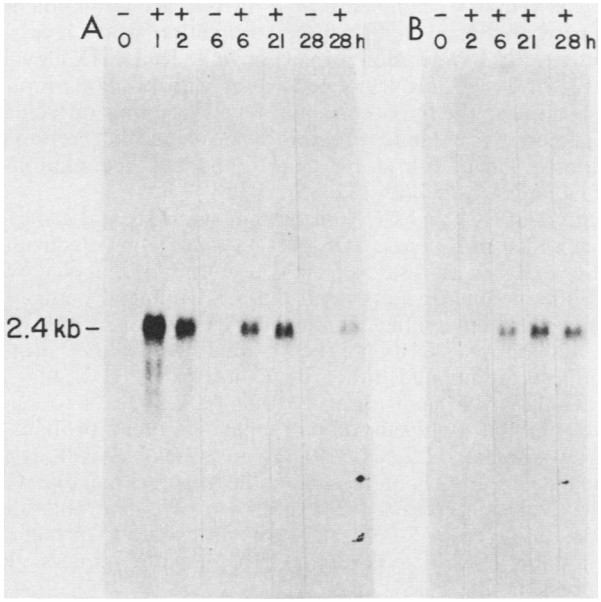

FIG. 2. Time course of c-*myc* mRNA accumulation in L2 cells stimulated with rIL2 or with ConA. L2 cells (5×10^6/ml) were cultured for various times in culture medium with 200 U of purified rIL2 (+) per ml or excipient control (−) (A) or with 10 μg of ConA (+) per ml or medium alone (−) (B). Total cellular RNA isolated from 5×10^6 L2 cells was denatured and size-fractionated by electrophoresis through formaldehyde-containing 1% agarose gels. After transfer of RNA to Gene Screen Plus membranes and prehybridization, the blots were hybridized with 10^6 cpm of a ^{32}P-labeled c-*myc* cDNA probe ($\approx 10^9$ cpm/μg) per lane. After washing, hybridized RNA blots were exposed to Kodak XAR film to −70°C with an intensifying screen for 4 days. The size of the transcript corresponding to the band in these autoradiograms was determined from the relative position of the 28S and 18S RNA and agrees well with previous reports (35). A 2.4-kilobase (kb) marker is shown.

cells at various times after stimulation with purified rIL2 (44, 51) by measuring red fluorescence after staining with propidium iodide (25). Analysis was accomplished with a FACS IV (15). At 7 days after previous antigenic stimulation and before the addition of rIL2 to cultures (T_0), approximately 97% of L2 cells had G_1 DNA content (Fig. 1). By 24 h (T_{24}) after stimulation with rIL2, 35% of the L2 cells had entered the *S* phase, and by 48 h (T_{48}), L2 cells were in all phases of the cell cycle (G_1, 34%; *S*, 50%; G_2/*M*, 16%).

In one set of IL2-stimulated L2 cells, colchicine (10^{-7} M) was added 22 h after initiation of cultures to prevent *M*-phase cells from reentering the G_1 phase of the cell cycle. DNA content was then measured at 48 h. rIL2 (100 U/ml) drove approximately 85% of the L2 cells into the *S*, G_2, or *M* phases of the cell cycle (Fig. 1). The concentration of rIL2 used in these experiments produced maximal thymidine incorporation in cultures of L2 cells (data not shown).

Time course of c-*myc* mRNA accumulation in L2 cells stimulated by IL2 or by ConA. Relative levels of accumulated c-*myc* mRNA were measured in L2 cells at various times after stimulation with purified rIL2 by RNA (Northern) blot analysis (48) with a cDNA probe specific for exons 2 and 3 of the human c-*myc* gene (1). c-*myc* mRNA reached peak levels within 1 h of exposure of L2 cells to rIL2 (200 U/ml) and gradually declined thereafter (Fig. 2A).

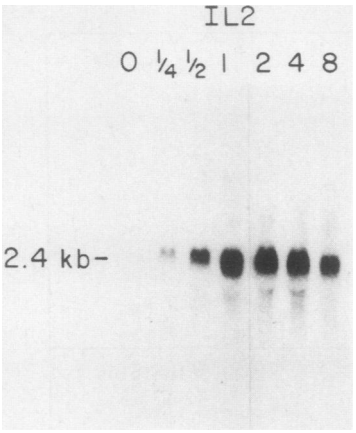

FIG. 3. Time course of c-*myc* mRNA accumulation in L2 cells stimulated with rIL2. L2 cells (7.5×10^6/ml) were cultured for various times in the presence of purified rIL2 (10^3 U/ml) before lysing cells in 4 M guanidine isothiocyanate solution (5) and isolating total cellular RNA by centrifugation through 5.7 M cesium chloride (18). After ethanol precipitation, purified RNA was quantified by determining A_{260} and was analyzed for relative levels of c-*myc* mRNA by RNA (Northern) blotting (10 μg per lane) as described in the legend to Fig. 1. A 2.4-kilobase (kb) marker is shown.

Because ConA has been reported to induce rapid elevations in c-*myc* mRNA levels in primary cultures of (G_0) murine splenocytes (26), we also investigated the ability of ConA to stimulate the accumulation of c-*myc* mRNA levels in L2 cells. The time course of c-*myc* mRNA accumulation induced by ConA (10 μg/ml) (Fig. 2B) was contrasted with that observed in rIL2-stimulated cultures of L2 cells (Fig. 2A). The concentration of ConA used here has been shown to induce maximal lymphokine production by L2 cells (11). As shown, ConA stimulated elevations in c-*myc* mRNA levels in L2 cells at a much slower rate than rIL2. Accumulation of c-*myc* transcripts was easily detectable by 6 h and reached maximal levels at 21 h after stimulation with ConA (Fig. 2B), whereas c-*myc* mRNA levels became maximal at 1 h in L2 cells stimulated by rIL2 (Fig. 2A).

In a separate experiment, we investigated more carefully the time course of c-*myc* mRNA accumulation induced by rIL2. The response to rIL2 was very rapid (Fig. 3), with elevations in c-*myc* mRNA levels becoming detectable within 15 min and reaching maximal levels, as before (see Fig. 2A), within 1 h after stimulation. The concentration of rIL2 in cultures of L2 cells was adjusted according to the cell density to ensure saturation of IL2 receptors on L2 cells.

c-*myc* mRNA accumulation in L2 cells can be stimulated via two pathways. ConA stimulates L2 cells to produce IL2 and other lymphokines (38). Given the data in Fig. 2 and 3 showing that rIL2 induced very rapid accumulation of c-*myc* mRNA, we wondered whether ConA might indirectly induce c-*myc* gene expression in L2 cells via an IL2-dependent pathway. To investigate this possibility, we used two inhibitors of lymphokine production, CsA and CHX. Both CsA and the protein synthesis inhibitor CHX suppress lymphokine production by L2 cells stimulated with ConA (39; J. M. Ely, Ph.D. thesis, University of Chicago, Chicago, Ill., 1981). If production of IL2 explains the ability of ConA to stimulate c-*myc* mRNA accumulation in L2 cells, both inhibitors would be expected to ablate the ConA-induced increase in c-*myc* mRNA levels.

Relative levels of c-*myc* mRNA were measured by RNA blot analysis of total cellular RNA (10 μg per lane) from L2 cells cultured with ConA (10 μg/ml), rIL2 (10^3 U/ml), CsA (1 μg/ml), CHX (15 μg/ml), or various combinations of these reagents. At the concentrations used in these experiments, both inhibitors reduced by >90% the elaboration of lymphokines into culture supernatants (39; data not shown). RNA blot analysis was done with RNA harvested either 8 h after stimulation, when elevations in c-*myc* mRNA levels were easily detectable in ConA-stimulated L2 cells (see Fig. 2B), or at 1 h after stimulation, when c-*myc* mRNA levels became maximal in rIL2-stimulated cultures (see Fig. 3).

CsA completely abrogated the ability of ConA to induce the accumulation of c-*myc* mRNA in L2 cells (Fig. 4A). However, it probably did not globally impair gene transcription, RNA processing, or RNA stability, since levels of actin mRNA and of phosphoglycerol kinase mRNA were not diminished in L2 cells exposed to CsA (data not shown). In contrast to the effects of CsA, the protein synthesis inhibitor CHX caused a small increase in the accumulation of c-*myc* RNA induced by ConA. Though shown here only for ConA at 8 h, CHX enhanced the accumulation of c-*myc* mRNA induced by ConA at several other times (data not shown). Taken together, these data indicate that ConA-induced expression of the c-*myc* proto-oncogene occurs via a CsA-sensitive, IL2-independent pathway.

In contrast to the effects of CsA on ConA-stimulated L2 cells, the simultaneous addition of CsA and rIL2 to L2 cell cultures only slightly inhibited the accumulation of c-*myc* mRNA at 1 h (Fig. 4B). Adding CsA to cultures of L2 cells several minutes before rIL2 did not result in a significant decrease in levels of c-*myc* mRNA induced by rIL2 (data not shown). As with ConA-stimulated L2 cells, there was a slight increase in the levels of c-*myc* mRNA at 1 h after stimulation with rIL2 and CHX (Fig. 4B). The augmentation by CHX of rIL2-induced c-*myc* mRNA accumulation was more pronounced at 8 h after stimulation when levels of c-*myc* mRNA were decreasing (Fig. 4C). That CHX elevates levels of c-*myc* mRNA is consistent with previous reports and supports the hypothesis that levels of c-*myc* mRNA are regulated by a labile protein or proteins that represses transcription of the c-*myc* gene, enhances degradation of c-*myc* mRNA, or both (26, 35).

Effect of CsA and CHX on protein synthesis and cell cycle progression in L2 cells. SDS-polyacrylamide gel electrophoretic analysis of lysates from L2 cells pulse-labeled with [^{35}S]methionine demonstrated that CsA minimally impaired cellular protein synthesis induced by ConA or by IL2 (Fig. 5). In contrast, CHX markedly inhibited cellular protein synthesis stimulated either by ConA or by IL2 (Fig. 5). Previously, we have observed that CsA appears to selectively inhibit synthesis of particular secretory proteins in ConA-stimulated L2 cells (39). Given these observations and the data in Fig. 5, it is reasonable to presume that CsA selectively suppresses the expression of c-*myc* and other genes involved in T-cell activation. In contrast, through its action on protein synthesis, CHX globally impairs gene expression in L2 cells.

It has been demonstrated previously that CsA blocks proliferation in primary cultures of splenocytes stimulated with antigen or with lectin mitogen (32, 52) but does not inhibit IL2-induced proliferation of long-term cultured T cells (37). Cell cycle studies with propidium iodide staining verified that CsA did not impair the cell cycle progression of L2 cells stimulated with rIL2. At T_{48} after stimulation with rIL2, L2 cells were in all stages of the cell cycle (41%, G_1; 49%, S; 10%, G_2-M). Likewise, 48 h after stimulation with

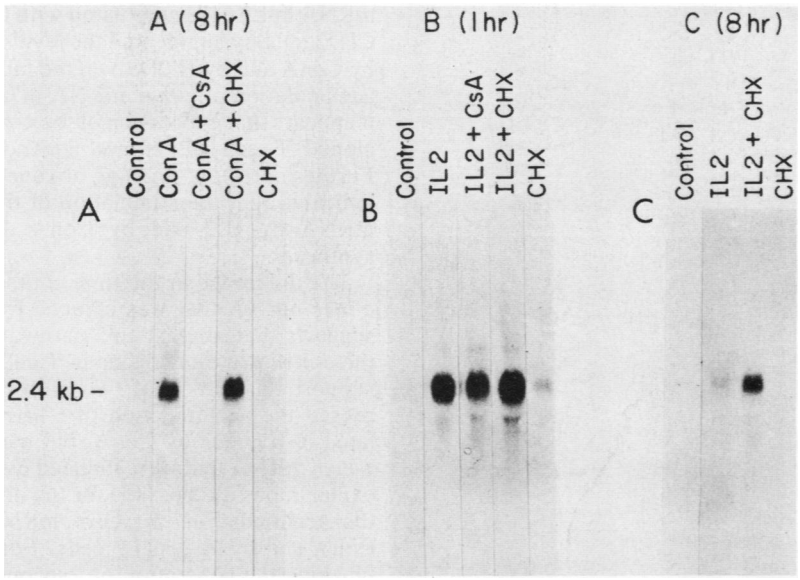

FIG. 4. Effects of CsA and CHX on the accumulation of c-*myc* mRNA induced in L2 cells by rIL2 and by ConA. L2 cells (5×10^6 to 10×10^6 ml) were cultured for either 1 h (panel B) or 8 h (panels A and C) with rIL2 (10^3 U/ml), ConA (10 μg/ml), CHX (15 μg/ml), CsA (1 μg/ml), or various combinations of these reagents as shown. Total cellular RNA (8 μg per lane) was analyzed for relative levels of c-*myc* mRNA by RNA (Northern) blot analysis with a ^{32}P-labeled c-*myc* cDNA probe ($\approx 5 \times 10^8$ cpm/μg of specific activity) as described in the legend to Fig. 1. Autoradiograms represent 5-day exposures. A 2.4-kilobase (kb) marker is shown.

rIL2 plus CsA, L2 cells were in all phases of the cell cycle (43%, G_1; 47%, S; 10%, G_2-M). In a separate experiment, CHX completely inhibited cell cycle progression of rIL2-stimulated L2 cells. At T_{48} after stimulation with rIL2, L2 cells were found in all stages of the cell cycle (63%, G_1; 26%, S; 11%, G_2-M). At 48 h after stimulation with rIL2 plus CHX, L2 cells were limited primarily to the G_1 all phase of the cell cycle (90%, G_1; 7%, S; 2%, G_2-M).

DISCUSSION

Cloned T lymphocytes possess many of the characteristics of normal activated T lymphocytes in that they maintain their specific antigen reactivity, they produce lymphokines in response to stimulation with antigen, and they proliferate in response to IL2 alone (13, 39). Cloned T cells are maintained in culture by weekly stimulation with antigen and growth factor (13, 17). During this weekly cycle, the L2 clone produces lymphokines during the first few days, proliferates during the middle of the week, and returns to a resting state at the end of the week (17, 38). In the resting state, L2 cells are larger, have more RNA than resting splenocytes (G_0) (G. Otten, personal communication), and, unlike resting splenocytes, express receptors for IL2. Thus, by several criteria, L2 cells in the resting state are best regarded as G_1-phase cells and not as G_0-phase cells.

The ability of purified rIL2 to induce c-*myc* mRNA accumulation in L2 cells indicates that c-*myc* gene expression can be stimulated not only during the $G_0 \rightarrow G_1$ transition, as in primary cultures of (G_0) splenocytes (27), but also during the G_1 phase of the cell cycle. Previous investigations from our laboratory with human peripheral blood mononuclear cell cultures support the idea that the accumulation of c-*myc* mRNA can be induced at more than one point in the cell cycle (40). In those studies, stimulation of T cells in peripheral blood mononuclear cell cultures with the lectin mitogen phytohemagglutinin resulted in rapid accumulation of c-*myc* mRNA that plateaued within 1 to 2 h and remained elevated for more than 24 h. Purified rIL2 augmented levels of c-*myc* mRNA in phytohemagglutinin-stimulated peripheral blood mononuclear cell cultures at 24 h, after T cells had entered the G_1 phase of the cell cycle and had acquired IL2 receptors, but not at 3 h. Similarly, a monoclonal antibody that blocks IL2 receptors on activated human T cells diminished levels of c-*myc* mRNA at 24 h but not at 3 h after initiation of phytohemagglutinin cultures. The combined evidence from murine T-cell clones (L2 cells) and from human peripheral blood lymphocyte cultures (40) thus demonstrates that c-*myc* mRNA accumulation is stimulated in T cells both during the $G_0 \rightarrow G_1$-phase transition and later during the G_1 phase of the cell cycle.

The time course of the accumulation of c-*myc* mRNA that was induced by rIL2 in L2 cells (see Fig. 3) is very similar to the results reported for the induction of c-*myc* gene expression by growth factors such as platelet-derived growth factor and fibroblast growth factor in fibroblast cell lines (35). In addition, a temporal sequence of cellular proto-oncogene activation has been elucidated in platelet-derived growth factor-stimulated fibroblasts with, in order, c-*fos*, c-*myc*, and c-*ras* expression occurring during the G_0/G_1 phase of the cell cycle (3, 6, 21, 29, 35). Furthermore, in regenerating liver after partial hepatectomy, the activation of the c-*myc* gene has been shown to precede the activation of the c-*ras* gene (20). Based on the time course for the activation of cellular oncogenes in fibroblasts and hepatocytes, others have argued that c-*myc* may be important for early events of the cell cycle, whereas c-*ras* may play an essential role at later points in the cell cycle (3, 20). In contrast, Thompson et al. (49) have shown that levels of c-*myc* mRNA do not vary through the cell cycle in exponentially growing cells. As reported by others, however, these investigators found that when growth-arrested cells were stimulated to divide, there was a transient increase in c-*myc* mRNA. Taken together, the available data suggest that a burst of c-*myc* expression occurs during the initiation of growth, and that subsequently a homeostatic level of c-*myc* expression is maintained during exponential growth.

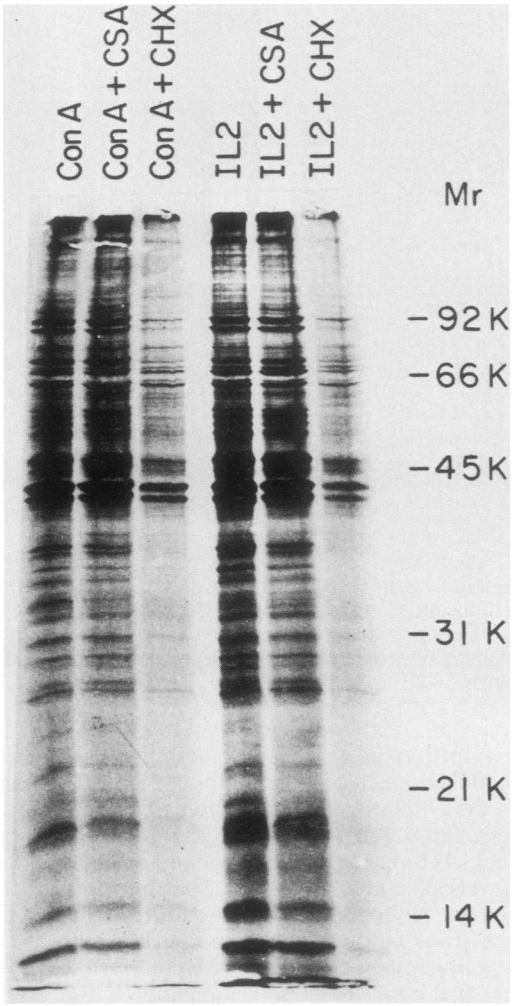

FIG. 5. Protein synthesis in L2 cells. L2 cells, cultured at 10^6 per ml with rIL2 (100 U/ml), ConA (10 μg/ml), CHX (15 μg/ml), CsA (1 μg/ml), or various combinations of these reagents (for 8 h), were pulse-labeled with 50 μCi of [^{35}S]methionine (specific activity, >800 Ci/mmol) in methionine-free medium for 1 h immediately before termination of cultures. Cell lysates (40 μl) were analyzed by SDS-polyacrylamide gel electrophoresis (12% polyacrylamide, 0.1% SDS), and bands were visualized by fluorography. Based on liquid scintillation counting of 5 μl of cell lysate, CHX and CsA inhibited de novo protein synthesis in L2 cells by approximately 75 and 25%, respectively. Relative molecular weight markers are shown, e.g., 92,000 (92 K).

The difference in the time course for the accumulation of c-*myc* mRNA induced by ConA versus that induced by rIL2 suggested initially that ConA might increase c-*myc* expression in L2 cells through an IL2-dependent mechanism. To address this possibility, two inhibitors of lymphokine production, CsA and CHX, were used. These compounds exert their inhibitory effects on gene expression at different levels. Because CsA diminished the accumulation of IL2 mRNA in lectin mitogen-stimulated T cells (28) and had minimal effects on cellular protein synthesis (Fig. 5), it probably acts somewhere between membrane signal transduction and translation. In contrast, CHX acts later by inhibiting translation. Though both inhibitors blocked ConA-induced lymphokine production in L2 cells by ≥90% (39; data not shown), CHX did not reduce the accumulation of c-*myc* mRNA in L2 cells stimulated with ConA (see Fig. 4). In fact, CHX actually augmented the levels of c-*myc* mRNA induced by ConA and by IL2. Given the failure of CHX to inhibit the accumulation of c-*myc* mRNA in ConA-stimulated L2 cells, it appears that expression of the c-*myc* proto-oncogene in the cloned T cell L2 is not dependent on IL2 production. Furthermore, the findings obtained with CHX (see Fig. 4) indicated that the stimulation of the accumulation of c-*myc* mRNA by IL2 and by ConA does not require protein synthesis.

The difference in the time course for the accumulation of c-*myc* mRNA that was induced by ConA and by rIL2 also suggests that at least two pathways regulate expression of the c-*myc* proto-oncogene in T cells. Our findings with CsA support this notion of two pathways. CsA markedly suppresed the accumulation of c-*myc* mRNA that was stimulated in L2 cells by ConA, but had little effect on levels of c-*myc* mRNA that were induced by rIL2 (see Fig. 4). Several explanations are possible for the differential effect of CsA on the accumulation of c-*myc* mRNA that was induced by ConA and by IL2 in L2 cells. For example, CsA could be blocking the generation of "second messengers" that were induced by the binding of ConA to L2 cells but not those that were induced by the binding of IL2 to IL2 receptors on L2 cells. Alternatively, CsA may more directly influence gene expression. Like steroid hormones (50), CsA binds to cytosolic receptors in T cells (22). Possibly, CsA-receptor complexes directly influence gene expression, in analogy to steroid hormone-receptor complexes. Differential regulation by CsA-receptor complexes of c-*myc* gene expression that was induced by IL2 or by ConA would require that the DNA sequences involved in IL2-mediated c-*myc* gene expression be different from the sequences involved in ConA-mediated expression of the c-*myc* proto-oncogene in L2 cells. It is therefore of interest that the c-*myc* gene contains two promoters from which transcription can be initiated independently (23, 46).

In contrast to the rapid induction of the accumulation of c-*myc* mRNA in ConA-stimulated (G_0) murine splenocytes (26) and in phytohemagglutinin-stimulated human peripheral blood mononuclear cells (40), the time course of the accumulation of c-*myc* mRNA in lectin mitogen-stimulated L2 cells is slower (see Fig. 2). Since accessory cells are necessary for the growth of lectin-stimulated T cells, perhaps accessory cells (macrophages or other non-T cells) in unfractionated primary splenocyte cultures or the soluble factors of accessory cells accelerate the rate of accumulation of c-*myc* mRNA induced in T cells by ConA. Nevertheless, our finding that ConA-stimulated L2 cells expressed c-*myc* but did not proliferate suggests that c-*myc* expression may be necessary but insufficient for lymphocyte growth. In this respect, Land et al. (30, 31) have shown that cotransfection of a *myc* gene and a *ras* gene is required for tumorigenesis in primary cultures of embryo fibroblasts. Transfecting a *myc* gene alone did not induce tumors in these experiments (30, 31). The results of these transfection studies are thus consistent with the idea that activation of the c-*myc* gene by itself is insufficient for cell growth.

IL2 is the major physiological growth factor for mature T lymphocytes. Exactly how IL2 stimulates the proliferation of activated T cells is unknown, but our results suggest that induction of c-*myc* expression may represent at least one important IL2-mediated event. If IL2 does exert its proliferative effects on T cells by stimulating c-*myc* expression, one could predict that activation of the *myc* gene in T cells would allow cell growth in an IL2-independent fashion. Unregu-

lated expression of the c-*myc* gene may play an important role in the etiology of mature T-cell leukemias that proliferate in the absence of IL2.

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