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Research Article

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Expression of c-*myc* Proto-Oncogene in Normal Human Lymphocytes

Regulation by Transcriptional and Posttranscriptional Mechanisms

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Abstract

Aberrant expression of the c-*myc* gene results from nonrandom chromosomal translocations involving the transcriptionally active antigen receptor gene loci, in particular lymphocytic leukemias and lymphomas, and is believed to contribute to the etiology of these neoplasms. In addition to its expression in abnormal lymphocytes, increased accumulation of c-*myc* mRNA occurs rapidly in normal B- and T-lymphocytes after stimulation with appropriate mitogens. The mechanisms that mediate these mitogen-induced elevations in c-*myc* mRNA levels, however, have not been determined for normal B and T cells. By using enriched populations of B- and T-lymphocytes obtained from freshly isolated human tonsils and stimulated with *Staphylococcus*-A or with phytohemagglutinin, respectively, we observed marked elevations (20–40-fold) in the steady state levels of accumulated c-*myc* messenger RNA (mRNA) within 1 h of exposure of cells to mitogens; modest increases (three- to fivefold) in the relative rate of transcription of the c-*myc* gene through protein synthesis-independent (cycloheximide-insensitive) mechanisms; and rapid rates of degradation of mature c-*myc* mRNAs through protein synthesis-dependent (cycloheximide-sensitive) mechanisms. These findings corroborate previous studies in other cell types and provide evidence for both transcriptional and posttranscriptional control of c-*myc* proto-oncogene expression in normal human lymphocytes.

Introduction

The c-*myc* proto-oncogene encodes a 65–68-kd (kilodalton) phosphoprotein that resides in the nucleus of cells and, at least in vitro, binds DNA with high affinity (1). High levels of constitutive expression of this gene have been observed in lymphoid neoplasms as a result of chromosomal translocations involving immunoglobulin gene loci in human and murine B cell tumors (Burkitt's lymphoma, murine plasmacytomas) (2); translocations involving T cell antigen receptor genes in occasional cases of acute T-lymphocytic leukemia in humans (3); and retrovirus-mediated promoter/enhancer insertion mechanisms in avian leukosis virus–induced B cell tumors and in Moloney murine leukemia virus–induced murine T cell neoplasms (4, 5). More rarely, increased expression of the c-*myc* proto-oncogene can occur in nonlymphoid neoplasms because of gene amplification, as in HL60 acute promonomyelocytic human cells, in a polyoma virus-transformed osteosarcoma and in some Abelson leukemia virus–transformed murine National Institutes of Health (NIH) 3T3 fibroblast cell lines (6–8). In the majority of cases, no alterations in the coding sequences of the c-*myc* gene have been detected, thus providing at least correlative evidence that deregulated production of the normal c-*myc* gene product contributes to the etiology of these neoplasms.

In addition to constitutive expression in particular leukemias and lymphomas, transient and inducible expression of the c-*myc* proto-oncogene occurs in several types of normal cells when stimulated to proliferate. Typically, levels of c-*myc* messenger RNA (mRNA) rise rapidly, peaking within 1–3 h, then gradually decline, and return to baseline 1–2 d later in normal cells. This time course of c-*myc* gene expression has been observed in B-lymphocytes stimulated with lipopolysaccharide (LPS) or anti-immunoglobulin antibodies (9, 10), resting T-lymphocytes stimulated with mitogenic lectins (9, 11), activated T cells treated with interleukin 2 (IL-2, T cell growth factor) (12), quiescent fibroblasts induced to proliferate with serum (9), and hepatocytes after partial hepatectomy (13). These findings have suggested that expression of the c-*myc* gene plays a role in normal cellular proliferation. Indeed, gene transfer experiments in fibroblasts (NIH 3T3 cells) have shown that c-*myc* can reduce the requirement for platelet-derived growth factor for proliferation (14), and microinjection studies have similarly shown that c-*myc* protein can render these cells "competent" to respond to progression factors present in platelet-poor plasma (15). The c-*myc* proto-oncogene product thus may serve some important function during early stages of normal cellular growth. However, a role for c-*myc* during later phases of the cell cycle has also been suggested by a recent report demonstrating that microinjection of c-*myc* protein can modulate DNA polymerase activity and is required for DNA synthesis in isolated nuclei (16).

Given the above evidence that c-*myc* may participate in the regulation of normal and abnormal cellular growth, it is important to understand the mechanisms that control the expression of this proto-oncogene. Despite extensive investigations in a variety of normal and neoplastic cells, the fundamental aspects of the regulation of c-*myc* gene expression remain undetermined for normal T- and B-lymphocytes: the cellular lineages wherein alterations in c-*myc* expression have most clearly been associated with human carcinogenesis. For this reason, we have investigated the regulation of c-*myc* mRNA levels in mitogen-stimulated T and B cells at the levels of transcription, steady state mRNA accumulation, and degradation. The findings begin to delineate the mechanisms controlling expression of the c-*myc* proto-oncogene in human lymphocytes.

Methods

Preparation of cells. Lymphocytes were isolated from freshly obtained human tonsils essentially as described (17). In brief, immediately after

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routine tonsillectomy, tonsillar tissue was placed into Hanks' balanced salt solution under sterile conditions and subsequently passed through a sieve to disperse the cells. Mononuclear cells were then purified by Ficoll–Hypaque density centrifugation and the resultant cells were enriched for B- or T-lymphocytes by two sequential rosettings with neuraminidase-treated sheep erythrocytes. After an overnight adherence step to remove residual contaminating monocyte/macrophage cells, enriched populations of B- (nonrosetted) and T- (rosetted) lymphocytes were analyzed by immunofluorescence assay (18) using OKB7 (B cell) and OKT11 (T cell) monoclonal antibodies (Ortho Diagnostics Systems Inc., Johnson & Johnson, Raritan, NJ). Purity of B and T cell preparations was routinely > 90%, with > 95% viability as determined by trypan blue dye exclusion.

Culture conditions. Enriched populations of B- and T-lymphocytes were cultured at 37°C in 5% CO_2–95% air in RPMI 1640 medium (10^6 viable cells/ml) containing 10% vol/vol heat-inactivated fetal calf serum, 50 U/ml penicillin, 100 μg/ml streptomycin, 1.0% vol/vol mycostatin suspension (Gibco, Grand Island, NY), and 2.0 mM glutamine. Replicate cultures of lymphocytes were incubated with 0.1% (vol/vol) *Staphylococcus aureus* Cowan I strain (SAC)[1] (Pansorbin; Calbiochem-Behring Corp., LaJolla, CA) 0.1% vol/vol phytohemagglutinin-*P* (PHA-*P*) (Difco Laboratories Inc., Detroit, MI), 10 ng/ml phorbol myristic acetate (PMA) (Chemicals for Cancer Research, Inc., Chanhassen, MN), 15–20 μg cycloheximide (CHX) (Sigma Chemical Co., St. Louis, MO), 5 μg/ml actinomycin D (Sigma Chemical Co.), or various combinations of these reagents. After various times, cells were recovered from cultures by centrifugation for RNA isolation and nuclear transcription assays.

Lymphocyte proliferation assay. Lymphocyte proliferation was indirectly assessed by measuring relative levels of DNA synthesis as described previously (18). Briefly, 200 μl aliquots of lymphocyte cultures were added in triplicate to 96-well flat-bottom microtiter plates (Linbro, Flow Laboratories, Hamden, CT; Costar, Data Packaging Corp., Cambridge, MA). 8 h before termination of cultures, wells were pulsed with 0.5 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA; sp act 5–10 Ci/mmol) and harvested onto fiberglass filters. Dried filters were counted in a liquid scintillation counter and the data expressed as mean counts per minute of triplicate cultures.

RNA blot analysis. Relative levels of accumulated mRNAs were measured by Northern blot analysis exactly as described (11, 12). Briefly, total cellular RNA was isolated from lymphocytes by a guanidium isothiocyanate method with cesium chloride modification, and equal amounts of RNA (10 μg/lane) were size-fractionated by electrophoresis through formaldehyde-containing 1% agarose gels. Before transfer to nylon membranes (Gene Screen Plus, DuPont Co., Diagnostic & Bio-Research Systems, Wilmington, DE), ethiudium-stained gels were visualized under ultraviolet illumination to determine the positions of 28S and 18S ribosomal RNA (rRNA) bands, to assess integrity of RNA, and to verify equal amounts of RNA were loaded. Membranes were then hybridized under high-stringency conditions (50% formamide, 1 M NaCl; 42°C) with various ^{32}P-labeled DNA probes (~ 10^9 cpm/μg) including pRYC7.4 (human c-*myc*), He7 control probe (pHe7), and pA1 (chicken β-actin) (19–21). After 24 h, membranes were washed as described previously (final washes in 15 mM NaCl, 1.5 mM sodium citrate, and 0.1% sodium dodecyl sulfate [SDS] at 50°C) and exposed to Kodak XAR film at −70°C with intensifying screens. Relative intensities of bands on autoradiograms were quantified by scanning densitometry using an ultrascan (LKB Instruments, Inc., Gaithersburg, MD).

Nuclear transcription assays. Measurements of relative rates of c-*myc* gene transcription were conducted by nuclear transcription ("run off") assays essentially as described (22). After stimulation of lymphocytes for various times, nuclei were isolated by incubating cells for 5 min on ice in lysing solution (0.3 M sucrose, 60 mM KCl, 15 mM Hepes [pH 7.5], 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, and 14 mM 2-mercaptoethanol) containing 0.5% Nonidet Pluronic 40 detergent and then layering the lysate onto a cushion of 30% sucrose (same as lysing solution except for adjustment of sucrose concentration) for centrifugation at 3,000 rpm for 10 min. The resultant nuclei pellets were resuspended in storage buffer (50% glycerol, 20 mM Tris [pH 7.9], 75 mM NaCl, 0.5 mM EDTA, 0.85 mM dithiothreitol (DTT) 0.125 mM para-methysulfonic acid, and 100 U/ml RNasin [Promega Biotec, Madison, WI]) and were frozen at −70°C.

Transcription reactions were conducted at 26–28°C with 25×10^6–50×10^6 freshly thawed nuclei resuspended in 100 μl of a solution containing 0.3 M $(NH_4)_2SO_4$, 100 mM Tris (pH 7.9), 4 mM $MgCl_2$, 4 mM $MnCl_2$, 0.2 M NaCl, 0.4 mM EDTA, 0.1 mM para-methysulfonic acid, 1.2 mM DTT, 10 mM creatine phosphate, 20 U/ml RNasin, 1 mM each of GTP, ATP, and CTP (Boeringer Manneheim Diagnostics, Inc., Houston, TX), 29% glycerol, and [α-^{32}*P*] uridine triphosphate (UTP) (> 600 Ci/mM; New England Nuclear). After 25–30 min (the minimum time necessary for sufficient incorporation of [^{32}P]UTP into c-*myc* transcripts for detection), reactions were stopped by two sequential 10 min incubations with 100 μg/ml DNase I (RNase-free; Bethesda Research Laboratories, Gaithersburg, MD).

After adjustment to 10 mM Tris (pH 7.9), 10 mM EDTA, 0.5% SDS, incubation for 30 min at 42°C with 200 μg/ml proteinase K (Bethesda Research Laboratories), and extraction twice with (1:1) chloroform/phenol, samples were passed through 1-ml Sephadex G50 columns (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ), treated for 20 min on ice with 0.2 M NaOH, neutralized with 1 M Hepes, and ethanol-precipitated in the presence of 1 mM UTP. The resultant precipitates were washed in 80% ethanol, dried, and resuspended in hybridization solution (50 mM Hepes [pH 7.0], 0.75 M NaCl, 50% [vol/vol] formamide, 0.5% SDS, 2 mM EDTA, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 20 μg/ml polyadenylate [Pharmacia Fine Chemicals, Div. of Pharmacia Inc.], and 500 μg/ml denatured salmon sperm DNA). Subjecting an aliquot of samples to agarose gel electrophoresis verified the absence of contaminating DNA in these preparations.

Volumes of transcription reaction products containing equal amounts of radioactivity (based on scintillation counting of an aliquot) were then brought to 3 ml in hybridization solution, heated at 80°C for 7 min, cooled on ice for 1 min, and incubated at 42°C with prehybridized nitrocellulose filters into which various linearized plasmid DNAs (2.7 μg pRYC7.4 [human c-*myc*], 5.5 μg pHe7 [positive control], 5.5 μg pBR322 [negative control] [23], and 1.8 μg A1 [28S rRNA] [24]) had been immobilized with the use of a slot–blot apparatus (Bethesda Research Laboratories) in amounts equivalent to 0.4 μg of insert DNA (Preliminary experiments indicated that these amounts of DNA were in excess of [^{32}P]RNA derived from these transcription reactions.). After hybridization for 2–3 d, filters were washed three times for 20 min in 2× standard saline citrate (SSC) (300 mM NaCl, 30 mM sodium citrate) and 0.1% SDS at room temperature, followed by two washes for 30 min in 0.1× SSC and 0.1% SDS at 50°C. Filters were then dried and exposed to Kodak XAR film with intensifying screens at −70°C for 1 wk.

Quantification of the data was performed by scanning densitometry wherein background hybridization to pBR322 was subtracted from all values and then the data corrected for slight inequalities in the amounts of [^{32}P]RNA added to filters by normalization relative to the 28S rRNA signal. Rates of c-*myc* gene transcription were then calculated relative to a constitutively transcribed control gene (He7) by dividing the corrected c-*myc* value by the corrected He7 value.

Results

Time courses of c-myc mRNA accumulation in mitogen-stimulated B- and T-lymphocytes. For investigating the regulation of c-*myc* gene expression in normal lymphocytes, we first determined the steady state levels of accumulated c-*myc* mRNA by

1. *Abbreviations used in this paper:* CHX, cycloheximide; PHA-P, phytohemagglutinin-P; pHe7, ^{32}P-labeled He7 control probe; SAC, *Staphylococcus aureus* Cowan strain I; UTP, uridine triphosphate.

standard Northern blot analysis (11, 12) in mitogen-stimulated B- and T-lymphocytes. For these experiments, we obtained lymphocytes from freshly isolated human tonsils and enriched for either B or T cells by standard techniques. Maximal proliferation of these lymphocytes (determined in preliminary experiments) was then induced by culturing B and T cells, respectively, with 10 ng/ml PMA (a phorbol ester) and either 0.1% (vol/vol) formalin-fixed SAC (a B cell–specific mitogen) or 0.1% (vol/vol) PHA-P (a relatively T cell–specific mitogen). Total cellular RNA was isolated from B and T cells at various times (0, 1, 8, 24, or 48 h) after stimulation. Because levels of rRNA increase after mitogenic stimulation of lymphocytes (25), we compared equal amounts of total RNA (10 μg/lane) rather than RNA from equivalent numbers of cells. The small amounts of RNA present in these freshly isolated lymphocytes (~ 0.2 μg total RNA per 10^6 resting cells) precluded routine use of purified polyadenylated mRNA.

As shown in Fig. 1 (*top* and *middle*), stimulation of either B cells with SAC and PMA or T cells with PHA-P and PMA produced similar time courses of c-*myc* mRNA accumulation. In both cases, steady state levels of a 12.4-kb (kilobase) c-*myc* mRNA rose typically 20-fold (range 10–40-fold, based on scanning densitometry) within 1–6 h after stimulation, then gradually declined, reaching baseline unstimulated levels by ~ 48 h. Eluting [^{32}P]c-*myc* probe from this RNA blot and rehybridizing with a control probe pHe7 demonstrated the specificity of the mitogen-induced changes in c-*myc* mRNA levels and verified equivalent loading and transfer of RNAs for each sample.

Several other mRNAs were examined as controls, including those for β-actin, phosphoglycerate kinase, and class I human leukocyte antigen, but none of these was maintained at levels as constant as He7. (These mRNAs typically underwent gradual and transient three to fivefold increases in their steady state levels after stimulation with mitogens.) Thus He7 was used as a control for many of the experiments described herein.

Comparison of the time course of c-*myc* mRNA accumulation with that of DNA synthesis (Fig. 1, *bottom*) in mitogen-stimulated lymphocytes revealed that steady state levels of accumulated c-*myc* mRNA peaked ~ 1 d before the onset of DNA synthesis and declined toward baseline coincident with maximal DNA synthesis in these cultures. These results thus agree well with previous reports in lymphocytes and other types of cells (9–13).

Regulation of c-myc mRNA degradation in normal B- and T-lymphocytes. As a first step towards delineating the mechanisms that account for the changes in steady state levels of accumulated c-*myc* mRNA observed in mitogen-stimulated lymphocytes, we determined the rate of degradation of c-*myc* mRNA in the presence and absence of the protein synthesis inhibitor CHX. Previous reports have demonstrated that treatment of resting lymphocytes with CHX results in increased accumulation of c-*myc* mRNA (9, 11, 12). This finding has been taken as putative evidence for a labile protein (or proteins) that negatively regulates the accumulation of c-*myc* mRNA.

Further evidence that labile proteins contribute to the regulation of c-*myc* mRNA levels in normal B and T cells is provided by the data in Fig. 2 *A*. As shown, addition of 15–20 μg/ml CHX (sufficient to diminish [^{35}S]methionine incorporation by ≥ 95%) to cultures of enriched B or T cells produced marked increases in the levels of c-*myc* mRNA. Though cells were treated for 6–8 h with CHX in the experiment, the results of which are shown in Fig. 2 *A*, increased accumulation of c-*myc* mRNA was

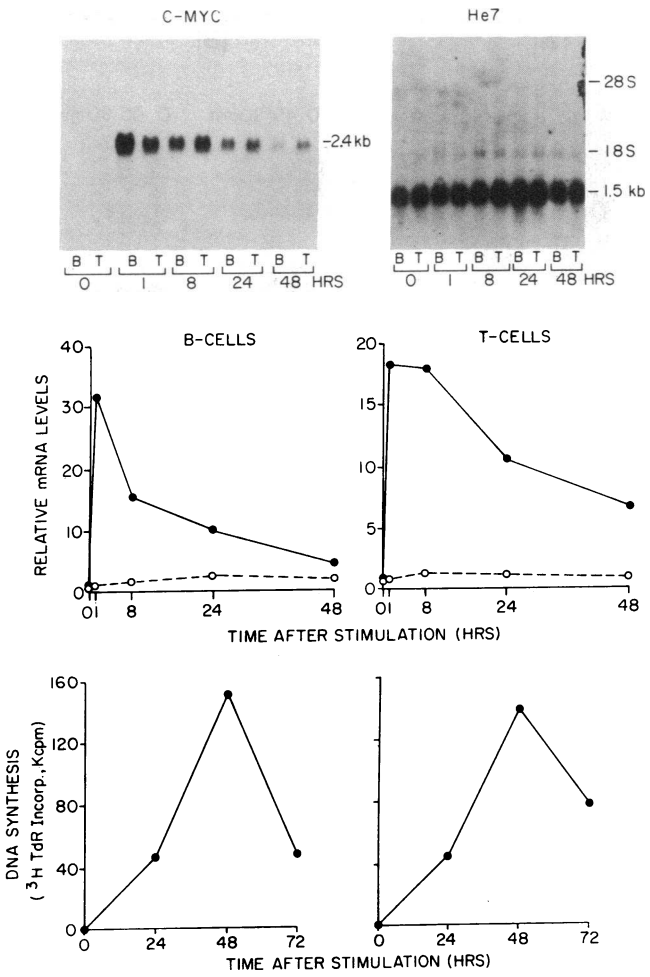

Figure 1. Course of time for c-*myc* mRNA accumulation in mitogen-stimulated normal human lymphocytes. Enriched populations of B cells and T cells were stimulated with 10 ng/ml PMA and either 0.1% SAC or 0.1% PHA-P, respectively, for either 0, 1, 8, 24, or 48 h before isolating total cellular RNA and determining relative levels of c-*myc* or He7 (control) mRNAs as described (11, 12). The top figures show typical data wherein an RNA blot was first hybridized with [^{32}P]c-*myc* probe (pRYC7.4) before eluting probe from the filter (1 h incubation in 10 mM Tris [pH 7.4] and 0.2% SDS at 85°C) and rehybridizing with pHe7. Autoradiograms represent overnight exposures at −70°C using Kodak XAR film with intensifying screens. Differences in the scales of the autoradiograms shown here for c-*myc* and for He7 mRNAs were created during photographing of original data that were derived from the same RNA blot. Data from autoradiograms (*top*) were quantified by scanning densitometry (*middle*) and the relative levels of c-*myc* (●) and He7 (○) mRNAs expressed relative to unstimulated cells (time 0 h). Note the difference of scale for the abscissae for B and T cell data. Though mitogen-stimulated B cells accumulated higher levels of c-*myc* mRNA than T cells in the particular experiment shown, this was not a general finding. For the data in the bottom figures, enriched populations of B and T cells were cultured as described above at 2×10^5 cells per flat-bottom microtiter well and 8 h before termination of 1, 2, or 3 d cultures, 0.5 μCi of [^{3}H]thymidine was added to individual wells, and relative levels of DNA synthesis were determined by scintillation counting. Data points represent means of triplicate samples (standard deviations ≤ 15% of means).

detectable within 1 h after blocking protein synthesis in resting lymphocytes with CHX (not shown).

To determine the rate of c-*myc* mRNA degradation in mi-

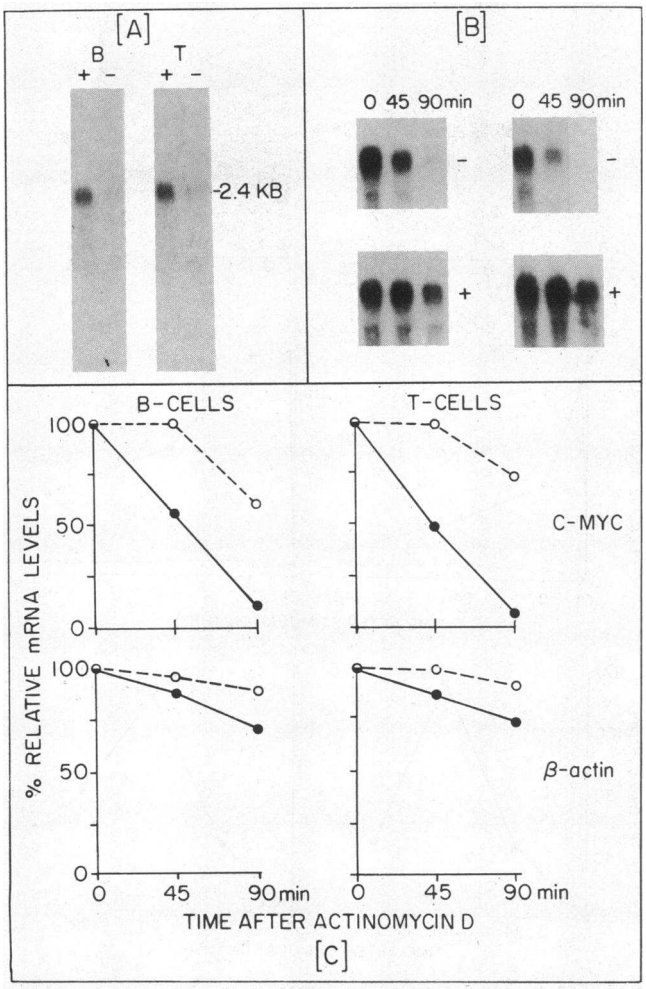

Figure 2. Degradation rate of c-*myc* mRNA in mitogen-stimulated lymphocytes. (*A*) enriched populations of tonsillar B and T cells were treated (+) or not treated (−) for 6–8 h with 15–20 μg/ml of CHX before isolating total cellular RNA (10 μg/lane) and measuring relative levels of accumulated c-*myc* mRNA by RNA blot analysis (11, 12). Rehybridization of these blots with pHe7 probe verified equivalent amounts of RNA in all lanes (not shown). (*B*) B (*left*) and T (*right*) cells were stimulated for 8 h with 10 ng/ml PMA and either 0.1% SAC or 0.1% PHA-P, respectively, before adding 5 μg/ml actinomycin D and harvesting RNA from aliquots of cells 0, 45, or 90 min later. Duplicate cultures were treated (+) or not treated (−) with 15–20 μg/ml CHX 0.5 h before addition of actinomycin D. Cell viability was routinely ≥ 90% for all culture conditions. Some lanes in autoradiograms from RNA blot analysis were reordered for clarity of presentation. (*C*) Scanning densitometry quantification of data shown in (*B*) are represented. In addition to c-*myc*, data are also shown for β-actin mRNA wherein the RNA blot in (*B*) was incubated in 0.2% SDS and 10 mM Tris (pH 7.4) at 85°C for 1 h to elute [^{32}P]pRYC7.4 probe before rehybridizing with [^{32}P]pA1 (β-actin) DNA. Densitometry data are expressed as a percentage of 0 h mRNA levels for lymphocytes treated (○) or not treated (●) with CHX.

togen-stimulated B and T cells in the presence or absence of CHX, we first stimulated lymphocytes with appropriate mitogens for several hours, thereby inducing accumulation of c-*myc* mRNA. Actinomycin D was then added at concentrations sufficient to block all further RNA synthesis (determined by [^{3}H]UTP incorporation; not shown) and RNA was subsequently isolated from aliquots of cells at various times thereafter (0, 45, and 90 min; see Figs. 2 *B* and *C*). Determination of the relative levels of c-*myc* mRNA before and at various times after actinomycin D, allowed us to estimate the rate of degradation of mature c-*myc* messages. Where indicated in Fig. 2, CHX was added to cultures of mitogen-stimulated lymphocytes 0.5 h before treatment with actinomycin D.

As demonstrated in Figs. 2 *B* and *C*, steady state levels of c-*myc* mRNA declined rapidly upon inhibition of further RNA synthesis in mitogen-stimulated B and T cells. The $t_{1/2}$ of c-*myc* mRNA under these conditions was estimated at ~ 45 min for both B and T cells. Addition of CHX to cultures markedly prolonged the $t_{1/2}$ of mature c-*myc* mRNA by two- to fourfold ($t_{1/2}$ was ~ 150 min by extrapolation). In contrast to c-*myc* mRNA, levels of β-actin mRNA (examined here as a control) declined more slowly after treatment of cells with actinomycin D and were influenced to a lesser extent by CHX throughout the course of these experiments (Fig. 2 *C*).

The data in Fig. 2 indicate that c-*myc* mRNA undergoes rapid turnover in mitogen-stimulated lymphocytes and provide presumptive evidence that CHX augments levels of c-*myc* mRNA, at least in part, by diminishing its rate of degradation. Though CHX may have some nonspecific stabilizing effects on mRNA $t_{1/2}$ (see actin data on Fig. 2 *C* and [26]), this seems an inadequate explanation for the marked increase in the $t_{1/2}$ of c-*myc* mRNA in CHX-treated cells.

Analysis of c-myc gene transcription in mitogen-stimulated B- and T-lymphocytes. Because steady state levels of accumulated mRNA reflect the net of RNA synthesis and RNA degradation, we next examined directly relative rates of c-*myc* gene transcription with the use of nuclear (run off) transcription assays (22). For these experiments (Fig. 3), nuclei were isolated from B and T cells after treatment with mitogens, CHX, or various combinations of these reagents, and nascent transcripts were elongated in vitro in the presence of [^{32}P]UTP. The resultant ^{32}P-transcripts were then hybridized to nitrocellulose filters onto which various plasmid DNAs (pRYC7.4 [human c-*myc*], pHe7, A1 [28S rRNA]) has been immobilized by using a slot–blot apparatus.

Fig. 3 shows transcription data derived from B and T cells stimulated for 0, 1, 6, or 24 h with appropriate mitogens before isolation of nuclei. As shown, transcription of the c-*myc* gene was barely detectable in resting lymphocytes. After treatment with mitogens, relative rates of c-*myc* transcription increased approximately threefold, becoming maximal between 1 and 6 h and declining toward baseline levels by 24 h. Unlike experiments that measured steady state levels of mRNA (Fig. 2), CHX had little effect on the rate of c-*myc* gene transcription in mitogen-stimulated lymphocytes (Fig. 3) and in resting lymphocytes (not shown). Thus, the CHX-sensitive mechanisms that regulate levels of c-*myc* mRNA in normal lymphocytes appear to operate post-transcriptionally.

Discussion

Here we have investigated the fundamental mechanisms that regulate the levels of c-*myc* mRNA in normal human B- and T-lymphocytes. Given that steady state levels of c-*myc* mRNA rose 20–40-fold in mitogen-stimulated lymphocytes (Fig. 1), whereas relative rates of c-*myc* transcription increased only about threefold (Fig. 3), our findings corroborate previous reports sug-

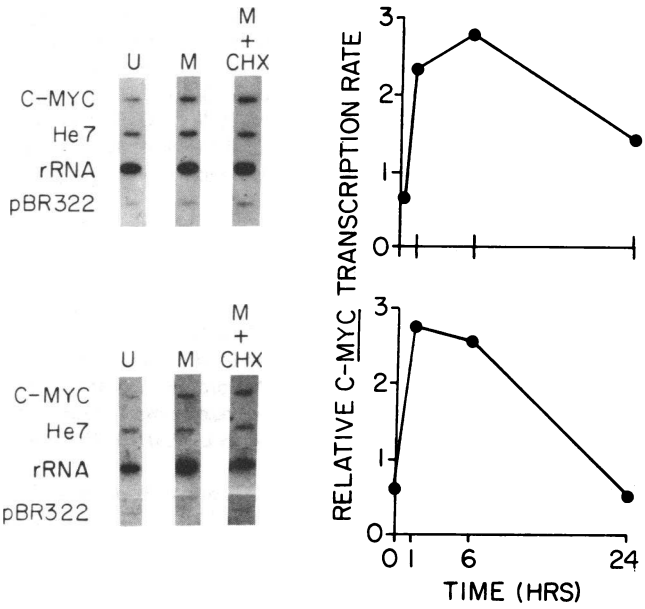

Figure 3. Relative rates of c-*myc* gene transcription in mitogen-stimulated lymphocytes. Nuclear transcription assays (22) were performed with nuclei isolated from lymphocytes at various times after culture in medium alone (U, unstimulated) or medium with mitogens (M) or with mitogens plus CHX (M + CHX). CHX was added 0.5 h before addition of mitogens, either 10 ng/ml PMA and 0.1% (vol/vol) SAC for B cells or 10 ng/ml PMA and 0.1% (vol/vol) PHA-P for T cells. Typical autoradiograms (*left*) are shown for B cells (*top*) stimulated for 1 h or for T cells (*bottom*) stimulated for 6 h. Plasmid DNAs slot-blotted onto nitrocellulose filters included pRYC7.4 (human c-*myc* [19]), pHe7 (positive control [20]), pBR322 (negative control [23]), and A1 (28S rRNA [24]). In some cases, slots were reordered for clarity of presentation. In preliminary experiments (not shown), adding 2 μg/ml α-amanitin to transcription reactions, specifically impaired transcription of genes for c-*myc* and He7 but not for 28S rRNA. Scanning densitometric quantification of nuclear transcription data are shown (*right*) for B cells (*top*) and T cells (*bottom*) stimulated for 0, 1, 6, or 24 h with appropriate mitogens. Data are expressed as the ratio of c-*myc* to He7 after subtraction of background hybridization (pBR322) and normalization to the 28S rRNA signal.

gesting that c-*myc* mRNA accumulation is regulated primarily through posttranscriptional mechanisms in normal cells (27–31). It should be noted, however, that our results with primary cultured human B- and T-lymphocytes differ from several previous investigations that used long-term cultured cells. In those studies, relatively high rates of c-*myc* transcription were present in resting cells and little or no increase above the baseline rate of transcription was detected in BALB/c mouse 3T3 fibroblast cells treated with serum, platelet-derived growth factor, or PMA (28); in CCL39 Chinese hamster lung fibroblast cells stimulated with thrombin and insulin (31); and in MSB-1–transformed avian T-lymphocytes released from density arrest (29). In contrast to these studies in fibroblasts and transformed T cells, our transcription data are similar to those showing an increase in c-*myc* transcription in primary cultures of mitogen-stimulated human T-lymphocytes (32) and in long-term cultured CT6 cells, a cloned murine T cells, stimulated with IL-2 (33). Thus, increased transcription may play a greater role in the regulation of c-*myc* mRNA levels in normal lymphocytes than in some other types of cultured cells after induction of their proliferation.

Probe selection must be considered when comparing c-*myc* transcription data since recent reports have demonstrated a regulatable block in the elongation of transcripts from this gene between exons 1 and 2 (22, 34). We purposely avoided measuring prematurely terminated transcripts by selecting a complementary DNA probe (pRYC7.4), encoding only the second and third exons of the c-*myc* gene (19). Though a variety of c-*myc* probes were used in the above mentioned studies of long-term cultured cells (27–31), in at least one of these reports (31) the same pRYC7.4 probe was employed. Thus, differences in the probes selected for transcription studies cannot fully account for the discrepancy between findings in normal lymphocytes and in other types of cells.

Our investigations of the effects of CHX on relative rates of c-*myc* transcription in normal human B and T cells demonstrated that synthesis of new proteins is not required for mitogen-induced increases in the transcription of this proto-oncogene (see Fig. 3). These findings (Fig. 3) are consistent with reports in other types of cells (28, 29, 31, 32) and indicate that the activity of preexisting regulators of c-*myc* gene transcription in normal lymphocytes is controlled through protein synthesis-independent mechanisms.

In contrast to its actions on c-*myc* transcription, CHX markedly decreased the turnover of mature c-*myc* mRNAs in B- and T- lymphocytes (see Fig. 2). Steady state levels of accumulated c-*myc* mRNA therefore appear to be regulated, at least in part, through posttranscriptional mechanisms in normal lymphocytes. Previous investigations in a variety of cells have suggested an important role for posttranscriptional mechanisms in the control of c-*myc* mRNA levels (27–31). For example, induction of differentiation of Daudi cells (Burkitt's lymphoma cell line) with gamma interferon produces decreases in the levels of accumulated c-*myc* mRNA that are associated with diminished c-*myc* mRNA stability without a change in the rate of c-*myc* transcription (30). With regard to investigations in Daudi cells, normal lymphocytes, and other cells, however, it should be noted that regulation at additional posttranscriptional steps, such as processing, cannot be excluded.

Though the mechanisms that influence the degradation of c-*myc* mRNA remain to be elucidated fully, the ability of CHX to augment levels of c-*myc* mRNA in resting lymphocytes (Fig. 2 *A*) suggests that c-*myc* degradation is regulated by a labile protein that is constitutively present in lymphocytes, rather than by a protein whose synthesis is induced after stimulation with mitogens. Use of preexisting proteins to regulate c-*myc* expression would be expected to allow for more rapid alterations in the levels of c-*myc* mRNA than if new protein synthesis were required.

In summary, our findings here demonstrate that levels of c-*myc* mRNA are regulated similarly in normal B- and T-lymphocytes and that both transcriptional and posttranscriptional mechanisms are involved. Though many details remain unknown, these studies begin to define the molecular events that control the expression of the c-*myc* proto-oncogene in normal human lymphocytes.

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