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Cyclin mRNA and Protein Expression in Recombinant Interleukin 2-Stimulated Cloned Murine T Lymphocytes

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Expression of cyclin, a non-histone nuclear protein, during recombinant interleukin 2 (rIL2)-driven cell-cycle progression of cloned T lymphocytes has been assessed. We found that expression of cyclin protein, as detected by immunofluorescence, is tightly associated with proliferation, and not merely S-phase, of L2 cells stimulated with rIL2. Cyclin immunofluorescence was detected in all cell-cycle phases ($G_1/S/G_2/M$, as detected by flow cytometry) of proliferating L2 cells. Accumulation of cyclin mRNA levels was induced as early as 1 h after stimulation, was maximal at 25–49 h, and remained elevated throughout stimulation, as detected by Northern blot analysis. A cDNA- encoding murine cyclin was cloned from a cDNA library prepared from IL2-stimulated cloned T cells. The sequence of the 5′ end of the murine cyclin cDNA was determined and found to be 88% and 82% similar to the sequences of cDNA clones encoding rat and human cyclin, respectively. The present studies demonstrate that cyclin protein and mRNA accumulation are highly regulated during IL2-induced proliferation of a cloned T cell. These data provide a framework for addressing the molecular mechanisms regulating cyclin gene expression during cellular proliferation.

Key words: cyclin, proliferating cell nuclear antigen, cloned T lymphocytes, interleukin 2

Cellular proliferation is the consequence of a complex array of biochemical and molecular events generally initiated in response to membrane-associated signals. The nature of many of these intracellular events has been described. For example, gene transcription [1] and translation [2] are required for cell-cycle progression. Further, a variety of "early" events have been associated with cellular proliferation, including changes in intracellular ion concentrations [3], phospholipid metabolism [4], and protein phosphorylation [5]. However, the mechanisms by which transcriptional and

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postranscriptional events are regulated and related to these "early" events remain enigmatic. A general approach to the study of regulation of cellular proliferation has been the examination of genes whose expression is required for entry into the cell cycle. A more specific approach is focused on analyzing molecules which may control DNA synthesis [6]. The nuclear protein cyclin (proliferating cell nuclear antigen, PCNA) has been studied using both approaches. Initial studies showed that cyclin expression is highly regulated in proliferating cells [7,8]. Recent evidence indicates that cyclin functions as part of a multi-enzyme complex responsible for the replication of DNA [9,10].

T lymphocytes are induced to proliferate in a two-signal manner. The first signal for T-cell proliferation is the interaction of antigen (in the context of self major histocompatibility molecules) with its receptor which results in lymphokine production (e.g., interleukin 2 [IL2]) and IL2 receptor expression. The second signal is provided by the interaction of IL2 and the IL2 receptor which drives G_1 progression. Unstimulated cloned T lymphocytes exist in a G_1 resting stage and can be induced to progress through cell division upon stimulation with IL2 [11], thus providing a model system for the evaluation of biochemical and molecular regulation of T-lymphocyte proliferation. Our laboratory has previously demonstrated that biosynthesis of cyclin, as assessed by biosynthetic labeling and SDS-PAGE, increases 1,000-fold during the mid-G_1 phase of the cell cycle in an IL2-stimulated cloned T lymphocyte, L2 [12]. Immunofluorescence studies have demonstrated that cyclin accumulates in the nucleus of L2 cells during IL2-induced proliferation [13]. Thus, the present investigations were designed to characterize further modifications in expression of cyclin protein as well as mRNA as a function of progression through the cell cycle.

MATERIALS AND METHODS

Cell Line

Derivation and maintenance of the cloned murine T-helper cell line, L2, has been previously described [14]. Cells were used 6–8 days following exposure to antigen and IL2.

Human IL2

Purified human recombinant IL2 (rIL2), a product of recombinant DNA technology in *Escherichia coli* [15], was a gift of Cetus (Emeryville, CA). This substance is 98% pure as assessed by SDS-PAGE analysis and contains .01 ng endotoxin per 3.6×10^6 units of IL2 according to the Limulus assay. Specific activity was assessed by the manufacturer, using a standard bioassay for IL2.

Immunofluorescence

Cyclin antiserum obtained from a patient with systemic lupus erythematosus was used as the primary antibody for indirect immunofluorescence, according to the method of Clevenger et al. [16]. L2 cells ($1–3 \times 10^6$) were washed in PBS then fixed for 10 min with 10 ml of 1.0% paraformaldehyde (Polysciences, Warrington, PA) in PBS. The cells were centrifuged (200*g*, 10 min), resuspended in 0.1% Triton X-100 (Sigma, St. Louis, MO) in PBS for 3 min, and recentrifuged. Cells were incubated in a 1:400 dilution of cyclin antiserum for 1 h, washed, and incubated with a 1:20 dilution of fluorescein-conjugated rabbit antimouse immunoglobulin (Antibodies Inc.,

Davis, CA) for 30 min. After washing, the cells were incubated in 2 ml of 150 U RNAse A/ml PBS (Worthington, Freehold, NJ) for 20 min at 37°C, centrifuged, and resuspended in 50 ug/ml propidium iodide (Calbiochem-Behring, San Diego, CA) in PBS at least 1 h prior to flow cytometry. Unless otherwise specified, all procedures took place at 4°C.

Flow Cytometry

Fluorescence-activated cell sorter (FACS) analysis of the cell cycle was performed as described [16].

cDNA Library Construction

Total cellular RNA was isolated by lysis of L2 cells in guanidinium isothiocyanate and density gradient ultracentrifugation over CsCl [17]. Polyadenylated RNA was selected by two passages over an oligo (dT) column and the cDNA library was constructed according to described methods [17].

Screening

Fifty thousand phage from the murine cDNA library were plated on agar plates. DNA was transferred to Colony/Plaque Screen hybridization membranes (NEN, Dupont, Boston, MA) and probed with a ^{32}P-labeled rat cDNA encoding cyclin [kindly provided by P. Nakane, Tokai University School of Medicine, Japan; 18], as described previously [19]. cDNA probes were labeled using the random primer extension method [20] with deoxycytidine 5′-[alpha-^{32}P]triphosphate (3,000 Ci/mmol; Amersham, Arlington Heights, IL). Hybridization with the rat cDNA probe in a secondary screening was confirmed by Southern blot analysis.

Subcloning and Sequencing

Inserts from cDNA clones hybridizing with the rat cyclin cDNA were isolated by agarose gel electrophoresis and electroelution and were subcloned into M13mp18 [17]. Clones were partially sequenced by the dideoxy chain termination method using Sequenase, a modified form of T7 DNA polymerase [21; United States Biochemical Corp., Cleveland, OH]. Sequence analysis and comparison with the European Molecular Biology Laboratories database was carried out using the Intelligenetics Software Program (Mountain View, CA) on Bionet^{tm}.

Northern Blot Analysis

RNA was prepared from L2 cells and Northern blot analysis was performed as previously described [22]. Probes were prepared as for screening and included cyclin cDNA and a murine cDNA encoding the beta chain of the T-cell receptor (TcR) (pUC25, a gift of Y. Hashimoto, University of Pennsylvania). Radioactivity on autoradiographs was quantified by scanning laser densitometry, using an LKB Ultroscan XL densitometer.

RESULTS

Previous immunofluorescence and biosynthetic analyses of cyclin during cellular proliferation have demonstrated cyclin expression varies in the cell population as a function of time following rIL2 stimulation [12,13]. Progression through the cell

cycle in these studies was based on a separate previous examination of DNA content in the total cell population with time following stimulation. However, a precise description of cyclin expression during the cell cycle requires simultaneous analyses of cyclin immunofluorescence and DNA content in individual cells. Figure 1 illustrates the results of simultaneous analyses of cyclin immunofluorescence and DNA content in IL2-stimulated cloned T cells during late G_1, G_1 to S phase transition, and active proliferation. L2 cells were maintained in culture by weekly stimulation with alloantigen and rIL2. The DNA content in 88% of the unstimulated cells, at the end of such a weekly cycle, was consistent with that of G_1 cells containing a normal diploid amount of DNA (Fig. 1). Twelve percent of quiescent L2 cells have apparent tetrapoloid DNA content, which is probably due to doublets for which no correction was made in these studies. Cyclin fluorescence was negligible in quiescent L2 cells. Previous experiments from this laboratory demonstrated synthesis of p36 protein [12], later shown to be cyclin [13], during the initial G_1 stage in IL2-stimulated L2 cells [13]. We therefore chose to begin our examination of cyclin expression at a period encompassing late G_1. Cyclin immunofluoresence was strikingly different at 21 h,

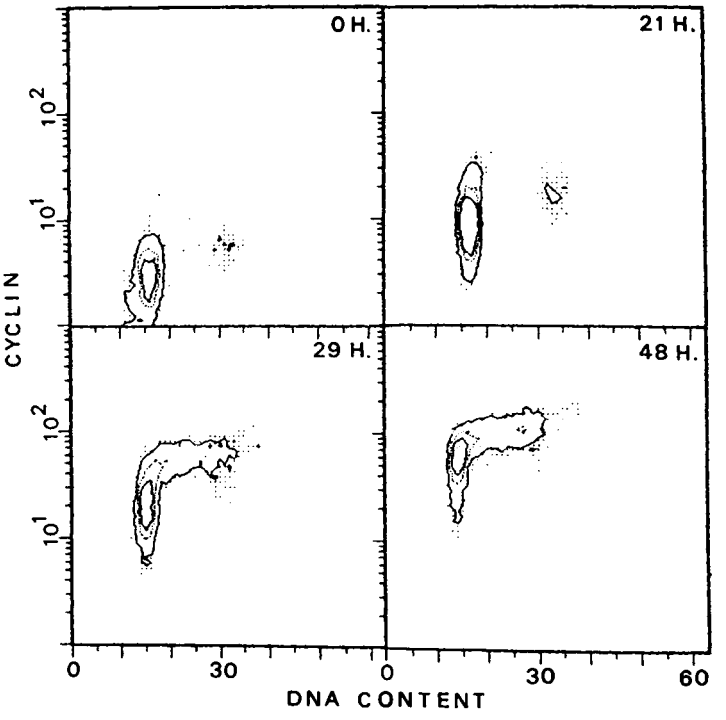

Fig. 1. Cell cycle analysis and cyclin immunofluorescence of rIL2-stimulated L2 cells. L2 cells were stimulated with 100 U/ml human recombinant IL2. At the indicated times, cells were harvested from culture, washed, and stained with human cyclin antiserum; this was followed by fluorescein-conjugated rabbit antihuman globulin and propidium iodide, as described [16]. The intensity of cyclin-associated fluorescence was plotted on a logarithmic scale on the ordinate. Propidium iodide fluorescence (DNA content) was plotted on a linear scale in arbitrary units on the abscissa. At 0 h, the cyclin fluorescence was identical to that obtained following control staining of cells with the rabbit antihuman globulin alone and thus represents background fluorescence. Each panel represents the analysis of 10,000 cells, and the contour lines represent the number of cells at each level (5, 10, 40, and 70).

compared to unstimulated cells. Approximately 66% of the cells containing a G_1 DNA content expressed cyclin and all of the cells at the G_1 to S phase transition or in S phase expressed cyclin. The mean increase in cyclin expression at 21 h compared to unstimulated cells was approximately fourfold. Eight hours later, at 29 h, 30% of the cells were in $S/G_2/M$ phases, and, again all of these cells expressed cyclin (Fig. 1). Further, the intensity of staining for cyclin was increased approximately fourfold over that seen at 21 h. At 48 h, the population was cycling asynchronously and cells could be identified in all phases of the cell cycle (Fig. 1). All cells at 48 h expressed cyclin, though the intensity of staining was greatest in those cells with the largest DNA content (Fig. 1), with a mean fluorescence intensity approximately 20-fold greater than that of unstimulated cells.

Human and rat cDNA encoding cyclin have recently been identified and their full sequences determined [18,25]. A murine cyclin cDNA would provide a tool for examining cyclin expression at the levels of mRNA accumulation, stability, and ultimately, transcription, as well as identification of cyclin clones from murine genomic libraries. In order to isolate a cDNA-encoding murine cyclin, we screened a cDNA library from IL2-stimulated L2 cells with the rat cDNA as a probe. Six of these clones (.01% of plated clones) hybridized with the rat cDNA in secondary screenings and Southern blot analysis. One of these clones, H5.3, was subcloned into M13mp18, and the 5′ end sequence was determined. This partial sequence, and its comparison to the relevant sequences of human and rat cyclin cDNA, is shown in Figure 2. The partial murine sequence was compared with all nucleotide sequences in the European Molecular Biology Laboratories (EMBL) and National Institutes of Health (NIH) databases and showed similarity only to the human and rat cyclin sequences. The 5′ partial murine sequence was slightly more similar to the rat than the human sequence (88% compared to 82% nucleotides matched, respectively). This clone was 1.1 kb in length, approximately the length of the rat and human clones (1.145 kb and 1.1 kb, respectively), and may therefore represent a full-length clone. Further sequence analysis will be required to determine the extent to which the 5′ end of the murine sequence is represented in this clone.

The murine cDNA clone was used as a probe for the determination of steady-state cyclin mRNA levels during IL2-driven L2 cell proliferation. Total RNA was isolated from L2 cells at various times following IL2 stimulation and subjected to Northern blot analysis. In unstimulated cells, cyclin mRNA was barely detectable (Fig. 3A,B). Cyclin message was increased as early as 1 h following stimulation and

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                                                                    64
HUMAN    ATGTTCGAGGCGCGCCTGGTCCAGGGCTCCATCCTCAAGAAGGTGTTGGAGGCACTCAAGGACC
                :    :::: :::::::::::::::::::::::::::::: ::::::: ::::: ::::
MOUSE         AGCTCGAATCCCTGATCCAGGGCTCCATCCTGAAGAAGGTGCTGGAGGCTCTCAAAGACC
                  : :  :::::::::::::::::::::::::::::::::::::::::: ::::::::::
RAT      ATGTTTGAGGCACGCCTGATCCAGGGCTCCATCCTGAAGAAGGTGCTGGAGGCCCTCAAAGACC

                                                                   126
HUMAN      TCATCAACGAGGCCTGCTGGGATATTAGCTCCAGCGGTGTAAACCTGCAGAGCATGGACTCG
           ::::::: ::::::::::::::: :::::::: :::: :: ::::::::::::::::::::::
MOUSE      TCATCAATGAGGCCTGCTGGGACGTCAGCTCGGGCGGCGTGAACCTGCAGAGCATGGACTCG
           :::::::::::::::::::::: :::::::::::::::::::::::::::::::::::: :::
RAT        TCATCAATGAGGCCTGCTGGGACATCAGTTCGGGCGGCGTGAACCTACAGAGCATGGATTCG
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Fig. 2. Partial nucleotide sequence of the coding strand of the murine cDNA (H5.3) encoding cyclin and comparison with the human and rat cyclin sequences [18,25]. Nucleotides are numbered in the 5′ to 3′ direction and begin with the first residue of the initiation codon ATG (human and rat). Spaces represent areas where the nucleotides are different between species.

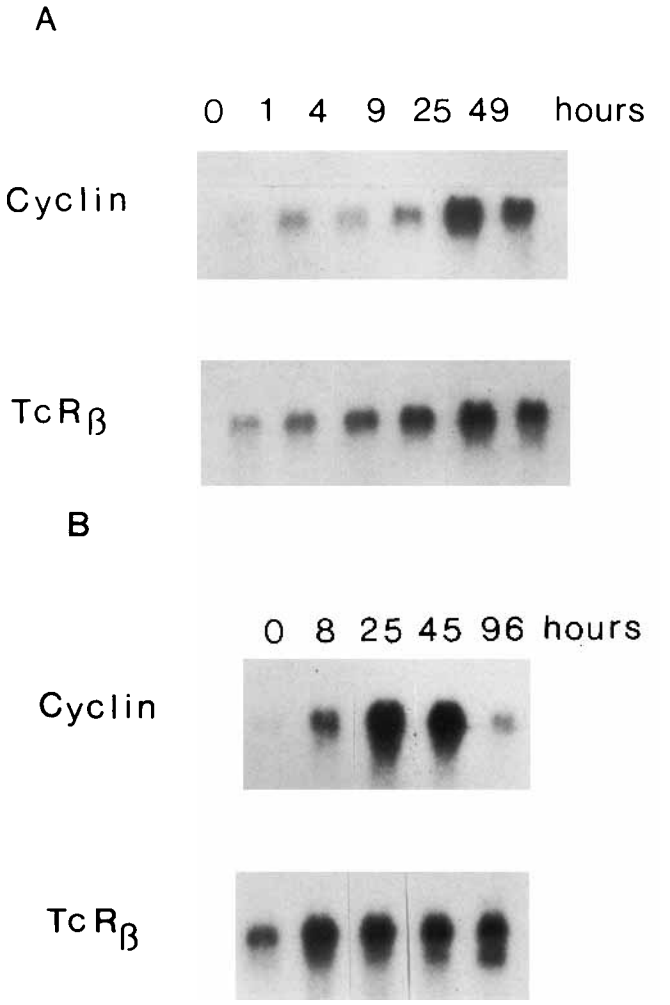

Fig. 3. Kinetics of accumulation of cyclin or the beta chain of the T cell receptor mRNA in L2 cells stimulated with 100 U/ml rIL2. Total RNA was isolated at 0, 1, 4, 9, 25, and 49 h **(A)** or 0, 8, 25, 45, and 96 h **(B)** after stimulation and subjected to Northern blot analysis as described in Materials and Methods.

increased with time reaching a maximum (24-fold stimulation compared to unstimulated cells) at 25 h after IL2 stimulation (Figs. 3A, 4A). We next examined cyclin mRNA accumulation at times corresponding to four discrete periods in the cell cycle: mid-G_1 (8 h), G_1 to S phase transition (25 h), active proliferation (45 h), and return to quiescence (96 h). At 8 h, cyclin mRNA levels were elevated nearly ten-fold above those seen in unstimulated cells (Figs. 3B, 4). Maximal cyclin mRNA accumulation (at 25 and 45 h) was greater than 30-fold above unstimulated levels. At 96 h, when the majority of cells had returned to a postmitotic G_1 state [26], cyclin mRNA levels dropped to approximately fivefold above unstimulated cells. The difference in the time of maximal cyclin message accumulation in the two experiments probably represents a difference in the degree to which the L2 cells were stimulated.

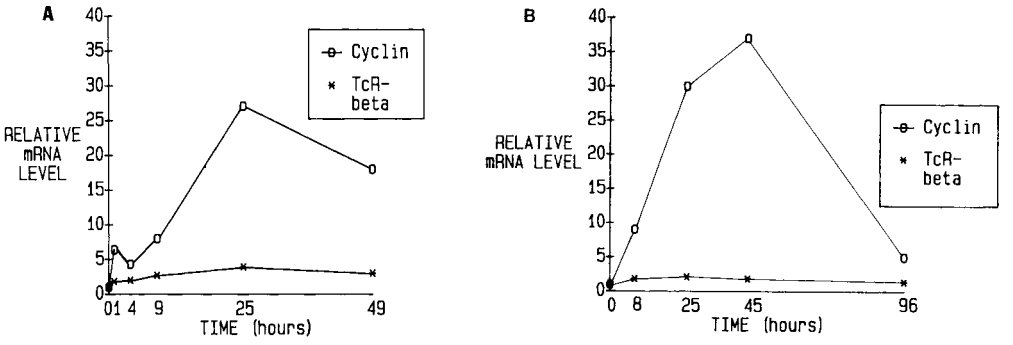

Fig. 4. Densitometric analysis of autoradiograms from Northern blots illustrated in Figure 3, probed with cDNA for cyclin or TcR-beta. Panels **A** and **B** are graphic representations of the data presented in Fig. 3, A and B, respectively. Data are presented as the relative increase in mRNA accumulation in stimulated compared to unstimulated cells.

To confirm that the amounts of RNA were relatively constant, the gels were stained with ethidium bromide and examined under ultraviolet illumination. The 28S and 18S ribosomal RNA bands were identified and appeared quantitatively similar on each lane (data not shown). To assess the relative change in cyclin RNA accumulation, levels of cyclin message were compared to the levels of message encoding the beta chain of the T-cell receptor (TcR-beta). The TcR-beta mRNA levels were only slightly elevated (2–3-fold) in stimulated compared to unstimulated cells (Figs. 3, 4), consistent with previous reports [27]. Thus, the increase in cyclin expression in Figure 3 represents a genuine response to rIL2 stimulation.

DISCUSSION

Cyclin, also called proliferating cell nuclear antigen, is a nuclear protein of 36,000-dalton molecular weight, first identified as an antigen recognized by autoantibodies produced in approximately 3% of patients with systemic lupus erythematosus [28]. These antibodies reacted preferentially with nuclei of proliferating normal and transformed cells [28]. Expression of cyclin, as identified by immunofluorescence, closely parallels ^{3}H-thymidine incorporation in proliferating cells [7]. In order to study the regulation of cyclin expression, and, conversely, how cyclin may possibly regulate DNA synthesis, the expression of cyclin must be examined at several levels. These levels may be considered to include the distribution of cyclin as identified by antibodies, cyclin protein synthesis and transcription of the cyclin gene.

Early studies suggested that cyclin expression was restricted to S phase [7,28]. However, cyclin can be detected by immunofluorescence at low levels in formaldehyde-fixed quiescent 3T3 cells [29]. Flow cytometric analysis of lectin-stimulated lymphocytes by Kurki et al. [23] suggested that cyclin was present later during G_1 activation but highest in S phase. Our previous studies indicated that cyclin biosyn-

thesis was initiated as early as 4 h after stimulation with IL2 [12] but only suggested that cyclin was present in all phases of the cell cycle in actively proliferating L2 cells [13]. Thus, we chose a time course for analyzing cyclin immunofluorescence and DNA content to demonstrate that cyclin levels are increased after IL2 stimulation in essentially all proliferating cells during all phases of the cell cycle.

Figure 1 clearly demonstrates that cyclin protein expression is tightly associated with proliferation, and not merely S phase, of L2 cells stimulated with rIL2; that is, cyclin levels are high in all cell-cycle phases ($G_1/S/G_2/M$) of actively proliferating cells. Cyclin detected in these experiments may be considered to include newly synthesized protein as well as accumulated protein. The flow cytometric experiments are considerably more sensitive than experiments in which cyclin-associated immunofluorescence is evaluated by microscopy. Further, fixation in formaldehyde reveals cyclin immunofluorescence at times when cyclin is undetected in methanol-fixed cells (i.e., in unstimulated cells) [29]. These methodological differences provide an explanation for the contrasting results of Celis et al. [7] who detected extremely weak cyclin immunofluorescence in G_2 and M phases of the cell cycle. The idea that cyclin protein expression is proliferation specific rather than S phase specific is supported by the findings of Bravo and Bravo [29], who also detected cyclin during the transition of 3T3 cells from a growing to a quiescent state, by pulse-chase experiments and immunoblotting. Kurki et al. [23] also detected significant levels of cyclin immunofluorescence at G_2/M in mitogen-stimulated splenocytes, though these levels were reduced compared to S-phase; G_1- associated fluorescence was minimal in those studies, in contrast to our findings.

Northern blot analysis of cyclin mRNA levels (Fig. 3) demonstrates that cyclin expression is also highly regulated throughout the cell cycle in IL2-stimulated L2 cells at the level of mRNA accumulation. Matsumoto et al. [18] detected significant levels of cyclin mRNA at 48 h, corresponding to maximal DNA synthesis in PHA-stimulated human peripheral blood lymphocytes. However, in contrast to our results, they did not detect cyclin message in unstimulated cells or in cells that were incorporating thymidine at 72 h after stimulation. Similarly, Almendral et al. [25] did not detect cyclin message in unstimulated quiescent 3T3 cells or in 3T3 cells until 12 h following serum stimulation. In those studies, maximal accumulation was detected at 16–18 h and cyclin levels declined dramatically at 24 h after stimulation, though histone H3 levels remained high, providing evidence that the cells were in S phase [30]. Our data correlate closely with previous cyclin protein synthesis data in IL2-stimulated L2 cells [12] and the present observation on cyclin immunofluorescence, since significant cyclin protein synthesis, immunofluorescence and RNA accumulation were detected at all phases of the cell cycle. It is puzzling to us that the cyclin message could not be detected at the later time points analyzed by Matsumoto et al. [18] and Almendral et al. [25], since our studies clearly demonstrate that the message remains elevated after S phase in IL2-stimulated L2 cells. An examination of cyclin mRNA expression in several types of cells stimulated to proliferate with various ligands may provide insight into the contrast between these findings. The differences in kinetics of the onset of expression of cyclin mRNA in IL2-stimulated L2 cells compared to 3T3 cells and PHA-stimulated human peripheral blood lymphocytes may be related to the relatively greater degree of quiescence of the latter cell types compared to L2 cells; L2 cells are maintained at a G_1 stage prior to stimulation.

Cyclin protein synthesis [12] and cyclin mRNA accumulation could be detected during early G_1 (4 h following stimulation) and was reduced, though still detectable,

during the return to quiescence (at 96 h). This observation provides evidence, for the first time, that cyclin protein expression, which also decreases during the return to G_1, is directly related to the mRNA levels. Thus, cyclin expression is likely to be regulated at the level of mRNA accumulation (i.e., message stability and/or transcriptional activation).

Maximal cyclin mRNA accumulation was observed at 24–48 h after stimulation. Previous experiments published by us demonstrate that in IL2-stimulated L2 cells, c-myc mRNA rises rapidly, reaching a maximum at 1–4 h after stimulation, and is almost undetectable at 28 h [22]. Thus, maximum cyclin mRNA accumulation follows c-myc expression and remains elevated for a longer period of time than c-myc in IL2-stimulated L2 cells. The difference in kinetics of induction of cyclin and c-myc mRNA suggests different mechanisms of regulation between genes activated early in G_1 compared to those activated at the G_1 to S phase transition. Such mechanisms may include differences in the stability of cyclin and c-myc mRNA. C-myc mRNA is relatively short-lived [$T_{1/2}$ of 10–60 min depending on the cell type; 31,32] and message stability is considered to play an important role in regulating c-myc mRNA levels since transcription of the c-myc gene may be only slightly induced in proliferating cells [33]. Cyclin mRNA stability and its contribution to regulation of cyclin expression remains to be explored.

The partial sequence of the murine cDNA encoding cyclin was similar to that of rat and human cDNA clones. The identification of a murine cDNA provides a tool for the identification of murine cyclin genomic clones for study of the organization of the cyclin gene and its regulation at the transcriptional level.

The present studies have demonstrated that cyclin protein and mRNA accumulation are highly regulated throughout the cell cycle in a cloned T-cell stimulated with IL2. Previous studies have identified cyclin as a proliferation-associated protein and have highlighted the changes in cyclin nuclear distribution and/or antigenicity. Identification and characterization of the regulation of cyclin expression at multiple levels will provide a framework within which to address questions concerning the molecular mechanisms regulating cyclin gene expression during cellular proliferation.

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