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Metabolism of c-*myc* gene products: c-*myc* mRNA and protein expression in the cell cycle

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The presence and synthesis of c-*myc* protein and mRNA in the cell cycle has been studied. We find that c-*myc* mRNA is present, at equivalent levels, at all times in the cell cycle with the possible exception of mitosis. Furthermore, we demonstrate that this mRNA is transcribed in both G_1 and G_2 phases. An analysis of the c-*myc* protein *in vivo* shows that *de novo* synthesis occurs in G_1 and G_2 and the protein turns over with a half-life of ~20–30 min in both phases. Furthermore, the level of c-*myc* protein rapidly increases in cell populations when they re-initiate the cell cycle, thereafter decreasing as the culture reaches quiescence. The results therefore suggest that expression of c-*myc* can be rapidly modulated and that it is activated during the G_0 to G_1 transition, but is expressed thereafter in the cell cycle.

Key words: cell cycle/c-*myc*/mRNA/protein synthesis/transcription

Introduction

The c-*myc* gene is expressed at varying levels in a wide variety of actively growing cell types. This gene has, however, been implicated as a cellular oncogene in various situations such as after chromosomal translocation in Burkitt's lymphoma (Bernard *et al.*, 1983; Hamlyn and Rabbitts, 1983; Saito *et al.*, 1983; Marcu *et al.*, 1983) where the association of the c-*myc* gene with the immunoglobulin loci is thought to activate the oncogene. In normal spleen cells expression of the c-*myc* gene can be induced very rapidly upon activation of resting lymphocytes by polyclonal mitogens (Kelly *et al.*, 1983), and in fibroblast cell lines by growth factors (Greenberg and Ziff, 1984). On the other hand, comparison of c-*myc* mRNA in exponential and contact-inhibited fibroblast cultures indicated that c-*myc* is not transcribed in resting fibroblast (Campisi *et al.*, 1984). These results argued that the transcription of the c-*myc* gene is regulated in the cell cycle and only expressed in the early phases. We now present evidence that both c-*myc* mRNA and protein are present and synthesised in G_1, S plus G_2 phases of the cell cycle and that the turnover times are equivalent throughout these various phases.

Results

Cell cycle analysis of c-myc mRNA

We have used inhibitors to prepare cells arrested in the various stages of the cell cycle to study c-*myc* gene expression. Hydroxyurea (an inhibitor of DNA synthesis) was used to prepare cells arrested in G_1 and early S_1 phase and either colcemid or razoxane to prepare G_2 arrested cells (see Materials and methods). The quality of the cell populations was assessed by flow cytometry using ethidium bromide-stained cells which distinguishes between G_1 and G_2 DNA content. The cells used in this study include normal concanavalin A-stimulated T cells (conA T-cells), Epstein-Barr virus (EBV)-transformed non-tumorigenic B cell lines, tumorigenic lines such as HeLa cells and BL cell-lines. Figure 1 shows an example of the two populations of Daudi cells analysed in this way and the cell cycle data for the various cells are summarised in Table I.

RNA was prepared from the various cells and analysed for the presence of c-*myc* mRNA by Northern filter hybridisation (Figure 2). All the cell types (both normal T cells and the tumour cells) examined contain c-*myc* mRNA, at approximately equivalent levels, in G_1- or G_2-enriched populations, (the filters were normalised using actin mRNA as control, data not shown). It should be noted that the level of c-*myc* mRNA in exponentially growing conA T-cells was found to be approximately equivalent to that of the arrested populations shown here (unpublished). [A

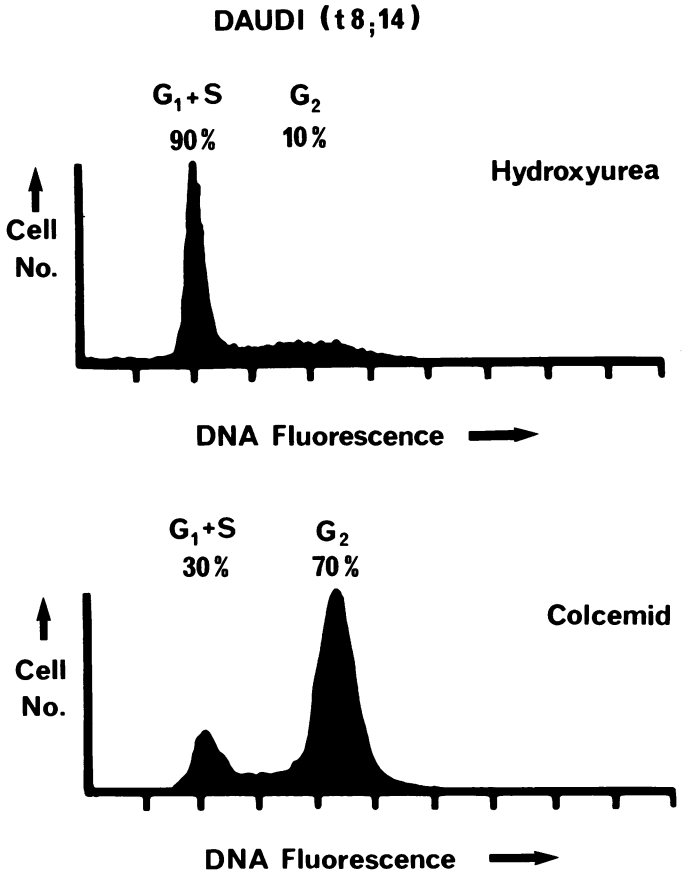

Fig. 1. Flow cytometry of cell cycle-arrested Daudi cells. Daudi cells were arrested in G_1/S with hydroxyurea and in G_2 with colcemid (as described in Materials and methods). The graphical representation shows the calculated percentage of cells in G_1/S and G_2 based on DNA content.

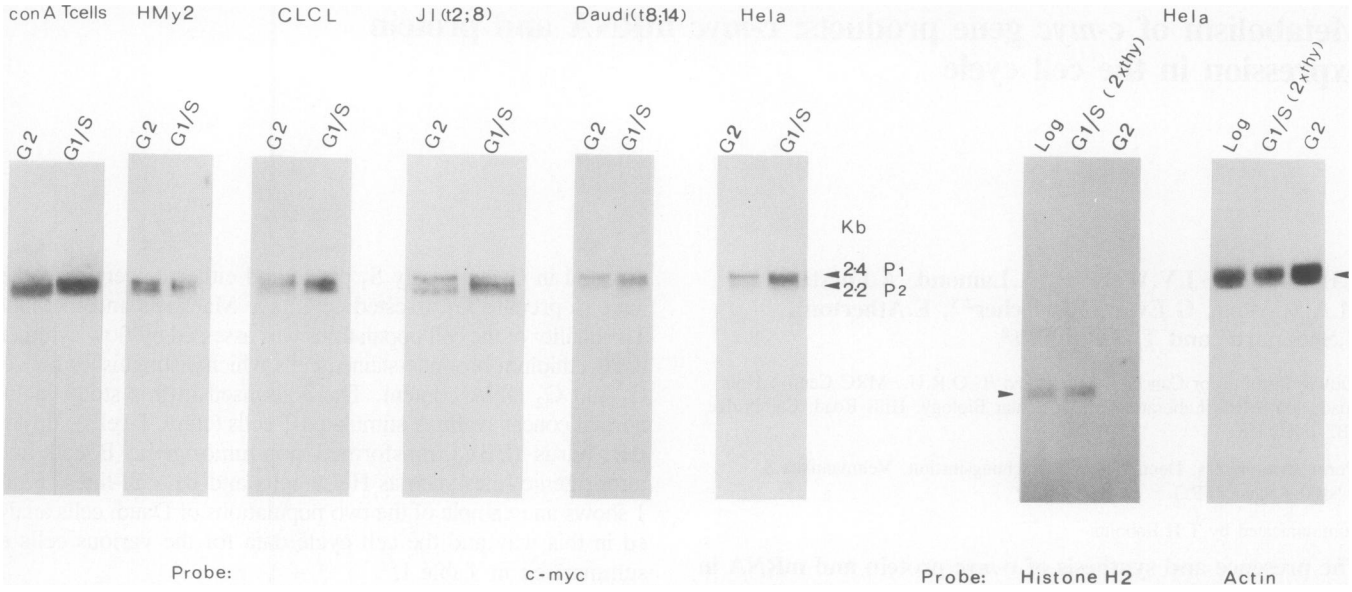

Fig. 2. Cell cycle analysis of mRNA in Burkitt's lymphoma and other cell lines. The cell lines indicated were arrested with cell cycle inhibitors as described in G_1/S or G_2, RNA extracted and 10 μg aliquots analysed by Northern filter hybridisation as described Rabbitts *et al.*, (1984). Northern filters were probed with c-*myc* cDNA clone pUCCDIA (Rabbitts *et al.*, 1983), the actin clone pRT3 or a histone H2 clone, pH2A (a gift from Dr M.Waye). The two mRNA species which derive from P1 (2.4 kb) or P2 (2.2 kb) of the c-*myc* gene are indicated. 2 x thy = double thymidine block.

Table I. Flow cytometry data of DNA content in cell cycle populations

Cell line	G_1 arrested		G_2/M arrested	
	% in G_1/S	G_2	% in G_1/S	G_2
Daudi	90	10	30	70
J1	95	5	13	87
Hmy2	91	9	58	42[a]
HeLa	85	15	20	80
CLCL	80	20	20	80
ConA T-cells	80	20	30	70

[a]In this preparation of Hmy2 G_2-enriched cells the number of cells in S phase was unusually high. If only the G_1 and G_2 cells are scored, the proportion of G_1 (excluding S) to G_2 cells is ~20–80%.

feature of some of the tumour cell lines used here is that the G_2 mRNA population is enriched for the type of transcript which comes from the P1 promoter of c-*myc*, e.g., HeLa (Figure 2).] The HeLa cell mRNA was also analysed with a histone H2 probe as a control for the cell cycle populations since H2 mRNA is closely linked to DNA synthesis in HeLa cells (Plumb *et al.*, 1984). In agreement with the previous data, we found that HeLa cells, arrested at the G_1/S boundary by double thymidine block, contained histone H2 mRNA, but those cells arrested in G_2 contained no detectable histone mRNA. These results show that histone gene transcription does show cell cycle control and support the validity of our blocked populations. We can conclude from these data, therefore, that c-*myc* mRNA persists in the G_2 phase of the cell cycle in a variety of cell types. Therefore, although c-*myc* mRNA is induced in early G_1 after mitogen stimulation of normal T cells (Kelly *et al.*, 1983), its presence persists into the G_2 phase. Although c-*myc* mRNA has a short half-life *in vivo* (Dani *et al.*, 1984; McCormack *et al.*, 1984) abnormal stabilities may occur in some situations (T.H.Rabbitts, A.Forster, M.A.Stinson, A.Lamond and P.H.Rabbitts, in

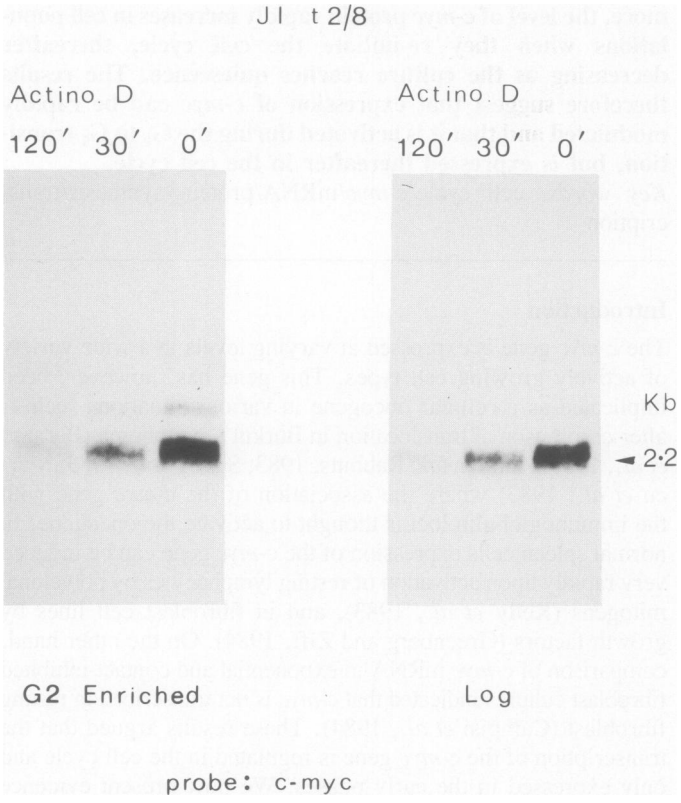

Fig. 3. Turnover of c-*myc* mRNA in exponential and G_2-arrested Burkitt's lymphoma cells. J1 cells were grown in exponential (log) phase and an aliquot arrested in G_2 using colcemid as described in Materials and methods. The log phase and G_2-arrested cultures were treated with 5 μg/ml actinomycin D, aliquots removed at the indicated times and RNA prepared. 10 μg amounts of RNA were analysed by Northern filter hybridisation (Thomas, 1980) using pUCCDIA c-*myc* cDNA (Hamlyn and Rabbitts, 1983).

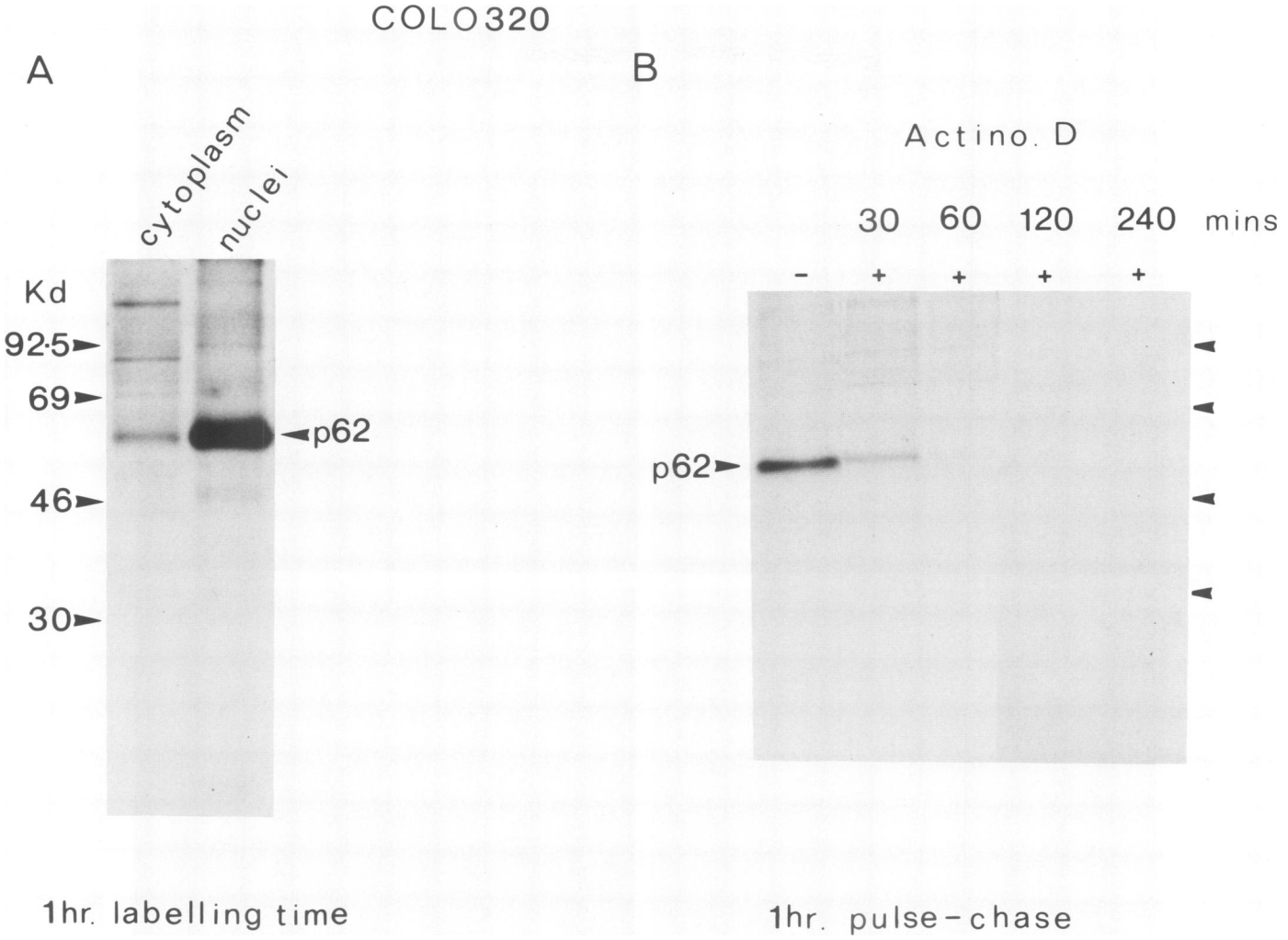

Fig. 4. c-*myc* protein turnover *in vivo*. **(A)** Colo320 cells were incubated for 30 min in lysine-free medium and for 1 h in the presence of [^{3}H]lysine prior to preparation of nuclei and cytoplasmic fractions from disrupted cells. c-*myc* protein was precipitated from the separated fractions using the anti-*myc* antiserum. The size of the c-*myc* protein (p62) was estimated by reference to co-electrophoresed ^{14}C-labelled mol. wt. markers. **(B)** Colo320 cells were labelled for 1 h with [^{3}H]lysine, the cells washed and an aliquot removed for c-*myc* protein precipitation (t_0). The remaining cells were incubated (in the absence of [^{3}H]lysine) for 30 min, 60 min, 120 min and 240 min in the presence of 5 μg/ml actinomycin D. After the respective incubation time, cells were collected, proteins extracted and immunoprecipitated using the anti-*myc* antiserum. Identical results were obtained in analogous pulse-chase experiments in the absence of actinomycin D.

preparation). Therefore we tested whether the G_2 c-*myc* mRNA was due to transcription of the gene. Accordingly exponential (log) cells or G_2-arrested JI cells were treated with actinomycin D to inhibit RNA transcription and samples removed at various times for RNA extraction. The RNA samples were analysed by filter hybridisation for the presence of c-*myc* mRNA (Figure 3). Two points emerge from this experiment. First, the level of c-*myc* mRNA observed in log phase or G_2 phase cells is similar and second, the mRNA turnover pattern and kinetics are very similar in log or G_2 cells. This shows that the *myc* mRNA we observe in log phase cells (predominantly G_1) and in the G_2 cells is a *de novo* transcription product of the c-*myc* gene.

c-myc protein metabolism in vivo

The experiments described in the previous section indicated that G_1 and G_2 cells transcribe c-*myc* mRNA. To eliminate any possibility that this results from artefacts due to the use of cell cycle inhibitors, we undertook to study the c-*myc* protein expression and turnover in the cell cycle. Initially we studied the *in vivo* longevity of c-*myc* protein in colo320 DM using an anti-*myc* antiserum raised against a synthetic peptide representing the carboxy 15 amino acids of the predicted human c-*myc* protein (peptide sequence E-Q-L-K-H-K-L-E-Q-L-R-N-S-C-A). This antiserum precipitates the c-*myc* protein which has a mol. wt. of ~62 000 daltons [corresponding to the c-*myc* protein previously reported (Hann and Eisenman, 1984; Persson *et al.*, 1984)]. Colo320 DM cells were labelled for 1 h in the presence of [^{3}H]lysine and the cells were fractionated into nuclei and cytoplasm after breaking the cells by nitrogen cavitation. The c-*myc* protein was subsequently precipitated from both cellular fractions and analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 4A). These experiments showed that, after labelling for 1 h, the vast majority of c-*myc* protein can be found within the nuclear fraction. Since isotope was continuously present throughout the labelling time and since a complete protein can be made, per ribosome, in between 1 and 7 min, the lack of cytoplasmic labelled protein indicates rapid transport of the c-*myc* protein to the nuclear compartment.

The stability of the c-*myc* protein was tested by carrying out pulse-chase protein labelling experiments with colo320 cells. Cells were labelled for 1 h, recovered by centrifugation, washed and resuspended. An aliquot of the labelled cells was removed

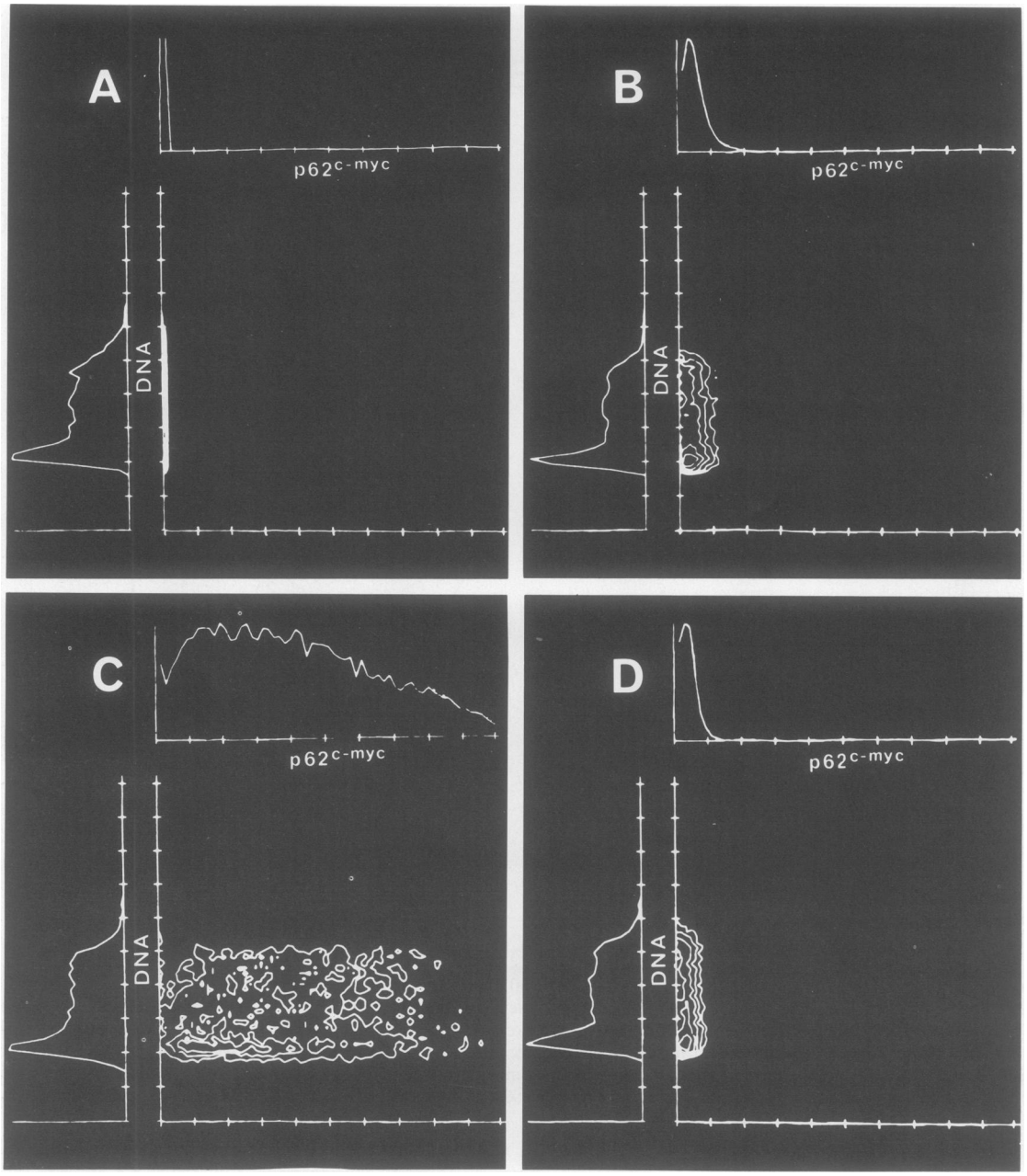

Fig. 5. Detection of c-*myc* protein in NIH 3T3 cells using anti-*myc* antibody. Exponentially growing NIH 3T3 cells were treated by the freeze-thaw procedure and simultaneously assayed for DNA content and c-*myc* protein. c-*myc* protein was detected using 6E10 anti-*myc* monoclonal antibody binding followed with fluorescent anti-mouse immunoglobulin; fluorescence due to *myc* protein was determined by flow cytometry as described in Materials and methods. Each panel of the figure shows DNA content on the ordinate and c-*myc* content on the abscissa. Each experiment was carried out in the presence of propidium iodide and with the antibody additions shown below. **Panels A** and **B** show the propidium iodide (PI) only stained control and the PI plus second antibody (FITC-conjugated) control, respectively. **Panel C** shows the results with the 6E10 anti-*myc* antibody plus FITC-conjugated second antibody and PI. **Panel D** shows that the specific c-*myc* fluorescence was completely blocked by incubating 6E10 with 1.0 μg of the immunising peptide.

and proteins extracted; the remaining cells were subsequently incubated in the absence of [^{3}H]lysine (in the presence or absence of actinomycin D). Aliquots of this culture were removed at 30, 60, 120 and 240 min for protein extraction. The c-*myc* protein was precipitated from the various samples with the anti-*myc* antiserum and analysed by SDS-PAGE (Figure 4B). We observed a rapid decrease of c-*myc* protein with ~50% of the labelled protein remaining after 30 min chase and no detectable protein after a 2 h chase. We can estimate that, assuming linear turnover of c-*myc* protein, the half-life is ~30 min or less. These results are consistent with the results of Hann and Eisenman (1984).

The analogous situation was investigated in NIH 3T3 cells using an assay for c-*myc* protein which involves the binding of a monoclonal antibody (designated 6E10, raised using a synthetic peptide corresponding to a central region of exon 2; Evan, Lewis, Ramsay and Bishop, in preparation) to the c-*myc* protein *in vivo* and visualisation with fluorescent-developing antibody. The detection of c-*myc* protein in NIH 3T3 cells is illustrated in Figure 5 where patterns of DNA fluorescence are plotted against antibody fluorescence. The DNA pattern breaks down into G_1, S and G_2 phases based in the cellular DNA; panel C shows the fluorescence associated with the *myc* protein present in G_1, S and G_2 cells. This fluorescence is reduced to background levels (shown in panels A and B) by addition of excess of the immunis-

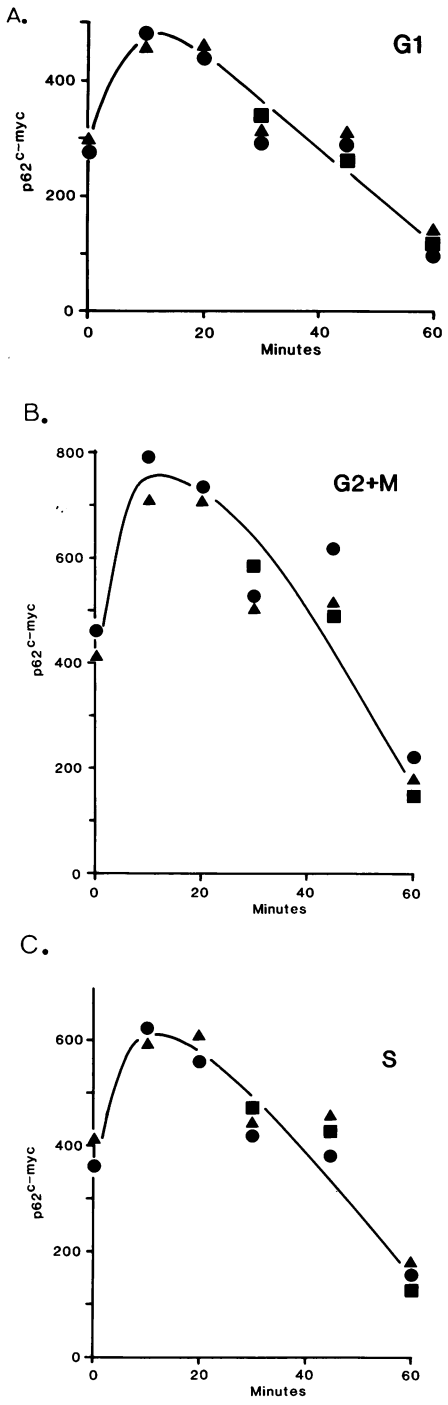

Fig. 6. Turnover of c-*myc* protein in the cell cycle of NIH 3T3 cells. NIH 3T3 cells were grown in exponential phase, pooled and seeded into 75 cm^2 tissue culture flasks (3 x 10^5 cells per flask). After overnight growth, medium was removed and fresh medium, supplemented with 100 μg/ml cycloheximide, added. Samples were subjected to the freeze-thaw procedure at the various times indicated and subsequently analysed as a batch. For each time point, antibody fluorescence was measured by the flow cytometer method in the presence and absence of anti-*myc* 6E10 for background subtraction. The fluorescence value for a given time point was calculated using 'in-house' computer programming. DNA content was measured using propidium iodide staining and the peaks of G_1, S and G_2/M DNA content were separately analysed for c-*myc* protein fluorescence. **(A)** Time course of c-*myc* decay in G_1 phase cells. **(B)** Time course of c-*myc* decay in mid-S phase cells. **(C)** Time course of c-*myc* decay in G_2/M phase cells. The data are duplicate samples for time zero, 10 and 20 min and triplicate samples for the remaining points.

ing peptide as a competitor (panel D). Thus c-*myc* protein, at least as defined by the epitope recognised by the 6E10 anti-*myc* antibody, exists in NIH 3T3 cells in G_1, S and in G_2.

The synthesis and turnover time of the c-*myc* protein detected in this assay was examined by blocking protein synthesis with cycloheximide (CHI) followed by a quantitative determination of the anti-*myc* fluorescence. These results are graphically shown, in Figure 6, where the amount of fluorescence (due to antibody binding) was calculated, as a function of time, for the cells in G_1 (Figure 6A), S (Figure 6B) or G_2 (Figure 6C). Interestingly, all three curves show an initial apparent superinduction of c-*myc* protein as judged by the fluorescence assay. This is presumably analogous to that observed for c-*myc* mRNA upon treatment of cells with CHI (Kelly *et al.*, 1983). The increased fluorescence is followed by a rapid decrease, consistent with *in vivo* turnover of the *myc* protein with a half-life of 20–30 min. This figure is very comparable with the observed time in the labelling experiments of colo320 cells (Figure 4) but does not exclude the possibility that differences may exist in other cells where it is conceivable that protein stabilising factors operate. A further conclusion is that since the c-*myc* protein turns over, after cessation of protein synthesis, the epitope detected by the antibody must be due to *de novo* protein synthesis. These data, therefore, validate the results obtained on mRNA present in cell cycle-arrested cells and show that c-*myc* expression occurs throughout G_1 and G_2 cell cycle phases.

c-myc protein expression during cell culture senescence

The rapidity with which c-*myc* mRNA can be modulated is suggestive of a regulatory system crucial for cell division. We have analysed c-*myc* protein expression in populations of NIH 3T3 starting from quiescent cells which are reactivated by plating at low density in the presence of serum and analysed as the cells passage through the quiescence. c-*myc* fluorescence in the G_1 population and the percentage of cells in S phase were monitored at times after replating quiescent cells. Figure 7 shows the graphical representation of these results. Within 24 h of replating, the level of c-*myc* protein reaches its maximum induced level. The level of c-*myc* protein in the population then plateaus for ~24 h and subsequently decays to the basal level within a further 2-day period. Interestingly, the curve for the increase and subsequent decrease in the proportion of cells undergoing DNA synthesis (i.e., involved in S phase) parallels the pattern of c-*myc* protein but the appearance of c-*myc* protein precedes the increase of S phase cells. This observation parallels the observed stimulation of c-*myc* mRNA in activation of lymphoid and fibroblast cells (Kelly *et al.*, 1983; Greenberg and Ziff, 1984) and formally demonstrates that the synchronised increase in mRNA is paralled by production of c-*myc* protein.

Discussion

Cell cycle expression of c-myc

Although data exist which show that c-*myc* transcription can be rapidly induced (Kelly *et al.*, 1983) and apparently switched off in stationary phase fibroblasts (Campisi *et al.*, 1984) we find that, in all cells tested, c-*myc* mRNA and protein occurs in both G_1 and G_2 phases of the cell cycle (Figure 1). The cells tested include mitogen-stimulated T cells as well as various tumour cells. Thus it would seem that c-*myc* mRNA can be induced early in G_1 phase of resting cells but that it persists (and as the gene is transcribed) through the division cycle, at least into G_2 when DNA replication is essentially complete (as judged by flow cytometric

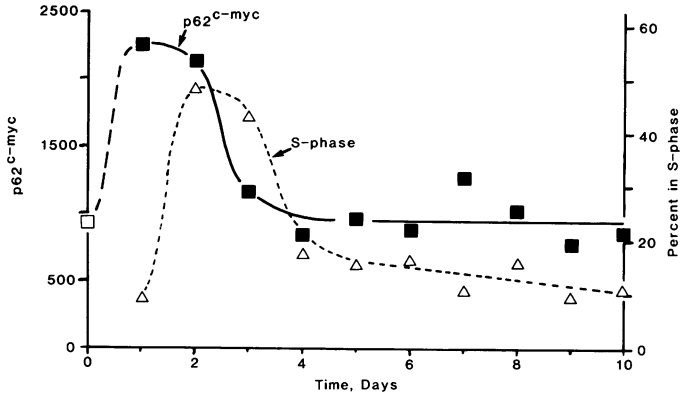

Fig. 7. Relationship between c-*myc* protein content and S phase cells in NIH 3T3 cultures. A culture of NIH 3T3 cells grown in RPMI plus 10% foetal calf serum was allowed to reach stationary phase for 4 days at confluence. The cells were split into a set of 75 cm^2 flasks (2 x 10^5 cells per flask) and grown for 24 h before replenishment of medium. At each time shown in the graph, a flask of cells was treated by the freeze-thaw method, DNA content and c-*myc* protein estimated by flow cytometry as described in Materials and methods. The proportion of cells in S phase and the amount of c-*myc* protein was calculated and displayed graphically. ■ c-*myc* protein fluorescence detection; △ percentage of cells in S phase.

analysis of DNA content in the G_2-arrested populations used here).

A key question is the function of the c-*myc* protein. The results described here and recently elsewhere (Thompson *et al.*, 1985; Hann *et al.*, 1985) show that c-*myc* mRNA and protein are available in G_1, S and G_2 stages of the cell cycle. We have extended these observations to show that the mRNA and protein present at the various stages result from *de novo* synthesis. Further, the rate of c-*myc* protein turnover in various cell cycle phases is similar (~20–30 min in NIH 3T3 cells), and the overall protein level can be shown to decay as mass cell cultures proceed to quiescence. Thus a picture emerges in which the c-*myc* gene (whose expression is capable of modulation post-transcriptionally by rapid turnover of either mRNA or protein) is activated in the G_0 to G_1 transition by external stimuli (e.g., growth factors). Each round of cell division may then require external stimuli to produce a new round of DNA synthesis. Extrapolating to tumour cells such as Burkitt's lymphoma, it is possible that translocation of the c-*myc* gene circumvents the need for additional external stimuli by allowing constitutive c-*myc* gene transcription or by altering mRNA longevity *in vivo* (T.H.Rabbits, A.Forster, M.A.Stinson, A.Lamond and P.H.Rabbitts, in preparation). The availability of antibodies to the c-*myc* protein should now allow us to isolate the eukaryotic c-*myc* protein and to carry out biochemical studies which should help to elucidate the function of this protein.

Materials and methods

Preparation of cell cycle populations

Cells were inhibited in the cell cycle by a number of procedures. Log phase cultures of J1, Daudi, CLCL and conA T-cells were treated for 17 h with 1 mM hydroxyurea to produce G_1/S populations. G_2/M cells were obtained by releasing from hydroxyurea arrest for 7 h followed by incubation for 17 h in the presence of 0.06 μg/ml colcemid or 200 μg/ml razoxane. ConA stimulation of mouse splenic T cells was carried out as described in Kelly *et al.* (1983). G_1/S HeLa cells were obtained by incubation of log phase cells with 2 mM thymidine for two 17 h periods with 2 h release in normal medium between. G_2/M HeLa cells were prepared by releasing from the double thymidine block for 7 h followed by colcemid arrest as above. The cell content of DNA was estimated by flow cytometry. Cells were stained for DNA content with ethidium bromide (EB) using a modification of the method described (Taylor, 1980). Briefly, 125 μl of staining solution containing 45 mg/100 ml EB in 1% (v/v) Triton X-100 were added to 0.1 ml aliquots of cells in growth medium containing between 10^5 and 10^6 cells. This procedure releases isolated nuclei thus removing the majority of the RNA which would otherwise also fluoresce with EB. After a minimum staining time of 5 min, the samples were analysed on the Cambridge MRC custom-built flow cytometer (Watson, 1980, 1981). The data are presented as percentage values calculated from histograms of cell number *versus* DNA fluorescence.

Analysis of RNA

RNA was prepared, fractionated and analysed by filter hybridisation as described (Rabbitts *et al.*, 1984).

Analysis of protein

Cell labelling. Cells were labelled with [^{3}H]lysine for 1 h (10^7 cells/ml). Nuclei were prepared from these cells disrupted by nitrogen cavitation, extracted with RIPA buffer (Abrams *et al.*, 1982) and the c-*myc* protein precipitated with the anti-*myc* peptide antiserum (see below). Antigen-antibody complex was recovered using protein A-Sepharose and labelled protein released by boiling in the presence of SDS and β-mercaptoethanol. Proteins were analysed by SDS-PAGE using 10% polyacrylamide. Sizes of marker proteins (M) are indicated.

The anti-*myc* antiserum was a rabbit antiserum raised against a 15 amino acid long peptide from the carboxy terminus of the predicted c-*myc* protein (E-Q-L-K-H-K-L-E-Q-L-R-N-S-C-A). The peptide was prepared according to previously published solid-phase procedures using a polar, polydimethylacrylamide support and Nα-fluorenylmethoxycarbonyl amino acids. Protection of amino acid side chain functional groups was by the t-butyl group, apart from cysteine, histidine and arginine which were protected by acetamidomethyl, fluorenyl-methoxycarbonyl and bis-adamantyloxycarbonyl groups, respectively. Couplings were achieved by the preformed symmetrical anhydride method apart from the glutamine and lysine which were added as *p*-nitrophenyl esters catalysed by addition of hydroxybenzotriazole. After cleavage from the resin the desired arginine peptide product was separated from an ornithinine-yielding contaminant by ion-exchange chromatography on carboxymethyl cellulose CM52 and isolated in an overall yield of 40% (found Asp, 1.00; Ser, 0.48; Glu, 4.31; Ala, 0.98; Cys, n.d.; Leu, 2.76; His, 0.97; Lys, 1.95; Arg, 0.9). H.p.l.c. analysis showed the peptide to be at least 95% pure; the low analytical results for Ser adjacent to Cys has also been observed in other instances (unpublished). The peptide was coupled to keyhole limpet haemocyanin and rabbits were immunised as described (Green *et al.*, 1982). The crude antiserum from the animals was pooled (after radioimmunoassay for peptide binding activity) and protein precipitated with ammonium sulphate. The precipitated material was affinity-purified by binding to protein A-Sepharose.

Fluorescence detection of c-myc protein. NIH 3T3 cells, grown in RPMI plus 10% FCS were prepared by the freeze-thaw technique previously described (Ganesan *et al.*, 1981; P.J.Smith, C.O.Anderson, J.Milner and J.V.Watson, in preparation). Treated cells were resuspended in 20 μl of 20 μg/ml 6E10 monoclonal anti-*myc* antibody (in PBS). After 45 min at room temperature, samples were centrifuged and the pellets incubated with 10 μl of fluorescein isothiocyanate-conjugated rabbit immunoglobulin. (Controls were carried out with only second antibody to assess background fluorescence.) After a further 60 min, 0.5 ml of 50 μg/ml propidium iodide was added for fluorescence assay of DNA. Fluorescence assays were carried out in the MRC custom-built flow cytometer. The A164-05 Spectra-Physics argon ion laser (Mountainview, CA, USA) tuned to the 488 nm line at a light power of 100 mW was used to excite green fluorescence from fluorescein-tagged immunoglobulin and red from propidium iodide-stained DNA. The fluorescence signals from each cell together with forward and 90° light scatter were quantitated by photodetectors. The red and green signals were separated by a dichroic beam splitter which reflects light below and transmits above 580 nm. The green and red photomultipliers were additionally guarded by a 515–560 nm band pass and 630 nm long pass filter, respectively.

Cell lines

J1 (Burkitt's lymphoma t2;8) (Bernheim *et al.*, 1981). Daudi (BL t8;14) (Klein *et al.*, 1968). DHLCL and CLCL (EBV-transformed lymphoblastoid cell lines). Hmy2 (lymphoblastsoid tumorigenic cell line). Colo320 (colon carcinoma) (Alitalo *et al.*, 1983).

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