MOLECULAR AND CELLULAR BIOLOGY, June 1988, p. 2504–2512
0270-7306/88/062504-09$02.00/0

Vol. 8, No. 6

c-*myc* and c-*myb* Protein Degradation: Effect of Metabolic Inhibitors and Heat Shock

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Received 18 December 1987/Accepted 9 March 1988

The proteins encoded by both viral and cellular forms of the c-*myc* oncogene have been previously demonstrated to have exceptionally short in vivo half-lives. In this paper we report a comparative study on the parameters affecting turnover of nuclear oncoproteins c-*myc*, c-*myb*, and the rapidly metabolized cytoplasmic enzyme ornithine decarboxylase. The degradation of all three proteins required metabolic energy, did not result in production of cleavage intermediates, and did not involve lysosomes or ubiquitin. A five- to eightfold increase in the half-life of c-*myc* proteins, and a twofold increase in the half-life of c-*myb* proteins was detected after heat-shock treatment at 46°C. In contrast, heat shock had no effect on the turnover of ornithine decarboxylase. Heat shock also had the effect of increasing the rate of c-*myc* protein synthesis twofold, whereas c-*myb* protein synthesis was decreased nearly fourfold. The increased stability and synthesis of c-*myc* proteins led to an overall increase in the total level of c-*myc* proteins in response to heat-shock treatment. Furthermore, treatments which reduced c-*myc* and c-*myb* protein turnover, such as heat shock and exposure to inhibitors of metabolic energy production, resulted in reduced detergent solubility of both proteins. The recovery from heat shock, as measured by increased turnover and solubility, was energy dependent and considerably more rapid in thermotolerant cells.

The modulation of expression of the c-*myc* gene in response to changes in growth state and the genetic rearrangements known to occur at this locus in a wide variety of neoplasms have led to the notion that the c-*myc* gene products play a role in the control of cell proliferation and differentiation (for reviews, see references 7, 8, and 12). Although attempts have been made to relate c-*myc* function to DNA replication (47), gene expression (25), and nuclear structure (11, 13, 46), none of the results has been definitive.

The production of *myc*-specific antisera has allowed the identification of several major protein species with apparent molecular sizes of 59 and 62 kilodaltons (kDa) in avian cells and 64 and 67 kDa in human cells (2, 17, 18, 27, 37, 39). These major forms are the result of two distinct translation-initiation sites (19). The minor forms are most likely due to phosphorylation or other, as yet unidentified, posttranslational modifications (B. Lüscher and D. Gillespie, unpublished observation). All of the different protein species were found to be phosphorylated and localized predominantly in the nucleus and to possess DNA-binding properties in vitro (1, 10, 11, 18, 39).

One of the most striking features of *myc* proteins is their rapid turnover rate. The c-*myc* proteins in all cell types analyzed to date exhibit a rapid rate of degradation relative to most cellular proteins. This has been demonstrated in different cell types from as evolutionarily widely separated species as humans, rodents, birds, and amphibians (3, 18, 27, 33, 41). In general, it appears that this short half-life ($t_{1/2}$) is not significantly affected by alterations in c-*myc* gene structure brought about by translocation, amplification, and retroviral insertion or transduction. This high turnover rate of c-*myc* proteins is also maintained throughout the cell cycle (33). The $t_{1/2}$ for *myc* proteins in all of these systems falls in the range of 15 to 50 min as determined by sequential immunoprecipitations of c-*myc* proteins after pulse-labeling of cells with radioactive amino acids (pulse) followed by continued growth in unlabeled medium (chase).

The c-*myc* mRNA has also been reported to have a short $t_{1/2}$ on the order of 10 to 30 min (9, 32). The rapid degradation rates of c-*myc* mRNA and proteins indicate tight control over steady-state protein levels in the cell. One might expect that changes in the transcription rate and/or in the $t_{1/2}$ of the mRNA would lead to rapid fluctuations in the levels of c-*myc* proteins in the cell. The possibility that the degradation of *myc* proteins may be linked to their biochemical function as well as to their regulation has led us to undertake experiments aimed at defining some of the basic characteristics of c-*myc* protein turnover.

In this paper we report the effects of metabolic and proteolytic inhibitors as well as heat shock on *myc* protein metabolism. The results obtained for c-*myc* proteins were compared with those for v-*myc* and two other short-lived proteins, c-*myb* and ornithine decarboxylase (ODC). c-*myb* is a nuclear phosphoprotein with an apparent molecular size of 75 and 80 kDa in avian and human cells, respectively (4, 28, 29). c-*myb* protein has DNA-binding properties and a $t_{1/2}$ of around 60 min (28). ODC is a cytoplasmic enzyme which catalyzes the conversion of ornithine to putrescine, the first and rate-limiting step in polyamine synthesis (48). ODC has an apparent molecular size of 51 kDa and a $t_{1/2}$ of 10 to 20 min (22, 24). Our results indicate both shared and unique features in the metabolism of these three proteins.

MATERIALS AND METHODS

Cell lines and chemicals. The tumor-derived chicken bursal lymphoma cell line BK3A was obtained from M. Linial (Fred Hutchinson Cancer Research Center). All drugs were from Sigma Chemical Co. (St. Louis, Mo.).

Antisera. The preparation of anti-*myc* sera recognizing the C-terminal 12 amino acids of chicken and human *myc* proteins, respectively, and a serum raised against bacterially expressed human c-*myc* proteins and their specificities have been described (17, 18, 27). Anti-*myb* sera raised against a

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N-terminal 115-amino-acid polypeptide BP52 were obtained from J. Lipsick (University of California, San Diego) (4). An antiserum raised against BP52 in our laboratory had the same specificity and was used for the results shown in Fig. 1B, 3, and 8. Anti-ODC serum was obtained from O. Jänne (Rockefeller University, New York) (24).

Metabolic cell labeling, immunoprecipitation, and gel electrophoresis. Exponentially growing cells were preincubated in methionine-free medium for 10 min before the addition of [^{35}S]methionine. After a 20-min pulse-labeling, cells were diluted 10-fold into complete medium and pelleted (pulse). The cells were then sampled (10 to 15 $\times$ 10^6 cells per sample) and harvested after further incubation as described in the figure legends (chase). Drugs were usually added from 100$\times$ or 1,000$\times$ stock solutions. For heat-shock treatment, samples were shifted to and incubated in a water bath at 46°C. Incubations were stopped by diluting the samples into ice-cold complete medium. Cells were either lysed in Ab buffer (20 mM Tris hydrochloride [pH 7.4], 50 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 [NP-40], 0.5% deoxycholate [DOC], 0.5% sodium dodecyl sulfate [SDS], 0.5% aprotinin, 10 mM iodoacetamide) and further processed for immunoprecipitation (18) or lysed in STM-N/D (250 mM sucrose, 50 mM Tris hydrochloride [pH 7.5], 5 mM $MgCl_2$, 0.5% aprotinin, 10 mM iodoacetamide, 0.5% NP-40, 0.5% DOC) for subcellular fractionation. Cells in STM-N/D were incubated on ice for 5 min, and the residual nuclei were pelleted and lysed in Ab buffer. The supernatants were adjusted to 0.5% SDS. The processing of the samples for immunoprecipitation was as described above. Immunoprecipitations were done sequentially with 1 μl of anti-ODC serum, 5 μl of anti-*myb* serum, and 5 μg of affinity-purified anti-*myc* antibodies. Absorption and washing of the immunocomplexes and SDS-polyacrylamide gel electrophoresis were as described previously (18). The following prestained molecular size markers (Bethesda Research Laboratories, Inc.) were used: myosin (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (46 kDa), α-chymotrypsinogen (25.7 kDa), β-lactoglobulin (18.4 kDa), and lysosyme (14.3 kDa).

Determination of $t_{1/2}$. To determine the $t_{1/2}$ of a particular protein, fluorographs of the appropriate gel were densitometrically scanned (see Fig. 1), or the bands were cut out from the dried gel, counted, and corrected for background (see Fig. 2, 3, 5, 7, and 8). The $t_{1/2}$ of *myc* proteins detected by immunoblotting was determined by densitometric scanning of photographic negatives (see Fig. 6). The $t_{1/2}$ was calculated as $(0.693 \times t)/\ln(N_0/N)$, where t is the chase time and N_0/N is the fraction of initially labeled protein (N_0) remaining (44).

Immunoblotting. BK3A cells (3 μg of packed cells) were solubilized in SDS-polyacrylamide gel electrophoresis sample buffer, separated on a 10% discontinuous SDS-polyacrylamide gel, and transferred to nitrocellulose as described previously (50). The c-*myc* proteins were visualized by staining with antipeptide serum and goat anti-rabbit alkaline phosphatase conjugate. The antibody incubations and the washings were done in phosphate-buffered saline containing 5% milk powder and 0.5% NP-40 (23).

RESULTS

General characteristics of c-*myc* protein turnover. We examined the rapid degradation of c-*myc* proteins by analysis of radiolabeled proteins in the avian bursal lymphoma cell line BK3A (32) because it produces high levels of apparently normal c-*myc* proteins. BK3A cells were pulse-labeled for 20 min with [^{35}S]methionine (Fig. 1, lane 1) and chased in excess cold methionine for the times indicated (lanes 2 through 4) before lysis and immunoprecipitation with specific anti-*myc* antisera (see Materials and Methods). The two major c-*myc* protein species, p59 and p62, were degraded with an approximate $t_{1/2}$ of 20 min as determined by densitometric scanning of fluorographs. The two minor protein species p61 and p65 exhibited a slightly longer $t_{1/2}$. The apparent decrease in degradation rate seen for c-*myc* proteins during the longer chase times (Fig. 2B; 120 to 240 min; longer exposed fluorographs were used to localize the protein bands) may reflect greater stability of a small portion of the proteins or, alternatively, may be due to a low level of incorporation of residual label. We note the absence of any major immunoprecipitable fragments resulting from proteolytic degradation. In kinetic experiments carried out with [^{3}H]leucine to uniformly label the *myc* protein and antisera reactive against both N- and C-terminal domains of c-*myc* we also were unable to detect *myc*-specific degradation intermediates (data not shown).

To establish whether the disappearance of [^{35}S]methionine-labeled c-*myc* proteins in pulse-chase experiments reflected actual degradation rather than sequestration into a compartment inaccessible to antibody after lysis, we measured turnover of total c-*myc* proteins by using immunoblotting. Unlabeled BK3A cells were chased in complete medium in the presence of emetine, a protein synthesis inhibitor, to allow decay of steady-state c-*myc* proteins in the absence of new protein synthesis. After different chase times, cells were pelleted and lysed in SDS-polyacrylamide gel electrophoresis sample buffer (containing 5% SDS and 4% β-mercaptoethanol) and boiled, and the amount of c-*myc* protein was determined by immunoblotting (see Fig. 6). The degradation rate of c-*myc* proteins detected by blotting after treatment with protein synthesis inhibitors (approximately 20 min) was comparable to the degradation rate of [^{35}S]methionine-labeled c-*myc* proteins. Similar $t_{1/2}$s for c-*myc* proteins were obtained when [^{35}S]methionine-labeled BK3A cells were lysed in 5% SDS and diluted before immunoprecipitation (data not shown). These results demonstrated that immunoprecipitation measures a representative fraction of *myc* molecules, independent of their age.

Degradation of *myc* proteins is energy dependent. It has been suggested that the degradation of proteins, in particular short-lived proteins, requires metabolic energy even though the cleavage of peptide bonds per se is exergonic (for a review, see reference 21). To establish whether metabolic energy is required for c-*myc* protein turnover, we first pulse-labeled cells and then chased in the presence of NaF, NaN_3, NaCN, or 2-deoxyglucose, agents which are known to interfere with intracellular ATP production (21). Different concentrations and combinations of these inhibitors interfered to various extents with c-*myc* protein degradation (Fig. 1A); 50 mM NaF–50 mM NaN_3 (Fig. 1A, lanes 10 through 12) and 10 mM NaCN–20 mM 2-deoxyglucose (Fig. 1A, lanes 5 through 7) were found to be the most effective, with nearly complete inhibition of turnover. Somewhat less effective was 20 mM 2-deoxyglucose on its own (Fig. 1A, lanes 15 through 17) or 10 mM NaF–10 mM NaN_3 (data not shown). When after the 30-min chase the inhibitors were washed out and the cells were incubated for another 30 or 90 min in regular medium, the degradation of c-*myc* proteins returned rapidly to its control rate (Fig. 1A, lanes 8 and 9, 13 and 14, and 18 and 19). This demonstrated that energy is required for the turnover of c-*myc* proteins and that they can be revers-

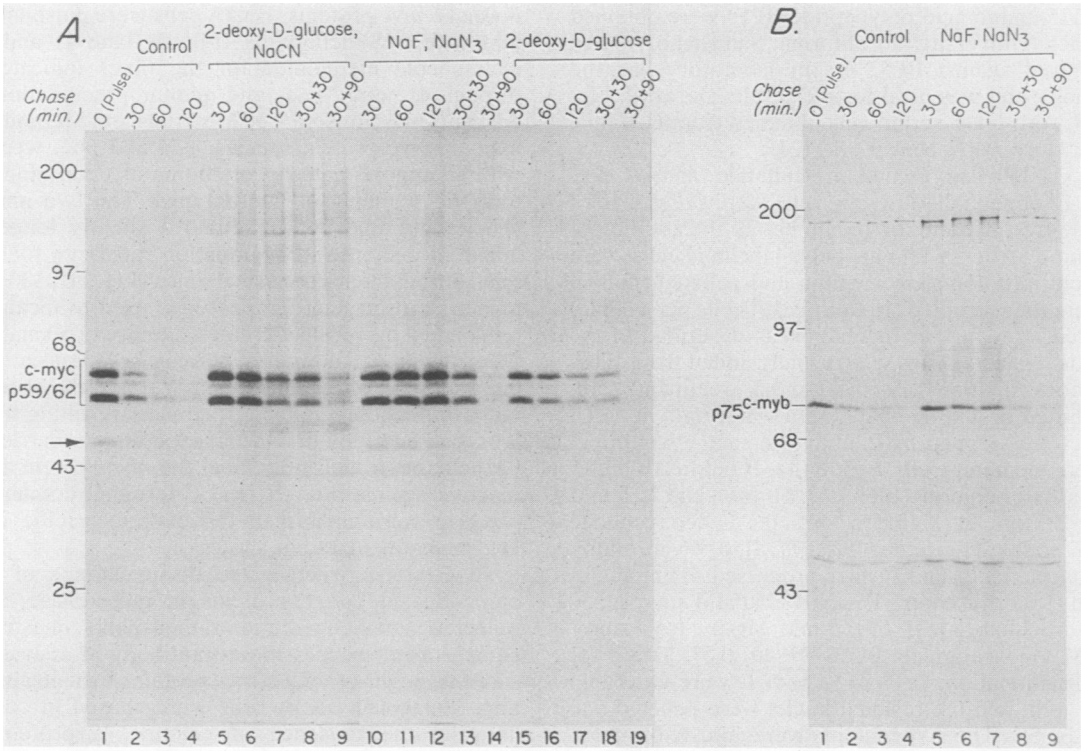

FIG. 1. Effect of metabolic energy inhibitors on the degradation of c-*myc* (A) and c-*myb* (B) proteins. BK3A cells were pulse-labeled for 20 min with [^{35}S]methionine as described in Materials and Methods and chased in unlabeled medium for the indicated times before lysis in Ab buffer and immunoprecipitation with anti-*myc* peptide serum or anti-*myb* expression vector serum. The concentrations of the metabolic inhibitors were 20 mM 2-deoxy-D-glucose, 10 mM NaCN, 50 mM NaF, and 50 mM NaN_3. In panel A, lanes 8, 9, 13, 14, 18, and 19, and in panel B, lanes 8 and 9, the cells were washed after 30 min to remove inhibitors and further incubated as indicated in normal medium. The arrow points to a c-*myc*-specific polypeptide, probably resulting from initiation of protein synthesis at an internal AUG (unpublished observation).

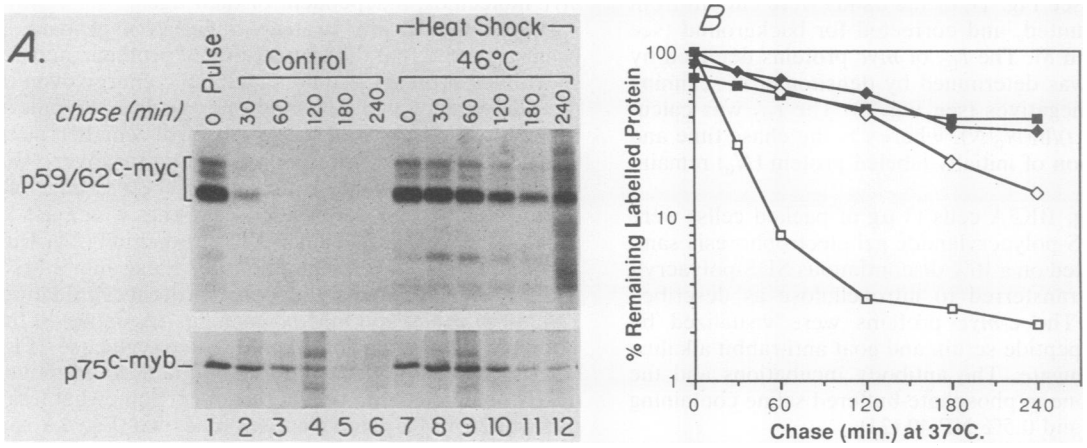

FIG. 2. Effect of heat shock on c-*myc* and c-*myb* protein turnover. (A) BK3A cells were pulsed for 20 min with [^{35}S]methionine and chased at 37°C without further treatment (control) or chased at 37°C after a 30-min heat shock at 46°C. Cell lysates were sequentially immunoprecipitated with anti-c-*myb* and anti-c-*myc* antibodies. Only the relevant portions of the gels are shown. (B) Graphic representation of the degradation of c-*myc* and c-*myb* proteins shown in panel A. Relevant bands were cut out from the dried gels and counted. The data from the pulse served as the reference (100%) for all time points. The first time point (0 min) for the heat-shocked samples represents the degradation observed during the heat-shock treatment. Symbols: c-*myc* protein control (□), c-*myc* protein after heat shock (■), c-*myb* protein control (◇), and c-*myb* protein after heat shock (◆).

ibly stabilized by the inhibition of metabolic energy production. Consistent with the fact that these agents effectively inhibited energy production, we found that [^{35}S]methionine incorporation into total cellular proteins dropped over 20-fold within 10 min after cells were pretreated with 10 mM NaF–10 mM NaN_3 or 10 mM NaCN–20 mM 2-deoxyglucose (data not shown). However, the block to protein synthesis itself is not responsible for the stabilization of c-*myc* proteins, since inhibitors of protein synthesis had only a marginal effect on the c-*myc* $t_{1/2}$ (see Fig. 6) (data not shown).

The two other short-lived proteins, p75$^{c\text{-}myb}$ (Fig. 1B) and p51^{ODC} (data not shown), also revealed an ATP dependent degradation. Again, 50 mM NaF–50 mM NaN_3 and the combination of 2-deoxyglucose and NaCN were the most effective. However, whereas c-*myc* and c-*myb* protein turnover was nearly completely inhibited, ODC turnover was less affected, with only a twofold increase in $t_{1/2}$ (data not shown). The block in c-*myb* protein turnover by ATP production inhibitors was reversible (Fig. 1B), similar to the result obtained for c-*myc* proteins.

Effect of heat shock on *myc* protein turnover. Since a number of studies have suggested a connection between heat shock and intracellular protein degradation (for reviews, see references 31 and 43), we became interested in determining the possible effect of heat shock treatment on the turnover of c-*myc*, c-*myb*, and ODC proteins. A heat shock at 46°C for 30 min after pulse-labeling of BK3A cells had a profound effect on c-*myc* protein turnover, increasing the $t_{1/2}$ to >3 h, whereas in non-heat-shocked cells c-*myc* proteins turned over with the expected $t_{1/2}$ of 15 to 20 min (Fig. 2). The same stabilization after heat-shock treatment was observed when degradation of total c-*myc* proteins was assayed by immunoblotting. BK3A cells were either mock treated at 37°C or heat shocked at 46°C for 30 min and further incubated at 37°C in the presence of emetine (see Fig. 6). In control cells c-*myc* proteins were undetectable after a chase of 4 h, whereas after the heat-shock treatment c-*myc* proteins were detectable even after 6 h of chase ($t_{1/2}$, >3 h). The heat-shock treatment also affected c-*myb* protein turnover but not as drastically as for c-*myc* protein. The c-*myb* protein half-life (80 min in control cells) was reproducibly increased about twofold (Fig. 2).

We have shown that under our conditions (30 min, 46°C), the 24-, 70-, and 89-kDa heat shock proteins are synthesized in BK3A cells (data not shown) (26). The treatment did not cause cell death; however, a delay in cell growth was noted (data not shown).

We next determined to what extent the time of exposure to high temperature affected the degradation rate of c-*myc* and c-*myb* proteins. BK3A cells were exposed for various lengths of time to heat shock temperature (46°C) after pulse-labeling (Fig. 3). The cells were then further incubated at 37°C up to a total chase time of 2 h, and levels of labeled c-*myc* and c-*myb* proteins were determined. Increased length of exposure to heat-shock temperature resulted in a decreased degradation rate of c-*myc* proteins. Whereas a 10-min incubation at 46°C increased the stability slightly, incubation for 20 or 30 min decreased the turnover rate of c-*myc* proteins significantly, with $t_{1/2}$s of around 90 and 220 min, respectively. Again, the effect on c-*myb* protein turnover, although significant, was less substantial, increasing the $t_{1/2}$ from around 75 min in the control sample to 150 min after a 30-min exposure to heat shock. Further increasing the length of heat-shock treatment had little additional effect on the degradation of the two nuclear proteins. In contrast, heat shock treatment at 46°C had no effect on the rate of degradation of ODC (Fig. 4). During the incubation at heat shock temperature the rate of ODC turnover was essentially the same as that in non-heat-shocked cells.

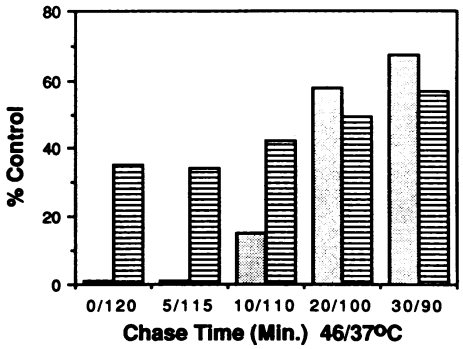

FIG. 3. Turnover of c-*myc* and c-*myb* proteins after exposure for different lengths of time to heat-shock temperature. BK3A cells were pulsed for 20 min at 37°C with [^{35}S]methionine and chased for different lengths of time at 46°C as indicated. Cell samples were then shifted to 37°C and incubated up to 120 min (total chase time). Cell lysates were sequentially immunoprecipitated with anti-c-*myc* and anti-c-*myb* antibodies. The relevant bands were cut out of the dried gels and counted. The data from the pulse represents 100% (control). A graphic representation of these results is displayed: c-*myc* protein (□), c-*myb* protein (▤).

Effect of heat shock on c-*myc* protein synthesis. To determine whether heat shock affected synthesis as well as turnover of c-*myc* proteins, we allowed heat-shocked BK3A cells (30 min, 46°C) to recover for different lengths of time at 37°C before pulse-chase labeling. In a typical experiment, the rate of protein synthesis (as measured by [^{35}S]methionine uptake) was reduced to 20 to 30% of control levels when cells were labeled immediately after the heat-shock treatment. During the recovery, protein synthesis gradually increased, reaching control levels at about 150 min after heat shock. In addition, increased synthesis of heat shock proteins was observed when total [^{35}S]methionine-labeled cell lysates were analyzed by SDS-polyacrylamide gel electro-

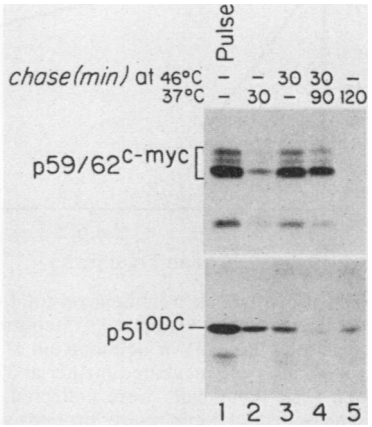

FIG. 4. Effect of heat shock on ODC turnover in comparison to c-*myc* proteins. BK3A cells were pulsed for 20 min with [^{35}S]methionine and chased at 46°C or at 37°C as indicated. Cell lysates were sequentially immunoprecipitated with anti-ODC and anti-c-*myc* antibodies. Only the relevant portions of the gels are shown.

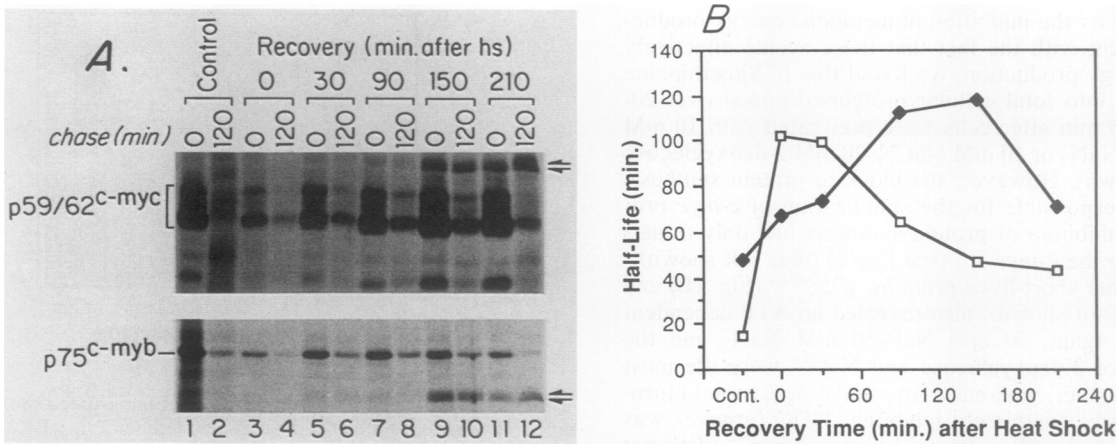

FIG. 5. Analysis of synthesis and turnover of c-*myc* and c-*myb* proteins during the recovery after heat-shock treatment. BK3A cells were either untreated (control) or heat shocked for 30 min at 46°C and allowed to recover as indicated. The samples were then pulsed for 20 min at 37°C with [^{35}S]methionine and chased as indicated. (A) Cell lysates were sequentially immunoprecipitated with anti-c-*myb* and anti-c-*myc* antibodies. Arrows point to proteins comigrating with the 70- and 71-kDa heat shock proteins, which are nonspecifically coimmunoprecipitated. Only the relevant portions of the gel are shown. (B) The $t_{1/2}$s of c-*myc* (□) and c-*myb* (◆) proteins were determined according to the formula $t_{1/2} = (0.693 \times t)/\ln(N_0/N)$, where t is the chase time, N_0 is the counts per minute of pulse samples, and N is the counts per minute of chase samples. Data are based on 120-min chase time.

phoresis (data not shown). c-*myc* protein synthesis, after an initial drop, reached its highest levels (1.5- to 2-fold over those of controls) around 120 min after heat shock treatment and then returned to control levels (Fig. 5A). The turnover rate of c-*myc* proteins during the recovery was significantly decreased, with $t_{1/2}$ increasing to >100 min immediately after the heat-shock treatment (Fig. 5B). Increased stability of c-*myc* proteins in response to heat shock was also evident by following the quantity of c-*myc* proteins by immunoblotting in the presence of emetine (Fig. 6). With increasing length of recovery time, the $t_{1/2}$ slowly decreased, reaching about 50 min after 210 min. In contrast, c-*myb* protein behaved differently. The synthesis decreased similarly to c-*myc* and total protein syntheses. However, during the course of the experiment, the synthesis of c-*myb* protein never reached control levels (two- to fourfold reduced) (Fig. 5A). The turnover rate increased gradually to about 100 to 120 min (Fig. 5B).

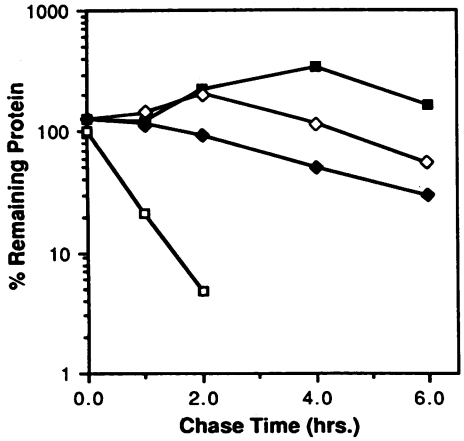

FIG. 6. Effect of heat-shock treatment on total c-*myc* proteins. BK3A cells were incubated for 30 min at 37°C (control) or 46°C (heat shock). Then emetine (100 μM) or actinomycin D (10 μg/ml) was added, and the samples were incubated further at 37°C as indicated (chase). At each time point cells were collected, lysed in SDS-sample buffer, and boiled for 5 min. c-*myc* proteins were detected by immunoblotting with anti-c-*myc* antibodies, followed by goat anti-rabbit coupled alkaline phosphatase. The immunoblots were photographed and the intensity of the bands determined by densitometric scanning of the negatives. Symbols: control, emetine (□); heat shock, emetine (◆); heat shock, untreated (■); heat shock, actinomycin D (◇).

When heat-shocked BK3A cells were allowed to recover for much longer periods (18 h), they attained degradation rates comparable to those of control cells (data not shown).

The 1.5- to 2-fold-increased rate of c-*myc* protein synthesis over that of the control and the decreased degradation rate would predict an increased total amount of c-*myc* proteins after heat shock treatment. As expected, we could detect increased levels of c-*myc* proteins by immunoblotting after recovery from heat shock (three- to fourfold at 4 h) (Fig. 6). This increase in steady-state c-*myc* proteins was dependent on new RNA synthesis, since it was inhibited by actinomycin D treatment (Fig. 6), i.e., after an initial increase in total amount, the degradation rate was similar to emetine-treated, heat-shocked cells. Northern blot analysis revealed no differences in c-*myc* and c-*myb* mRNA levels in response to heat shock treatment in BK3A cells (data not shown).

We conclude from these experiments that the accumulation of c-*myc* proteins in response to heat shock treatment results mainly from changes in its $t_{1/2}$ and to a lesser extent from increased translation.

***myc* protein extractability in response to heat shock.** In a recent paper, Evan and Hancock (13) describe the reduced extractability of c-*myc* proteins after heat shock treatment. They found that c-*myc* proteins were resistant to high salt extraction from DNase-treated nuclei prepared from heat-shocked cells, whereas c-*myc* proteins were readily solubilized from control nuclei. We have used detergent extraction to evaluate the solubility of c-*myc* proteins and the possible relationship of solubility to c-*myc* protein metabolism. This was carried out by assaying for the amount of *myc* proteins in the detergent supernatant and the nuclear pellet fraction

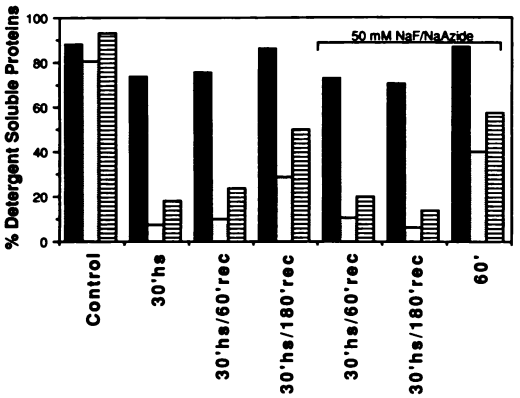

FIG. 7. Detergent extractability of c-*myc*, c-*myb*, and ODC proteins. BK3A cells were pulsed for 20 min with [^{35}S]methionine and chased under different conditions as indicated. At each time point, cells were lysed in 0.5% NP-40–DOC, and the residual nuclei were pelleted and dispersed in lysis buffer. The supernatants were adjusted to 0.5% SDS. Total labeled proteins from the supernatant and the nuclear fractions were determined by trichloroacetic acid precipitation. The distribution of specific proteins was determined by immunoprecipitation in both fractions. Relevant bands were then cut out of the dried gels and counted. The graph displays percent detergent-soluble proteins of the combined supernatant and nuclear pellet fractions. Abbreviations: chase times in minutes ('), pulse-labeled cells (control), heat shock at 46°C (hs), recovery at 37°C (rec). Symbols: total protein (■), c-*myc* protein (□), c-*myb* protein (▤).

during a pulse-chase experiment. The nonionic detergent NP-40 at a concentration of 0.5% solubilized 30 to 50% of pulse-labeled c-*myc* proteins, whereas a 0.5% NP-40–DOC mixture solubilized 80 to 90%. (Our usual lysing buffer containing 0.5% NP-40–DOC–SDS used for immunoprecipitation solubilizes more than 95% *myc* proteins from both control and heat-shocked cells.)

We then determined the effect of heat shock before lysis on the detergent solubility of c-*myc* proteins. Heat shock treatment of cells for 30 min at 46°C reduced the soluble fraction of c-*myc* proteins in NP-40–DOC to less than 10% (Fig. 7). During the recovery period after the heat shock, the solubility increased to around 30% after 3 h. This resolubilization was completely blocked when the recovery occurred in the presence of 50 mM NaN_3–50 mM NaF. Similar results were obtained with c-*myb* protein; a significant decrease in solubility was also observed after heat shock treatment, and resolubilization during the recovery was energy dependent. In general c-*myb* protein appeared slightly more soluble in NP-40–DOC than c-*myc* protein under all conditions tested (see also below). Using the same extraction procedure we determined the amount of [^{35}S]methionine-labeled proteins associated with the residual nuclear structures before and after heat shock (Fig. 7). This treatment decreased the NP-40–DOC-soluble fraction from 88 to 74%. This amount increased during recovery and reached control levels after 3 h. Again, no resolubilization was observed when energy was depleted. It therefore appeared that heat shock treatment resulted in the reduced solubility in NP-40–DOC of a significant fraction of total proteins and, in particular, in the almost complete insolubility of c-*myc* and c-*myb*, followed by an energy-dependent resolubilization during recovery.

To establish the effect of metabolic energy inhibitors on protein solubility, pulse-labeled BK3A cells were chased for 60 min in 50 mM NaF–NaN_3. The solubility of c-*myc* and c-*myb* proteins was reduced by about 50%, whereas no or only little effect was seen for total proteins (Fig. 7). Little reduction in solubility of c-*myc* and c-*myb* proteins was observed when BK3A cells were heat shocked before pulse-labeling and subsequent lysis in 0.5% NP-40–DOC (data not shown). These results demonstrated that the solubilities of c-*myc* and c-*myb* proteins synthesized before or after the heat-shock treatment were quantitatively different. Since heat shock before or after metabolic labeling as well as a chase in the presence of energy production inhibitors interfered profoundly with c-*myc* and c-*myb* protein turnover, we tested the two other turnover-inhibiting drugs, chloroquine and *o*-phenanthroline (33), for their effects on extractability of the two nuclear proteins. c-*myc* proteins were insoluble after an exposure of cells to either of the two drugs for 30 min, whereas in control cells (pulsed or pulse-chased) the majority were soluble (data not shown). Again, c-*myb* protein behaved similarly to c-*myc* proteins.

We conclude from these results that all the treatments affecting c-*myc* and c-*myb* protein turnover were accompanied by a reduction in solubility in NP-40–DOC.

Adaptation of *myc* protein metabolism to heat shock. The results shown in Fig. 7 demonstrated a reduced solubility of c-*myc* and c-*myb* proteins in response to heat shock followed by slow resolubilization which was inhibited by energy inhibitors. It has been suggested that protein resolubilization after heat shock may be mediated by hsp70 (38). Therefore it was of interest to examine the kinetics of *myc* protein resolubilization in thermotolerant cells (20); i.e., in cells which have been subjected to an initial heat shock before labeling with [^{35}S]methionine, followed by a second heat shock. Since such cells contain preexisting heat-shock proteins at the time of labeling (30, 31), we reasoned that their presence might affect the kinetics and energy dependence of c-*myc* protein resolubilization. The results (Fig. 8) indicate that this is the case. Although in thermotolerant cells there was still a significant decrease in solubility of c-*myc* and c-*myb* proteins, this decrease was less marked than that in control cells. In addition, the resolubilization of both proteins appeared to be accelerated. Metabolic energy was required for c-*myc* and c-*myb* resolubilization, as shown by its inhibition in the presence of NaF-NaN_3. The analysis of total proteins revealed a smaller decrease in solubility after heat shock in thermotolerant cells compared with that in control cells (8 versus 15%). Within an hour, the percentage of soluble proteins returned to control levels (Fig. 8), whereas in nonthermotolerant cells 3 h of recovery was required (Fig. 7). In addition, the turnover rate after heat shock appeared to be accelerated in thermotolerant cells (around 60 min for both c-*myc* and c-*myb* proteins) compared with control cells after heat-shock treatment (Fig. 8).

We conclude that the resolubilization of c-*myc* and c-*myb* proteins is dependent on energy metabolism. Also the presence of heat shock proteins in thermotolerant cells resulted in a quicker recovery from the second heat shock as determined by the resolubilization of c-*myc*, c-*myb*, and total proteins and by the turnover rates of the two nuclear proteins.

DISCUSSION

The proteins encoded by the nuclear protooncogene c-*myc* analyzed in the bursal lymphoma cell line BK3A exhibit a rapid turnover ($t_{1/2}$, 15 to 20 min). This characteristic feature of c-*myc* and v-*myc* proteins has been established in many different cell lines (3, 17, 27, 33, 41). Our turnover studies

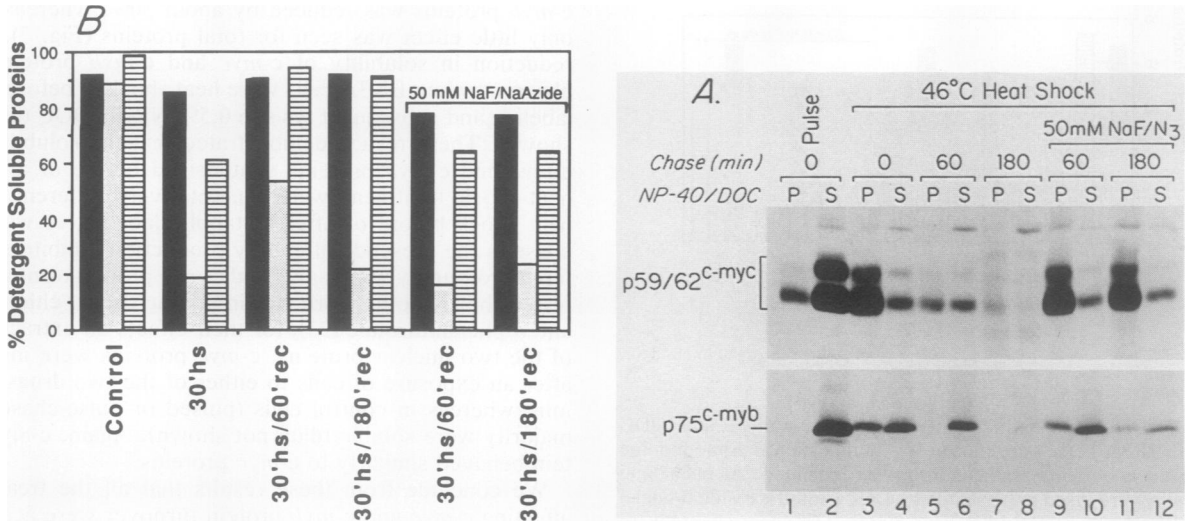

FIG. 8. Detergent extractability of c-*myc* and c-*myb* proteins in thermotolerant cells. BK3A cells were heat shocked at 46°C for 30 min, recovered at 37°C for 4 h, labeled for 20 min, heat shocked a second time, and chased as indicated. Cells were lysed, and total labeled proteins were determined as described in the legend to Fig. 7. (A) Both fractions (P, pelleted fraction of NP-40–DOC-lysed cells; S, supernatant) were sequentially immunoprecipitated with anti-c-*myc* and anti-c-*myb* antibodies. Only the relevant portions of the gels are shown. (B) The relevant bands of the gels shown in panel A were cut out and counted. Abbreviations and symbols are as described in the legend to Fig. 7.

with different antibodies and different cell lysis and labeling conditions and immunoblotting show that *myc* proteins are completely degraded. We were unable to detect either degradation intermediates or a population of c-*myc* molecules which were protected from degradation. In addition, no qualitative differences were found when the results on degradation of c-*myc* and v-*myc* proteins isolated from several other cell lines (bursal lymphomas, Burkitt's lymphomas, fibroblasts) were compared with the results described in this report.

Energy requirement for c-*myc* protein degradation. All three proteins analyzed in this study, c-*myc*, c-*myb*, and ODC, are degraded by an energy-dependent mechanism. The complete inhibition of turnover by 50 mM NaF–50 mM NaN_3 was immediately relieved by washout of the drugs, indicating that we are not simply limiting *myc* and *myb* protein turnover by inducing cell death (Fig. 1). NaF and NaN_3 at 10 mM, which completely inhibited protein synthesis within 10 min, had only a small effect on c-*myc* and c-*myb* and apparently no effect on ODC turnover (data not shown). This suggests that the proteolytic degradation of the three proteins has an ATP requirement well below that of protein synthesis. Our results on energy dependence of c-*myc* turnover appeared to hold true for different rearranged and mutated c-*myc* proteins as well as for v-*myc* proteins. This is in contradiction to a recent paper by Bader et al. (3), who concluded that the MC29 v-*myc* protein is degraded by an ATP-independent mechanism. However, the treatment they used to inhibit metabolic energy production, reducing the ATP level to less than 10% in 1 h, may not have been sufficient for an effective inhibition of *myc* protein turnover, reflecting the low-level ATP requirement demonstrated by our data.

In an attempt to identify a protease(s) involved in the degradation of one or several of the proteins studied, we screened a number of drugs with the potential capacity to inhibit different types of proteases. These studies did not yield a clear picture of a specific proteolytic system involved, since no specific inhibitors were found (data not shown). However, some general conclusions could be made. The use of lysosomal inhibitors revealed that the degradation of neither c-*myc*, c-*myb*, nor ODC was mediated by an acidic membrane-bound compartment. Therefore we suggest that the three proteins are degraded by nonlysosomal nuclear or cytoplasmic systems (40).

Very little information is available about nuclear proteases. However, a recent report identifies ubiquitin, which has been detected in both nucleus and cytoplasm, as a protease (15). We have tested whether ubiquitin (6, 14) is involved in the c-*myc* degradation pathway by using anti-ubiquitin serum to detect c-*myc*-ubiquitin conjugates. We failed to detect any such conjugates in either untreated or heat-shocked BK3A cells under conditions which would have detected 1 to 2% of c-*myc* proteins linked to ubiquitin (data not shown). In addition, vanadate, which has been described as an inhibitor of the ubiquitin pathway (49), had no effect on turnover of c-*myc*, c-*myb*, and ODC (data not shown). We conclude that ubiquitin is unlikely to play a significant role in c-*myc* protein degradation.

Few other studies have been published on the degradation of short-lived proteins. Woods and Lazarides (51) have analyzed the degradation of unassembled α- or β-spectrin and demonstrated lysosomal involvement in α- but not β-spectrin. In addition, inhibitors of energy metabolism at relatively low doses had no effect on β-spectrin, whereas α-spectrin turnover was inhibited. The inability to inhibit β-spectrin turnover with low doses of metabolic energy inhibitors, lysosomal inhibitors, ionophores, and ion-transport inhibitors is very similar to our findings for the degradation of c-*myc*, c-*myb*, and ODC proteins; none of multiple drugs belonging to one of the four classes had any effect on the degradation of the three proteins tested. Gronostajski et al. (16) analyzed the turnover of p53, a tumor-associated nuclear phosphoprotein with a short $t_{1/2}$ (~30 min). They found that the degradation of p53 is energy dependent and nonlysosomal. Interestingly, the $t_{1/2}$ increases to more than 20 h when p53 is complexed to the large T antigen in simian virus 40-transformed 3T3 cells, and the greater stability is believed to be responsible for the increase in steady-state p53 levels in these cells (35). Covalent modifications of

proteins have also been suggested as a mechanism to alter the susceptibility to degradation (40). We have found two exceptions to the rapid degradation of c-*myc* proteins, coinciding with covalent modification. First, c-*myc* proteins in metaphase-arrested cells revealed a prolonged $t_{1/2}$, which correlates with additional phosphorylation (unpublished results). In addition, we have found evidence for stabilization of a hyperphosphorylated form of c-*myc* in the Manca Burkitt lymphoma cell line, which has multiple point mutations in c-*myc* (Hayday, personal communication) but not in other Burkitt's lymphomas or in any other cell line tested. These modifications are currently under study.

Relationship between c-*myc* and c-*myb* protein solubility and degradation. It has been speculated that heat shock results in the denaturation of proteins which then become substrates for proteolysis. However, recent data on this subject are controversial (5, 36). The finding that aberrant proteins, which are highly unstable in control cells, are efficiently stabilized after heat shock treatment (34) has been taken as an argument for an alteration of the proteolytic system in response to heat shock. However, it may be concluded from our data that not all proteolytic systems involved in the turnover of short-lived proteins are affected by heat shock. In particular, the turnover of ODC in BK3A is unaltered after exposure to heat shock at 46°C (however, ODC is stabilized by heat-shock treatment at 49°C), whereas c-*myc* and c-*myb* proteins are stabilized to different degrees (Fig. 2). Evan and Hancock originally demonstrated that heat shock had the effect of rendering *myc* and *myb* proteins insoluble to high salt extraction (13). We show here that such insolubilization correlates with the inability to degrade these proteins (Fig. 2 and 7).

We have also demonstrated that the total amount of c-*myc* proteins is increased three- to fourfold in response to heat-shock treatment (Fig. 6). A similar increase was also observed for v-*myc* proteins (data not shown). The main cause for this increase seems to be a decreased degradation rate (five- to eightfold) of this protein, although we also detected an increased rate of synthesis. This increased synthesis of c-*myc* protein in heat-shocked cells may reflect a critical need for this protein during the recovery period or for the reinitiation of proliferation.

Since heat shock resulted in both stabilization and insolubilization of c-*myc* and c-*myb* proteins, we tested other agents which inhibited turnover for their effects on detergent solubility. We found that the inhibitors of metabolic energy production reduced solubility of c-*myc* and c-*myb* proteins but only by about 50%, suggesting that the effect of these drugs on the turnover of these proteins could be at least in part on the proteolytic system. In contrast, chloroquine and *o*-phenanthroline treatment reduced the solubility of c-*myc* and c-*myb* proteins drastically (over 90%) and increased the $t_{1/2}$ of both proteins, followed by slow recovery of turnover for both proteins. In any case, the reduced solubility after these treatments was due not to covalent modification of the proteins (data not shown), but rather was the result of heat denaturation (heat shock) or alteration of the metal composition in nuclei and thus interference with ionic interactions (*o*-phenanthroline). The decreased turnover rate may therefore reflect, at least in part, the inaccessibility of the proteins for a particular proteolytic system. However, a direct effect on the degradation machinery cannot be excluded. The quantitative difference in the stabilization of c-*myc* and c-*myb* proteins in response to heat shock may reflect the finding that c-*myb* protein appeared to be more soluble than c-*myc* in detergent.

The slow recovery of turnover after chloroquin and *o*-phenanthroline treatment (data not shown), as well as after heat shock, could be due to the need for an activity to resolubilize c-*myc* and c-*myb* proteins. In fact, Pelham (38) proposed that the major heat shock protein, hsp70, catalyzes the ATP-dependent reassembly of preribosomes and other ribonucleoparticles after stress treatment. Our data in primary heat-shocked and thermotolerant cells are consistent with the requirement for such an enzymatic activity.

Are the two nuclear oncogenes and ODC degraded by different nonlysosomal systems? One pattern which emerges from our studies was that inhibitors of c-*myc* and c-*myb* protein turnover did not always correlate with inhibition of ODC turnover. Several experiments suggested that ODC turnover was distinct from the degradation of the two nuclear oncoproteins. First, even though the three proteins were degraded by an energy-dependent pathway, the ATP requirement for ODC appeared substantially lower than that for c-*myc* and c-*myb* proteins (Fig. 1) (data not shown). Second, chloroquine had no appreciable effect on ODC turnover, whereas under the same conditions c-*myc* and c-*myb* protein turnover was effectively inhibited (33) (data not shown). Third, heat shock treatment at 46°C resulted in inhibition of c-*myc* and c-*myb* protein turnover and had no effect on ODC degradation (Fig. 2 and 4). At this point we cannot be certain that such differences reflect distinct proteolytic systems as opposed to differences in the nature of the substrates. However, taking the different subcellular localizations into account we would favor distinct proteolytic systems for c-*myc*, c-*myb*, and ODC proteins. Clearly one aim of future studies will be to isolate and characterize a protease or proteolytic system which may control the metabolism of these nuclear regulatory proteins.

ACKNOWLEDGMENTS

We thank Linda Akiyama and Susan Fisher for expert technical assistance and Jonathan Cooper, Juliane Firzlaff, David Gillespie, and Michael King for helpful comments during the course of this work and for critical readings of the manuscript. We are grateful to Joe Lipsick for providing the *myb* antiserum and the *myb* expression vector clone and to Olli Jänne for the ODC antiserum. We also thank Volker Vogt and Dan Putterman for the ubiquitin blots and Kendall, Audeen Wagner, and Judy Pirotte for word processing assistance.

This project was supported by Public Health Service grants to R.N.E. from the National Cancer Institute. B.L. is a recipient of postdoctoral fellowship 83.503.0.87 from the Swiss National Fund.

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