Determination of Copy Number of c-Myc Protein per Cell by Quantitative Western Blotting

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The protooncogene *c-Myc* plays a key role in growth control, differentiation, and apoptosis. An abnormally high expression of c-myc has been found to be associated with many neoplasms. *c-Myc* gene expression is usually measured at the mRNA level. Few studies have been published on quantitative Myc protein determination. A major drawback of ELISA (enzyme-linked immunosorbent assay) methods is the uncertainty of the specificity of the antibody reaction. In contrast, antibody specificity can be easily controlled by Western/immunoblotting. Here we describe a method to quantify c-Myc protein in primary human IMR90 lung fibroblasts based on Western blotting. Using a high-resolution polyacrylamide gel, we were able to differentiate the cellular c-Myc protein (64 kDa) from a c-Myc internal standard (65 kDa). We determined both the total c-Myc protein content per cell and its distribution in the cytoplasmic and nuclear fractions. About 4000 c-Myc protein molecules were detected in the cytoplasmic fraction and 29,000 copies in the nuclear fraction for proliferating human lung fibroblasts IMR90. The ratio of nuclear (active) to cytoplasmic (inactive) c-Myc protein changed from 17:1 for proliferating cells to 2.5:1 for confluent cells. © 1999 Academic Press

c-myc is a key regulator of cell growth and differentiation and myc oncoproteins are frequently activated in human cancer (for review see 1 and 2). c-myc is required for cellular proliferation and is rapidly downregulated upon differentiation and concomitant inhibition of cell proliferation (3, 4). Consistent with its role as a regulator of cell cycle progression (5, 6), the levels of c-myc mRNA and protein were found to increase rapidly in response to growth factors, with a peak in G1 and a gradual return to low levels for the rest of the cell cycle (7, 8). c-Myc protein has been demonstrated to be short-lived, phosphorylated, and mainly localized to the cell nucleus (9). As a transcription-activating factor c-Myc acts in concert with its dimerization partner Max by binding to the DNA motif CACGTG (10–12). Only a few genes have been suggested to be directly regulated by Myc/Max: ODC (ornithine decarboxylase; 13–15), α-prothymosin (16), the CAD gene (17), the cdc25 gene (18), the gene encoding the mRNA cap-binding protein (19), and the lactate dehydrogenase-A gene (LDH-A; 20). c-Myc regulates gene expression both directly (see above) or indirectly by binding to other factors such as TfII-I (21), YY1 (22), Miz-1 (23), BIN1 (24), Nmi (25), Rb- and Rb-related p107 protein (26, 27). This complexity might be considered as a "c-myc network." To date there are no reports demonstrating a precise quantitative correlation of the c-Myc expression with the expression of directly and indirectly controlled genes. It was therefore of interest to be able to determine the actual number of c-Myc protein molecules per cell. Due to the function of c-Myc as a transcription factor (9, 10, 12), the protein localized to the nucleus might be considered as "active c-Myc" in comparison to the "inactive" cytoplasmic form. Because of this and data indicating a compartmentalization of c-Myc protein (28), we determined also its distribution in nuclear and cytoplasmic compartments.

In most cases, c-myc expression has been quantified at the mRNA level (29–31), but Moore *et al.* determined total c-Myc protein molecules per cell by enzyme-linked immunosorbent assay (ELISA)[3] (32). A

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[3] Abbreviations used: ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco's Modification of Eagle Medium; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; GST, glutiathione *S*-transferase; BSA, bovine serum albumin; CPD, cumulative population doublings; PAA, polyacrylamide

disadvantage of the very sensitive ELISA procedure resides in the relative difficulty in assessing its specificity. We have tried to overcome this problem by developing an immunoblot-based quantification method for c-Myc. After standardizing the cell lysis method we were able to determine reproducibly in addition to "total c-Myc" the protein in both the cytoplasmic and nuclear fractions.

MATERIALS AND METHODS

Cell culture. IMR 90 (human lung fibroblasts; ATCC No. CCL 186) were cultivated in DMEM with 10% FCS (both Gibco, Eggenstein, FRG) and subcultivated as described before (33). Cells were seeded at 3×10^3 cells/cm^2 and for every protein preparation the cell density number (cells/cm^2) was determined. Cumulative population doublings (CPD) were evaluated according to Hayflick's procedure (34, 35).

Total protein extraction. We followed the preparation method of Kumar and Chambon (36). Briefly, the cells were washed twice with cold CMF–PBS (0.136 M NaCl, 2 mM KCl, 6.46 mM Na$_2$HPO$_4 \times$ H$_2$O, 1.47 mM KH$_2$PO$_4$, pH 7.2), scraped off with 1.5 ml CMF–PBS into a reaction tube, followed by a short centrifugation. The supernatant was reused for further scraping off residual cells. This procedure was repeated. After a centrifugation of 10 s (room temperature; 14,000g) the supernatant was well discarded and the pellet was resuspended to about 7×10^4 cells/μl in 600 mM KCl, 20 mM Tris–HCl, pH 7.8, 20% glycerol, 0.5 mM PMSF (phenylmethylsulfonyl fluoride), 2 mM DTT (dithiotreitol), 1 ng/μl aprotinin, 1 ng/μl leupeptin by flicking the tube. The suspension was frozen briefly in liquid N$_2$ and was allowed to thaw slowly on ice (about 10 min). The freeze/thaw cycle was repeated once. After vortexing for 10 s the lysates were stored at -70°C.

Protein extraction of the cytoplasmic (without nuclei) and nuclear fractions (nuclei). We used the method of Andrew and Faller (37). The cells were scraped off as described for total protein extraction, but the pellet was resuspended in 400 μl of cold buffer A (10 mM Hepes–KOH, pH 7.9, at 4°C, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 1 ng/μl aprotinin, 1 ng/μl leupeptin) by flicking the tube and was then incubated on ice for 10 min. The hypotonic buffer A caused the cells to swell and burst. The lysates were vortexed for 10 s and centrifuged (14,000g) for 10 s at room temperature. The supernatant represented the cytoplasmic fraction and was removed. For nuclear extraction the pellet was resuspended in a cold (4°C) high salt buffer C (20 mM Hepes–KOH, pH 7.9, at 4°C, 1.5 mM MgCl$_2$, 420 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 1 ng/μl aprotinin, 1 ng/μl leupeptin) and then incubated for 20 min on ice.

After vortexing for 10 s this nuclear lysate and the cytoplasmic fraction were frozen at -70°C.

c-Myc standard and antibody. The c-Myc standard used (Santa Cruz Biotechnology; Heidelberg, FRG) is an amino terminal segment (amino acids 1–262) of human c-Myc, produced as a 65-kDa GST-tagged fusion protein. It is provided as 10 μg protein/0.1 ml samples in SDS–PAGE loading buffer. Immediately before its use, the standard was diluted to stock solutions of 1 and 0.25 ng/μl in loading buffer as recommended by the supplier and containing in addition 50 ng/μl BSA. For detection, the rabbit polyclonal anti-human c-Myc IgG antibody (N-262; sc-764) from Santa Cruz was used.

PAA gel electrophoresis. A modified procedure of Laemmli (38) was used. The separation gel (10%) contained 15% glycerol. To increase the separation of the 65-kDa standard from the 64-kDa c-Myc band, a ratio of acrylamide to N',N'-methylene-bis-acrylamide of 30:0.3 (w/w) was chosen. The ratio for the stacking gel (3%) was 30:0.8 (w/w). Directly before loading, lysates from about 5×10^5 cells were treated for 5 min at room temperature with 10 μl benzonase (Merck, FRG; 25 U/μl), mixed (1:1) with loading buffer and supplemented with 2.5 or 5 μl of diluted standard (see above). After denaturation for 5 min at 65°C the samples were loaded onto the gel and electrophoresed at 16 V/cm until the blue marker of the loading buffer reached the bottom of the 12-cm gel.

Western blot with chemiluminescence detection. Proteins were transferred onto nitrocellulose membrane Immobilon-P/PVDF (Millipore; Eschborn, FRG) by semidry blotting as described elsewhere (39). After blocking overnight (4°C) with blocking solution (2% BSA in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.2% NP-40), the sheets were incubated with c-Myc antibody (1:100 dilution in blocking solution). After washing six times for 10 min with washing solution (0.5% BSA in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.2% NP-40), the sheets were treated with the secondary peroxidase-coupled anti-rabbit antibody (1:5000, in blocking solution; Dianova, Hamburg, FRG), followed by further washing (six times for 10 min). Detection was done using a commercial chemiluminescence kit (Amersham, Braunschweig, FRG). The band-intensity on the film (MP-Hyperfilm, Amersham) was determined densitometrically and analyzed by specific computer software (Image-Quant; Molecular Dynamics).

RESULTS AND DISCUSSION

Because changes of c-Myc protein levels in many cases do not correlate with changes in the mRNA levels (40–42), we decided to develop a method (1) to quantify the number of c-Myc proteins within a cell and (2) to

differentiate between the nuclear and cytoplasmic fractions.

Accuracy of the method. We used two different lysis methods—one for total protein content and one for cytoplasmic/nuclear fraction. Cell lysis methods such as shearing with fine-gauge needles or other mechanical homogenization procedures (e.g., with Dounce homogenizer) lead in most cases to losses of material (32, 43, 44). Therefore, after scraping off the cells as described under Materials and Methods, they were either completely lysed by repeated freeze/thaw cycles for extraction of total cellular protein or gently lysed in hypotonic buffer for separation of cytoplasma from intact nuclei. Lysis of nuclei was then achieved in high salt buffer. These procedures avoid losses of cells. To exclude loss of c-Myc protein bound to precipitated proteins or DNA the lysates were not centrifuged. During cell lysis, separation of the cytoplasm from intact nuclei or lysis of nuclei was controlled by microscopy. With the procedures as described the loss of cells or an incomplete extractability has largely been eliminated as a source for incorrect determination of c-Myc molecules per cell.

Specific detection of the p64 c-Myc protein. In primary human IMR-90 fibroblasts we could only detect the predominant p64 c-Myc protein. A second primary translation product, p67, translated from an ORF beginning at an unconventional CUG initiation codon at the 3′ end of exon 1, contains 15 additional amino acids at the amino terminus (45). This 67-kDa c-Myc protein, known to be present in small amounts in some cells (45), was below the detection limit of our assay. The bands of the standard (65 kDa) and the cellular c-Myc protein (64 kDa) were well separated (Fig. 1). No proteins with lower molecular weights were detected, indicating that neither degradation nor unspecific antibody binding had occurred (Fig. 1A). The molecular weights were determined using a standard proteinmarker mixture (Pharmacia; Ratingen, FRG).

Recovery efficiency of standard protein. The dilution of the c-Myc standard for Western blotting directly into the protein lysate or in sample buffer without other proteins led to intensity differences of a factor of up to 17:1 (cf. lanes 1 and 3 in Fig. 1B). In contrast, dilution of the same amount of standard c-Myc in loading buffer supplemented with 50 ng/μl BSA showed no significant difference in comparison to dilution in a protein lysate (lanes 1 and 2; Fig. 1). Therefore, to avoid the problems caused by adherence of proteins to the wall of the reaction tube, we strongly recommend dilution of the standard protein directly into the protein lysates or into loading buffer supplemented with 50 ng/μl BSA.

Importance of protease inhibitors during cell lysis. Because the c-Myc protein is very short-lived in cells (46), active protease activity during cell lysis can be a source for errors. We therefore tested the influence of aprotinin and leupeptin in addition to the PMSF used in standard protocols. These compounds inhibit a variety of proteases including kallikrein, trypsin, chymotrypsin, plasmin, papain, cathepsin B, and proteinase K. Indeed, as shown in Fig. 1B and Table 1, without the additional inhibitors aprotinin and leupeptin a 40% loss of detectable c-Myc protein was observed (even in the presence of PMSF). Therefore, both aprotinin and leupeptin were included in all experiments shown here.

Determination of the number of c-Myc protein molecules per cell by linear regression analysis. Per lane the intensity ratio of the standard (65 kDa) to the cellular c-Myc (64 kDa) was determined densitometrically and was quantified by specific computer software (Image-Quant, Molecular Dynamics). According to procedures used for quantification of competitive RT–PCR (47) the amount of the standard (added to the lysate) can then be plotted versus the intensity ratio of standard/"lysate" c-Myc. The point of equivalence (i.e., the intensity of the standard equals that of cellular c-Myc) represents the amount of the protein in the unknown probe. Since the amount of cells in the lanes differed slightly, the results were normalized according to the cell number and a 1:1 ratio was determined by linear regression analysis through the origin. From this amount of standard protein, the content of cellular c-Myc protein was calculated with 65 kDa = 1.08×10^{-19} g and thus 1 ng = 9.26×10^9 molecules for the standard.

An example of such a linear regression analysis is presented in Fig. 1C which shows satisfactory linearity using three standard concentration points (5, 2.5, and 1.25 ng) and the origin as a fourth fixed point. By using c-Myc internal standard protein losses during gel loading are not relevant and small differences in absolute intensities from lane to lane do not represent a problem. Furthermore, since such an analysis was carried out for each preparation, linearity could be checked individually.

Based on such a linear regression the results of the Western blot in Fig. 1B are shown in Table 1. We detected about 29,000 c-Myc molecules in the nucleus and about 4000 in the cytoplasm. These values are in agreement with the general observation of preferred nuclear location of c-Myc (9). Furthermore, the results for the preparation of total cellular protein and the sum of the cytoplasmic and nuclear fraction are comparable within the range of error: 46,037 ± 10,726 is statistically not different from 32,690 ± 6812 (*t* test).

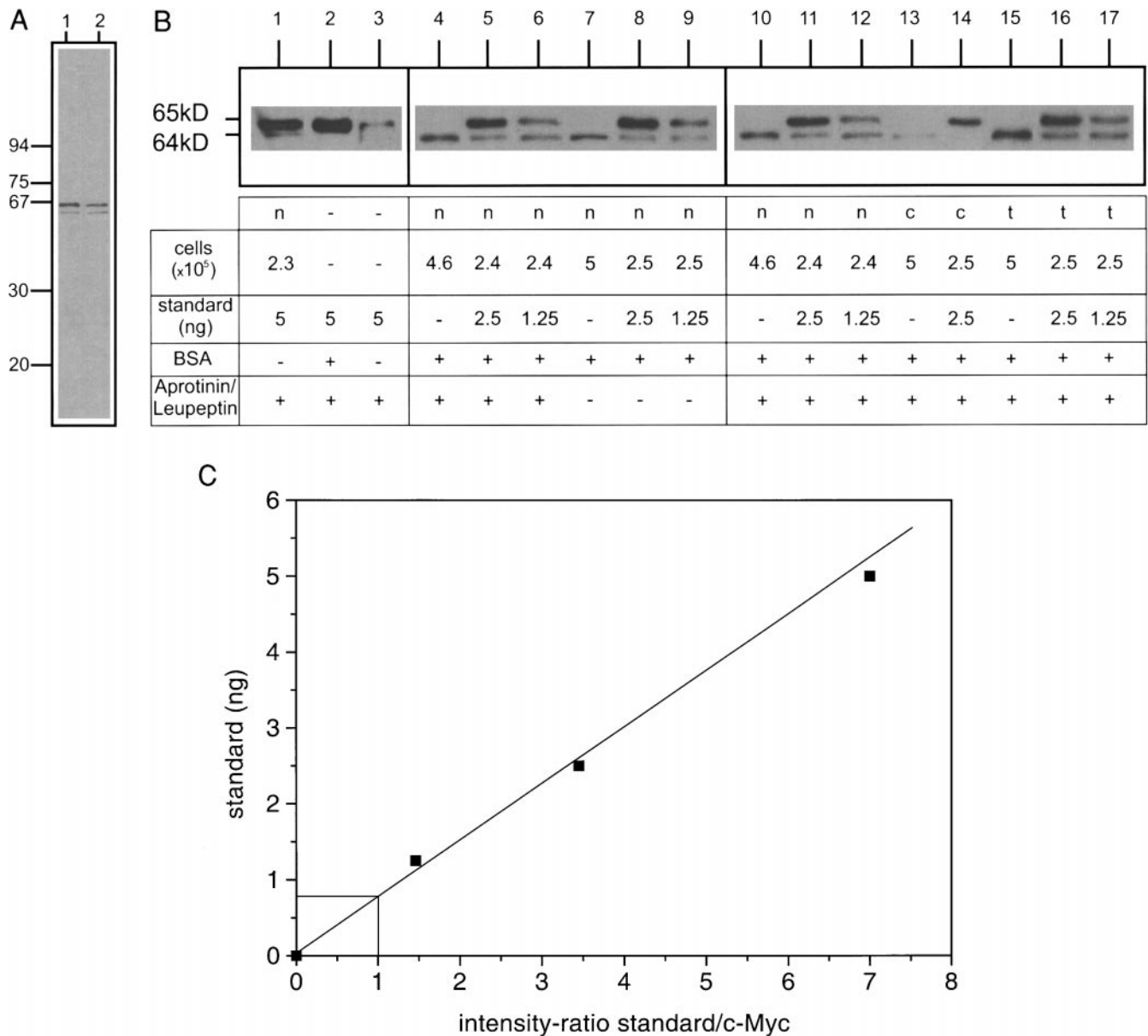Moore *et al.* measured 6300 total c-Myc protein mol-

FIG. 1. Western blot. The Western blot was carried out as described under Materials and Methods. (A) To demonstrate the specificity of the detection of cellular c-Myc (64 kDa) and the c-Myc standard (65 kDa) the whole PAA gel is shown with lane 1, 1.25 ng standard, 5×10^5 cells; lane 2, 0.625 ng standard, 5×10^5 cells; both cytoplasmic fractions. Due to the specificity of the blots, we show in B only the two bands of 65 and 64 kDa for whole cell preparations, cytoplasmic and nuclear fractions. The preparations were made from exponentially growing IMR90 lung fibroblasts with 41.56 CPD and a cell density of 3.8×10^4 cells/cm^2. The presence of BSA during dilution of the standard and the presence of the protease inhibitors aprotinin/leupeptin during the protein preparation (PMSF was always present) is indicated with (+). n, nuclear fraction; c, cytoplasmic fraction; t, total protein. (C) Example for linear regression analysis. The amount of the standard (added to the lysate) is plotted versus the intensity ratio of standard/"lysate" c-Myc. The point when the intensity of the standard equals that of cellular c-Myc (1:1 ratio) is indicated. For further details see text.

ecules per cell in serum-stimulated human MRC5 fibroblasts (33). This smaller amount could be cell type specific. With our procedure using exponentially growing human IMR90 fibroblasts, for which until now no quantitative determinations of c-Myc proteins per cell exist, we detected an average of about 35,000 c-Myc molecules per cell in several independent preparations.

To exclude the possibility that the differences between our work and Moore *et al.* are due to the use of a polyclonal antibody (sc-764; Santa Cruz) and a GST–c-Myc fusion protein as standard, while Moore *et al.* used the monoclonal 3C7 antibody and a bacterially expressed human c-Myc protein as standard, we also tested the 3C7 antibody. In the nuclear fraction of

TABLE 1

c-Myc Protein Molecules per Cell, Cytoplasm, and Nucleus in IMR90 Lung Fibroblasts

	c-Myc proteins per cell ± SD
I total cellular protein	46,037 ± 10,726
II cytoplasmatic fraction	3,961 ± 238
III nuclear fraction	28,729 ± 6,808
Σ = II + III, i.e., total cellular protein	32,690 ± 6,812
IV nuclear fraction without proteinase inhibitors	16,593 ± 1,626

Note. The results of the preparations shown in the Western blot of Fig. 1B are presented after regression analysis. All preparations were carried out with the proteinase inhibitors aprotinin and leupeptin but the preparation IV (for further explanations see text).

young (low CPD) exponentially growing IMR90 fibroblasts we measured about 45,000 c-Myc molecules per cell with the polyclonal Santa Cruz antibody and about 55,000 with the monoclonal 3C7 antibody. These results are comparable within the range of error. Since both antibodies only detected the standard, did not show any unspecific binding, and yielded comparable results, we also conclude for both the same affinity for the standard and the "lysate" c-Myc.

Another possible reason for the difference may be the lysis method. Moore *et al.* sheared the cells for cell lysis through a fine needle which could have led to losses of cells as also mentioned by these authors. They also discussed the possibility of systematic error by a factor of 5 in measuring the concentration of the standard. Including possible errors of our determination, our results and those of Moore *et al.* are comparable in the range of errors and in consideration of two different cell lines used.

In addition to the determination of the total number of c-Myc molecules per cell, we differentiated between cytoplasmic and nuclear c-Myc protein. Figure 2B shows the distribution of c-Myc proteins in cytoplasmic and nuclear fractions, determined for different points of a growth curve (Fig. 2A), i.e., during exponential growth and at confluence (see also 48). The number of total c-Myc molecules per cell decreases significantly from exponential phase to confluence. This reduction of c-Myc protein expression was also reflected by a threefold reduction of c-myc mRNA expression in this stage (48 and manuscript in preparation). Furthermore, the ratio of nuclear to cytoplasmic c-Myc protein changes significantly from 17.3 ± 2.04 during exponential growth to 2.46 ± 0.71 at confluence. Thus, in addition to the total decrease of c-Myc protein, a shift toward cytoplasmic location occurs, emphasizing the decline of available nuclear c-Myc for transcription. These changes reflect a reduced capacity to activate c-Myc target genes and thereby cell proliferation.

These changes in the distribution of nuclear and cyto-

plasmic protein in different growth states suggest that cells also regulate c-Myc function by compartmentalization, presumably through modulation of nuclear/cytoplasmic translocation of the protein. Therefore, when studying the regulation of c-Myc function not only the number of total c-Myc proteins per cell but also their distribution between nuclear and cytoplasmic compartment should be taken into account. Our procedure might turn out to be useful for this type of studies.

In summary, we have standardized a procedure to determine quantitatively and specifically the number of c-Myc proteins per cell in human IMR90 lung fibroblasts by a Western blot method. By using the internal standard and its excellent separation from the cellular c-Myc, this method might find application beyond the use in cell culture, e.g., in clinical samples. It could be a preferable alternative to an ELISA-based method due to specific detection of c-Myc proteins and possible protein degrada-
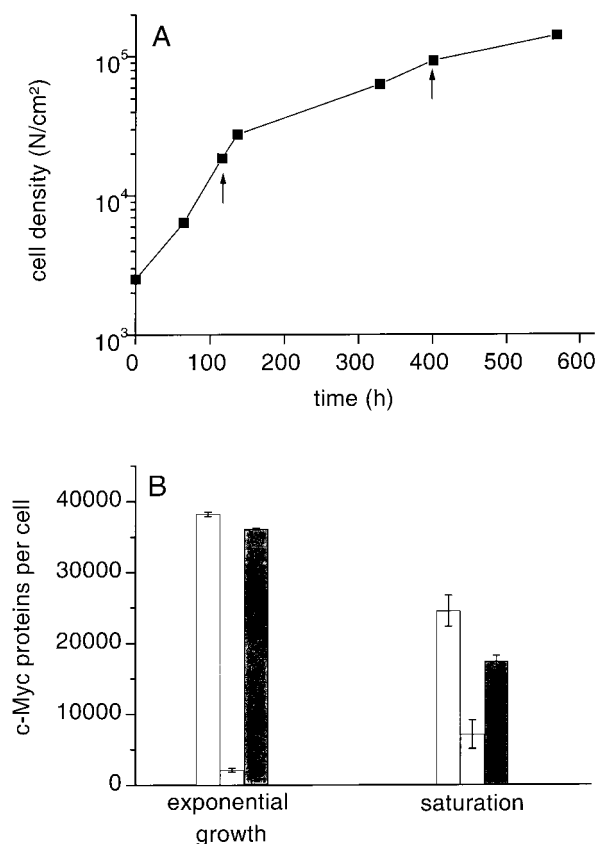

FIG. 2. Changes of c-Myc protein expression with growth conditions. (A) Growth curve of IMR90 fibroblasts as presented in Fig. 1 and Table 1, but at a CPD of 35 (cumulative population doublings). At indicated time points (arrows), c-Myc protein molecules per cell (± SD) were determined (per c-Myc determination, 3–4 different concentrations of the standard for linear regression). (B) The results during exponential growth (2.77×10^4 cells/cm²) and confluence (9.4×10^4 cells/cm²) are shown. White bars, total cellular c-Myc protein; light gray bars, cytoplasmic c-Myc protein; dark gray bars, nuclear c-Myc protein.

tions. Because we were also able to differentiate between cytoplasmic (nonfunctional) and nuclear (functional) c-Myc protein, our procedure might be suitable for further investigations on c-Myc protein expression and compartmentalization in different cell lines, growth situations, and pathological conditions.

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