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# The N-Terminal Domain of c-Myc Associates with α-Tubulin and Microtubules In Vivo and In Vitro

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**The polymerization of α- and β-tubulin into microtubules results in a complex network of microfibrils that have important structural and functional roles in all eukaryotic cells. In addition, microtubules can interact with a diverse family of polypeptides which are believed to directly promote the assembly of microtubules and to modulate their functional activity. We have demonstrated that the c-Myc oncoprotein interacts in vivo and in vitro with α-tubulin and with polymerized microtubules and have defined the binding site to the N-terminal region within the transactivation domain of c-Myc. In addition, we have shown that c-Myc colocalizes with microtubules and remains tightly bound to the microtubule network after detergent extraction of intact cells. These findings suggest a potential role for Myc-tubulin interaction in vivo.**

The product of the c-*myc* gene is a nuclear phosphoprotein that has been implicated in the regulation of cell differentiation, apoptosis, and the development of human tumors (5, 19, 28). The c-Myc protein includes multifunctional domains that include an N-terminal transactivation domain within exon II and a C-terminal domain, within exon III, which is necessary for heterodimerization (5, 19). These observations have supported the model that c-Myc functions as a transcription factor whose activity can be regulated by its protein binding partners. In addition, the N-terminal transactivation region of c-Myc binds to the retinoblastoma protein-related tumor suppressor protein p107, and it has been recently shown that this interaction can suppress the activity of the c-Myc transactivation domain (12). Further, the observation that Burkitt's lymphoma frequently contains naturally occurring somatic mutations within the transactivation domain (3, 34) which result in loss of p107 suppression confirms that this is an important functional domain of c-Myc.

Although c-Myc is characterized as a nuclear protein (10, 15), it has recently been proposed that the subcellular localization of c-Myc can vary according to the proliferation state of the cell. For example, in actively growing NIH 3T3 cells c-Myc is predominantly found in the nucleus, while a shift to the cytoplasm occurs in contact-inhibited cells (31). An increased cytoplasmic/nuclear ratio for c-Myc has also been noted following monocytic differentiation of human myeloid leukemia cells (8) and in nondividing prefertilized *Xenopus* oocytes (14). In addition, comparison of c-Myc localization patterns of normal tissue, adenomas, and colorectal tumors showed that tumor progression was associated with an accumulation of cytoplasmic c-Myc protein (27). These experiments suggested that nucleus-cytoplasm exchanges of c-Myc protein play an important functional role in cell proliferation. Since it had been proposed that tubulin mediates the cytoplasm-to-nucleus translocation of the glucocorticoid and vitamin D receptors (2, 25), we examined whether c-Myc could also interact with tubulin and microtubules. This hypothesis was also suggested by the observation that the large T antigen of simian virus 40, which shares several functional properties with the c-*myc* protein, was recently shown to associate with microtubules (22). We have demonstrated that c-Myc can interact with α-tubulin and with polymerized microtubules in vivo and in vitro through the N-terminal functional domain. In addition, our findings suggest that c-Myc is stored bound to microtubules which may act as a reservoir to sequester the c-Myc protein.

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## MATERIALS AND METHODS

**Construction of pGEX-2T plasmids.** Glutathione *S*-transferase (GST)-Myc II was constructed by an in-frame insertion of a PCR-generated exon II fragment (873 bp) into the *Sma*I site of pGEX-2T vector (Pharmacia). The 5′ and 3′ PCR primers were 5′CGCGACGATGCCCCTCAACG3′ (coordinates 4514 to 4533) and 5′TCTGCTATCTCTCCTTCCTA3′ (coordinates 5368 to 5387), respectively. GST-Myc II contains 252 amino acids encoded by exon II and 36 amino acids from intron II. GST-Myc IIa, containing only 252 amino acids encoded by exon II, was constructed similarly to GST-Myc II except that the 3′ primer used was 5′CAGAGTCGCTGCTGGTGGTGG3′ (coordinates 5256 to 5277). Both GST-Myc II and GST-Myc IIa gave identical results in a tubulin binding assay. GST-Myc III was constructed by an in-frame insertion of the blunt-end *Cla*I-*Eco*RI fragment of exon III into the *Sma*I site of the pGEX-2T vector. GST-Myc Δ51–252 and Δ135–252 were constructed by in-frame insertion of PCR-generated exon II fragments into the *Sma*I site of pGEX-2T vector. The 3′ primers were at coordinates 4651 to 4671 (5′TCCAGATATCCTCGCTGGGC3′) and 4903 to 4922 (5′CATACAGTCCTGGATGATGA3′), respectively, and the 5′ PCR primer was identical to the primer used for the GST-Myc II construct. The GST-Myc Δ51–252 and Δ135–252 mutants both initiate at the beginning of the c-Myc protein (exon II) and terminate at amino acid 50 or 134, respectively. GST-Myc Δ1–48 and Δ1–101 were constructed by in-frame insertion of *Eco*RV-*Bgl*II and *Bst*EII-*Bgl*II blunt-ended fragments into *Eco*RI-cut and blunt-ended pGEX-2T. The junction of each of the GST-Myc mutants was subjected to nucleotide sequencing to confirm the correct reading frame.

**Cell lines and antibodies.** The HL60 human myeloid leukemia cell line and the HeLa cervical carcinoma cell line were obtained from the American Type Culture Collection and grown in RPMI and Dulbecco's modified Eagle's medium, respectively, in 10% fetal calf serum (ICN Biochemicals). Anti-c-Myc clone 9E10 (11) and anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (Ab-1) were purchased from Oncogene Science. For in vivo immunoprecipitation, anti-α-tubulin and -β-tubulin monoclonal antibodies (clones DMIA and DMIB, respectively) were obtained from either Oncogene Science (purified Ab-1) or Amersham (ascites fluid) and were used with identical results. For immunoblot analysis, the anti-α-tubulin from Amersham was used. Immunofluorescence analysis was performed with rat antitubulin monoclonal antibody (YL1/2; Accurate).

**Precipitation of tubulin from cell lysates with GST-Myc.** The GST fusion proteins were expressed and purified from bacteria with glutathione-Sepharose beads as previously described (17). HL60 cells were metabolically labelled with [$^{35}$S]methionine as previously described (17). Cellular proteins were extracted from labelled and unlabelled HL60 cells in ELB buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM NaF, 0.5 mM sodium orthovanadate) containing leupeptin and aprotinin (10 μg/ml each). Labelled cells ($10^7$) and 1 mg of unlabelled cellular proteins per lane were incubated with the GST-Myc fusion proteins in 1 ml of ELB buffer for 2 h at 4°C. Following incubation the pellets were washed six times with cold buffer and subjected to sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS–7.5% PAGE). In experiments with unlabelled cellular lysates, precipitated proteins were transferred to nitrocellulose filters, immunoblotted with anti-α-tubulin, and detected with $^{125}$I-protein A as described previously (9). [$^{35}$S]methionine-labelled extracts were visualized by SDS-PAGE followed by fluorography.

**Precipitation of in vitro-transcribed and -translated tubulin with GST-Myc.** In vitro transcription and translation with [$^{35}$S]methionine of the α-tubulin cDNA clone HHCJ32 linearized with *Nco*I was performed with rabbit reticulocyte lysate as recommended by the supplier (Promega). $^{35}$S-labeled in vitro-translated α-tubulin (25 μl) was incubated with the GST fusion proteins in 0.5 ml of ELB buffer per lane as described above. Bound proteins were analyzed by SDS-PAGE followed by fluorography.

**Immunoprecipitation analysis.** HL60 protein lysate (1 mg) was immunoprecipitated with α- or β-tubulin antibodies by using agarose G beads as recommended by the manufacturer (Oncogene Science). Precipitated proteins were resolved by SDS–7.5% PAGE, transferred to the nitrocellulose filters, and immunoblotted with anti-c-Myc (clone 9E10).

**Two cycles of temperature-dependent depolymerization and polymerization of microtubules.** Tubulin purification through two cycles of polymerization was performed as previously described (22, 30). HL60 cells ($6 \times 10^8$) were sonicated in 3 ml of microtubule stabilization buffer [0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.6), 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM $MgSO_4$, 1 mM GTP, 1 mM phenylmethylsulfonyl fluoride, and 10 μg (each) of leupeptin and aprotinin per ml], incubated for 15 min at 2°C, and centrifuged for 1 h at 50,000 × *g* at 2°C. The cleared supernatant was incubated at 37°C for 30 min to allow polymerization of tubulin into microtubules (first cycle). The polymerized microtubules were pelleted at 50,000 × *g* at 37°C for 1 h, and the pellet was resuspended in 500 μl of the above-described buffer (2% each of the pellet and supernatant after the first round of polymerization [pellet I and Supt I] were loaded into the gel). The pellet from the first cycle was sonicated in microtubule solubilization buffer and incubated on ice to depolymerize microtubules. The cold insoluble fraction (CIF) was removed by centrifugation, and 100% of the CIF was loaded into the gel. The cleared supernatant was incubated at 37°C for 30 min to allow the second polymerization, generating pellet II and Supt II (100% each of pellet II and Supt II were loaded). The indicated amounts of protein fraction from each cycle were resolved by SDS-PAGE, and proteins were transferred to nitrocellulose filters and immunoblotted with anti-c-Myc. The membrane was stripped in 0.2 M glycine-HCl (pH 2.3)–0.5 M NaCl and incubated sequentially with anti-α-tubulin (Amersham) and anti-PCNA (Oncogene Science).

**Subcellular fractionation, immunoblot, and competition analysis.** HeLa cells (50% confluent) were collected by scraping and fractionated as described by Vriz (31). Cytoplasmic and nucleoplasmic fractions (50 μg each) were resolved by SDS–7.5% PAGE, transferred to nitrocellulose filters, and incubated sequentially with α-tubulin and c-Myc antibodies. Blocking of the c-Myc antibody was performed by preincubating the c-Myc antibody (clone 9E10) with Myc epitope-tagged ADP-ribosylation factor (ARF) protein (1:10 molar ratio) (26) in a total volume of 200 μl of phosphate-buffered saline (PBS) for a 2 h at room temperature.

**Colocalization of c-Myc and tubulin by double immunofluorescence analysis.** HeLa cells ($10^4$ per well) were plated on multiwell slide, grown overnight, and then fixed with methanol at −20°C and permeabilized with 0.1% Triton as previously described (36). The fixed cells were blocked with 3% bovine serum albumin–10% goat serum in PBS for 30 min and then incubated sequentially with the indicated primary and secondary antibodies for 1 h each. c-Myc or PCNA (10 μg/ml) was used first and detected with Texas red-conjugated goat antibody $F(ab')_2$ to mouse immunoglobulin G (IgG) (Oncogene Science). Cells were washed three times with PBS, incubated with rat monoclonal antitubulin antibody (YL1/2; Accurate) (10 μg/ml), and detected with fluorescein-conjugated goat antibody to rat IgG (Oncogene Science). The specificity of the c-Myc antibody was demonstrated by preincubating the c-Myc antibody with Myc epitope-tagged ARF (26) in blocking buffer (1:10 ratio) for 2 h at 4°C. In blocking experiments the c-Myc was detected with fluorescein-conjugated goat antibody to mouse IgG (Cappel).

Detergent extraction of cells under conditions that stabilize or destabilize microtubules was performed as previously described (7). Cells were extracted in microtubule-stabilizing buffer (0.1 M PIPES [pH 6.9], 2 mM EGTA, 4% polyethylene glycol, 0.2% Triton X-100, 2 mM dithiothreitol) at 37°C. Extractions were also carried out under microtubule-destabilizing conditions (1 mM EDTA, 2 mM dithiothreitol, and 1% Nonidet P-40 in PBS) at 0°C. Extracted cells were fixed in −20°C methanol and sequentially stained with anti-c-Myc and antitubulin antibodies as described above. Slides were mounted with 90% glycerol in PBS and were examined and photographed at a magnification of ×40 in a Zeiss Axiophot Photomicroscope fluorescence system.

## RESULTS

**Specific binding of c-Myc to tubulin in vitro.** To investigate whether c-Myc can associate with tubulin, the N-terminal and C-terminal domains of c-Myc were expressed separately as GST-Myc fusion proteins and were designated GST-Myc II and GST-Myc III, representing c-*myc* exon II and exon III, respectively. Both fusion proteins were incubated with HL60 cell lysates, and the bound proteins were separated by SDS-PAGE and immunoblotted with anti-α-tubulin. We observed that GST-Myc II precipitated α-tubulin from the cell lysate (Fig. 1A, lane 3) while GST-Myc III and the GST leader peptide had no binding activity (Fig. 1A, lanes 2 and 4). Specific binding was also obtained when an α-tubulin cDNA clone, HHCJ32, was translated in vitro in the presence of [$^{35}$S]methionine and incubated with GST-Myc II (Fig. 1B, lane 3), while no binding was observed with GST-Myc III and the GST leader peptide (Fig. 1B, lanes 2 and 4). These results suggested that c-Myc interacts with tubulin in vitro through its N-terminal domain.

To confirm that tubulin forms specific complexes with the N-terminal domain of c-Myc, we precipitated metabolically [$^{35}$S]methionine-labelled and unlabelled HL60 cell lysates with either the GST-Myc II fusion protein or the GST leader peptide. The washed pellets were resolved by SDS-PAGE and transferred to nitrocellulose filters. The lanes corresponding to the unlabelled proteins were immunoblotted with either α- or β-tubulin antibody, while the lanes corresponding to the $^{35}$S-labelled lysate were exposed directly to autoradiography. We observed that the two predominant bands precipitated when the GST-Myc II was incubated with $^{35}$S-labelled extracts comigrated with α- and β-tubulins (Fig. 1C). Taken together, these results indicate that the N-terminal domain of c-Myc (amino acids 1 to 252) forms a specific complex with tubulin in vitro.

**Deletion mutant analysis for tubulin binding.** To further define the c-Myc binding domain, we constructed a series of GST-Myc deletion mutants for tubulin binding assays (Fig. 2B) and analyzed each fusion protein by SDS-PAGE and Coomassie staining (Fig. 2C). HL60 cell lysates were precipitated with the c-Myc fusion proteins, and the washed pellets were immunoblotted with α-tubulin antibody (Fig. 2A). This experiment suggested that the region containing c-Myc amino acids 48 to 134 was necessary for binding to α-tubulin. Amino acids 1 to 143 in c-Myc are required for cotransformation with *ras* in the rat embryo fibroblast cell system, and amino acids 1 to 103 are required for the transcription regulatory activity of the c-Myc (18, 24). In addition, transactivation of gene expression by Myc is inhibited by mutations at the phosphorylation sites Thr-58 and Ser-62 (13). These two mutations contribute to increased transformation potential as measured by the soft-agar cloning assay (16) and are frequently targeted in Burkitt's lymphoma cells (3, 34). These results suggest that tubulin regulates the c-Myc protein by binding to its N-terminal functional domain.

**Copolymerization of c-Myc with microtubules in temperature-dependent oligomerization of tubulin.** To determine whether c-Myc can also associate with microtubules, we examined whether Myc retained tubulin binding activity under conditions that favor tubulin polymerization. Microtubules were induced to self-assemble in cytosolic extracts at 37°C in the presence of GTP and $MgCl_2$ and were depolymerized when

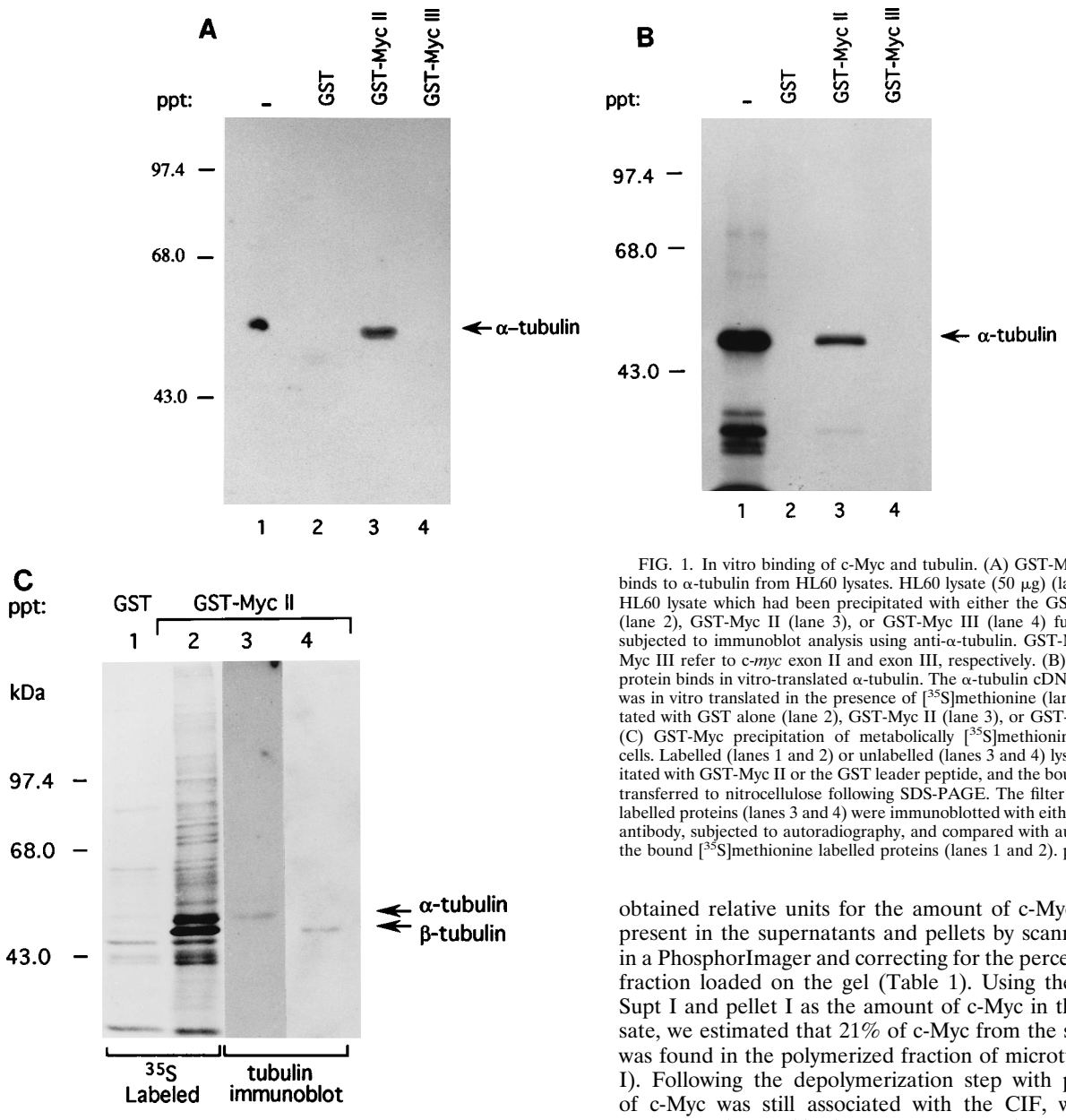


FIG. 1. In vitro binding of c-Myc and tubulin. (A) GST-Myc fusion protein binds to α-tubulin from HL60 lysates. HL60 lysate (50 μg) (lane 1) or 1 mg of HL60 lysate which had been precipitated with either the GST leader peptide (lane 2), GST-Myc II (lane 3), or GST-Myc III (lane 4) fusion protein was subjected to immunoblot analysis using anti-α-tubulin. GST-Myc II and GST-Myc III refer to c-*myc* exon II and exon III, respectively. (B) GST-Myc fusion protein binds in vitro-translated α-tubulin. The α-tubulin cDNA clone HHCJ32 was in vitro translated in the presence of [$^{35}$S]methionine (lane 1) and precipitated with GST alone (lane 2), GST-Myc II (lane 3), or GST-Myc III (lane 4). (C) GST-Myc precipitation of metabolically [$^{35}$S]methionine-labelled HL60 cells. Labelled (lanes 1 and 2) or unlabelled (lanes 3 and 4) lysates were precipitated with GST-Myc II or the GST leader peptide, and the bound proteins were transferred to nitrocellulose following SDS-PAGE. The filter was cut, and unlabelled proteins (lanes 3 and 4) were immunoblotted with either α- or β-tubulin antibody, subjected to autoradiography, and compared with autoradiographs of the bound [$^{35}$S]methionine labelled proteins (lanes 1 and 2). ppt, precipitate.

extracts were incubated at 0°C (30). This temperature-dependent cycle purification of tubulin has previously allowed the identification of microtubule-associated proteins (30). Using HL60 cell extracts, we performed two rounds of assembly-disassembly of microtubules, and the polymerized tubulin was collected by centrifugation as previously described (22, 30). The solubilized pellets and proteins from the supernatants from both rounds of polymerization (Fig. 3A) were separated by SDS-PAGE and immunoblotted with anti-c-Myc (Fig. 3B). We observed that c-Myc cofractionated with the microtubule pellets after both rounds of polymerization (pellet I and pellet II) and with the CIF (Fig. 3B, lanes 2 to 4; see quantitation in Table 1) but was no longer detected in the supernatant following the second round of polymerization (Supt II) (Fig. 3B, lane 6). In contrast, PCNA did not fractionate with polymerized tubulin in the pellets and in the CIF (Fig. 3B, lanes 2 and 4) and remained in the supernatant after both rounds of polymerization (Fig. 3B, lane 6). We obtained relative units for the amount of c-Myc and tubulin present in the supernatants and pellets by scanning the blots in a PhosphorImager and correcting for the percentage of each fraction loaded on the gel (Table 1). Using the total of the Supt I and pellet I as the amount of c-Myc in the starting lysate, we estimated that 21% of c-Myc from the starting lysate was found in the polymerized fraction of microtubules (pellet I). Following the depolymerization step with pellet I, 73% of c-Myc was still associated with the CIF, while 27% of c-Myc was detected in the supernatant. In the second polymerization cycle, 100% of c-Myc associated with the polymerized fraction (pellet II) and no Myc protein was detected in Supt II.

**c-Myc associates with tubulin in vivo and colocalizes with microtubules in intact cells.** To determine whether c-Myc binds in vivo to tubulin, we immunoprecipitated a protein extract from HL60 cells with a monoclonal antibody directed against either α- or β-tubulin and then immunoblotted it with anti-c-Myc (Fig. 4A). The immunoprecipitation experiments were performed using two different sources of antibodies to α- and β-tubulin (see Materials and Methods). We found that the α-tubulin but not the β-tubulin antibody coprecipitated c-Myc and tubulin from cell lysates, demonstrating that c-Myc interacts with α-tubulin in vivo (Fig. 4A). To address why the β-tubulin antibody did not precipitate c-Myc since α- and β-tubulin exist in vivo as dimers, we immunoprecipitated metabolically $^{35}$S-labelled HL60 cell lysate with each of the antibodies. We found that the anti-α-tubulin used for these experiments

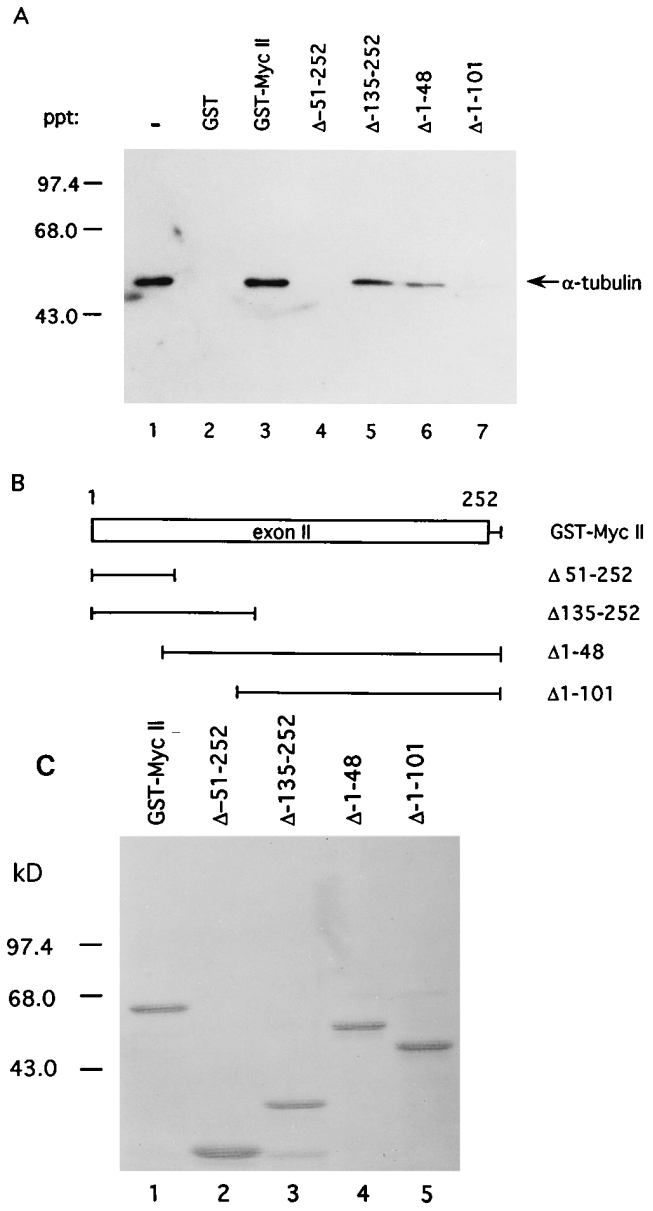


FIG. 2. Localization of the c-Myc binding domain. (A) HL60 protein extract (1 mg) was precipitated with either GST-Myc II or a series of GST-Myc deletion mutants. The bound proteins were analyzed by SDS–7.5% PAGE, transferred to nitrocellulose, and immunoblotted with α-tubulin antibodies. (B) Schematic representation of the GST-Myc deletion mutants. (C) Coomassie blue-stained GST-Myc II and the deletion mutants. ppt, precipitate.

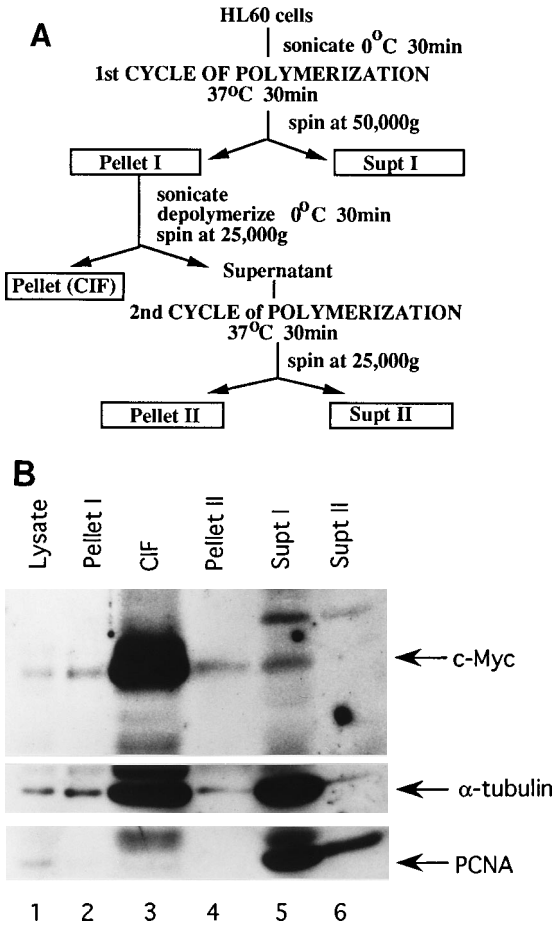


FIG. 3. Association of tubulin and c-Myc through two cycles of temperature-dependent depolymerization and polymerization of microtubules. (A) Schematic representation of the microtubule polymerization and depolymerization protocol. (B) Polymerized tubulin from pellet I, CIF, pellet II, and supernatants from the first and second cycles of polymerization (Supt I and II) were separated by SDS-PAGE and immunoblotted with c-Myc antibodies. Following autoradiography the membrane was stripped and incubated sequentially with anti-α-tubulin and anti-PCNA. The c-Myc ($M_r$, 64,000), tubulin ($M_r$, 50,000), and PCNA ($M_r$, 36,000) bands are indicated.

precipitated only the α-tubulin subunit (Fig. 4B, lane 1), while the anti-β-tubulin precipitated only the β-tubulin subunit (Fig. 4B, lane 2). This is consistent with our observation that c-Myc interacts with the α-tubulin subunit and could not be coprecipitated with the antibody to β-tubulin used for this study since the β-tubulin antibodies did not coprecipitate the α-tubulin subunit (Fig. 4B, lane 2).

The subcellular localization of c-Myc and tubulin and the specificity of the c-Myc antibody were determined by immunoblot analysis. Although we detected c-Myc protein predominantly in the nucleus, we also demonstrated cytoplasmic components in exponentially growing HeLa cells (Fig. 5A). Tubulin was localized predominantly to the cytoplasmic compartment (Fig. 5A, lane 1). To test the specificity of the c-Myc antisera, we performed the immunoblot in the presence and absence of a blocking c-Myc epitope (Fig. 5B). Since competition with the immunizing oligopeptide was inefficient, we

TABLE 1. Quantitative analysis of tubulin and c-Myc association through two cycles of temperature-dependent depolymerization and polymerization of microtubules

| Purification of microtubules | Fraction analyzed | Quantitation[a] | | Myc/tubulin ratio |
|---|---|---|---|---|
| | | Tubulin | Myc | |
| First cycle of polymerization | Supt I | 182,920,000 | 12,510,850 | 0.06 |
| | Pellet I | 8,156,500 | 3,346,300 | 0.4 |
| CIF of microtubules | Pellet | 2,998,710[b] | 2,462,972[b] | 0.8 |
| Second cycle of polymerization | Supt II | 134,708 | 0 | 0 |
| | Pellet II | 141,004 | 172,730 | 1.2 |

[a] Relative units were obtained from the immunoblot shown in Fig. 3B by scanning with a PhosphorImager (Molecular Dynamics) and by correcting for the dilution factor used for loading samples as described in Materials and Methods.

[b] Values reflect the amount of CIF which could be solubilized in SDS sample buffer. The remaining insoluble material was removed by centrifugation before loading the sample on the gel.

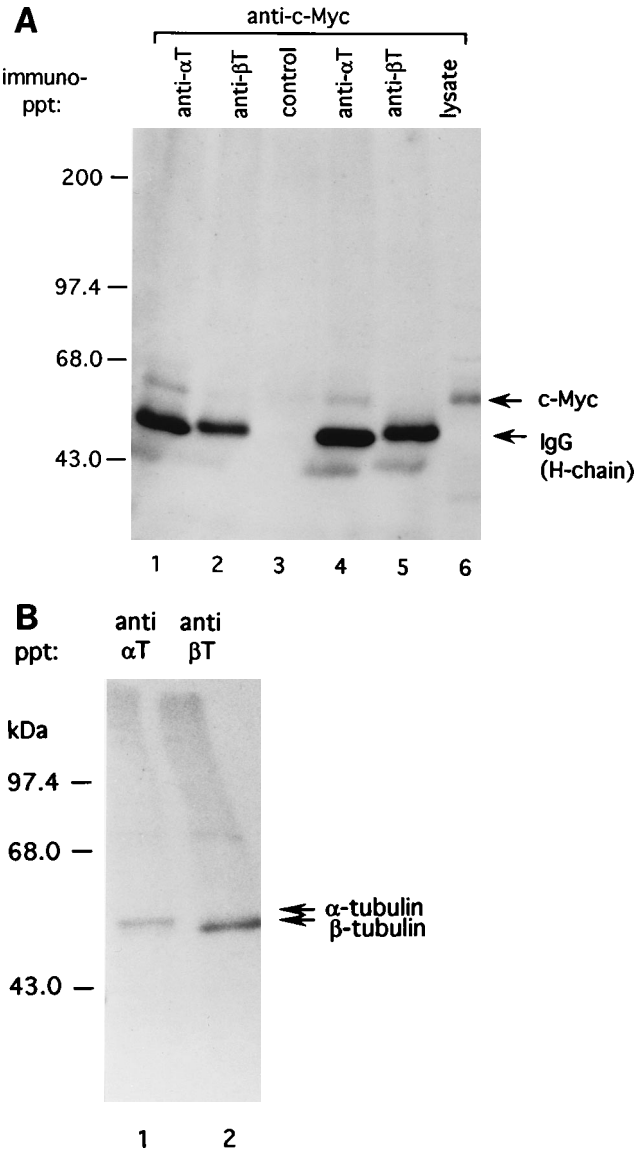


FIG. 4. In vivo binding of c-Myc and α-tubulin. (A) HL60 protein lysate (1 mg) was immunoprecipitated with anti-α-tubulin or -β-tubulin (anti-αT and anti-βT) from Amersham (lanes 1 and 2) or anti-α-tubulin or -β-tubulin from Oncogene Science (lanes 4 and 5). Beads incubated with lysate only are shown in lane 3. The washed pellets or 50 μg of whole-cell protein (lane 6) was separated on SDS-PAGE and immunoblotted with anti-c-Myc. (B) Immunoprecipitated α- and β-tubulin from [$^{35}$S]methionine-labelled HL60 cell lysates are shown as controls (lanes 1 and 2). ppt, precipitate.

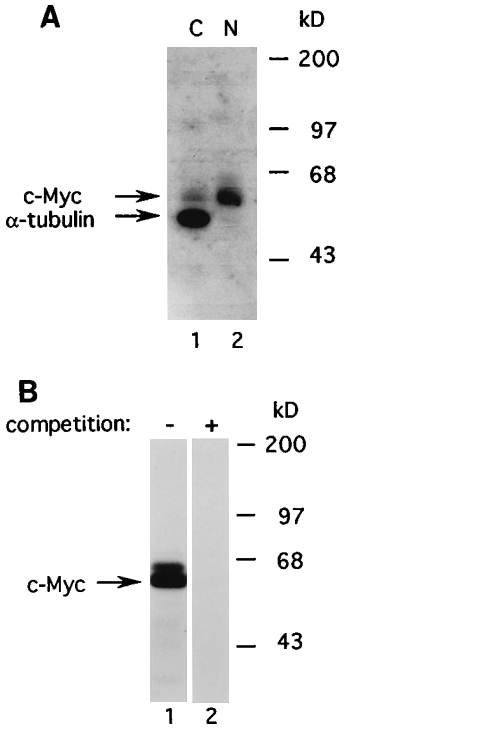


FIG. 5. Localization of c-Myc and α-tubulin. (A) HeLa cells were fractionated as described in Materials and Methods. Cytoplasmic (C) and nucleoplasmic (N) fractions (50 μg) were analyzed by immunoblotting with anti-c-Myc and anti-α-tubulin. (B) Competition analysis of anti-c-Myc with c-Myc epitope-tagged ARF protein (200 μg of HeLa protein extract loaded per lane). Incubation was with anti-c-Myc (lane 1) and with anti-c-Myc blocked with c-Myc epitope-tagged ARF protein (lane 2).

utilized a recombinant Myc epitope-tagged ARF protein (26) for the blocking experiment and observed full competition of the c-Myc signal, confirming the specificity of the c-Myc antibody (Fig. 5B, lane 2).

To examine whether c-Myc colocalizes with microtubules in vivo, we performed double immunofluorescence in HeLa cells with c-Myc and α-tubulin monoclonal antibodies (Fig. 6). We found that during interphase, the c-Myc staining pattern coincided with the microtubule staining (Fig. 6A, panels a and c). In contrast, the PCNA staining pattern did not colocalize with microtubules (Fig. 6A, panels b and d). In addition, we observed full competition of c-Myc staining when we performed the immunofluorescence experiment in the presence of the c-Myc epitope-tagged ARF protein (Fig. 6C).

To demonstrate that c-Myc behaves as a microtubule-binding protein in vivo and to exclude the possibility that the c-Myc staining pattern was a fixation artifact, we extracted the HeLa cells with microtubule-stabilizing buffer containing Triton X-100 before fixation as previously described (7). This extraction preserves microtubular structures but removes cytoplasmic proteins which are not associated with microtubules. We observed that the extracted cells continued to stain for both microtubules and c-Myc (Fig. 6B, panels a and c) suggesting that c-Myc is associated with microtubules in vivo and was not artifactually bound to microtubules as a result of fixing intact cells. In addition, detergent extraction of depolymerized cytoplasmic microtubules at 0°C resulted in the removal of both tubulin and c-Myc staining (Fig. 6B, panels b and d).

## DISCUSSION

We have demonstrated that c-Myc interacts with α-tubulin and with polymerized microtubules in vivo and have localized the tubulin binding site to the N-terminal domain of c-Myc. We observed that a fusion protein including the N terminus, but not the C terminus, of c-Myc could precipitate α-tubulin from cellular lysates as well as from in vitro-translated α-tubulin. Using a series of deletion mutants generated within the N-terminal domain of c-Myc, we have demonstrated that amino acids 48 to 134, spanning the first homology domain in exon II, are necessary for binding to α-tubulin. This region overlaps a domain previously identified as necessary for transactivation and for complementing activated *ras* in rat embryo

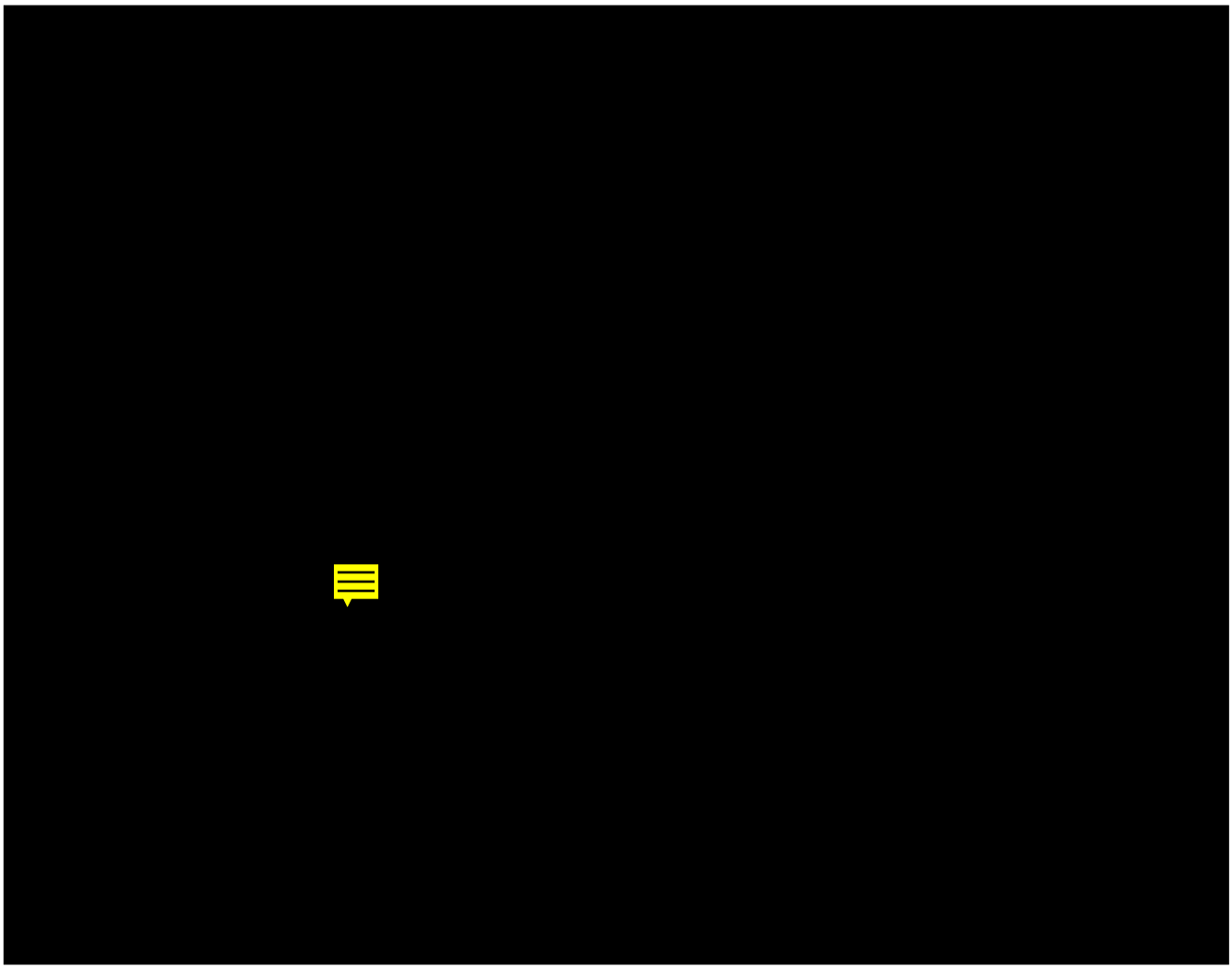

FIG. 6. Colocalization of c-Myc and tubulin by double immunofluorescence analysis. (A) HeLa cells were fixed and permeabilized with 0.1% Triton as described in Materials and Methods. c-Myc (a) and PCNA (b) were detected with mouse monoclonal antibody and Texas red-conjugated goat antibody $F(ab')_2$ to mouse IgG. Tubulin was detected with a rat monoclonal antibody (YL1/2; Accurate) and fluorescein-conjugated goat antibody to rat IgG (c and d). The same field of cells is shown in panels a and c and in panels b and d. (B) Detergent extraction of cells under conditions that stabilize or destabilize microtubules. Column 1, cells extracted in microtubule-stabilizing buffer at 37°C; column 2, extractions carried out under microtubule-destabilizing conditions at 0°C. Extracted cells were fixed and sequentially stained with anti-c-Myc (a and b) and antitubulin (c and d) antibodies. (C) Competition analysis for the c-Myc staining pattern. c-Myc was detected with fluorescein-conjugated goat antibody to mouse IgG. (Panel 1) Anti-c-Myc; (panel 2) anti-c-Myc blocked with c-Myc epitope-tagged ARF protein.

cell transformation assays (amino acids 1 to 143) (18, 29). Recent data have shown that Myc can heterodimerize with a cellular protein, designated Max, through its basic-helix-loop-helix-zipper region in exon III to enhance DNA binding and concomitant transactivation activity (4, 20, 21). This transactivation, however, could be partially suppressed by overexpressing the p107 protein product which can bind in vivo to this N-terminal polypeptide sequence of c-Myc (12). The importance of this sequence for Myc function was further suggested by the observation of clonal point mutations clustering at codons 56 to 64 in c-Myc from Burkitt's lymphoma tumor tissue (3, 34) which disrupt the ability of p107 to suppress Myc transcriptional activity (12). Therefore, the N-terminal domain of c-Myc may be critical for normal wild-type activity and may also be a common target for mutations in human tumors other than Burkitt's lymphoma (12).

The mechanism by which tubulin could regulate c-Myc function may differ from that proposed for p107. Tubulin is a heterodimer composed of α- and β-tubulin subunits. These subunits multimerize to generate microtubules which provide a cytoskeletal framework and also associate with a diverse family of proteins designated microtubule-associated proteins. While c-Myc is predominantly a nuclear protein (10, 15), it has recently been reported that it can be found in the cytoplasm in serum-starved and contact-inhibited cells and can be rapidly translocated to the nucleus in actively growing cultures (31). In addition, it has been shown that c-Myc is localized to nuclei in the proliferation zones of normal mucosae; however, it accumulates in the cytoplasm during progression to the neoplastic disease. This may be related to the contrast between the rapid but regulated division of the normal mucosa cells and the slower but continuous division of their tumor counterparts (27). Using immunoblot analysis, we detected c-Myc protein predominantly in the nucleus, although we also demonstrated presence of the c-Myc in the cytoplasm of exponentially growing HeLa cells. In the immunofluorescence analysis we also

observed nuclear, perinuclear, and cytoplasmic staining with anti-c-Myc. Although we cannot rule out the possibility that with our fixation condition there is some leakage of c-Myc protein to the cytoplasm, we demonstrated exclusively nuclear staining for PCNA using the same fixation conditions. In addition, we chose methanol as a fixative because it preserved the microtubular network (23, 33). Therefore, these results suggest that under the conditions used in our assays, c-Myc colocalized with microtubules in HeLa cells. We also showed that c-Myc colocalizes with microtubules under microtubule-stabilizing conditions and remains bound to microtubule structures after detergent extraction of intact cells. However, whether c-Myc-tubulin interaction preferentially takes place in resting cells remains to be established, since it has been shown that c-Myc translocates to the cytoplasm in serum-starved cells (31). Therefore, c-Myc may be stored in the cytoplasm bound to microtubules, which may then act as a reservoir to sequester and release the c-Myc protein.

Tubulin and microtubules have also been shown to interact with other viral and cellular oncogene products as well as with regulatory components of the cell cycle apparatus. For example, tubulin has been associated in complexes with signal-transducing G proteins (32) and with neurofibronin to inhibit Ras GAP activity and to regulate the Ras signaling pathway (6). The simian virus 40 large T antigen and p53 have also been shown to be microtubule-associated proteins (22). Whether these binding interactions with tubulin are direct or are mediated by other components remains to be established. In addition, the *mos* oncogene product pp39$^{mos}$, which phosphorylates tubulin in vitro (36), forms a complex with both microtubules and the p34$^{cdc2}$ kinase (35, 36). Microtubules were also shown to be required for tyrosine dephosphorylation and activation of p34$^{cdc2}$ kinase as well as for degradation of B-type cyclin in the fission yeast *Schizosaccharomyces pombe* (1). Thus, microtubules may serve as a network to bring molecules together to perform specialized functions. The association of tubulin and microtubules with the transactivation domain of the c-Myc oncoprotein suggests an important mechanism in the regulation of c-Myc activity, its interaction with other regulatory molecules, and its control of cell proliferation.

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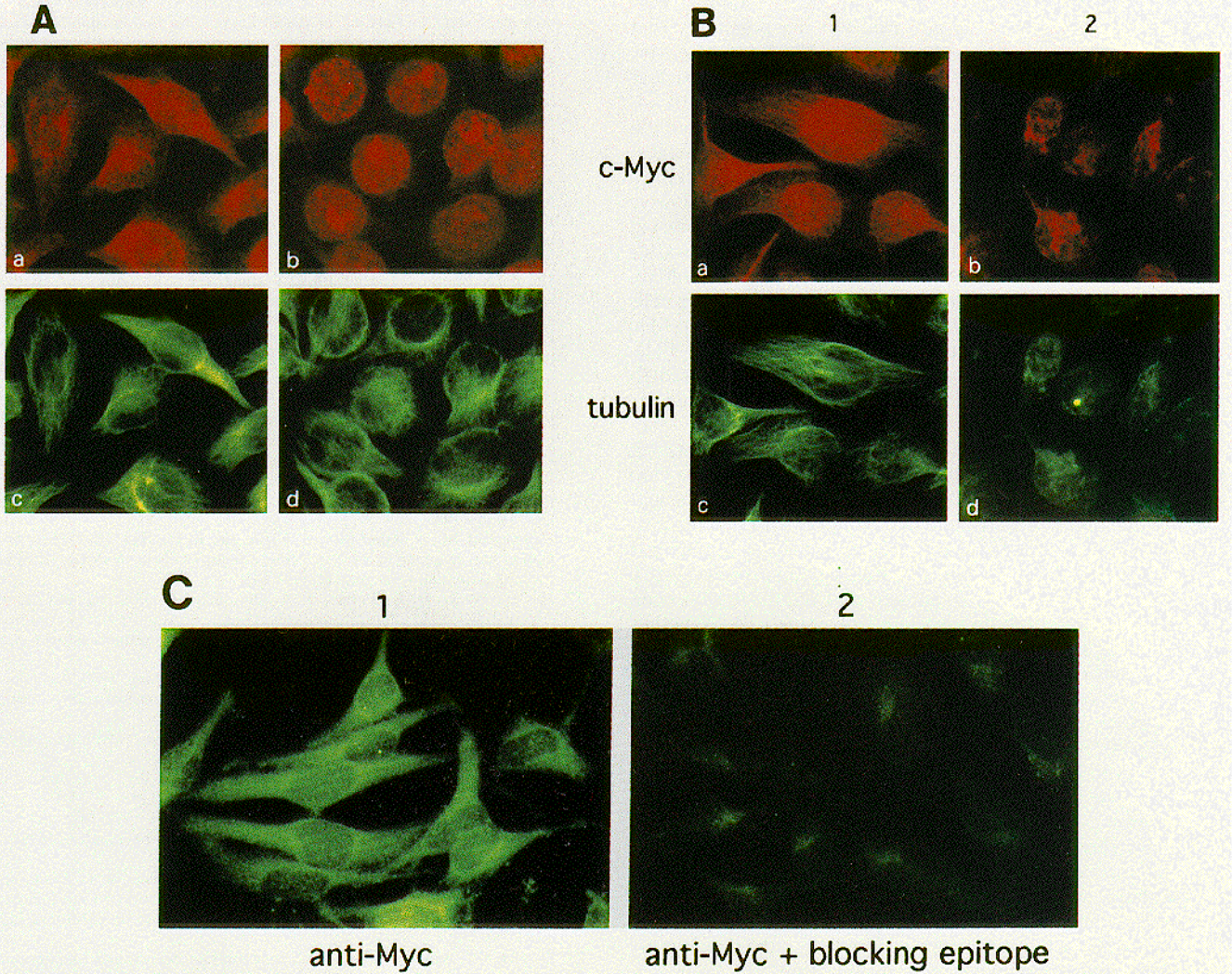
## REFERENCES

1. **Alfa, C. E., B. Ducommun, D. Beach, and J. S. Hyams.** 1990. Distinct nuclear and spindle pole body population of cyclin-cdc2 in fission yeast. Nature (London) **347:**680–682.
2. **Amizuka, N., and H. Ozawa.** 1992. Intracellular localization and translocation of 1α, 25-dihydroxyvitamin D3 receptor in osteoblasts. Arch. Histol. Cytol. **55:**77–88.
3. **Bhatia, K., K. Huppi, G. Spangler, D. Siwarski, R. Iyer, and I. Magrath.** 1993. Point mutations in the c-Myc transactivation domain are common in Burkitt's lymphoma and mouse plasmacytomas. Nat. Genet. **5:**56–61.
4. **Blackwood, E. M., and R. N. Eisenman.** 1991. Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science **251:**1211–1217.
5. **Blackwood, E. M., L. Kretzner, and R. N. Eisenman.** 1992. Myc and Max function as a nucleoprotein complex. Curr. Opin. Genet. Dev. **2:**227–235.
6. **Bollag, G., F. McCormick, and R. Clark.** 1993. Characterization of full-length neurofibromin: tubulin inhibits Ras GAP activity. EMBO J. **12:**1923–1927.
7. **Brockman, J. L., S. D. Gross, M. R. Sussman, and R. A. Anderson.** 1992. Cell cycle-dependent localization of casein kinase I to mitotic spindles. Proc. Natl. Acad. Sci. USA **89:**9454–9458.
8. **Craig, R. W., H. L. Buchan, C. I. Civin, and M. B. Kastan.** 1993. Altered cytoplasmic/nuclear distribution of the c-myc protein in differentiating ML-1 human myeloid leukemia cells. Cell Growth Differ. **4:**349–357.
9. **Davis, L. G., M. D. Dibner, and J. F. Battey.** 1986. Basic methods in molecular biology. Elsevier, New York.
10. **Eisenman, R. N., C. Y. Tachibana, H. D. Abrams, and S. R. Hann.** 1985. v-*myc*- and c-*myc*-encoded proteins are associated with the nuclear matrix. Mol. Cell. Biol. **5:**114–126.
11. **Evan, G. I., G. K. Lewis, G. Ramsay, and J. M. Bishop.** 1985. Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. Mol. Cell. Biol. **5:**3610–3616.
12. **Gu, W., K. Bhatia, I. T. Magrath, C. V. Dang, and F. R. Dalla.** 1994. Binding and suppression of the Myc transcriptional activation domain by p107. Science **264:**251–254.
13. **Gupta, S., A. Seth, and R. J. Davis.** 1993. Transactivation of gene expression by Myc is inhibited by mutation at the phosphorylation sites Thr-58 and Ser-62. Proc. Natl. Acad. Sci. USA **90:**3216–3220.
14. **Gusse, M., J. Ghysdael, G. Evan, T. Soussi, and M. Méchali.** 1989. Translocation of a store of maternal cytoplasmic c-*myc* protein into nuclei during early development. Mol. Cell. Biol. **9:**5395–5403.
15. **Hann, S. R., H. D. Abrams, L. R. Rohrschneider, and R. N. Eisenman.** 1983. Proteins encoded by v-myc and c-myc oncogenes: identification and localization in acute leukemia virus transformants and bursal lymphoma cell lines. Cell **34:**789–798.
16. **Henriksson, M., A. Bakardjiev, G. Klein, and B. Luscher.** 1993. Phosphorylation sites mapping in the N-terminal domain of c-myc modulate its transforming potential. Oncogene **8:**3199–3209.
17. **Kaelin, W. J., D. C. Pallas, J. A. DeCaprio, F. J. Kaye, and D. M. Livingston.** 1991. Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product. Cell **64:**521–532.
18. **Kato, G. J., J. Barrett, M. Villa-Garcia, and C. V. Dang.** 1990. An amino-terminal c-Myc domain required for neoplastic transformation activates transcription. Mol. Cell. Biol. **10:**5914–5920.
19. **Kato, G. J., and C. V. Dang.** 1992. Function of the c-Myc oncoprotein. FASEB J. **6:**3065–3072.
20. **Kato, G. J., W. M. Lee, L. L. Chen, and C. V. Dang.** 1992. Max: functional domains and interaction with c-Myc. Genes Dev. **6:**81–92.
21. **Kretzner, L., E. M. Blackwood, and R. N. Eisenman.** 1992. Myc and Max proteins possess distinct transcriptional activities. Nature (London) **359:**426–429.
22. **Maxwell, S. A., S. K. Ames, E. T. Sawai, G. L. Decker, R. G. Cook, and J. S. Butel.** 1991. Simian virus 40 large T antigen and p53 are microtubule-associated proteins in transformed cells. Cell Growth Differ. **2:**115–127.
23. **Osborn, M., and K. Weber.** 1982. Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: tubulin-containing structures. Methods Cell Biol. **24:**97.
24. **Penn, L. J. Z., M. W. Brooks, E. M. Laufer, T. D. Littlewood, J. P. Morgenstern, G. I. Evan, W. M. F. Lee, and H. Land.** 1990. Domains of human c-*myc* protein required for autosuppression and cooperation with *ras* oncogenes are overlapping. Mol. Cell. Biol. **10:**4961–4966.
25. **Pratt, W. B., E. R. Sanchez, E. H. Bresnick, S. Meshinchi, L. C. Scherrer, F. C. Dalman, and M. J. Welsh.** 1989. Interaction of the glucocorticoid receptor with the Mr 90,000 heat shock protein: an evolving model of ligand-mediated receptor transformation and translocation. Cancer Res. **49:**2222s–2229s.
26. **Randazzo, P. A., O. Weiss, and R. A. Kahn.** 1992. Preparation of recombinant ADP-ribosylation factor. Methods Enzymol. **219:**362–369.
27. **Royds, J. A., R. M. Sharrard, B. Wagner, and S. V. Polacarz.** 1992. Cellular localisation of c-myc product in human colorectal epithelial neoplasia. J. Pathol. **166:**225–233.
28. **Spencer, C. A., and M. Groudine.** 1991. Control of c-myc regulation in normal and neoplastic cells. Adv. Cancer Res. **56:**1–48.
29. **Stone, J., T. de Lange, G. Ramsay, E. Jakobovits, J. M. Bishop, H. Varmus, and W. Lee.** 1987. Definition of regions in human c-*myc* that are involved in transformation and nuclear localization. Mol. Cell. Biol. **7:**1697–1709.
30. **Vallee, R. B.** 1986. Reversible assembly purification of microtubules without assembly-promoting agents and further purification of tubulin, microtubule-associated proteins, and MAP fragments. Methods Enzymol. **134:**89–104.
31. **Vriz, S., J.-M. Lemaitre, M. Leibovici, N. Thierry, and M. Méchali.** 1992. Comparative analysis of the intracellular localization of c-Myc, c-Fos, and replicative proteins during cell cycle progression. Mol. Cell. Biol. **12:**3548–3555.
32. **Wang, N., K. Yan, and M. M. Rasenick.** 1990. Tubulin binds specifically to the signal-transducing proteins, Gsα and Giα1. J. Biol. Chem. **265:**1239–1242.

33. **Wang, Y.** 1992. Fluorescence microscopic analysis of cytoskeletal organization and dynamics, p. 1–22. *In* D. Rickwood and B. D. Hames (ed.), The cytoskeleton: a practical approach. IRL Press, Oxford.
34. **Yano, T., C. A. Sander, H. M. Clark, M. V. Dolezal, E. S. Jaffe, and M. Raffeld.** 1993. Clustered mutations in the second exon of the MYC gene in sporadic Burkitt's lymphoma. Oncogene **8:**2741–2748.
35. **Zhou, R., I. Daar, D. K. Ferris, G. White, R. S. Paules, and G. F. Vande Woude.** 1992. pp39$^{mos}$ is associated with p34$^{cdc2}$ kinase in c-*mos*$^{xe}$-transformed NIH 3T3 cells. Mol. Cell. Biol. **12:**3583–3589.
36. **Zhou, R., M. Oskarsson, R. S. Paules, N. Schulz, D. Cleveland, and G. F. Vande Woude.** 1991. Ability of the c-mos product to associate with and phosphorylate tubulin. Science **251:**671–675.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