neoplasms may represent a stage in the evolution of a malignant neoplasm and in other cases they may be 'end points' which do not readily undergo transition to malignant neoplasms."

In this report, we have used the term "neoplasm" freely to describe both benign and malignant neoplasms.

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15. In today's phraseology, this report has been concerned with scientific risk assessment and not with risk management. The process of risk management, by definition, begins after risk assessment has determined that a risk to a human population exists. Whereas "assessment" deals with biological significance, "management" deals with the possible alternative regulatory actions. Included in risk management may be evaluations of costs, feasibilities, risk-benefit ratios, availability of replacement substances or processes, and the level of risk that is acceptable to the society in question. Management of risks is a political, social, and economic issue. Scientists acting as scientists have a role in this phase, but it is limited to ensuring that the biological meaning of the risk is understood throughout the process.

RESEARCH ARTICLE

Antibodies to Human c-*myc* Oncogene Product: Evidence of an Evolutionarily Conserved Protein Induced During Cell Proliferation

Håkan Persson, Lothar Hennighausen, Rebecca Taub
William DeGrado, Philip Leder

The viral oncogene v-*myc*, which is harbored by avian myelocytomatosis viruses, is derived from a cellular gene (c-*myc*) found in all vertebrates (*1*). The cellular *myc* gene contains three exon sequences transcribed from two promoters located either just 5′ of or just within the first exon (*2–4*). Considerable interest has been shown in the c-*myc* gene since Burkitt lymphoma cells show translocations that have brought the c-*myc* gene in close proximity to the immunoglobulin heavy chain locus, t(8;14), or the immunoglobulin light chain loci, t(2;8) and t(8;22) (*5*). The molecular mechanism by which c-*myc* oncogenicity can occur, however, remains obscure although several mechanisms regarding its activation have been proposed. These include a transcriptional activation of the c-*myc* gene resulting from the chromosomal translocation (*6*), a deregulation of the c-*myc* gene allowing constitutively high levels of expression (*7*), a removal of an untranslated 5′ exon, thus facilitating c-*myc* gene expression at the translational level (*8*), release of a transcriptional repressor (*9*), a differential usage of promoters in normal and malignant cells or somatic mutations occurring at a high level as a result of its proximity to the immunoglobulin locus (*10*).

The protein product of the c-*myc* gene is, most likely, responsible for c-*myc* oncogenicity, and some information on potential properties of the c-myc protein has been obtained from studies with the myelocytomatosis viruses. The transformation specific protein from MC29-type viruses is synthesized as part of a polyprotein with a molecular weight of 110,000 (110K), in which v-myc protein is fused to the gag protein (*11*). The p110K$^{gag\text{-}myc}$ polyprotein is found largely in the cell nucleus, binds to double-stranded DNA, and at least a fraction of the protein is associated with chromatin (*11*). Two other members of the myelo-

Håkan Persson, Lothar Hennighausen, Rebecca Taub, and Philip Leder are associated with the Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115; William DeGrado is associated with the Central Research and Development Department, Experimental Station E328/229, E. I. DuPont deNemours and Company, Wilmington, Delaware 19898.

Summary. Antisera to a synthetic c-myc peptide and to c-myc antigens synthesized from various portions of the human gene expressed in *Escherichia coli* were used in order to characterize the protein product of the human c-*myc* oncogene. Although the deduced molecular weight of the human c-myc protein is 49,000, these antisera precipitate a protein from human cells that migrates in sodium dodecyl sulfate–polyacrylamide gel as if its molecular weight were 65,000. In addition, the mouse c-myc protein, whether synthesized in cells or in a cell-free system directed by pure, synthetic messenger RNA, has analogous properties and is immunoprecipitated by the antiserum to the human c-myc protein. Similar proteins are immunoprecipitated from monkey, rat, hamster, and frog cells, suggesting evolutionary conservation of antigenic structure of the c-myc protein among vertebrates. In addition, and in a manner consistent with the behavior of its messenger RNA, the immunoprecipitable c-myc protein is sharply induced by the action of mitogens on resting human T cells.

cytomatosis virus family, OK10 and MH2, generate myc proteins unlinked to gag through subgenomic messenger RNA's (mRNA's). Unlike studies with the MC29 p110K$^{gag\text{-}myc}$ polyprotein in which available antisera to gag have been used, studies with OK10 and MH2 viruses require a specific antiserum to v-myc protein. An antiserum to a synthetic peptide representing the COOH-terminal region of MC29 v-myc and an antiserum to the peptide fragment representing amino acids 107 to 228 of v-myc expressed in and prepared from bacteria have therefore been developed (*12*). Both antisera immunoprecipitated v-myc proteins having molecular weights of 58K to 63K which were found mainly in the nucleus of OK10 or MH2 transformed cells (*12*).

In our present study we used different antisera to the human c-myc product to identify an evolutionarily conserved 65K human c-myc protein. Furthermore, the 65K c-myc protein was shown to be an inducible gene product in the sense that its synthesis was greatly enhanced after activation of cells with mitogens. Recently, Eisenman and Hann informed us of an antibody that they have prepared to human c-myc that immunoprecipitates a protein of approximately the same apparent molecular weight as that reported here (*13*).

In order to obtain large amounts of c-myc antigen for antiserum production, we used hybrid plasmids in *Escherichia coli* to synthesize different portions of the human c-myc protein as a β-galactosidase c-myc fusion protein. Two distally located fragments from the human c-*myc* gene were chosen (Fig. 1A); the first was a Pst I fragment encoding amino acids 42 to 180 of c-myc protein (pURmycI) (Fig. 1A), and the second, a Sau 3A fragment encoding amino acids 380 to 439 (pURmycIII, Fig. 1A). These fragments were inserted into the expression vectors pUR290 and pUR292, respectively. The increase in size of the β-galactosidase protein when these constructions were expressed in *E. coli* was demonstrated by immunoprecipitation with an antiserum to β-galactosidase and by subsequent sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1A).

The Sau 3A fragment encoding the 59 COOH-terminal amino acids of the human c-myc protein utilizes a translational termination codon in the c-*myc* gene, whereas expression of the Pst I fragment from the second c-*myc* exon uses a termination codon within the vector sequences 3′ of the Pst I fragment. The COOH-terminal of the c-myc protein was stable as a fusion protein with β-galactosidase (Fig. 1A, lane h); whereas the protein translated from the c-*myc* second exon was degraded to some extent in *E. coli* (Fig. 1A, lane d). Large amounts of both β-galactosidase c-myc fusion proteins were isolated by preparative SDS-PAGE; the isolated protein was mixed with Freund's complete adjuvant and injected into rabbits. Sera were collected from the rabbits after three to five booster injections and immunoglobulin G fractions were prepared. The sera were affinity purified by repetitive adsorption to a column of Sepharose-linked *E. coli* cell extract containing large amounts of β-galactosidase. This procedure depleted the sera of antibodies against β-galactosidase and resulted in immunoglobulin G fractions containing antibodies to two different portions of the human c-myc protein. These antisera are hereafter referred to as "anti(c-myc 5′ exon)" for the antiserum to amino acids 42 to 180 of the human c-myc protein and "anti(c-myc C-ter)" for the antiserum to the COOH-terminal portion of the human c-myc protein.

A peptide corresponding to residues 24 to 40 (Fig. 1B) was synthesized by the Merrifield solid phase method, as described (*14*). This sequence was chosen because it spanned a minimum in the hydration profile (*15*) of the c-myc protein sequence, and hence would be expected to be on the surface of the native protein. A glycine residue was added at the COOH-terminus of the synthesized peptide as a spacer. The peptide was purified by size exclusion column chromatography on Sephadex G-25 (2.5 by 100 cm; 50 percent aqueous acetic acid as diluting buffer). Reversed-phase high-performance liquid chromatography (HPLC) of the purifed material gave a single major peak corresponding to approximately 80 percent of the ultraviolet absorbing material. Amino acid and sequence analyses confirmed the structure of the peptide.

The peptide was conjugated via its cysteine residue to keyhole limpet hemocyanin (KLH) (*16*); it was then used to immunize rabbits and the antiserum was collected after four booster injections. The immunoglobulin G fraction of this serum was used for immunoprecipitations and is referred to as "anti(c-myc N-ter)." The presence of antibodies to the peptide was confirmed by immunoprecipitation of a β-galactosidase c-myc fusion protein containing the peptide sequence used for immunization. A Bss HII fragment overlapping the first intron and second exon of the human c-*myc* gene was inserted into a single Bss HII restriction site within the expression vector pUR290 (Fig. 1B shows a schematic representation). This construction, pURmycII, resulted in a fusion protein where the 156 NH_2-terminal residues of the human c-myc protein were integrated in the middle of β-galactosidase.

This protein as well as the β-galactosidase c-myc fusion protein containing amino acids 42 to 180 of the c-myc protein (pURmycI) was immunoprecipitated with an antiserum to β-galactosidase (Fig. 1B, lanes a and g, respectively). The β-galactosidase c-myc fusion protein containing the amino acid sequence represented in the c-myc synthetic peptide was immunoprecipitated with anti(c-myc N-ter), whereas the fusion protein lacking this sequence was not immunoprecipitated (Fig. 1B, lanes b and f, respectively). This immunoprecipitation was blocked by first incubating the serum with synthetic human c-myc peptide (Fig. 1B, lane c) or with the same peptide linked to KLH (Fig. 1B, lane e), whereas blocking with KLH alone (Fig. 1B, lane d) had no effect on the immunoprecipitation of the 136K fusion protein. These results demonstrate that the anti(c-myc N-ter) contains antibodies to the synthetic human c-myc peptide and,

moreover, that the antiserum is capable of recognizing the peptide sequence when it is embedded as a part of a 136K protein.

Identification of c-myc–specific proteins. Human promyelocytic leukemia cells (HL60) have a stable 20- to 30-fold amplification of the c-*myc* gene (*17*). Most likely as a result of this, HL60 cells show a marked increase in the amount of c-*myc* mRNA compared with most other neoplastic or normal cells. Because of the high c-*myc* mRNA expression in HL60 cells, we used them in our initial attempts to identify the human c-myc protein.

Cell extracts from HL60 cells labeled with [^{35}S]methionine were immunoprecipitated with the different antisera to human c-myc protein. The anti(c-myc N-ter) specifically immunoprecipitated a 65K protein, and this precipitation was decreased by prior incubation of the serum with the synthetic peptide (Fig. 2, lanes a and b). The anti(c-myc 5′ exon) also specifically immunoprecipitated a 65K protein as well as a 49K protein (Fig. 2, lane c); prior incubation of this antiserum with a Sepharose-linked bacterial extract containing the human c-myc 5′ exon β-galactosidase fusion protein abolished the precipitation of both the 65K and the 49K proteins (data not shown). The anti(c-myc C-ter) specifically immunoprecipitated a 68K and a 65K protein; the latter comigrated in SDS-PAGE with the 65K protein immunoprecipitated with the anti(c-myc N-ter) (Fig. 2, lanes d and e). Immunoprecipitation of the 65K protein with the anti(c-myc C-ter) was greatly decreased when the cell extract was first incubated with the anti-

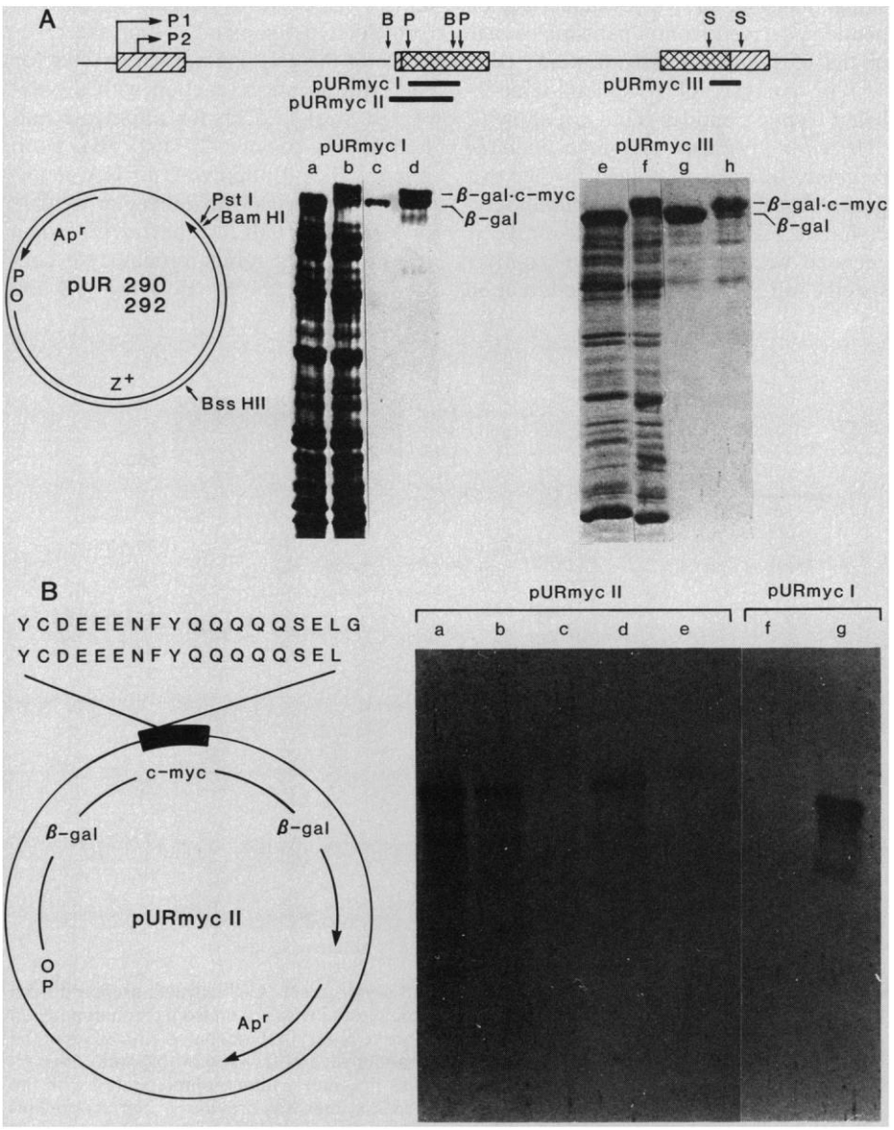


Fig. 1. Synthesis of human c-myc antigens for production of specific antisera to them. (A) At the top is the structure of the human c-*myc* gene. The three exons are indicated by boxes. Cross bars represent untranslated exon sequences and regions predicted by DNA sequence analysis (*2*) to encode the 439–amino acid c-myc protein in exons 2 and 3 are shown with cross-hatched bars. P1 and P2 denote the two promoters used for transcription of the c-*myc* gene. Restriction sites relevant for construction of β-galactosidase c-myc fusion proteins are indicated [B, Bss HII; P, Pst I; and S, Sau 3A]. Shown below is the structure of the expression vectors pUR290 and pUR292 (*28*). The promoter and operator region of the β-galactosidase gene (Z^+) is indicated by P and O, respectively. The Pst I and Bam HI restriction sites are part of a polylinker replacing three in-phase occurring termination triplets in the wild-type β-galactosidase gene. The 414-bp Pst I fragment and the 255-bp Sau 3A fragment indicated in the figure were purified from appropriate subclones of the 12.5-kb human c-*myc* Eco RI fragment [see Battey *et al.* (*2*)]. The purified Pst I fragment was ligated into Pst I restricted and alkaline phosphatase–treated pUR290 DNA and the ligation mixture was transformed in *E. coli* strain RRΔ M15. Transformants were tested by restriction analysis and a plasmid with the c-*myc* Pst I fragment in the correct orientation pURmycI was tested for expression of a β-galactosidase c-myc fusion protein. The purified Sau 3A fragment was inserted into the Bam HI site of pUR292 and the resulting plasmid was named pURmycIII. For expression studies RRΔ M15 cells containing the appropriate plasmid was grown to standard density (A 600 nm = 0.8), isopropylthio-β-D-galactoside (IPTG) was added to a final concentration of 0.2 m*M*, and 30 minutes later the cells were labeled with [^{35}S]methionine (25 μCi/ml) for 5 minutes. The labeled cells were sedimented and resuspended to one-tenth the original volume in 0.05*M* tris-HCl (*p*H 7.5) and 10 percent sucrose; and frozen and thawed once. Lysozyme (200 μg/ml, final) and 0.05*M* NaCl were added, and the sample was incubated on ice for 45 minutes and centrifuged at 20,000*g* for 30 minutes. The supernatant was either analyzed by SDS-PAGE or immunoprecipitated with the indicated sera. Samples were analyzed on 10 percent SDS-polyacrylamide gels (*29*) and subsequent fluorography. Cell extracts analyzed before immunoprecipitation from cells containing the following plasmids: (a) pUR290, (b) pURmycI, (e) pUR292, (f) pURmycIII. Immunoprecipitation of cell extracts with an antiserum against β-galactosidase from cells containing (c) pUR290, (d) pURmycI, (g) pUR292, (h) pURmycIII. (B) The 552-bp Bss HII c-*myc* fragment indicated at the top of (A) was purified and inserted into the single Bss HII site within the vector pUR290 (*30*), generating plasmid pURmycII. The structure of the synthetic human c-myc peptide representing amino acids 24 to 40 is shown where it occurs in the β-galactosidase c-myc fusion protein. The glycine residue was added as a spacer for coupling and is therefore not present in the pURmycII plasmid. The pURmycI and the pURmycII plasmids were expressed in *E. coli* strain RRΔ M15 as described above, and cell extracts were immunoprecipitated with the indicated sera followed by SDS-PAGE: (a) antiserum to β-galactosidase; (b) anti(c-myc N-ter); (c) anti(c-myc N-ter) incubated with 80 μg of the human c-myc peptide; (d) with 80 μg of KLH; (e) 80 μg of KLH conjugated with the c-myc peptide; (f) anti(c-myc N-ter) added; and (g) antiserum to β-galactosidase. Single letter abbreviations for the amino acid residues are as follows: Y, Tyr; C, Cys; D, Asp; E, Glu; N, Asn; F, Phe; Q, Gln; S, Ser; L, Leu; and G, glycine.

(c-myc N-ter) serum, suggesting identity between the two 65K proteins (Fig. 2, lane f). Furthermore, the 65K proteins which immunoprecipitated with the anti(c-myc N-ter)- or the anti(c-myc C-ter)-generated *Staphylococcus aureus* V8 proteolytic peptides that were indistinguishable from the V8 peptides generated by the 68K protein immunoprecipitated with the anti(c-myc C-ter) serum (Fig. 3B). By the same analysis, the 49K protein immunoprecipitated with the anti(c-myc 5′ exon) serum showed no peptide homology with the 65K protein. This result was also confirmed by two-dimensional analysis of [^{35}S]cysteine-labeled peptides derived from a pepsin cleavage of the 65K and the 49K proteins (Fig. 3A) or analysis of [^{35}S]methionine-labeled tryptic peptides (data not shown).

In vitro synthesis of c-myc *encoded proteins*. In order to collect further evidence that the 65K protein immunoprecipitated with our antisera to c-myc is encoded within the c-*myc* gene, c-*myc*-specific mRNA was synthesized in an in vitro transcription system with the use of the bacteriophage SP6 promoter. A 1.85-kb Hind III fragment containing the mouse complementary DNA (cDNA) sequence from the second c-*myc* promoter (*18*) or a 1.5-kb Sst I–Hind III fragment containing a mouse c-*myc* cDNA lacking the c-*myc* first exon were fused to the SP6 promoter (Fig. 4A). (Mouse mRNA was used because of the availability of the mouse cDNA clone.) The two plasmid constructions were transcribed in vitro with the use of an RNA polymerase specific for SP6 promoters and subsequently processed with guanylyl transferase. Gel electrophoresis (agarose-formaldehyde) showed, within the resolution of the gel, one mRNA species for each transcription reaction with the expected sizes (1.5 kb for pSpmyc-1 and 1.85 kb for pSpmyc-2) (Fig. 4B). Both pSpmyc-1 and pSpmyc-2 mRNA generated two proteins with molecular weights of 62K and 60K, respectively, when translated in a rabbit reticulocyte cell-free system (Fig. 4B). Both the 62K and 60K mouse proteins were immunoprecipitated with the human anti(c-myc N-ter) (Fig. 4B) as well as with the anti(c-myc C-ter) (data not shown). The encoded 62K c-myc protein comigrated in SDS-PAGE with a 62K c-myc protein immunoprecipitated with anti(c-myc N-ter) or anti(c-myc C-ter) from mouse cells labeled in vivo with [^{35}S]methionine (Fig. 4B).

An evolutionarily conserved protein. Many of the oncogenes identified from different species show extensive DNA sequence homology (*19*). This homology is reflected in the ability of the human antibody to cross-react with the mouse protein as indicated above. The different antisera to c-myc were therefore used to assess the extent of this homology and to immunoprecipitate cell extracts from different species in order to identify c-myc proteins. Anti(c-myc N-ter) specifically immunoprecipitated ~65K proteins from monkey, mouse, and hamster as well as from human cells (Fig. 5). Like the results obtained for mouse c-*myc* encoded proteins synthesized in vitro, the c-myc protein immunoprecipitated from various mouse cells migrated somewhat faster in SDS-PAGE than the human protein, and an apparent molecular weight of 62K was indicated. A similar size c-myc protein was also detected in rat cells (data not shown). Immunoprecipitation of [^{35}S]methionine-labeled cell extracts prepared from frog oocytes also revealed a 65K protein, further emphasizing evolutionary conservation of this protein. A similar set of ~65K proteins was also immunoprecipitated with the anti(c-myc C-ter), whereas anti(c-myc 5′ exon) showed no or very weak cross-reactivity for the 49K protein between different species (data not shown).

Enhanced expression of the 65K c-myc protein after activation of cells with mitogens. Recently Kelly *et al.* (*20*) showed that c-*myc* RNA expression is induced by agents that induce a proliferative response in resting cells. Experiments were therefore performed in order to establish whether expression of the identified c-myc proteins was enhanced after activation of peripheral blood cells with the mitogen phytohemagglutinin (PHA). Peripheral blood cells were collected, the erythrocytes were removed, and of the remaining cell population 30 to 50 percent of the cells were T lymphocytes. These quiescent cells were stimulated to proliferate by addition of PHA, and 2 hours after stimulation the cells were labeled with [^{35}S]methionine. Control cells not stimulated with PHA were analyzed in parallel. Cell extracts were prepared and an equal amount of acid precipitable radioactivity from each sample

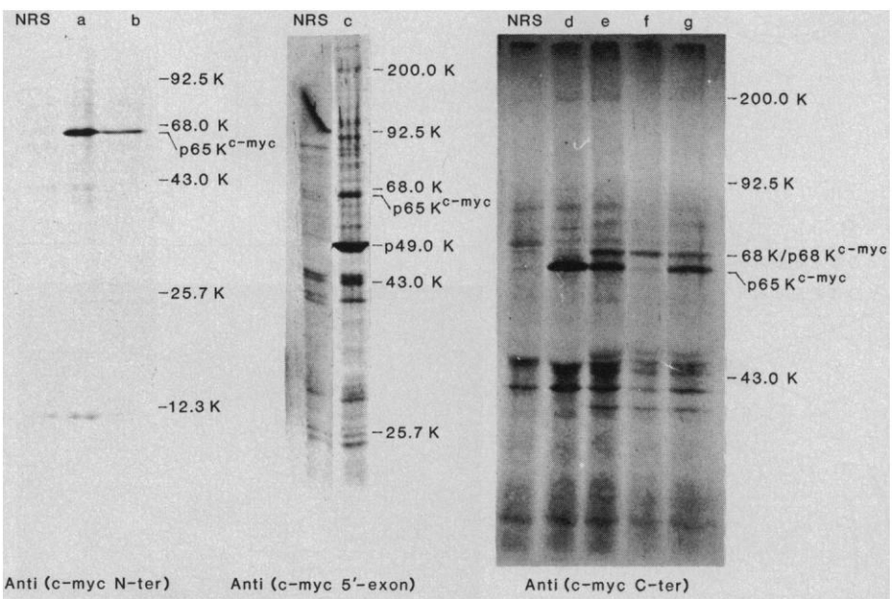


Fig. 2. Immunoprecipitation with antisera to human c-*myc* genes. Cell extracts prepared from [^{35}S]methionine-labeled HL60 cells were immunoprecipitated with serum from a nonimmunized rabbit (NRS) or with (a) anti(c-myc N-ter); (b) anti(c-myc N-ter) first incubated with 80 μg of the human c-myc peptide; (c) anti(c-myc 5′ exon); (d) anti(c-myc N-ter) serum; (e) anti(c-myc C-ter); (f) anti(c-myc C-ter) with a cell extract that was previously immunoprecipitated with the anti(c-myc N-ter); (g) anti(c-myc C-ter) with a cell extract that was previously immunoprecipitated with antiserum to β-galactosidase. The washed immunoprecipitates were analyzed on a 13 percent SDS-polyacrylamide gel (far left panel) or 10 percent SDS-polyacrylamide gels. HL60 cells at 10^6 per milliliter were labeled with [^{35}S]methionine (50 μCi/ml) in RPMI 1640 medium containing fetal calf serum (10 percent) and 1/20 the normal concentration of methionine for 15 hours. The cells were washed once in ice-cold phosphate buffered saline (PBS) and resuspended by brief sonication at a density of 2×10^7 cells/ml in RIPA buffer [0.05*M* tris-HCl, *p*H 7.5, 0.15*M* NaCl, 0.1 percent SDS, 1 percent Triton X-100, 0.5 percent sodium deoxycholate, and 1 m*M* phenylmethylsulfonyl fluoride (PMSF)]. The cell extract was clarified by centrifugation at 16,000*g* for 10 minutes and the supernatant was diluted ten times with PBS containing 1 percent Triton X-100 and 1 m*M* PMSF. The diluted sample was immunoprecipitated with the indicated sera for 1 hour at room temperature and incubated overnight at 4°C. Immunoprecipitates were collected by addition of Pansorbin (Calbiochem), and the samples were washed five times in RIPA buffer. Unless otherwise stated washed immunoprecipitates were analyzed on 10 percent SDS-polyacrylamide gels as described by Laemmli (*29*). This and subsequent figures show fluorograms of the dried gels.

was used for immunoprecipitation. No significant increase in the expression of the 49K protein was observed (data not shown). By contrast, expression of the 65K c-myc protein was significantly increased, and densitometer tracing of the autoradiogram (Fig. 6) revealed about a 20-fold enhancement compared with nonstimulated cells. This 20-fold increase in expression of the 65K c-myc protein was correlated with an increase in c-*myc* RNA levels as measured by S1 endonuclease sensitivity (data not shown).

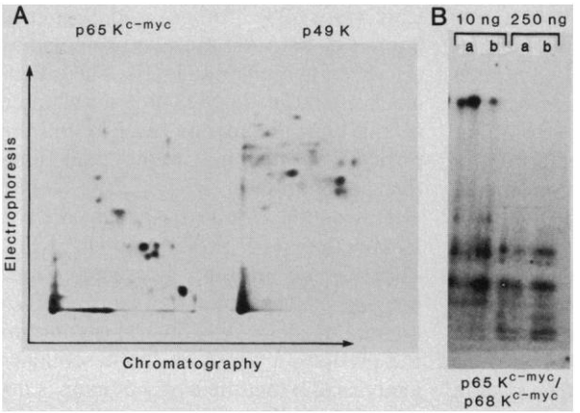


Fig. 3. Peptide analysis of c-myc proteins. (A) Cell extracts from [^{35}S]cysteine- or (B) [^{35}S]-methionine-labeled HL60 cells were immunoprecipitated with indicated antisera to c-myc protein (legend to Fig. 2). (A) Indicated proteins were excised from the gel by electrophoresis and digested with pepsin (*31*). [^{35}S]Cysteine-labeled peptides were separated in two dimensions by electrophoresis followed by ascending chromatography. The peptides were visualized by autoradiography. (B) The p65K$^{c\text{-}myc}$ protein immunoprecipitated with anti(c-myc N-ter) and the p68K$^{c\text{-}myc}$ protein immunoprecipitated with the anti(c-myc C-ter) were digested with the indicated amounts of *Staphylococcus aureus* V8 protease (*32*). Partial V8 protease peptides were analyzed in a 15 percent SDS-polyacrylamide gel with subsequent fluorography.

Discussion

A human protein with an apparent molecular weight of 65K in SDS-PAGE was immunoprecipitated with either of two antiserums to the NH_2-terminal portion or with an antiserum to the COOH-terminal portion of the human c-myc protein (Fig. 2). The 65K protein immunoprecipitated with the anti(c-myc N-ter) was shown to be identical to the 65K protein immunoprecipitated with the anti(c-myc C-ter) serum in that the NH_2-terminal serum abolished immunoprecipitation of the 65K protein with the COOH-terminal serum (Fig. 2). The demonstration that these two antisera to each of two widely separated parts of the human c-myc protein can immunoprecipitate the same 65K protein suggests that this protein is encoded within the human c-*myc* gene. The antiserum to the peptide fragment consisting of amino acid 42 to 180 of the human c-myc protein also showed a high affinity for a 49K protein (Fig. 2). A comparison of peptides generated after cleavage by pepsin or V8 protease showed no common peptides between the 65K and the 49K proteins (Fig. 3A). A precursor-product relation between the 49K and the 65K protein therefore appears unlikely. The possibility that the 49K protein might be encoded within the c-*myc* gene is unlikely because mouse c-*myc* mRNA synthesized in vitro with SP6 promoters, when translated in a cell-free system, generated two proteins of significantly larger sizes than 49K (Fig. 4B). The immunoprecipitation of the 49K protein was, however, clearly specific, suggesting the possibility that the 49K protein is synthesized from a gene that belongs to a family that includes the c-*myc* gene. It is possible that this 49K protein is identical to that described by Giallongo *et al.* (*21*), a 48K protein immunoprecipitated with an antiserum to a COOH-terminal human c-myc peptide. It appears, however, that it is not a product of the c-*myc* gene.

The result that our antisera c-myc im-

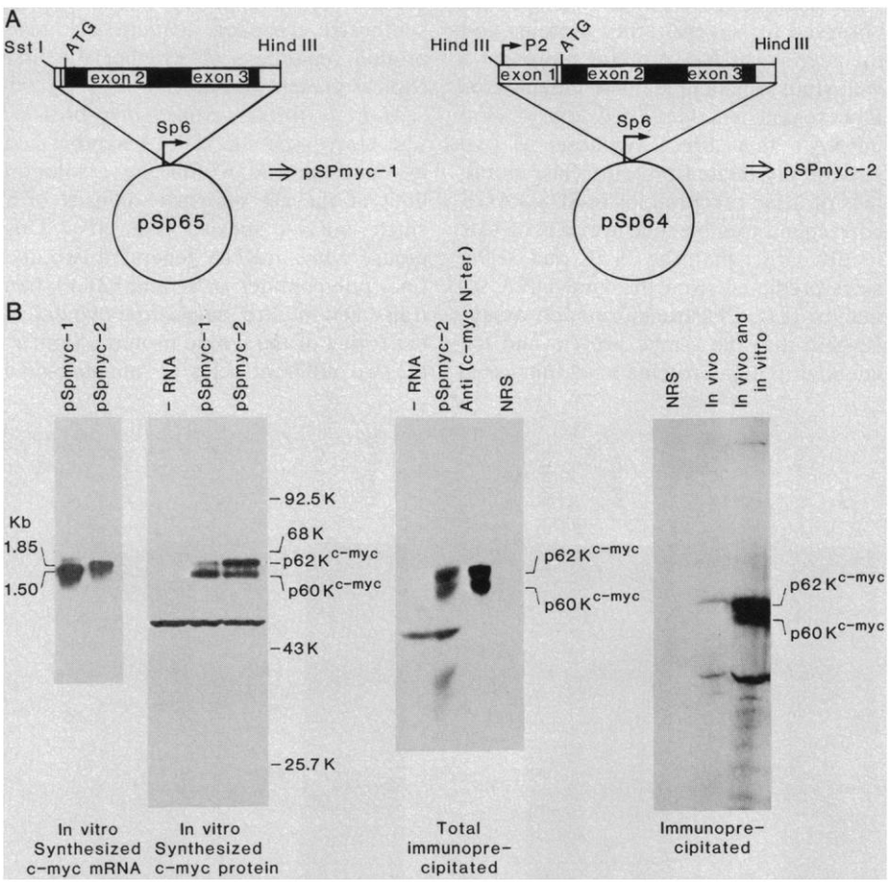


Fig. 4. In vitro synthesis of c-myc specific mRNA and protein. (A) The 1.5 kb Sst I–Hind III fragment containing only 60 bp of the untranslated region and all of the coding region (exons 2 and 3) of a mouse c-*myc* cDNA (*18*) was cloned into the Sst I–Hind III site of the pSP65 M13 polylinker (Promega Biotec). The 1.85-kb Hind III fragment containing the untranslated region from promoter 2 (P2) and all of the coding region of the mouse c-*myc* DNA was cloned in the correct orientation into the Hind III site of the pSP64 cloning vector (Promega Biotec). (B) pSmyc-1 was linearized with Hind III and pSpmyc-2 was linearized with Eco RI. Both constructs (0.1 to 0.5 μg) were transcribed in vitro in the presence of trace amounts of (α-^{32}P)-labeled GTP with the use of phage SP6 RNA polymerase (*33*). Isolated RNA was processed (capped) with guanylyl transferase (Bethesda Research Laboratories), and the capped products were subjected to electrophoresis on a 1 percent agarose–6.5 percent formaldehyde gel including λ Hind III standards. The gel was dried and analyzed by autoradiography. Approximately 0.1 μg of each capped RNA was translated in a rabbit reticulocyte cell-free system, and the translational products were analyzed on a 10 percent SDS-PAGE. (−RNA) denotes no RNA added to the cell-free system. The translational products from pSpmyc-2 RNA were immunoprecipitated with the indicated sera. Mouse erytholeukemia cells (JM979) were labeled in vivo for 4 hours with [^{35}S]methionine and the cell extract was immunoprecipitated with NRS or with anti(c-myc N-ter) (in vivo). The same immunoprecipitate was also mixed with c-myc proteins synthesized in vitro from pSpmyc-2 RNA and analyzed together in a 10 percent SDS-PAGE (in vivo and in vitro).

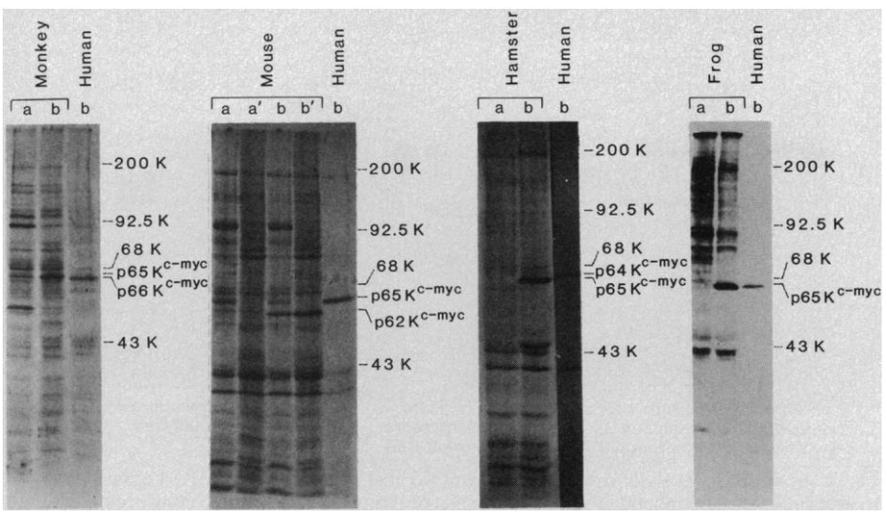
munoprecipitated the mouse c-*myc* encoded 60K and 62K proteins synthesized in vitro (Fig. 4B) provides additional evidence that the antisera to c-myc used in our study recognize the c-myc protein. The mouse c-myc–specific 62K protein immunoprecipitated from in vivo labeled cells comigrated in SDS-PAGE with the mouse c-*myc* encoded 62K protein. The size of this c-myc protein is significantly larger than the 49K predicted from the c-*myc* DNA sequence (*22*). Since no post-translational modifications are known to occur in the reticulocyte cell-free system and yet the c-*myc* encoded protein has the same size as seen in vivo, it appears likely that the size discrepancy is due to an abnormal migration of the c-myc protein in SDS-PAGE.

Anomalous protein migration has been observed for several other proteins and the adenovirus E1a proteins represent a well-studied example. The adenovirus E1a region encodes two early viral mRNA's that direct synthesis of two structurally related proteins. The mobilities of these two proteins in SDS-PAGE correspond to molecular weights of 15K to 30K larger than the 26.5K and 31.9K sizes predicted from the viral DNA sequence (*23*). Cell transformation assays suggest that the c-myc protein and the adenovirus E1a proteins have functional similarities (*24*). Amino acid sequence homology between the two proteins has also been demonstrated (*25*). The anomalous migration in SDS–polyacrylamide gels of both the adenovirus E1a and the $p65K^{c\text{-}myc}$ protein may reflect their functional similarities.

Translation of c-*myc*–specific mRNA lacking the first 5′ exon (pSpmyc-1) (Fig. 4B) resulted in the same size c-myc proteins as translation of a c-*myc* mRNA containing all three exons. This confirms the prediction based on DNA sequence analysis (*22*) that the c-*myc* 5′ exon is not used for translation of the c-myc protein. Furthermore, the presence of the c-*myc* 5′ exon does not seem to affect the translation efficiency of the c-myc protein in vivo since cell lines expressing comparable amounts of c-*myc* mRNA synthesize the same amount of c-myc protein regardless of whether the first exon is present or not (*26*).

Multiple forms of the c-myc protein. The c-*myc*–specific mRNA synthesized in vitro appears within the resolution limit of the gel system to consist of a single mRNA species (Fig. 4B). This mouse c-*myc* mRNA generated two distinct polypeptides (60K and 62K) when translated in vitro, suggesting two different forms of the c-myc protein. Similarly, two different sizes of human c-myc protein with apparent molecular weights of 68K and 65K were observed in vivo after immunoprecipitation and SDS-PAGE. This was most clearly demonstrated with anti(c-myc C-ter), which readily immunoprecipitated the $p68K^{c\text{-}myc}$ protein as well as the $p65K^{c\text{-}myc}$ protein (Fig. 2). Peptide analysis revealed identity between the 68K and the 65K proteins (Fig. 3B), also suggesting that the human c-myc protein exists in multiple forms. The sequence of the human c-myc protein predicted from the DNA sequence (*22*) suggests possible ways by which this can occur. The protein is rich in proline residues and several of these are clustered as for instance in the NH_2-terminal part where 7 of 22 residues are prolines. This, in combination with clustered charged amino acids, may create several compact and different secondary structures for the c-myc protein that may also result in anomalous migration in SDS-polyacrylamide gels.

Mitogen induced expression of an evolutionarily conserved $p65K^{c\text{-}myc}$ protein. Anti(c-myc N-ter) identified c-myc–specific proteins from several different species, with apparent molecular weights from 62K to 68K (Fig. 5). The sizes of these c-myc proteins as revealed by SDS-PAGE is in reasonable agreement with the sizes of the identified 58K to



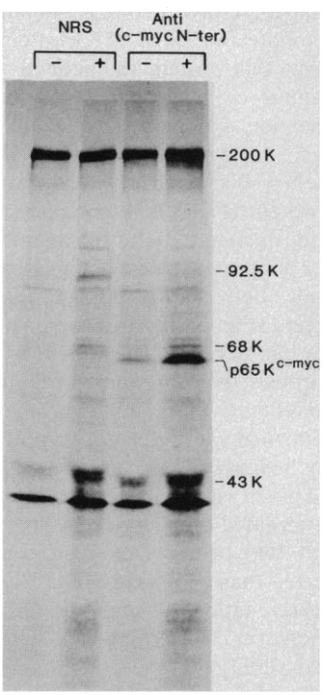


Fig. 5 (left). Conservation of the 65K c-myc protein between species. Cell extracts labeled with [^{35}S]methionine were prepared from the indicated species as described in Fig. 2 and immunoprecipitated with (a) NRS or with (b) anti(c-myc N-ter). Samples were analyzed on 10 percent SDS-polyacrylamide gels and then subjected to fluorography. The human cell extract was prepared from the lymphoblastoid cell line IARC 100. Monkey extracts were from African green monkey kidney cells; mouse extracts were from either Friend erythroleukemia cells (JM979) (a and b in mouse lanes) or from a mouse lymphoblastoid cell line (M12) (a′ and b′ in mouse lanes); hamster extracts were from fibroblasts (E_36), and frog extracts were from oocytes. Fig. 6 (right). Induction of the 65K c-myc protein after activation of cells with mitogens. Human peripheral blood cells were depleted of erythrocytes by incubation with 0.1*M* NH_4Cl at 4°C for 10 minutes and then washed in PBS. The washed cells were suspended in RPMI 1640 medium containing 5 percent nonmitogenic fetal calf serum and divided into two equal parts. One half of the cells were treated with PHA (0.25 μg/ml, final concentration) and the other half was left untreated. Both cell cultures were washed with RPMI 1640 medium without methionine 2 hours after the addition of PHA and labeled for 20 hours with [^{35}S]methionine in medium with (+) or without (−) PHA as described in Fig. 2. An equal amount of acid precipitable radioactivity from each cell extract was immunoprecipitated with the serum indicated. Stimulated cells incorporated approximately twice as much radioactivity as nonstimulated cells in the experiment shown. The samples were analyzed on 10 percent SDS–polyacrylamide gels followed by fluorography.

63K v-myc proteins (*12*). Studies with antiserum to v-myc have also identified proteins of similar sizes in uninfected or malignant chicken cells (*12*). The ability of the anti(c-myc N-ter) to immunoprecipitate c-myc–specific proteins of the 65K size from cells isolated from a number of different species shows that this protein has been conserved during evolution. Accordingly, DNA sequence analysis shows more than 90 percent sequence homology at the amino acid level between chicken, mouse, and human c-myc proteins (*22*). The ability to immunoprecipitate the $p65K^{c\text{-}myc}$ protein from frog oocytes (Fig. 5) suggests that the c-*myc* gene is expressed early during development and also suggests a crucial function for the c-myc protein in a large number of cell types.

Labeling of cells with [^{35}S]methionine followed by immunoprecipitation revealed a strong correlation between cell growth and expression of the $p65K^{c\text{-}myc}$ protein. This was directly demonstrated by induction of quiescent cells with the mitogen PHA (Fig. 6). The large increase in expression of the $p65K^{c\text{-}myc}$ protein after activation with PHA suggests an important role for the $p65K^{c\text{-}myc}$ protein during cell proliferation. Abnormal regulation of the $p65K^{c\text{-}myc}$ protein that allows high levels of expression throughout the cell cycle may therefore disturb normal cell growth, thus leading to cell transformation. The availability of specific antisera to c-myc should be most useful in studying the function of the $p65K^{c\text{-}myc}$ protein in the cell cycle, thus leading to an understanding of its role in malignant transformation of cells. In this respect, we show in an accompanying report (*27*) that the human c-myc protein shares two properties with the MC29 transformation specific $p110K^{gag\text{-}myc}$ polyprotein believed to be relevant for MC29 transformation of cells, namely, a nuclear localization in combination with a DNA binding property.

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