MOLECULAR AND CELLULAR BIOLOGY, Dec. 1986, p. 4450–4457
0270-7306/86/124450-08$02.00/0

Vol. 6, No. 12

Human Proto-Oncogene N-*myc* Encodes Nuclear Proteins That Bind DNA

GARY RAMSAY, LAWRENCE STANTON, MANFRED SCHWAB, AND J. MICHAEL BISHOP*

Department of Microbiology and Immunology and The G. W. Hooper Research Foundation, University of California, San Francisco, California 94143

Received 28 May 1986/Accepted 18 August 1986

N-*myc* is a gene whose amplification has been implicated in the genesis of several malignant human tumors. We have identified two proteins with molecular weights of 65,000 and 67,000 encoded by N-*myc*. The abundance of these proteins in tumor cells was consonant with the extent of amplification of N-*myc*. The two proteins apparently arose from the same mRNA, were phosphorylated, were exceptionally unstable, were located in the nucleus of cells, and bound to both single- and double-stranded DNA. These properties suggest that the products of N-*myc* and of the related proto-oncogene c-*myc* may have similar biochemical functions and that N-*myc* may be a regulatory gene. Our findings sustain the view that inordinate expression of N-*myc* may contribute to the genesis of several different human tumors.

N-*myc* is a gene whose amplification has been demonstrated in three forms of malignant human tumors—neuroblastoma (23, 28, 39), retinoblastoma (27), and small cell carcinoma of the lung (30). In the absence of amplification, the gene is expressed in a limited variety of cells and tissues (17, 40, 48), but its function in either normal or malignant cells is not known.

Three points of evidence suggest that N-*myc* may contribute to tumorigenesis. First, studies on the structure of N-*myc* have revealed a remarkable kinship with the proto-oncogene c-*myc*, which has also been implicated in tumorigenesis (24, 43). Second, amplification of N-*myc* in human neuroblastomas occurs principally in rapidly progressing forms of the tumor, as if abundant expression of N-*myc* might exacerbate the malignant phenotype of the tumor (3). Third, molecular clones of N-*myc* can cooperate with a mutant version of the proto-oncogene c-Ha-*ras* to transform embryonic rat cells in culture (41, 47). These findings suggest that N-*myc* is an authentic proto-oncogene and that elucidation of its biochemical function may provide insight into the genesis of several different human tumors.

To explore the function of N-*myc*, we produced rabbit antisera that allowed the identification of two related proteins encoded by the gene. Both proteins bear striking resemblances to the products of c-*myc*, and their properties suggest that N-*myc* may be a regulatory gene. Slamon et al. (42) have recently described products of human N-*myc* that are apparently identical to those reported here.

MATERIALS AND METHODS

Cells. The human colonic apudoma cell line COLO320-HSR, the three neuroblastoma lines Kelly, IMR-32, and NMB, the retinoblastoma line Y79, and the cervical carcinoma line HeLa were all obtained from the American Type Culture Collection. Human skin fibroblasts and rat embryo cultures were obtained from the University of California at San Francisco Cell Culture Facility. The rat embryo cell line transformed by cotransfection with the oncogenes *ras* and N-*myc* has been described previously (11).

Peptide coupling and immunization. Three peptides were synthesized according to the human N-*myc* nucleotide sequence and supplied by Sequemat, Inc. Peptide R (residues 104 to 113) and G (residues 44 to 58) have complete homology with regions in the human c-*myc* protein, whereas peptide Y (residues 23 to 34) was unique for N-*myc*. The peptides were coupled to tuberculin-purified protein derivative (TPPD) by glutaraldehyde (45). The TPPD was dialyzed to remove glutaraldehyde and peptide. Each coupled peptide was injected subcutaneously into three rabbits (female New Zealand White, 2 to 4 kg). Rabbits were immunized on the following schedule: 200 μg of peptide-coupled TPPD in complete Freund adjuvant on day 0; 200 μg in incomplete Freund adjuvant subcutaneously on day 14; and 200 μg with 4 mg of alum subcutaneously on day 21. After the initial course of injections, rabbits were boosted with 200 μg of peptide-coupled TPPD with 4 mg of alum at four 1-week intervals and bled 1 to 2 weeks following injection.

Cell labeling, immunoprecipitation, and electrophoresis. Immunoprecipitation from labeled cell lysates was performed as described previously (36). Briefly, cells were labeled for 1 h in methionine-free medium containing 250 μCi of [^{35}S]methionine (Amersham Corp.) per ml. Cells were then washed once in ice-cold phosphate-buffered saline, lysed in lysis buffer (25 mM Tris hydrochloride, 150 mM NaCl, 0.5% Nonidet P-40 [NP40], 0.5% sodium deoxycholate, and 0.2% sodium dodecyl sulfate [SDS], pH 8.0), sheared through a fine-gauge needle until no longer viscous, and clarified by centrifugation at 10,000 × *g* for 30 min at 4°C. The supernatant was first cleared of nonspecific material by preincubation with Formalin-fixed *Staphylococcus aureus* cells and centrifugation. The resulting supernatants were immunoprecipitated, washed, and extracted as described previously (36). For competitive inhibition of antibody precipitation (blocking) experiments, 20 μg of peptide per μl of sera were incubated together for 30 min at 4°C and then used directly in the immunoprecipitation assay. The released proteins were electrophoresed on discontinuous 10% SDS–polyacrylamide gels and fluorographed (36).

***S. aureus* V8 protease mapping.** The proteins of interest were solubilized, immunoprecipitated, and electrophoresed as described above. The gel was dried down and exposed for autoradiography. The bands of interest were identified and

* Corresponding author.

cut out, and the proteins were then subjected to partial V8 proteolytic analysis as described by Cleveland et al. (5).

In vitro synthesis of N-*myc*-specific mRNA and proteins. A 1.6-kilobase-pair *Xba*I-*Eco*RI fragment from a human N-*myc* cDNA clone was inserted into the corresponding restriction sites of the polylinker in vector pSP64, giving rise to the construct pcN64RX (see Fig. 4A). The *Eco*RI site of the cDNA that was used in this construction is artificial, having been introduced during the cDNA cloning procedure. N-*myc* RNA was synthesized from pcN64RX as follows. A 50-μl reaction containing 2 μg of plasmid (linearized at the *Eco*RI site), 40 mM Tris hydrochloride (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol (DTT), 50 U of RNasin (Promega Biotec), 500 μM each ATP, CTP, UTP, and GTP, and 10 U of SP6 RNA polymerase (Promega Biotec) was incubated at 37°C for 90 min. The reaction mixture was then treated with 2 U of pancreatic DNase at 27°C for 15 min, followed by equal-volume extractions, once with phenol and twice with chloroform. The RNA was then precipitated by ethanol in the presence of 2 M ammonium acetate. The yield of RNA was approximately 10 μg. The in vitro-transcribed N-*myc* RNA was then translated in a rabbit reticulocyte lysate (Promega Biotec), and the translational products were analyzed on a 10% SDS-polyacrylamide gel.

Subcellular fractionation. Approximately 2×10^7 cells were labeled as described above and then fractionated by a method similar to that described by Klempnauer et al. (21). Cells were washed twice in ice-cold hypotonic buffer containing 5 mM KCl, 1 mM $MgCl_2$, and 25 mM Tris hydrochloride (pH 7.4) and then incubated in that buffer for 15 min. Then an equal volume of hypotonic buffer containing 1% NP40 was added, and the cells were incubated for a further 5 min. The nuclei were separated from the cytoplasmic fraction by centrifugation at $1,000 \times g$ for 5 min and then washed twice in hypotonic buffer plus 0.5% NP40 before solubilization in lysis buffer. The wash fractions were combined with the cytoplasmic fraction and then adjusted to the same salt and detergent concentration as the nuclear fraction. All fractions were then analyzed by immunoprecipitation as described above.

DNA-cellulose chromatography. The DNA-binding experiment was performed as described elsewhere (34). Cells that had been labeled with $^{32}P_i$ were washed with Tris-saline and lysed in 200 μl of buffer A (10 mM Tris hydrochloride, pH 8.0, 1 mM $MgCl_2$, 0.5% NP40, 0.45 M NaCl). The lysate was then clarified by centrifugation at $10,000 \times g$ for 10 min at 4°C. The supernatant was removed and immediately diluted 10-fold with loading buffer (10 mM potassium phosphate, pH 6.2, 1 mM $MgCl_2$, 0.5% NP40, 1 mM DTT, 10% glycerol) without salt and adjusted to pH 6.2 by the addition of 0.01 M HCl. The extract was then loaded onto a column of single- or double-stranded calf thymus DNA-cellulose (bed volume approximately 1 ml; P-L Biochemicals, Inc., Milwaukee, Wis.) which had been equilibrated in loading buffer with 50 mM NaCl. The column was then washed with 5 bed volumes of loading buffer to remove any unbound protein. Bound protein was removed by washing the column with elution buffer (10 mM Tris hydrochloride, pH 8.0, 1 mM DTT, and increasing concentrations of NaCl). Samples of the flowthrough and gradient fractions were analyzed by immunoprecipitation.

RESULTS

Identification of proteins encoded by N-*myc*. We used three synthetic oligopeptides as antigens (Materials and Methods). Two of these represent regions that are shared between the proteins encoded by c-*myc* and N-*myc*; the third is unique to N-*myc*. All are located in the amino-terminal domain of the N-*myc* protein, the only portion of the protein for which we had deduced the amino acid sequence at the time the choices of antigens were made. When coupled to carrier protein, all three oligopeptides elicited rabbit antibodies that reacted specifically with the homologous immunogen in enzyme-linked immunosorbent assays. Only the antiserum induced by the unique peptide immunoprecipitated cellular proteins that might be encoded by N-*myc* (data not shown). We designated this antiserum Y3 and proceeded to characterize its reactivity in detail.

The N-*myc* gene is expressed at very low levels or not at all in most established lines of human cells that have been examined (22, 40). We therefore performed our preliminary tests with the neuroblastoma cell line Kelly, in which N-*myc* RNA is abundant by virtue of a 200-fold amplification of the gene (40). When reacted with extracts of Kelly cells, the Y3 antiserum immunoprecipitated two proteins with molecular weights of 65,000 and 67,000 (Fig. 1A, lane 4), whose resolution is better illustrated in Fig. 1C. Precipitation of both proteins could be blocked by competition with the peptide used to induce the antisera (Fig. 1A, lane 3). Since both proteins were phosphorylated and since both were encoded by N-*myc* (see below), we designated them pp65/pp67$^{\text{N-}myc\text{(human)}}$.

We examined a series of cell lines to determine whether the amounts of the putative N-*myc* proteins reflected the previously determined abundance of N-*myc* RNA (Fig. 1B, lanes 1 to 10). We used three cell lines that display amplification of N-*myc* (the neuroblastoma lines IMR-32 and NMB and the retinoblastoma line Y79) and two sorts of cells in which N-*myc* is not amplified (HeLa cells and human skin fibroblasts). All of the cell lines with amplification of N-*myc* contained readily detectable pp65/67$^{\text{N-}myc}$, whereas no N-*myc* protein could be detected in the cells with diploid copies of N-*myc*. These results exemplify our subsequent experience with numerous human cell lines: N-*myc* protein was detectable only in cells with amplification of the gene; amplification of N-*myc* had no effect on the molecular weights of the gene products; and in no instance were the relative amounts of pp65 and pp67 changed (data not shown).

We made a preliminary comparison with products of c-*myc* by immunoprecipitating pp62/66$^{\text{c-}myc\text{(human)}}$ from the cell line COLO320 (Fig. 1A, lane 2) with a previously characterized antiserum (35). The results demonstrated three similarities between the products of c-*myc* and N-*myc*. (i) The products of the two genes had similar molecular weights. (ii) The proteins ran at anomalous positions in SDS-polyacrylamide gels: the predicted molecular weights of the two gene products predicted from nucleotide sequencing should be 48,000 (c-*myc*) (6) and 49,000 (N-*myc*) (24, 43). (iii) Two forms of product from each gene could be detected.

The peptide against which our antiserum was raised has no homology with c-*myc*, and no cross-reactivity was observed in immunoprecipitations with cells containing high levels of c-*myc* protein (data not shown). Similarly, our previously described antisera directed against the products of c-*myc* failed to react with the proteins allegedly encoded by N-*myc*.

Authentication of the N-*myc* products by gene transfer. It is possible to establish transformed cells from primary rat embryo cells after cotransfection with the N-*myc* and *ras* genes (41, 47). We made use of cells transformed in this way to show that introduction of N-*myc* into a cell not previously

expressing the gene results in the production of pp65/67$^{N\text{-}myc(human)}$. We used cell lines transfected with c-Ha-*ras* (EJ) in combination with a construction containing the two coding exons of N-*myc* linked to the promoter-enhancer of Moloney murine leukemia virus (41). The Y3 antiserum did not precipitate proteins resembling the products of N-*myc* from rat embryo cells (Fig. 2, lane 2). Thus, either the antiserum does not react with the rat N-*myc* protein or the level of that protein in the cells is so low it is undetectable. (The latter possibility is likely to be correct, since N-*myc* RNA cannot be detected in cultures of rat embryo cells [41].) With the cell lines cotransfected with N-*myc* and *ras*, however, pp65 and pp67 were specifically precipitated and found to comigrate with the proteins detected in Kelly cells (Fig. 2, lanes 4 and 6). We conclude that pp65 and pp67 are probably encoded by human N-*myc*.

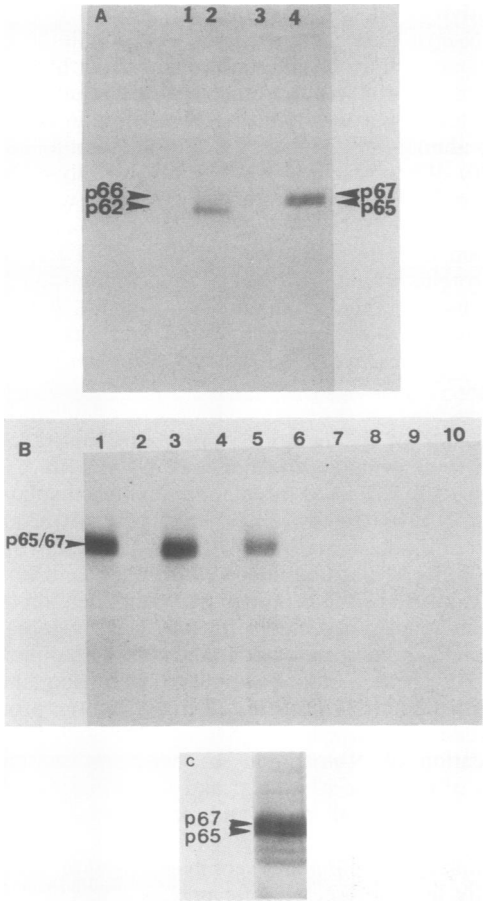

FIG. 1. Comparative immunoprecipitation of N-*myc* proteins from a variety of human cell lines. (A) Approximately 10^7 cells from the human cell line COLO320HSR (lanes 1 and 2) and the neuroblastoma cell line Kelly (lanes 3 and 4) were labeled for 60 min with [^{35}S]methionine (500 μCi), and the c-*myc* and N-*myc* proteins were immunoprecipitated as described in Materials and Methods. Lanes: 1, c-*myc*-specific serum G10 preincubated with the peptide against which the serum was raised; 2, c-*myc*-specific serum G10; 3, Y3 serum preincubated with the Y peptide; 4, Y3 serum. (B) Approximately 10^7 cells were labeled with [^{35}S]methionine, and the N-*myc* protein was immunoprecipitated. The amount of radioactivity incorporated into total cellular protein was determined by precipitation onto filters with 10% trichloroacetic acid, and the quantity of lysate used for immunoprecipitation was adjusted to give equal counts per minute. Lanes: 1 and 2, retinoblastoma line Y79; 3 and 4, neuroblastoma line 1MR32; 5 and 6, neuroblastoma line NMB; 7 and 8, HeLa; 9 and 10, human skin fibroblasts. Lanes 1, 3, 5, 7, and 9 were immunoprecipitated with the anti-human N-*myc* serum Y3; lanes 2, 4, 6, 8, and 10 were immunoprecipitated with Y3 serum preincubated with peptide Y. (C) Neuroblastoma cell line Kelly was labeled for 60 min with [^{35}S]methionine (500 μCi), and the N-*myc* proteins were immunoprecipitated with Y3 serum.

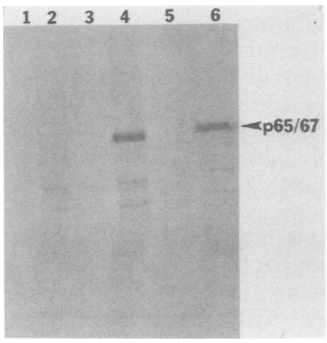

FIG. 2. Detection of the human N-*myc* proteins in cells transformed by DNA-mediated gene transfer. Kelly cells, rat embryo fibroblasts, and the rat cell line V1-d [established by cotransfection with c-Ha-*ras* (EJ) and exons 2 and 3 of human N-*myc*] were labeled for 1 h with [^{35}S]methionine (500 μCi) and then immunoprecipitated. Lanes: 1, rat embryo cultures immunoprecipitated with Y3 serum preincubated with peptide Y; 2, rat embryo cultures immunoprecipitated with Y3 serum; 3, V1-d cells immunoprecipitated with Y3 serum preincubated with peptide Y; 4, V1-d cells immunoprecipitated with Y3 serum; 5, Kelly cells immunoprecipitated with Y3 serum preincubated with Y peptide; 6, Kelly cells immunoprecipitated with Y3 serum.

N-*myc* products generated by translation in vitro. We used translation in vitro to authenticate further the genetic origin of pp65 and pp67 and to address the genesis of the two forms of the protein. The SP6 bacteriophage promoter was used to transcribe a cDNA containing the entire coding unit of N-*myc* (Fig. 3A). Translation of the resulting RNA produced two proteins with electrophoretic mobilities identical to those of the candidate products of N-*myc* and two smaller proteins with molecular weights of ca. 55,000 (Fig. 3B, lane 2). To determine whether the pp65/67 synthesized in vivo was related to the pp65/67 seen in vitro, we carried out partial proteolytic mapping with *S. aureus* V8 protease. pp65 and pp67 made in vivo gave maps identical to those of the pp65 and pp67 synthesized in vitro (Fig. 3C, lanes 1 through 4).

We conclude that we have identified authentic products of N-*myc*. The two larger proteins (pp65 and pp67) must arise from the same mRNA and the same reading frame, perhaps by virtue of modification subsequent to translation: the cDNA contains only one reading frame capable of encoding these proteins, and splicing to give different mRNAs is unlikely to occur in the transcriptional reaction with the SP6 promoter. The two smaller proteins produced in abundance in vitro have not been found in cells (data not shown). They could be the products of either proteolytic cleavage or translational initiation within the reading frame.

Products of N-*myc*(human) are unstable phosphoproteins. Labeling cells with $^{32}P_i$ revealed that both forms of the protein encoded by N-*myc* were phosphorylated (Fig. 4, lane 4). The ratio of ^{32}P-labeled pp65 to pp67 was the same as that when the proteins were labeled with [^{35}S]methionine. It is therefore possible that the difference between the two forms is not due to phosphorylation, as is also the case for the products of c-*myc* (35).

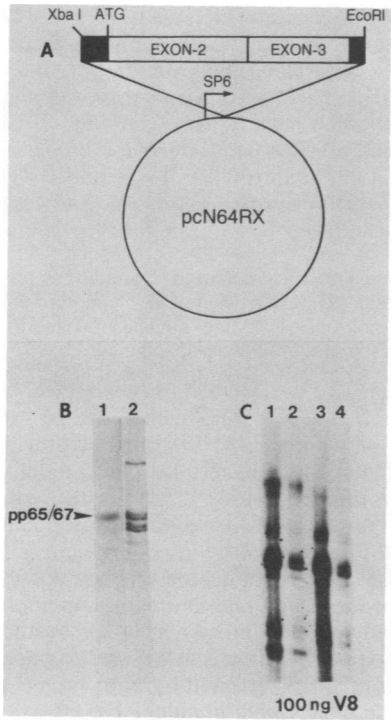

FIG. 3. Comparative analysis of N-*myc* proteins synthesized in vitro and in vivo. (A) Schematic of plasmid pcN64RX used for in vitro production of N-*myc* RNA. The amino acid-coding portion of a human N-*myc* cDNA clone was inserted distally to the SP6 promoter in the transcription vector pSP64. The plasmid, pcN64RX, generates translatable N-*myc* RNA in an SP6 transcription system when the plasmid is linearized at the *Eco*RI site. The entire coding portion of N-*myc* contained within exon 2 and 3 is present in the construction, along with 140 and 70 base pairs of 5′ and 3′ untranslated sequences (blackened bars). (B) The construct was transcribed and translated as described in Materials and Methods. Lane 1 shows the N-*myc* gene product immunoprecipitated from Kelly cells; lane 2 shows the in vitro product. (C) Partial V8 proteolytic mapping of the in vitro and in vivo products of the N-*myc* gene. The in vivo and in vitro N-*myc* gene products were electrophoresed and eluted from a 10% SDS–polyacrylamide gel, incubated with 100 ng of V8 protease, and electrophoresed on a 14% polyacrylamide gel as described in Materials and Methods. Lanes: 1, in vivo p65; 2, in vitro p65; 3, in vivo p67; 4, in vitro p67.

We used pulse-chase analysis to determine the half-lives of pp65/67$^{N\text{-}myc(human)}$ and explore the relationship of the two proteins. Both forms were labeled and chased in a parallel fashion, demonstrating that neither protein is a precursor of the other (Fig. 5A). These data allowed us to determine that the half-lives of pp65 and pp67 are ca. 30 min (Fig. 5B). This is in remarkable agreement with the half-lives of pp62/66$^{c\text{-}myc(human)}$ and is in accord with the view that the *myc* gene family encodes proteins that are regulatory rather than structural elements in the cell (18).

N-*myc* proteins are located in the nucleus. The proteins encoded by v-*myc* and a variety of c-*myc* genes have been found in the cell nucleus by both biochemical fractionation and immunofluorescence microscopy (1, 2, 9, 15). To determine whether the product of N-*myc* was similarly located, we prepared subcellular fractions from two cell lines (Kelly and Y79) and assayed the fractions for pp65/67$^{N\text{-}myc(human)}$ by immunoprecipitation. When cells were lysed by hypotonic shock and then fractionated, more than 95% of the pp65/67 was found in the nuclear fraction (Fig. 6). We attempted to extend these observations by the use of immunofluorescence microscopy but were unable to obtain a persuasive signal, perhaps because the N-*myc* proteins were not sufficiently abundant in any of the cells we examined (data not shown). We have encountered similar difficulties with the products of c-*myc* with cells in which the abundance of c-*myc* protein resembled that of N-*myc* protein in the cells examined here.

N-*myc* protein binds to DNA. The viral and cellular *myc* gene products bind to both single- and double-stranded DNA (9, 10, 32, 46). We therefore tested the N-*myc* product for similar capability. The DNA binding was performed by DNA-cellulose chromatography as previously described for simian virus 40 large tumor antigen (34). A column containing cellulose without bound DNA did not retain pp65/67$^{N\text{-}myc(human)}$ (data not shown). By contrast, columns containing cellulose linked to calf thymus single- or double-stranded DNA retained a high proportion of pp65/67$^{N\text{-}myc(human)}$ (Fig. 7A and B). The N-*myc* protein could be eluted with NaCl concentrations ranging from 0.3 to 2.0 M (Fig. 7A and B). When the material present in the column flowthrough was rechromatographed, most of the N-*myc* protein bound to the DNA, suggesting that we exceeded the capacity of the column in the initial fractionation. We conclude that the N-*myc* proteins can bind to DNA, but the specificity and mechanism of that binding remain to be evaluated.

DISCUSSION

Identification of the proteins encoded by human N-*myc*. We used an oligopeptide as an antigen to obtain an antiserum that reacts with the proteins encoded by human N-*myc*. Two of the peptides tested originally as antigens represented amino acid sequences found in the proteins encoded by both c-*myc* and N-*myc*. Neither of these peptides elicited a useful antiserum. The successful immunizations were performed with an oligopeptide that is unique to the N-*myc* protein. We chose this peptide for use because it contained a small hydrophobic region that in the full N-*myc* protein is embedded within a large hydrophilic domain. We reasoned that the entire domain was likely to be on the surface of the protein and that the small hydrophobic region might therefore be

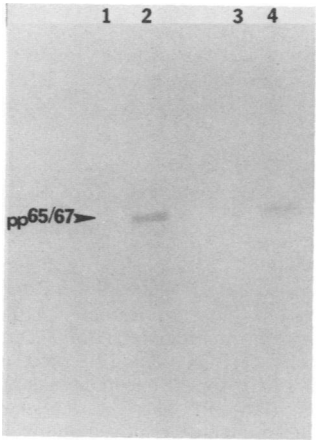

FIG. 4. Phosphorylation of the human N-*myc* gene product. Kelly cells were labeled for 1 h with [^{35}S]methionine (500 μCi) (lanes 1 and 2) or for 3 h with 1 mCi of $^{32}P_i$ (lanes 3 and 4) and then analyzed with either Y3 serum preincubated with peptide Y (lanes 1 and 3) or Y3 serum (lanes 2 and 4).

protuberant and hence a particularly accessible epitope. However arbitrary this reasoning might seem, the peptide did indeed elicit high-titer antibodies that reacted satisfactorily with proteins encoded by N-*myc*.

Several independent lines of evidence indicate that we have identified the authentic products of N-*myc*. (i) Immunoprecipitation of the proteins could be blocked by competition with an excess of the appropriate peptide. (ii) Cells with high levels of N-*myc* RNA (e.g., Kelly and Y79) synthesized large quantities of pp65/67$^{N\text{-}myc(human)}$; in contrast, cells containing very low levels of N-*myc* RNA contained little or no pp65/67. (iii) Introduction of the coding domain of N-*myc*(human) into rat embryo cells engendered the synthesis of pp65/67$^{N\text{-}myc(human)}$. (iv) Proteins apparently identical to pp65/67$^{n\text{-}myc}$ could be translated from N-*myc* mRNA synthesized in vitro.

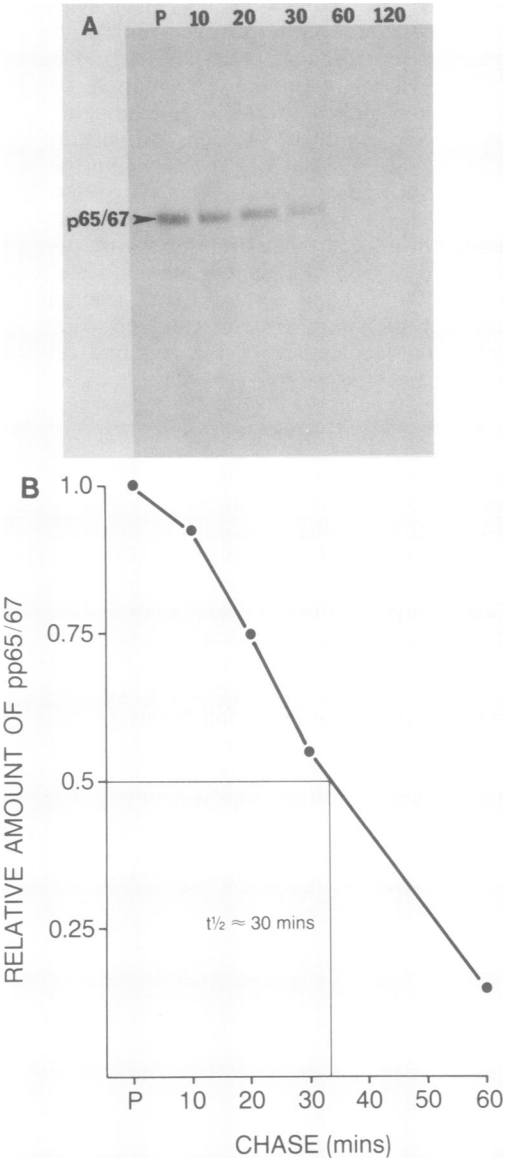

FIG. 5. Products of N-*myc* are unstable. (A) Pulse-chase analysis of the N-*myc* proteins immunoprecipitated from Kelly cells. Six samples of Kelly cells, each containing 10^7 cells, were labeled with [^{35}S]methionine (500 μCi) for 30 min. One sample was lysed and immunoprecipitated (lane P), and the remaining five had the [^{35}S]methionine-labeled medium removed and were then incubated at 37°C for the indicated times (in minutes) in chase medium containing excess cold methionine. (B) Analysis of a pulse-chase experiment obtained by densitometric scanning of the fluorogram shown in panel A. The rate of synthesis of the N-*myc* proteins was arbitrarily set at 1.0.

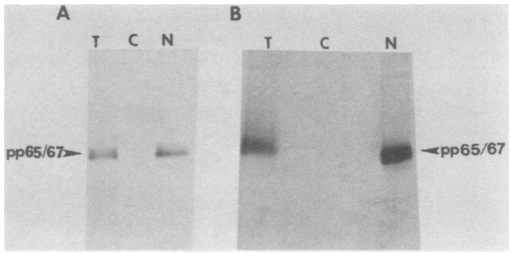

FIG. 6. Subcellular fractionation of human N-*myc* proteins. Y79 (A) or Kelly cells (B) were labeled with [^{35}S]methionine, divided into total (T), cytoplasmic (C), and nuclear (N) fractions, and then immunoprecipitated with N-*myc*-specific serum Y3. Sample T represented 50% (A) or 20% (B) of the labeled cells.

From these data, we conclude that our antisera recognize authentic products of human N-*myc*. Slamon et al. (42) have also identified products of human N-*myc* with antisera that were raised against antigen synthesized in bacteria and that reacted with proteins encoded by both N-*myc* and c-*myc*.

The products of N-*myc* in cells have always appeared as two proteins, with apparent molecular weights of 65,000 and 67,000. By contrast, the nucleotide sequence of N-*myc* encodes a protein with a calculated molecular weight of 49,000 (24, 43). A similar size anomaly has been observed for the products of c-*myc* (16, 31, 36). No effort to explain this anomaly by a modification subsequent to translation has succeeded. Instead, it appears likely that the chemical compositions of c-*myc* and N-*myc* proteins affect their mobilities during electrophoresis through polyacrylamide.

How do the two forms of c-*myc* and N-*myc* proteins arise? Previous efforts to attribute the two c-*myc* proteins to different mRNAs have now been refuted (31). We are similarly certain that the two forms of N-*myc* protein are produced from a single mRNA because transcription of N-*myc* in vitro, under circumstances in which splicing is unlikely to occur, produces mRNA that can be translated into both forms of the protein. These findings leave the duality of both c-*myc* and N-*myc* proteins unexplained, although it is unlikely that they are due to differences in either phosphorylation or glycosylation of the proteins (16, 35; present data).

***myc* gene family.** Early efforts to isolate human genes related to v-*myc* revealed a family of genes with several members (12). Three of these have now been well defined: c-*myc* (26), L-*myc* (29), and N-*myc* (24, 43). Each is distinctive, but the three are nevertheless closely related.

The similarities between c-*myc* and N-*myc* have been especially well documented. The two genes have remarkably similar topographies and coding domains (24, 43); each can cooperate with a mutant version of the proto-oncogene c-Ha-*ras* to transform embryonic cells in culture (41, 47); expression of either gene declines in certain lines of tumor cells when they are induced to differentiate (37, 44); and the proteins encoded by the two genes share several prominent biochemical properties and may therefore possess related functions. The two genes differ in their expression, however: c-*myc* is expressed in a wide variety of cells (14, 33), whereas expression of N-*myc* has been detected in only a limited variety of cells and tissues (17, 48). We therefore

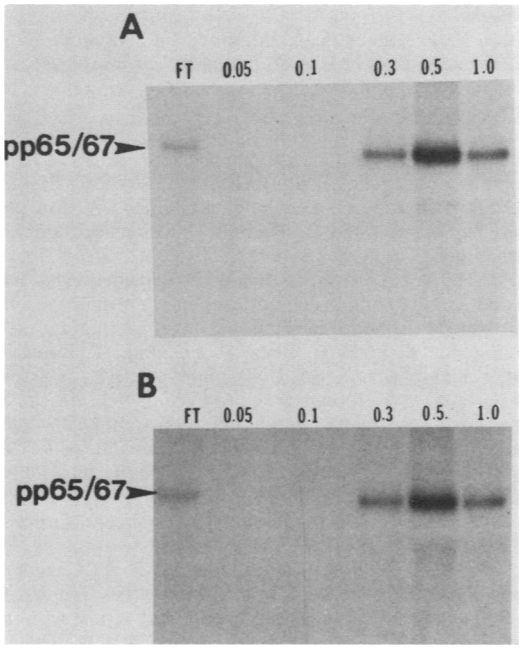

FIG. 7. DNA-cellulose chromatography of p65/67$^{N\text{-}myc}$. Nuclei from approximately 2×10^7 Kelly cells labeled with $^{32}P_i$ were incubated in the presence of 0.4 M NaCl. In separate experiments, the extract diluted in buffer without salt was passed through columns containing either (A) single- or (B) double-stranded calf thymus DNA linked to cellulose. Samples were applied in buffer containing 0.015 M NaCl, and the columns were washed batchwise with buffers containing the indicated salt concentrations. The flow-through (FT) and gradient fractions were then analyzed by immunoprecipitation.

suspect that the two genes serve different physiological purposes, in contrast to the biochemical similarities of their products.

N-*myc* in tumorigenesis. Amplification or abundant expression of N-*myc* has been reported to be a frequent feature of three human malignancies—neuroblastoma (23, 39), retinoblastoma (27), and small cell carcinoma of the lung (30). These reports were based solely on the analysis of nucleic acids. Here we show that the production of N-*myc* protein by tumor cells is consonant with the abundance of RNA transcribed from the gene and that amplification of N-*myc* therefore augments expression of the gene.

What is the significance of amplification and inordinate expression of N-*myc* in tumor cells? The most provocative clue has come from the finding that amplification of N-*myc* in human neuroblastomas occurs principally in rapidly progressing forms of the tumor that are resistant to therapy (3). These findings have prompted two suggestions: that inordinate expression of N-*myc* may exacerbate the malignant phenotype of the tumor, and that amplification of N-*myc* can be used as a diagnostic and prognostic device. The antisera we have described here should permit these suggestions to be explored in greater detail.

What are the functions of *myc* genes? The biochemical and physiological functions of the proteins encoded by *myc* genes are not known. We have three clues to these functions, however, in the form of properties shared by all of the *myc* proteins studied to date.

First, the proteins are located in the nucleus of both normal and transformed cells (1, 2, 9, 15; present data). The consistency and rapidity with which *myc* proteins reach the nucleus suggest that the principal site of action for these proteins must be in that organelle. There is disagreement, however, over which compartment within the nucleus contains the site of action: some workers have assigned the proteins to the "nuclear matrix" (10), and others have questioned this assignment (11). We have not as yet examined this issue in detail for the N-*myc* proteins, but others have assigned the products of N-*myc* to the nuclear matrix (42).

Second, the proteins are decidedly unstable, a property they share with nuclear proteins encoded by other oncogenes and proto-oncogenes, such as *fos* (7) and *myb* (G. Ramsay, unpublished observations). Cellular proteins typically have half-lives that range from 10 h to several days (13). By contrast, the half-lives of the proteins encoded by *fos*, *myb*, and *myc* are on the order of 30 to 60 min. This property suggests that the proteins are designed to undergo rapid changes in abundance, a conclusion that is further substantiated by the remarkable instability of the mRNA for c-*myc* (8).

Third, the proteins encoded by c-*myc* and N-*myc* bind to both single- and double-stranded DNA (9, 10, 31, 46; present data). The products of *myb* genes have the same property (20, 28; G. Ramsay, unpublished data). The binding of *myc* protein to DNA is presently regarded as nonspecific (46), and its functional significance is therefore open to question. In addition, it is possible that the binding is mediated by other proteins. We consider this an unlikely possibility because preliminary experiments have shown that N-*myc* protein still binds to DNA after purification by immunoaffinity chromatography, as is also the case for proteins encoded by c-*myc* and v-*myc* (9, 44). The domain within the proteins responsible for the binding has not been directly identified, although the region that is most conserved between the products of N-*myc* and c-*myc* has chemical qualities resembling those of proteins whose functions require interaction with DNA (24, 43).

We conclude that N-*myc* and its kin are more likely to serve regulatory than structural purposes within the cell. There is only one hint, however, as to what those purposes might be: the claim that c-*myc* can act in *trans* to augment transcription from another gene (19). Our preliminary efforts to demonstrate a similar property for N-*myc* have failed (N. Hay, personal communication), but the reagents and information described here provide a point of departure from which the function of N-*myc* can now be explored in detail.

ACKNOWLEDGMENTS

We thank our colleagues for many useful discussions and Lynn Vogel for her preparation of the manuscript.

Work summarized here was supported by Public Health Service grants CA-12705 and SO7-RR05355 from the National Institutes of Health and by funds from the G. W. Hooper Research Foundation. G.R. is a fellow of the Weingart Foundation, and L.W.S. is a fellow of the Leukemia Society of America.

LITERATURE CITED

1. **Abrams, H. D., L. R. Rohrschneider, and R. N. Eisenman.** 1982. Nuclear location of the putative transforming protein of avian myelocytomatosis virus. Cell **29**:427–439.
2. **Alitalo, K., G. Ramsay, J. M. Bishop, S. Ohlsson, W. W. Colby, J. P. McGrath, and A. D. Levinson.** 1983. Identification of nuclear proteins encoded by viral and cellular *myc* oncogenes. Nature (London) **306**:274–277.
3. **Brodeur, G. M., R. C. Seeger, M. Schwab, H. E. Varmus, and**

J. M. Bishop. 1984. Amplification of N-*myc* in untreated human neuroblastomas correlates with advanced disease stage. Science **224**:1121–1124.
4. **Campisi, J., H. E. Gray, A. B. Pardee, M. Dean, and G. E. Sonenshein.** 1984. Cell-cycle control of c-*myc* but not c-*ras* expression is lost following chemical transformation. Cell **36**:241–247.
5. **Cleveland, D., S. Fischer, M. Kirschner, and U. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
6. **Colby, W. W., E. Y. Chen, D. H. Smith, A. D. Levinson, D. J. Slamon, T. C. Boone, R. C. Seeger, D. E. Keith, V. Chazin, H. C. Lee, and L. M. Souza.** 1986. Identification and characterization of the protein encoded by the human N-*myc* oncogene. Science **232**:768–777.
7. **Curran, T., A. D. Miller, L. Zokas, and I. M. Verma.** 1984. Viral and cellular *fos* proteins: a comparative analysis. Cell **36**:259–268.
8. **Dani, C., J. M. Blanchard, M. Piechaczyk, S. El Sabouty, L. Marty, and P. Jeanteur.** 1984. Extreme instability of *myc* mRNA in normal and transformed human cells. Proc. Natl. Acad. Sci. USA **81**:7046–7051.
9. **Donner, P., I. Greiser-Wilke, and K. Moellig.** 1982. Nuclear localization and DNA binding of the transforming gene product of avian myelocytomatosis virus. Nature (London) **296**:262–265.
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., and D. C. Hancock.** 1986. Studies on the interaction of the human c-*myc* protein with cell nuclei: $p62^{c\text{-}myc}$ as a member of a discrete subset of nuclear proteins. Cell **43**: 253–261.
12. **Favera, R. D., E. P. Gelmann, S. Martinotti, G. Franchini, T. S. Papas, R. C. Gallo, and F. Wong-Staal.** 1982. Cloning and characterization of different human sequences related to the *onc* gene (v-*myc*) of avian myelocytomatosis virus (MC29). Proc. Natl. Acad. Sci. USA **79**:6497–6501.
13. **Goldberg, A. L., and A. C. St. John.** 1976. Intracellular protein degradation in mammalian and bacterial cells. Annu. Rev. Biochem. **45**:747–804.
14. **Gonda, T. J., D. K. Sheiness, and J. M. Bishop.** 1982. Transcripts from the cellular homologs of retroviral oncogenes: distribution among chicken tissues. Mol. Cell. Biol. **2**:617–624.
15. **Hann, S. R., H. D. Abrams, L. R. Rohrschneider, and R. N. Eisenman.** 1983. Proteins encoded by the v-*myc* and c-*myc* oncogenes: identification and localization in acute leukemia virus transformants and bursal lymphoma cell line. Cell **34**:789–798.
16. **Hann, S. R., and R. N. Eisenman.** 1984. Proteins encoded by the human c-*myc* oncogene: differential expression in neoplastic cells. Mol. Cell. Biol. **4**:2486–2497.
17. **Jakobovits, A., M. Schwab, J. M. Bishop, and G. R. Martin.** 1985. Expression of N-*myc* in teratocarcinoma stem cells and mouse embryos. Nature (London) **318**:188–191.
18. **Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder.** 1983. Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. Cell **35**:603–610.
19. **Kingston, R. E., A. S. Baldwin, Jr., and P. A. Sharp.** 1984. Regulation of heat shock protein 70 gene expression by c-*myc*. Nature (London) **312**:280–282.
20. **Klempnauer, K.-H., and A. E. Sippel.** 1986. Subnuclear localization of proteins encoded by the oncogene v-*myb* and its cellular homolog c-*myb*. Mol. Cell. Biol. **6**:62–69.
21. **Klempnauer, K.-H., G. Symonds, G. Evan, and J. M. Bishop.** 1984. Subcellular location of proteins encoded by viral and cellular myb genes. Cell **37**:537–547.
22. **Kohl, N. E., C. E. Gee, and F. W. Alt.** 1984. Activated expression of the N-*myc* gene in human neuroblastomas and related tumors. Science **226**:1335–1337.
23. **Kohl, N. E., N. Kanda, R. R. Schreck, G. Burns, S. A. Latt, F. Gilbert, and F. W. Alt.** 1983. Transposition and amplification of oncogene-related sequences in human neuroblastomas. Cell **35**:359–367.
24. **Kohl, N. E., E. Legouy, R. A. DePinho, P. E. Nisen, R. K. Smith, C. E. Gee, and F. W. Alt.** 1986. Human N-*myc* is closely related in organization and nucleotide sequence to c-*myc*. Nature (London) **319**:73–77.
25. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature (London) **304**:596–601.
26. **Leder, P. J. Battey, G. Lenoir, C. Moulding, W. Murphy, H. Potter, T. Stewart, and R. Taub.** 1984. Translocations among antibody genes in human cancer. Science **222**:765–771.
27. **Lee, W.-H., A. L. Murphree, and W. F. Benedict.** 1984. Expression and amplification of the N-*myc* gene in primary retinoblastoma. Nature (London) **309**:458–460.
28. **Moelling, K., E. Pfaff, H. Beug, P. Beimling, T. Bunte, H. E. Schaller, and T. Graf.** 1985. DNA-binding activity is associated with purified *myb* proteins from AMV and E26 viruses and is temperature-sensitive for E26 ts mutants. Cell **40**:983–990.
29. **Nau, M. M., B. J. Brooks, J. Battey, E. Sausville, A. F. Gazdar, I. R. Kirsch, O. W. McBride, V. Bertness, G. F. Hollis, and J. D. Minna.** 1985. L-*myc*, a new *myc*-related gene amplified and expressed in human small cell lung cancer. Nature (London) **318**:69–73.
30. **Nau, M. M., J. B. Burke, D. N. Carney, A. F. Gazdar, J. F. Battey, E. A. Sausville, and J. D. Minna.** 1986. Human small-cell lung cancers show amplification and expression of the N-*myc* gene. Proc. Natl. Acad. Sci. USA **83**:1092–1096.
31. **Persson, H., L. Hennighausen, R. Taub, W. DeGrado, and P. Leder.** 1984. Antibodies to human c-*myc* oncogene product: evidence of an evolutionarily conserved protein induced during cell proliferation. Science **225**:687–693.
32. **Persson, H., and P. Leder.** 1984. Nuclear localization and DNA binding properties of a protein expressed by human c-*myc* oncogene. Science **225**:718–721.
33. **Pfeifer-Ohlsson, E., A. S. Goustin, J. Rydnert, L. Bjersing, D. Stehelin, and R. Ohlsson.** 1984. Spatial and temporal pattern of cellular *myc* oncogene expression in developing human placenta: implication for embryonic cell proliferation. Cell **38**:585–596.
34. **Prives, C., B. Barnet, A. Scheller, G. Khoury, and J. Gilbert.** 1982. Discrete regions of simian virus 40 large T antigen are required for nonspecific and viral origin-specific DNA binding. J. Virol. **43**:73–82.
35. **Ramsay, G., G. I. Evan, and J. M. Bishop.** 1984. The protein encoded by the human proto-oncogene c-*myc*. Proc. Natl. Acad. Sci. USA **81**:7742–7746.
36. **Ramsay, G., M. J. Hayman, and K. Bister.** 1982. Phosphorylation of specific sites in the *gag-myc* polyproteins encoded by MC29-type viruses correlates with their transforming ability. EMBO. J. **1**:1111–1116.
37. **Reitsma, P. H., P. G. Rothberg, S. M. Astrin, J. Trial, Z. Bar-Shavit, A. Hall, S. L. Teitelbaum, and A. J. Kahn.** 1983. Regulation of *myc* gene expression in HL-60 leukaemia cells by a vitamin D metabolite. Nature (London) **306**:492–494.
38. **Schwab, M.** 1985. Amplification of N-*myc* in human neuroblastomas. Trends Genet. **1**:172–175.
39. **Schwab, M., K. Alitalo, K.-H. Klempnauer, H. E. Varmus, J. M. Bishop, F. Gilbert, G. Brodeur, M. Goldstein, and J. Trent.** 1983. Amplified DNA with limited homology to the *myc* cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumor. Nature (London) **305**:245–248.
40. **Schwab, M., J. Ellison, M. Busch, W. Rosenau, H. E. Varmus, and J. M. Bishop.** 1984. Enhanced expression of the human gene N-*myc* consequent to amplification of DNA may contribute to malignant progression of neuroblastoma. Proc. Natl. Acad. Sci. USA **81**:4940–4945.
41. **Schwab, M., H. E. Varmus, and J. M. Bishop.** 1985. Human N-*myc* gene contributes to neoplastic transformation of mammalian cells in culture. Nature (London) **316**:160–162.
42. **Slamon, D. J., T. C. Boone, R. C. Seeger, D. E. Keith, V. Chazin, H. C. Lee, and L. M. Souza.** 1986. Identification and characterization of the protein encoded by the human N-*myc* oncogene. Science **232**:768–777.

43. **Stanton, L. W., M. Schwab, and J. M. Bishop.** 1986. Nucleotide sequence of the human N-*myc* gene. Proc. Natl. Acad. Sci. USA **83**:1772–1776.
44. **Thiele, C. J., C. P. Reynolds, and M. A. Israel.** 1985. Decreased expression of N-*myc* precedes retinoic acid-induced morphological differentiation of human neuroblastoma. Nature (London) **313**:404–406.
45. **Walter, G., and R. Doolittle.** 1983. Antibodies against synthetic peptides, p. 61–91. *In* J. K. Setlow and A. Hollaender (ed.), Genetic engineering. Academic Press, Inc., New York.
46. **Watt, R. A., A. R. Shatzman, and M. Rosenberg.** 1985. Expression and characterization of the human c-*myc* DNA-binding protein. Mol. Cell. Biol. **5**:448–456.
47. **Yancopoulos, G. D., P. D. Nisen, A. Tesfaye, N. E. Kohl, M. P. Goldfarb, and F. W. Alt.** 1985. N-*myc* can cooperate with *ras* to transform normal cells in culture. Proc. Natl. Acad. Sci. USA **82**:5455–5459.
48. **Zimmerman, K. A., G. D. Yancopoulos, R. G. Collum, R. K. Smith, N. E. Kohl, K. A. Denis, M. M. Nau, O. N. Witte, D. Toran-Allerand, C. E. Gee, J. D. Minna, and F. W. Alt.** 1986. Differential expression of *myc* family genes during murine development. Nature (London) **319**:780.