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The protein encoded by the human proto-oncogene c-*myc*

(chicken c-*myc*/Burkitt lymphoma/gene amplification/chromosomal translocation)

GARY RAMSAY, GERARD I. EVAN*, AND J. MICHAEL BISHOP

Department of Microbiology and Immunology and The Hooper Research Foundation University of California Medical Center, San Francisco, CA 94143

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ABSTRACT **The proto-oncogene c-*myc* may play a role in controlling the growth and division of normal cells, and abnormalities of the gene have been implicated in the genesis of a substantial variety of human tumors. To facilitate further study of these issues, we developed antisera that permit the identification and isolation of the protein encoded by the human and other mammalian versions of c-*myc*. We found that c-*myc*(human) gives rise to at least two phosphoproteins with apparent molecular weights of 62,000 [pp62$^{c\text{-}myc(human)}$, the major product] and 66,000 [pp66$^{c\text{-}myc(human)}$, produced in smaller quantities and possibly a modified version of the M_r 62,000 protein]. Both proteins have relatively short half-lives of ≈30 min. Mouse c-*myc* encodes similar proteins with molecular weights of 64,000 and 66,000. The use of cells transformed by DNA-mediated gene transfer sustained previous deductions that the entire coding domain of c-*myc*(human) is contained in the second and third exons of the gene and resolved previous ambiguities by showing that analogous exons specify the entire protein product of c-*myc*(chicken). Tumor cells containing amplification of c-*myc*(human) produce relatively large amounts of pp62/pp66$^{c\text{-}myc(human)}$. By contrast, translocations of c-*myc* found in cells derived from Burkitt lymphoma appear merely to sustain expression of c-*myc*(human) at levels found also in nontumorigenic lymphoblastoid cells, rather than to increase expression of the gene to manifestly abnormal levels.**

The oncogene v-*myc* of the avian myelocytomatosis viruses arose by transduction of a cellular gene known as c-*myc* (1). Although tumorigenicity was first attributed to the viral form of *myc*, circumstantial evidence has now also implicated abnormalities of c-*myc* in the genesis of diverse forms of tumors (1). First, integration of retroviral DNA in the vicinity of c-*myc* can apparently activate the gene to engender the lymphomas induced by infection with avian leukosis virus (2, 3), murine leukemia virus (4), and feline leukemia virus (5). Second, amplification of c-*myc* and consequent increased expression of the gene have been found in several varieties of human malignancies (6–8). Third, chromosomal translocations in the cells of mouse plasmacytomas and human Burkitt lymphoma often relocate c-*myc* to the vicinity of an immunoglobulin gene (9, 10). The effects of these translocations on the structure, expression, and function of c-*myc* are for the moment controversial (11).

The boundaries and topography of mammalian c-*myc* have been defined with considerable precision (12, 13). In both mice and humans, the gene is composed of three exons and two intervening introns. The leftmost exon comprises only untranslated regions of the gene, whereas both the second and third exons encode portions of the c-*myc* protein (12, 13). By contrast, the structure of chicken c-*myc* remains ambiguous because only two exons (analogous to the second and third exons of murine and human c-*myc*) have been identified with any assurance (14, 15); it has therefore been difficult to define the boundaries of the coding domain for avian c-*myc*.

Previous work has demonstrated that avian c-*myc* encodes a phosphoprotein whose electrophoretic mobility suggests a M_r of ≈58,000 (16, 17). By contrast, the product of human c-*myc* has been described as a M_r 48,000 protein (18)—a claim that is in accord with predictions from the nucleotide sequence of human c-*myc* (19, 20) but that stands in unexpected contrast to the results with the chicken c-*myc* protein. In an effort to resolve this conflict and to obtain reagents that will allow the role of c-*myc* in tumorigenesis to be examined in greater detail, we have used oligopeptides to prepare antisera that should react with the protein encoded by human c-*myc*.

MATERIALS AND METHODS

Cells. The cell lines Daudi, Raji, COLO 320, HeLa, and human skin fibroblasts were obtained from the American Type Tissue Culture Collection. The small cell carcinoma of the lung N417 and the lymphoblastoid cell line Bristol-8 were kindly provided by J. Minna and P. Parham, respectively. The rat embryo culture cell lines transformed by cotransfection with the oncogenes *myc* and *ras* were kindly provided by W. Lee, M. Schwab, and H. E. Varmus.

Peptide Coupling and Immunization. Seven peptides were synthesized according to the human c-*myc* nucleotide sequence (19) and were kindly supplied by R. Lerner. Peptides A (residues 25–40), B (residues 43–55), C (residues 56–70), D (residues 171–188), E (residues 208–231), F (residues 272–300), and G (residues 408–439) were coupled to keyhole limpet hemocyanin with m-maleimidobenzoyl *N*-hydroxysuccinimide ester as the coupling reagent (21). Rabbits were divided into three groups and immunized with peptide F, G, or a mixture of all seven peptides according to the procedure described (21).

Cell Labeling and Immunoprecipitation. Cells were labeled with [^{35}S]methionine or [^{32}P]orthophosphate and were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis as described (22).

RESULTS

Testing the Antisera. Rabbits were immunized with the peptides as described above and the antisera obtained were first tested for reactivity with previously identified products of *myc* genes—pp110$^{gag\text{-}myc}$ encoded by the genome of MC29 virus (23) and pp58$^{c\text{-}myc(chicken)}$ (16, 17). Seven of the 12 antisera that we tested precipitated both of these proteins. Fig. 1 (lanes 1, 2, and 3) illustrates these results for the viral protein using three different anti-peptide antisera: G10, raised against the carboxyl-terminal peptide; F1, raised against peptide F; and CK1, raised against a mixture of all seven peptides. It therefore appeared that the peptide antigens had elicited immune responses to highly conserved epitopes in the *myc* proteins.

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*Present address: Ludwig Institute for Cancer Research, Medical Research Council Centre, Hills Road, Cambridge, U.K.

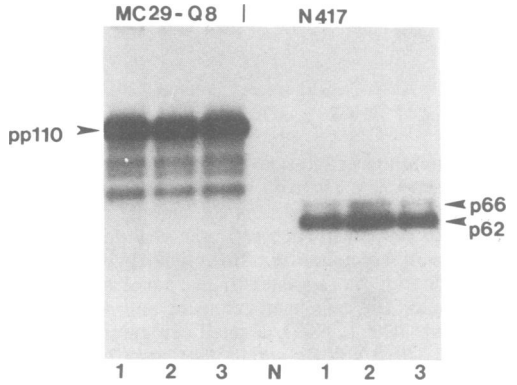

FIG. 1. Immunoprecipitation of the human c-*myc* protein. The MC29-transformed quail cell line Q8 and the human cell line N417 were labeled for 60 min with 1 mCi of [^{35}S]methionine (1 Ci = 37 GBq) and were immunoprecipitated with the following antisera: lane 1, G10 raised against residues 408–439 of human c-*myc* protein; lane 2, F1 raised against residues 272–300; lane 3, CK1 raised against all seven *myc* peptides. Lane N, normal rabbit serum. The samples were analyzed on a NaDodSO$_4$/10% polyacrylamide gel. The apparent molecular weights of polypeptides were interpolated from molecular weight standards.

We then used the antisera to search for product(s) of c-*myc*(human). Since c-*myc* is expressed at relatively low levels in most human cells, we first analyzed the cell line N417 in which expression of c-*myc* RNA is very high due to a 40-fold amplification of the gene (24). The antisera recognized two proteins—a major form of M_r 62,000 and a minor form of M_r 66,000 (Fig. 1).

The fact that antisera directed against different epitopes within the polypeptide encoded by c-*myc*(human) reacted with the M_r 62,000 and M_r 66,000 proteins provided initial evidence that both are products of c-*myc*(human). The specificity of immunoprecipitation was tested further by showing that the reactions of antisera G10 and F1 with the M_r 62,000 protein could be blocked by competition with the appropriate peptide (Fig. 2). Similar results could be perceived for the M_r 66,000 protein on longer exposures of the autoradiograms (data not shown).

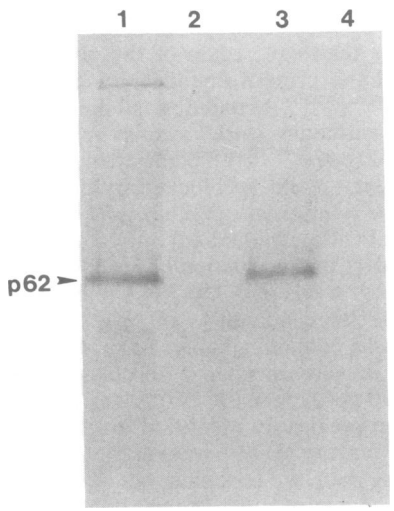

FIG. 2. Specificity of anti-*myc* antisera. N417 cells were labeled for 60 min with 1 mCi of [^{35}S]methionine and were immunoprecipitated with the following: lane 1, anti-*myc* antiserum G10; lane 2, anti-*myc* antiserum G10, after preincubation with peptide G; lane 3, anti-*myc* antiserum F1; and lane 4, anti-*myc* antiserum F1, after preincubation with peptide F.

The Products of c-*myc*(Human) Are Phosphoproteins with Relatively Short Half-Lives. Labeling of cells with [^{32}P]orthophosphate revealed that both forms of protein encoded by c-*myc*(human) are phosphorylated (Fig. 3). We therefore tentatively designated these proteins as pp62/pp66$^{c\text{-}myc(human)}$. We used pulse–chase labeling to explore the relationship between pp62 and pp66. The two proteins labeled in a parallel manner, demonstrating that neither protein is likely to be a precursor of the other (data not shown). The data also allowed us to estimate half-lives of ≈30 min for each of the proteins, in accordance with suggestions that the products of c-*myc* are regulatory rather than structural elements in the cell (25).

Further Identification of the c-*myc*(Human) Protein by DNA-Mediated Gene Transfer. Recently, Land *et al.* (26) showed that it was possible to establish transformed cell lines from primary rat embryo cells after cotransfection with the oncogenes *myc* and *ras*. We have made use of cells transformed in this manner (kindly provided by W. Lee, M. Schwab, and H. E. Varmus) to show that introduction of c-*myc*(human) into a heterologous cell results in the production of pp62/pp66$^{c\text{-}myc(human)}$. The transformed cells were established by transfection of primary rat embryo cells with combinations of two oncogenes: c-Ha-*ras*(EJ), a mutant version of c-Ha-*ras* isolated from the EJ line of human bladder carcinoma cells (27); and c-*myc*(human), either the entire gene or a portion of the gene that includes only the second and third of the three exons isolated from the colonic carcinoma COLO 320 (7). Transcription from c-Ha-*ras*(EJ) was under the control of the gene's natural promoter, whereas both versions of c-*myc*(human) were linked to the promoter/enhancer from Moloney murine leukemia virus (28) to achieve vigorous gene expression.

Cell lines transfected with c-Ha-*ras*(EJ) in combination with either two or three exons of c-*myc*(human) were examined (Fig. 4*A*; unpublished data). The cell lines established by transfection contained pp62, whereas no candidate *myc* protein could be found in primary rat embryo cells. This result further establishes that pp62 is encoded by human c-*myc*.

Identification of the Coding Domain in c-*myc*(Chicken). Ambiguities persist regarding the coding domain of c-*myc*(chicken). In particular, it has not been clear whether the protein is entirely encoded in the two exons that are analogous to exons 2 and 3 of mouse and human c-*myc*, or whether additional coding sequence might lie upstream of these domains in the chicken gene (29). To address this problem,

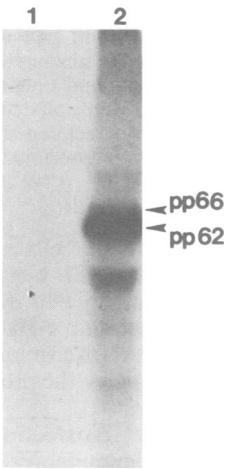

FIG. 3. Phosphorylation of the human c-*myc* gene product. N417 cells were labeled for 3 hr with 1 mCi of [^{32}P]orthophosphate. Detergent lysates were made and immunoprecipitated with normal rabbit serum (lane 1) and anti-*myc* G10 (lane 2).

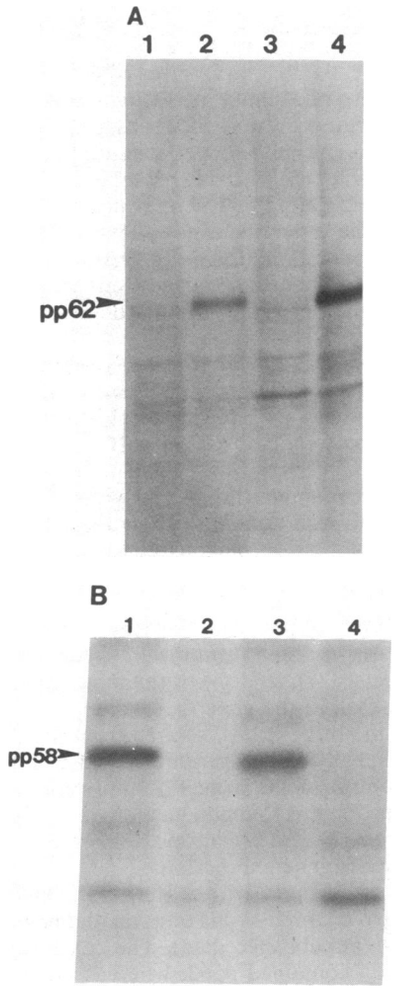

FIG. 4. Detection of human and chicken c-*myc* proteins in cells transformed by DNA-mediated gene transfer. (*A*) Identification of the c-*myc*(human) protein by DNA-mediated gene transfer. N417 cells and the rat cell line M2 [established by cotransfection with c-Ha-*ras*(EJ) and exons 2 and 3 of human c-*myc*] were labeled for 1 hr with 0.5 mCi of [^{35}S]methionine and were immunoprecipitated with antiserum. Lanes: 1, M2 cells immunoprecipitated with normal rabbit serum; 2, M2 cells immunoprecipitated with anti-*myc* antiserum G10; 3, rat embryo cells immunoprecipitated with anti-*myc* antiserum G10; 4, N417 cells immunoprecipitated with anti-*myc* antiserum G10. (*B*) Identification of the coding domain in c-*myc*(chicken). The avian lymphoma cell line R2B and the rat cell line B1 [established by cotransfection with c-Ha-*ras*(EJ) and LL4 c-*myc*(chicken)] were labeled as described above and were immunoprecipitated with antiserum. Lanes: 1, R2B cell line immunoprecipitated with anti-*myc* antiserum G10; 2, B1 cell line immunoprecipitated with abnormal rabbit antiserum; 3, B1 cell line immunoprecipitated with anti-*myc* antiserum G10; 4, rat embryo cells immunoprecipitated with anti-*myc* antiserum G10.

we examined a line of rat embryo cells established by transfection with c-Ha-*ras*(EJ) and LL4 c-*myc*—the two identified exons of c-*myc*(chicken) isolated from the bursal lymphoma LL4 (3). Transcription from c-Ha-*ras*(EJ) was driven as described above, whereas transcription from the exons of c-*myc*(chicken) was controlled by the promoter/enhancer of avian leukosis virus.

The cells established by transfection contained a M_r 58,000 protein that could be immunoprecipitated by our antisera (Fig. 4*B*, lane 3). The protein possessed an electrophoretic mobility identical to that of authentic pp58$^{c\text{-}myc(chicken)}$, immunoprecipitated from an extract of avian lymphoma cells (Fig. 4*B*, lane 1). No similar protein could be isolated from

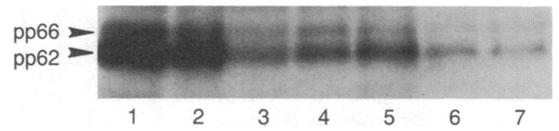

FIG. 5. Immunoprecipitation of c-*myc* protein in normal and neoplastic human cells. The cells were incubated for 60 min with 0.5 mCi of [^{35}S]methionine, lysed, and the amount of radioactivity incorporated into protein in each cell type was determined after trichloroacetic acid precipitation. The quantity of lysate used for immunoprecipitation was adjusted to give equal amounts of radioactivity per sample. The cells analyzed using anti-*myc* antiserum G10 were as follows: lane 1, N417, a small cell carcinoma of the lung; lane 2, COLO 320, a colonic carcinoma; lanes 3 and 4, Daudi and Raji, Burkitt lymphomas; lane 5, Bristol-8, a lymphoblastoid cell line; lane 6, HeLa, an ovarian carcinoma; lane 7, human skin fibroblasts.

rat embryo cells that had not been transfected (Fig. 4*B*, lane 4). We conclude that the two identified exons of c-*myc*(chicken) encode the entirety of the gene product.

Analysis of c-*myc* Protein in Normal and Neoplastic Human Cells. Alterations in the c-*myc* gene have been implicated in the genesis of a variety of tumors. To investigate the effect these changes have had on the product of c-*myc*, we analyzed two cell lines with amplification of c-*myc* (COLO 320 and N417), two cell lines with the *t*(8;14) translocation of c-*myc* (Daudi and Raji), and three lines with no detectable rearrangements in c-*myc* gene (the Epstein–Barr virus-transformed lymphoblastoid cell line Bri-8, human skin fibroblasts, and HeLa cells).

As shown in Fig. 5, all the cells examined synthesized pp62/pp66$^{c\text{-}myc(human)}$. There were notable quantitative differences among the cell lines, however. The amplified lines contained 10-fold more pp62/pp66$^{c\text{-}myc(human)}$ than the three lymphoid lines, and the lymphoid cells in turn contained 5-fold more of the *myc* proteins than did the human fibroblasts and HeLa cells.

DISCUSSION

Identification and Preliminary Characterization of the Protein Encoded by c-*myc*(Human). We have used a series of oligopeptides as antigens to obtain a set of antisera that react with the gene product(s) of c-*myc*(human). Several independent points of evidence indicate that our antisera react with authentic products of c-*myc*(human). (*i*) Antisera raised against more than one region of the predicted amino acid sequence of the protein encoded by c-*myc* reacted with pp62/pp66$^{c\text{-}myc(human)}$. In addition, all members of a series of monoclonal antibodies raised against peptides E and G recognized pp62/pp66$^{c\text{-}myc(human)}$. (*ii*) Immunoprecipitation by various antisera could be blocked by competition with an excess of the appropriate peptide. (*iii*) Introduction of the coding domain of c-*myc*(human) into rat embryo cells apparently engenders the production of pp62/pp66$^{c\text{-}myc(human)}$. (*iv*) Cells with high levels of c-*myc* RNA (e.g., N417 and COLO 320) produce large quantities of pp62/pp66$^{c\text{-}myc(human)}$; by contrast, cells containing only small quantities of c-*myc* RNA (e.g., human embryonic fibroblasts and HeLa cells) produce little pp62/pp66$^{c\text{-}myc(human)}$. (*v*) The antisera immunoprecipitate previously identified products of both v-*myc* (pp110$^{gag\text{-}myc}$ of the MC29 virus) and c-*myc*(chicken).

From these data, we conclude that our antisera recognize authentic products of c-*myc*(human) and that the principal product of this gene is a M_r 62,000 phosphoprotein. The gene also gives rise to smaller amounts of a M_r 66,000 phosphoprotein whose nature has yet to be clarified. We cannot account for the previous report that the product of c-*myc*(human) might be a M_r 48,000 protein (18).

Pulse–chase labeling revealed that pp62 and pp66 are syn-

thesized and degraded in a parallel manner, with the relatively short half-life of ≈30 min. It therefore appears that neither protein is a precursor of the other and that the expression of c-*myc* must be exquisitely sensitive to regulation of its transcription. These suggestions are in accord with recent findings that implicate c-*myc* in the governance of the cellular division cycle (25).

We presume that pp62 and pp66 are differently modified versions of the same gene product. The difference is probably not attributable to phosphorylation, however, because the pp62/pp66 ratio remained the same when the proteins were labeled with either [^{32}P]orthophosphate or [^{35}S]methionine (unpublished results). The antisera described here also react with two mouse phosphoproteins of M_r 64,000 and 66,000 (data not shown). Our provisional evidence indicates that these are products of c-*myc*(mouse).

The Coding Domain of c-*myc*. Previous studies have established that the proteins encoded by both mouse and human c-*myc* almost certainly arise from a coding domain located in the second and third exons of the genes (12, 13). The results reported here are in accord with that conclusion. By contrast, the topography of chicken c-*myc* has remained ambiguous because of failure to decisively locate either the transcriptional boundaries of the gene or a domain analogous to the first exon of mouse and human c-*myc*.

We have made use of cells transformed by DNA-mediated gene transfer to demonstrate that previously identified product of c-*myc*(chicken) is encoded entirely by the two authenticated exons of the gene (14, 15). The topographies of the coding domains in chicken, mouse, and human c-*myc* are therefore all similar to one another, and the proteins encoded by the three versions of c-*myc* all display the same discrepancy between molecular weights predicted from the nucleotide sequence of the coding domains (M_r, 46,000–48,000) and the apparent molecular weights deduced from electrophoretic mobilities of the proteins (M_r, 58,000–66,000). We presume, but cannot prove, that this discrepancy reflects unusual physical properties arising from the amino acid sequence of the c-*myc* proteins. It is also now apparent that the exons encoding c-*myc* proteins have been conserved over a broad range of vertebrate evolution, whereas divergence has occurred between the chicken and mammalian genes upstream of the coding exons.

The Products of c-*myc* in Tumor Cells Containing Abnormalities of c-*myc*. There is substantial circumstantial evidence to implicate abnormalities of c-*myc* in the genesis of a diverse variety of human tumors. At least two sorts of abnormalities have been found: amplification of c-*myc* (8), and translocation of the gene to a new chromosomal location (9–11). Amplification of c-*myc* increases the quantity of RNA transcribed from the gene (8), but the consequences of translocation remain in dispute (11). With the antisera described here, it is now possible to examine the impact of these abnormalities on the amounts and structure of the protein encoded by c-*myc*.

Although our results to date are limited in scope, they reveal several important principles. First, neither amplification nor translocation of c-*myc* has grossly altered the structure of the protein encoded by the gene in the instances we have examined (our data cannot exclude the presence of point mutations and other small changes). Second, gene amplification does indeed greatly increase the quantity of protein produced from c-*myc*. The amounts observed here in cells containing amplified c-*myc* are greater than those found in any other setting to date: only cells transformed by v-*myc* contain more *myc* protein. Third, translocation of c-*myc* to the vicinity of an immunoglobulin gene (as observed in mouse plasmacytomas and Burkitt lymphoma) may merely sustain rather than increase the expression of the gene. The two examples of Burkitt tumor cells examined here (the Daudi and Raji lines) both contain translocations of c-*myc* from its native position on chromosome 8 to an immunoglobulin heavy-chain locus on chromosome 14 (30); yet the tumor cells produce no more pp62/pp66$^{c\text{-}myc(human)}$ than do lymphoblastoid cells transformed by Epstein–Barr virus, which are not tumorigenic and in which there is no identified structural abnormality of c-*myc*. The quantity of pp62/pp66$^{c\text{-}myc(human)}$ produced in the lymphoblastoid and Burkitt cells is probably sufficient to have functional consequences, however, because it is in considerable excess of the quantities found in human fibroblasts and HeLa cells (a human carcinoma cell line).

Our findings are in accord with the suggestion that expression of c-*myc* is part of the chain of command that directs the growth and division of lymphoid and other cells (25). The unregulated sustained expression of c-*myc* in otherwise normal amounts might therefore suffice to explain the role of c-*myc* in the genesis and maintenance of both lymphoblastoid cell lines and the neoplastic cells of Burkitt lymphoma (31).

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