Isolation and Characterization of the Human Cellular *myc* Gene Product†

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ABSTRACT: Antibodies against the product of the human cellular *myc* gene (c-*myc*) were prepared against a bacterially expressed human c-*myc* protein by inserting the *ClaI/BclI* fragment of the human c-*myc* DNA clone in an expression vector derived from pP_LC24. These antibodies cross-react with viral-coded *myc* (v-*myc*) proteins from MC29 and OK10 viruses. Furthermore, IgGs specific for synthetic peptides, corresponding to the 12 carboxy-terminal amino acids of the human c-*myc* gene and 16 internal amino acids, were isolated. By use of the various *myc*-specific antisera or IgGs, a protein of M_r 64 000 was detected in several human tumor cell lines including Colo320, small cell cancer of the lung (417d), HL60, Raji, and HeLa. This protein is larger than the corresponding v-*myc* or chicken c-*myc* proteins from avian virus transformed cells or avian bursa lymphoma cells (RP9), both of which are proteins of M_r 55 000. The human c-*myc* protein is located in the nucleus of Colo320 cells, exhibits a half-life of about 15 min, and is expressed at significantly lower levels than the viral protein. The human c-*myc* protein was enriched about 3000-fold from Colo320 cells using c-*myc*-specific IgG coupled to Sepharose beads. The protein binds to double-stranded DNA in vitro, a reaction that can be inhibited to more than 90% by c-*myc* specific IgG.

The oncogene v-*myc* of the avian myelocytomatosis (MC29) virus family is closely related to the cellular *myc* gene, c-*myc*, found in all vertebrate cells [for a review, see Bishop (1983)]. Altered expression of the c-*myc* gene is an important event in malignant transformation. Possible mechanisms by which changes in c-*myc* gene expression have occurred include insertion of the retroviral promoter in the vicinity of c-*myc* (Hayward et al., 1981), amplification of c-*myc* DNA (Dalla Favera et al., 1982), and chromosomal translocations [for a review, see Robertson (1983)]. The protein product of the v-*myc* oncogenes of various myelocytomatosis virus isolates, which are expressed either as *gag-myc* polyproteins or as v-*myc* proteins, have been shown to be nuclear antigens (Donner et al., 1982; Abrams et al., 1982; Alitalo et al., 1983a,b), except for the large polyprotein *gag-pol-myc* coded for by the OK10 virus which is cytoplasmic (Bunte et al., 1984). The purified *gag-myc* and v-*myc* proteins bind to double-stranded DNA in vitro (Donner et al., 1982, 1983; Bunte et al., 1982, 1983). DNA binding in vitro is considered important for transformation since deletion mutants code for deleted v-*myc* proteins which have a significantly reduced ability to bind to DNA in vitro, even though their proteins still reside in the nucleus of fibroblasts [Donner et al., 1983; for a review, see Moelling (1985)].

Several groups have prepared v-*myc*-specific antisera and described the v-*myc* proteins as molecules ranging in size from M_r 55 000 to 62 000 (Alitalo et al., 1983a,b; Bunte et al., 1984; Hann et al., 1983; Moelling et al., 1984a,b). Recently also the human c-*myc* gene product has been identified (Giallongo et al., 1983; Persson et al., 1984; Moelling et al., 1984a,b; Eisenman et al., 1984; Hann & Eisenman, 1984). The sizes reported are M_r 48 000, 65 000, 60 000–65 000 and a doublet of 64 000/67 000, respectively. The size predicted from the nucleotide sequence would be M_r 49 000 (Reddy et al., 1983; Colby et al., 1983). The size differences could be due to different gel systems, markers, or the antibodies.

The present study was undertaken to compare the human c-*myc* gene product with the viral one by using antisera against bacterially expressed v-*myc* and human c-*myc* gene products and various synthetic peptides. We purified the *hu*-c-*myc* gene product from Colo320 by means of c-*myc*-specific IgG and demonstrate its ability to bind to DNA in vitro. This binding is inhibited to more than 90% by c-*myc*-specific IgG, indicating that DNA binding is an intrinsic property of the protein and not mediated through cellular factors.

Experimental Procedures

Materials

Cells. The human tumor cell lines were grown in RPMI medium supplemented with 10% fetal calf serum (FCS) except for HeLa cells which were kept in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS. RP9 cells were grown in DMEM supplemented with 8% FCS and 2% heat-inactivated chicken serum (CHS). MC29-transformed quail fibroblasts (Bister et al., 1977) were kept in DMEM–RPMI (1:1), 5% tryptose phosphate broth (TPB), 0.5% dimethyl sulfoxide (Me_2SO), 5 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (Hepes), 5% newborn calf serum (NCS), and 1% CHS. OK10-transformed quail fibroblasts obtained the same medium as MC29 cells except for NCS which was replaced by FCS.

Cells were labeled metabolically with [^{35}S]methionine (250–500 μCi/mL; Amersham Buchler, England) for 90–240 min by using methionine-free medium supplemented with dialyzed FCS.

Antigens and Antisera. Antibodies against p19 were prepared in rabbits or from a hybridoma as described (Greiser-Wilke et al., 1981). Antiserum against the bacterially expressed v-*myc* and v-*myc*-specific synthetic peptides were prepared as described (Bunte et al., 1984; Moelling et al., 1984b). Antibodies against the human cellular C-terminal peptide (12 amino acids and additional tyrosine) were obtained after several injections of the peptide coupled to keyhole limpet hemocyanine (KLH) by glutaraldehyde. A 4-mg sample of peptide was coupled to 30 mg of KLH in 6 mL of PBS buffer by 7.5% glutaraldehyde and 0.5 mL injected in the presence

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of Freund's adjuvant (Pfaff et al., 1982). Antiserum against MS2 alone was obtained as described (Bunte et al., 1984). Antiserum against the MS2-hu-c-*myc* protein was obtained by immunization of the bacterially expressed protein which was either isolated from bacteria as described below or eluted from gels. For that purpose bacteria were solubilized in sodium dodecyl sulfate (SDS) sample buffer and loaded onto a preparative polyacrylamide gel. After electrophoresis a narrow vertical strip of the gel was excised and stained with Coomassie brilliant blue. The stained gel strip served as a guide to excise the MS2-fusion protein from the remainder of the unfixed and unstained gel, which was homogenized and covered with PBS buffer. The protein was eluted by rocking for 12 h at 10 °C. A 100–200-μg sample of MS2-fusion protein was injected in 2-week intervals in the presence of Freund's adjuvant. Ten days after each immunization the rabbits were bled from an ear vein and the sera stored at −20 °C.

IgG specific for C_{16} and C_{12} were isolated from polyvalent human c-*myc* serum by using the appropriate peptides coupled to carrier beads as described (Bunte et al., 1984).

Methods

Indirect Immunoprecipitation. Indirect immunoprecipitation was performed as described previously (Donner et al., 1982), except with the following modifications: 10^7 cells were lysed in 2 mL of lysis buffer containing 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, 50 mM NaCl, 0.5% NP-40, 0.5% deoxycholate (DOC), and 0.5% sodium dodecyl sulfate (SDS) supplemented with protease inhibitors [1 mM phenylmethanesulfonyl fluoride (PMSF) and 100 units/mL trasylol). The lysate was agitated through a Pasteur pipet to shear the high molecular weight DNA. Insoluble material was pelleted by centrifugation at 10000 rpm in a Sorvall HB-4 rotor for 30 min at 4 °C. The supernatant was used immediately for indirect immunoprecipitation. It was pretreated with 25 μL of *Staphylococcus aureus* cells (Pansorbin, Calbiochem), centrifuged, and then treated with 5 μL of serum for 20 min at 4 °C. Subsequently 25 μL of *Staphylococcus aureus* bacteria was added for 10 min at 4 °C. The precipitate was collected by short spin and washed: 3 times using RIPA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% DOC, and 0.1% SDS), 1 time with high-salt buffer (10 mM Tris-HCl, pH 7.2, 1 M NaCl, and 0.1% NP-40), and again with RIPA buffer. The pellet was processed for gel electrophoresis.

Molecular Cloning of the hu-c-myc Gene. Previously a fragment of the viral *myc* gene (*Sal*I/*Bam*HI) has been cloned into a derivative of the expression vector pP_Lc24 which contained v-*myc* sequences (Bunte et al., 1984). A *Cla*I/*Bam*HI fragment of this vector was excised and the *Cla*I/*Bcl*I fragment of the human c-*myc* DNA clone inserted. This fragment was obtained from the human c-*myc* DNA clone pMC41-3rc (Dalla Favera et al., 1982). The resulting plasmid coded for 99 amino acids of the replicase gene of the bacteriophage λ, 20 amino acids of v-*myc*, and 178 amino acids of human c-*myc*. Details of the cloning procedure have been published (Bunte et al., 1984; Moelling et al., 1985). The MS2-hu-c-*myc* fusion protein was purified from bacteria similarly as described by Kuepper et al. (1982) and Moelling et al. (1985). A 20-mL culture was grown overnight at 28 °C, diluted 1:5 with Ty buffer (Kuepper et al., 1982), and shifted to 42 °C for 3 h. The bacteria were centrifuged at 5000 rpm for 15 min at 4 °C in a Sorvall GSA rotor; the pellet was washed with phosphate-buffered saline (PBS) and either stored dry at −20 °C or processed. The pellet was suspended in 3 mL of 10% sucrose, and then 750 μL of lysozyme (10 mg/mL, stored at −20 °C) and 750 μL of ethylenediaminetetraacetic acid (EDTA) (0.2 M) were added. The solution was kept for 30 min at 4 °C until it was highly viscous as a result of high molecular weight DNA. The DNA was sheared by sonification (6 times, 10 s, 50 W). Then 1% Triton X-100 was added (30 min at 4 °C), and the solution was centrifuged at 10000 rpm for 30 min at 4 °C. The pellet was suspended in 10 mL of 1 M urea (in 50 mM Tris-HCl, pH 7.5, and 1% Triton X-100) for 30 min at 4 °C. The suspension was centrifuged at 10000 rpm for 30 min at 4 °C. This step removed significant amounts of soluble bacterial proteins. The resulting pellet was suspended in 10 mL of 6 M urea (in 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, and 200 mM NaCl). At this stage a 200000*g* centrifugation was performed in a 50Ti rotor at 45000 rpm for 2 h at 4 °C. The supernatant was dialyzed against PBS (50 mM, Na_2HPO_4/KH_2PO_4 and 120 mM NaCl, pH 7.2) resulting in partial precipitation of the protein. The pellets were used for immunization and were kept frozen at −20 °C. The solution contained about 1 mg/mL MS2-*myc* protein. This was used for coupling to Sepharose beads (see below).

Preparation of Immunoaffinity Columns. To set up an immunoaffinity column, anti-*myc*-specific IgG was isolated from anti-MS2-hu-c-*myc* hyperimmune serum. This was achieved by a two-step procedure, which has been described in detail recently for the anti-MS2-*myb* serum (Moelling et al., 1985). In summary, the unfused MS2 protein was purified from bacterial lysates and coupled to activated CH-Sepharose 4B. This column binds MS2-specific IgG from the serum. The flow-through material from this column containing residual IgGs was applied to an MS2-hu-c-*myc*-specific column prepared from bacterially expressed and purified MS2-hu-c-*myc* protein coupled to Sepharose beads. The hu-c-*myc*-specific IgG was eluted from this column with 1.5 M NaSCN and dialyzed. Twenty milliliters of hyperimmune serum allowed the isolation of about 3 mg of specific IgG. Coupling of IgG to protein A–Sepharose was performed as described (Donner et al., 1982).

Isolation of p64 hu-c-myc Protein. To purify the p64 hu-c-*myc* protein by the anti-hu-c-*myc* immunoaffinity column, 10^8 Colo320 cells were labeled for 90 min with [^{35}S]methionine (500 μCi/mL), washed twice in PBS (50 mM Na_2HPO_4/KH_2PO_4 and 120 mM NaCl, pH 7.2), and lysed in 20 mL of lysis buffer (see above) supplemented with protease inhibitors. After a short sonification treatment, the solution was clarified (10000 rpm, Sorvall rotor HB-4, 30 min, 4 °C) and applied to the immunoaffinity column. The column was washed with 50 mL of RIPA, 10–20 mL of high-salt buffer, and 10–20 mL of PBS. The protein was then eluted with low pH buffer (0.1 M citric acid, pH 3.5, 300 mM NaCl, 0.1% Triton X-100, and 50% ethylene glycol) which was neutralized immediately. The eluted protein was stored at −20 °C. The specific activity of the eluted proteins was about 20000 cpm/μg and amounted to about 5 μg per preparation. The purification was controlled by analyzing an aliquot (75 μL) of the eluted fractions (1 mL) on an SDS–polyacrylamide gel and autoradiography (for about 5 days).

DNA-Binding Assay and Inhibition of DNA–Protein Interaction by IgG in Vitro. DNA binding was tested by a filter-binding assay similar to that as previously described (Donner et al., 1982; Bunte et al., 1984). ^{3}H-Labeled double-stranded normal chicken cell DNA (200000 cpm/μg) was sheared to about 20 kilobases. Approximately (5–20) × 10^4 cpm of DNA was used per assay. The incubation mixture (500 μL) contained 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 50

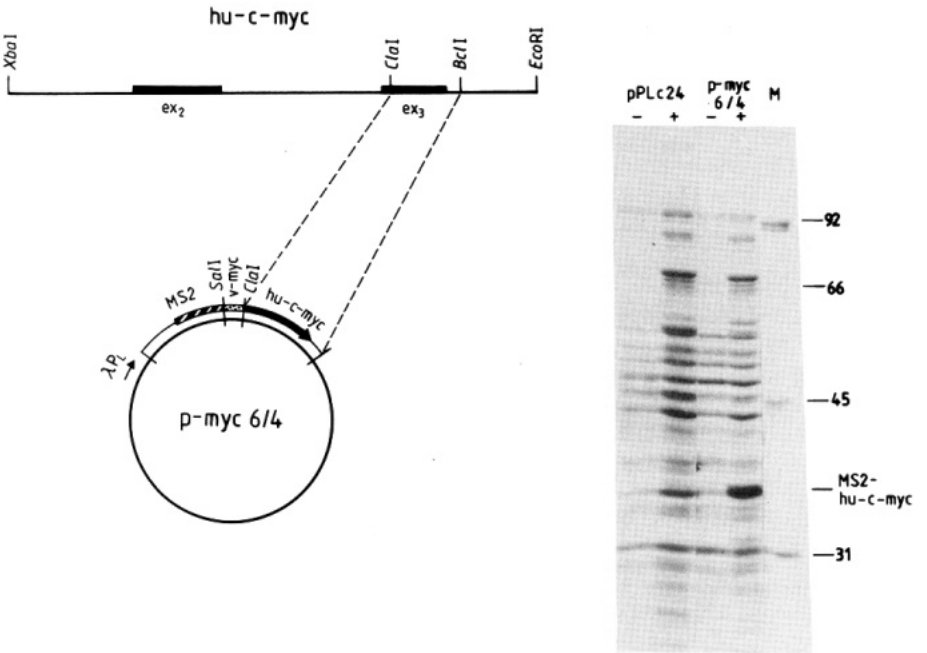

FIGURE 1: From the human cellular *myc* DNA clone pMC41 (Dalla Favera et al., 1982) a *Cla*I/*Bcl*I fragment was isolated and inserted into a previously described expression vector p-*myc* 5/30 Sal (Bunte et al., 1984), containing a *Sal*I/*Bam*HI viral *myc* gene. The *Cla*I/*Bam*HI viral *myc* gene was excised and replaced by the *Cla*I/*Bcl*I human cellular *myc* gene fragment. The vector, designated p-*myc* 6/4, codes for a portion of the MS2 polymerase and was derived from the P_Lc24 vector (Remaut et al., 1981). The clone p-*myc* 6/4 was transferred to bacteria that carry a thermolabile λ repressor (Remaut et al., 1981). Protein expression was regulated by thermoinduction. Protein patterns of bacteria carrying the vector pP_Lc24 or clone p-*myc* 6/4 are shown by Coomassie blue staining before (−) and after (+) induction of protein synthesis by a shift from 28 to 42 °C. M indicates molecular weight markers. MS2-hu-c-*myc* indicates the expressed fusion protein.

mM NaCl, [^{3}H]DNA, and [^{35}S]methionine-labeled protein as indicated. Incubation was for 10 min at 37 °C. Then the reaction mixture was run through a filter (BA85, Schleicher & Schuell). [^{3}H]DNA retained on the filter was determined after subtraction of radioactivity contributed by the protein and background radioactivity contributed by the DNA alone (1000 cpm).

Inhibition of DNA binding was tested by preincubation of purified [^{35}S]methionine-labeled proteins with purified IgGs. The IgGs were previously tested for the absence of intrinsic DNA binding. If the IgG itself bound to DNA, this contamination was removed by DNA–cellulose chromatography. The IgG was then preincubated with the protein for 15 min at 4 °C in the buffer of a DNA-binding assay. (Donner et al., 1982). After this period the ^{3}H-labeled DNA was added, and a filter-binding assay was performed.

RESULTS

Preparation of myc-Specific Antibodies. To identify the human cellular *myc* gene product, antibodies against the bacterially expressed protein were prepared. A fragment of the human c-*myc* DNA clone, *Cla*I/*Bcl*I, which corresponds approximately to the $exon_3$ (Dalla Favera et al., 1982; Colby et al., 1983), was inserted into an expression vector. The expression vector was a derivative of pP_Lc24 originally described by Remaut et al. (Kuepper et al., 1982; Remaut et al., 1981). It carries a strong leftward promoter of the bacteriophage λ. The activity of the promoter can be controlled by using host strains that carry the gene for a temperature-sensitive λ repressor. The expression vector carries information for the first 99 amino acids of the replicase gene of the bacteriophage MS2. The vector has been previously used for expression of v-*myc* (Bunte et al., 1984). A *Cla*I/*Bam*HI fragment of this vector was excised and the *Cla*I/*Bcl*I fragment of the human c-*myc* DNA clone inserted (Figure 1). A protein of about M_r 40000 is expressed after temperature shift for 2 h. The polypeptide consists of 99 amino acids of the MS2 replicase, 20 amino acids of v-*myc*, and 178 amino acids of the human c-*myc* gene. The 20 v-*myc* amino acids differ from the human sequence in this region only by the inversion of two amino acids. The bacterially expressed protein is rather insoluble and more rapidly degraded than its v-*myc* counterpart. The protein was eluted from polyacrylamide gels as well as partially purified and used for immunization (Bunte et al., 1984; Moelling et al., 1985).

A second antibody was prepared against a synthetic peptide, corresponding to the 12 carboxy-terminal amino acids of the human cellular *myc* gene (Lys-His-Lys-Leu-Glu-Gln-Leu-Arg-Asn-Ser-Cys-Ala) to which Tyr was added at the amino terminus. This peptide was used for immunization after coupling to KLH carrier protein (for details, see Experimental Procedures).

Identification of the Human c-myc Gene Product. The antibodies described above were applied to human tumor cell lines in order to detect the human c-*myc* gene product by indirect immunoprecipitation analysis. Experimental conditions that allow the detection of the v-*myc* gene product failed with the human protein. Therefore, conditions for radioactive labeling and cellular lysis were modified, precautions against proteolysis were improved, and washing procedures of the immunocomplexes were extended (for details, see Experimental Procedures).

The human c-*myc* gene product was analyzed in Colo320 cells, a cell line derived from a colon carcinoma that possesses amplified *myc*–DNA sequences and high levels of *myc*–mRNA (Alitalo et al., 1983a,b). These cells were treated with various sera and purified IgGs for indirect immunoprecipitation. The human c-*myc* protein was identified as a molecule of M_r 64000 shown in Figure 2. Precipitation of p110$^{gag\text{-}myc}$ from MC29 quail fibroblast cells is shown for comparison. The total protein inputs of Colo320 and MC29 lysates were standardized (200 μg/assay). The specific activities of the

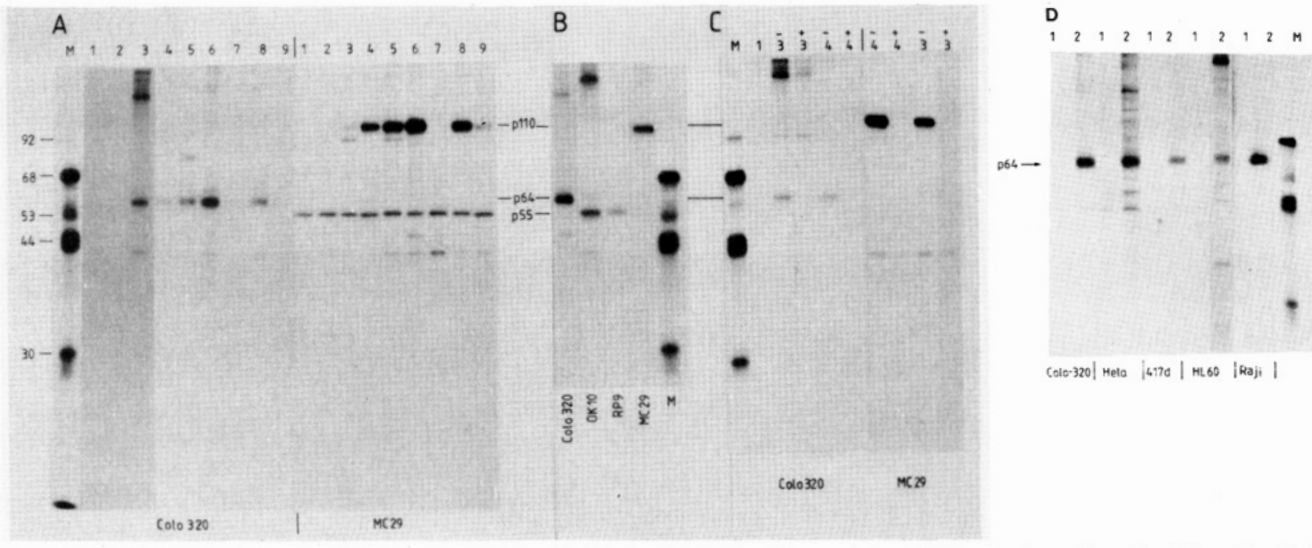

FIGURE 2: (A) Indirect immunoprecipitation was performed with the Colo320 cell line and the MC29-transformed quail cell line Q8. Cells were labeled for 90 min with [^{35}S]methionine, lysed, and processed for immunoprecipitation (10^7 cpm/assay). The following antisera (5 μL each) were used. (1) normal rabbit serum, (2) rabbit serum against the 99 MS2-specific amino acids present in the fusion protein, (3) rabbit serum against MS2-hu-c-*myc* shown in Figure 1, (4) rabbit serum against the viral *myc* gene product, MS2-v-*myc*, (5) IgG specific for amino acids 253–268 (C_{16}) of the human cellular *myc* gene product (10 μg), (6) IgG specific for amino acids 239–254 (C_{16}) of the viral *myc* gene product (8) (20 μg), (7) rabbit antiserum against the carboxy-terminal amino acids (C_{12}) of the human cellular *myc* gene product, (8) IgG isolated from serum 3 by using the C_{12} peptide (10 μg), and (9) rabbit antiserum against the carboxy-terminal amino acids of v-*myc* (C_9). M indicates molecular weight markers as indicated. Exposure time for Colo320 cells was 15 days and for MC29 cells, 3 days. (B) Indirect immunoprecipitation of [^{35}S]methionine-labeled cells was performed with serum against the MS2-hu-c-*myc* gene product [lane 3 in (A)] with Colo 320 cells and serum against the viral *myc* gene product [lane 4 in (A)] for MC29-Q8 cells, RP9 cells, and OK10-transformed quail cells (for details, see Methods). M as in (A). (C) Competition of antibodies by bacterial proteins. Indirect immunoprecipitation experiments were performed with Colo320 and MC29 cellular lysates using antisera 3 and 4 as described in (A). A total of 10 μg each of bacterial v-*myc* and c-*myc* proteins was allowed to react with the antibodies for 10 min at room temperatures in the reactions indicated by (+) and then processed for indirect immunoprecipitation as in the absence (−) of competing proteins. (D) Human tumor cell lines were labeled with [^{35}S]methionine for 90 min and processed directly for indirect immunoprecipitation using normal serum (1) and anti-MS2-hu-c-*myc* serum (2). M as in (A). The cellular inputs were standardized to 1.7×10^8 cpm/assay. Exposure time was for 12 days.

lysates were also similar (0.5×10^6 cpm/μg of protein). Antibodies against the bacterially expressed human c-*myc* gene product (MS2-hu-c-*myc*) cross-reacted with the v-*myc* protein (slot 3, Figure 2A) and vice versa: antibodies against the bacterially expressed v-*myc* protein precipitate the c-*myc* protein (slot 4, Figure 2A). Antiserum against the carboxy terminus of the v-*myc* protein (C_9) is of low titer and recognizes only its homologous v-*myc* gene product (slot 9, Figure 2A). It differs only in its next to last position from the human cellular carboxy terminus and contains there an arginine instead of a cysteine (Alitalo et al., 1983a,b; Colby et al., 1983). Immunization with the carboxy-terminal human c-*myc* peptide (C_{12}) gave rise to a very low-titered antibody (slot 7, Figure 2A). An internal synthetic peptide, C_{16}, corresponding to amino acids 239–254 of v-*myc* (Bunte et al., 1984), which has a high degree of homology to c-*myc* (amino acids 253–268 with three amino acid exchanges and two amino acids in reversed order), was coupled to activated CH-Sepharose. Polyvalent antiserum against the MS2-hu-c-*myc* protein was passed over this column and C_{16}-specific IgG isolated which precipitated c-*myc* as well as v-*myc* proteins.

In previous studies we have demonstrated the v-*myc* gene product to be a molecule of M_r 55000. Figure 2B shows v-*myc* and human c-*myc* proteins in a direct size comparison. Furthermore, the chicken cellular *myc* gene product is shown. It was precipitated from the avian bursa lymphoma cell line RP9 (Hayward et al., 1981). While both the viral and the chicken c-*myc* gene products are identical in size which was determined here as M_r 55000, the human c-*myc* protein is larger and measures M_r 64000.

In order to demonstrate the specificities of the sera that were prepared against the bacterially expressed v-*myc* and hu-c-*myc* proteins, precipitation of $p64^{hu\text{-}c\text{-}myc}$ from Colo320 cells and $p110^{gag\text{-}myc}$ from MC29 cells was performed in the absence and presence of the appropriate bacterial proteins. Both of them inhibit the precipitation reactions (Figure 2C).

Analysis of Various Tumor Cell Lines for the Presence of the Human c-myc Protein. Previously a variety of human tumor cell lines have been characterized with respect to their levels of *myc*-specific mRNA transcripts. Among the ones tested were the human colon carcinoma cells Colo320 (Alitalo et al., 1983a,b), the human promyelocytic cell line HL60 (Dalla Favera et al., 1982; Collins & Groudine, 1982), Raji, and the small cell cancer of the lung SCCL-417d (Little et al., 1983). Colo320, HL60, and SCCL-417d carry normal amplified *myc* genes (Erikson et al., 1981; Hamlin et al., 1983). Raji carries the chromosomal translocation t(8;14) (Zech et al., 1976). We have labeled the above-mentioned cell lines with [^{35}S]methionine and processed cellular lysates with equal amounts of radioactivity (1.7×10^9 cpm/assay) for indirect immunoprecipitation. The result is shown in Figure 2D. High hu-c-*myc* protein levels were detected in Colo320, Raji, and HeLa cells. HeLa cells always exhibited more nonspecific background. The amount of c-*myc* protein in HL60 cells was lower than expected. Two out of three other HL60 lines exhibited even less c-*myc* protein, indicating that properties of the cells vary with their history. This may also explain why c-*myc* was not detected in HeLa cells by others (Eisenman & Hann, 1984). In contrast to recently published results (Hann & Eisenman, 1984), the sera used here precipitate only one hu-c-*myc*-specific protein.

Turnover Rate of the Human myc Gene Product. We experienced difficulties in identifying the human cellular *myc* gene product which led us to optimize labeling conditions. While MC29 quail fibroblasts and other virally transformed cells were efficiently labeled during a 4-h labeling period (not

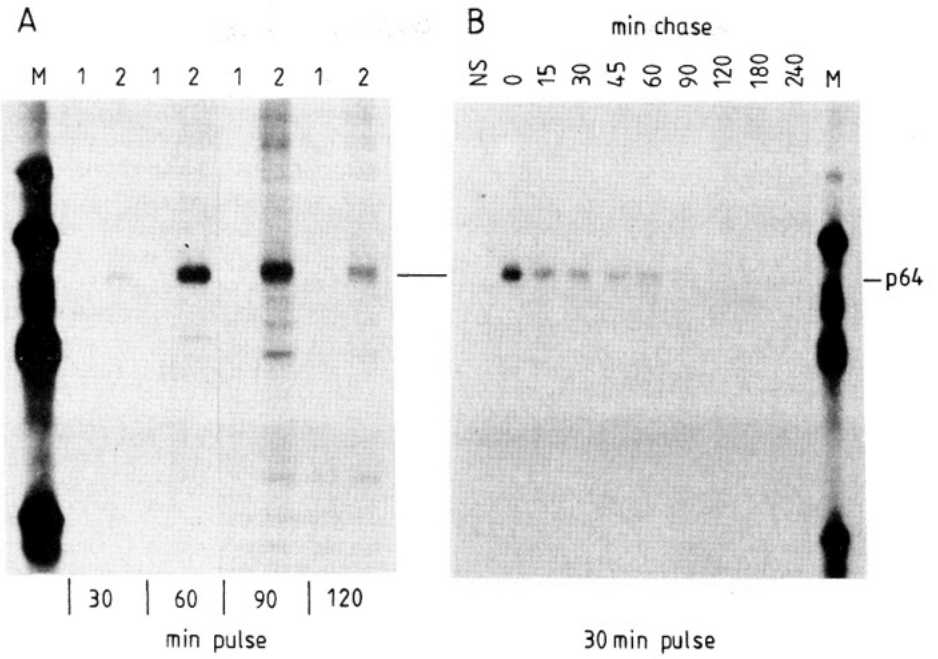

FIGURE 3: Pulse–chase analysis of the human-c-*myc* protein. (A) Colo320 cells were labeled with [^{35}S]methionine for 30, 60, 90, and 120 min and subsequently processed for indirect immunoprecipitation with normal rabbit (1) and rabbit anti-MS2-hu-c-*myc* sera (2). Exposure time: 3 days. M indicates molecular weight markers as in Figure 2. (B) Colo320 cells were labeled with [^{35}S]methionine for a 30-min pulse and subsequently chased with normal medium for the indicated periods of time. The cells were lysed and processed for indirect immunoprecipitation as described in (A). NS indicates normal serum control. Exposure time: 18 days.

shown), the human c-*myc* gene product exhibited optimal incorporation of radioactivity after 60–90 min. Incorporation declined thereafter (Figure 3A).

The human c-*myc* gene product was analyzed by pulse–chase experiments. When Colo320 cells were labeled for 30 min and subsequently chased with nonradioactive medium for 15, 30, 45, and 60 min, the amount of the M_r 64 000 protein rapidly decreased and was nondetectable thereafter (Figure 3B). These results suggest a short half-life of approximately 15 min.

Nuclear Localization. The antibodies against the bacterially expressed hu-c-*myc* protein have previously been demonstrated to give rise to *myc*-specific nuclear fluorescence with HeLa cells (Moelling et al., 1984a,b). Colo320 cells were analyzed here for the localization of the human c-*myc* gene product by cell fractionation analysis. The result, which is shown in Figure 4, indicates that the hu-c-*myc* protein is predominantly located in the nuclear fraction. The immunoprecipitates shown in Figure 4 were matched so that each fraction corresponded to identical aliquots of the total.

Isolation and DNA Binding in Vitro of the hu-c-myc Protein. The location of the hu-c-*myc* protein in the nucleus raised the question whether or not it interacted with DNA. The viral *myc* gene product is a DNA-binding protein in vitro. However, deletion mutants of MC29 code for truncated *myc* proteins which—even though they are located in the nucleus—exhibit a greatly reduced DNA-binding ability (Donner et al., 1983). Therefore, the nuclear location does not necessarily correlate with the ability of the protein to interact with DNA in vitro. To investigate the properties of the human c-*myc* protein, we purified it about 3000-fold from Colo320 cells by immunoaffinity chromatography. The column material was prepared by covalently coupling IgG specific for the human c-*myc* gene product to protein A–Sepharose. The isolated protein was analyzed by gel electrophoresis. The result is shown in Figure 5A. The hu-c-*myc* protein showed in addition to the prominent p64 protein a minor band of M_r 67 000. A doublet seems to be more readily detectable by differently derived

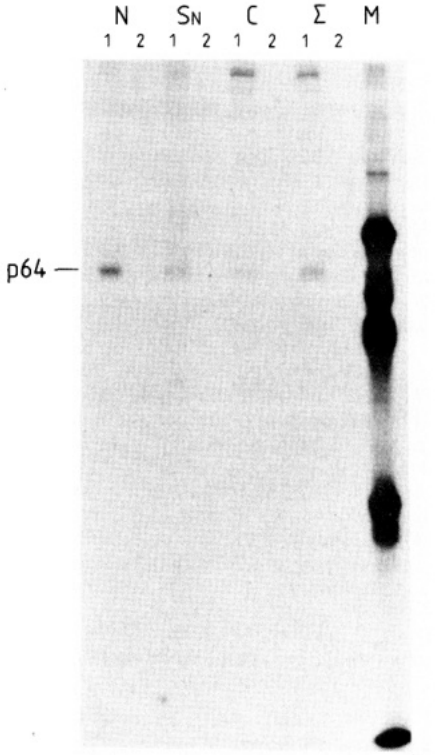

FIGURE 4: Cell fractionation analysis. Colo320 cells labeled for 90 min with [^{35}S]methionine were fractionated into nuclear (N) and cytoplasmic (C) fractions. Furthermore, the nuclear strip (S_N) obtained by detergent treatment of the nuclei (Donner et al., 1982) and total cellular lysate ($\sum$) are shown. The immunoprecipitates were matched so that each fraction corresponded to identical aliquots of the total. For details, see Methods and Donner et al. (1982). (1) Normal serum control; (2) anti-hu-c-*myc* serum.

antibodies (Persson et al., 1984; Hann & Eisenman, 1984).

The purified c-*myc* protein was analyzed with ^{3}H-labeled double-stranded cellular DNA for its ability to bind to DNA in vitro. The DNA-binding assay was performed in parallel

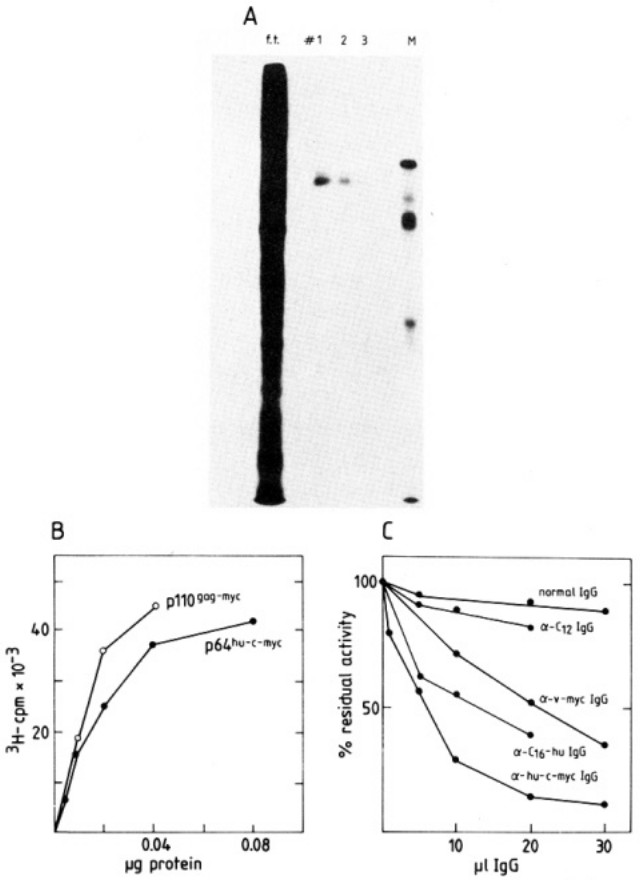

FIGURE 5: Purification of the human c-*myc* gene product from Colo320 cells. Cells (1×10^8) were labeled with [^{35}S]methionine (500 µCi/mL) for 90 min, lysed, and processed immediately for immunoaffinity column purification. The column was prepared by immunoglobulins specific for the human c-*myc* gene product. MS2-specific and non-specific immunoglobulins had been removed beforehand [for details of the procedure, see Methods and Bunte et al. (1984)]. After extensive washing, the human c-*myc* gene product was eluted from the column. A total of 75 µL of each fraction (1 mL) 1–3 was applied to the SDS–polyacrylamide gel. f.t. indicates 25 µL of 20 mL of flow through. Exposure time: 6 days. M indicates molecular weight markers as in Figure 2. (B) The protein purified as shown in (A) was tested in an in vitro filter-binding assay (Donner et al., 1982) for its ability to bind to ^{3}H-labeled double-stranded normal cellular DNA (5×10^4 cpm per assay, with a specific activity of 2×10^5 cpm/µg). The p110$^{gag\text{-}myc}$ protein from MC29-transformed fibroblasts, purified analogously, is shown as the control. (C) Inhibition of DNA binding in vitro. The purified human c-*myc* gene product shown in (A) was incubated with various IgGs for 15 min at 4 °C. Subsequently the residual DNA-binding ability was determined by filter-binding assay. The IgGs used were normal IgG, IgG specific for C_{16}, and C_{12}, v-*myc*, and human c-*myc* (for details see text and Figure 2). The amounts of IgG are as indicated (0.03 mg/mL). The initial activity designated as 100% corresponded to about 40 000 cpm bound by 0.04 µg of the human c-*myc* protein as shown in (B).

with the p110$^{gag\text{-}myc}$ protein. Both proteins bind to DNA with similar efficiencies. The result is shown in Figure 5B.

In order to further investigate the DNA-binding ability of the isolated hu-c-*myc* protein, DNA binding was tested in the presence of various immunoglobulin fractions. Normal IgG and IgGs directed against hu-c-*myc*, v-*myc*, C_{12}, and C_{16} were tested. C_{12}-specific IgG was isolated from polyvalent anti-hu-c-*myc* serum and not anti-C_{12} serum, since C_{12} itself was of low immunogenicity. While the normal IgG and C_{12}-specific IgG did not significantly inhibit DNA binding, hu-c-*myc*- and v-*myc*-specific IgGs inhibited DNA binding significantly. C_{16}-specific IgG also shows a strong inhibitory effect (Figure 5C). We have previously described an analogous phenomenon with the v-*myc* protein (Bunte et al., 1984).

Discussion

The data presented here indicate that the human c-*myc* gene product is a molecule of M_r 64 000 which copurifies with a minor protein of M_r 67 000. Two independently derived antisera and peptide-specific immunoglobulins prove that the protein is *myc* specific. The antibodies directed against the bacterially expressed v-*myc* and c-*myc* proteins cross-react and thus deliver additional proof that the protein is *myc* specific. The size determinations reported here are in agreement with those reported by Persson et al. (1984) and Hann & Eisenman (1984) even though the v-*myc* protein was reported to be M_r 62 000 by the latter group (Hann et al., 1983), whereas we assigned a molecular weight of 55 000 to the v-*myc* protein (Bunte et al., 1984).

The M_r 67 000 protein was not detected in the immunoprecipitation analyses of various human tumor cell lines shown in Figure 2D. Therefore, we cannot confirm with our antibodies the prevalence of the M_r 67 000 protein in Raji cells reported by others (Hann & Eisenman, 1984). It is possible that C-terminal peptide antibodies used by others (Hann & Eisenman, 1984) exhibit different specificities for the two forms of the *myc* protein than the antibodies against the bacterially expressed protein used here. The protein used as antigen was partially denatured during its isolation. The antibodies may therefore more efficiently recognize the M_r 64 000 protein than the M_r 67 000 one which probably exhibites a different conformation due to modifications.

A comparison of the hu-c-*myc* and v-*myc* sequences (Alitalo et al., 1983a,b; Colby et al., 1983) indicates that the v-*myc* gene codes for 422 amino acids and hu-c-*myc* has 18 amino acid deletions and 42 additions. Therefore, the hu-c-*myc* protein is longer by 24 amino acids. The additional amino acids and several amino acid substitutions do not account for the larger apparent molecular weight determined for hu-c-*myc* than for v-*myc*. It has been noted previously that v-*myc* shows an abnormal migration behavior and appears larger than its predicted size (Hann et al., 1983). This may be true for hu-c-*myc* as well (Persson et al., 1984). From the nucleotide sequence it appears reasonable that v-*myc* and chicken c-*myc* comigrate since they both code for 422 amino acids (Figure 2).

The human c-*myc* protein is a nuclear antigen and exhibits a high turnover. We have purified the protein and compared its ability to bind to DNA with that of the v-*myc* protein. Both purified proteins show close similarities. In a recent report DNA binding of the human c-*myc* protein has been demonstrated by indirect immunoprecipitation from a mixture of proteins bound to a DNA–cellulose column (Persson & Leder, 1984). The protein described here is soluble and was characterized by several specific IgGs. It can be concluded from the in vitro inhibition studies that DNA binding is an intrinsic property of the c-*myc* protein and is not mediated through cellular factors. The high degree of inhibition caused by the c-*myc*-specific IgG also suggests that the DNA-binding region of the c-*myc* protein is probably located in the 3′ domain of the molecule, corresponding to exon$_3$, since the c-*myc* IgG is directed only against this domain.

Further studies are under way to determine by which molecular mechanism the c-*myc* protein interacts with DNA, whether it acts as transcriptional activator (Newmark, 1984), or how it regulates gene expression in normal and tumor cells.

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