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Protein Product of Proto-Oncogene c-*mil*

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Using antipeptide antibodies with specificity for the carboxyl termini of v-*raf* and v-*mil* protein products, two proteins with apparent molecular weights of approximately 71,000/73,000 and 215,000 were detected in immunoprecipitates from normal uninfected chicken cells. The 71,000/73,000-molecular-weight protein was identified as the product of the c-*mil* proto-oncogene by the close structural relationship of its 42,000-molecular-weight carboxyl-terminal domain to the v-*mil*-encoded domain of the hybrid protein p100$^{gag\text{-}mil}$ specified by the avian retrovirus MH2. The amino-terminal domain of the cellular protein is encoded by 5′ c-*mil* sequences that have not been transduced into the genome of MH2. The c-*mil* protein (p71/73$^{c\text{-}mil}$) was found to be phosphorylated in vivo, and homologous proteins were detected at variable levels in a variety of vertebrate cells, including human cells.

The genome of the highly oncogenic avian retrovirus MH2 contains two cell-derived sequences, termed v-*mil* and v-*myc* (4, 9, 11, 13). The v-*myc* oncogene of MH2 is expressed via a subgenomic mRNA (17) as a 59,000/61,000-molecular-weight protein (5, 18) and is closely related to v-*myc* alleles present in the genomes of three other independently isolated avian RNA tumor viruses (1; K. Bister and H. W. Jansen, Adv. Cancer Res., in press). The v-*mil* gene of MH2 is expressed as a *gag*-related 100,000-molecular-weight hybrid protein (p100$^{gag\text{-}mil}$) containing amino-terminal sequences encoded by a partial complement of the viral *gag* gene and carboxyl-terminal sequences encoded by v-*mil* (4, 6, 9, 11, 13). Analyses of the biological properties of spontaneous and constructed deletion mutants of MH2 have revealed that v-*myc* is the basic oncogenic principle of this virus and that v-*mil* apparently has auxiliary functions in oncogenicity (10; T. Graf, F. von Weizsäcker, S. Grieser, J. Coll, D. Stehelin, T. Patschinsky, K. Bister, C. Bechade, G. Calothy, and A. Leutz, submitted for publication). The close structural relationship between v-*mil* and v-*raf*, the single transforming gene of the highly oncogenic murine sarcoma virus 3611 (3611-MSV) (20), also supports the view that v-*mil* may enhance or enlarge the oncogenic potential of MH2 (2, 8, 22). The cellular progenitor of v-*mil* is the proto-oncogene c-*mil* which is unrelated and physically unlinked to c-*myc* (11, 12). The regions of c-*mil* with homology to v-*mil* are organized in 11 exons (7). Nucleotide sequence analysis revealed that the predicted sequences of c-*mil* and v-*mil* protein products differ by only five amino acid substitutions within the shared domain corresponding to a 42,000-molecular-weight polypeptide (7). The sequence analysis also predicted that the carboxyl terminus of the c-*mil* product is identical to that of the v-*mil* product, while the amino terminus of the cellular protein is apparently encoded by sequences extending 5′ to the region of homology to v-*mil*. Hence, it was necessary to identify the protein encoded by c-*mil* to get insight into the full coding capacity of the cellular gene and to facilitate studies on the function of c-*mil*.

For the identification of the c-*mil* protein product, we made use of an antiserum [anti-*raf*(SP63)] directed against a synthetic peptide (SP63) with a sequence corresponding to the predicted sequence of 12 amino acids from the carboxyl terminus of the v-*raf* protein product (19). Ten of these amino acids are shared between the carboxyl-terminal sequences of v-*raf* and v-*mil* proteins (22). Accordingly, we have previously shown that not only *gag-raf* hybrid proteins encoded by 3611-MSV but also *gag-raf-mil* hybrid proteins with v-*mil*-specific carboxyl termini specified by constructed recombinant viruses are specifically recognized by the anti-*raf*(SP63) serum (19). Furthermore, the data in Fig. 1 directly demonstrate that anti-*raf*(SP63) specifically precipitated p100$^{gag\text{-}mil}$ from extracts of MH2-transformed quail fibroblasts. Hence, the antiserum appeared to be suitable for the identification of the avian c-*mil* protein for which carboxyl-terminal amino acid sequences identical to those of the v-*mil* protein product have been predicted from DNA sequence analyses (7). Uninfected chicken embryo fibroblasts were labeled with [^{35}S]methionine, and proteins were immunoprecipitated from detergent extracts as described previously (9, 18), using anti-*raf*(SP63) or anti-*raf*(SP63) preadsorbed to the SP63 peptide. Two proteins were specifically precipitated: a heterogeneous species with an apparent molecular weight of 71,000/73,000 and a 215,000-molecular-weight species (Fig. 1). Both proteins could also be labeled in vivo with radioactive phosphate (Fig. 1). The heterogeneity in electrophoretic mobility of the 71,000/73,000-molecular-weight protein is clearly visible for the ^{32}P-labeled proteins, indicating that it may be due to different degrees of phosphorylation. The complexity of the 71,000/73,000-molecular-weight protein agrees well with the size of a protein predicted from the sequence analysis of a human c-*raf*(*mil*) cDNA clone (T. I. Bonner, H. Oppermann, P. Seeburg, S. B. Kerby, M. A. Gunnell, A. C. Young, and U. R. Rapp, Nucleic Acids Res., in press) and is also compatible with the coding capacity of the chicken c-*mil* mRNA with a complexity of 4.0 kilobases (4). Hence, based on these considerations and on direct structural evidence (see below), the 71,000/73,000-molecular-weight protein appears to be the protein product (p71/73$^{c\text{-}mil}$) of the chicken c-*mil* gene. The 215,000-molecular-weight protein may cross-react with the serum owing to fortuitous homology to the peptide used for immunization or to its origin from a gene distantly related to c-*mil*. Alternatively, it may form a complex with p71/73$^{c\text{-}mil}$.

To provide direct evidence for the genetic origin of p71/73$^{c\text{-}mil}$, the structural relationship of this protein to the

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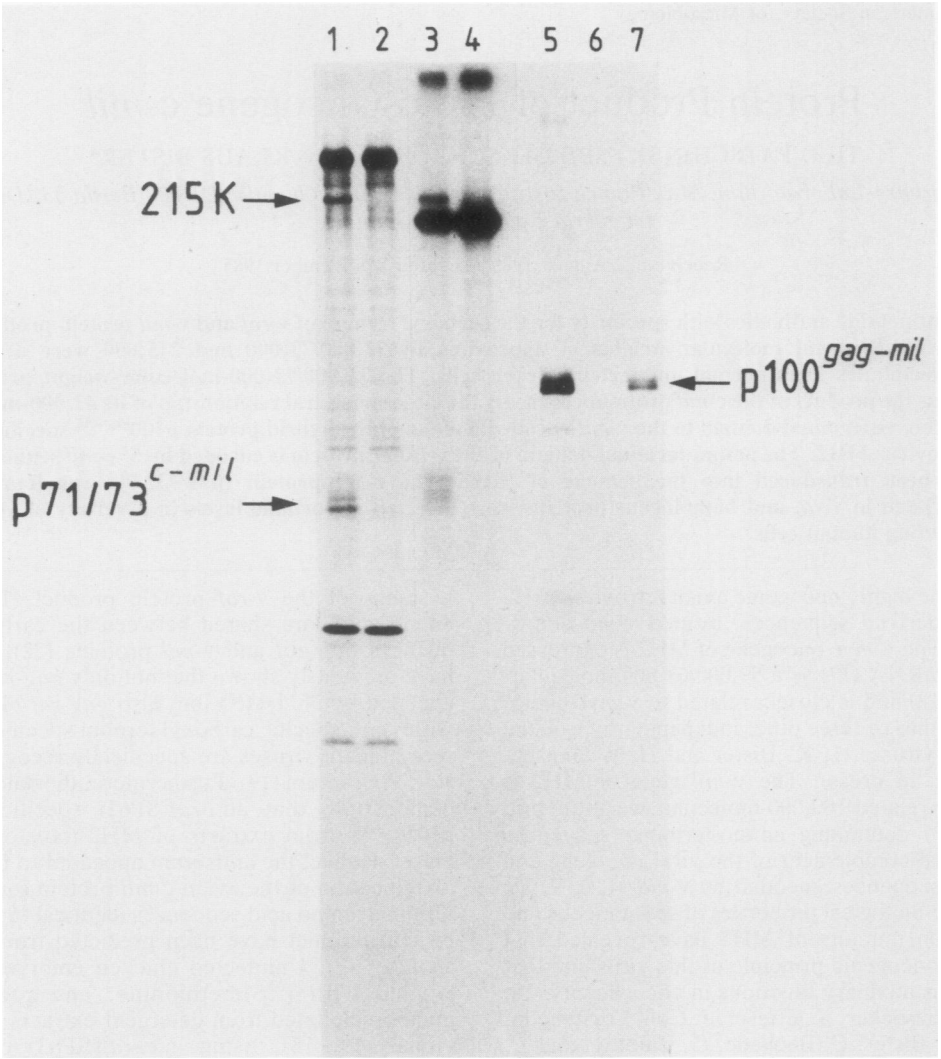

FIG. 1. Identification of the c-*mil* protein product. Lanes 1 and 2: Chicken embryo fibroblasts on two 100-mm dishes were labeled for 2 h with 400 μCi of [^{35}S]methionine (~1,000 Ci/mmol; Amersham Buchler GmbH, Braunschweig) per dish as described previously (9, 18). Cells were lysed in 1 ml of RIPA buffer (9) per dish. Samples (1 ml) of the lysate were incubated for 60 min at 4°C with either 1 μl of anti-*raf*(SP63) (lane 1) or with 1 μl of anti-*raf*(SP63) preadsorbed for 45 min at 4°C to 5 μg of the SP63 peptide (lane 2). Immune complexes were precipitated by the addition of 200 μl of a 1% (wt/vol) suspension of *Staphylococcus aureus* cells (Pansorbin; Calbiochem GmbH, Frankfurt) in RIPA buffer containing 1 mg of bovine serum albumin per ml. Precipitates were washed and processed as described previously (9, 18), and samples corresponding to equal aliquots (10%) of the total lysate were analyzed on a 12.5% polyacrylamide-sodium dodecyl sulfate gel. Lanes 3 and 4: Chicken embryo fibroblasts on two 100-mm dishes were labeled for 2 h with 5 mCi of $H_3{}^{32}PO_4$ (carrier-free; Amersham) per dish. All other procedures were as described above, with lane 3 showing ^{32}P-labeled proteins precipitated by anti-*raf*(SP63) and lane 4 showing those precipitated by the preadsorbed anti-*raf*(SP63). Lanes 5, 6, and 7: Cells from the MH2-transformed nonproducer quail fibroblast line A103 (10) on a 35-mm dish were labeled for 2 h with 200 μCi of [^{35}S]methionine. Proteins were precipitated from a 50-μl sample of the 1-ml cellular lysate with 1 μl of an anti-*gag* protein serum (9) (lane 5) and from 450-μl samples with either 1 μl of anti-*raf*(SP63) (lane 7) or 1 μl of preadsorbed anti-*raf*(SP63) (lane 6). Samples applied to the gel correspond to equal aliquots (1%) of the original lysate. The fluorograph of the gel was exposed for 18 h. 215K, 215,000-molecular-weight protein.

v-*mil*-encoded domain of p100$^{gag\text{-}mil}$ was analyzed by two-dimensional tryptic peptide mapping. Cells were labeled with [^{35}S]methionine, proteins were isolated by immunoprecipitation and preparative gel electrophoresis, and tryptic digests of these proteins were analyzed as described previously (9) and in the legend to Fig. 2. To facilitate comparison of p71/73$^{c\text{-}mil}$ with the v-*mil*-encoded domain of p100$^{gag\text{-}mil}$, *gag*-related peptides of the viral hybrid protein were first identified by comparison of the map of p100$^{gag\text{-}mil}$ with that of the *gag* gene precursor protein Pr76^{gag} of the MH2-associated helper virus MHAV (Fig. 2B and D) and by analysis of an appropriate mixture of digests from both proteins (data not shown). On the map of p100$^{gag\text{-}mil}$, eight major and six minor methionine-containing peptides could be identified which have homologous counterparts on the map of Pr76 (indicated by arrows in Fig. 2). Subtraction of these peptides from the map of the viral hybrid protein allowed the identification of 11 v-*mil*-specific peptides, in good agreement with the predictions from the amino acid sequence deduced from the nucleotide sequence of v-*mil* (22) and with a previous analysis of p100$^{gag\text{-}mil}$ (9). Comparison of the map of p71/73$^{c\text{-}mil}$ with that of p100$^{gag\text{-}mil}$, and analysis of a map containing a mixture of peptides derived from both proteins revealed that 11 methionine-containing peptides

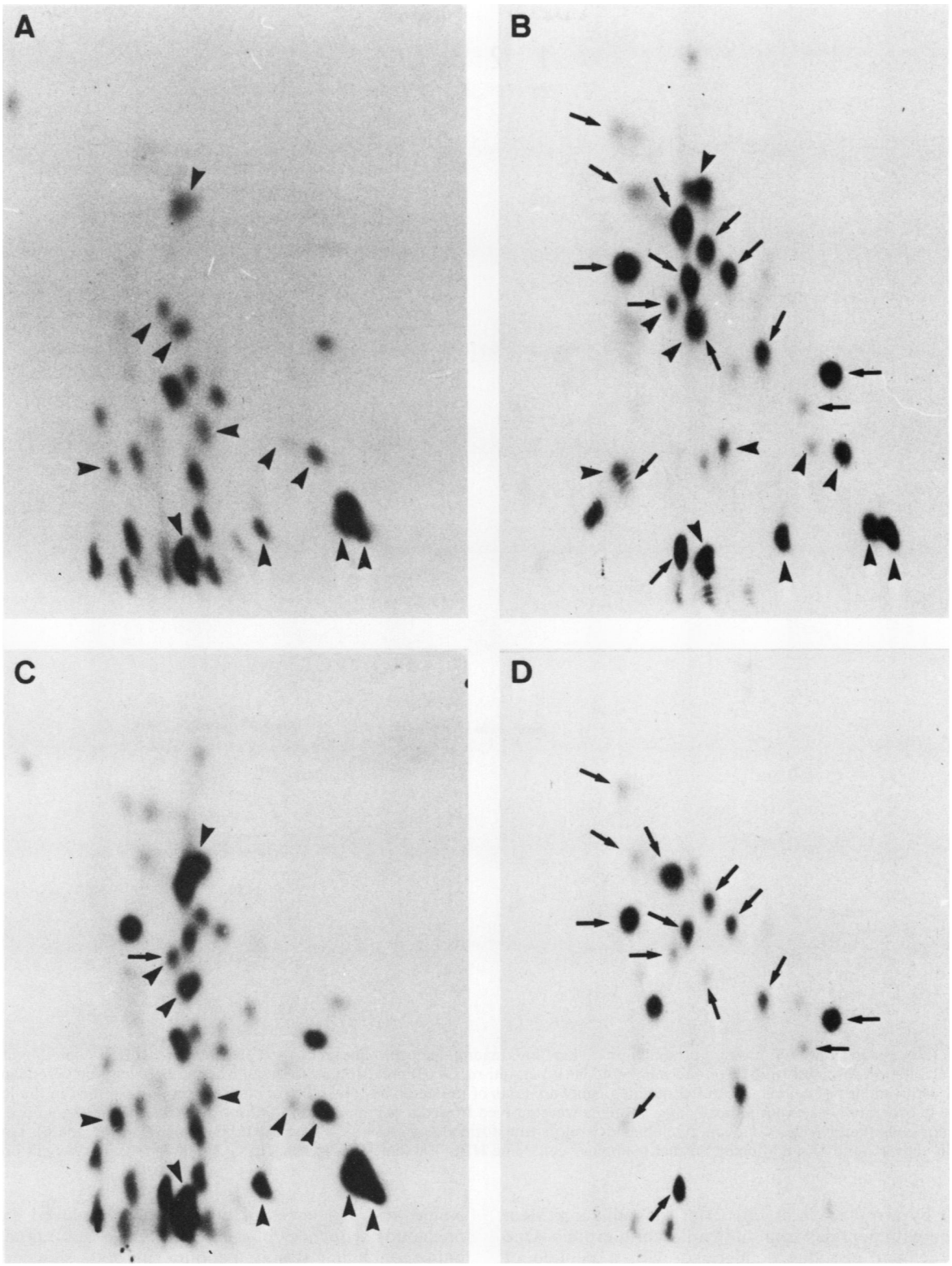

FIG. 2. Structural relationship between p71/73$^{c\text{-}mil}$ and p100$^{gag\text{-}mil}$. Tryptic peptide maps of p71/73$^{c\text{-}mil}$ from chicken embryo fibroblasts (A), of p100$^{gag\text{-}mil}$ from the MH2-transformed nonproducer quail fibroblast line A103 (B), of a mixture of digests from both proteins (C), and of Pr76^{gag} from the MH2(MHAV)-infected producer quail fibroblast line A10 (9) (D) are shown. Proteins were isolated by immunoprecipitation from [^{35}S]methionine-labeled cells with anti-*raf*(SP63) (for p71/73$^{c\text{-}mil}$) or anti-*gag* serum (for p100$^{gag\text{-}mil}$ and Pr76^{gag}), followed by preparative gel electrophoresis as described in the legend to Fig. 1 and elsewhere (9, 18). Analysis of tryptic digests was performed as described previously (9) by electrophoresis at pH 4.7 with the origin at the lower left and the negative electrode at the right, followed by ascending chromatography from bottom to top of the panels. [^{35}S]methionine-containing peptides were visualized by fluorography. The amount of radioactivity loaded and the exposure times were: (A) 6,500 cpm, 18 days; (B) 24,200 cpm, 5 days; (C) 7,000 cpm (p71/73$^{c\text{-}mil}$) plus 4,000 cpm (p100$^{gag\text{-}mil}$), 18 days; (D) 12,900 cpm, 5 days. Arrows indicate peptides shared between p100$^{gag\text{-}mil}$ and Pr76^{gag}, and arrowheads indicate peptides shared between p71/73$^{c\text{-}mil}$ and p100$^{gag\text{-}mil}$ (see text).

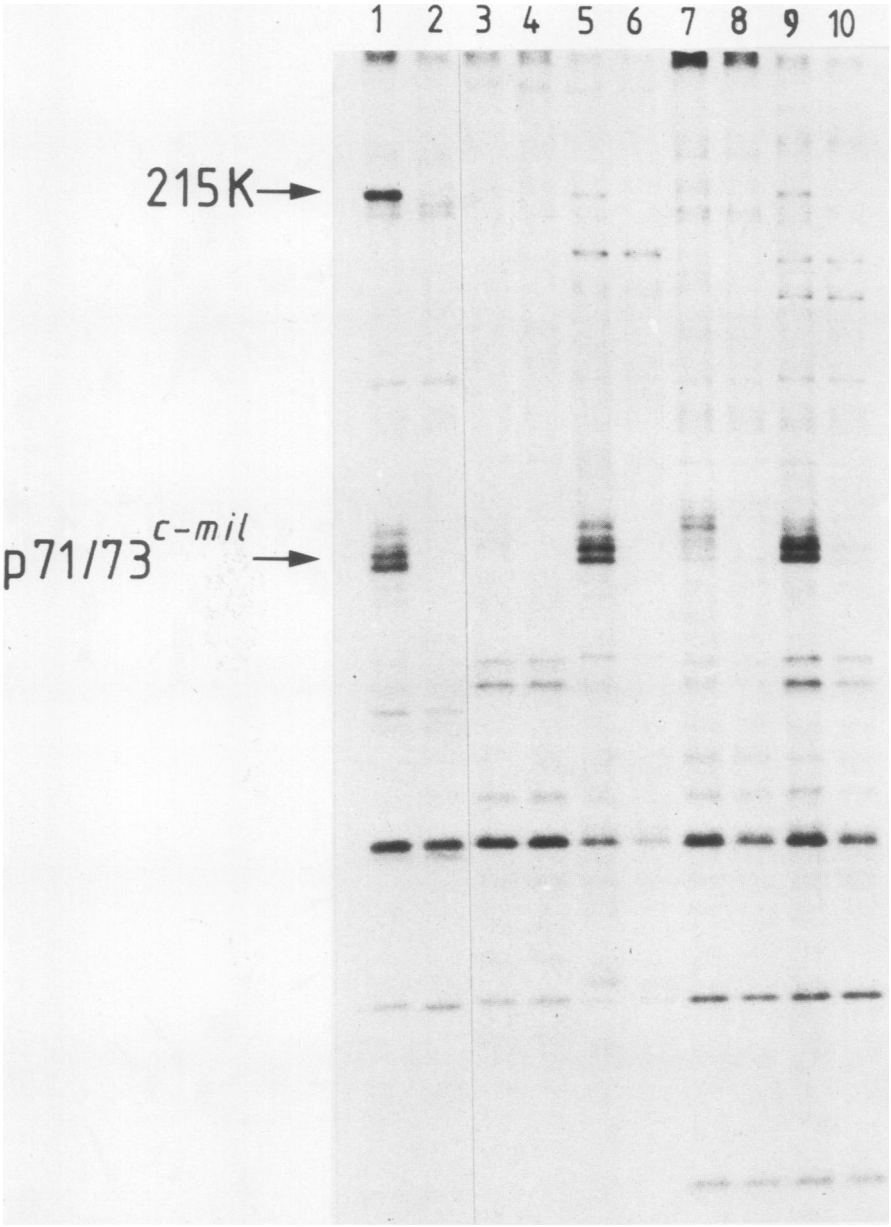

FIG. 3. Expression of c-*mil* protein products in avian and mammalian cell lines. Cells were labeled with [^{35}S]methionine, and immunoprecipitation with anti-*raf*(SP63) (odd-numbered lanes) or anti-*raf*(SP63) preadsorbed to the SP63 peptide (even-numbered lanes) was performed with samples of cellular lysates containing equal amounts of radioactivity. Gel electrophoresis was as described in the legend to Fig. 1. The fluorograph was exposed for 4 days. Proteins were derived from normal quail embryo fibroblasts (lanes 1 and 2), mouse Ehrlich ascites tumor cells (lanes 3 and 4), avian myeloblastosis virus-transformed chicken myeloblast cells, line BM-2 (lanes 5 and 6), normal rat kidney cells (lanes 7 and 8), and human normal mammary cells, line HBL-100 (lanes 9 and 10). 215K, 215,000-molecular-weight protein.

(indicated by arrowheads in Fig. 2) of the cellular protein comigrated with peptides from the viral hybrid protein. One of these cellular peptides comigrated with a peptide of p100$^{gag\text{-}mil}$ (indicated by both arrowhead and arrow in Fig. 2B and C) which in turn comigrated with a *gag*-specific peptide of Pr76^{gag}. This comigration is likely to be fortuitous, and p100$^{gag\text{-}mil}$ contains either the c-*mil*- or the *gag*-specific peptide or both. Hence, at least 10 or possibly 11 peptides are shared between the c-*mil* protein and the v-*mil*-encoded domain of p100$^{gag\text{-}mil}$ as determined by comigration in two-dimensional tryptic peptide maps. This number is in good agreement with predictions based on the amino acid sequences of both proteins deduced from the nucleotide sequences of c-*mil* and v-*mil* (7, 22): nine methionine-containing tryptic peptides in both sequences are either identical (seven peptides) or differ by a single amino acid substitution (two peptides). One major non-*gag*-specific peptide of p100$^{gag\text{-}mil}$ (in the lower left corner of Fig. 2B) was not found in the map of p71/73$^{c\text{-}mil}$. It is possible that it represents the hybrid peptide that is predicted from the sequence analyses to be generated at the *gag-mil* junction (7, 22). About 12 peptides of p71/73$^{c\text{-}mil}$ (unmarked in Fig. 2A) were not found in the map of p100$^{gag\text{-}mil}$. These are presumably derived from the amino-terminal domain of the cellular

protein (corresponding to a 30,000-molecular-weight polypeptide) which is encoded by 5′ c-*mil* sequences that have not been transduced into the genome of MH2 (7). The immunological relationship and the structural homology between the cellular 71,000/73,000-molecular-weight protein and the v-*mil*-encoded domain of $p100^{gag\text{-}mil}$ strongly indicate that $p71/73^{c\text{-}mil}$ is the authentic protein product of c-*mil*.

The c-*mil* gene is highly conserved among avian and mammalian species (2, 8; Bister and Jansen, in press) and is transcribed into mRNA species of similar sizes (3.4 to 4.0 kilobases) in different vertebrate cells (4). The data in Fig. 3 present a survey of c-*mil* expression at the protein level in normal and transformed cells of different species including normal quail embryo fibroblasts, mouse Ehrlich ascites tumor (EAT) cells, cells from the avian myeloblastosis virus-transformed chicken myeloblast line BM-2 (3), normal rat kidney (NRK) cells, and cells from the human normal mammary cell line HBL-100 (21). Immunoprecipitation of [^{35}S]methionine-labeled proteins was carried out with anti-*raf*(SP63) or with anti-*raf*(SP63) preadsorbed to the SP63 peptide. Pronounced expression of $p71/73^{c\text{-}mil}$ was observed in the quail fibroblasts, and in cells from the chicken line BM-2 and the human line HBL-100 (Fig. 3). Relatively low amounts of the protein were detected in EAT and NRK cells. In the latter, the higher-molecular-weight forms are more abundant than the lower-molecular-weight species, possibly owing to more extensive phosphorylation (compare Fig. 1). Further experiments demonstrated that $p71/73^{c\text{-}mil}$ is expressed at levels similar to those shown in Fig. 3 in chemically transformed or sarcoma virus-transformed quail fibroblasts and in different mouse and rat cell lines (data not shown).

The identification of the c-*mil* protein product as a 71,000/73,000-molecular-weight phosphoprotein confirmed the previous conclusion derived from nucleotide sequence data (7) that the v-*mil* oncogene of MH2 represents a truncated form of the cellular c-*mil* gene. Activation of the mouse c-*mil* homolog, i.e., of the c-*raf* gene, either by retroviral transduction (in the genesis of 3611-MSV [20]) or by insertion mutagenesis (in transfection experiments with DNA from retroviral long terminal repeat sequences [15]) also involves truncation of the c-*raf* gene. Interestingly, all three oncogenic mutant alleles of c-*mil*(*raf*) genes, i.e., v-*mil*, v-*raf*, and long terminal repeat-c-*raf*, have retained similar complements from the 3′ half of the gene and hence encode polypeptides with similar molecular weights ranging from 36,000 to 45,000 which all share the carboxyl terminus with the normal cellular protein. Extensive truncations of coding domains have been observed in the activation (either by transduction or insertion mutagenesis) of other cellular oncogenes, such as the c-*erbB* or the c-*myb* gene (14, 16, 23, 24; Bister and Jansen, in press). It is conceivable that the removal of protein-coding domains from cellular oncogenes is a critical step in changing their function from physiological to oncogenic. Naturally, the relevant biochemical functions of the protein products of normal and mutant alleles have to be identified to assess this possibility in a qualitative or quantitative way.

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