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Identification of the leukemogenic protein of avian myeloblastosis virus and of its normal cellular homologue

(avian myeloblastosis virus oncogene/immunoprecipitation/anti-peptide antibodies)

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ABSTRACT **The genome of the replication-defective avian myeloblastosis virus (AMV) contains an inserted cellular sequence (*amv*) that is part of the oncogene responsible for acute myeloblastic leukemia in chickens infected with AMV. Three antisera raised against distinct synthetic peptides predicted from the long open reading frame of *amv* specifically precipitated the same 48-kilodalton protein (p48^amv^) from leukemic myeloblasts but not from normal hematopoietic tissue, fibroblasts, or from fibroblasts infected with the AMV helper virus, MAV-1 (myeloblastosis-associated virus type 1). p48^amv^ is not glycosylated or phosphorylated and does not appear to act as a protein kinase *in vitro*. The same three antisera that recognized p48^amv^ also specifically precipitated a common 110-kilodalton protein from normal uninfected hematopoietic tissue. This normal cellular homologue of the AMV leukemogenic protein, p110^*proto-amv*^, was not present in normal fibroblasts, MAV-1 infected fibroblasts, or, interestingly, in some leukemic myeloblasts. We conclude that p48^amv^ is the leukemogenic product of an altered, transduced, partial protooncogene. Short helper-virus sequences provide its carboxyl terminus and also may provide the amino terminus.**

Avian myeloblastosis virus (AMV) causes acute myeloblastic leukemia in chickens and *in vitro* transforms myeloid hematopoietic cells but not fibroblasts (1). The oncogene responsible for leukemogenesis consists mostly of chicken genetic elements inserted into the viral genome (2, 3). The acquired cellular sequences (*amv*) have replaced the 3′-terminal portion of the reverse transcriptase gene and most of the gene encoding retroviral envelope proteins (*env*) (4). As a result, AMV is replication defective and requires a helper virus for the synthesis of infectious progeny virus. The natural helper for AMV is myeloblastosis-associated virus type I (MAV-1) from which AMV was generated by recombination with host genetic material (3, 4).

Nucleotide sequence analyses of the AMV transforming gene region and of the corresponding region in MAV-1 have revealed the following information (4). The cellular insert consists of (*i*) a 5′-terminal open reading frame of 81 base pairs (bp) plus the termination codon TAG, (*ii*) an adjacent sequence of 340 bp that contains consensus transcription regulatory elements but no initiation or termination codon, and (*iii*) a continuation of the open reading frame for 762 bp up to the 3′ terminus. In AMV the 5′ recombination site of the cellular insert is located 111 bp upstream from the termination codon for the MAV-1 reverse transcriptase gene. The 3′ recombination site of the insert is positioned 33 bp upstream from the termination codon of the MAV-1 envelope gene. Thus, although the location of the amino terminus of the AMV transforming gene is unknown, its carboxyl terminus presumably represents the 11 carboxyl-terminal amino acids of the MAV-1 envelope gene.

The structure of the AMV genome resembles that of the defective Bryan strain of Rous sarcoma virus (5–7), and leukemic myeloblasts transformed in the absence of helper virus release replication-defective virions (8). Also the viral oncogene product is not expressed as a polyprotein with fused viral proteins, such as a *gag* fusion protein (*gag* is the gene encoding the structural proteins of the retroviral core) (9). Consequently, the AMV oncogene product, like that of Rous sarcoma virus, cannot be detected with antisera directed against subviral components but requires an antiserum directed against the transforming protein itself (10). The preparation of such antisera is complicated because AMV is defective and induces acute myeloblastic leukemia only in chickens, and the chicken genome contains gene(s) homologous to *amv*.

Anti-peptide antibodies have been successful in detecting proteins deduced from their nucleic acid sequences and have been used to identify DNA and RNA tumor virus proteins responsible for cellular transformation (11, 12). In this report we describe the identification of a protein of 48 kilodaltons (kDa) as the AMV oncogene product by using antisera generated by immunization of rabbits with synthetic peptides of 15–19 amino acids representing different regions of the AMV leukemogenic protein predicted from the nucleotide sequence of the AMV oncogene.

The normal cellular sequences homologous to *amv* (*proto-amv*) are interrupted by several regions of nonhomology and distributed over 8–9 kilobases (kb) of cellular DNA (13, 14) and are expressed as part of a 4.5-kb mRNA in certain hematopoietic tissues (refs. 15 and 16; unpublished data). The antisera prepared against the synthetic *amv* peptides described above also have identified a protein of 110 kDa as the normal product of the cellular gene that contains the *proto-amv* sequences.

MATERIALS AND METHODS

Cells and Viruses. The BAI strain A of AMV was obtained from J. Beard (Life Sciences, St. Petersburg, FL). An established clonal line of leukemic myeloblasts (BM2) (17) was provided by C. Moscovici (Veterans Administration Hospital, Gainesville, FL). The BM2 myeloblasts contain the AMV provirus but no helper provirus and produce replication-defective AMV virions (8). Leukemic myeloblasts were isolated from leukemic chickens as described (18). Chicken embryo fibroblast (CEF) cultures were prepared from the thigh muscles of 11-day-old chicken embryos (C/E, negative for group-specific antigen

Abbreviations: AMV, avian myeloblastosis virus; *amv*, cellular insert in AMV genome; *proto-amv*, normal cellular homologue of *amv*; CEF, chicken embryonic fibroblast(s); MAV-1, myeloblastosis-associated virus type 1; bp, base pair(s); kb, kilobase(s); kDa, kilodalton(s); P1, P2, P3, and P4, synthetic peptides 1, 2, 3, and 4.

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and chicken helper factor; Spafas, Roanoke, IL). Embryonic thymus cells were obtained from 17-day-old C/E Spafas chicken embryos. All cells were cultured as described (19).

Preparation of Anti-Peptide Antisera. The synthetic peptides described in *Results* were purchased from Peninsula Laboratories (San Carlos, CA) with a purity of 80% or greater. Carboxyl-terminal cysteine was added to peptides 1 and 2 because initially we planned to conjugate the peptides to bovine thyroglobulin (Sigma) through the cysteine sulfhydryl group (20). However, because of poor conjugation, we switched to the carbodiimide method. To prepare the peptide-bovine thyroglobulin conjugates, 10 mg of synthetic peptide and 3 mg of bovine thyroglobulin were dissolved in 3 ml of 0.1 M borate buffer (pH 9.5). To the solution, 8 mg of 1-ethyl-3(3-dimethylamino)propylcarbodiimide·HCl (Sigma, E7750) was added dropwise at 4°C over 30–60 min. The mixture was then incubated at 25°C for 24 hr and used directly for immunization. The coupled protein (100 μg) with complete Freund adjuvant was injected intraperitoneally into rabbits at 14-day intervals. Animals were bled 1 wk after injection. The various bleedings were tested for antibody activity by using ^{125}I-labeled peptides in a radioimmunoassay. To assay binding of peptides 1, 2, and 3, which do not contain tyrosine, a small amount of these peptides were synthesized with tyrosine at the carboxyl terminus, iodinated with ^{125}I, and mixed with the unlabeled natural peptides. The titer for 20% precipitation ranged between 1:2,500 and 1:5,000 for the second and third bleedings. The antisera were used after the fourth, fifth, and sixth bleedings. Serum was obtained from each rabbit before any injection was administered (preimmune serum).

Preparation of Radioactively Labeled Cell Lysates. Cells to be analyzed were preincubated in either methionine-free or phosphate-free medium for 60 min. Cells were biosynthetically labeled by addition of 250 μCi of [^{35}S]methionine (specific activity, 1,105 Ci/mmol; Amersham; 1 Ci = 3.7×10^{10} Bq) or 500 μCi of [^{32}P]orthophosphate (Amersham) per 5×10^7 cells. After incubation for 90 min, the cells were washed twice in phosphate-buffered saline (Dulbecco) and lysed at 10^7 cells per ml of detergent buffer (100 mM sodium chloride/10 mM sodium phosphate, pH 7.5/0.5% sodium deoxycholate/1% Triton X-100/0.1% sodium dodecyl sulfate/0.1 mM leupeptin/5 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride/0.4 trypsin-inhibiting units per ml of aprotinin. These cell lysates were frozen at −20°C, thawed to 4°C, and then centrifuged at 100,000 $\times$ *g* for 60 min.

Immunoprecipitation. The immunoadsorbant used in precipitations was first treated with unlabeled cell lysates to decrease the nonspecific binding of radioactively labeled cell proteins. Pansorbin (10% suspension of *Staphylococcus aureus* cells bearing protein A; Calbiochem) was incubated with the appropriate unlabeled cell lysate for 30 min at 37°C and then washed twice with detergent buffer. Preimmune serum (10 μl) was added to a 500-μl sample of labeled protein cell lysate. Immune complexes were precipitated by a 120-min incubation in presence of 60 μl of (pretreated) Pansorbin. After centrifugation, 10 μl of the pertinent anti-peptide antiserum was added to the supernatant and incubated overnight at 4°C. Anti-peptide-specific immune complexes were precipitated and pelleted in the same manner as were the preimmune serum complexes and then were washed twice with detergent buffer and once with 0.5 M lithium chloride/0.1 M Tris/base, pH 8.0. The immune complexes were eluted from *S. aureus* by resuspension in 50 μl of elution buffer (62.5 mM Tris·HCl, pH 6.8/5% $NaDodSO_4$/5% 2-mercaptoethanol/2 M urea/100 mM dithiothreitol/1 mM EDTA), boiling for 5 min, and centrifugation.

Polyacrylamide Gel Electrophoresis. Immune precipitates were analyzed on 7.5% polyacrylamide gels by using a discontinuous buffering system (21) containing 0.1% $NaDodSO_4$ and 0.5 M urea. After electrophoresis, the gels were fixed in 50% methanol/10% glacial acetic acid and then processed for fluorography by the method of Chamberlain (22). Gels were exposed to Kodak XAR-5 x-ray film for time periods ranging from 12 hr to 3 days at −76°C with Dupont Cronex intensifying screens.

RESULTS

Identification of the AMV Oncogene Product in Chicken Leukemic Myeloblasts. Four peptides with a potentially hydrophilic amino acid sequence were selected from four domains of the putative protein of 265 amino acids predicted from the long open reading frame in the cellular insert of AMV (4). Synthetic peptide 1 (P1) of 15 amino acids from *amv* and an additional Cys-COOH has the sequence NH_2-Pro-Gln-Glu-Ser-Ser-Lys-Ala-Gly-Pro-Pro-Ser-Gly*-Thr-Thr-Gly-Cys-COOH and is located between nucleotide positions 546 and 590 from the *Kpn* I site in AMV (4). (The Gly* at position 12 resulted from a typographic error, G instead of C, in the nucleotide sequence and should have been an alanine residue.) Synthetic peptide 2 (P2) of 15 *amv* amino acids plus an added Cys-COOH has the sequence NH_2-Met-Ala-Phe-Ala-His-Asn-Pro-Pro-Ala-Gly-Pro-Leu-Pro-Gly-Ala-Cys-COOH and is located between nucleotide positions 612 and 656. Synthetic peptide 3 (P3) of 16 *amv* amino acids with the sequence NH_2-Pro-Pro-Val-Asp-His-Gly-Cys-Leu-Pro-Glu-Glu-Ser-Ala-Ser-Pro-Ala-COOH is located between nucleotide positions 1,023 and 1,070. Synthetic peptide 4 (P4) with the 19-amino-acid sequence NH_2-Pro-Phe-His-Lys-Asp-Gln-Thr-Phe-Thr-Glu-Tyr-Arg-Lys-Met-His-Gly-Gly-Ala-Val-COOH is located between nucleotide positions 1,254 and 1,310. Peptide P4, the presumed carboxyl terminus of the AMV oncogene product, overlaps the 3′-terminal recombination site and contains the 11 carboxyl-terminal amino acids of the MAV-1 envelope gene (4). Each synthetic peptide was coupled to thyroglobulin as carrier and injected into a rabbit to raise antibodies (20).

The source of AMV leukemogenic product was an established line of chicken leukemic myeloblasts (17), which had been transformed by AMV but were not infected by helper virus. They transcribe a high level of 2.5-kb mRNA homologous to *amv* (data not shown) and produce defective AMV virions (8). A lysate of these cells biosynthetically labeled with [^{35}S]methionine was treated with each anti-peptide antiserum after the respective preimmune serum (Fig. 1). Antiserum against synthetic peptide P1, P2, or P4 but not that against peptide P3 immunoprecipitated a common molecule of 48 kDa ($p48^{amv}$). In addition to the bands revealed by preimmune serum from the same rabbit, anti-P1 antiserum and anti-P4 antiserum immunoprecipitated only $p48^{amv}$, whereas anti-P2 antiserum specifically precipitated three molecules of 56, 68, and 90 kDa in addition to $p48^{amv}$.

Specificity was demonstrated by complete inhibition of immunoprecipitation of $p48^{amv}$ only if the antisera were preincubated with their respective peptide haptens (Fig. 1). Preincubation with peptide P2 abolished precipitation of the 56-, 68-, and 90-kDa molecules by anti-P2 antiserum (Fig. 1). Thus, the cellular molecules other than $p48^{amv}$ immunoprecipitated by anti-P2 antiserum must share antigenic sites with $p48^{amv}$. This crossreactivity phenomenon has been noted previously with antisera against other short synthetic peptides but does not necessarily imply any functional or evolutionary relationship between the crossreacting cellular proteins (23–25).

To demonstrate the generality that $p48^{amv}$ is AMV encoded,

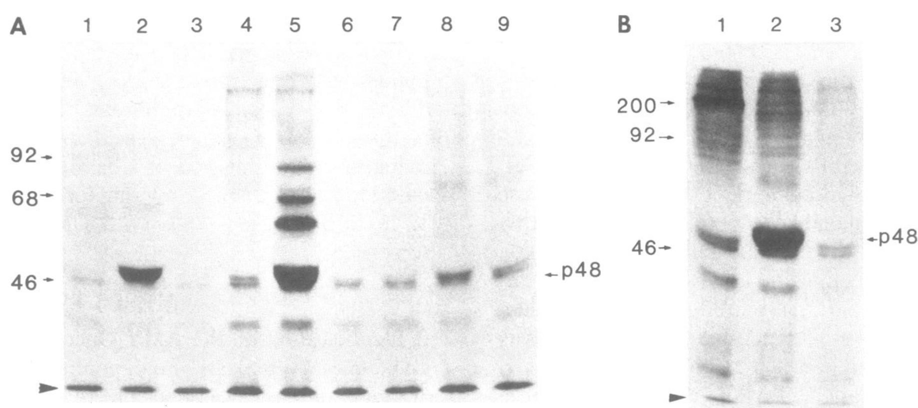

FIG. 1. Identification of p48^{amv} in the lysate of a clonal line of chicken leukemic myeloblasts not harboring the helper provirus. Individual samples of lysate labeled with [^{35}S]methionine (4×10^6 cpm of trichloroacetic acid precipitable material) were sequentially immunoprecipitated with preimmune serum followed by either anti-peptide antiserum or anti-peptide antiserum that had been preincubated with 2–5 μg of the corresponding peptide. Immune complexes bound to *S. aureus* were eluted and then analyzed on 7.5% $NaDodSO_4$/urea polyacrylamide slab gels, and radiolabeled polypeptides were identified by fluorography. (*A*) Lanes: 1, 4, and 7, precipitates with individual rabbit preimmune sera; 2, 5, and 8, precipitates with anti-P1, anti-P2, and anti-P3 antisera, respectively; 3, 6, and 9, precipitates with anti-P1, anti-P2, and anti-P3 antisera preincubated with peptides P1, P2, and P3, respectively. (*B*) Lanes: 1, precipitate with preimmune serum for anti-P4 antiserum; 2, precipitate with anti-P4 antiserum; 3, precipitate with anti-P4 antiserum preincubated with P4. The large solid arrowhead indicates the bromophenol blue dye front. Molecular size markers were a mixture of [^{14}C]methylated proteins (Amersham): 46 kDa, ovalbumin; 68 kDa, bovine serum albumin; 92 kDa, phosphorylase B; and 200 kDa, myosin.

a ^{35}S-labeled lysate was prepared from leukemic myeloblasts obtained from the peripheral blood of a leukemic chicken (no. 21803). Again a molecule of 48 kDa (p48^{amv}) was specifically precipitated by antisera against peptides P1, P2, and P4 (Fig. 2*A*) but not by antiserum against peptide P3 (not shown). Also the same crossreactive cellular molecules detected above were specifically immunoprecipitated by anti-P2 antiserum. In addition to p48^{amv}, anti-P1 antiserum specifically precipitated a molecule of approximately 110 kDa.

E26 virus, a replication-defective acute leukemia virus which causes erythroblastosis and myeloblastosis in chickens, shares transformation-specific RNA sequences with AMV (26). However, in contrast to the AMV genome, E26 virus contains a cellular insert in the *gag* gene, and its presumptive 135-kDa transforming protein is a fusion of a partial *gag* gene product and an *amv*-related E26 virus-specific gene product. Thus, E26 virus resembles the MC29 virus in both genome structure and transforming protein structure (see ref. 26). Because the E26 virus transforming protein should be related to the AMV oncogene product, E26 virus-transformed chicken leukemic cells offer an ideal control for the specificity of our anti-peptide antisera. Biosynthetically labeled lysates of such cells were treated with anti-*gag* antiserum or anti-P2 antiserum, and a common 135-kDa protein was immunoprecipitated (Fig. 2*B*). The anti-P2 antiserum immunoprecipitation of the E26 *gag*-fusion protein was inhibited by preincubation with P2. The anti-*gag* antiserum also precipitated Pr180, the *gag–pol* precursor (*pol* is the gene encoding the retroviral polymerase gene) and Pr76, the *gag* precursor, whereas the anti-P2 antiserum did not, as expected (Fig. 2*B*).

The specificity of the anti-peptide antisera was further tested in lysates of CEF labeled biosynthetically with [^{35}S]methionine. Sister CEF cultures, uninfected or infected with MAV-1 helper virus and subcultured four times after infection, were immunoprecipitated with antisera against peptides P1, P2, and P4 and with their respective preimmune sera. None of the antisera precipitated p48^{amv} from CEF (Fig. 3) or from CEF infected with MAV-1 (data not shown).

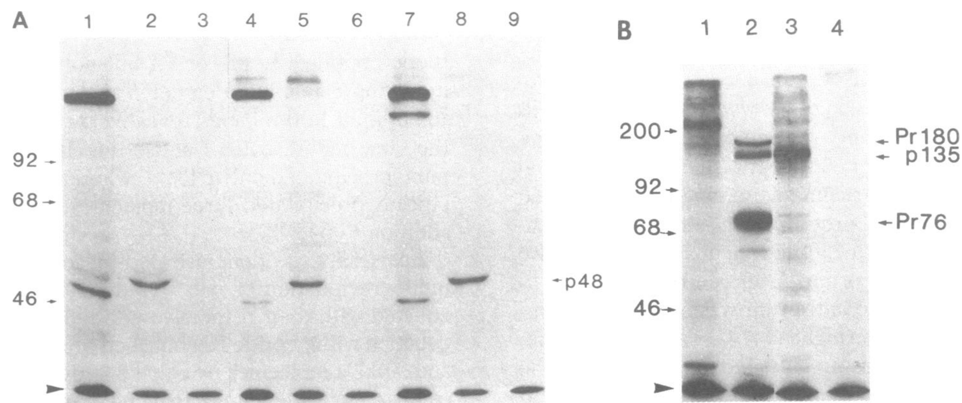

FIG. 2. (*A*) Identification of p48^{amv} in a lysate of peripheral blood chicken leukemic myeloblasts. Analysis of the immunoprecipitates labeled with [^{35}S]methionine was performed as in Fig. 1. Lanes: 1, 4, and 7, preimmune sera for anti-P1, anti-P2, and anti-P4 antisera, respectively; 2, 5, and 8, anti-P1, anti-P2, and anti-P4 antisera, respectively; 3, 6, and 9: anti-P1, anti-P2, and anti-P4 antisera preincubated with peptides P1, P2, and P4, respectively. (*B*) Immunoprecipitation of the E26 *gag*-fusion protein, p135, containing related AMV sequences from a lysate of E26 virus-transformed chicken leukemic cells labeled with [^{35}S]methionine. Lanes: 1, pooled rabbit preimmune sera; 2, anti-AMV p19 antiserum; 3, anti-P2 antiserum; 4, anti-P2 antiserum preincubated with peptide 2. Sizes are shown in kDa.

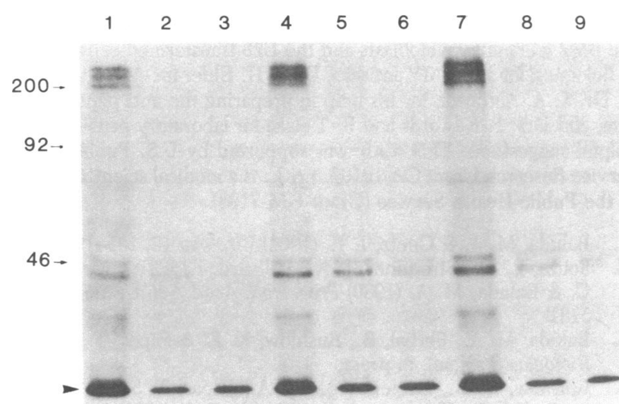

FIG. 3. Absence of p48^{amv} in chicken embryonic fibroblasts. Semiconfluent cultures were biosynthetically labeled with [^{35}S]methionine for 60 min and lysed in the dish with detergent buffer. Analysis of immunoprecipitates was performed as in Fig. 1. Lanes: 1, 4, and 7, preimmune sera for anti-P1, anti-P2, and anti-P4 antisera, respectively; 2, 5, and 8, anti-P1, anti-P2, and anti-P4 antisera, respectively; 3, 6, and 9, anti-P1, anti-P2, and anti-P4 antisera preincubated with peptides P1, P2, and P4, respectively. Sizes are shown in kDa.

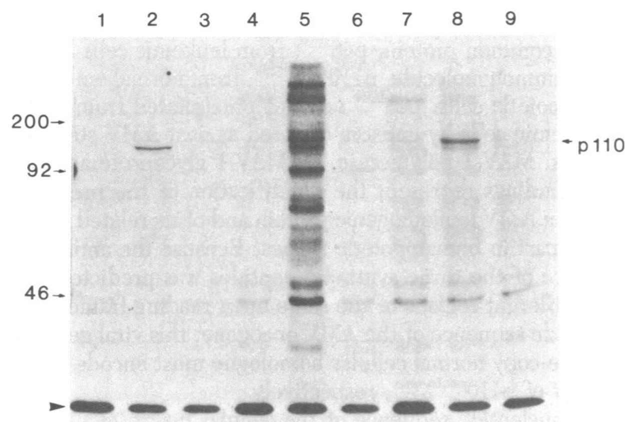

FIG. 5. Specific immunoprecipitation of a cellular protein, p110$^{proto\text{-}amv}$, in a lysate of normal chicken embryonic thymocytes labeled with [^{35}S]methionine. Analysis of immunoprecipitates was performed as in Fig. 1. Lanes: 1, 4, and 7, preimmune sera; lanes 2, 5, and 8, anti-P1, anti-P2, and anti-P4 antisera, respectively; lanes 3, 6, and 9, anti-P1, anti-P2, and anti-P4 antisera preincubated with peptides P1, P2, and P4, respectively. Sizes are shown in kDa.

The AMV Oncogene Product Is a Nonphosphorylated Protein Without Kinase Activity. The transforming proteins of several acutely oncogenic retroviruses are phosphoproteins with associated protein kinase activity. To determine whether or not p48^{amv} was phosphorylated *in vivo*, leukemic myeloblasts were biosynthetically labeled with [^{32}P]orthophosphate, and the cellular lysate was analyzed by immunoprecipitation with anti-P2 antiserum and gel electrophoresis. Phosphorylation of p48^{amv} was not detected, whereas the cellular molecules of 56 and 68 kDa specifically precipitated by anti-P2 antiserum were phosphorylated (Fig. 4). The *in vitro* protein kinase activity of immunoprecipitated p48^{amv} was tested (27) and found to be negative (data not shown).

Because the normal cellular homologue of the AMV oncogene appears to be expressed only in hematopoietic tissues and primarily in very immature blood cells, it might be involved in hematopoietic cell regulation (refs. 15, 16, and 28; unpublished data). Because many hematopoietic regulatory molecules are glycoproteins (29, 30), we investigated whether or not p48^{amv} was glycosylated. Three different types of experiments indicated that it was not (data not shown): (*i*) immunoprecipitation with anti-P1, anti-P2, and anti-P4 antisera of leukemic cell lysates biosynthetically labeled with [^{14}C]glucosamine did not reveal a radioactive band with a molecular size of 48 kDa after gel electrophoresis; (*ii*) treatment of a [^{35}S]methionine-labeled lysate from leukemic cells with the endoglycosidase F (31) and immunoprecipitation with anti-P2 or anti-P4 antiserum did not reveal a decreased electrophoretic mobility for p48^{amv}; and (*iii*) labeling of leukemic cells with [^{35}S]methionine in the presence of tunicamycin (2 μg/ml) also did not decrease the electrophoretic mobility of p48^{amv}. Thus, it appears that p48^{amv} is neither phosphorylated nor glycosylated and does not possess protein kinase activity.

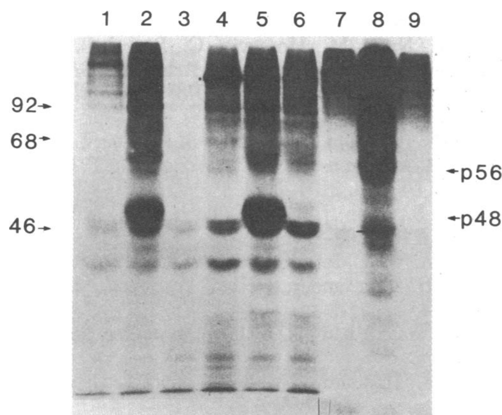

FIG. 4. The AMV oncogene product p48^{amv} is not phosphorylated. Analysis of immunoprecipitates from lysates of BM2 leukemic myeloblasts labeled with either [^{35}S]methionine (lanes 1–6) or [^{32}P]orthophosphate (lanes 7–9). Lanes: 1, 4, and 7, preimmune sera; 2, 5, and 8, anti-P2, anti-P4, and anti-P2 antisera, respectively; 3, 6, and 9, anti-P2, anti-P4, and anti-P2 antisera preincubated with peptides P2, P4, and P2, respectively. Sizes are shown in kDa.

Identification of the Normal Cellular Homologue of the AMV Leukemogenic Protein. Normal chicken hematopoietic tissues including embryonic yolk sac, thymus, and spleen and adult thymus and bone marrow express a 4.5-kb mRNA species that hybridizes to *amv*-specific probes (refs. 15 and 16; unpublished data). In contrast, nonhematopoietic tissues do not contain *amv*-related mRNA (ref. 16; unpublished data). Therefore, it might be expected that hematopoietic tissues would contain the normal homologue of p48^{amv}. This was tested by immunoprecipitation with our anti-peptide antisera of cellular lysates prepared from freshly explanted chicken embryonic thymus cells that had been biosynthetically labeled *in vitro* with [^{35}S]methionine. Specific precipitation of a molecule with a molecular size of 110 kDa (p110$^{proto\text{-}amv}$) but not of p48^{amv} occurred with antisera against peptides P1, P2, and P4 (Fig. 5) but not with antiserum against P3 (data not shown). As happened with p48^{amv}, the precipitation of p110$^{proto\text{-}amv}$ was inhibited by preincubation of each antiserum with its respective peptide (Fig. 5). The finding that three independent antisera, which were directed against peptides predicted from a nucleotide open reading frame, that specifically immunoprecipitated p48^{amv} also precipitated p110$^{proto\text{-}amv}$ strongly suggests that p110$^{proto\text{-}amv}$ is the normal cellular homologue of p48^{amv}

DISCUSSION

Antibodies from three rabbits individually immunized with a conjugate of bovine thyroglobulin and one of three synthetic peptides representing different regions of the protein deduced

from the *amv* nucleotide sequence specifically immunoprecipitated a common protein, p48^{amv}, from leukemic cells and another common molecule, p110$^{proto\text{-}amv}$, from normal embryonic hematopoietic cells. p48^{amv} was not precipitated from lysates of leukemic cells by antisera directed against AMV structural proteins, MAV-1 polymerase, or MAV-1 glycoproteins (8, 9). These findings represent the identification of the previously unknown AMV leukemogenic protein and of its related normal counterpart in hematopoietic tissues. Because the amino acid sequence of the three synthetic peptides was predicted from three different regions of the same open reading frame in the nucleotide sequence of the AMV oncogene, this viral gene and its single-copy normal cellular homologue must encode p48^{amv} and part of p110$^{proto\text{-}amv}$, respectively.

The nucleotide sequence of the cellular insert (*amv*) in the AMV genome reveals a single long open reading frame, but it is interrupted once by a TAG codon at position 162 from the *Kpn* I site (4). Also, there is no apparent initiation codon 5′ to position 516 in that reading frame of *amv* (4). From the nucleotide 516 initiation codon to the MAV-1 envelope termination codon, there is an open reading frame of 795 bases that could code for a protein of ≈32,000 Da. The amino acid sequence predicted from this reading frame suggests the presence of two potential glycosylation sites (Asn-X-Thr/Ser) at positions 906 and 1,164 (32). Consequently, because each glycoprotein carbohydrate chain could add 8 kDa to the estimated molecular weight, a glycoprotein of 48 kDa might be generated from this coding region. However, as we have shown, p48^{amv} is not glycosylated and, therefore, is too large to be encoded solely by this region of *amv*.

The size of p48^{amv} implies an approximate coding region of 1,200 nucleotides, which equals the total length of *amv*. Therefore, it appears that, whereas translation of the AMV transforming protein almost certainly terminates with the 11 codons of the MAV-1 envelope followed by the TAG at position 1,311, the unknown initiation site probably resides 5′ to the entire cellular insert. This initiation codon may be provided by the leader sequence from the viral long-terminal-repeat region, which contains several initiation codons (4) and is spliced to the transforming protein mRNA of AMV (refs. 33 and 34; unpublished data). This implies that the termination codon at position 162 must be either read through or bypassed by RNA splicing.

Furthermore, the p48^{amv} mRNA splicing site that joins the leader sequence derived from the viral long terminal repeat to the *amv* coding sequence should occur 5′ to the *amv Hae* II site located at position 294. Two pieces of evidence support this hypothesis: (*i*) the *Hae* II–*Xba* I fragment between positions 294 and 1,309 did not transform myeloid target cells *in vitro* when cotransfected with the entire MAV-1 proviral DNA, whereas the *Kpn* I–*Xba* I fragment, which includes *amv* in its entirety, caused transformation (unpublished data); and (*ii*) the 2.5-kb *amv*-specific mRNA in leukemic myeloblasts contains *amv* nucleotide sequences located upstream (5′) from the *Hae* II site as well as sequences from the MAV-1 long terminal repeat (unpublished data). Thus, the AMV oncogene product appears to be a fusion protein with short MAV-1 sequences at both its amino and carboxyl termini. This further supports our previous conclusion that the AMV oncogene represents an altered cellular protooncogene (3, 14).

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