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Monoclonal Antibodies to the p21 Products of the Transforming Gene of Harvey Murine Sarcoma Virus and of the Cellular *ras* Gene Family

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We have isolated eight rat lymphocyte-myeloma hybrid cell lines producing monoclonal antibodies that react with the 21,000-dalton transforming protein (p21) encoded by the v-*ras* gene of Harvey murine sarcoma virus (Ha-MuSV). These antibodies specifically immunoprecipitate both phosphorylated and non-phosphorylated forms of p21 from lysates of cells transformed by Ha-MuSV. All eight react with the products of closely related *ras* genes expressed in cells transformed by two additional sarcoma viruses (rat sarcoma virus and BALB sarcoma virus) or by a cellular Harvey-*ras* gene placed under the control of a viral promoter. Three of the antibodies also react strongly with the p21 encoded by the v-*ras* gene of Kirsten MuSV. These same three antibodies immunoprecipitate the predominant p21 species synthesized normally in a variety of rodent cell lines, including the p21 produced at high levels in 416B murine hemopoietic cells. This suggests that an endogenous gene closely related to Kirsten-*ras* is expressed in these cells. The monoclonal antibodies have been used to confirm two properties associated with p21: localization at the inner surface of the membrane of Ha-MuSV-transformed cells, assayed by immunofluorescence microscopy, and binding of guanine nucleotides.

The characterization of the transforming proteins encoded by oncogenic viruses has depended heavily on the use of specific antisera. In many cases, animals challenged with viral-induced tumors develop antibodies to proteins implicated in transformation. Thus, the T antigens of papovaviruses, such as simian virus 40 (4), the $p60^{src}$ protein of Rous sarcoma virus (6), and the *onc* proteins of several mammalian retroviruses (3, 31, 37, 40), can be precipitated by sera from appropriate tumor-bearing animals, even after removal from the sera of antibodies directed against viral structural proteins. This affords an assay for the transforming protein and for related cellular proteins (8, 9, 26, 27, 30).

The Harvey murine sarcoma virus (Ha-MuSV) (16) encodes a 21,000-dalton protein (p21) that can be precipitated by sera from rats immunized with syngeneic viral-transformed cells (37). This p21 is the product of the viral transforming gene (13, 38) now designated v-*ras* (7). The Kirsten murine sarcoma virus (Ki-MuSV) (22) also encodes a 21,000-dalton transforming protein, and this p21 is immunologically related to that of Ha-MuSV (37, 38). Although the Harvey and Kirsten v-*ras* genes (v-Ha-*ras* and v-Ki-*ras*) share some homology, they are clearly distinct and in fact derive from divergent members of a family of normal vertebrate genes (14). Sera from some rats bearing Ha-MuSV-induced tumors precipitate p21 proteins expressed at low levels in a variety of uninfected vertebrate cells (26). Cells of an unusual murine hemopoietic precursor cell line, 416B, express very high levels of p21 (34). To date, it has not been possible to identify these cellular p21 species as the products of specific members of the *ras* gene family.

The available antisera have guided the partial purification and biochemical characterization of p21. The protein, or a factor closely associated with it, binds guanine nucleotides (33, 36). The protein can be assayed by incubating it in the presence of labeled nucleotide and antiserum and by determining the amount of nucleotide retained with antigen-antibody complexes on an immunoadsorbent protein A-Sepharose (33, 36). Immunocytochemical studies of cells transformed by Ha-MuSV, using sera from tumor-bearing rats, suggest that p21 is localized to a region just below the surface of the cell membrane (39).

Because the sera from tumor-bearing rats are likely to contain antibodies to proteins in addition to p21, some care must be exercised in assigning properties to p21 itself. To test more rigorously the conclusions obtained with these sera and to facilitate future biochemical studies,

we sought to isolate monoclonal antibodies (23, 24) directed against p21. If the p21 species encoded by divergent *ras* genes carry unique antigenic determinants, monoclonal antibodies might also permit discrimination among these gene products.

We report here the isolation of eight hybrid myeloma cell lines producing monoclonal antibodies to p21 encoded by Ha-MuSV. We have used these antibodies to test the association of guanine nucleotide binding activity with p21 and the localization of the protein to the cell membrane and to attempt to discriminate among various p21 species.

MATERIALS AND METHODS

Cells and viruses. Fibroblasts and epithelial cells were grown in Dulbecco-Vogt-modified Eagle minimum essential medium (DMEM) containing penicillin (10 U/ml) and streptomycin (100 μg/ml) and supplemented with 10% fetal calf serum (Meloy Laboratories, Springfield, Va.). 416B hemopoietic cells were grown in a 1:1 mixture of DMEM and Ham F12 medium supplemented with antibiotics and 20% horse serum (Flow Laboratories, Rockville, Md.). All cells were grown in humidified incubators in an atmosphere of 10% CO_2.

Uninfected fibroblast and epithelial cell lines have been described previously: NIH 3T3 murine fibroblasts (19), Fisher rat embryo fibroblasts (35), normal rat kidney (NRK) fibroblasts (12), and canine kidney epithelial (MDCK) cells (American Type Culture Collection, Rockville, Md., line CCL34). 416B hemopoietic cells, which produce Friend murine leukemia virus but do not contain a sarcoma virus genome, were described in a recent publication (11).

The sources of sarcoma viruses which code for *ras* gene products have been detailed in prior publications and are listed with the current nomenclature for their transforming genes: (i) Harvey murine sarcoma virus (Ha-MuSV) (16), v-Ha-*ras*; (ii) Kirsten murine sarcoma virus (Ki-MuSV) (22); v-Ki-*ras*; (iii) rat sarcoma virus (Ra-SV) (29), *gag*-v-Ha-*ras* (encoding a fusion protein containing both *gag* and *ras* sequences); and (iv) BALB sarcoma virus (BALB-SV) (28), *bas*, a murine-derived Harvey-related *ras* gene (2).

A variety of transformed cells were prepared by using sarcoma virus complexes containing either Friend murine leukemia virus, Moloney murine leukemia virus, or 4070A amphotrophic virus as helper. Methods for infection and isolation of nonproducer clones have been described previously (37, 38, 41, 42). Some nonproducer transformed cells have been obtained by DNA transfection of NIH 3T3 cells with molecularly cloned forms of Harvey DNA (10, 13, 17). The SD-1 and 4NQ rat cells lines are those cocultivated by Rasheed et al. (29) to isolate Ra-SV in cell culture. The cells used for particular experiments are noted in each figure legend.

The rat myeloma cell line 210RCY3 Ag 1.2.3 (Y3) (15) was obtained from C. Milstein, Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom. The cells are deficient in hypoxanthine-guanine phosphoribosyl transferase activity and so fail to grow in medium containing aminopterin or methotrexate. The myeloma cells were grown in suspension in roller bottles in DMEM supplemented with 10% fetal calf serum. The use of these cells for fusion with immune lymphocytes is described below. Lymphocyte-myeloma hybrids were grown initially in a 1:1 mixture of DMEM and Ham F12 medium (DMEM:F12) supplemented with antibiotics, 1 mM sodium pyruvate, HAT (3.9×10^{-7} M methotrexate, 8.1×10^{-5} M hypoxanthine and 1.6×10^{-5} M thymidine) or HT (8.1×10^{-5} M hypoxanthine and 1.6×10^{-5} M thymidine), and 16.7% fetal calf serum. After the cells were cloned and stable lines were established, the cells were grown in DMEM:F12 supplemented with antibiotics and 10% fetal calf serum. It was also possible to grow the hybrids in a serum-free medium under conditions similar to those used for murine B cells (18). The growth medium was either DMEM:F12 or the modification of the DMEM used by Iscove and Melchers (18) supplemented with antibiotics and 400 μg of bovine serum albumin per ml, 5 μg of insulin per ml, 5 μg of transferrin per ml, and 5 ng of sodium selenite per ml (Collaborative Research, Inc., Waltham, Mass.). We are grateful to G. Sato and J. Mather for introduction to techniques for growth of cells in serum-free culture at the 1981 Cold Spring Harbor Cell Culture Course.

Metabolic labeling of cells and immunoprecipitation. Cells were labeled in 60-mm Falcon petri dishes with [^{35}S]methionine or $^{32}P_i$ by minor variations of procedures previously published (37). All labeling was carried out at 37°C for 18 to 24 h, and cell lysates were prepared as follows. For [^{35}S]methionine-labeled cells, the radioactive medium was removed, and the cells were washed once in phosphate-buffered saline (PBS). The cells were scraped from the plate into 1 to 2 ml of a buffer (at 4°C) containing 0.02 M Tris-hydrochloride (pH 7.4), 0.005 M $MgCl_2$, 0.10 M NaCl, 1% (vol/vol) Triton X-100, 0.5% recrystallized sodium deoxycholate, and 3 mM phenylmethylsulfonyl fluoride. For phosphate-labeled cells, the same buffer was used with the addition of 0.02 M sodium phosphate (pH 7.2) and 0.02 M sodium fluoride. The cells were subjected to agitation by a Vortex mixer to facilitate lysis, and the extracts were centrifuged at 100,000 × *g* for 30 min at 4°C. The supernatant (S100) was aspirated and either used immediately or stored at −170°C.

Each immunoprecipitation reaction contained 5×10^6 trichloroacetic acid-precipitable cpm (^{35}S or ^{32}P). Most of the ^{32}P in the phosphate-labeled lysates was incorporated into nucleic acids rather than protein, but this method was used to standardize approximately the input for each experiment.

Immunoprecipitation was carried out in the lysis buffer used to prepare each extract. Each reaction mixture contained in 0.30 ml extract as described above and antibody as indicated in the figure legends. The reaction was incubated for 2.0 h at 4°C, and 0.05 ml of a 10% (vol/vol) suspension of Formalin-fixed *Staphylococcus aureus* (20) was added, and the reaction incubated for an additional 2.0 h at 4°C to render insoluble the antigen-antibody complexes. Because many rat immunoglobulin molecules do not bind well to *S. aureus* protein A, when monoclonal antibodies were used the fixed *S. aureus* cells were first coated with rabbit antibodies against rat immunoglobulin G (IgG) (Cappel Laboratories, Cochranville, Pa.). Per 0.1 ml of packed cells, 0.25 ml of anti-rat IgG serum

diluted to 1.0 ml with PBS was added and incubated for at least 2.0 h at 4°C. The coated *S. aureus* cells were then washed three times by centrifugation in PBS and resuspended at a 10% suspension in PBS. Immunoprecipitates were collected by centrifugation and washed four to five times in a buffer containing 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.5), 0.10 M NaCl; 1.0% (vol/vol) Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, and 0.001 M EDTA. Immunoprecipitates were dissolved and subjected to electrophoresis on 10.5% polyacrylamide gels as previously described (25, 37). Protein bands were identified by fluorography for the ^{35}S label (5) or autoradiography with intensifier screens for the ^{32}P label.

GDP binding assay. The GDP binding assay for p21 was carried out with minor modifications of procedures detailed earlier (33, 36). Reaction mixtures were incubated at 4°C and contained in 0.30 ml 0.02 M Tris-hydrochloride (pH 7.5), 0.003 M magnesium chloride, 0.02% recrystallized sodium deoxycholate, 0.1% Triton X-100, 2×10^{-6} M [^{3}H]GDP (specific activity, approximately 10 Ci/mmol), partially purified p21 from membranes of cells transformed by the v-Ha-*ras* gene as indicated in Fig. 1, antisera or monoclonal antibodies as indicated in Fig. 1, and 0.065 ml of a 1:14 (wt/vol) suspension of protein A-Sepharose (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). All components were added within a few minutes of each other, and the reaction mixtures were then shaken vigorously for 90 min. The last addition was always the protein A-Sepharose, but we have found it unnecessary to carry out a long preincubation of antigen and antibody before the addition of the immunoadsorbent. As described above for the immunoprecipitation assays, the protein A-Sepharose was coated with rabbit antibodies against rat IgG (Cappel Laboratories) before use. After incubation, the immune complexes were washed with reaction mixture buffer lacking GDP twice by centrifugation. The complexes were then collected on 0.45-μm Millipore filters and washed three times with 5.0 ml of the same buffer. All washes were carried out at 4°C. Filters were dissolved in Aquasol, and the amount of [^{3}H]GDP retained was determined by liquid scintillation counting. In most experiments, 1 pmol of [^{3}H]GDP represents 8,200 cpm.

The partially purified p21 used in these experiments was obtained by modifications of techniques described by Shih et al. (37). The experiments of Fig. 1 and Table 1 used p21 isolated from membranes of NIH 3T3 cells carrying multiple copies of v-Ha-*ras* introduced by transfection of molecularly cloned DNA fragments (17). Membranes were solubilized with 1% Nonidet P-40, and a 0 to 40% ammonium sulfate precipitate fraction containing p21 activity was obtained by procedures that will be described in detail elsewhere.

Immunofluorescence. For screening of supernatants of lymphocyte-myeloma hybrid cells for production of antibodies to p21, we used Ha-MuSV-transformed MDCK cells (35) as indicators (35, 39). Cells were grown overnight on 8- or 15-well multitest slides (Flow Laboratories, Rockville, Md.), washed with PBS, fixed for 4 min with absolute methanol at −10°C, and air dried. In some cases, cells were first fixed with 3.7% formaldehyde in PBS for 10 min at room temperature, then with methanol as described above, and finally rinsed with acetone at −10°C. The cells were then incubated with antibody for 30 to 60 min at 37°C, washed with PBS, and incubated for a further 30 to 60 min at 37°C with a fluorescent labeled antibody to rat IgG. For routine screening, we used fluorescein isothiocyanate-labeled anti-rat IgG prepared in rabbits (Cappel Laboratories), absorbed on fixed cells as described by Willingham et al. (39). For photography, we used rhodamine-labeled anti-rat IgG prepared in goats (Cappel Laboratories), affinity purified on a column of immobilized rat IgG. The latter reagent was a generous gift of J. Wheland. Cells were washed with PBS, mounted under cover slips with Elvanol, and observed with a Zeiss microscope equipped with epifluorescence optics, with appropriate filter combinations for fluorescein or rhodamine.

Preparation of monoclonal antibodies. Previously, we have shown that the p21 transforming protein encoded by the v-*ras* gene of Ha-MuSV is antigenic in Osborne-Mendel rats carrying tumors of syngeneic nonproducer NRK cells transformed by Ha-MuSV (37). To obtain monoclonal antibodies to p21, we injected rats subcutaneously with Ha-MuSV-transformed NRK cells at 10 days of age (1×10^6 cells per rat) and screened sera of individual animals approximately 4 weeks later to find those rats making the strongest immune response against p21, as determined by activity in the GDP binding assay. Such rats were boosted by injection of approximately 350 μg of a crude preparation containing sufficient p21 to bind approximately 55 pmol of [^{3}H]GDP. We estimate that this corresponds to at least 1 μg of p21. The p21 derived from a 1.0% Triton X-100 extraction of concentrated membranes and virus released into the supernatant from Fisher rat embryo cells producing Ha-MuSV and Moloney murine leukemia virus helper. We found such preparations to be a convenient source of p21 which was approximately 10-fold more purified than whole lysates of virus-infected cells. The lysate was heated at 90°C for 5 min and injected intraperitoneally in incomplete Freund adjuvant 4 days before fusion. We have no direct evidence that this boost contributed to the successful recovery of hybrids producing antibodies to p21.

Cell fusion and selection of lymphocyte-myeloma hybrids were carried out by minor modifications of techniques described by Kohler and Milstein (24), as adapted for the rat myeloma system by Galfre et al. (15). Spleens were removed from immunized animals, and cell suspensions containing splenic lymphocytes were prepared by disruption on a wire screen. The splenocytes were fused with hypoxanthine-guanine phosphoribosyl transferase-deficient Y3 rat myeloma cells at a ratio of 5:1 by incubation with 50% polyethylene glycol (PEG 1450, Eastman Organic Chemicals, Rochester, N.Y.) for 2 min at 37°C, diluted, and plated in 1-ml samples in 24-well culture trays (Linbro Scientific Inc., Hamden, Conn.) on mitomycin-treated NIH 3T3 feeder cells. Approximately 1×10^8 splenocytes and 2×10^7 myeloma cells were used for each fusion and were divided into 200 to 300 cultures. After 1 day the cell population was subjected to selection in HAT medium (3.9×10^{-7} M methotrexate, 8.1×10^{-5} M hypoxanthine, and 1.6×10^{-5} M thymidine). The medium was changed at intervals of 2 to 3 days. Hybrid cells grew to confluence in almost all wells after 7 to 14 days of incubation. At this point, methotrexate was eliminated from the growth medium, and

cells were fed with medium containing HT (8.1×10^{-5} M hypoxanthine and 1.6×10^{-5} M thymidine). Supernatants were harvested and assayed for antibodies to p21. The initial screening was carried out by using the GDP binding assay or immunofluorescence on Ha-MuSV-transformed MDCK cells, or both. Supernatants scoring positively in these assays were then tested for the ability to immunoprecipitate p21, either from crude lysates of transformed cells labeled with [^{35}S]methionine or from partially purified preparations labeled in the in vitro autophosphorylation reaction by using [γ-^{32}P]GTP as donor, as described by Shih et al. (36). In each of two fusion experiments, six to eight cultures contained antibodies to p21, as determined by at least two independent assays. Cells from the positive cultures were cloned in soft agar (0.25% over a base layer of 0.5%) containing complete growth medium. After 7 to 10 days of incubation, individual colonies were transferred to 1-ml cultures with mitomycin-treated NIH 3T3 feeders and grown to confluence, and supernatants were tested for antibodies to p21 by immunofluorescence. Positive cultures were cloned a second time in soft agar and established as stable cell lines. Antibodies were obtained from the spent medium of cells grown in suspension culture in roller bottles. Supernatants from overgrown cultures, either from 10% fetal calf serum-supplemented medium or from serum-free medium, were harvested and subjected to precipitation with 50% saturated ammonium sulfate. The precipitates were resuspended and dialyzed against PBS. Preparations from serum-free medium generally contained at least 50% immunoglobulin by weight at this stage, as determined by gel electrophoresis. We estimate that most lines produce 10 to 50 μg of immunoglobulin per ml at saturation under our standard growth conditions. Each of the anti-P21 monoclonal antibodies characterized was of the IgG class; we have not determined subclass specificities.

RESULTS

GDP binding by p21 assayed with monoclonal antibodies. From two cell fusion experiments using lymphocytes from individual rats, we isolated eight hybrid myeloma lines producing monoclonal antibodies to the p21 transforming protein encoded by Ha-MuSV. Several other antibodies were identified that do not react with p21, but bind to other components of both normal and transformed cells, including intermediate filaments, stress fibers, and nuclei. These will be described in a separate communication.

Antisera from tumor-bearing rats (TBR sera) have been used to associate a guanine nucleotide-binding activity with p21 (33, 36, 38) and to localize the protein to the inner surface of the membrane of Ha-MuSV-transformed cells (39). However, there remained a formal possibility that these properties ascribed to p21 actually are associated with other proteins bound by antibodies in TBR sera. The isolation of monoclonal antibodies not directed to p21 reinforces the general concern that sera from animals bearing virus-induced tumors contain antibodies to a variety of antigens in addition to the product of the specific viral oncogene. The monoclonal antibodies to p21 allow a stringent test for the correlation of antigenic determinants on this protein with the associated guanine nucleotide-binding activity and with the antigen detected by immunocytochemistry on the inner surface of the cell membrane.

When antisera from rats bearing Ha-MuSV-induced tumors are incubated with labeled guanine nucleotide, partially purified p21, and an immunoadsorbent (protein A-Sepharose), the label is retained in a complex that can be assayed by collecting the Sepharose beads on a filter (32, 33). A monoclonal antibody to Ha-MuSV-encoded p21 permits the retention of as much bound [^{3}H]GDP in this assay as does TBR serum (Fig. 1). The amount of bound nucleotide depends strictly on the amount of added extract containing p21. Extract from uninfected NIH 3T3 cells fails to replace the extract from transformed cells (data not shown). These data confirm that p21 or a component directly associated with p21 in an immunoprecipitable complex

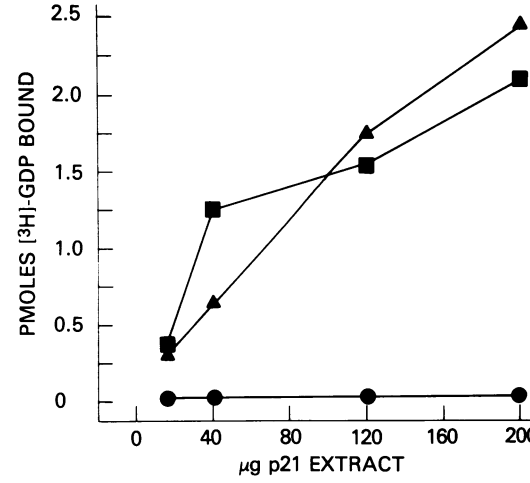

FIG. 1. Use of monoclonal antibody in guanine nucleotide binding assay for p21. The GDP binding assay was carried out as described in Materials and Methods. Each reaction contained 5 μl of serum or monoclonal antibody. The monoclonal antibody Y13-238 was used as a 0 to 50% (wt/vol) $(NH_4)_2SO_4$ precipitate fraction prepared from spent culture medium and dialyzed against PBS to give a 10-fold concentrate. An identical titration curve was obtained when this antibody preparation was used at 0.5 μl per reaction (approximately 0.05 μg of IgG). Symbols: ●, serum from normal rats; ■, immune serum from Osborne-Mendel rats bearing tumors of Ha-MuSV-transformed NRK cells (TBR serum); and ▲, Y13-238 monoclonal antibody. Several control monoclonal antibodies, such as one to the *thy*-1 antigen, tested in amounts equivalent or greater than that used for Y13-238, gave results identical to normal rat serum (data not shown).

binds guanine nucleotides. All eight monoclonal antibodies to p21, including four that were identified initially by other assays, permit specific retention of bound guanine nucleotide (Table 1). The antibody Y13-259 consistently gives lower levels of GDP binding than do the other monoclonal antibodies; the explanation for this observation will be discussed in a separate communication.

Localization of p21 by immunofluorescence. Ha-MuSV-transformed cells fixed with methanol or acetone show specific surface-related staining when treated with TBR serum and fluorescent anti-rat IgG antibody (39). Transformed MDCK cells display characteristic rings of fluorescent staining, particularly at the borders between cells (Fig. 2A). Electron microscopic immunocytochemistry showed that most of the antigen detected by TBR sera was localized to the inner surface of the cell membrane and that the level was especially high in short microvilli observed on surfaces and interdigitating in the regions between cells in monolayers, a characteristic feature of these cells derived from renal epithelium. When culture supernatants of hybrid cells obtained by fusion of rat myeloma cells to lymphocytes from rats immunized against p21 were screened by immunofluorescence on Ha-MuSV-transformed MDCK cells, several supernatants gave staining very similar or identical to that observed with TBR antisera. All but one of these supernatants contained antibodies capable of precipitating p21 from labeled cell extracts. Conversely, those supernatants which gave the highest level of p21-dependent GDP binding also gave the characteristic fluorescent staining pattern on Ha-MuSV-transformed MDCK cells. This pattern is observed with each of the eight monoclonal anti-p21 antibodies. The intense surface-related fluorescence is observed with Ha-MuSV-transformed MDCK cells, but not with the uninfected MDCK cells (compare Fig. 2A and C). Normal rat sera and irrelevant monoclonal antibodies (e.g., an antibody directed to a hemopoietic cell surface antigen) fail to stain these cells (Fig. 2B and D). Electron microscopic immunocytochemistry on Ha-MuSV-transformed NIH 3T3 fibroblasts using the monoclonal antibodies to p21 confirms the localization of this protein to the inner surface of the cell membrane, as previously inferred from studies with TBR sera (M. C. Willingham and M. E. Furth, unpublished data).

TABLE 1. p21-Dependent guanine nucleotide binding with monoclonal antibodies[a]

Antibody	[^{3}H]GDP bound (pmol)
Normal rat serum (5 μl)	<0.05
YA6-40	2.0
YA6-92	1.8
YA6-165	1.8
YA6-172	2.2
Y13-4	1.3
Y13-128	2.0
Y13-238	2.1
Y13-259	0.35

[a] [^{3}H]GDP binding with monoclonal antibodies was carried out as described in Materials and Methods. Each reaction mixture contained 150 μg of total protein containing partially purified p21 from membranes of cells transformed by the v-Ha-*ras* gene. Titrations were performed with ammonium sulfate concentrates of spent culture medium from cells producing each monoclonal antibody (0.1 to 50 μl). The indicated values represent the maximal binding at saturation levels of monoclonal antibody.

Reaction of genetically distinct forms of p21 with monoclonal antibodies. The monoclonal antibodies obtained in this study all derive from lymphocytes of rats immunized against cells transformed by Ha-MuSV. Do these antibodies also recognize the p21 encoded by Ki-MuSV? Figure 3 shows the result of immunoprecipitation of [^{35}S]methionine-labeled proteins from lysates of NRK rat fibroblasts and of NRK cells transformed by Ha-MuSV and by Ki-MuSV, using four anti-p21 monoclonal antibodies and a control antibody directed against an unrelated cellular protein. Each of the anti-p21 antibodies precipitates a characteristic doublet band from Ha-MuSV-transformed cells (Fig. 3, lanes 1 to 4). In contrast, only two of these four monoclonal antibodies precipitate the Ki-MuSV-encoded p21, which appears as a single band (Fig. 3, lanes 6 and 8). The same two antibodies precipitate a small amount of a 21,000-dalton protein from uninfected NRK cells (barely visible in Fig. 3, lanes 11 and 13).

Both the Ha-MuSV and the Ki-MuSV-encoded p21 species can be metabolically labeled with ^{32}P. Monoclonal antibodies efficiently bind the phosphorylated forms of these proteins (Fig. 4). As previously observed with TBR sera, only the more slowly migrating form of Ha-MuSV-encoded p21 is phosphorylated (Fig. 4A). Again, of four representative antibodies two cross-react strongly with the Ki-MuSV-encoded p21 (Fig. 4B). No phosphorylated p21 is detected in the parent fibroblast cell line, in this case NIH 3T3 (Fig. 4C). As expected from previous studies (37), the viral-encoded p21 species detected with monoclonal antibodies are phosphorylated on a threonine residue (data not shown).

The v-Ha-*ras* and v-Ki-*ras* genes, carried by Ha-MuSV and Ki-MuSV, respectively, are two representatives of a conserved cellular gene family. Related genes are carried by two addi-

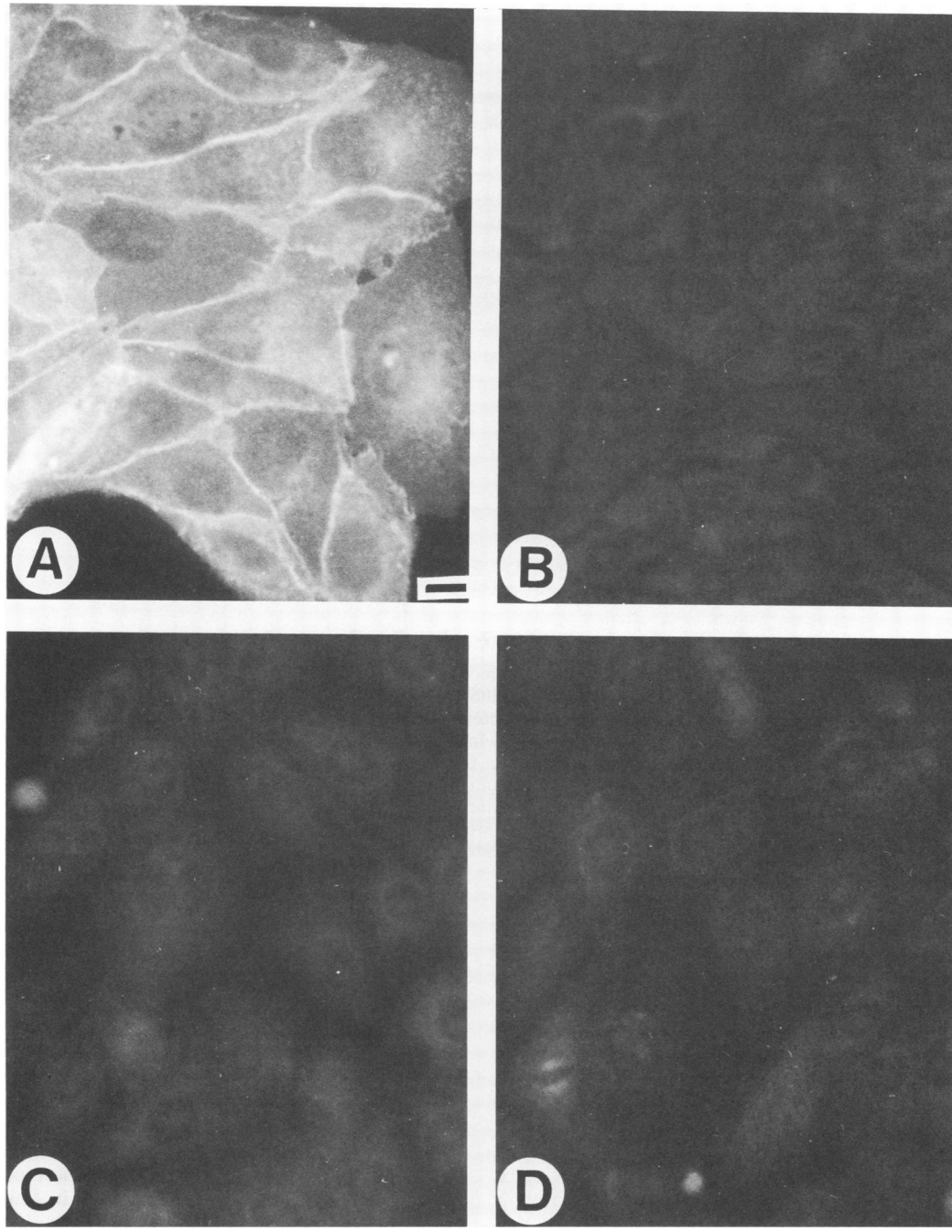

FIG. 2. Immunofluorescence with monoclonal antibody to p21. MDCK cells and Ha-MuSV-transformed MDCK cells (viral nonproducers) were grown on multitest slides and fixed by sequential treatment with formaldehyde, methanol, and acetone as described in Materials and Methods. Cells were incubated either with monoclonal antibody YA6-172, specific for Harvey-type p21, or with a control rat monoclonal antibody YBM-42.2 to a murine hemopoietic cell surface antigen. The YA6-172 antibody was purified by ammonium sulfate fractionation from serum-free medium, whereas the control antibody was purified from ascitic fluid by chromatography on DEAE Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.) to better than 90% of homogeneity. Each antibody was used at a final concentration of 25 μg/ml in PBS with 0.4% (wt/vol) bovine serum albumin as carrier. The bound monoclonal antibody was detected by incubation with rhodamine-labeled, affinity-purified goat antibodies to rat IgG. The control YBM-42.2 antibody was shown to give very bright surface staining of mouse thymocytes when used at the same concentration (data not shown). Cells were observed by using a Zeiss 63× Planapochromat objective, numerical aperture 1.4, and were photographed under UV light (epifluorescence). Photographs were matched for magnification and for times of exposure and development. (A) Ha-MuSV MDCK cells stained with anti-p21 monoclonal YA6-172; (B) Ha-MuSV MDCK cells stained with control monoclonal YBM-42.2; (C) MDCK cells stained with anti-p21 monoclonal YA6-172; (D) MDCK cells stained with control monoclonal YBM-42.2. The bar in (A) = 10 μm.

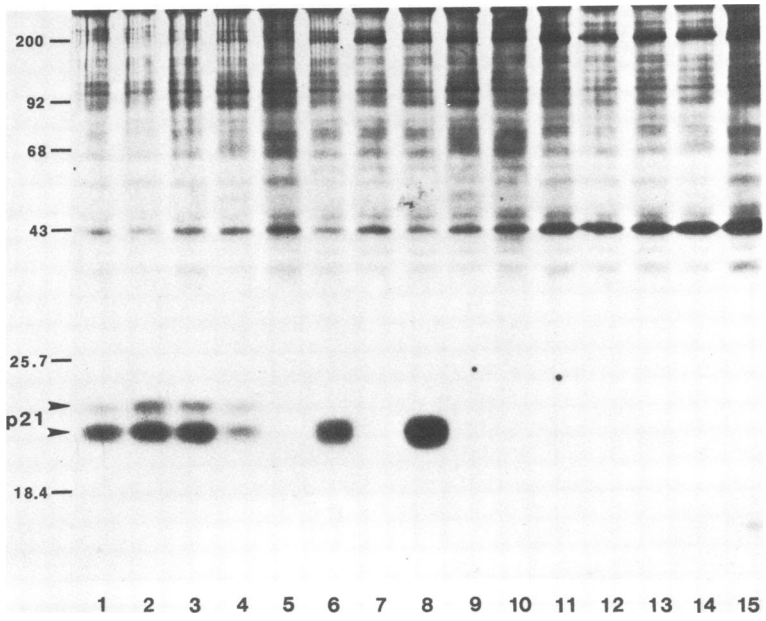

FIG. 3. Immunoprecipitation of p21 from transformed and uninfected NRK cells. NRK rat fibroblasts and clonal derivatives transformed by Ha-MuSV and Ki-MuSV (viral nonproducers) were metabolically labeled with [^{35}S]methionine, and lysates were prepared and subjected to immunoprecipitation as described in Materials and Methods. Each antibody was added as 5 μl of a 10-fold concentrate of spent culture medium prepared by ammonium sulfate precipitation. Lanes 1 to 5, extract from Ha-MuSV NRK immunoprecipitated with Y13-4, Y13-238, Y13-259, YA6-172, and control monoclonal antibody YA6-83, respectively. Lanes 6 to 10, extract from Ki-MuSV NRK immunoprecipitated with monoclonal antibodies in the same order as for lanes 1 to 5. Lanes 11 to 15, extract from uninfected NRK immunoprecipitated with monoclonal antibodies in the same order as for lanes 1 to 5. Arrowheads indicate p21 species. Bars indicate the relative mobilities of marker proteins of known molecular weight ($\times 10^{-3}$).

tional retroviruses: a murine sarcoma virus, BALB-MuSV (1, 2, 28), and rat sarcoma virus, Ra-SV (29, 41, 42). Each of these viruses carries a *ras* gene that is closely related to v-Ha-*ras*. All eight of the monoclonal antibodies to the Ha-MuSV-encoded p21 (Fig. 5A) react efficiently with the p21 encoded by BALB-MuSV (Fig. 5B). Similarly, all eight immunoprecipitate the 29,000-dalton *gag-ras* fusion protein encoded by Ra-SV (data not shown). In contrast, much less p21 is detected in uninfected NIH 3T3 cells, although three antibodies clearly precipitate low levels of a cellular protein of about 21,000 daltons (Fig. 5C).

The normal rat genome contains two genes that are very closely related to v-Ha-*ras* (c-Ha-*ras1* and c-Ha-*ras2*) (10). The c-Ha-*ras1* gene can transform NIH 3T3 fibroblast when ligated to the terminal repeat region of a murine leukemia virus. Cells transformed in this manner express at high levels a p21 that can be immunoprecipitated by TBR sera (10). All eight monoclonal antibodies to p21 react with the c-Ha-*ras1*-encoded p21, although one antibody (YA6-165) does so relatively inefficiently (Fig. 5D). All eight antibodies also recognize the p21 encoded by c-Ha-*ras2* (N. Rosenthal, T. Shih, and M. Furth, unpublished data).

Another cellular p21 species is expressed at high levels in an unusual murine hemopoietic cell line, 416B (34). Three of the eight monoclonal antibodies permitted efficient immunoprecipitation of p21 from these cells (Fig. 5E). A fourth antibody (YA6-165) did so less efficiently. In each case, the reaction of the antibody with the p21 of 416B cells correlated with the reaction with p21 encoded by Ki-MuSV. It therefore seems likely that the protein expressed in 416B cells was encoded by an endogenous Ki-MuSV-related gene (c-Ki-ras^{mus}). Studies of the messenger RNA encoding p21 in 416B cells directly support this hypothesis. The cells contain substantial amounts of mRNA homologous to a v-Ki-*ras* cloned DNA probe, and much less RNA homologous to a v-Ha-*ras* probe (R. W. Ellis, D. Defeo, M. E. Furth, and E. M. Scolnick, manuscript in preparation).

A large number of cell lines from many species contain small amounts of a protein of about 21,000 daltons that can be immunoprecipitated by TBR sera (26). Can this protein be identified as the product of any of the known c-*ras* genes?

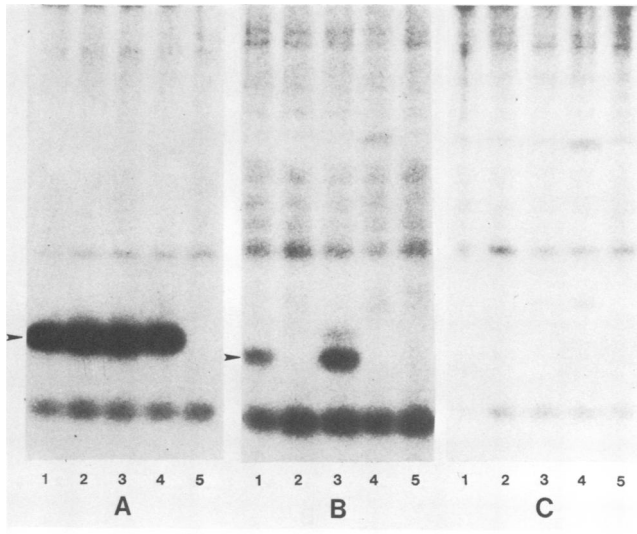

FIG. 4. Monoclonal antibodies precipitate phosphorylated form of p21. NIH 3T3 cells and transformed derivatives were grown to 75% of confluence in 100-mm dishes and were labeled with 800 μCi of $^{32}P_i$ for 16 h. Lysates were prepared and immunoprecipitation was carried out as described in Materials and Methods. Monoclonal antibodies were added as 50 μl of culture medium from cells grown in saturation. Lanes 1 to 4, the monoclonal antibodies to Ha-MuSV-encoded p21 were Y13-128, Y13-238, Y13-259, and YA6-172, respectively. Lane 5, 5 μl of normal rat serum was diluted with 45 μl of tissue culture medium. Lysates were prepared from (A) NIH 3T3 cells transformed by v-Ha-*ras* DNA (17), (B) NIH 3T3 cells transformed by Ki-MuSV (viral nonproducer), and (C) uninfected NIH 3T3 cells. Arrowheads indicate the phosphorylated p21 species.

Three of the eight monoclonal antibodies clearly recognize a p21 species in several uninfected rodent, carnivore, and primate cell lines (Fig. 3, Fig. 5C, Fig. 6, and unpublished data), although the protein is present in much lower amounts than in transformed cells (Fig. 6, compare lanes 1, 3, 6, 8, 11, 13, and 18 with lanes 16 and 17). The antibodies that precipitate this endogenous p21 species (Y13-4, Y13-128, and Y13-259) are the same antibodies that efficiently immunoprecipitate the p21 encoded by Ki-MuSV and the p21 of 416B cells. The monoclonal antibodies that efficiently bind only the Ha-*ras* p21 species (e.g., Y13-238 and YA6-172, Fig. 6, lanes 2, 4, 7, 9, 12, and 14) immunoprecipitate much less p21 from uninfected rat cells. These data suggest strongly that the major endogenous p21 of rodents is encoded by a c-Ki-*ras* gene. However, it remains possible that this p21 is encoded by an as yet unidentified member of the *ras* gene family and shares common determinants with p21 encoded by Ha-*ras* or Ki-*ras*.

It is significant that of the rat cell lines neither 4NQ nor SD-1 produces more Ha-*ras*-type p21 than do other rodent lines (Fig. 6, lanes 2, 4, 7, and 9), and both express the usual Kirsten-related p21 (Fig. 6, lanes 1, 3, 6, and 8). These two cell lines, after cocultivation, gave rise to the rat sarcoma virus which expresses a Ha-*ras*-related transforming protein (29, 41, 42). The formation of Ra-SV, therefore, cannot be readily explained by an altered expression of a Ha-*ras* gene either in the chemically transformed 4NQ cells or in the cells producing a rat leukemia virus.

DISCUSSION

The specificity of binding of monoclonal antibodies to unique antigenic determinants (23) makes them ideal reagents for the detailed analysis of protein structure and function. The isolation of monoclonal antibodies to the 21,000-dalton transforming protein (p21) encoded by the Ha-MuSV allows a critical test of the association of guanine nucleotide binding with this protein (32, 36) and of its localization to the inner surface of the cell membrane (39). Our studies with eight independent monoclonal antibodies to p21 confirm that these properties are characteristic of the protein and do not reside in other antigens detected by contaminating specificities in sera from tumor-bearing rats. Each of the monoclonal antibodies immunoprecipitates p21, mediates the binding to an immunoadsorbent of a complex containing p21 and a guanine nucleotide, and gives a characteristic surface-related pattern in immunofluorescent staining of cells transformed by Ha-MuSV. We now hope to use these antibodies to probe the structural determinants responsible for guanine nucleotide binding and membrane association and for the

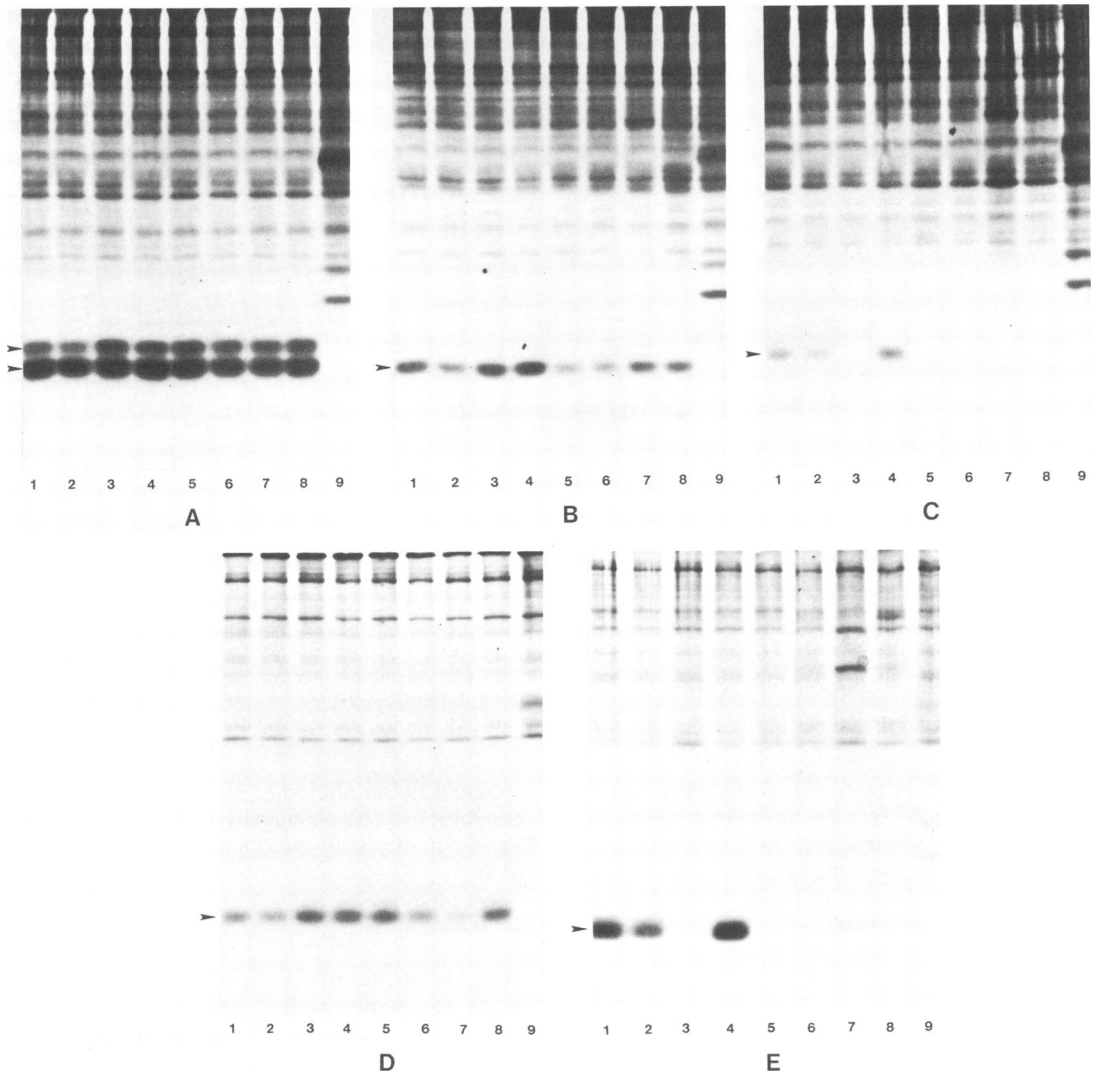

FIG. 5. Immunoprecipitation of viral and endogenous p21 species. Immunoprecipitation and analysis of [^{35}S]methionine-labeled proteins was carried out as described in Materials and Methods. Lanes 1 to 8, monoclonal antibodies to HaSV-encoded p21, added as 5 μl of ammonium sulfate concentrate of spent culture medium, were Y13-4, Y13-28, Y13-238, Y13-259, YA6-40, YA6-92, YA6-165, and YA6-172, respectively. Lane 9, rat monoclonal antibody to α-tubulin, YL1-2 (a generous gift of J. Kilmartin) was added. Lysates were prepared from (A) NIH 3T3 cells transformed by v-Ha-*ras* gene (13), (B) NIH 3T3 cells transformed by BALB-SV, (C) uninfected NIH 3T3 cells, (D) NIH 3T3 cells transformed by rat c-Ha-*ras1* ligated to the long terminal repeat region of Ha-MuSV (10), and (E) 416B murine hemopoietic cells. NIH 3T3 cells transformed by Rous sarcoma virus gave results indistinguishable from those of panel C (NIH 3T3) (data not shown). Film exposures: (A, D, and E) 1 day, (B and C) 7 days. Arrowheads indicate the p21 species.

GTP-specific autophosphorylation activity of the Ha-MuSV p21 (36).

Monoclonal antibodies also facilitate the resolution of related but distinct antigens. The antibodies to the p21 of Ha-MuSV allow us to discriminate among the protein products of the *ras* gene family (14). Direct analysis of the genomes of several sarcoma viruses and of normal cells revealed two classes of *ras* genes: those very closely related to v-Ha-*ras* and those more closely related to v-Ki-*ras*, the oncogenes of Ha-MuSV and Ki-MuSV, respectively (10, 14). Although sera from many rats bearing Ha-MuSV-induced tumors will precipitate a p21 species from Ki-MuSV-transformed cells, the Ha-MuSV- and Ki-MuSV-encoded proteins differ significantly in their tryptic peptide maps (14). The monoclonal antibodies to the p21 en-

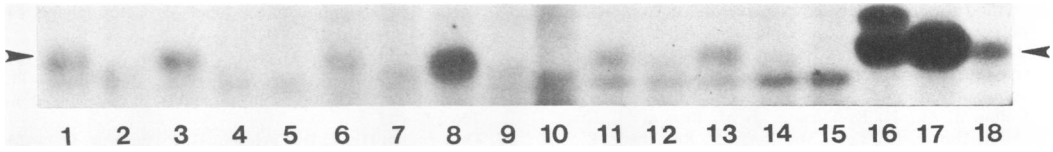

FIG. 6. Immunoprecipitation of endogenous p21 from rat cell lines. Immunoprecipitation of [^{35}S]methionine-labeled cell lysates was carried out as described in Materials and Methods, and antibodies were added as 5 μl of ammonium sulfate concentrate from spent culture medium. For clarity, the photograph shows only the region of the autoradiogram corresponding to proteins of mobility close to the region of p21. Antibodies Y13-4 and Y13-259 precipitate the p21 encoded by Ki-MuSV, whereas Y13-238 and YA6-172 do not (Fig. 3). Lanes 1 to 5, extract from SD-1 cells immunoprecipitated with Y13-4, Y13-238, Y13-259, YA6-172, and control monoclonal YA6-83, respectively. Lanes 6 to 10, extract from 4NQ cells immunoprecipitated with monoclonal antibodies in same order as for lanes 1 to 5. Lanes 11 to 15, extract from Fisher rat embryo cells immunoprecipitated with monoclonal antibodies in same order as for lanes 1 to 5. Lanes 16 to 18, immunoprecipitation with monoclonal antibody Y13-259 of lysates from Ha-MuSV NRK cells, Ki-MuSV NRK cells, and uninfected NRK cells, respectively. Arrowheads indicate the position of the major endogenous p21 species detected in these cells.

coded by the v-Ha-*ras* gene were tested for their ability to immunoprecipitate genetically distinct forms of p21. Each of these antibodies reacts with all of the products of genes closely related to v-Ha-*ras*: the p21 encoded by the *bas* gene of BALB-SV (2), the p29 encoded by Ra-SV (41, 42), and the p21 encoded by the cellular gene c-Ha-*ras*1 (10). However, only three of the eight monoclonal antibodies efficiently immunoprecipitate the p21 encoded by Ki-MuSV, whereas one does so less well.

The specificity of the monoclonal antibodies should allow us to determine whether a given p21 species is encoded by a Harvey- or a Kirsten-related *ras* gene. Cells of the murine line 416B, described initially by Dexter et al. (11) as primitive hemopoietic precursors, express high levels of an endogenous p21 (34). The observation that the p21 of 416B cells can be immunoprecipitated by the same monoclonal antibodies that react with the Ki-MuSV-encoded p21, but not by the monoclonal antibodies that react well only with Harvey-related p21 species, implies strongly that the 416B protein is the product of a c-Ki-*ras* gene. This inference is supported by direct analysis of polysomal messenger RNA in 416B cells (Ellis et al., manuscript in preparation). Immunoprecipitation studies suggest that most of the p21 expressed in a variety of rodent cells is encoded by a c-Ki-*ras* gene.

We do not yet know the chemical nature of the antigenic determinants that are recognized by the various monoclonal antibodies, or whether each antibody recognizes primary amino acid sequence or a conformational determinant. Thus, we do not know what differences in protein structure among diverse p21 species account for selective reactivity with particular antibodies. In principle, the monoclonal antibodies should permit the purification of each of the genetically distinct p21 species. This should, in turn, help us to assess what features of p21 structure are critical for its function as a guanine nucleotide-binding protein, a membrane-associated protein, a GTP-utilizing autokinase, and a cellular transformation-mediating protein. The antibodies should also help to define the biological role of the endogenous p21 species expressed in uninfected cells. We anticipate that microinjection into cells transformed by Ha-MuSV of antibodies specific to Harvey-related p21 might inactivate the viral protein but not the majority of the cell-encoded p21, whereas injection of antibodies with broader specificity would inactivate both viral and cell-encoded p21. Thus, the antibodies might provide important clues about the significance of the diversity of p21 species in normal cells and about the mechanism by which qualitative and quantitative changes in p21 expression can lead to malignant transformation.

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