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Immunology

Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse

(tumor antigen/immunoprecipitation/p53)

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ABSTRACT **Antisera prepared against BALB/c Meth A sarcoma in syngeneic or compatible F_1 mice recognize a protein with an apparent molecular weight of 53,000 in extracts of [^{35}S]methionine-labeled transformed BALB/c cells. This component, designated p53, was not detected in normal adult mouse fibroblasts, lymphoid cells, or hematopoietic cells or in mouse embryo cells or 3T3 cells. An extensive variety of antisera, including alloantisera and heterologous antisera directed against structural antigens of murine leukemia viruses, was tested for reactivity with p53; other than Meth A antisera, only comparably prepared antisera against another BALB/c sarcoma, CMS4, had anti-p53 activity. All transformed mouse cells tested were found to express p53; these tests included chemically induced sarcomas, leukemias, spontaneously transformed fibroblasts, and cells transformed by simian virus 40 and murine sarcoma virus. The presence of p53 in tumors of no known viral etiology indicates coding by resident cellular genes; this does not exclude endogenous viruses as the source of coding sequences or the possibility that transforming viruses code directly for p53.**

Mouse sarcomas induced by polycyclic hydrocarbons, such as methylcholanthrene, have been a favorite object of study by tumor immunologists. The first and still most compelling evidence for the existence of tumor-specific antigens comes from the demonstration that these tumors are immunogenic in their inbred strain of origin (1–5). Mice can be immunized in several ways: e.g., prior growth and removal of tumor transplants or injection of irradiated tumor cells, tumor cell fractions, or solubilized antigens. After immunization, mice become resistant to subsequent transplants of the same tumor. A remarkable and characteristic feature of these transplantation antigens of chemically induced sarcomas is their diversity; each tumor appears to express a distinctive antigen or set of antigens not shared with any other tumor. Thus, immunization with a particular tumor generally elicits transplantation immunity to that tumor but not to other similarly induced tumors, and this is true even if the different tumors are independently induced in the same mouse. Although these antigens of chemically induced tumors have been recognized for over 30 years, little is known of their biochemical nature or genetic origin, and this slow progress can be ascribed to difficulties in using an immunological assay that depends on resistance to transplants as the end point. Serological identification of these antigens would clearly facilitate their analysis, but this has proved to be more difficult than expected. Mouse sarcomas frequently express antigens related to murine leukemia viruses (MuLV) and mouse sera frequently contain natural antibodies to MuLV-related antigens (6–9). Unless steps are taken to control for MuLV-related reactions, they can introduce considerable confusion into the serological analysis of mouse sarcomas. Another problem relates to the greater difficulty of defining cell surface antigens of solid tumors that require enzymes for the preparation of single cells and that grow as attached cell populations in culture. It is for this reason that far more is known about the surface antigens of normal and malignant lymphoid cells than about any other cell population in the mouse, as these cells can be easily obtained in suspension from *in vivo* or *in vitro* sources (10). Finally, the most difficult problem of all has been the generation of antibody directed to the individually distinct antigens of chemically induced sarcomas. Despite prolonged immunization with strongly immunogenic tumors, resulting in a high level of transplantation resistance, the sera of such mice generally lack antibody having specificity for the immunizing tumor. In this regard, these tumor-specific antigens behave like antigens determined by certain *H-2* mutants; recognition of these mutant *H-2* products appears to be entirely in the province of cellular immunity and does not result in the production of a humoral immune reaction (11). Nevertheless, extensive immunization with two antigenically unrelated BALB/c sarcomas, Meth A and CMS4, has given rise to cytotoxic sera that define two noncrossreacting systems of cell surface antigens on these tumors (8, 12). Absorption tests identified the Meth A antigen on 1 of 20 BALB/c sarcomas (Meth A) and the CMS4 antigen on 2 of 20 BALB/c sarcomas (CMS4 and 11); neither antigen could be detected on a wide range of normal cells or other types of malignant cells, nor do these antigens appear to bear any relation to MuLV-related antigens.

To identify the molecules bearing the Meth A and CMS4 determinants, we immunoprecipitated [^{35}S]methionine-labeled sarcoma cell extracts with antisera recognizing these two antigens. In contrast to the individually distinct antigens defined in cytotoxic tests with these antisera, immunoprecipitation tests have detected a crossreacting antigen that, from present evidence, appears to be transformation related.

MATERIALS AND METHODS

Cells. The following cells of BALB/c origin were studied. *Methylcholanthrene (MC)-induced sarcomas:* Meth A, CMS4, CMS11, and CMS17 (ref. 8); CI-3 and CI-4 (ref. 13); C-1 (ref. 14). *Leukemias:* LSTRA, Moloney MuLV-induced leukemia (15); RLδ1, x-ray-induced leukemia (10). *Virus-transformed cell lines:* MCS, Moloney murine sarcoma virus (MuSV)-transformed embryo cells; 11A, Moloney MuSV-transformed

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Abbreviations: SV40, simian virus 40; MuLV, murine leukemia virus; MuSV, murine sarcoma virus; αMeth A, antiserum against Meth A sarcoma; $NaDodSO_4$, sodium dodecyl sulfate; MC, methylcholanthrene; C, BALB/c; B6, C57BL/6; $CB6F_1$, (BALB/c × C57BL/6)-F_1.

3T3 cells (16); K234 and 50, Kirsten MuSV-transformed 3T3 cells (17); SV4RL-2, simian virus 40 (SV40)-transformed 3T3 cells (18). *Spontaneously transformed fibroblasts:* CTF, derived from adult lung fibroblasts (8). *Nontransformed cells:* 3T3 cells (19); 3T3 cells productively infected with MuLV from a BALB/c MuLV$^+$ sarcoma; adult lung fibroblasts (*in vitro* passage 1 or 2); 10- to 15-day-old embryo cells (*in vitro* passage 1); spleen cells, peritoneal cells, and bone marrow cells from 2-month-old female mice.

Sera. The following sera were studied. *Antisera to BALB/c MC-induced sarcomas* (8, 12): (*i*) (BALB/c × C57BL/6)F_1 ($CB6F_1$) anti-Meth A (αMeth A); (*ii*) $CB6F_1$ anti-CMS4 (αCMS4); (*iii*) $CB6F_1$ anti-CMS5 (αCMS5); (*iv*) BALB/c (C) anti-Meth A (αMeth A); (*v*) C anti-CMS3 (αCMS3); (*vi*) C anti-CMS4 (αCMS4); and (*vii*) C anti-CMS5 (αCMS5). *Alloantisera:* (*i*) C57BL/6 (B6) anti-Meth A (αH-2^d); (*ii*) $CB6F_1$ anti-C3H sarcoma BP8 (αH-2^k); (*iii*) BALB/c anti-129 thymocytes (αH-2^b); (*iv*) (B6 × DBA/2)F_1 anti-MOPC-70A (αPC.1); and (*v*) C3H anti-CE thymocytes (αLyt-1.2). *Antisera to MuLV-related antigens:* (*i*) rat (W/Fu × BN)F_1 anti-W/Fu leukemia (C58NT)D (αNTD, a polyvalent αMuLV); (*ii*) goat anti-Kirsten MuSV/MuLV (αMuLV, a polyvalent αMuLV); (*iii*) goat anti-Rauscher MuLV gp70 (αgp70); (*iv*) goat anti-Rauscher MuLV p30 (αp30); (*v*) goat anti-AKR MuLV p30 (αp30); (*vi*) goat anti-Rauscher MuLV p15 (αp15); (*vii*) goat anti-AKR MuLV p15 (αp15); (*viii*) goat anti-Rauscher MuLV p12 (αp12); (*ix*) goat anti-AKR MuLV p10 (αp10); (*x*) B6 anti-Moloney MuLV leukemia EδM2 (αFMR); (*xi*) B6 anti-Abelson MuLV lymphoma B6T1 (αAbelson, αFMR); (*xii*) B6 anti-AKR leukemia K36 (αGCSA, αH-2^k); and (*xiii*) $CB6F_1$ anti-RLδ1 (αX.1). *Normal sera*: obtained from BALB/c or $CB6F_1$ mice (6–8 weeks old).

Labeling of Cells with [^{35}S]Methionine. Subconfluent cell monolayers or cell suspensions (approximately 20×10^6 cells) were labeled with 50 μCi of L-[^{35}S]methionine per ml (700–1000 Ci/mmol, 1 Ci = 3.7×10^{10} becquerels) in Earle's balanced salt solution containing 10% Eagle's minimum essential medium and 2% fetal bovine serum. After a 2-hr incubation at 37°C, during which time there was a linear incorporation of the radiolabel, approximately 8–10% of the total label was incorporated. The cells were washed and lysed with Tris-buffered saline containing 1% Nonidet P-40 and 2 mM phenylmethylsulfonyl fluoride. In general, the cell extracts had a specific activity of $0.5–1.5 \times 10^7$ cpm/mg of protein.

Immunoprecipitation and Sodium Dodecyl Sulfate ($NaDodSO_4$)/Polyacrylamide Gel Analysis. The procedure for immunoprecipitation with antiserum and heat-inactivated *Staphylococcus aureus* has been described (20). Immunoprecipitation was carried out with an aliquot of cell extract containing approximately 2×10^6 cpm and 1 μl of undiluted antiserum in a volume of not more than 200 μl. Immunoprecipitates (in a volume of 15 μl) were analyzed by electrophoresis in 10% $NaDodSO_4$/polyacrylamide slab gels (21). Two-dimensional gel analysis was performed by isoelectric focusing in the presence of ampholine (pH 3.5–10), followed by $NaDodSO_4$/polyacrylamide slab gel electrophoresis (20). Radiolabeled proteins were detected by fluorography (22). The protein markers used as molecular weight standards were proteins induced by the Ad2$^+$ND2 hybrid virus in human KB cells (21).

Cell-Free Synthesis of Kirsten MuSV Proteins. Kirsten MuSV proteins were synthesized in lysates of rabbit reticulocytes treated with micrococcal nuclease as described (23), with RNA extracted from purified Kirsten MuSV (24).

RESULTS

Reactions of Meth A Antisera with [^{35}S]Methionine-Labeled Meth A Sarcoma and Normal BALB/c Fibroblasts. Extracts of radiolabeled Meth A sarcoma and BALB/c lung fibroblasts were incubated with either Meth A antiserum (αMeth A) or normal mouse serum. The resulting immune complexes were precipitated with protein A-bearing *S. aureus* and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1). Four proteins with apparent molecular weights (M_r) of >200,000, 120,000, 93,000, and 53,000 were precipitated from the Meth A cell extract by αMeth A but not by normal mouse serum. The >200,000, 120,000, and 93,000 M_r proteins, but not the 53,000 M_r protein, were also detected by αMeth A in normal fibroblasts. Because the 53,000 M_r component (p53) appeared to distinguish Meth A sarcoma cells from normal cells, its occurrence was followed in subsequent studies.

Reactions of Other Hyperimmune Sera with [^{35}S]Methionine-Labeled Meth A Sarcoma. As one approach to defining the specificity of the reaction of αMeth A with the p53 component of Meth A, a variety of other hyperimmune sera was tested for p53 reactivity (Fig. 2 and see *Sera* in *Materials and Methods*). The sera included (*i*) antisera recognizing alloantigenic determinants—e.g., H-2, PC.1, and Lyt; (*ii*) antisera recognizing MuLV structural and cell surface determinants—e.g., gp70, p30, p15, p12, GCSA, and G_{IX}; and (*iii*) antisera prepared against three recently derived MC-induced sarcomas of BALB/c origin, CMS3, CMS4, and CMS5, by hyperimmunization of BALB/c or $CB6F_1$. Of the 25 antisera tested, only antisera prepared against Meth A or CMS4 in syngeneic or compatible F_1 mice had anti-p53 activity.

Reactions of αMeth A and αCMS4 with [^{35}S]Methionine-Labeled Normal and Transformed Cells. A range of normal and transformed cell types of BALB/c origin were assayed for the presence of p53 by immunoprecipitation with αMeth A and αCMS4 (Fig. 3 and see *Cells* in *Materials and Methods*). Because these two antisera gave parallel results, only reactions obtained with αMeth A will be illustrated.

The following BALB/c cell types were p53 positive: (*i*) seven of seven independently derived MC-induced sarcomas; (*ii*) four

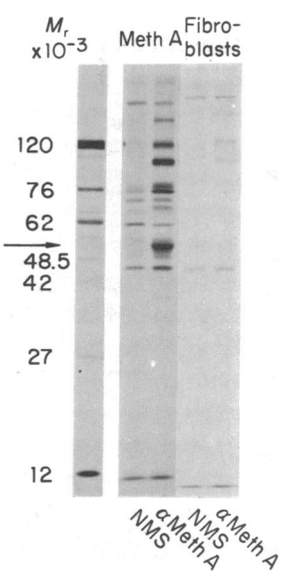

FIG. 1. Autoradiogram of [^{35}S]methionine-labeled proteins immunoprecipitated from extracts of Meth A sarcoma and adult lung fibroblasts by normal mouse serum (NMS) or Meth A antiserum (αMeth A) and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis.

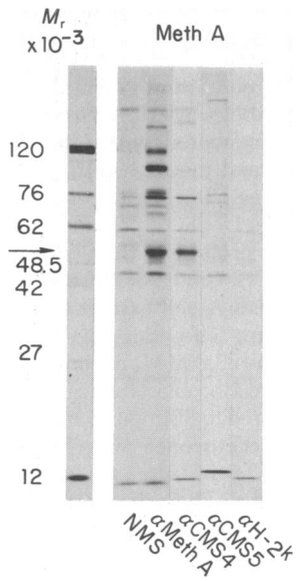

FIG. 2. Autoradiogram of [^{35}S]methionine-labeled proteins immunoprecipitated from extracts of Meth A sarcoma by normal mouse serum (NMS) or (BALB/c × C57BL/6)F_1 antisera against Meth A (αMeth A), CMS4 (αCMS4), CMS5 (αCMS5), and C3H sarcoma BP8 (αH-2^k); immunoprecipitates were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis.

of four cell lines transformed by SV40, Moloney MuSV, or Kirsten MuSV; (*iii*) a line of spontaneously transformed adult lung fibroblasts (CTF); and (*iv*) two leukemias, one induced by Moloney MuLV and one induced by x rays.

The following BALB/c cell types were p53 negative: (*i*) eight of eight cultures of adult lung fibroblasts (passages 1–2); (*ii*) eight of eight cultures of mouse embryo cells (passage 1) derived from 10- to 15-day-old embryos; (*iii*) 3T3 cells; (*iv*) 3T3 cells productively infected with MuLV from a MuLV$^+$ MC-induced sarcoma; and (*v*) spleen cells, peritoneal cells (macrophages), and bone marrow cells from normal 2-month-old female mice (each cell population tested individually).

Two-Dimensional Polyacrylamide Gel Electrophoresis of Meth A p53 and CMS4 p53. Extracts of [^{35}S]methionine-labeled Meth A and CMS4 were incubated with αMeth A, αCMS4, or normal mouse serum and the resulting immunoprecipitates were analyzed by two-dimensional polyacrylamide gel electrophoresis. The p53 components recognized by αMeth A or αCMS4 in extracts of Meth A or CMS4 were indistinguishable, having in each case an isoelectric point of pH 6.3. This analysis with radiolabeled Meth A and αMeth A or normal mouse serum is illustrated in Fig. 4.

Comparison of Meth A p53 and the 53,000 M_r Protein Coded for by Kirsten MuSV. *In vitro* translation of Kirsten MuSV RNA has shown that the viral genome codes for a nonstructural component with an apparent M_r of approximately 50,000 (24). To examine the relationship of this protein, which migrates as a 53,000 M_r component in our gel system (Fig. 5), and the p53 component detected by αMeth A and αCMS4 in transformed cells (including Kirsten MuSV-transformed cells), we compared the two components by two-dimensional polyacrylamide gel electrophoresis and reactions with αMeth A. According to these criteria, the two products appear unrelated. The Kirsten MuSV 53,000 M_r product has a pI outside the pH 3.5–8.0 range (data not shown), in contrast to a pI of 6.3 for Meth A p53, and is not precipitated by αMeth A (Fig. 5).

DISCUSSION

These studies have defined a new class of antigen in transformed cells of the mouse. The p53 component recognized by antisera prepared against sarcoma Meth A or CMS4 has been found in cells transformed by chemicals, irradiation, or viruses, as well as in spontaneously transformed cells. Although p53 appears to be widely represented in mouse tumors, antibody to p53 is not commonly found in antisera prepared against tumor cells. Of the 15 antisera prepared against 11 different tumors, only those against Meth A and CMS4 resulted in p53 antibody. This would indicate that the p53 molecule either is generally poorly immunogenic or requires special conditions of immunization to elicit antibody (e.g., syngeneic or semisyngeneic immunization) or that the p53 components of Meth

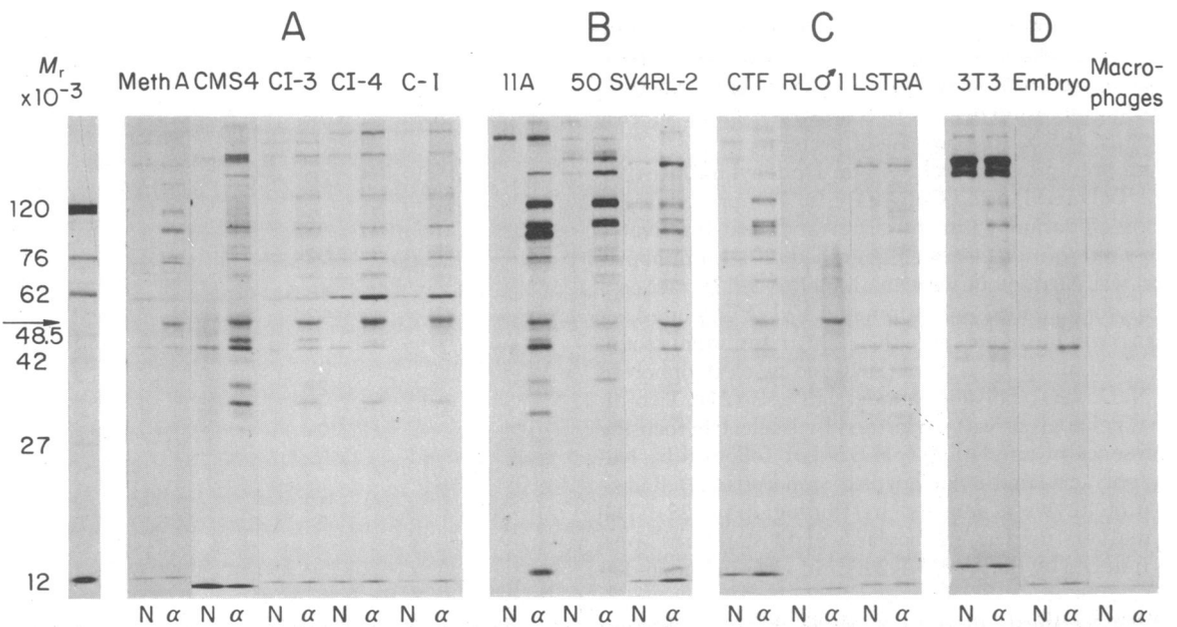

FIG. 3. Autoradiograms of [^{35}S]methionine-labeled proteins immunoprecipitated from extracts of transformed or nontransformed BALB/c cells by normal mouse serum (N) or Meth A antiserum (α) and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. (*A*) Methylcholanthrene-induced sarcomas; (*B*) MuSV- and SV40-transformed cell lines; (*C*) spontaneously transformed fibroblasts (CTF) and leukemias RLδ1 and LSTRA; (*D*) nontransformed cells.

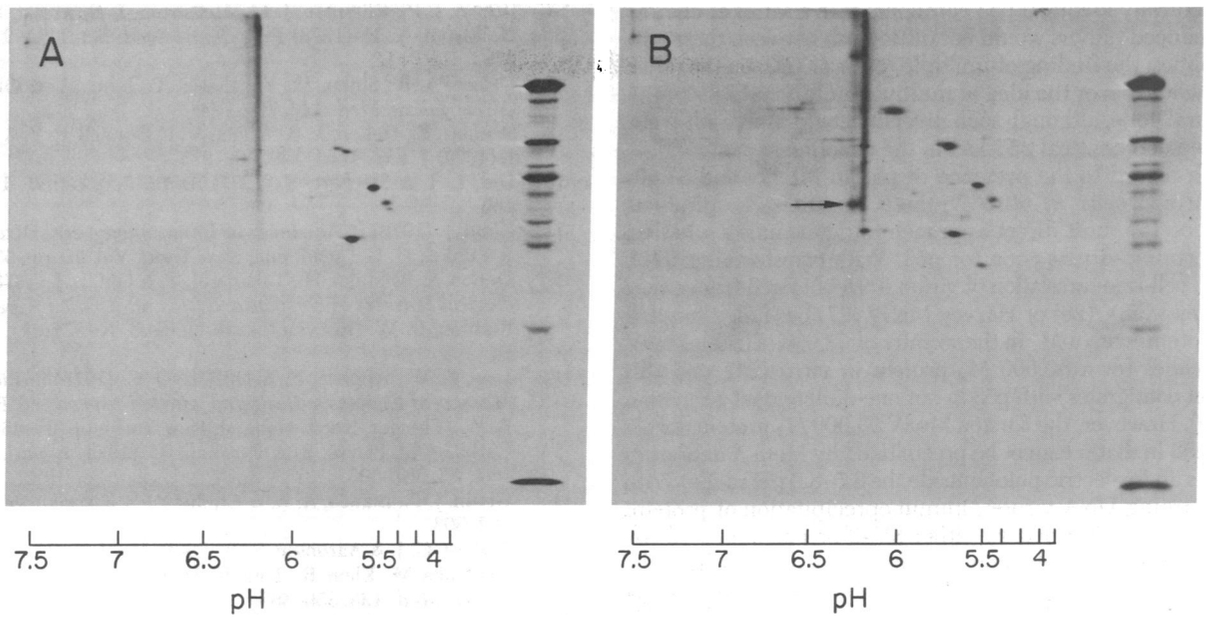

FIG. 4. Autoradiogram of [^{35}S]methionine-labeled proteins immunoprecipitated from extracts of Meth A sarcoma by normal mouse serum (*A*) or Meth A antiserum (*B*) and analyzed by two-dimensional polyacrylamide gel electrophoresis. Molecular weight markers are shown on the right side of each gel. The p53 component is indicated by the arrow.

A and CMS4 are particularly immunogenic. An important next step will be to prepare peptide maps of the p53 components from different tumors to see whether they represent a family of antigenically related but structurally distinguishable molecules or a single molecular entity. Another critical task is to develop more sensitive assays for p53 (e.g., radioimmunoassays with mouse or heteroantibody to p53) to determine whether the apparent absence of p53 in normal cells reflects a quantitative or a qualitative difference in comparison with malignant cells.

The antisera found to have reactivity with p53 components also have cytotoxic antibodies that identify two systems of cell surface antigens on chemically induced sarcomas—the Meth A antigen and the CMS4 antigen (8, 12). Absorption studies indicate that these two antigens, in contrast to p53, have an exceedingly limited distribution among mouse tumors and, in this respect, resemble the individually distinct or unique transplantation antigens that characterize chemically induced sarcomas. Whether the serologically defined Meth A and CMS4 antigens are identical or related to the transplantation antigens of these tumors is not known. However, recent work with the Meth A sarcoma leads to the surprising suggestion that they may be different. Fractionation of solubilized membranes of Meth A cells has led to a preparation that is highly active in inducing transplantation resistance in syngeneic hosts; immunization with 2.5 μg of this fraction protects against Meth A challenge but not against several other syngeneic tumors (13, 25). This active Meth A fraction does not absorb cytotoxic activity from Meth A antisera nor do Meth A antisera precipitate radiolabeled Meth A transplantation antigen (unpublished data). However, before any firm conclusion can be drawn about this matter, comparable studies with other sarcomas need to be performed. With regard to the relation of the p53 component of sarcomas and the serologically defined Meth A and CMS4 antigens, it appears likely that the p53 precipitating antibody in Meth A or CMS4 antisera is distinct from the cytotoxic antibodies in these antisera. This can be tested directly by isolating p53 components from Meth A, CMS4, and other sarcomas and determining whether these can or cannot absorb cytotoxic antibody and, if they do, whether the absorption pattern seen with p53 corresponds to the pattern obtained with viable sarcomas cells.

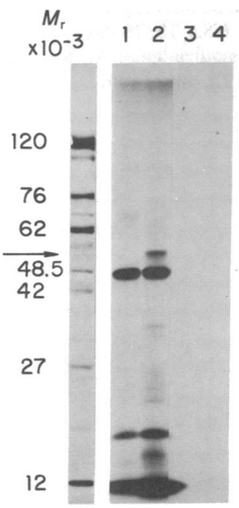

FIG. 5. Cell-free synthesis of Kirsten-MuSV proteins in lysates of rabbit reticulocytes. Autoradiogram of synthesized [^{35}S]methionine-labeled products analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. 1, No RNA added; 2, Kirsten-MuSV RNA added; 3, immunoprecipitate of Kirsten-MuSV RNA product with normal mouse serum; and 4, immunoprecipitate of Kirsten-MuSV RNA product with Meth A antiserum.

Insights into the genetic origin of p53 components should result from an understanding of their structure. The presence of p53 in tumors of no known viral etiology indicates that resident cellular genes can code for these components; this would not exclude endogenous viral genes as the source of coding sequences or the possibility that transforming viruses share these sequences conceivably as a consequence of a past recombinational event with host genes. The fact that p53 has not been detected in nontransformed cells suggests that its expression is tightly regulated. Its appearance in transformed cells could result, therefore, from an alteration, mutational or otherwise, at a regulatory locus, allowing the structural locus to be expressed constitutively, or a mutation at a structural locus that would free it or its product from regulatory control. The finding

of structurally identical p53 components in a series of chemically induced tumors would not distinguish between these two possibilities; the finding of multiple forms of p53, on the other hand, would favor the idea of multiple mutational sites in the structural gene, although such diversity could also result from multiple nonidentical *p53* loci in the genome.

With regard to the presence of p53 in MuSV- and SV40-transformed cells, *in vitro* synthesis of viral gene products would be the most direct approach to determining whether transforming viruses code for p53. With transforming RNA viruses, cell-free translation of virion RNA obtained from either Moloney MuSV (26) or Harvey MuSV (27) has failed to yield any proteins with a M_r in the vicinity of 53,000. Kirsten MuSV RNA codes for a 50,000 M_r protein *in vitro* (24), and this product comigrates with p53 in our one-dimensional gel system (Fig. 5). However, the Kirsten MuSV 53,000 M_r protein differs from p53 in that it cannot be precipitated by Meth A antiserum and has an isoelectric point outside the 3.5–8.0 pH range. With transforming DNA viruses, immunoprecipitation of proteins from cells transformed by either SV40 or polyoma virus by using homologous antitumor sera has yielded, apart from the virus-coded T antigens (28, 29), a 55,000 M_r protein of unknown origin (29–31). At least in polyoma-transformed cells, this 55,000 M_r protein does not appear to be virus coded (29). Tests are under way to see whether the 55,000 M_r protein in polyoma- and SV40-transformed cells is related to p53. An alternative, therefore, to transforming viruses having coding sequences for p53 would be direct or indirect activation or derepression of *p53* cellular genes by a viral gene product. Virus-transformed cells with temperature-sensitive viral gene products should allow exploration of these possibilities and provide ways to relate p53 expression to the initiation and maintenance of transformation.

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