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Immune Response to the *src* Gene Product in Mice Bearing Tumors Induced by Injection of Avian Sarcoma Virus-Transformed Mouse Cells

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A single subcutaneous injection of 10^7 live cells of the highly tumorigenic avian sarcoma virus (Schmidt-Ruppin strain, subgroup D)-transformed BALB/c line into BALB/c mice resulted in the production of an antiserum specific for the avian sarcoma virus gene product pp60^{src}. All sera taken from mice 3 weeks after injection of tumor cells contained antibodies to pp60^{src}. Immunoprecipitation experiments showed that all sera precipitated pp60^{src} from Schmidt-Ruppin-infected chicken cells, but only a portion of these sera precipitated pp60^{src} from chicken cells infected with other strains of avian sarcoma virus, i.e., Prague and Bratislava-77. Analysis of the cross-reactivity patterns of these antisera demonstrated a minimum of three to four antigenic determinants on pp60^{src}. The findings reported here should facilitate the production of monoclonal antibodies to pp60^{src}, which in turn will provide highly specific probes for further investigations into the structure and function of this protein.

Avian sarcoma viruses (ASV) induce tumors in avian species and in several mammalian species, as well as transform avian fibroblasts in vitro. Genetic analyses of deletion and temperature-sensitive mutants of ASV have defined a region of the ASV genome (the *src* gene) as being necessary for both the initiation and maintenance of neoplastic transformation (14). Recently, the product of the *src* gene has been identified by using antisera from rabbits bearing ASV-induced tumors. Brugge and Erikson (4) have shown that such antisera can immunoprecipitate a 60,000-molecular-weight phosphoprotein (pp60^{src}) from radiolabeled extracts of chicken or hamster cells transformed by the Schmidt-Ruppin strain of ASV (SR-ASV), but not from extracts of chicken embryo fibroblasts (CEF) infected with a transformation-defective (td) mutant of SR-ASV or from uninfected cells. The inability of disrupted virions to block the immunoprecipitation of pp60^{src} by these rabbit antisera demonstrated that pp60^{src} was not a viral structural protein or its metabolic precursor. Tryptic peptide analyses of the pp60^{src} proteins from SR-ASV, subgroup D (SR-ASV-D)-transformed chicken, hamster, and field vole cells have shown that the proteins are quite similar to one another, yet each is distinct from the ASV structural protein precursor pr76 (3). Similar analyses of pp60^{src} produced by various strains of ASV reveal only minor differences in tryptic peptide patterns and migration rates upon gel electrophoresis (2, 12, 13).

In vivo and in vitro labeling experiments have shown that pp60^{src} is a phosphoprotein containing two sites of phosphorylation. In addition, immune complexes containing pp60^{src} exhibit a protein kinase activity which appears to be clearly attributable to the pp60^{src} protein. Analysis of a temperature-sensitive mutant of ASV has shown that both the extent of phosphorylation of pp60^{src} and its protein kinase activity were temperature sensitive, whereas the synthesis of the protein was not (6, 7, 10).

By using sera from either tumor-bearing rabbits (TBR sera) or tumor-bearing marmosets, a cross-reactive protein similar in molecular weight to pp60^{src} has been immunoprecipitated from a number of uninfected avian and mammalian cells. This protein also appears to be a phosphoprotein and exhibits a protein kinase activity (2, 5; R. Erickson, personal communication).

In this paper we describe murine antisera to pp60^{src} obtained by the injection of SR-ASV-transformed BALB/c cells into BALB/c mice. These antisera are specific for pp60^{src} in that they contain little, if any, antibody to viral structural proteins, yet can cross-react with sarcoma gene products from other strains of ASV.

MATERIALS AND METHODS

Cells and viruses. Primary cultures of CEF cells were prepared from 10-day-old gs-negative/chf-nega-

tive/Marek-negative embryos (Spafas, Norwich, Conn.). Cultures were maintained in Dulbecco-modified Eagle medium containing high glucose (Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% tryptose phosphate and 5% calf serum (Flow Laboratories) and subcultured every 3 to 5 days. Secondary cultures of CEF cells were infected with ASV, using a multiplicity of 0.1 to 0.5 focus-forming unit per cell in the presence of 2 μg of Polybrene (Aldrich Chemical Co., Milwaukee, Wis.) per ml. Infected cells were subcultured every 3 to 5 days, and the medium was changed daily. The following strains of ASV were used: SR-ASV-A or SR-ASV-D; td-SR-ASV-A; the Prague strain of ASV, subgroup C (Pr-ASV-C); and the Bratislava-77 strain of ASV, subgroup C (B_{77}-ASV-C).

The SR-ASV-D-transformed BALB/c (SR-BALB) cell line was originally obtained in the laboratory of D. Bolognesi by injecting SR-ASV-D-transformed chicken cells into neonatal BALB/c mice. Tumors which developed were excised and passaged several times by reinjection into mice before being established in cell culture. All mouse cell lines were maintained in Dulbecco-modified Eagle medium supplemented with 10% calf serum.

Preparation of antisera. TBR sera were prepared by the method of Brugge and Erikson (4). Approximately 10^8 focus-forming units of partially purified SR-ASV-D were injected subcutaneously and intramuscularly at multiple sites into each of eight neonatal New Zealand rabbits. Tumors arose 3 to 4 weeks after inoculation. Thereafter, the rabbits were bled weekly until the tumors regressed. Tumor-bearing mouse (TBMo) sera were obtained by the subcutaneous injection of 10^7 SR-BALB cells into 6- to 7-week-old BALB/c mice (Flow Laboratories). The cells were prepared for injection by scraping confluent monolayers in Dulbecco-modified Eagle medium, washing cells, and suspending them in 100 to 300 μl of the medium. Palpable tumors appeared 5 to 7 days after injection. Massive tumor growth and metastasis were followed by death in 5 to 6 weeks. The animals were routinely sacrificed and bled before this time.

Purification of virus. SR-ASV-D was purified by concentrating the virus from culture fluids of SR-ASV-D-infected CEF cells. Cellular debris was removed from the culture fluids by centrifugation at 10,000 × *g* for 10 min, and the virus was pelleted in a Beckman 45 Ti rotor for 40 min at 42,000 rpm at 4°C. The virus pellets were suspended in TEN buffer (10 mM Tris-hydrochloride, 1 mM EDTA, 0.1 M NaCl, pH 7.2) and subjected to two sequential purifications by equilibrium centrifugation on a 15 to 60% sucrose gradient containing TEN buffer. The virus was subsequently pelleted as described above and suspended in 0.5 to 1.0 ml of TEN buffer, and the protein concentration was measured by a modification of the Lowry method (11).

Immunoprecipitation of labeled cell proteins. Labeling of cells with [^{35}S]methionine was carried out as follows. Cells, grown in 100-mm culture dishes, were washed once with labeling medium (Hams F-10) containing 1% calf serum and incubated for 2 h in the same medium. Cells were then incubated for 3 h in fresh labeling medium containing 100 μCi of [^{35}S]methionine (1,200 to 1,400 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per ml.

To label cells with $^{32}P_i$, we washed cell cultures three times with medium 199 minus phosphate (Flow Laboratories), supplemented with 2% dialyzed calf serum and 1% dimethyl sulfoxide. The cells were then incubated for 2 h in the same medium containing 500 μCi of carrier-free $^{32}P_i$ (New England Nuclear Corp., Boston, Mass.) per ml.

Cell extracts were prepared essentially as described by Brugge and Erikson (4). Cells were washed three times in STE buffer (150 mM NaCl, 50 mM Tris-hydrochloride, 1 mM EDTA, pH 7.2) and lysed in 2 ml of RIPA buffer (150 mM NaCl, 50 mM Tris-hydrochloride, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1% trasylol, 2 mM phenylmethylsulfonyl fluoride, 2 mM iodoacetamide, pH 7.2). Extracts were then clarified by centrifugation at 100,000 × *g* for 30 min at 4°C. For immunoprecipitation, samples of the labeled cell extract were incubated for 30 min at 4°C with either 2 to 10 μl of TBMo serum or 15 to 50 μl of TBR serum. Blocking experiments were carried out by preincubation of the antiserum with 125 to 150 μg of purified SR-ASV-D (disrupted in RIPA buffer) before the addition of the labeled cell extract. When TBMo sera were used, rabbit antiserum to either mouse immunoglobulin or to mouse kappa light chains was then added. The resulting immune complexes were removed from solution by the addition of either the fixed Cowan I strain of *Staphylococcus aureus* as described by Kessler (8) or excess protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.). The adsorbed complexes were washed and subsequently eluted into 75 μl of sample buffer (0.07 M Tris-hydrochloride, 11% glycerol, 3% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol, pH 6.8) by heating for 3 min in a boiling water bath. Analyses of such eluted immunoprecipitates were carried out by SDS-polyacrylamide slab gel electrophoresis as described below.

Polyacrylamide gel electrophoresis and autoradiography. Polyacrylamide slab gel electrophoresis was carried out by using the discontinuous buffer system described by Laemmli (9). The stacking gel was 3.0% acrylamide–0.08% bisacrylamide, pH 6.8. The separating gel was 7.5% acrylamide–0.2% bisacrylamide, pH 8.8. Gels were stained in 0.1% Coomassie brilliant blue–50% methanol–7.5% acetic acid and destained in 5% methanol–7.5% acetic acid. Gels containing [^{35}S]methionine-labeled proteins were prepared for photofluorography (1), dried on Whatman 3MM paper, and exposed to Kodak XRP film. The gels containing ^{32}P-labeled proteins were dried and exposed to Kodak XRP film, using DuPont Cronex Lightning Plus intensifying screens.

RESULTS

Immunoprecipitation of the *src* gene product from ASV-infected chicken cells with sera from BALB/c mice bearing ASV-BALB tumors. Injection of 10^7 SR-BALB cells into BALB/c mice resulted in the formation of tumors within 7 to 10 days. TBMo sera immunoprecipitated a 60,000-dalton polypeptide (p60) from [^{35}S]methionine-labeled extracts of SR-ASV-infected chicken cells but not from labeled

extracts of either td-SR-ASV-infected cells or normal cells (Fig. 1). Preincubation with nonlabeled, disrupted virus did not reduce the ability of such antisera to immunoprecipitate p60 (Fig. 1, tracks 14 and 16), suggesting that this polypeptide was not a viral structural protein or a precursor thereof. Similar results were obtained with TBR antisera (Fig. 1). The electrophoretic mobility of the p60 protein precipitated by TBMo sera was identical to that of the $pp60^{src}$ precipitated by the TBR sera. In contrast to the TBR sera, only a few of the many TBMo sera tested immunoprecipitated significant amounts of a viral structural protein precursor. In those instances, small amounts of a polypeptide having the same mobility as pr76 were precipitated in the absence of any detectable immunoprecipitation of its structural protein products, i.e., p27, p19, p15, and p12 (Fig. 1, track 15).

Both TBMo and TBR sera immunoprecipitated a phosphorylated protein of identical molecular weight (i.e., 60,000) from $^{32}P_i$-labeled extracts of SR-ASV-infected CEF cells but not from similarly labeled extracts of either td-SR-ASV-infected cells or normal cells (Fig. 2). Preincubation of either TBMo or TBR sera with disrupted ASV virions did not block subsequent immunoprecipitation of the labeled p60 polypeptide. Only the TBR sera precipitated $^{32}P_i$-labeled viral structural protein precursors, i.e., pr76.

To confirm the identity of the polypeptide precipitated by TBMo sera and that precipitated by TBR sera, sequential immunoprecipitation experiments were performed as follows. Samples of [^{35}S]methionine-labeled extracts of SR-ASV-infected CEF cells were incubated with an excess of the TBR serum, and the resulting immune complexes were removed by absorption to protein A-Sepharose and centrifugation. The supernatant was subsequently incubated with an amount of TBMo serum previously determined to immunoprecipitate readily detectable quantities of the p60 polypeptide. Any immune complexes formed during this second immunoprecipitation were also removed by absorption to protein A-Sepharose and centrifugation. The resultant immunoprecipitates, as well as those

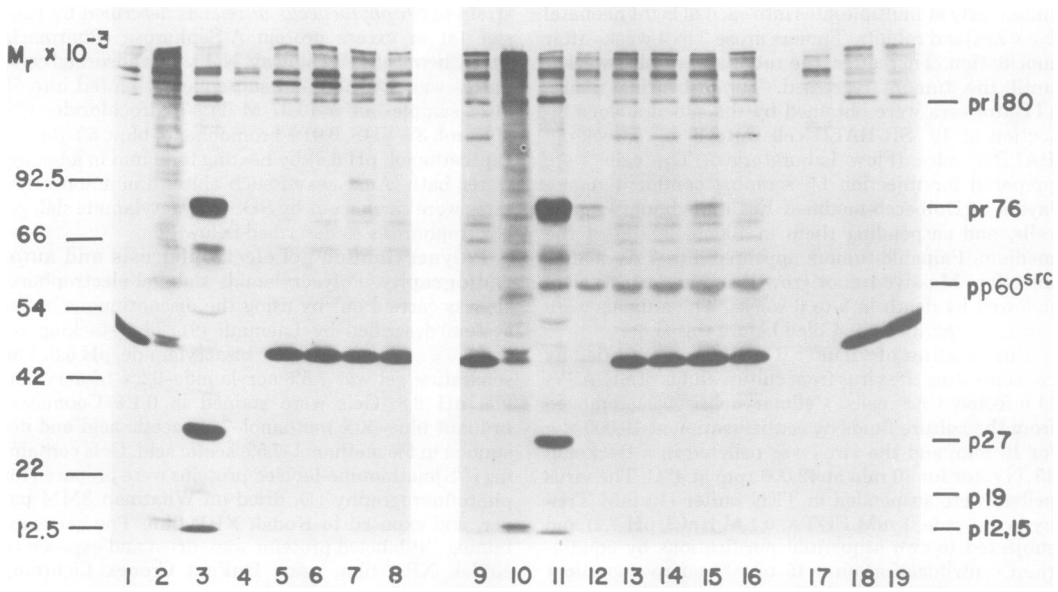

FIG. 1. *Immunoprecipitation of the src gene product from [^{35}S]methionine-labeled ASV-infected CEF. [^{35}S]methionine-labeled cell lysates were aliquoted, and immunoprecipitates were then prepared and analyzed by SDS-polyacrylamide gel electrophoresis and photofluorography. Tracks 1 to 8 contain immunoprecipitates from td-SR-ASV-A-infected CEF; tracks 9 to 16 contain immunoprecipitates from SR-ASV-D-infected CEF; and tracks 17 to 19 contain immunoprecipitates from normal CEF. Antisera tested were the following: TBMo preimmune serum, tracks 1 and 9; normal rabbit serum, tracks 2 and 10; TBR serum, tracks 3, 11, and 17; TBR serum preabsorbed with disrupted virus, tracks 4 and 12; TBMo-1 serum, tracks 5, 13, and 18; TBMo-1 serum preabsorbed with disrupted virus, tracks 6 and 14; TBMo-2 serum, tracks 7, 15, and 19; TBMo-2 serum preabsorbed with disrupted virus, tracks 8 and 16. Molecular weight standards used were the following: (i) rabbit muscle phosphorylase, M_r 92,500; (ii) bovine serum albumin, M_r 66,000; (iii) human immunoglobulin, heavy chain, M_r 54,000; (iv) ovalbumin, M_r 42,000; (v) human immunoglobulin, light chain, M_r 22,000; and (vi) cytochrome c, M_r 12,000.*

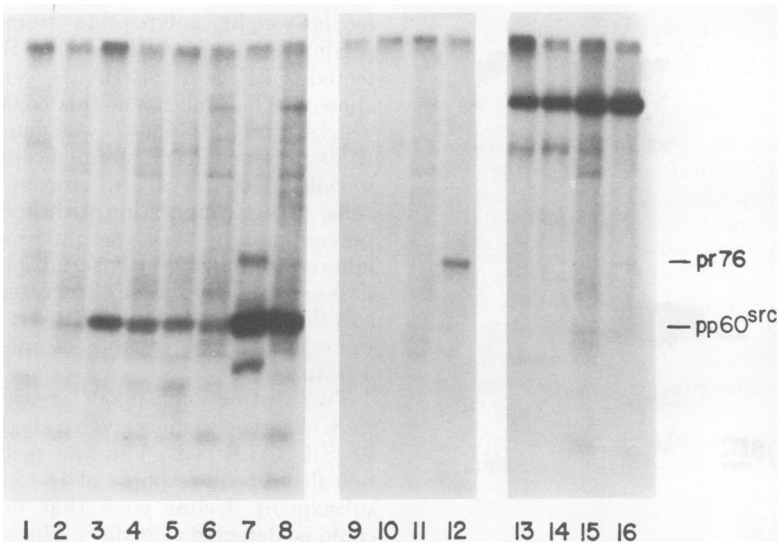

FIG. 2. *Immunoprecipitation of the src gene product from $^{32}P_i$-labeled ASV-infected CEF. $^{32}P_i$-labeled cell lysates were aliquoted, and immunoprecipitates were then prepared and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Immunoprecipitates, using extracts of SR-ASV-D-infected CEF, are shown in tracks 1 to 8; of td-SR-ASV-A-infected CEF in tracks 9 to 12; and of normal CEF in tracks 13 to 16. Antisera tested were: normal mouse serum, tracks 1, 9, and 13; normal mouse serum preabsorbed with SR-ASV-D, track 2; TBMo-3 serum, track 3; TBMo-3 serum preabsorbed with SR-ASV-D, track 4; TBMo-4 serum, tracks 5, 10, and 14; TBMo-4 serum preabsorbed with SR-ASV-D, tracks 6, 11, and 15; TBR serum, tracks 7, 12, and 16; TBR serum preabsorbed with SR-ASV-D, track 8.*

from the appropriate controls, were subjected to SDS-polyacrylamide gel electrophoresis and photofluorography. Figure 3 shows that prior precipitation of such extracts with TBR sera effectively removed all of the p60 polypeptide recognized by TBMo sera. This result, together with the data of Fig. 1 and 2, demonstrates that the p60 polypeptide specifically immunoprecipitated by TMBo sera is identical to pp60^{src}.

Frequency of production of anti-pp60^{src} by mice bearing SR-BALB-induced tumors. Of 57 mice bled at weekly intervals after a single subcutaneous injection of 10^7 SR-BALB cells, 33 produced anti-pp60^{src}. Of the 33 positive sera, 19 were strongly positive, as assessed by the intensity of the pp60^{src} band on autoradiograms. All sera (22 of 22) taken 3 weeks after the injection of tumor cells contained anti-pp60^{src}. Of these latter sera, 59% were strongly positive. Most of the negative sera were obtained from mice bled within 2 weeks of the injection of tumor cells.

The optimum time for bleeding such mice after a single injection of 10^7 SR-BALB tumor cells was determined with sera taken by tail bleeding at approximately 3-day intervals from individual BALB/c mice. Two distinct anti-pp60^{src} response patterns became apparent from such kinetic studies (Fig. 4). Anti-pp60^{src} was first detected 10 to 17 days after tumor cell injection, and the peak of the antibody response was reached by 18 to 22 days. However, in some mice the quantity of anti-pp60^{src} in the sera gradually decreased such that no antibody was detectable in those sera taken at 28 days (the time of sacrifice). In all other mice the quantity of anti-pp60^{src} reached peak levels and remained at that concentration in all subsequent sera taken. No correlation appeared to exist between the pattern of the anti-pp60^{src} antibody response and tumor size.

Cross-reaction between anti-pp60^{src} and pp60^{src} from various strains of ASV. Antisera from 15 mice obtained 18 to 22 days after injection of SR-BALB cells were tested for their capacity to immunoprecipitate pp60^{src} from $^{32}P_i$-labeled extracts of chicken cells infected with SR-ASV-D, SR-ASV-A, Pr-ASV-C, or B_{77}-ASV-C. All sera precipitated pp60^{src} from the SR-ASV-D- and SR-ASV-A-infected cells. Some sera (9 of 15) precipitated pp60^{src} from cells infected with Pr-ASV-C (Fig. 5), and 4 of these 9 sera also precipitated pp60^{src} from B_{77}-ASV-C-infected cells (data not shown).

DISCUSSION

A single subcutaneous injection of 10^7 live SR-BALB cells into BALB/c mice resulted in the production of an antiserum specific for the sarcoma gene product pp60^{src}. All sera taken from

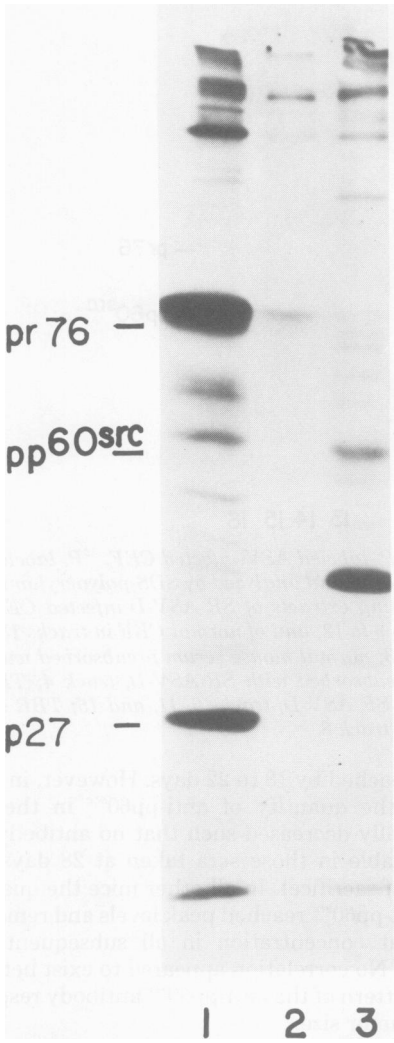

FIG. 3. *Identity of pp60^{src} precipitated by TBR serum and the p60 polypeptide precipitated by TBMo serum. Identical portions of [^{35}S]methionine-labeled SR-ASV-D-infected CEF extracts were immunoprecipitated with either 50 μl of TBR serum (track 1) or 10 μl of TBMo serum (track 3). The supernatant from the TBR reaction was then immunoprecipitated with 10 μl of TBMo serum (track 2). The samples were subjected to SDS-polyacrylamide gel electrophoresis and photofluorography.*

mice 3 weeks after injection of tumor cells contained antibodies to SR-ASV pp60^{src}. The antisera analyzed in this study fall into three categories: (i) those specific for the SR-ASV, (ii) those cross-reactive with other strains of ASV (Pr-ASV-C and B_{77}-ASV-C), and (iii) those cross-reactive with normal cell *sarc* protein (R. Erickson, personal communication).

TBMo sera immunoprecipitated a 60,000-molecular-weight polypeptide from extracts of [^{35}S]methionine or $^{32}P_i$-labeled SR-ASV-D-infected CEF and SR-BALB cells (data not shown). The identity of this 60,000-molecular-weight protein to pp60^{src} was demonstrated by: (i) its presence in transformed cells and absence in cells infected with a td virus or in uninfected cells, (ii) its comigration with pp60^{src} immunoprecipitated by TBR serum prepared in our laboratory and by TBR serum provided by R. Erikson, and (iii) by the observation that preabsorption of extracts with TBR serum completely removed all p60 capable of interacting with TBMo sera.

Two distinct anti-pp60^{src} response patterns were observed in mice given a single injection of 10^7 SR-BALB cells. The first response pattern revealed a peak response at 18 to 22 days and a subsequent decline such that no anti-pp60^{src} could be detected at 28 days. The second pattern showed a similar peak response, with no subsequent decline in antibody level. No correlation was found between antibody titer, response pattern, and tumor size. As noted above, all sera taken 3 weeks after injection contained anti-pp60^{src}. Sixty percent of these sera were strongly positive and had titers three to four times greater than those detected in our TBR sera. Most of the negative sera were taken within 2 weeks after injection. We have also examined several other regimens for the induction of pp60^{src} antisera, including a primary injection of a subtumorigenic dose of SR-BALB cells, followed by a secondary challenge with a tumorigenic dose. None of the protocols was as effective as the single-injection method.

More than 90% of the sera examined showed no antibody to viral structural proteins or their precursors. This probably reflects the low levels of synthesis of viral structural proteins by the SR-BALB tumor cells (e.g., by radioimmunoassay pr76 is detectable at only 1 to 2% of the level found in infected chicken cells [V. Volk, personal communication]).

The cross-reactivity patterns observed with these murine antisera (Fig. 5) are consistent with at least three antigenic determinants on pp60^{src}: (i) one which is specific to SR-ASV, (ii) one which is common to SR-ASV and Pr-ASV-C, and (iii) one which is common to the three strains of ASV used in these studies (SR-ASV, Pr-ASV-C, and B_{77}-ASV-C). The presence of a fourth antigenic determinant is suggested by the cross-reactivity of some mouse antisera with normal cell *sarc* protein (R. Erikson, personal communication). TBR sera and sera from tumor-bearing marmosets show similar patterns of cross-reactivity (2, 12, 13; S. Parsons, unpublished data).

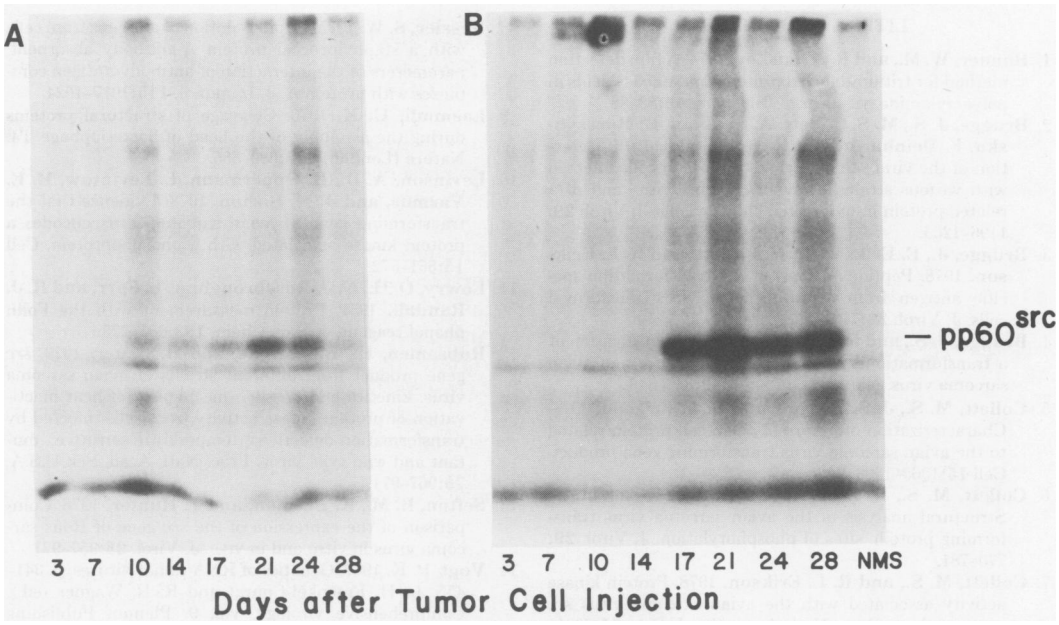

FIG. 4. *Kinetics of anti-pp60^{src} antibody production by BALB/c mice bearing SR-BALB cell-induced tumors. After a single injection of 10^7 SR-BALB cells, sera were taken from two individual mice on the days indicated. These sera were tested for anti-pp60^{src} antibody by immunoprecipitation of extracts of $^{32}P_i$-labeled SR-ASV-D-infected CEF. The immunoprecipitates were analyzed by polyacrylamide gel electrophoresis and autoradiography. (A) TBMo-5 serum; (B) TBMo-6 serum. NMS, Normal mouse serum control.*

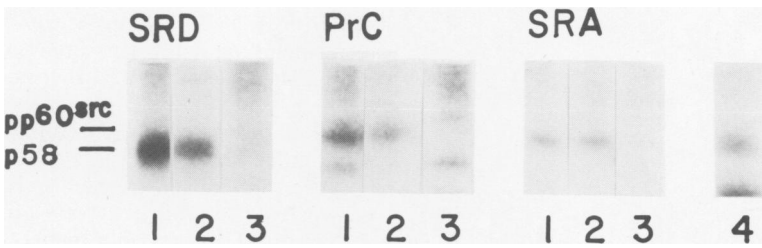

FIG. 5. *Cross-reaction of TBMo antisera with pp60^{src} from SR-ASV and Pr-ASV-C. CEF cells infected with either SR-ASV-D, Pr-ASV-C, or SR-ASV-A were labeled with $^{32}P_i$, and equal portions of the extracts were immunoprecipitated with the following antisera: TBMo-2 serum (track 1), TBMo-7 serum (track 2), or preimmune mouse serum (track 3). pp60^{src} precipitated by TBR sera from an extract of SR-ASV-D-infected CEF is shown in track 4. p58 is a nonspecific band seen in some immunoprecipitates of Pr-ASV-C-infected CEF, using preimmune sera as well as TBMo sera.*

Somatic cell fusion methods should enable the production of hybridoma cell lines, with each cell line producing monoclonal antibody specific for a single antigenic determinant on pp60^{src}. Such studies are underway in our laboratory, using BALB/c mice immunized with SR-BALB tumor cells, and should facilitate a more thorough characterization of the structure of the sarcoma gene product and its function.

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