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Rat Sarcoma Virus: Further Analysis of Individual Viral Isolates and the Gene Product

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Rasheed rat sarcoma virus, derived by in vitro cocultivation of two rat cell lines (Rasheed et al., Proc. Natl. Acad. Sci. U.S.A. **75**:2972-2976, 1978), has been reported to code for a protein of 29,000 M_r, immunologically related to the 21,000 M_r *src* gene product of Harvey and Kirsten sarcoma viruses. Rat sarcoma virus p29 was thought to contain at least part of a rat type C virus structural protein, since antiserum prepared against whole rat virus was able to immunoprecipitate rat sarcoma virus p29 but not Harvey or Kirsten sarcoma virus p21 (Young et al., Proc. Natl. Acad. Sci. U.S.A. **76**:3523-3527, 1979). We now report that antiserum directed against rat type C virus p15, but not viral p12, p10, or p27, immunoprecipitated rat sarcoma virus p29. The p15 antiserum was also able to immunoprecipitate both denatured p29 and a peptide derived by V-8 protease cleavage of p29, indicating that this antiserum contains antibodies directed against primary amino acid determinants. Finally, five separate isolates of rat sarcoma virus were found to code for p29, which indicates that a highly specific site of recombination is involved in the generation of sarcoma viruses in rat cells.

During the past few years, a great deal of work has focused on the gene products of those retroviruses capable of cellular transformation both in vitro and in vivo. With respect to mammalian sarcoma viruses, a number of these gene products have been well characterized. Among these, the genomes of feline sarcoma virus (3, 18) and Abelson leukemia virus (24) have all been shown to encode a polypeptide containing a portion of the *gag* gene of their respective helper virus fused to an additional protein sequence also encoded in normal cellular genetic material. In addition, woolly monkey sarcoma virus has been found to contain a portion of the *gag* gene of woolly monkey helper virus linked to some presumably cellular sequence (1), although the transforming ability of these cellular sequences has yet to be proven. In contrast to these viruses, Harvey and Kirsten murine sarcoma viruses (Ha-MuSV and Ki-MuSV, respectively), which are stable recombinants consisting of both rat and mouse genetic information, have been shown to code for a protein of 21,000 M_r (p21) which is unrelated to any of the retrovirus structural proteins and is found in low levels in normal cells (12); viral p21 is thought to represent the *src* gene product of these viruses (20, 21). Additionally, a third transforming virus of rat origin, the Rasheed rat sarcoma virus, has been found to code for a protein of 29,000 M_r (p29) which cross-reacts with antisera directed against either the Ha-MuSV or Ki-MuSV p21 gene product (26). This protein p29 has been found to share common V-8 protease-derived peptides with Ha-MuSV p21, and Ha-MuSV-transformed cell extracts can block the immunoprecipitation of rat sarcoma virus p29 by p21 antiserum (26). We have also previously reported that antiserum prepared by the injection of either whole or disrupted rat type C virus into rabbits can precipitate rat sarcoma virus p29, but not Ha-MuSV or Ki-MuSV p21, thus suggesting that a portion of p29 was of rat type C viral origin (26). We now report that antiserum prepared by injection into rabbits of a 15,000 M_r purified from intact rat type C virus is capable of immunoprecipitating rat sarcoma virus p29, rat type C virus *gag* precursor, and a leucine- but not a methionine-containing protein of 15,000 M_r from radiolabeled rat type C virus. In addition, five separate isolates of rat sarcoma virus each were found to code for an immunologically cross-reactive protein of 29,000 M_r.

MATERIALS AND METHODS

Cells. SD-1T, a Sprague-Dawley rat cell line spontaneously transformed and expressing rat type C virus, a Fischer rat embryo (FRE) cell line (C16E) trans-

formed by the Rasheed sarcoma virus (RaSV), a normal rat kidney (NRK) cell line (NST-5) transformed by RaSV, and NRK cells have been described previously (7, 16, 17, 26). Ha-NRK, a nonproducer NRK cell transformed by Ha-MuSV, was obtained from Edward Scolnick, National Cancer Institute, Bethesda, Md. All cell lines were grown on Dulbecco-modified Eagle medium containing 5 or 10% fetal bovine serum at 37°C in an atmosphere of 8 to 10% CO_2. Cells were examined routinely for mycoplasma contamination by Richard DelGiudice, Frederick Cancer Research Center, Frederick, Md., and all cells were found to be negative.

Viruses. Rat sarcoma viruses were isolated from two chemically transformed cell lines (NQ and HTC) and one polyoma virus-transformed cell line (PY-T). NQ cells were derived from serially passaged rat tumors induced by rat embryo cells transformed in vitro by 4-nitroquinoline oxide. HTC (clone of Morris hepatoma) cultures were established from tumors induced in vivo by *N,N*-2-7-fluorenylenebis-2-2-2-trifluoroacetamide and passaged in rats for many years before culturing in vitro. The rat cell line PY-T was obtained from tumors induced by rat embryo cells transformed in vitro by polyoma virus. NQ, HTC, and PY-T cells were separately cocultivated with Sprague-Dawley rat leukemia virus-productive cells (SD-1T), and supernatant virus was assayed on NRK or FRE cells. Foci of transformed cells were isolated and designated as NST or FST for NRK or FRE transformed cells, respectively.

Radiolabeling. For labeling of *src* proteins, 60-mm petri dishes containing confluent cell cultures were incubated with either 250 μCi of [^{35}S]methionine (800 to 1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) or 500 μCi of [^{3}H]leucine (110 Ci/mmol; New England Nuclear Corp., Boston, Mass.) for 5 to 6 h in 1 ml of methionine- or leucine-free medium containing 1% dialyzed fetal bovine serum. For labeling of the rat type C virus *gag* precursor, SD-1T cells were incubated for 1 h with 500 μCi of [^{35}S]methionine. For preparation of labeled virus, confluent 60-mm petri dishes of SD-1T cells were incubated with 500 μCi of [^{35}S]methionine in 1 ml of methionine-free medium or 500 μCi of [^{3}H]leucine in 1 ml of leucine-free medium for 7 to 8 h. At that time, 4 ml of complete Dulbecco-modified Eagle medium containing 5% fetal bovine serum was added, and incubation was continued for an additional 18 h. The medium was then removed and centrifuged at 5,000 × *g* for 10 min to pellet cells and then at 100,000 × *g* for 3 h to pellet virus. Tubes were carefully drained to remove the excess medium, and the virus was suspended in a phosphate-detergent buffer, pH 7.4, containing 0.01 M sodium phosphate, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.1 M NaCl, 1 mM EDTA, and 1% Aprotinin (Sigma Chemical Co., St. Louis, Mo.) (26).

Immunoprecipitation. Immunoprecipitation of radiolabeled extracts or radiolabeled virus was performed with Formalin-treated *Staphylococcus aureus* Cowan strain I containing protein A (10) as previously reported (26). Radiolabeled cell extracts were precleaned with 100 μl of 10% *S. aureus* Cowan strain I containing protein A per 60-mm dish before immunoprecipitation.

V-8 protease digestion. Digestion with *S. aureus* V-8 protease (6) was carried out as described previously (26), except that the respective proteins were eluted from the gel slices with 0.01% sodium dodecyl sulfate for 48 h at room temperature before V-8 protease digestion. For the location of [^{3}H]leucine-containing proteins, approximately 100,000 cpm of [^{35}S]-methionine-labeled extract was mixed with approximately 10^6 cpm of [^{3}H]leucine-labeled extract before immunoprecipitation. Using this ratio, the eluted [^{3}H]leucine-labeled proteins contained less than 10% [^{35}S]methionine radioactivity. V-8 protease digestion was halted by the addition of β-mercaptoethanol to a final concentration of 5%, the addition of sodium dodecyl sulfate to a final concentration of 1%, and placement of the reaction mixture in a boiling water bath for 2 min (8). For immunoprecipitation experiments, V-8 protease proteolysis was halted by the addition of phenylmethylsulfonyl fluoride to a 0.1 mM final concentration (C. Heilman, personal communication). After incubation for 30 min at 4°C, 2× phosphate-detergent buffer was added, and immunoprecipitation was performed as described previously (26).

Purification of viral proteins. Viral proteins were purified by high-pressure, reversed-phase liquid chromatography (HPLC) as described elsewhere (L. E. Henderson, R. Sowder, and S. Oroszlan, *in* D. T. Liu, A. N. Schechter, and R. Heinrikson, ed., *Proceedings of the International Conference on Chemical Synthesis and Sequencing of Peptides and Proteins*, in press). Approximately 20 to 30 mg of total viral protein was used for initial chromatographic separation. Briefly, sucrose density gradient-purified virus was extracted twice with butanol to remove lipids, dissolved in 8.4 M guanidine hydrochloride, and passed over a μ Bondapak C-18 column (Waters Associates Inc., Milford, Mass.). Protein was eluted in 1 h at a rate of 1 ml/min with a 0 to 70% acetonitrile gradient in 0.05% trifluoroacetic acid and monitored at 230 nm with a model 450 variable wavelength detector (Waters Associates, Inc.). Protein-containing fractions were then monitored by polyacrylamide gel electrophoresis, and those fractions containing the desired proteins were subjected to another round of chromatography on the HPLC column.

Immunizations. One- and two-year old, 2-kg rabbits were prebled and inoculated intradermally at multiple sites on the back with equal parts of antigen and complete Freund adjuvant, using 0.10 to 0.20 ml per site and a total of 100 μg of antigen per rabbit.

Second inoculations were given on day 14, and third inoculations were given on day 28, with a similar amount of antigen in an equal amount of incomplete Freund adjuvant. Approximately 50% of the inoculum was given subcutaneously, and the remainder was given intramuscularly. All subsequent inoculations were also similar to the second inoculation.

Test bleeds were taken beginning 4 to 5 days after the second inoculation and at periodic intervals thereafter until the animals were exsanguinated.

Immunization with gel slices. p15 and p27 preparations obtained after HPLC were subjected to electrophoresis on 15% polyacrylamide gels. After electrophoresis, gels were stained for 5 min with a 2% solution

of Coomassie brilliant blue in 5% acetic acid–50% methanol and immediately washed with distilled water. Sections of the gel containing p15 or p27 were soaked two times in phosphate-buffered saline for 10 min to remove excess staining solution. These gel strips were mechanically disrupted to small (<1-mm^2) pieces with a Dounce homogenizer. A minimal amount of phosphate-buffered saline was used to avoid sticking of gel material to the glass homogenizer. After disruption, the gel solution was dialyzed against phosphate-buffered saline (250 volumes overnight at 4°C). The final volume of material, usually about 2 ml, was passed through 18- and 21-gauge needles before injection into the rabbits. Total protein ranged from 50 to 100 μg per inoculation.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as previously described (11, 26). For V-8 protease analysis, a 17% acrylamide gel containing an acrylamide/bisacrylamide ratio of 30:0.1 was used (6).

Fluorography. Polyacrylamide gels were treated with En^{3}Hance (New England Nuclear Corp.), dried, and exposed to Kodak X-Omat film at −70°C.

RESULTS

Immunoprecipitation of viral proteins. Radiolabeled rat type C virus yielded a low-molecular-weight protein pattern very similar to that of many type C viruses upon immunoprecipitation with whole rat virus antiserum (Fig. 1). Proteins of M_r equivalent to p27, p15, p12, and p10 were detected in rat virus preparations, but not in supernatants from rat cells not producing a type C virus (data not shown). When comparing [^{35}S]methionine- and [^{3}H]leucine-labeled virus preparations, proteins with approximate molecular weights of 15,000 and 10,000 were observed to contain leucine, but not methionine (data not shown), which is similar to results observed with both Rauscher and AKR leukemia viruses (13). Through the use of HPLC and polyacrylamide gel electrophoresis, the various low-molecular-weight viral proteins were purified from sucrose density gradient-banded virus, and antisera were prepared against these proteins. These antisera specifically immunoprecipitated the appropriate rat viral proteins (Fig. 1, lanes 3 to 6). Additionally, each antiserum immunoprecipitated a protein of 65,000 M_r from short-term (1-h)-labeled SD-1T cells (Fig. 2). The size of this protein (~65,000 M_r) indicated that it was a viral *gag* precursor similar to that seen in mouse type C virus-producing cells (4). In addition to the immunoprecipitation of low-molecular-weight proteins, a protein of approximately 35,000 to 40,000 M_r was precipitated by both p27 and p10 antisera (Fig. 1, lanes 5 and 6), indicating that this molecule was probably an uncleaved p27-p10 intermediate (13; A. Schultz, personal communication). p15 and p12 sera both precipitated a protein of 27,000 M_r which ran slightly slower than viral p27 (Fig. 1, lanes 3 and 4). The size of this protein suggests that it represented the intact p15-p12 intermediate. In further support of this observation, it has been found that p12 is the major phosphoprotein of the rat type C virus and that p15 and p12 sera but not p27 and p10 sera immunoprecipitate a phosphorylated protein of approximately 27,000 M_r (unpublished data). The presence of these intermediates suggests that the processing of the rat type C *gag* precursor resembles that of the mouse type C *gag* precursor (4).

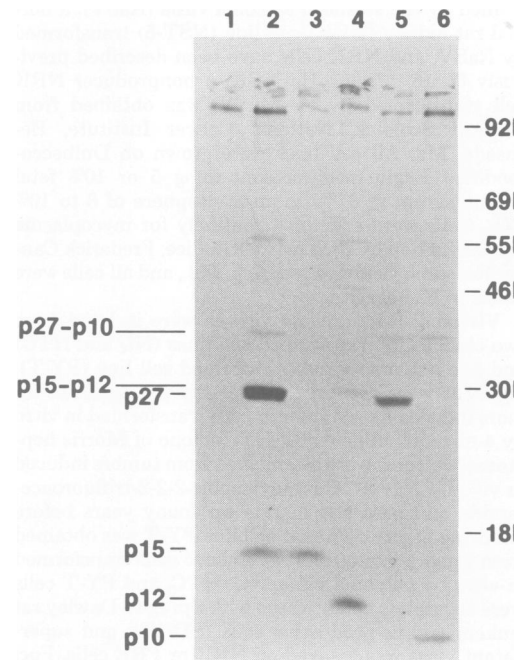

FIG. 1. *Immunoprecipitation of rat type C virus low-molecular-weight proteins. Rat type C virus was labeled with [^{3}H]leucine as described in the text. Each lane represents approximately 100,000 to 150,000 ^{3}H cpm added before immunoprecipitation and electrophoresis on a 13% polyacrylamide gel. Lane 1, Normal rabbit serum; lane 2, SD-1T whole virus antiserum; lane 3, p15 antiserum; lane 4, p12 antiserum; lane 5, p27 antiserum; lane 6, p10 antiserum. The markers and their molecular weights are as follows: phosphorylase b, 92,000 (92K); bovine serum albumin, 69K; immunoglobulin G, 55K; ovalbumin, 46K; carbonic anhydrase, 30K; lactoglobulin A 18K.*

Immunoprecipitation of RaSV p29 by viral antisera. Each of the viral *gag* gene product-specific antisera was then tested for immunoreactivity to RaSV p29 (Fig. 3). Only whole virus antiserum (lane 4), the serum which was reactive with viral p15 (lane 5), and tumor-bearing rat serum (lane 9) were able to immunoprecipitate RaSV p29 (Fig. 3). This indicated that the additional determinants found in RaSV p29

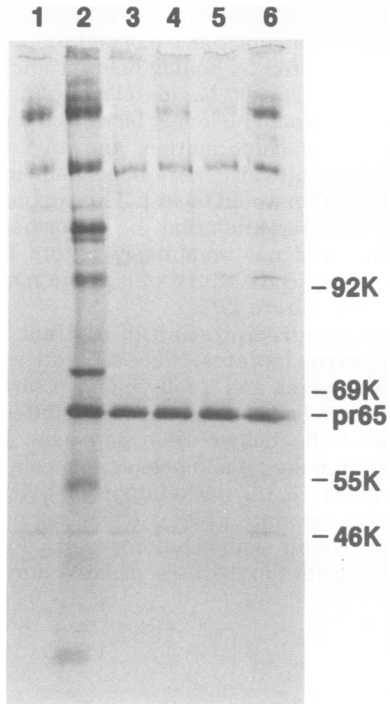

FIG. 2. *Immunoprecipitation of rat type C virus gag precursor. Labeling of SD-1T cells with [^{35}S]-methionine for 1 h was performed as described in the text. Each lane represents approximately 2 × 10^6 to 3 × 10^6 ^{35}S cpm added before immunoprecipitation and electrophoresis on an 8% polyacrylamide gel. Lane 1, Normal rabbit serum; lane 2, SD-1T whole virus antiserum; lane 3, p15 antiserum; lane 4, p12 antiserum; lane 5, p27 antiserum; lane 6, p10 antiserum. Markers are the same as in the legend to Fig. 1.*

originated from viral p15. No additional protein bands representing additional *gag* proteins were detected in RaSV nonproducer cells with any of the sera tested.

Immunoprecipitation of V-8 protease-digested p29 and Ha-MuSV p21. We previously reported that V-8 protease digestion of RaSV p29 and Ha-MuSV p21 yielded very similar patterns (26). With the use of higher V-8 protease concentrations and elution of p29 and p21 from the gel slices, we detected small differences between p29 and p21 (Fig. 4). Four bands appeared in V-8 protease-digested p29 and p21 (Fig. 4, lanes 2, 4, 6). Doublets always appeared upon immunoprecipitation of intact p29 and p21 (Fig. 4, lanes 1, 3, and 5) (12, 20, 21, 25), and although they are not easily seen in Fig. 4 due to overexposure of the gel, we were unable to separate these bands after immunoprecipitation. This observation of p29 and p21 doublets may account for the heterogeneity seen in V-8 protease-digested bands A and C in p29 (Fig. 4, lanes 2 and 4) and region A (Fig. 4, lane 6) in p21. These bands appeared to be very diffuse and could contain modified regions of the *src* proteins. The reason for the doublet in the intact and protease-treated protein is unclear at this time but probably represents phosphorylation of only one of the two bands since only a single band of both p29 and p21 is phosphorylated (12; unpublished data). Moreover, band C appeared in p29 but not in p21, whereas band E was present in p21 but not in p29 (Fig. 4, lanes 2, 4, and 6). Furthermore, there was no large extra [^{3}H]leucine-labeled band in p29 as might be expected if the p15 portion of p29 contained a V-8 protease site (Fig. 4, lane 4).

With p29 and p21 eluted from gel slices (Fig. 4), immunoprecipitation of intact p29 and p21 molecules or the V-8 protease fragments of p29 and p21 produced a somewhat unexpected result

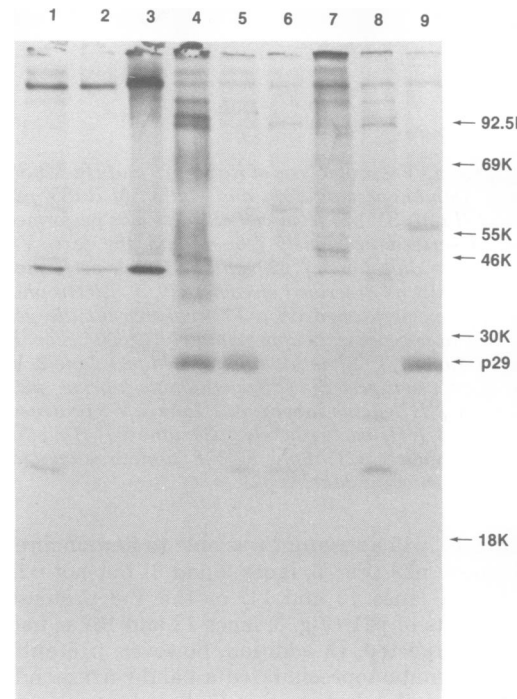

FIG. 3. *Immunoprecipitation of RaSV p29 by low-molecular-weight viral protein antiserum. Labeling of RaSV-transformed NRK cells with [^{35}S]methionine was performed as described in the text. Each lane represents approximately 5 × 10^6 ^{35}S cpm added before immunoprecipitation and electrophoresis on a 12% acrylamide gel. Lanes 1 and 2, Normal rabbit serum; lane 3, normal rat serum; lane 4, SD-1T whole virus antiserum; lane 5, p15 antiserum; lane 6, p12 antiserum; lane 7, p27 antiserum; lane 8, p10 antiserum; lane 9, tumor-bearing rat antiserum. Markers are the same as in Fig. 1.*

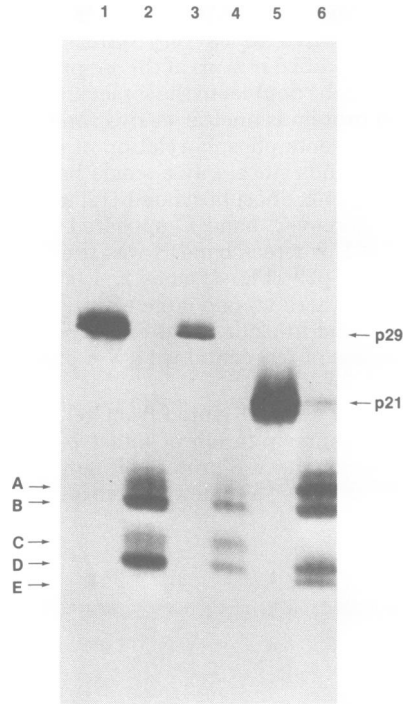

FIG. 4. *V-8 proteolysis of RaSV p29 and Ha-MuSV p21. Immunoprecipitation and elution of RaSV p29 and Ha-MuSV p21 from cell extracts was performed as described previously (26) and in the text. V-8 protease digestion of p29 and p21 was performed essentially as described elsewhere (6, 8). Electrophoresis was performed on a 17% polyacrylamide gel. Each lane represents approximately 10,000 ^{35}S or ^{3}H cpm. Lane 1, [^{35}S]methionine-labeled p29; lane 2, V-8 protease-digested, [^{35}S]methionine-labeled p29; lane 3, [^{3}H]leucine-labeled p29; lane 4, V-8 protease-digested, [^{3}H]leucine-labeled p29; lane 5, [^{35}S]methionine-labeled p21; lane 6, V-8 protease-digested, [^{35}S]methionine-labeled p21.*

(Fig. 5). p15 antiserum was able to immunoprecipitate p29 (Fig. 5, lanes 2 and 6) but not p21 (Fig. 5, lanes 10 and 14) or the V-8 protease products of p21 (Fig. 5, lanes 12 and 16) as had been expected. In addition, however, p15 antiserum immunoprecipitated a band corresponding most closely to region C (based on relative migration of p29 and the various V-8 protease bands shown in Fig. 4) of RaSV p29 (Fig. 5, lanes 4 and 8). Surprisingly, the tumor-bearing rat serum was unable to immunoprecipitate either intact p29 (Fig. 5, lanes 1 and 5), p21 (Fig. 5, lanes 9 and 13), or any of the V-8 protease peptides from either protein (Fig. 5, lanes 3, 7, 11, and 15). This suggested that the tumor-bearing rat serum contained antibodies directed against conformational determinants of p21, whereas p15 serum reacted with primary amino acid determinants. Since p29 and p21 molecules were denatured by boiling before sodium dodecyl sulfate-polyacrylamide electrophoresis, the molecules would no longer be expected to retain their native conformation, and any antigenic determinants which were dependent upon this conformation would be lost. This was consistent with the observation that the tumor-bearing rat serum could not immunoprecipitate temperature-sensitive Ki-MuSV p21 at the nonpermissive temperature (21).

Immunoprecipitation of distinct rat sarcoma virus isolates. Since separate isolates of feline sarcoma and woolly monkey sarcoma viruses have been shown to contain different portions of the helper virus *gag* gene products linked to nonviral and presumably cellular gene products (1, 3, 18), it was important to determine whether separate isolates of rat sarcoma virus would exhibit similar heterogeneity. Five separate transforming viruses, isolated during sepa-

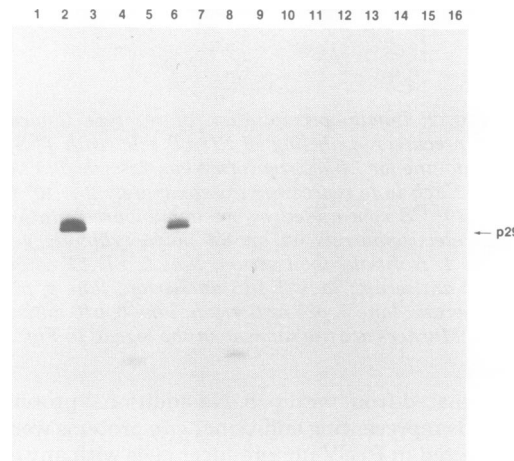

FIG. 5. *Immunoprecipitation of V-8 protease digestion of p29 and p21 peptides. Immunoprecipitation and elution of RaSV p29 and Ha-MuSV p21 from cell extracts was performed as described previously (16) and in the text. V-8 protease digestion of p29 and p21 and immunoprecipitation of V-8 peptides were performed essentially as described elsewhere (6, 8). Electrophoresis was performed on a 17% polyacrylamide gel. Odd-numbered lanes, Tumor-bearing rat serum; even-numbered lanes, rabbit p15 antiserum. Lanes 1 and 2, [^{35}S]methionine-labeled p29; lanes 3 and 4, V-8 protease-digested, [^{35}S]methionine-labeled p29, lanes 5 and 6, [^{3}H]leucine-labeled p29; lanes 7 and 8, V-8 protease-digested, [^{3}H]leucine-labeled p29; lanes 9 and 10, [^{35}S]methionine-labeled p21, lanes 11 and 12, V-8 protease-digested, [^{35}S]-methionine-labeled p21; lanes 13 and 14, [^{3}H]leucine-labeled p21; lanes 15 and 16, V-8 protease-digested, [^{3}H]leucine-labeled p21.*

rate cocultivations of SD-1T cells with other transformed rat cells by both S. Rasheed and H. Young (see above), were examined. All five isolates clearly contained the p29 doublet, although the intensity of the upper and lower bands differed (Fig. 6, lanes 6 to 10). As stated earlier, the reason for the doublet and the variation in intensity of the two bands is probably due to differences in phosphorylation of the protein, since phosphate labeling experiments show only a single p29 band (unpublished data); however, neither of these two bands was precipitated by normal rat sera (Fig. 6, lanes 1 to 5). Although it is not shown in this figure, p15 antiserum was also able to immunoprecipitate both p29 bands from each of the five isolates, indicating that the structure of p29 is identical in each case (i.e., a portion of viral p15 fused to *src* p21).

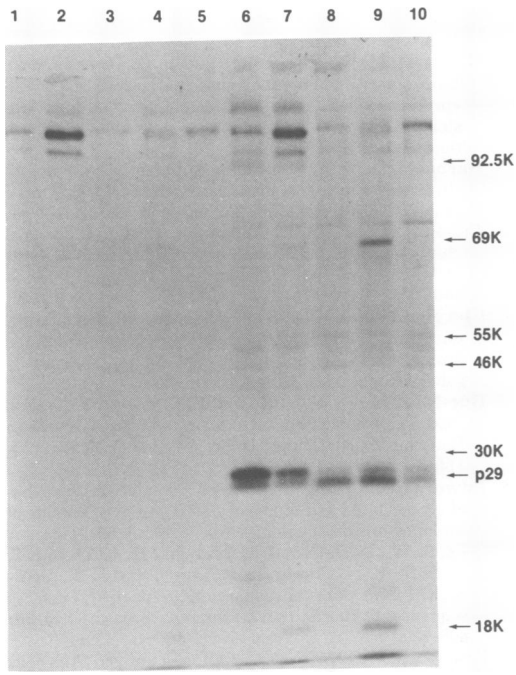

FIG. 6. *Immunoprecipitation of cell extracts obtained from cells transformed by separate isolates of rat sarcoma virus. Labeling of cells with [^{35}S]methionine was performed as described in the text. Each lane represents approximately 5 × 10^6 ^{35}S cpm added before immunoprecipitation and electrophoresis on a 10% polyacrylamide gel. Lanes 1 through 5, Normal rat serum; lanes 6 through 10, tumor-bearing rat serum. Lanes 1 and 6, NST-6 (NRK cell transformed by NQ plus SD-1T-rescued RaSV) (Rasheed); lanes 2 and 7, NST-7 (NRK cell transformed by PY-T plus SD-1T-rescued RaSV) (Rasheed); lanes 3 and 8, C16E (FRE cell transformed by NQ plus SD-1T-rescued RaSV) (Young); lanes 4 and 9, FST-9 (FRE cell transformed by PY-T plus SD-1T-rescued RaSV) (Rasheed); lanes 5 and 10, FST-2 (FRE cell transformed by HTC plus SD-1T-rescued RaSV) (Rasheed). Markers used are the same as described in Fig. 1.*

DISCUSSION

We have demonstrated that RaSV p29 represents a fusion product of rat type C virus p15 and p21 rat transforming gene product. Antiserum directed against viral p15, but not the other viral *gag* gene products, was capable of immunoprecipitating rat sarcoma virus p29, a V-8 protease cleavage product of p29, and a rat type C virus *gag* precursor. Interestingly, each of five separate isolates of rat sarcoma virus codes for p29, suggesting either that this region of the rat type C viral genome represents a selective spot for genetic recombination or that the structure of the viral *src* protein (p15-p21) is important in the transformation event. With regard to the latter possibility, it has been previously reported that Ha-MuSV p21 is located on the inner surface of the cell membrane (23), suggesting that p21 or a precursor of p21 might contain certain hydrophobic regions required for membrane association. Since murine type C virus p15 is highly hydrophobic (2) and can be cross-linked to lipids (14), this RaSV p29 portion may facilitate the insertion of this *src* protein into the cell membrane. Since fibroblast transformation was the criterion used during the in vitro isolation of this virus, those viruses which contained a p15-*src* configuration might be selected for, since this configuration of the *src* protein would be necessary for transformation.

This model does not explain the fact that Ha-MuSV and Ki-MuSV apparently code only for p21 *src* protein. These viruses were derived by the passage of mouse type C viruses through rats and mice and represent recombinants between the mouse type C viruses, the rat endogenous replication-defective 30S genome, and the p21 *sarc* gene (9, 22, 25). Since it is uncertain as to how many rounds of recombination were necessary to derive these viruses, it might be argued that Ha-MuSV and Ki-MuSV represent deletion mutants of a larger *src* protein and that the p21 portion of the gene is required for transformation and thus retained. Evidence for the generation of numerous deletion mutants during infection in vitro has recently been reported for Moloney murine sarcoma virus (5). However, it should also be noted that both heteroduplex and restriction enzyme studies indicate that the transforming region of Ha-MuSV is approximately 1 kilobase (9, 25), a region of DNA larger than that needed to code for a protein of 21,000 M_r. The extent and location of the protein-coding infor-

mation in this gene remain to be elucidated and will require sequence analysis of this DNA.

A second important question with regard to RaSV is how the virus is generated during the cocultivation experiment. As stated earlier, it may be due to a rare recombination event which occurs during the cocultivation or the possibility that the sarcoma virus genome preexists in one of the two cells utilized in the experiment. In the latter case, expression of the sarcoma virus genome could be repressed in the parent cells and may be transient during cocultivation. Such transient expression of transforming genomes has been suggested by Rapp and Todaro in studies involving the generation of organ-specific, mouse transforming retroviruses (15). It should be noted that neither parent cell produces any detectable amounts of p29, although both cells express low levels of p21 (H. Young, unpublished data). Furthermore, although both of the rat cell lines express the replication-defective 30S genome (the rat genetic information in Ha-MuSV and Ki-MuSV), the relationship of 30S RNA expression to the p21 gene expression is unclear, since no 30S RNA has been detected in RaSV and no correlation of 30S RNA expression and p21 has been observed (19, 25; unpublished data). In other studies, we have been unable to generate a sarcoma virus by direct cell fusion experiments between the two parental rat cell lines or by infecting other rat cells with rat type C virus from the SD-1T cells. The complete analysis of how RaSV was generated will require genetic analysis of the integration sites of both the p21 gene and the rat type C genome in each of the parental cells. These studies are currently in progress, and preliminary analysis indicates that the *sarc* gene arrangement in SD-1T cells appears to be the same as normal rat DNA (R. Ellis and H. Young, unpublished data). This would suggest that rat sarcoma virus does not preexist in SD-1T cells and that recombination is essential for the generation of this virus. We cannot, however, completely rule out the possibility that the sarcoma virus genome does preexist in a minor subpopulation of SD-1T cells.

In summary, the gene product of RaSV represents a fusion product between a rat type C virus *gag* gene protein and a gene product of cellular origin. Similar fusion genes have been observed in a number of other mammalian transforming viruses (1, 3, 18, 24), but in contrast to these other viruses, RaSV appears to be highly specific in the composition of its gene product. Furthermore, RaSV gene product structure may provide an important model for insertion of proteins into cellular membranes and the subsequent events which result in cellular transformation.

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