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Different rat-derived transforming retroviruses code for an immunologically related intracellular phosphoprotein

(sarcoma virus/immunoprecipitation/helper virus)

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ABSTRACT **Kirsten sarcoma virus (Ki-MSV) and Harvey sarcoma virus (Ha-MSV) are mouse–rat recombinant viruses that were originally isolated by experimental inoculation of rats with helper-independent mouse type C viruses. We have recently identified in cells transformed by Ki-MSV or Ha-MSV, a phosphoprotein, p21, coded for by Ki-MSV and Ha-MSV [Shih, T. Y., Weeks, M. O., Young, H. A. & Scolnick, E. M. (1979) *Virology* 95, in press]. The p21, which is not a virion structural protein, was identified with antisera prepared by transplantation in rats of syngeneic Ha-MSV- or Ki-MSV-transformed nonproducer cells. In this study, we have applied the same methodology to examine a purely rat sarcoma virus (RaSV), which was isolated in cell culture by using helper-independent rat type C viruses [Rasheed, S., Gardner, M. B. & Huebner, R. J. (1978) *Proc. Natl. Acad. Sci. USA* 75, 2972–2976]. We report here that this new, purely rat sarcoma virus apparently codes for a p29, which shares immunological determinants and common V-8 protease-generated peptides with the p21 of Ha-MSV. The data suggest that the RaSV has acquired genetic information with similar coding capacity to some rat genetic information in the mouse–rat recombinant viruses, Ki-MSV and Ha-MSV. Based on data obtained on the p21 of a mutant of Ki-MSV temperature-sensitive for the maintenance of transformation, we suggest that the gene in RaSV that codes for the p29 is also required for the maintenance of RaSV-induced fibroblast transformation.**

Type C retroviruses with the potential to transform fibroblasts or more specialized target cells have been isolated from several vertebrate species (1–3). Except for certain strains of Rous sarcoma virus, all the isolates are replication-defective viruses. In most cases, these transforming viruses have been shown to be recombinants between portions of the genomes of helper-independent type C viruses and other genetic information derived from the host species from which the transforming virus was originally isolated. In studies on isolates derived from a given species, one of the interesting facts that has emerged is the diversity in the newly acquired host genetic information among the isolates (4, 5).

In the case of viruses of mammalian origin, we have been especially interested in two independent viral isolates from rats, Kirsten sarcoma virus (Ki-MSV) and Harvey sarcoma virus (Ha-MSV). Both of these viruses have been shown to be mouse–rat recombinant viruses and detailed maps of the viral genomes have been reported (6). Recently, a third isolate of a retrovirus from rats with the ability to transform fibroblasts has been reported (7). This rat transforming virus (RaSV) was isolated by cocultivating a transformed Sprague–Dawley cell line producing helper-independent rat type C viruses with a Fischer rat tumor cell, originally transformed after treatment with 4-nitroquinoline. The Fischer rat cell line had been transplanted in syngeneic Fischer rats and then reestablished in cell culture prior to cocultivation.

With the isolation of the RaSV in cell culture, we had the opportunity to compare the genome of the new RaSV to the genomes of Ki-MSV and Ha-MSV isolated by experimental passage of helper-independent mouse type C viruses in rats (8, 9). We report here (*i*) that RaSV contains sequences homologous to helper-independent rat type C virus and, as expected, no sequences of helper-independent mouse type C viruses, (*ii*) that we cannot detect with currently available cDNA probes homology between Ki-MSV and RaSV, and (*iii*) that RaSV codes for a phosphoprotein, p29, which immunologically crossreacts and shares *Staphylococcus aureus* V-8 protease-generated peptides with the p21 nonvirion phosphoprotein, recently shown to be coded for by Ha-MSV and Ki-MSV (10). The significance of the p29 of RaSV and the p21 of Ha-MSV and Ki-MSV with regards to the oncogenic potential of these viruses is discussed, based on studies of the p21 in a mutant of Ki-MSV temperature-sensitive for the maintenance of transformation.

MATERIALS AND METHODS

Cells and Viruses. *Mouse cells.* The cell line C127 is contact-inhibited and derived from an RIII mouse by procedures developed to isolate contact-inhibited cells (11). An Ha-MSV-transformed nonproducer cell derived from this cell was the gift of Harry Langbeheim (National Cancer Institute). He obtained this nonproducer cell by using a limiting dilution of an ecotropic Moloney leukemia virus pseudotype of Ha-MSV grown in C127 cells. Procedures employing Falcon microtest II plates to isolate the nonproducer cell have been described (12). An NIH nonproducer cell transformed by Ha-MSV has been described (10) and an NIH nonproducer cell transformed by Schmidt–Ruppin avian sarcoma virus was the gift of Geoffrey Cooper (Harvard Medical School).

Rat cells. Uninfected rat cells used include NRK cells (13); a contact-inhibited cell derived from a Fischer rat embryo, FRE clone 2; and a transformed cell, 4NQ. The 4NQ cell was derived after treatment of other Fischer rat embryo cells with 4-nitroquinoline (7). Transformed cells derived after this treatment were transplanted in syngeneic rats, and a cell line was reestablished after tumor development. The characterization of this cell will be detailed elsewhere; it has only low levels of RNA ($C_rt_{1/2} > 5000$ mol·sec·liters^{-1}) homologous to helper-inde-

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Abbreviations: Ki-MSV, Kirsten sarcoma virus; Ha-MSV, Harvey sarcoma virus; RaSV, rat "sarcoma" virus; p, phosphoprotein; $C_rt_{1/2}$, initial concentration of RNA (moles of nucleotide per liter) × time (seconds) necessary for 50% hybridization; Mo-MuLV, Moloney murine leukemia virus; $NaDodSO_4$, sodium dodecyl sulfate; Staph A, protein A.

pendent rat type C viruses, and high levels of RNA ($C_rt_{1/2}$, 100–200 mol·sec·liters^{-1}) homologous to the rat 30S RNA subunit (unpublished observations).

Rat cells either producing virus or infected exogenously with virus include: a transformed Sprague–Dawley cell producing rat type C viruses (SD-1); NST-6, a transformed NRK cell producing SD-1 rat leukemia virus and RaSV; NST-5, an NRK nonproducer cell transformed by RaSV and obtained as described (7). Other infected cells include FRE clone 2 cells producing Sprague–Dawley rat type C virus, which was obtained by subjecting the original SD-1 virus to two cycles of limiting dilution on the FRE clone 2 cells. This cell is called FRE/SD-1. An FRE clone 2 nonproducer cell transformed by RaSV was obtained by cocultivating the SD-1 and 4NQ cells, inoculating virus from this cocultivation on FRE cells, and isolating transformed cells as described (7). This cell is called FRE-6E. Other cells used include a nonproducer FRE cell transformed by the FBJ mouse sarcoma virus. The derivation of this cell will be described elsewhere. A culture of FRE cells producing Ha-MSV and Moloney murine leukemia virus (Mo-MuLV) with approximately a 20-fold excess of Ha-MSV has been described (10). It was used to produce virus for Ha-MSV viral RNA. All cells were routinely grown at 37°C in an atmosphere of 8–10% CO_2 in Dulbecco–Vogt modified Eagle's medium with 10% calf serum. Cells were assayed for aerobic and anaerobic mycoplasma by Flow Laboratories (McLean, VA) and found to be negative. Positive controls were employed in each mycoplasma test.

Preparation of Antiserum. Anti-rat helper virus serum was prepared by injecting sucrose gradient isopycnically banded SD-1 virus into adult white rabbits. Antiserum to the RaSV p29 or the Ha-MSV or Ki-MSV p21 was obtained by injecting 2×10^7 nonproducer NRK or FRE cells into syngeneic 10-day-old rats and bleeding the rats 3–6 weeks after tumor appearance (10).

[^{35}S]Methionine Labeling of Cells and Immunoprecipitations. Labeling of cells with [^{35}S]methionine was performed as described (10). Cell extracts were prepared in PD buffer [1.0% Triton X-100/0.5% deoxycholate/0.1% sodium dodecyl sulfate ($NaDodSO_4$)/0.1 M NaCl, 1mM EDTA/0.01 M sodium phosphate, pH 7.5/1.0% Trasylol]. For cells labeled with 500 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine per ml, $1–4 \times 10^8$ trichloroacetic acid-precipitable ^{35}S cpm were obtained using $1–2 \times 10^6$ cells. Immunoprecipitation was carried out at 4°C by the addition of 5 μl of serum to cell extracts containing 5×10^6 acid-precipitable [^{35}S]methionine cpm ($\approx 1 \times 10^5$ cells) in a final volume of 250 μl of PD buffer for 20 hr, and antigen-antibody complexes were then removed from the cell extract by the addition of 50 μl of a 10% suspension of formaldehyde-fixed *S. aureus* Cowan Strain I containing protein A (Staph A) (14). After incubation at 4°C for 60 min, the Staph A was removed by centrifugation at 3000 rpm for 10 min, the pellets were washed three times in PD buffer, and the precipitated proteins were prepared for electrophoresis by resuspending the pellets in 40 μl of buffer (0.05 M Tris·HCl, pH 6.8/1% $NaDodSO_4$/0.14 M 2-mercaptoethanol/20% glycerol/0.002% bromphenol blue). The resulting suspension was heated at 95°C for 3 min, insoluble material was removed by centrifugation, and the supernatants were subjected to electrophoresis (see below).

***In Vitro* Translation of Viral RNA.** *In vitro* translation of Ha-MSV viral RNA was carried out in nuclease-digested rabbit reticulocyte extracts as described (10). For the competition assay performed here, unlabeled cellular extracts were prepared essentially as described for preparation of labeled cellular extracts (10), except that the cells were disrupted in a buffer containing 0.02 M Tris·HCl (pH 7.2), 0.1 M NaCl, 5 mM $MgCl_2$, 1% Triton X-100, and 1% Trasylol, and the precleaning step was omitted. A limiting dilution of rat antiserum (1–2 μl) was incubated at 4°C for 4–6 hr with varying amounts of the cellular protein in a 0.20-ml reaction mixture containing PD buffer. Then, [^{35}S]methionine (4×10^5 acid-precipitable counts from *in vitro* translation of Ha-MSV RNA) was added to each tube in 70 μl of PD buffer, bringing the final reaction mixture volume to 0.27 ml. The incubation was continued for 18 hr at 4°C. Antigen–antibody complexes were recovered by using Staph A and dissolved (as above) for electrophoresis on 11.5% acrylamide gels.

Polyacrylamide Gel Electrophoresis. Discontinuous gel electrophoresis was carried out by using a 3.5% acrylamide stacking gel and a 10%, 11.5%, or 13% acrylamide separating gel containing 0.38 M Tris·HCl (pH 8.8), 0.1% $NaDodSO_4$, 0.5% linear polyacrylamide, 0.3% bis acrylamide, and a Tris glycine running buffer containing 0.025 M Tris·HCl (pH 8.8), 0.1% $NaDodSO_4$, and 0.19 M glycine (15). Fluorography was carried out as described (16) by using Kodak XR-1 film. Peptide analysis was carried out as described (17), using *S. aureus* V-8 protease.

Hybridization Analysis. Hybridization analysis with cDNAs from rat helper virus, Mo-MuLV, 3′ Mo-MuLV, or Ki-MSV was carried out at 67°C for 20 hr in 0.75 M NaCl as described (18) to RNA extracted from FRE cells or RaSV-transformed nonproducer cells (6E). Hybrids were analyzed by using S1 nuclease (19), and C_rt values were corrected to 0.18 M NaCl as described by Birnstiel *et al.* (20).

RESULTS

In order to determine whether RaSV and Ha-MSV coded for any common proteins, nonproducer cells transformed by RaSV were pulse labeled with [^{35}S]methionine and immunoprecipitated with rat sera made by syngeneic transplantation of Ha-MSV nonproducer cells. The results are shown in Fig. 1. As reported (10), mouse cells transformed by Ha-MSV contain a p21 that can be precipitated with this serum, and this p21 has been shown to be coded for by Ha-MSV by translation of Ha-MSV RNA (21). The same serum precipitated a p29 from nonproducer Fischer rat cells and Osborne Mendel rat cells transformed by RaSV (lanes 9 and 12), whereas no p29 was seen in control FRE cells (lane 10) or NRK cells (lane 7). In studies not shown, p29 was not detected in FRE cells transformed by FBJ sarcoma virus or NRK cells transformed by Schmidt–Ruppin virus or Moloney sarcoma virus. In other controls, no additional protein bands were detected in nontransformed FRE cells infected with SD-1 rat type C virus (lane 11) or in transformed Sprague–Dawley cells producing SD-1 virus (lane 8) compared to uninfected rat cells. The results suggested either that there was an immunological crossreaction between the p21 of Ha-MSV and the p29 of RaSV or that the same antiserum contained antibodies independently reactive with p21 and p29.

To approach this question, other Ha-MSV antisera with varying ability to precipitate Ha-MSV p21 were tested against cells transformed by RaSV and Ha-MSV. The results are shown in Fig. 2. In all, nine sera were tested. The same sera that precipitated the p21 (Fig. 2*B*) also precipitated p29 (Fig. 2*A*) (lanes 9–18), whereas sera that had low or undetectable titers of antibody to p21 (Fig. 2*B*) failed to precipitate the p29 (Fig. 2*A*) (lanes 1–8). In other studies, four antisera prepared to Ki-MSV nonproducer cells that contained anti Ki-MSV p21 antibodies also precipitated the p29 of RaSV. The results are consistent with the possibility that p21 and p29 share immunochemical determinants.

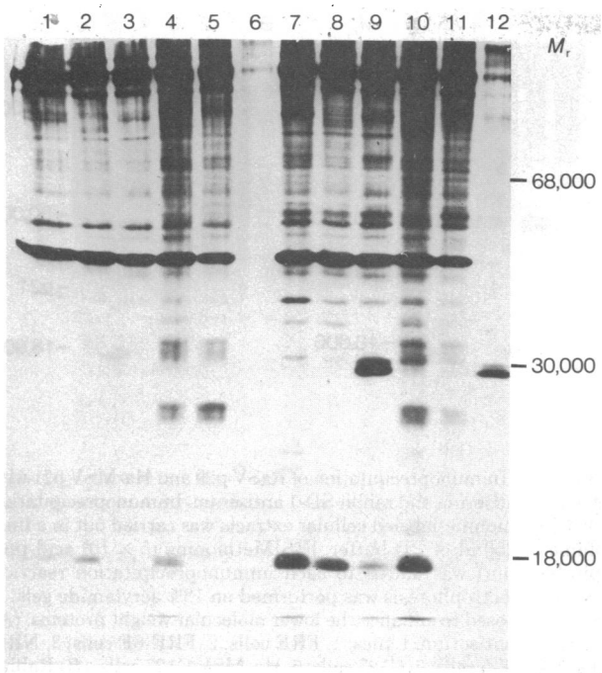

FIG. 1. Immunoprecipitation of p29 with antiserum prepared against Ha-MSV nonproducer cells. After immunoprecipitation of [^{35}S]methionine-labeled cellular extracts NaDodSO$_4$ gel electrophoresis was carried out on 10% polyacrylamide gels. Each immunoprecipitation reaction mixture contained approximately 5×10^6 acid-precipitable cpm. Lanes: 1, normal rat serum and NRK cells; 2, normal rat serum and SD-1 cells; 3, normal rat serum and NRK nonproducer cells transformed by RaSV (NST-5); 4, normal rat serum and FRE cells; 5, normal rat serum and FRE/SD-1 cells; 6, normal rat serum and FRE nonproducer cells (clone 6E) transformed by RaSV; 7, Ha-NRK rat serum and NRK cells; 8, Ha-NRK rat serum and SD-1 cells; 9, Ha-NRK rat serum and NST-5 cells; 10, Ha-NRK rat serum and FRE cells; 11, Ha-NRK rat serum and FRE/SD-1 cells; 12, Ha-NRK rat serum and FRE-6E cells.

Immunoprecipitation and Competition of *In Vitro* Synthesized Ha-MSV p21. To determine more directly whether there was crossreactivity between Ha-MSV p21 and RaSV p29, we first transplanted the FRE-6E cell in Fischer rats and produced an antiserum that precipitated RaSV p29 (see below for details). This antiserum was found to precipitate *in vitro* synthesized Ha-MSV p21, and a limiting dilution of this anti-p29 serum (2 μl) was used with *in vitro* synthesized [^{35}S]methionine-labeled Ha-MSV p21 in a competition immunoprecipitation assay. The competition assay is displayed on an acrylamide gel in Fig. 3. The immunoprecipitation of the Ha-MSV p21 by the RaSV antiserum was almost completely blocked by the addition of unlabeled cellular extract from 6E cells but not from FRE cells, thus indicating that the RaSV cellular extract contains a protein that interacts directly with the antibodies that are specific for Ha-MSV p21. Similar results were obtained when Ha-MSV antiserum replaced RaSV antiserum, because the extract from RaSV-transformed cells could compete effectively with this antibody–antigen complex as well.

Peptide Analysis of p21 and p29. To analyze the methionine-labeled peptides of the Ha-MSV p21 and RaSV p29, p21- and p29-immunoprecipitated protein bands like those shown in Fig. 1 were excised from the gel, treated with *S. aureus* V-8 protease, and electrophoresed on 17% acrylamide gels. The results are shown in Fig. 4. The p21 cleaved by V-8 protease yielded two peptide bands, slightly separated, which are visible in lane 4. The p29 yielded three peptide bands, two of which migrated identically to those observed with the Ha-MSV p21 (lane 3). The results using this one-dimensional peptide analysis

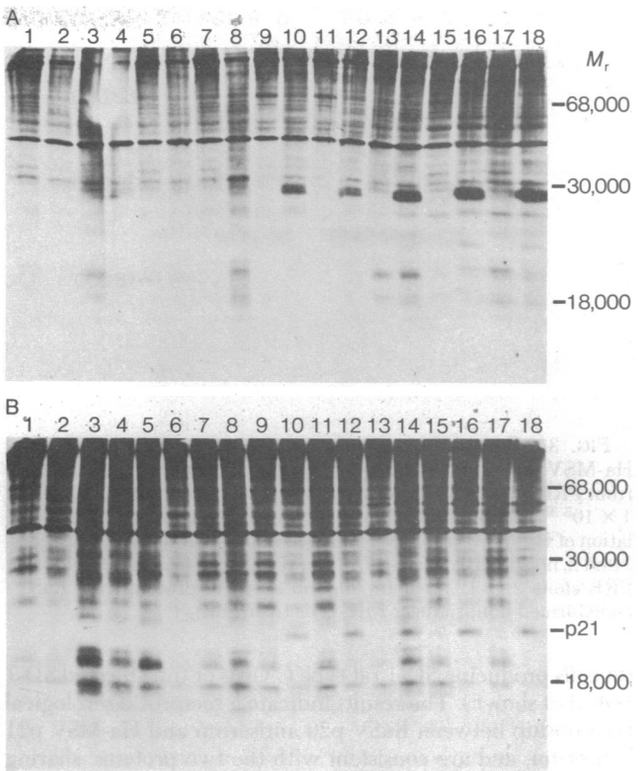

FIG. 2. Immunoprecipitation of RaSV p29 and Ha-MSV p21 with various rat antisera. Immunoprecipitation of [^{35}S]methionine-labeled cellular extracts were carried out in a final volume of 250 liters of PD buffer. [^{35}S]Methionine (5×10^6 acid-precipitable cpm) was added to each reaction mixture. Electrophoresis was performed on 12% acrylamide gels. (*A*) Lanes: 1, normal rat serum and FRE cells; 2, normal rat serum and FRE-6E cells; 3, rat serum 1 and FRE cells; 4, rat serum 1 and FRE-6E cells; 5, rat serum 2 and FRE cells; 6, rat serum 2 and FRE-6E cells; 7, rat serum 3 and FRE cells; 8, rat serum 3 and FRE-6E cells; 9, rat serum 4 and FRE cells; 10, rat serum 4 and FRE-6E cells; 11, rat serum 5 and FRE cells; 12, rat serum 5 and FRE-6E cells; 13, rat serum 6 and FRE cells; 14, rat serum 6 and FRE-6E cells; 15, rat serum 7 and FRE cells; 16, rat serum 7 and FRE-6E cells; 17, rat serum 8 and FRE cells; 18, rat serum 8 and FRE-6E cells.

(*B*) Lanes: 1, normal rat serum and Schmidt–Ruppin (SR)-NIH cells; 2, normal rat serum and Ha-MSV NIH cells; 3, rat serum 1 and SR-NIH cells; 4, rat serum 1 and Ha-MSV NIH cells; 5, rat serum 2 and SR-NIH cells; 6, rat serum 2 and Ha-MSV NIH cells; 7, rat serum 3 and SR-NIH cells; 8, rat serum 3 and Ha-MSV NIH cells; 9, rat serum 4 and SR-NIH cells; 10, rat serum 4 and Ha-MSV NIH cells; 11, rat serum 5 and SR-NIH cells; 12, rat serum 5 and Ha-MSV NIH cells; 13, rat serum 6 and SR-NIH cells; 14, rat serum 6 and Ha-MSV NIH cells; 15, rat serum 7 and SR-NIH cells; 16, rat serum 7 and Ha-MSV NIH cells; 17, rat serum 8 and SR-NIH cells; 18, rat serum 8 and Ha-MSV NIH cells.

is additional evidence for the structural similarities of these two proteins.

Further Characterization of p29. To further explore the relationship between the p29 found in RaSV-transformed cells and the Ha-MSV p21, the RaSV-transformed Fischer rat cells were transplanted in 10-day-old Fischer rats. An antisera (used above in the competition assay) obtained from a rat after 6 weeks of tumor growth was tested against Ha-MSV-transformed cells and RaSV-transformed cells and the results are shown in Fig. 5*A*. The antisera precipitated p29 from RaSV-transformed cells (lanes 2, 4) and p21 from Ha-MSV-transformed mouse cells (lane 6). As noted above, the same serum also precipitated the *in vitro* Ha-MSV p21. No additional rat helper virus-specific protein bands were precipitated from nontransformed Fischer

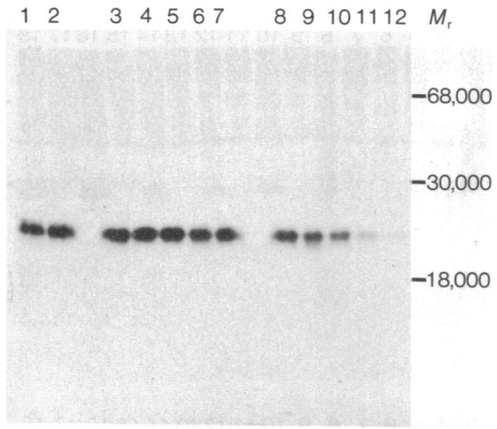

FIG. 3. Competition immunoassay with *in vitro* synthesized Ha-MSV p21. Competition assays with unlabeled cellular extracts from FRE cells and FRE-6E cells were performed. All lanes contain 4×10^5 ^{35}S acid-precipitable cpm obtained from the *in vitro* translation of Ha-MSV viral RNA and 2 μl of RaSV p29 antiserum. Lanes: 1 and 2, no added extract; 3–7, 3, 7.5, 15, 30, and 45 μg of protein from FRE clone 2; 8–12, 2, 5, 10, 20, and 30 μg of protein from RaSV-transformed nonproducer FRE cells (clone 6E).

rat cells producing SD-1 rat type C virus or transformed SD-1 cells (not shown). The results indicate a reciprocal serological relationship between RaSV p29 antiserum and Ha-MSV p21 antiserum, and are consistent with the two proteins' sharing common immunological determinants.

Because of the difference in molecular weight and peptide analysis between RaSV p29 and Ha-MSV p21, we tested rabbit antisera prepared against sucrose gradient isopycnically banded SD-1 rat leukemia viruses with [^{35}S]methionine-labeled extracts from RaSV- and Ha-MSV-transformed cells. The results are shown in Fig 5*B*. The SD-1 antiserum precipitated the p29 from the RaSV-containing cells (lanes 2, 4). In contrast to the RaSV p29 antiserum and the Ha-MSV p21 antiserum, the SD-1 virus antiserum did not precipitate the p21 of Ha-MSV (lane 6). This SD-1 antiserum was able to precipitate rat helper virus proteins from [^{35}S]methionine-labeled SD/FRE or SD-1 cellular extracts (not shown). The results with the SD-1 virus antiserum suggest that p29 contains determinants contained in a structural protein(s) of SD-1 rat type C virus, whereas p21 does not. These results do not, however, allow us to determine to which rat virus protein the p21-related peptides are linked. In other studies to characterize p29, cells transformed by RaSV were labeled for 2 hr with inorganic ^{32}P, and the extracts of these cells were

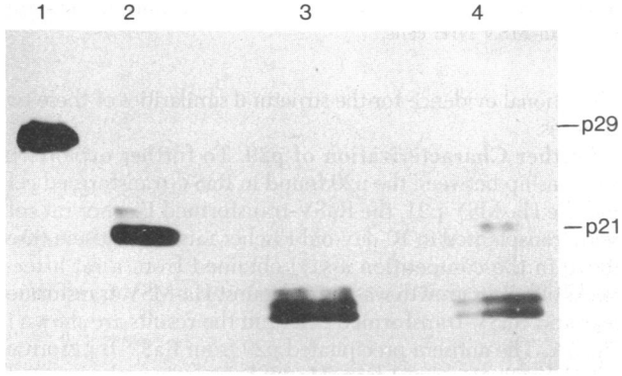

FIG. 4. Peptide analysis of RaSV p29 and Ha-MSV p21. Peptide analysis of RaSV p29 and Ha-MSV p21 was carried out as described (17). Lanes: 1, RaSV p29; 2, Ha-MSV p21; 3, RaSV p29 and 2 μg of V-8 protease; 4, Ha-MSV p21 and 2 μg of V-8 protease.

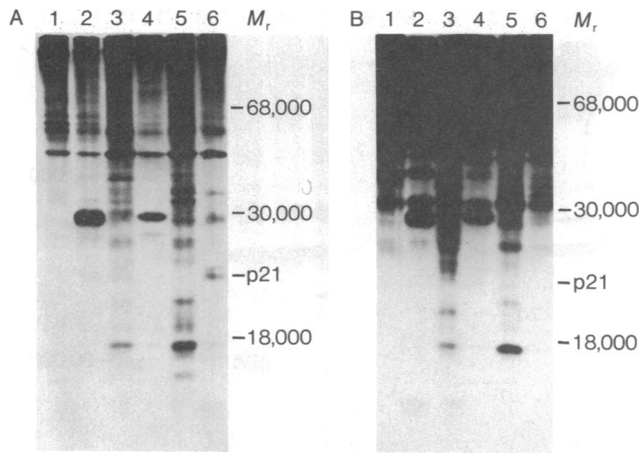

FIG. 5. Immunoprecipitation of RaSV p29 and Ha-MSV p21 with rat RaSV antiserum and rabbit SD-1 antiserum. Immunoprecipitation of [^{35}S]methionine-labeled cellular extracts was carried out in a final volume of 250 μl of PD buffer. [^{35}S]Methionine (5×10^6 acid-precipitable cpm) was added to each immunoprecipitation reaction mixture. Electrophoresis was performed on 13% acrylamide gels. *B* was overexposed to enhance the lower molecular weight proteins. (*A*) Rat RaSV antiserum. Lanes: 1, FRE cells; 2, FRE-6E cells; 3, NRK cells; 4, NST-5 cells; 5, C127 cells; 6, Ha-MSV C127 cells. (*B*) Rabbit SD-1 antiserum. Lanes: 1, FRE cells; 2, FRE-6E cells; 3, NRK cells; 4, NST-5 cells; 5, C127 cells; 6, Ha-MSV C127 cells.

immunoprecipitated with either Ha-MSV p21 antiserum or SD-1 antiserum. In results not shown, ^{32}P-labeled p29 was shown to be precipitable by the same Ha-MSV p21 antiserum, RaSV p29 antiserum, and SD-1 virus antiserum that precipitated the [^{35}S]methionine-labeled p29; therefore the p29, like the Ha-MSV and Ki-MSV p21, is a phosphoprotein.

Hybridization Analysis of RaSV. Because of the immunological relationships between RaSV p29 and Ha-MSV and Ki-MSV p21, we compared the genetic composition of RaSV to that of Ki-MSV by molecular hybridization. Hybridization analysis was performed to RaSV nonproducer cellular RNA with [^3H]cDNA homologous to either rat helper virus (RT21C) (18), Ki-MSV, Mo-MuLV, or the 3′ end of the Mo-MuLV (22). As can be seen in Fig. 6, considerable homology to rat helper virus cDNA was detected in the nonproducer cellular RNA whereas no significant homology to Ki-MSV or to either Mo-MuLV cDNA was detected. Though not shown in this figure, a Ki-MSV-specific probe cycled to remove the mouse sequences present in the Ki-MSV genome produced identical results to those with the Ki-MSV cDNA shown here. The results indicate that RaSV, as expected, contains no mouse genetic information and that we cannot detect homology between Ki-MSV (or Ha-MSV) and RaSV with our currently available cDNA probes.

DISCUSSION

Three distinct fibroblast-transforming retroviruses have been isolated from rats. Two of these viruses, Ki-MSV and Ha-MSV, were isolated by experimental inoculation of mouse type C viruses into rats (8, 9). These viruses were shown to be mouse–rat recombinant viruses, and a general model for generation of oncogenic retroviruses was proposed (23). The third virus, RaSV, was isolated in cell culture by cocultivation of a cell producing rat type C viruses and a Fischer rat cell transformed after exposure to 4-nitroquinoline (7). The current studies, and those recently reported by us (10), indicate that these three different retroviruses, each of which can transform fibroblasts in cell culture, code for a related intracellular phosphoprotein which is not a virion structural protein. Ki-MSV and Ha-MSV

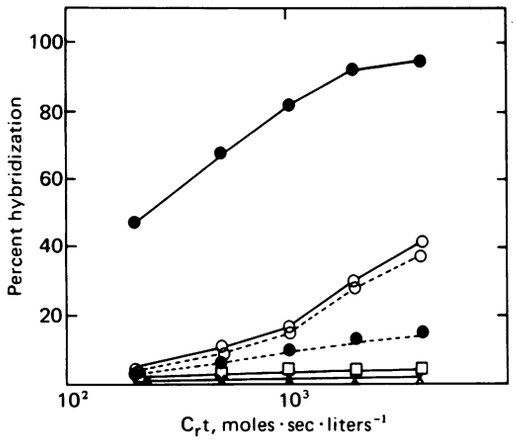

FIG. 6. Hybridization analysis of RaSV-transformed nonproducer cell RNA. Hybridization of RaSV-transformed nonproducer total cellular RNA (6E) to rat helper-independent virus (RT21c) [^{3}H]cDNA, Ki-MSV [^{3}H]cDNA, Mo-MuLV cDNA, and Mo-MuLV 3′ cDNA was carried out as described (18). The preparation of Mo-MuLV 3′ cDNA has also been described (22). [^{3}H]cDNA (≈2500 acid-precipitable cpm) was added to each hybridization reaction, and cDNA·RNA hybrids were analyzed by using S1 nuclease (19). Hybridization at 100% represents hybridization of each cDNA to saturating levels of viral RNA. ●—●, RT21c [^{3}H]cDNA to 6E RNA; o—o, Ki-MSV [^{3}H]cDNA to 6E RNA; ●---●, RT21c [^{3}H]cDNA to FRE RNA; o---o, Ki-MSV [^{3}H]cDNA to FRE RNA; □—□, Mo-MuLV [^{3}H]cDNA to 6E RNA; x—x, 3′ Mo-MuLV [^{3}H]cDNA to 6E RNA.

code for a phosphoprotein, p21, and RaSV for a phosphoprotein, p29. These proteins share immunological crossreactions and common V-8 protease-generated peptides. The p21 is not precipitated by any serum prepared against mouse or rat helper-independent type C virions, or mouse or rat type C virion structural proteins (10). The p29 is precipitated by various p21 antisera and also by antisera against rat helper-independent type C virus. The results suggest that the p29 is a p21-like protein fused to an unidentified rat leukemia virus structural protein. The biological method of isolation of RaSV and the molecular hybridization data presented herein indicate that RaSV is a purely rat sarcoma virus. Previous physical maps of Ki-MSV and Ha-MSV suggested that any proteins coded for by these viruses would of necessity be translated from rat-derived genes (6). The relationships between the p21 and p29 described herein strongly suggest that both the p21 of Ki-MSV and Ha-MSV and a portion(s) of the p29 of RaSV are translated from rat-derived gene(s) with related coding sequences and that this gene(s) has been introduced into each sarcoma virus by recombination involving different helper-independent type C viruses either in rats or in rat cells under *in vitro* cell culture conditions.

The results raise several questions that cannot be answered at this time: (*i*) What rat leukemia virus protein is the p21-like portion of the p29 fused to? Is the p29 then like other larger fusion proteins, called polyproteins, that have been described for various replication-defective transforming viruses (24–27)? (*ii*) Why can we not detect homology by molecular hybridization between Ki-MSV and RaSV even though we can demonstrate immunological relationships between the p21 and p29 proteins? This could be due to the inadequacies of our cDNA probes or to the fact that degeneracy of the genetic code could allow somewhat diverse genes with similar amino acid coding information to code for immunologically related proteins. Too much diversity might exist between such genes to allow nucleic acid cross-hybridization to be detected by our methods. We cannot resolve these possibilities at this time. (*iii*) What is the function of the p29 and is it active as p29, or after processing to its p21-like portion? Recently we have shown that the p21 of Ki-MSV demonstrated thermolability by *in vitro* heating studies in a mutant of Ki-MSV temperature-sensitive for the maintenance of transformation (28). These results would imply that the gene of RaSV that codes for the p29, by virtue of its p21 coding sequences, would be required for the maintenance of transformation induced by RaSV. (*iv*) Is a p21-related protein expressed in the cell transformed after exposure to 4-nitroquinoline that was then used in the cocultivations to isolate RaSV? Additional studies are necessary to test these hypotheses and answer the questions posed.

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