Characterization of Some Isolates of Newly Recovered Avian Sarcoma Virus

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We previously reported the isolation of a newly recovered avian sarcoma virus (rASV) from tumors of chickens injected with transformation-defective (td) mutants of the Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV). In this paper, we present further biological and biochemical characterization of the recovered sarcoma viruses. High titers of rASV's were generally obtained by cocultivation of tumor cells with normal chicken embryo fibroblasts or by homogenization of tumor tissues. Most rASV isolates were similar to SR-RSV, subgroup A (SR-RSV-A), in their growth characteristics and were nondefective in replication. The subgroup specificity of rASV's and the electrophoretic mobilities of their structural proteins were the same as those of parental td viruses. The nondefectiveness of rASV's was further substantiated by the size of their genomic RNA, which was indistinguishable from that of SR-RSV-A and substantially larger than that of parental td RNA. Molecular hybridization using complementary DNA specific to the src gene of SR-RSV ($cDNA_{src}$) showed that the RNAs of td mutants used in this study contained extensive deletions within the src gene (7 to 30% hybridization with $cDNA_{src}$); the same probe hybridized up to 90% with RNA from two isolates of rASV. These data indicate that rASV has regained genetic information which had been deleted in the td mutants and strongly suggest that the generation of rASV involves a genetic interaction between td virus and host cell genetic information.

Transformation-defective (td) mutants of Rous sarcoma virus (RSV) are viruses which arise spontaneously when RSV is passaged in tissue culture (16, 31). These td viruses retain all replicative functions but have lost the ability to transform chicken embryo fibroblasts (CEF) in tissue culture. Size analysis and RNA fingerprinting of such viruses have indicated that a deletion of 15 to 20% near the 3' end of the genome occurs during their generation (5, 13, 15, 19, 23, 33, 34). The fact that RNA sequences in this region are always present in sarcoma viruses and always absent in the genome of leukosis or td viruses provides the strongest evidence that this portion of the genome codes for the products essential for cell transformation. Thus, this segment of viral RNA has been called the src gene (34). The possible translational products of this gene have been described recently (2, 14, 24).

We previously reported (7) that when certain td mutants of the Schmidt-Ruppin strain of RSV (SR-RSV) were injected into the wing web of 1-day-old chicks, a significant fraction of birds developed tumors after a long latent period. All tumors examined were found to contain a new

virus, which we have named recovered avian sarcoma virus (rASV), since the virus was capable of producing sarcomas in chickens and transforming CEF in culture. The morphology of foci induced by rASV appeared different from those induced by SR-RSV, indicating that rASV's are unique viruses.

In this study, we further examined the biological and biochemical characteristics of rASV. Most of the rASV's were found to be nondefective in replication and to contain a 39S genomic RNA. These viruses contain src-specific genetic information, as shown by nucleic acid hybridization with complementary DNA specific for the src region of SR-RSV ($cDNA_{src}$). The structural proteins and subgroup specificity of rASV's were indistinguishable from those of the parental td mutants.

(Portions of the work presented here were submitted by C. C. Halpern in partial fulfillment of the Ph.D. degree at The Rockefeller University, 1978.)

MATERIALS AND METHODS

General. X-irradiation was performed using a Radiofluor 120 at 5 mA and 100 kV (Philips Electronic Instruments, Mt. Vernon, N.Y.). Neutralizing antisera

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against viruses were obtained from infected chickens (anti-SR-RSV, subgroup A [anti-SR-RSV-A] was obtained from a td107-injected bird). TEN is 0.01 M Tris-hydrochloride (pH 7.4), 0.001 M EDTA, 0.1 M NaCl; ETS is 0.01 M Tris-hydrochloride (pH 7.4), 0.01 M EDTA, 0.2% sodium dodecyl sulfate (SDS); NETS is ETS plus 0.1 M NaCl; phosphate-free medium is the Temin modification of Eagle minimal essential medium (MEM). ^{3}H-amino acid mixture, [^{3}H]uridine (40.4 Ci/mmol), and carrier-free ^{32}PO$_4$ were purchased from New England Nuclear Corp., Boston, Mass.; [^{14}C] uridine (486 mCi/mmol) was purchased from Amersham/Searle, Arlington Heights, Ill.; proteinase K was from EM Laboratories, Inc., Elmsford, N.Y.; sucrose was RNase-free and from Schwarz/Mann, Orangeburg, N.Y. Experiments involving viral RNA were performed using glassware baked at 150°C for 3 h.

Cells and viruses. CEF were cultured from individual embryos (SPAFAS, Inc., Norwich, Conn.) as described previously (6) and were generally used after the first or second passage. Quail cells were prepared from 11-day-old embryos (Shamrock Farms, North Brunswick, N.J., and Life Sciences, Inc., St. Petersburg, Fla., through courtesy of the Office of Program Resources and Logistics, National Cancer Institute).

Viruses used in these studies were: rASV as described previously (7; Table 1); td viruses were originally cloned by Kawai et al. (15); Rous-associated virus-1 (RAV-1), RAV-2, RAV-7, the chf pseudotype of the Bryan high-titer strain of RSV [(BH-RSV-(chf)], and SR-RSV-A and -B have been described elsewhere (9, 16, 27); RAV-0 was obtained from producer cell lines 7 × 15 generously provided by L. B. Crittenden (4).

Infective center assay. Two somewhat different methods were used for the infective center assay.

(i) A total of 10^6 chick cells were infected with 100 to 500 focus-forming units (FFU) of virus. After 10 to 12 h, half of the cultures were X-irradiated with 5,000 R. The control cultures were kept at room temperature during this time. After irradiation, the medium of both control and treated cultures was replaced by fresh culture fluid containing 10^6 normal chick cells. After cell attachment, the cultures were overlaid with hard agar; foci were counted 5 to 7 days later.

(ii) About 7 × 10^5 chick cells were infected with 10^2 to 10^3 FFU of virus. After 12 h, the cultures were incubated for 2 h in 1 ml of medium with or without 10 µg of mitomycin C. Cultures were then rinsed twice with fresh culture fluid and incubated for an additional

TABLE 1. *Origin of rASV's used in various experiments*[a]

td from which rASV was generated	rASV no.
101	50, 165, 1181, 1182, 1231, 1232, 122
107	233, 2931, 2932, 293
108	145, 148, 151, 1441, C1442, 1721, 1722
109	156, D157, 1701, 1702

[a] td and rASV's have been described previously (7, 15). The rASV number refers to the chicken number in which the tumor was found. rASV stocks are the supernatants from tumor cells cocultivated with CEF.

hour in fresh medium. Medium was poured off; culture fluid containing 10^6 normal chick cells was added to the mitomycin C-treated plates, and 2 × 10^5 cells were added to control cultures. After cell attachment, the cultures were overlaid with hard agar for focus assay.

Neutralization. Virus samples were incubated with antisera against SR-RSV-A, RAV-2, RAV-7, or RAV-60 for 45 min at 37°C. Control virus samples were incubated in an equivalent volume of medium. Focus formation was assayed on CEF or quail cells, as indicated in the figure legends.

Interference. CEF were infected with either RAV-1, RAV-2, RAV-7, or mock infected. After three transfers these cells were infected with various dilutions of rASV or various pseudotypes of BH-RSV. Interference was determined by the reduction in the number of foci formed on the RAV-infected cells compared with the control cells.

Growth curve. Chick cells were infected with rASV or SR-RSV-A at multiplicities of 1 or 0.05. After virus adsorption for 1 h, cells were rinsed with growth medium and fresh culture fluid was added. At various times after the initial virus addition, medium was removed. Cells and debris were removed by low-speed centrifugation, and supernatants were frozen at −70°C. When all samples were collected, supernatants were assayed on CEF for focus formation.

Focus isolation. Chick cells were infected with various dilutions of rASV and overlaid with hard agar containing antiserum against SR-RSV-A. After 6 to 7 days, plates were examined, and those which contained 10 or fewer foci were used for focus isolation. For this purpose the agar was removed, and a 0.05% trypsin solution containing antiserum was added to the plate. Transformed cells from individual foci were recovered with a capillary pipette and were added to another plate containing 10^6 normal CEF. After cell attachment, plates were overlaid with soft agar. The cultures were transferred every 3 to 4 days. When the majority of cells in the cultures became transformed, supernatants were assayed for focus-forming virus.

Labeling of viral RNA. Infected cells were incubated for one or two 12-h periods with MEM containing [^{3}H]- or [^{14}C]uridine (20 and 5 to 10 µCi/ml, respectively). The medium was replaced with MEM, and culture fluids were collected at 3- to 4-h intervals for up to 12 h. Fluids were pooled, and cells were removed by low-speed centrifugation (2,000 rpm for 10 min).

For ^{32}P labeling, cells were preincubated in phosphate-free medium for about 10 h. Cells were labeled for 12 h in phosphate-free medium containing 0.1 mCi of ^{32}P$_i$ (carrier-free) per ml. Culture fluids were collected every 3 h thereafter.

Labeling of viral proteins. Infected cells were labeled with ^{3}H-amino acids (20 µCi/ml) for 12 h. The medium was replaced with MEM, and culture fluids were collected at 12-h intervals for 24 h. During labeling and chase periods, the tryptose phosphate broth concentration was lowered to 1%.

Virus purification. Virus for protein or RNA analysis was purified as described previously (12), except that the Pronase and hyaluronidase treatments were omitted.

RNA analysis. Purified virus was pelleted in a

Beckman type 40 rotor (36,000 rpm, 2 h), suspended in 0.4 ml of TEN containing 0.2% SDS and 0.5 mg of proteinase K per ml, and incubated at 37°C for 30 min. For isolation of 60 to 70S RNA, the proteinase K-treated virus was layered on a 15 to 30% (wt/wt) sucrose gradient (sucrose made up in ETS buffer plus 0.05 M LiCl). Gradients were centrifuged in a Beckman SW40 rotor at 40,000 rpm for 3 h at 23°C. Fractions were collected from the bottom of the gradient, and 10-μl samples were used for scintillation counting. The peak of high-molecular-weight RNA was pooled, and the NaCl concentration was adjusted to 0.2 M; yeast 4S RNA (40 μg/ml) and 2 volumes of cold 95% ethanol were added.

For RNA sizing, 60 to 70S RNA from two different viruses labeled with different isotopes was pooled and pelleted. RNA was dissolved in 0.3 ml of ETS, heat denatured at 85°C for 90 s, and analyzed by rate zonal centrifugation on 15 to 30% sucrose gradients as previously described (11). Centrifugation was for 7 h at 40,000 rpm and 23°C in a Beckman SW40 rotor. Fractions were collected from the bottom of the tube (about 56 fractions were obtained). A 0.5-ml amount of water was added to each fraction, and the whole sample was used for scintillation counting, using 10 volumes of liquid scintillation cocktail (Biofluor; New England Nuclear Corp.). Data were corrected for spillover of ^{14}C or ^{32}P into the ^{3}H channel.

Protein analysis. Polyacrylamide gel electrophoresis was performed by using slab gels with minor modifications of the Laemmli method (17). Pelletized virus samples were suspended in 50 μl of 0.1 M Tris-hydrochloride (pH 6.7)–0.2% SDS–1% 2-mercaptoethanol–10% glycerol and boiled for 90 s. Gels were fixed and stained with 50% methanol–7% acetic acid–0.25% Coomassie brilliant blue. After destaining (30% methanol–7% acetic acid), gels were processed for fluorography as described previously (3).

Nucleic acid hybridization. Hybridization was performed in liquid, as previously described (11). The extent of hybridization was monitored by treatment with nuclease S1 (12), using 1,000 to 2,000 U of S1 (Miles Laboratories, Inc., Elkhart, Ind.) per ml, based on the manufacturer's specifications.

Preparation and characterization of cDNA$_{src}$ has been described in detail elsewhere (11). This probe has a genetic complexity of approximately 1,900 nucleotides, as determined by its ability to protect ^{32}P-labeled SR-RSV-B RNA from RNase digestion (11). Saturation in these experiments was attained at a molar ratio (DNA/RNA) of 3 to 4 (mass ratio of approximately 0.6), and 75% saturation was attained at a molar ratio of 1, indicating that the probe contains a fairly uniform representation of src-specific sequences.

RESULTS

Titers of recovered virus. The titer of various isolates of rASV was determined with culture fluids obtained from fully transformed cultures after cocultivation of tumor cells with normal chicken cells. As shown in Table 2, rASV preparations obtained from tumors originated by injection with td101, -107, and -108 had titers

of 10^5 to 10^6 FFU/ml. On the other hand, several rASV stocks obtained from td109-derived tumors had low titers of transforming virus (10^2 to 10^3 FFU/ml).

The titers of transforming virus shown in Table 2 were obtained by assaying on CEF without the addition of polycation at the time of infection. Subsequently, we found that certain recovered viruses had a higher titer of transforming virus when infection was carried out in the presence of DEAE–dextran. As indicated in Table 3, the titer of certain virus preparations could be increased from 3- to 14-fold in the presence of DEAE–dextran. The differences in the responsiveness to the addition of polycation seem to be correlated with the origin of rASV's. rASV 165, which was recovered from a tumor of a bird injected with td101, showed no enhancement of

TABLE 2. Titers of rASV's[a]

rASV no.	Titer (FFU/ml)
Tumors induced by td101	
122	2.2×10^6
165	2.0×10^6
1232	1.1×10^6
1182	5.4×10^5
1231	1.6×10^5
Tumors induced by td107	
293	3.0×10^5
2932	2.8×10^5
2931	1.0×10^3
Tumors induced by td108	
1721	1.1×10^6
1441	1.4×10^6
145	9.0×10^5
151	1.5×10^5
148	3.8×10^4
1722	1.6×10^4
Tumors induced by td109	
1702	7.8×10^3
157	3.3×10^2
1701	60
156	40

[a] CEF were infected with dilutions of various rASV isolates. After 10 to 15 h, the cultures were overlaid with hard agar and maintained at 41°C. Foci were counted 5 to 7 days later.

TABLE 3. Effect of DEAE–dextran on virus titers

| Virus | Virus titer (FFU/ml)[a] | |
	– Dextran	+ Dextran
165	2.9×10^5	2.2×10^5
293	5.0×10^4	1.6×10^5
1441	9.6×10^5	2.9×10^6
D157	1.3×10^3	1.9×10^4

[a] CEF were infected with dilutions of rASV either with or without the addition of 5 μg of DEAE–dextran per ml. Cultures were overlaid with hard agar, and foci were counted 5 days later.

infectivity in the presence of this polycation. The other viruses (293, 1441, and D157) were recovered from tumors of birds injected with td107, -108, and -109, respectively, and their infectivity was enhanced by the polycation. A difference in responsiveness to polycation among clonal isolates of SR-RSV-A was noted by Kawai (unpublished data). Two representative virus clones, 85-1, which is not dependent on DEAE–dextran, and 85-7, which is somewhat dependent (about 5 to 10 times enhancement), were isolated. Among td viruses used in this study, only td101 was derived from 85-1. Therefore, the above-described DEAE–dextran-dependent characteristics of rASV's obtained from td107, -108, and -109 appear to reflect the original property of 85-7.

The presence of nontransforming virus in rASV stocks was determined by end point dilution and assay for the presence of polymerase or interfering virus. We found that in most preparations the titer of nontransforming virus was lower than that of focus-forming virus. Some preparations (e.g., rASV 233 and 1231) did, however, contain nontransforming virus in excess of focus-forming virus.

Growth curve. As previously described (7), the morphology of foci formed by rASV is different from that of SR-RSV-A. Foci made by rASV are more compact than those generally observed with SR-RSV-A. The difference could be attributed to either the absence of the production of infectious virus by rASV foci or slower spread of rASV than SR-RSV from infected cells to neighboring cells. Therefore, the growth rate of one of the rASV isolates (rASV 165) was compared with that of SR-RSV-A. As shown in Fig. 1, the growth rate of rASV 165 was indistinguishable from that of SR-RSV-A at two multiplicities, 1 and 0.05. Therefore, these two viruses appear to be produced from infected cells and multiply by repeated infection of neighboring cells at approximately the same rate. As described below, rASV 165 was shown to be nondefective. Therefore, the compact appearance of foci induced by rASV 165 compared with those induced by SR-RSV-A must be explained by some characteristic other than growth rate or spread of infection and thus may reflect a specific property of the src gene of rASV's.

Subgroup specificity of rASV. All rASV isolates tested were found to belong to the same subgroup (subgroup A) as that of the td viruses from which they were derived. This was determined first by using viral interference, as shown in Table 4. When cells which had previously been infected with leukosis viruses of subgroup A, B, or C were superinfected with some representative samples of rASV, only RAV-1-infected
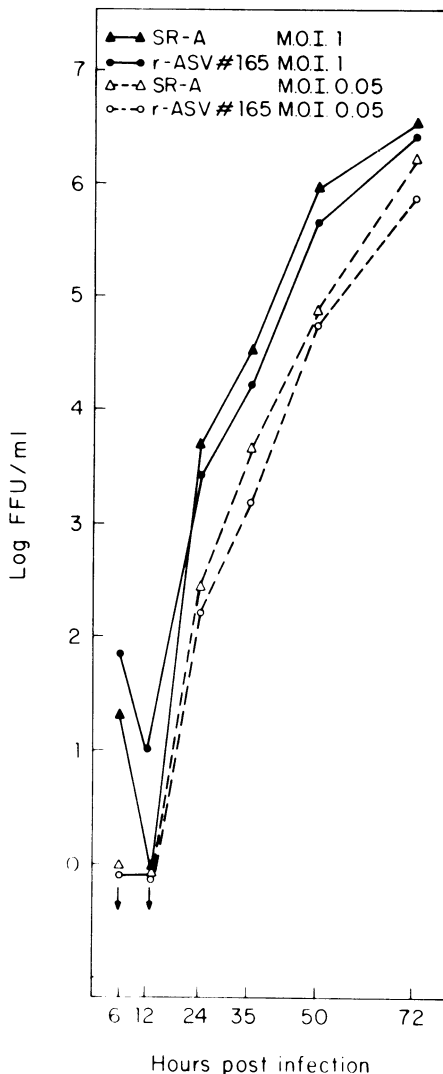

FIG. 1. Kinetics of virus replication. Parallel cultures of CEF were infected with SR-RSV-A (clone 85-1) and rASV 165 at multiplicities of 1.0 or 0.05. Culture fluids were harvested at the times indicated and assayed for the presence of focus-forming virus on CEF.

cells showed significant resistance to superinfection by rASV. Focus formation on the RAV-1-infected cultures was reduced by a factor of at least 10^4 with all seven rASV's.

rASV's were neutralized by antisera against subgroup A virus, but not by antisera against RAV-2 or RAV-7 (Table 5). Since rASV's appear to be derived by recombination between td virus and endogenous sequences, we were interested in determining whether endogenous env sequences or env gene products (which are of subgroup E specificity) are associated either ge-

TABLE 4. *Subgroup determination by interference*[a]

Virus	Fraction of focus-forming virus on cells preinfected with:		
	RAV-1	RAV-2	RAV-7
165	0.00001	0.2	0.6
1231	<0.00002[b]	0.2	0.8
293	<0.00001[b]	0.1	0.6
2932	<0.000004[b]	0.2	1.0
1441	0.00007	0.7	1.0
C1442	0.00009	1.0	1.0
D157	0.00004	0.3	0.5
SR-RSV-A	0.00001	1.0	1.0
RSV(RAV-1)	0.0001	0.7	1.0
RSV(RAV-2)	1.0	0.000004	1.0

[a] CEF were infected with leukosis viruses of subgroups A, B, and C. After three transfers, the cells were challenged with various rASV and RSV preparations. The number of foci was compared with those in control cultures which were not infected with leukosis virus, but were otherwise treated the same as RAV-infected cells.

[b] Since no foci were found in these cultures, the value given is calculated for 1 focus or less.

TABLE 5. *Subgroup determination by antibody neutralization*[a]

Virus	Fraction of focus-forming virus surviving after incubation with:		
	Anti-SR-RSV-A	Anti-RAV-2	Anti-RAV-7
165	0.000008	1.0	0.9
293	0.00009	3.3	2.2
1441	0.00001	0.8	0.6
D157	0.008	0.5	0.4

[a] A 0.2-ml amount of virus was mixed with an equal volume of each antiserum (antisera were used at concentrations previously determined to be neutralizing for 10^5 to 10^6 FFU of virus of the appropriate subgroup) and incubated at 37°C for 40 min. Control virus samples were incubated in 0.2 ml of medium for the same time. A 0.3-ml amount of virus-antibody mix was used to determine the titer of focus-forming virus on CEF.

TABLE 6. *Assay for subgroup E virus*[a]

Virus	Fraction of focus-forming virus surviving after incubation with:		
	Anti-SR-RSV-A	Anti-RAV-60	Anti-SR-RSV-A + anti-RAV-60
165	0.002	1.0	0.002
293	0.03	1.1	0.01
1441	0.01	1.3	0.0001
D157	0.08	NT[b]	0.05
RSV(chf)	1.2	0.0001	0.0002
SR-RSV-A	0.00006	1.1	NT[b]

[a] A 0.1-ml amount of virus was mixed with equal volumes of either anti-SR-RSV-A and media or anti-SR-RSV-A and anti-RAV-60 antisera and incubated at 37°C for 40 min. Control virus samples were incubated in 2 volumes of culture fluid for the same time. After incubation, 0.2 ml of virus-antiserum mix was assayed for focus-forming virus on quail embryo fibroblasts.

[b] NT, Not tested.

netically or phenotypically with rASV. As shown in Table 6, treatment of rASV's with anti-RAV-60 (subgroup E) did not reduce their plating efficiency on quail cells, indicating that the majority of the virus population was of subgroup A. However, when they were treated with antiserum against SR-RSV-A as well as antiserum against RAV-60, the infectivity of one isolate (rASV 1441) was significantly reduced below that obtained with anti-SR-RSV-A alone, suggesting the presence of a minor population of subgroup E virus in this preparation. It is likely that chf expression was positive in the chicken from which rASV 1441 was derived and that subgroup E pseudotypes were produced as a minor fraction of the virus progeny.

Protein analysis. The proteins of various rASV isolates were analyzed by SDS gel electrophoresis. The electrophoretic mobilities of the major structural proteins of these viruses were indistinguishable from those of SR-RSV-A and the *td* viruses derived from SR-RSV-A (Fig. 2). However, the p27 of rASV's was clearly distinguishable from that of RAV-0 (data not shown). RAV-0, a virus considered to be endogenous to chicken cells, contains a p27 detectably larger than that of all other known exogenous leukosis-sarcoma viruses (25). Thus, the *gag* gene of rASV's appears to be derived from the *td* virus, rather than from endogenous virus genes.

The migration of rASV gp85 may be retarded as compared with gp85 of *td* virus (Fig. 2). The slower migration of glycoproteins of tumor viruses grown in transformed cells compared with those of viruses grown in nontransformed cells has been reported (18) and attributed to more extensive glycosylation of the envelope glycoprotein in transformed cells.

Nondefectiveness of rASV. To determine whether single virions of rASV contain both transforming and replicative functions, individual foci were isolated from CEF infected with several rASV stocks. For these experiments, cells were infected with several dilutions of rASV. Foci were picked from cultures which contained 10 or fewer foci and had been kept overlaid with medium containing antiserum to SR-RSV-A. In the case of rASV 1231, for example, 15 out of 15 foci picked produced infectious rASV. Essentially the same results were obtained with rASV 50 and 165. Furthermore, no nontransforming virus was detectable beyond the end point dilution of transforming virus with the latter rASV preparations. These results strongly suggest that these isolates of rASV are nondefective.

It has previously been shown that when cells infected with a defective RSV are X-irradiated (8) or treated with mitomycin C (20, 36), no infectious centers can be registered after cocul-
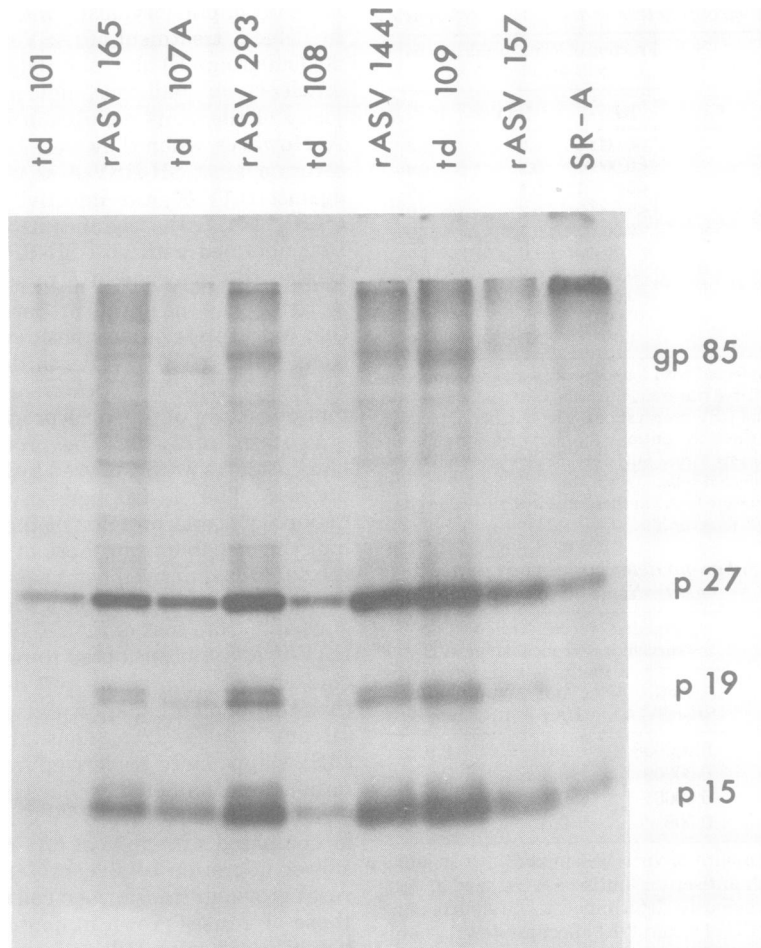
FIG. 2. *SDS–polyacrylamide gel electrophoresis of viral proteins. Purified, ³H-amino acid-labeled rASV's, td mutants, and SR-RSV-A were disrupted with SDS and coelectrophoresed in parallel slots on a 12.5% SDS–polyacrylamide slab gel. Samples were prepared as described in the text.*

tivation with fresh normal cells. On the other hand, these treatments do not cause a significant reduction in the number of infectious centers if cells are infected with nondefective RSV, since each infected cell releases infectious progeny even after blockage of cell division, resulting in the formation of an infectious center due to infection of neighboring normal cells. As seen in Table 7, cells infected with BH-RSV or with nondefective SR-RSV behaved as expected when low multiplicities of infection were used to avoid double infection with a helper virus. Results with cells infected with rASV 165 clearly showed that this virus is nondefective by this test. Four other rASV preparations (50, 1231, 1232, and 1721) gave essentially the same results as 165. However, several rASV isolates recovered from td109-infected chickens appear to be defective in replication (data not shown). The nature of the lesion in these td109-derived

rASV's is currently being investigated.

Size of viral RNAs. Kawai et al. (15) have shown by gel electrophoresis that the RNA of some *td* mutants is larger than standard leukosis virus RNA but significantly smaller than wild-type sarcoma virus RNA. The recovery of full-length (39S) RNA from rASV would immediately suggest the gain of genetic information during the generation of rASV. Such data would also strongly indicate the nondefectiveness of the particular isolate of rASV. In this study the size of RNA was analyzed by sedimentation in sucrose gradients; sedimentation values were calculated by comparison with 18S and 28S rRNA's, RAV-2 RNA (35S), and SR-RSV-B RNA (39S). Figure 3 and Table 8 show that the viral RNAs of *td*101 and -108 are somewhat larger (300 to 500 nucleotides) than that of RAV-2 35S RNA. These results are essentially the same as those described by Kawai et al. (15),

TABLE 7. *Infective center assay*[a]

Virus	Dilution	No. of foci			
		Control	X-irradiated	Mitomycin C	% of Control
SR-RSV-A	1×10^{-4}	440	330		75
SR-RSV-A	2×10^{-5}	159	101		64
RSV(RAV-1)	1×10^{-4}	255	4		2
RSV(RAV-1)	2×10^{-5}	87	4		5
rASV 165	1×10^{-4}	79	67		85
SR-RSV-A	1×10^{-3}	580		380	66
RSV(RAV-1)	4×10^{-3}	274		44	16
rASV 165	1×10^{-4}	284		164	58

[a] CEF were infected with 100 to 500 FFU of virus. At 10 to 12 h postinfection, half of the cultures were either X-irradiated (5,000 R) or incubated with 10 µg of mitomycin C. Cultures were rinsed, and fresh normal CEF were added to both control and treated cultures. Infective centers were scored 5 to 7 days later.

except that the size differences were slightly greater in this study and the size of *td*101 RNA was found larger than in the previous measurements. This could be due to the different effects of secondary structure on the sedimentation rates and electrophoretic mobilities. The size obtained in this study appears to be better correlated with the content of *src*-specific sequences in *td*101 (see below).

Results of analyses of the RNA of some representative rASV preparations are shown in Fig. 4 and 5. The rASV's from *td*101, -107, and -108 contained genomic RNA which was indistinguishable in size from that of SR-RSV-A (data on rASV's derived form *td*108 are not shown). While preparations such as rASV 1181 contained primarily 39S RNA, other preparations such as rASV 233 contained two discrete RNA species: one which cosedimented with SR-RSV-A RNA and the other which cosedimented with *td*107A. rASV 1231 has a minor peak of 39S RNA and a major peak cosedimenting with *td* virus RNA. As mentioned above, this virus preparation contained an excess of nontransforming virus. We have not yet determined whether the *td* virus present in certain rASV stocks resulted from segregation of *td* virus from rASV or had been present in the original tumors as a result of multiplication of *td* viruses originally injected into newborn chicks.

Previously, Kawai et al. (15) described the presence of RNAs of heterogeneous size in virions of *td*107 stock. This was confirmed by sedimentation analysis, as shown in Fig. 5. Furthermore, it was demonstrated that *td*107A (a virus isolated by end point dilution of *td*107) has a homogeneous RNA peak which cosediments with RAV-2 35S RNA. As we reported earlier (7), chickens injected with *td*107 developed tumors, whereas *td*107A-injected birds did not.

src-specific sequences in RNAs of *td* mutants and rASV's. RNAs from *td* mutants and

rASV's were tested for the presence of *src* sequences by hybridization to a *src*-specific cDNA probe (cDNA$_{src}$) prepared from SR-RSV-B (11). This probe does not hybridize to leukosis virus RNAs (less than 2% to RAV-0, RAV-2, RAV-7, and RAV-60), but hybridizes extensively with RNA from SR-RSV, BH-RSV, and the Prague strain of RSV, subgroup B (>87%) (11).

Hybridization of cDNA$_{src}$ to RNAs from various *td* viruses demonstrated that *td*101, -108, and -109 retain portions of the *src* region (24 to 30% hybridization), whereas *td*107A contains little or no *src*-specific information (7% hybridization) (Table 8). These values are compatible with the estimated size of the viral RNAs, based on their sedimentation values (Table 8), with the exception of *td*109, which has an RNA of roughly the same size as RAV-2, but appears to contain about 24% of the *src* region. Since this virus recombines with *ts*68 (15) and contains *src* sequences by hybridization assay, it seems likely that the deletion in this virus extends into some nonessential region outside the *src* gene.

cDNA$_{src}$ hybridized approximately 95% to RNAs from two independent isolates of rASV (165 and 1441). Thus, the generation of rASV from *td* viruses involves the acquisition of new *src*-specific information. This is presumably derived from the endogenous *src*-related information, which demonstrates extensive homology with the *src* region of RSV (Table 8; references 29, 30, and 35).

DISCUSSION

We previously described the isolation of a new sarcoma virus from tumors induced by *td* mutants of SR-RSV (7). In this paper we have presented an analysis of the biological and biochemical features of the isolates recovered from tumors induced by *td*101, -107, and -108.

Using neutralizing antisera and interference, we demonstrated that the rASV's are of the
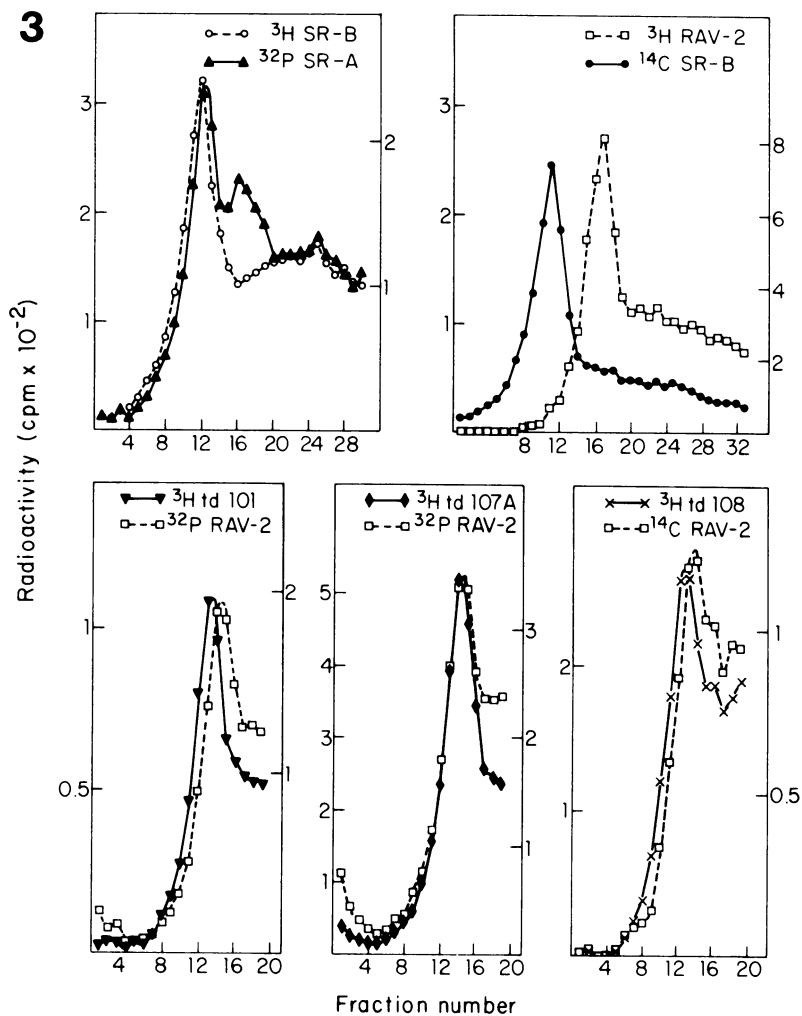
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Fig. 3 through 5. *Sedimentation analysis of heat-denatured viral RNAs. Purified 70S RNA of two viruses was coprecipitated and heat denatured in ETS buffer (90 s at 85°C). Samples were layered on a 15 to 30% sucrose-SDS gradient (0.2% SDS) and centrifuged for 7 h at 40,000 rpm in a Beckman SW40 rotor (23°C), as described in the text. Twenty-drop fractions were collected from the bottom of the gradient (total, 56 to 57 fractions). In each panel, the scale on the left represents the ^3H-labeled RNA.*

same subgroup specificity as are parental *td* viruses. Furthermore, the responsiveness to DEAE–dextran of individual rASV isolates correlated with that of the *td* viruses used to generate each isolate, providing further evidence that the rASV *env* gene is derived from the parental *td* virus.

Analysis of viral proteins by SDA-polyacrylamide gel electrophoresis indicates that the major proteins of rASV and parental *td* viruses are indistinguishable in their electrophoretic mobilities. It has been shown previously (25) that the p27 of RAV-0 (coded for by chick cell endogenous information) has an electrophoretic mobility slower than that of the p27 of several exogenous viruses. The fact that re-

covered virus p27 has an electrophoretic mobility identical to that of *td* viruses, but distinct from that of RAV-0 p27, provides evidence that at least a portion of the *gag* gene of rASV's is derived from parental *td* viruses rather than from endogenous virus.

We have demonstrated the nondefective nature of five recovered virus isolates, employing infective center assays and by individual focus isolation (representative data are shown in Table 7 for rASV 165). Therefore, these viruses appear to have retained most, if not all, of the genetic information of *td* viruses, but have gained additional information coding for the transforming function.
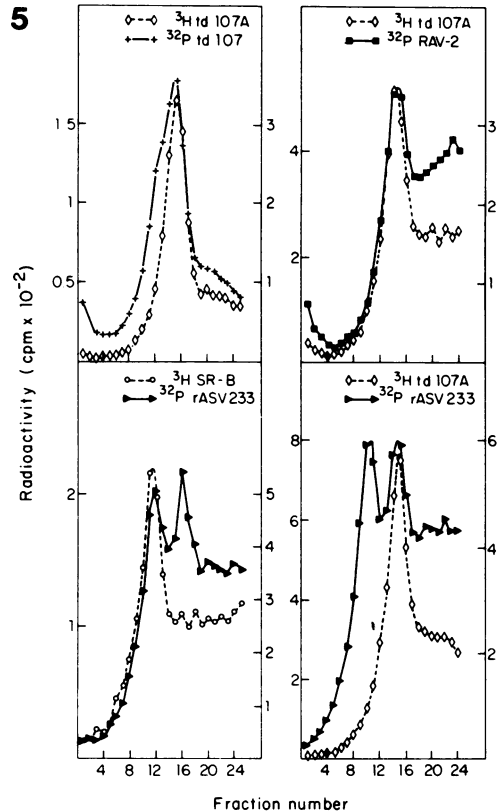
The size of recovered virus genomic RNA
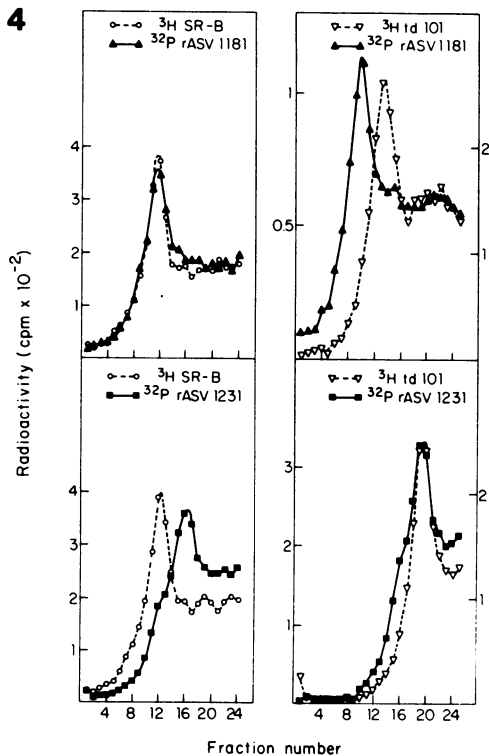
TABLE 8. src-Specific information in viral RNA

Virus	Sedimentation value of RNA ($s_{20,w}$)	% cDNA$_{src}$ hybridized[a]
RAV-2	35	<2
td101	36	26
td107A	35	7
td108	36.2	30
td109	35	24
Endogenous src	27	>80[b]
rASV 165	39	95
rASV 1441	39	96
SR-RSV-A	39	100

[a] Maximum levels of hybridization to viral RNAs, determined at C_rt values of 0.5 to 2 mol·s/liter. Values have been normalized to the level of hybridization obtained with SR-RSV RNA (actual value, 93%).

[b] Hybridization to polyadenylic acid-containing RNA from uninfected gs⁻ chf⁻ cells (36), at an effective C_rt value of 2×10^5. The hybridization level shown represents an underestimate of the extent of homology, since a stable plateau was not attained at this C_rt value due to the low concentration of src-specific RNA in these cells (29, 35).

analyzed by sedimentation in sucrose–SDS gradients provided further confirmation of this conclusion. As shown in Fig. 3 and Table 8, the size estimates of leukosis and td virus RNAs deter-

mined by this method are in good agreement with previous reports based on polyacrylamide gel electrophoresis and, in general, correspond to the expected molecular weights based on gene content (11, 15). Our data on the size of RNA of rASV (165 and 1441) have also been confirmed by SDS–polyacrylamide gel electrophoresis (L.-H. Wang, C. C. Halpern, M. Nadel, and H. Hanafusa, Proc. Natl. Acad. Sci. U.S.A., in press).

The sedimentation analysis of recovered virus RNA clearly indicates that these viruses contain genomic RNA with a sedimentation value (39S) characteristic of nondefective RSV and significantly larger than that of parental td viruses (35 to 36S). These findings apply to all recovered viruses tested which were derived from tumors induced by td101, -107, and -108. Some recovered virus preparations contained a second RNA peak (in addition to 39S) which cosedimented with td virus RNA. Since this second peak cosedimented with parental td virus RNA, it is likely that its presence in the virus stocks is due to the presence of td virus in the tumors from which rASV was isolated. An alternative explanation which we have not excluded is that td viruses segregate at high rates from certain rASV's.

The characteristics described above suggest that rASV's have gained a transforming gene analogous to the src gene present in known sarcoma viruses, since the genome length of recovered viruses is indistinguishable from that of SR-RSV. It is possible that the recovered virus src gene arose by recombination between endogenous src sequences (29, 30, 35) and the remaining src sequences in the partially deleted td viruses. Direct evidence that rASV's have a complete, or nearly complete, src gene is provided by our experiments in which recovered virus RNA was hybridized to a src-specific cDNA probe. In contrast to partenal td viruses, which show a maximum of 30% hybridization to this probe, two recovered viruses tested (1441 and 165) anneal to >90% of this SR-RSV-derived src probe. Preliminary experiments indicate that the src sequences of rASV are closely related to, but not completely homologous with, the src region of exogenous RSV (determined by thermal stability measurements of $cDNA_{src}$/RNA hybrids [unpublished data]). These data are compatible with the notion that the sarcomagenic information of rASV is derived from the src-related gene(s) present in the uninfected chicken cell.

An interesting correlation has emerged from some of our findings; namely, the retention of a certain portion of the src gene seems to correlate with the capacity of td virus to generate transforming virus. So far, a total of four td viruses have been analyzed for their src gene content by molecular hybridization. Three of these (td101, -108, and -109) hybridize 24 to 30% with the src-specific probe, and one, td107A, anneals to only 7% of the same cDNA. As we previously reported (7), td107A failed to produce tumors, whereas td101, -108, and -109 did. These results therefore suggest that a certain portion of the src gene (greater than 7%) is required for the generation of sarcoma virus from a td virus under the conditions of our experiments. If recovered viruses are generated by recombination between endogenous src sequences and td viruses, a minimal degree of homology between the interacting parental species may be required. The endogenous src-specific RNA transcript does not appear to contain sequences homologous with other viral genes (29, 35). Thus, retention of 24 to 30% of the src gene in some td viruses may greatly increase their specific interaction with endogenous src-specific sequences.

The generation of sarcoma viruses by passage of leukemia viruses in vivo has previously been reported with murine leukemia virus (10, 22, 26). Although the mechanism for the generation of murine sarcoma viruses is not entirely clear, the major difference between our findings and those reported with the murine system (26, 28) is that the recovered virus isolates described here have gained sarcomagenic information without the loss of replicative functions. It is interesting to note, however, that not all recovered viruses fall into the same category. Of notable exception are some isolates from tumors induced by td109. Preliminary results suggest that these viruses may be defective in replication, and thus their generation could be analogous to the formation of murine sarcoma viruses (26, 28).

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