JOURNAL OF VIROLOGY, May 1982, p. 602–611
0022-538X/82/050602-10$02.00/0

Vol. 42, No. 2

Tumorigenicity of Partial Transformation Mutants of Rous Sarcoma Virus

P. KAHN,[1] K. NAKAMURA,[3] S. SHIN,[1] R. E. SMITH,[2] AND M. J. WEBER[3]*

Department of Genetics, Albert Einstein College of Medicine, Bronx, New York 10461[1]; Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710[2]; and Department of Microbiology, University of Illinois, Urbana, Illinois 61801[3]

Received 6 November 1981/Accepted 21 January 1982

Chicken embryo cells infected with partial transformation mutants of Rous sarcoma virus were tested for tumor-forming ability in chickens and in nude mice. Cells transformed by each of these partial transformation mutants display different combinations of transformation parameters. They therefore present a potentially favorable system for analyzing which properties of transformed cells are necessary for tumor formation. We found that the relative tumorigenicity of the virus mutants was generally similar in chickens and in nude mice, except that certain temperature-conditional mutants appeared to be sensitive to the differences in body temperature of the two experimental animals. (The body temperature of nude mice is 4 to 5°C lower than that of chickens). Thus, the nude mouse appears to be a suitable system for testing the tumorigenicity of transformed chicken cells. Because mice are nonpermissive for Rous sarcoma virus infection and replication, it was possible to recover the transformed chicken cells from the tumors in this host and to determine what phenotypic changes they had undergone during tumor development. We also examined the relationship between various cellular properties of the virus-infected chicken cells in vitro and their tumorigenicity in nude mice. The combined results of these two studies indicated that anchorage independence and plasminogen activator production were highly correlated with the tumor-forming ability of these cells, whereas loss of fibronectin did not correlate with tumorigenicity. Furthermore, the inability of the least tumorigenic virus mutant to stimulate the phosphorylation of a 36,000-M_r target of pp60^{src} raises the possibility that the 36,000-M_r protein plays a role in tumor formation.

The transformation of fibroblastic cells results in a variety of metabolic, morphological, and regulatory changes which are collectively termed "the transformed phenotype." These alterations include loss of cell surface fibronectin, decreased adhesiveness, changes in cell shape, increased production of plasminogen activator, increased glucose transport, loss of density-dependent growth inhibition, and acquisition of the ability to grow in semisolid medium (reviewed in references 11 and 29). Although not all transforming agents induce all of these changes and not all cell types express the same phenotypic alterations, the frequency with which these alterations appear in cells transformed in vitro and in naturally occurring tumors suggests that at least some of these changes may play an important role in the development of tumors in vivo.

Because the specific manifestations of the transformed phenotype appear to be affected by both the transforming agent and the target cell, one approach to analyzing the role of a specific transformation parameter in tumor development is to study in detail the transformation of a single cell type by a single oncogenic agent. Investigations of this sort were performed by Shin et al. (27) and Pollack and Rifkin (21), who examined a series of simian virus 40-transformed mouse and rat cell clones. They found that only those transformed clones which were anchorage independent and produced plasminogen activator formed tumors in nude mice, whereas clones which were anchorage dependent and produced low levels of low plasminogen activator were not tumorigenic, regardless of whether they expressed other transformation-associated properties.

Rous sarcoma virus (RSV) is one of the most studied and best understood oncogenic agents. The viral gene (*src*) which is responsible for malignant transformation of susceptible cells in vitro and for tumor formation in suitable animal hosts has been identified (30), and the protein it codes for (pp60^{src}) has been found to possess a protein kinase activity which phophorylates tyrosine (3–5, 7, 12, 19, 22, 26). Thus, cellular proteins which become phosphorylated on tyrosine during transformation are candidate targets for pp60^{src}. Recently, we reported the isolation

and characterization of mutants of RSV which fully induce the appearance of some parameters of transformation in chicken cells but do not induce the appearance of others (1). Mutants of this type, which are called partial transformation mutants (2, 31), provide a favorable system for analyzing the role of specific transformation parameters in tumor formation.

The usual system for studying tumorigenicity of RSV is its natural host, the chicken. However, because chickens are permissive for replication of RSV, tumorigenicity studies in this host can be difficult to interpret for the following reasons. First, the ability of RSV to replicate in chickens permits recruitment of host cells at the site of initial virus injection as well as spread of the virus to secondary sites. It is therefore difficult to compare the biological properties of the virus-transformed cells in vitro with those of tumor-derived cells. Second, virus replication increases the likelihood of reversion of the virus to a wild-type phenotype and of recombination between the virus and endogenous host-cell information. Thus, a test animal which is nonpermissive for RSV replication (such as the nude mouse) has certain advantages for determining the tumorigenicity of RSV-transformed cells. On the other hand, nude mice, because of their lack of immunological responsiveness, provide a somewhat artificial environment for the determination of tumorigenicity.

We therefore investigated the tumorigenicity of RSV-transformed chicken cells in athymic nude mice and compared this with the tumorigenicity of various RSV mutants in chickens. We also analyzed the properties of cells derived from tumors induced in nude mice by the injection of virus-transformed chicken cells to determine which transformation parameters are associated with tumor-forming ability. Finally, we examined the total phosphotyrosine content of the mutant-infected cells and phosphorylation of a 36,000-dalton (36K) cellular protein which is a target of $pp60^{src}$ kinase (8, 23, 24). Our results indicate that the relative tumorigenicity of the mutants is similar in nude mice and chickens, and therefore the nude mouse is a suitable assay system for testing the tumorigenicity of transformed chicken cells. We also found that phosphorylation of the 36K protein, increased plasminogen activator production, and the acquisition of anchorage independence were correlated with tumorigenicity in this system.

MATERIALS AND METHODS

Viruses and cell culture. Primary cultures of chicken embryo cells were prepared by standard procedures (29). Fertile eggs from a gs^- $chf^{\pm}$ flock were obtained from SPAFAS, Inc. (Roanoke, Ill). Virus characterization is described in previous publications (1, 2, 31). Cells were cultured in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) with 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.), 4% calf serum, and 1% heat-inactivated chicken serum (GIBCO). Cultures were infected with virus at the second passage after being placed into cell culture.

Tumorigenicity in chickens. Fertile eggs were obtained from SPAFAS, Inc., Storrs, Conn. Chickens were hatched and reared in an isolation facility at Duke University designed to prevent spread of virus among groups of infected animals. Viruses were brought to the same concentration of particles based on reverse transcriptase. Virus was administered to juvenile chickens by intradermal injection of 0.1 ml into each wing web. Onset of tumor formation was determined by palpation, and tumor growth was assessed by measurement with calipers. Histological sections of representative tumors were prepared by fixation in 10% neutral buffered Formalin, paraffin embedding, sectioning, and staining with hematoxylin and eosin before light microscopic examination.

Breeding and maintenance of nude mice. The BALB/c nude mouse colony used in these studies was initiated in 1973 with +/nu breeding pairs originally obtained from GI. Bomholtgard, Ry, Denmark, and has been maintained at Albert Einstein College of Medicine by mating +/nu females with nu/nu males. The breeding stock was derived from the fourth backcross generation of the outbred nu/nu NMRI mice with BALB/c mice. The mice were kept in laminar flow racks (Lab Products, Garfield, N.J.). All cages, bedding, and other materials coming into direct contact with the mice were autoclaved before use. Under these conditions, nude mice in our colony had an average life span of more than 1 year.

For experiments with sublethally irradiated nude mice, animals were treated with a single dose (500 rads) of γ-irradiation from a cesium source (Gamma Cell 40, Atomic Energy of Canada, Ltd., Ottawa) 1 day before injection of the transformed cells.

Tumorigenicity in nude mice. Cells grown in monolayer cultures were trypsinized and suspended in 0.2 ml of phosphate-buffered saline; the desired number of cells was then injected subcutaneously in a volume of 0.2 ml into 4- to 8-week-old nude mice. For most experiments, cells were infected with virus as secondary cultures and harvested as tertiary cultures for injection. Mice were monitored for tumor development for at least 4 months after the injection.

Establishment of cell cultures from tumors. Tumor-derived cell cultures were prepared as described by Freedman and Shin (10). Briefly, a small piece of tumor tissue was surgically removed from the mouse, minced, and then passed through a fine stainless-steel mesh. The resulting cell suspension, consisting mostly of single cells, was plated in Dulbecco modified Eagle medium with 10% tryptose phosphate broth, 4% calf and 1% chicken serum and kept at 42°C. This procedure resulted in growing cultures after a few days.

Biochemical analyses. Electrophoresis for glucose-phosphate isomerase (GPI; D-glucose-6-phosphate ketoisomerase, EC 5.3.1.9) was carried out on lysates prepared from 5×10^6 cells (20) according to the method of Eicher and Washburn (6).

The phosphotyrosine content of total cells was determined by labeling cells with 0.5 to 1.0 mCi of $^{32}P_i$ for 10 to 16 h, lysing the cells with RIPA buffer (2%

sodium dodecyl sulfate, 1.5 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 1% Trasylol, and 40 mM NaF), and then boiling immediately. Proteins were precipitated with 20% trichloroacetic acid and hydrolyzed under nitrogen in distilled HCl for 2 h at 110°C, and the phosphoamino acids were separated by two-dimensional electrophoresis at pH 1.9 and 3.5.

To examine phosphorylation of the 36K protein, cells were labeled simultaneously with 70 μCi of [^{35}S]methionine per ml (>400 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and 1 mCi of $^{32}P_i$ per ml for 12 to 16 h. Cells were washed and lysed on the dishes in RIPA (3) supplemented with 1% Trasylol (FBA Pharmaceuticals, New York, N.Y.), 1 mM EDTA, and 40 mM NaF and then clarified at 30,000 × *g* for 30 min. The 36K protein was precipitated with rabbit anti-chicken 36K serum (a generous gift from R. Erikson, Denver, Colo.) and protein A-Sepharose (Sigma Chemical Co., St. Louis, Mo.). The complex was washed five times with RIPA plus Trasylol, boiled for 3 min, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography. [^{35}S]methionine and $^{32}P_i$ were quantitated from the film as described (K. Nakamura and M. J. Weber, Mol. Cell. Biol., in press). The data in Table 8 are derived from the $^{32}P/^{35}S$ ratio in the 36K protein.

RESULTS

In vitro properties of RSV mutants. Chicken cells infected in vitro with wild-type RSV, subgroup A (SR-A), at 36 or 42°C are fully transformed with respect to the following properties: loss of anchorage dependence, release from density-dependent inhibition of growth, increased levels of plasminogen activator production and hexose uptake, loss of cell surface fibronectin, altered morphology, and decreased adhesiveness to culture plates.

The partial transformation mutants of RSV carry lesions (some of which are temperature sensitive) in the *src* gene, as shown by both genetic analysis and measurement of pp60^{src} kinase activity (1, 2; M. J. Weber, unpublished data); consequently, they are defective in inducing the expression of one or more of these transformation parameters. Table 1 summarizes the biological properties of chicken cells infected in vitro with these mutants. Cells infected with CU2 form only very small "minicolonies" in soft agar and are thus partially defective for anchorage-independent growth. tsCU11 does not induce morphological changes in chicken cells, even at 36°C (the permissive temperature for expression of the other transformation parameters), whereas CU12 fails to induce the loss of surface fibronectin. Cells infected with tsGI251 are thermosensitive for most transformation parameters, but cold sensitive for the ability to grow in soft agar. Cells infected with tsNY68 are phenotypically fully transformed at the permissive temperature of 36°C, but are nearly normal at the nonpermissive temperature (42°C) (17). tdNY101 is a *src* deletion mutant of SR-A (16). Cells infected with this mutant are phenotypically untransformed with respect to all of the properties we examined.

Tumorigenicity of RSV mutants in chickens. Virus stocks were adjusted to the same number of virus particles per milliliter based on reverse transcriptase activity. Then 0.1 ml of each virus was injected into each wing web of 5-week-old chicks. The incidence of tumors and their progression were monitored for 35 days (Table 2; Fig. 1). It is clear that all the mutants were less tumorigenic than the wild-type virus, SR-A. tsGI251 rapidly induced tumors with an incidence similar to that of SR-A, but the tumors induced by tsGI251 uniformly regressed during the course of the experiment. In contrast, tumors induced by SR-A grew progressively, and only one chicken was observed to have a tumor which regressed. tsNY68 and tsCU11 induced tumors which enlarged slowly over the period of observation, and these tumors were clearly less progressive than those induced by wild-type SR-A. Our findings with tsNY68 are in agreement with those reported previously by Kawai and Hanafusa (17). CU12 induced tumors in most animals, but these tumors regressed in all cases. CU2 was clearly the least tumorigenic of this group of viruses. Tumors induced by CU2 were slow to appear and were very small, nonprogressing nodules. However, it should be noted that a single tumor nodule induced by CU2 was progressing at the termination of the experiment. Whether this tumor arose as a consequence of a virus revertant, or was due to some other cause, was not determined.

Histological examination of the tumor nodules revealed the presence of a loose network of fibrous cells, which varied in density. Tumors induced by mutants were rarely invasive, always contained pale-staining cells in a loose arrangement, and frequently contained collections of lymphoid cells. In contrast, tumors induced by SR-A were dense collections of anaplastic, dark-staining fibrous cells which were invasive (data not shown).

Tumorigenicity of mutant-infected chicken cells in nude mice. RSV does not readily infect mammalian cells. Therefore, to study tumorigenicity of the RSV mutants in nude mice, we infected chicken embryo cells with the viruses and then injected various numbers of infected cells into mice (Table 3). Cells infected with tsNY68 were the most tumorigenic of the mutant-infected cells, occasionally forming tumors even when only 10^5 cells were injected. Cells infected with CU12 or tsCU11 were not tumorigenic at this inoculum, but formed tumors in most of the animals when 10^6 cells were injected. tsGI251 was somewhat less tumorigenic than tsCU11,

TABLE 1. Properties of cells transformed by partial mutants[a]

Cellular Phenotype	Virus mutant				
	CU2	tsCU11 (36°C)	CU12	tsGI251	
				36°C	42°C
Transformed	Fibronectin; density inhibition	Fibronectin; anchorage dependence; casein plaques; plasminogen activator	Anchorage dependence; density inhibition; casein plaques; plasminogen activator	Plasminogen activator; hexose transport; fibronectin; morphology; adhesiveness	Anchorage dependence; density inhibition; morphology; adhesiveness
Intermediate or abnormal	Anchorage dependence; casein plaques; blebby morphology; plasminogen activator	Density inhibition; hexose transport	Hexose transport; fibronectin; fusiform morphology; adhesiveness[b]		Fibronectin
Untransformed	Hexose transport; adhesiveness[c]; focus formation	Morphology; adhesiveness; focus formation	Adhesiveness[b]; focus formation	Anchorage dependence; density inhibition	Plasminogen activator; hexose transport

[a] Data are from references 1 and 31.
[b] Value varied with assay conditions.
[c] Adhesiveness even greater than normal cell control.

whereas CU2 was the least tumorigenic of the partial mutants. Cells infected with tdNY101, a mutant in which the *src* gene is entirely deleted, were completely nontumorigenic.

Our results with wild-type virus, SR-A, deserve a special comment. We found that cells freshly infected with this virus were highly tumorigenic in nude mice, but that infected cells which were passaged more than once in vitro before injection were much less tumorigenic. In vitro passaging did not lead to a similar reduction in the tumorigenicity of cells infected with mutant viruses. The decreased tumorigenicity of the passaged SR-A-infected cells may have been due either to their more rapid senescence in vitro relative to mutant-infected cells (Weber, unpublished data; 18) or to a *src*-mediated cytotoxicity.

Tumorigenicity in sublethally irradiated nude mice. Although nude mice lack T-cell-mediated immunity, they possess T-cell-independent graft rejection mechanisms which can recognize and destroy certain highly malignant cells (25; reviewed in reference 13). In particular, nude mice are capable of rejecting certain virus-transformed cells (15; P. Kahn, W. Topp, and S. Shin, manuscript in preparation), perhaps because they recognize some virus-coded proteins. It was therefore possible that the different tumor-forming abilities of chicken cells infected with RSV mutants were a consequence of differential susceptibility to the graft rejection mechanisms in nude mice. To test this possibility, we compared the tumorigenicity of cells infected with tsNY68 (the most tumorigenic of the mutant viruses) and CU2 (the least tumorigenic mutant) in untreated and sublethally irradiated nude mice. Sublethal γ-irradiation of nude mice has been shown to greatly impair the T-cell-independent graft rejection process and to enhance tumor growth by certain otherwise poorly tumorigenic cells (14).

Irradiation of the host had little effect on the tumorigenicity of the RSV-transformed cells (Table 4). Other experiments have demonstrated that identical treatment would permit the growth and metastasis of tumors which would ordinarily be rejected by untreated nude mice (14). These results suggest that the poor tumorigenicity of CU2-infected cells is not predominantly due to the host graft rejection mechanism, but reflects the lower neoplastic potential of these cells.

Relative tumorigenicity of RSV mutants. In determining the relative tumorigenicity of the mutants, two factors need to be taken into account: (i) the initial incidence of tumors; and (ii) whether the tumor grows progressively, or whether it regresses. The initial formation of a tumor nodule may reflect the intrinsic oncogenicity of the transformed cells, whereas its growth or regression is likely to depend on both the cells' immunogenicity as well as their oncogenicity and perhaps on other factors as well. In Table 5 we list the relative tumorigenicity of the

TABLE 2. Tumorigenicity in chickens of partial transformation mutants of RSV

Virus	Tumorigenicity at given days postinfection															
	9		13		15		18		21		26		30		35	
	Tumor diam (mm)[a]	Incidence[b]	Tumor diam (mm)	Incidence	Tumor diam (mm)	Incidence	Tumor diam (mm)	Incidence	Tumor diam (mm)	Incidence	Tumor diam (mm)	Incidence	Tumor diam (mm)	Incidence	Tumor diam (mm)	Incidence
CU2	1.0	0/6	1.0	0/6	1.0	2/6	1.3 ± 0.5	4/6	1.2 ± 0.9	2/6	1.4 ± 1.4	2/6	1.8 ± 2.5	1/6	2.2 ± 3.8	1/6
tsCU11	1.0	0/4	1.0	1/4	1.4 ± 0.5	2/4	2.5 ± 1.9	4/4	3.4 ± 2.0	4/4	4.2 ± 3.6	4/4	5.3 ± 2.4	3/3	8.5 ± 3.4	3/3
CU12	1.6 ± 1.3	5/6	2.1 ± 1.4	4/6	1.9 ± 1.2	4/6	2.3 ± 1.6	4/6	1.5 ± 0.8	3/6	1.3 ± 1.1	1/6	1.0	0/6	1.0	0/6
tsNY68	1.6 ± 1.2	3/6	2.1 ± 1.9	3/6	2.2 ± 2.2	3/6	2.8 ± 2.7	4/6	3.5 ± 3.3	5/6	4.0 ± 3.5	4/6	4.8 ± 4.6	4/6	5.3 ± 6.0	4/6
tsGI251	3.6 ± 1.9	6/6	4.2 ± 2.2	6/6	3.7 ± 2.2	6/6	3.2 ± 2.4	6/6	2.7 ± 2.2	5/6	2.5 ± 2.5	2/6	1.7 ± 1.6	2/6	1.7 ± 1.6	1/6
SR-A	3.9 ± 1.8	5/5	8.7 ± 2.9	5/5	9.7 ± 4.5	5/5	9.5 ± 5.1	3/3	9.0 ± 5.9	3/3	8.5 ± 6.8	2/3	7.3 ± 5.5	2/3	7.3 ± 5.5	2/3

[a] Numbers represent mean tumor diameter ± standard error. The thickness of the uninfected wing web was 1.0 mm.

[b] Numbers reflect chickens with a tumor in one or both wing webs over total chickens observed. A decrease in the incidence reflects regression of the tumor; a decrease in the total number of chickens reflects a death due to tumor mass or metastasis. No nonspecific deaths were observed.

FIG. 1. Growth and regression of tumors induced in chickens by partial transformation mutants of RSV.

five RSV mutants both at early times (reflecting initial incidence) and at later times (reflecting growth versus regression). In nude mice, this rank ordering was not greatly affected by the time after injection at which the observations were made. This is consistent with the fact that nude mice cannot mount an effective immune response to these cells. In chickens, however, the rank ordering based on initial incidence was strikingly different from the rank ordering at the end of the experiment. This was because, although tsGI251 and CU12 were efficient at inducing tumors, the tumors that formed regressed (see Fig. 1).

Temperature of experimental animals. Examination of the relative tumorigenicity of the RSV mutants in nude mice and chickens at early times (Table 5) indicated that cells infected with the temperature-sensitive mutants tsNY68 and tsCU11 showed relatively greater initial tumorigenicity in mice than in chickens, whereas tsGI251, which was cold sensitive for anchorage-independent growth, was relatively less tumorigenic in mice. These differences would be explicable if the body temperature of mice was significantly lower than that of chickens. Therefore, we measured the body temperature of these two animals at various sites, as well as the intratumor temperatures, using an electronic thermometer fitted with a microprobe. Intratumor temperatures in nude mice were approximately 4 to 5°C lower than in chickens (Table 6).

Isozyme analysis of tumor-derived cell cultures. Since nude mice are nonpermissive for RSV

TABLE 3. Tumorigenicity in nude mice of chicken embryo cells transformed by partial transformation mutants of RSV

Virus	Tumorigenicity at given weeks postinjection		
	4	8	16
10^5 cells/site			
CU2	0/6	0/6	0/6
tsCU11	0/6	0/6	0/6
CU12	0/6	0/6	0/6
tsNY68	1/6[a]	2/6[a]	2/6[a]
tsGI251	0/6	0/6	0/6
tdNY101	0/6	0/6	0/6
SR-A	0/6	0/6	0/6
10^6 cells/site			
CU2	0/6	0/6	0/6
tsCU11	5/6	3/6[a]	2/4[a]
CU12	5/6	4/4[a]	2/2[a]
tsNY68	6/6	6/6[a]	6/6[a]
tsGI251	1/5	1/5[a]	1/5[a]
tdNY101	0/6	0/6	0/6
SR-A	7/10	6/6	4/4
5×10^6 cells/site			
CU2	4/6	4/6	4/6
tsCU11	6/6	5/6	5/6[a]
CU12	2/6	4/6	2/4[a]
tsNY68	6/6	6/6	6/6
tsGI251	5/5	5/5	2/2[a]
tdNY101	0/6	0/6	0/6
10^7 cells/site			
CU2	5/6[a]	5/6[a]	5/6[a]
tsCU11	6/6	5/6	5/6
CU12	5/6	3/6	3/6[a]
tsNY68	6/6	—[b]	—[b]
tsGI251	5/5	5/5	1/1[b]
tdNY101	0/6	0/6	0/6

[a] Small, nongrowing nodules.
[b] Hosts died from tumors.

infection and replication, it is possible to recover cells from tumors in this host and to initiate tumor-derived cultures containing descendants of the original chicken cell inoculum. To verify that such tumor-derived cultures do indeed consist of chicken cells, we screened tumor cultures derived from tsNY68-infected chicken cells for the chicken isozyme of GPI. The chickens and

TABLE 4. Tumorigenicity of mutant virus-infected chicken embryo fibroblasts in untreated and in sublethally irradiated nude mice

No. of cells injected	Tumorigenicity			
	Unirradiated mice		Irradiated mice	
	tsNY68	CU2	tsNY68	CU2
10^7	3/3	3/3[a]	6/6	4/4[a]
5×10^6	3/3	0/3	5/6	5/5[a]
10^6	3/3[a]	0/3	3/6[a]	0/3
10^5	0/3	0/3	0/5	0/3

[a] Small, nongrowing nodules.

TABLE 5. Relative tumorigenicity of RSV mutants[a]

Rank	Relative tumorigenicity in given host			
	Chicken		Mouse[b]	
	Early	Late	Early	Late
1	tsGI251	tsCU11	tsNY68	tsNY68
2	CU12	tsNY68	CU12	CU12
3	tsNY68	tsGI251	tsCU11	tsCU11
4	tsCU11	CU2	tsGI251	tsGI251
5	CU2	CU12	CU2	CU2

[a] Based on tumor incidence.
[b] Based on injection of 10^6 cells/site. This number of cells displayed the greatest differences in tumorigenicity of the mutants.

the BALB/c mice expressed electrophoretically distinguishable isozymes of GPI; the tumor-derived cultures showed only the chicken isozyme (Fig. 2).

Transformation parameters of tumor cells. To determine whether any transformation parameters of the chicken cells were altered during the growth of the cells as tumors in a mammalian host, cultures derived from nude mouse tumors induced by each of the virus mutants were examined for their transformation-associated properties (Table 7).

The most striking difference between virus-infected tertiary cultures of chicken embryo cells and cell cultures established from tumors was that the tumor-derived cells produced significantly more plasminogen activator than did

TABLE 6. Temperatures in vivo in a nude mouse or a chicken carrying a tumor of RSV-transformed chicken cells

Site of measurement	Temp (°C)[a]
Mouse	
Internal body (rectal)	35.5
Subcutaneous, flank	34.1
Intratumor, at various depths[b]	34.4
Chicken	
Internal body (cloacal)	41.5
Tumor and wing web	38.4

[a] Local temperatures in vivo were measured with a no. 26 gauge thermocouple, attached to an electronic digital thermometer (Bailey Instrument Co., Saddle Brook, N.J.), or with a thermocouple thermometer (Wescor, Inc., Logan, Utah) using a wire probe inserted into a 20-gauge needle. The temperatures in the tumors varied by 1°C in each direction during different measurements and were affected by the tumor size and depth of the measurement, as well as (most probably) vascularization and thermal regulation at the tumor site.
[b] Tumor size, 12 by 20 mm.

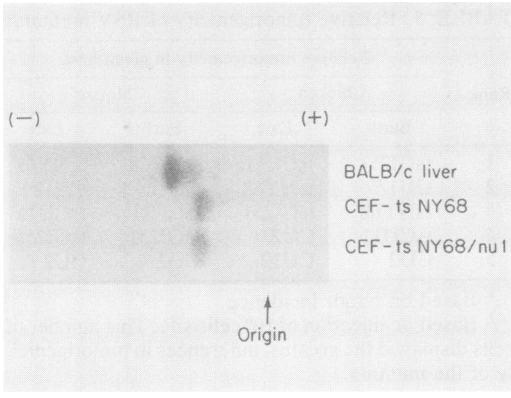

FIG. 2. GPI isozymes in (from top to bottom) mouse tissue, cultured chicken embryo cells transformed by tsNY68, and cultured cells from a nude mouse tumor initiated with tsNY68-transformed chicken embryo cells.

the parental cells before injection. Thus, cells with high levels of plasminogen activator appeared to have been selectively favored during tumor formation. Most of the tumor-derived cultures had more cell surface fibronectin than the corresponding parental cells before passage in vivo, indicating that a reduction in fibronectin was not associated with tumor growth by these cells. Tumor-derived cells showed a slight decrease in the efficiency of colony formation in soft agar relative to the same cells before passage in nude mice. This may have been a consequence of the tumor-derived cells undergoing senescence shortly after they were placed into culture.

The morphologies of the tumor-derived cells were similar to those previously reported for the mutant-infected cultured cells infected with three of the four mutants tested (a round morphology for tsNY68 at 36°C, blebs on the cell surface for CU2, fusiform for CU12). The one exception was that tumor-derived tsCU11-infected cells grown at 36°C frequently had a rounded, highly transformed appearance, whereas the morphology of the parental cultures was similar to that of normal, uninfected chicken embryo cells (data not shown). This morphological difference was seen with cultures derived from three independent tumors. We have sometimes observed a similar effect on cell morphology after repeated in vitro passaging of tsCU11 cells.

In contrast, several other transformation-associated properties were not significantly altered during tumor formation. There was no selection for cells with enhanced detachability (decreased adhesiveness) except for cells infected with tsCU11, as described above. A slight increase in hexose transport was seen in tumor-derived cells infected with CU2 but was not highly significant.

Transformation parameters of cells infected with viruses recovered from tumors. To determine whether the phenotypic changes exhibited by the tumor-derived cells were due to virus mutations, virus was harvested from the tumor

TABLE 7. Transformation parameters of tumor-derived and in vitro-transformed cells

Parameter	Value[a]							
	36°C				42°C			
	tsNY68	CU2	tsCU11	CU12	tsNY68	CU2	tsCU11	CU12
Tertiary cells infected with initial virus stocks								
Plasminogen activator	100	38	39	33	6	10	2	40
Detachability	100	16	22	9	14	11	9	18
2-Deoxyglucose transport	100	26	51	47	14	24	16	48
Fibronectin	100	58	93	413	480	29	439	243
Cultured cells from tumor								
Plasminogen activator	700	84	756	152	36	86	86	86
Detachability	65	22	97	30	27	16	57	27
2-Deoxyglucose transport	79	57	48	45	20	37	15	43
Fibronectin	50	240	515	808	1,300	365	1,125	5,000
Anchorage-independent growth	4	1	4	4	0.08	0.15	1	2
Tertiary cells infected with virus from tumor								
Plasminogen activator	61	82	94	40	10	5	33	25
Detachability	41	11	68	50	11	7	9	32
2-Deoxyglucose transport	118	54	100	85	41	31	61	54

[a] Expressed as percentage of the value obtained with tsNY68-infected tertiary cells at 36°C. Values given are the average of duplicate plates, which generally displayed less than 20% difference; however, variability between experiments was sometimes as much as twofold. Therefore, in comparing the mutants with one another, only differences greater than twofold should be considered significant. Methods for performing the assays are described in references 1 and 31.

cultures and used to infect normal chicken embryo cells (Table 7). The most striking difference between these cells and the original transformed cells was that tsCU11 recovered from tumor cells induced quantitatively greater changes in plasminogen activator production, detachability, and hexose transport relative to the initial virus stocks, although it was still temperature sensitive. This was true also for its ability to cause morphological transformation (data not shown). Thus, these changes were due to virus reversion. On the other hand, the virus produced by the tumor cells did not induce a particularly high level of plasminogen activator production. Thus, the very high plasminogen activator levels of the tumor cells must have been a consequence of selection for cellular variants, rather than a consequence of virus mutation.

Phosphorylation on tyrosine in cells infected with the partial transformation mutants. Tumorigenicity as well as the expression of the transformation parameters are dependent on the activity of $pp60^{src}$, the virus-coded transforming protein. $pp60^{src}$ has been shown to possess a protein kinase activity which phosphorylates on tyrosine residues (5, 12, 26). One of the major targets of $pp60^{src}$ kinase activity is a protein of 36,000 M_r (8, 23, 24). We therefore examined the phosphorylation of the 36,000-M_r protein as well as the total phosphotyrosine content in cells infected with the partial transformation mutants and held at 36°C (Table 8). It is striking that cells infected with CU2, which were the least tumorigenic, displayed the lowest 36K protein phosphorylation. It may be noteworthy that tsNY68 and CU12, which were the most tumorigenic in nude mice, caused the highest phosphorylation of the 36K protein. There was a much poorer relationship between total phosphotyrosine content and tumorigenicity.

DISCUSSION

Nude mice, which are congenitally deficient in thymus-dependent immunity and generally incapable of graft rejection, are presently the animals of choice for the assay of cellular tumorigenicity (reviewed in reference 10). These animals make it possible to compare the oncogenic potential of diverse types of tumor cells in a single host. With respect to the particular transformed cells used in our studies, nude mice have two additional advantages. First, they are nonpermissive for replication of RSV, and thus permit the direct descendants of the initial cell inoculum to be recovered from the tumors. Second, the body temperature is lower than that of chickens, so that the tumorigenicity of cells infected with temperature-sensitive virus mutants can also be assayed in nude mice.

Although nude mice are widely used in studies of the tumorigenicity of mammalian cells, such studies have not previously been reported for avian cells. Thus, we began our investigation by comparing the tumorigenicity of our partial transformation mutants in nude mice and in chickens. We found that the initial tumorigenicity of the mutants was similar in these two hosts, except for a few differences which could be due to the lower body temperature of nude mice compared with chickens. For example, tsNY68-infected cells injected into nude mice showed a high degree of tumorigenicity comparable to that of cells transformed with wild-type virus (SR-A), whereas in chickens, tsNY68 was considerably less tumorigenic than SR-A. This is consistent with the fact that at 34°C (the approximate internal temperature of the nude mouse tumors), tsNY68-infected cells are fully transformed by in vitro criteria, whereas at 38.4°C (the intratumor temperature in chickens), these cells are only partially transformed in vitro (31). A similar argument can be advanced with respect to tsCU11. tsGI251-infected cells, which are thermosensitive for most parameters of transformation in vitro and cold sensitive for anchorage-independent growth, were less tumorigenic (relative to SR-A-infected cells) in nude mice than in chickens, where this virus was nearly as tumorigenic as SR-A at early times. (At longer times after injection, some of the tumors induced in chickens regressed, whereas this was not a major factor with the tumors formed in mice.)

Comparison of the in vitro properties of the virus-infected cells with their tumorigenicity in vivo indicates that anchorage independence is the property which was best correlated with tumor-forming ability. For example, cells infected with CU2 are defective for growth in soft agar, forming only minicolonies which are barely visible to the unaided eye. CU2 is also the least tumorigenic mutant in both chickens and nude mice, capable of forming only small, nonprogressing nodules.

TABLE 8. Phosphotyrosine in cells infected with partial transformation mutants of RSV

Virus	Phosphorylation of 36K protein[a]	Total phosphotyrosine content[b]
CU2	0.07	0.69
tsCU11	0.51	0.62
CU12	1.09	1.32
tsNY68	0.78	0.78
tsGI251	0.59	0.74

[a] Expressed as fraction of wild-type SR-A.
[b] Expressed as percentage of total phosphoamino acids.

The relationship between in vitro properties and tumor-forming ability was also examined in cells derived from tumors in nude mice. Several lines of evidence indicate that the cells obtained after plating tumor cell suspensions in vitro are indeed chicken cells. (i) The cultures were initially grown at 42°C in growth medium containing 10% tryptose phosphate broth, 4% calf serum, and 1% chicken serum. In our experience, these conditions do not permit the survival of rodent cells. (ii) The cultured tumor cells produced RSV, and mouse cells are not readily infected by the SR-A strain of RSV. Moreover, mouse cells are nonpermissive for replication of the virus. (iii) The GPI isozyme pattern of the tumor cultures indicates that they are composed predominantly of chicken cells.

The tumor-derived cultures were found to produce extremely high levels of plasminogen activator relative to the corresponding cells before injection. This was not due to virus mutation, since normal chicken cells infected with viruses recovered from the tumors failed to produce equivalent levels of plasminogen activator. Instead, this increase may have been the consequence of selection for high levels of plasminogen activator production during tumorigenesis. In contrast, there was no selection in vivo for cells with reduced amounts of cell surface fibronectin, which is consistent with the idea that the absence of fibronectin in vitro is not necessary for tumorigenicity in vivo (13).

A 36,000-M_r protein has been described which is rapidly phosphorylated during RSV transformation and which may be a target for the kinase activity of pp60^{src} (8, 23, 24). This protein is poorly phosphorylated in CU2-infected cells. This result, taken together with the tumorigenicity data presented in this report, raises the possibility that the 36,000-M_r protein may be involved in tumor growth by RSV-transformed cells. Note, however, that CU12-infected cells display a level of 36K phosphorylation and total phosphotyrosine content at least as high as that shown by wild-type-infected cells, but nonetheless are less tumorigenic. This indicates that 36K phosphorylation may be necessary for tumorigenicity, but not sufficient.

In summary, we have shown that the nude mouse is a suitable animal model for testing the tumor-forming ability of transformed chicken cells. Using this host to analyse the tumorigenicity of cells transformed by partial transformation mutants of RSV, we found that anchorage-independent growth, plasminogen activator production, and the phosphorylation of a 36,000-M_r protein correlated with tumorigenicity.

ACKNOWLEDGMENTS

We thank Margaret Bruesch, Andre Brown, Helene Weiss, and Ted Wheeler for expert technical assistance.

This work was supported by Public Health Service research grants CA 12467 (to M.J.W.), CA 21054 (to S.S.), and CA 12323 and CA 14236 (to R.E.S.). P.K. was a predoctoral trainee supported by a Public Health Service training grant in viral oncology and tumor biology (CA 09060). All grants were from the National Institutes of Health.

LITERATURE CITED

1. **Anderson, D. D., R. P. Beckmann, E. H. Harms, K. Nakamura, and M. J. Weber.** 1981. Biological properties of "partial" transformation mutants of Rous sarcoma virus. J. Virol. **37**:445–458.
2. **Becker, D., R. Kurth, D. Critchley, R. Friis, and H. Bauer.** 1977. Distinguishable transformation-defective phenotypes among temperature-sensitive mutants of Rous sarcoma virus. J. Virol. **21**:1042–1055.
3. **Brugge, J. S., and R. L. Erikson.** 1977. Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature (London) **269**:346–348.
4. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. U.S.A. **75**:2021–2024.
5. **Collett, M. S., A. F. Purchio, and R. L. Erikson.** 1980. Avian sarcoma virus transforming protein shows protein kinase activity specific for tyrosine. Nature (London) **285**:167–169.
6. **Eicher, E. M., and L. L. Washburn.** 1978. Assignment of genes to regions of mouse chromosomes. Proc. Natl. Acad. Sci. U.S.A. **75**:946–950.
7. **Erikson, R. L., M. S. Collett, E. Erikson, and A. F. Purchio.** 1979. Evidence that the avian sarcoma virus transforming gene product is a cyclic-AMP-independent protein kinase. Proc. Natl. Acad. Sci. U.S.A. **76**:6260–6264.
8. **Erikson, E., and R. L. Erikson.** 1980. Identification of a cellular protein substrate phosphorylated by the avian sarcoma virus transforming gene product. Cell **21**:829–836.
9. **Freedman, V. H., A. L. Brown, H. P. Klinger, and S. Shin.** 1976. Mass production of animal cells in nude mice with retention of cell-specific markers. Exp. Cell Res. **98**:143–151.
10. **Freedman, V. H., and S. Shin.** 1978. Use of nude mice for studies on the tumorigenicity of animal cells, p. 353-384. *In* J. Fogh and B. C. Giovanella (ed.), The nude mouse in experimental and clinical research. Academic Press, Inc., New York.
11. **Hanafusa, H.** 1977. Cell transformation by RNA tumor viruses, p. 401–483. *In* H. Fraenkel-Conrat and R. Wagner (ed.), Comprehensive virology, vol. 10. Plenum Publishing Corp., New York.
12. **Hunter, T., and B. M. Sefton.** 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. **77**:1311–1315.
13. **Kahn, P., and S. Shin.** 1979. Cellular tumorigenicity in nude mice: test of associations among loss of cell surface fibronectin, anchorage independence, and tumor-forming ability. J. Cell Biol. **82**:1–16.
14. **Kahn, P., and S. Shin.** 1981. Thymus-independent tumor surveillance mechanisms, p. 417–424. *In* C. M. Steinberg and I. Lefkovits (ed.), The immune system, vol. 2. S. Karger, Basel.
15. **Kahn, P., R. S. Simon, A. S. Klein, and S. Shin.** 1980. Tumor formation by transformed cells in nude mice. Cold Spring Harbor Symp. Quant. Biol. **44**:695–702.
16. **Kawai, S., P. H. Duesberg, and H. Hanafusa.** 1977. Transformation defective mutants of Rous sarcoma virus with *src* gene deletions of varying length. J. Virol. **24**:910–914.
17. **Kawai, S., and H. Hanafusa.** 1971. The effects of reciprocal changes in temperature on the transformed state of cells infected with a RSV mutant. Virology **46**:470–479.
18. **Khoury, A. T., and H. Hanafusa.** 1976. Synthesis and integration of viral DNA in chicken cells at different times after infection with various multiplicities of avian oncor-

navirus. J. Virol. 18:383–400.
19. **Levinson, A. D., H. Opperman, L. Levintow, H. Varmus, and J. M. Bishop.** 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. Cell 15:561–572.
20. **Meera Khan, P.** 1971. Enzyme electrophoresis on cellulose acetate gel: zymogram patterns in man-mouse and man-Chinese hamster somatic cell hybrids. Arch. Biochem. Biophys. 145:470.
21. **Pollack, R., and D. Rifkin.** 1975. Actin-containing cables within anchorage dependent rat embryo cells are dissociated by plasmin and trypsin. Cell 6:495–506.
22. **Purchio, A. F., E. Erikson, J. S. Brugge, and R. L. Erikson.** 1978. Identification of a polypeptide encoded by the avian sarcoma virus *src* gene. Proc. Natl. Acad. Sci. U.S.A. 75:1567–1571.
23. **Radke, K., T. Gilmore, and G. S. Martin.** 1980. Transformation by Rous sarcoma virus: a cellular substrate for transformation-specific protein phosphorylation contains phosphotyrosine. Cell 21:821–828.
24. **Radke, K., and G. S. Martin.** 1979. Transformation by the Rous sarcoma virus: effects of *src* gene expression on the synthesis and phosphorylation of cellular polypeptides. Proc. Natl. Acad. Sci. U.S.A. 78:5212–5216.
25. **Reid, L. M., N. Minato, I. Gresser, J. Holland, A. Kadish, and B. R. Bloom.** 1981. Influence of anti-mouse interferon serum on the growth and metastasis of tumor cells persistently infected with virus and of human prostatic tumors in athymic nude mice. Proc. Natl. Acad. Sci. U.S.A. 78:1171–1175.
26. **Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart.** 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. Cell 20:807–816.
27. **Shin, S., V. H. Freedman, R. Risser, and R. Pollack.** 1975. Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth in vitro. Proc. Natl. Acad. Sci. U.S.A. 72:4435–4439.
28. **Tooze, J.** 1975. Molecular biology of tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Vogt, P. K.** 1977. The genetics of RNA tumor viruses, p. 341–455. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 9. Plenum Publishing Corp., New York.
30. **Weber, M. J.** 1973. Hexose transport in normal and in Rous sarcoma virus transformed cells. J. Biol. Chem. 248:2978–2983.
31. **Weber, M. J., and R. R. Friis.** 1979. Dissociation of transformation parameters using temperature-conditional mutants of Rous sarcoma virus. Cell 16:25–32.