Distinguishable Transformation-Defective Phenotypes Among Temperature-Sensitive Mutants of Rous Sarcoma Virus

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Eight transformation-defective, temperature-sensitive (*ts*) mutants of the Prague strain of Rous sarcoma virus, subgroup A, have been isolated after mutagenesis with 5-bromodeoxyuridine followed by selection on the basis of focus tests. Five of these mutants, *ts* GI201, GI202, GI203, GI204, and GI205, exhibit properties like most previously reported isolates in that they show a temperature-sensitive response to each of a variety of transformation-specific parameters tested. Interestingly, GI201, in addition to the temperature-sensitive defect, carries a lesion that was observed as a nonconditional loss of expression of plasminogen activator protease. Three mutants, *ts* GI251, GI252, and GI253, have been designated partial transformation-defective (PTD) mutants since they behave as *ts* mutants according to some tests for transformation and as wild type according to others. These three mutants fail to form foci at the nonpermissive temperature (41°C) and are nontumorigenic in 3-week-old chickens (body temperature, 42°C). The agglutinability by concanavalin A of cells infected with these mutants shows a definite temperature sensitivity, as do the rate of 2-deoxyglucose uptake and the disappearance of the 250,000-dalton normal cell glycoprotein (large, external, transformation sensitive [LETS]). Although the PTD mutant-infected cells, unlike cells infected with other transformation mutants, exhibit a cell-bound plasminogen activator protease at the nonpermissive temperature, this activator is not detectable as a free protease in the medium, as it is with wild-type, virus-infected cells. The PTD mutants behave like the wild-type parent in their ability to induce transformed growth properties in the infected cells, i.e., growth beyond normal cell saturation density with or without serum-supplemented medium and growth leading to colony formation in soft-agar- or methyl cellulose-containing suspension media.

A great variety of changes in cell behavior, form, and metabolic activity are characteristic of fibroblasts that have undergone virus-induced transformation. Transformed cells fail to respond to conditions that inhibit the growth of normal cells, such as high population density (39), absence of serum from the medium (13), or absence of a substrate for anchorage (23, 37). Formation of characteristic foci makes transformed cells apparent in a monolayer of normal cells (38). Striking changes in the rate of transport of certain sugars, which greatly exceed the range of values obtained with logarithmically growing or stationary normal fibroblasts (11,

43), have been observed in transformed cells. The increased mobility or altered distribution of appropriate receptors results in significantly enhanced agglutinability of transformed cells by concanavalin A and other lectins (5, 32). Increased expression of a protease measured as a plasminogen activator (27, 30, 40, 41) occurs as cells become transformed. It is probable that some proteolytic activity, in turn, influences the structure of the cell surface by degrading cell surface proteins, among them the 250,000-dalton large, external, transformation-sensitive (LETS) glycoprotein (12, 14, 34, 37, 45).

Various temperature-sensitive (*ts*) mutants of avian sarcoma viruses have been studied with respect to certain of these parameters of transformation (2, 4, 6, 9, 20, 22, 24, 47). In general, mutants isolated by selection, based on a focus test, have been temperature sensi-

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tive for all other parameters tested. Two notable exceptions have been reported in the literature: *ts* LA334 bears a transformation defect, which, although it prevents focus formation at the nonpermissive temperature, does not interdict formation of colonies in soft-agar suspension (9); and *ts* LA25, which, though defective for focus and colony formation at the nonpermissive temperature, does stimulate cells to grow to densities much higher than those obtained by normal cells (22, 47).

This paper will document the isolation of eight transformation-defective (TD) avian sarcoma virus mutants, three of which exhibit an interesting dissociation of transformation parameters, i.e., respond as temperature-sensitive mutants according to certain tests but behave like wild-type virus according to other tests. Hence, two phenotypically different categories emerged as analyses of these mutants were carried out, and the profiles of these two categories will be described.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo cell cultures were prepared from fertile white leghorn embryos after 9 to 10 days of incubation. Eggs for this purpose were generously supplied from a leukosis virus-free flock by E. Vielitz, Lohmann Tierzucht GmbH, Cuxhaven, West Germany. Tissue cultures of normal and infected chicken embryo cells were grown in Dulbecco-modified Eagle medium prepared from a powder (L and S Labor Service, Munich, West Germany) supplemented with antibiotics, 5% heat-inactivated calf serum, and 10% tryptose phosphate broth (Difco Corp., Ann Arbor, Mich.).

The parent virus used for the mutagenization and isolation of mutants was the Prague strain of Rous sarcoma virus, subgroup A. The original parent had been repeatedly cloned by isolation, with an aspirating pipette, of single foci occurring at high dilution in a focus test (33), and mutants subsequently obtained were routinely cloned at each stock preparation step. The mutant designations (*ts* GI201 through 205; *ts* GI251 through 253) conform to suggestions put forth by Vogt et al. (42).

The procedures for preparation of soft-agar suspension cultures (8) and methyl cellulose suspension cultures (31, 46) used in tests for colony formation have been described previously. The compositions and protocols for the agar casein plaque overlay test and the soft-agar casein suspension culture have been reported by Goldberg (10) and Balduzzi and Murphy (3), respectively. In all cases Dulbecco-modified Eagle medium was used with the appropriate supplements.

Test for in vivo tumorigenicity of mutants. Chickens were inoculated in the wing webs of both wings at 21 days of age and examined biweekly thereafter for appearance of tumors at the site of inoculation. Based on the method of Bauer (unpublished observation), it was found that both wings could be used, using different doses to give two independent titra-

tions of the inoculated sample in a single animal.

Concanavalin A agglutination test. The concanavalin A agglutination test used was a modification of that of Pollack and Burger (28), which has been described in detail elsewhere (21). It was found necessary to use plastic pipettes and test tubes for all manipulations to minimize damage to the cell plasma membranes. Briefly, subconfluent chicken embryo cells were washed twice for 5 min each with phosphate-buffered saline (PBS), pH 7.2, on a rocking platform. Cells were suspended by 15 min of incubation at 37°C in PBS containing 0.02% (wt/vol) EDTA and, subsequently, were washed once in warm serum-free medium followed by centrifugation for 5 min at $1,200 \times g$. The cell number was determined with an electronic cell counter and adjusted to 2×10^6 /ml in PBS. Lyophilized concanavalin A (Sigma, St. Louis, Mo.) was reconstituted in distilled water, diluted in PBS to the desired concentrations, and predispensed in 5- μ l quantities into wells of a Microtest plate (type I, Falcon Plastics). Each well then received 10^4 cells in a 5- μ l volume; microplates were inverted to produce hanging drops and incubated for 15 min at room temperature on a slowly rocking platform. Scoring was performed using a low-magnification microscope (Dynascope, Vision Engineering Ltd., Send, Surrey).

Determination of LETS glycoprotein. Chicken embryo cells grown in 90-mm dishes were washed three times with PBS and labeled by adding 1 ml of PBS containing 5 mM glucose, 0.1 U of glucose oxidase, 20 μ g of lactoperoxidase, and 400 μ Ci of carrier-free sodium [125 I]iodide. After 10 min at room temperature, the reaction was stopped by adding 8 ml of PBS containing 50 mM sodium iodide, and the cells were washed three times with the same buffer.

Cells were scraped into PBS containing 50 mM sodium iodide and 2 mM phenyl sulfonyl fluoride, pelleted, and dissolved at 100°C in 0.4 ml of sample buffer (0.1 M Tris, pH 6.8, 15% glycerol, 2% sodium dodecyl sulfate). Prior to electrophoresis, samples were made 0.1 M with respect to dithiothreitol and reheated to 100°C. The procedure has been described in detail elsewhere (17).

Proteins were separated by using discontinuous sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (17). Gels were fixed and stained in 30% methanol-10% acetic acid containing Coomassie blue, dried onto paper, and exposed to Kodak X-ray film.

Determination of plasminogen activator. Insoluble [125 I]fibrin plates were prepared as described by Unkeless et al. (41). Harvest fluids were taken from cell cultures that had been washed once with serum-free medium, incubated for 2 h with serum-free medium, and finally washed a third time with serum-free medium prior to incubation for 12 h in medium supplemented with 2.5% chicken serum (for cell-bound plasminogen activator determination) or in unsupplemented medium (for supernatant plasminogen activator determination). After collection of harvest fluids, the cell number was determined for each plate, and the harvest fluids were diluted with the appropriate unincubated medium so that the harvest unit could be defined as the activity in 1 ml of medium incubated over 10^6 cells for 12 h. Harvest

fluids were centrifuged for 15 min at $1,200 \times g$ prior to storage at -80°C until tested on insoluble [^{125}I]fibrin plates. The fibrinolytic reaction was always performed at 37°C , as previous experiments (Friis, unpublished observations) had shown that neither supernatant plasminogen activator nor plasmin from mutant preparations was more temperature sensitive than that from wild-type virus-infected preparations. The fibrinolytic reaction was performed with 1 ml of harvest fluid per plate containing, or supplemented with (according to the type of harvest fluid), 2.5% chicken serum as a source of plasminogen. The fibrinolytic reaction was monitored over a period of 12 h, with 0.1-ml samples taken at intervals and counted in a liquid scintillation counter. For evaluation of plasminogen activator activities, reaction times were chosen at which not more than 30 to 50% of the available insoluble [^{125}I]fibrin (according to trypsin digestion controls) had been released with the wild-type virus-infected preparations or mutant virus-infected preparations from cells grown at the permissive temperature.

RESULTS

Isolation of mutants after 5-bromodeoxyuridine mutagenesis. Cells infected with a stock of cloned Prague strain Rous sarcoma virus, subgroup A, were treated according to the method of Bader and Brown (1) in the dark from the time of infection until 12 h postinfection with $100 \mu\text{g}$ of 5-bromodeoxyuridine per ml in medium lacking the usual tryptose-phosphate broth supplement. At 40 h postinfection, the supernatants were harvested, and the titer of infectious virus in treated and untreated samples was determined in a focus test. It was found that 5-bromodeoxyuridine treatment reduced the titer of treated samples by a factor of about 20. The 5-bromodeoxyuridine-treated virus was plated at high dilutions in a focus test, and single foci were isolated as presumptive mutagenized virus clones, which were grown to small stocks in a Microtest plate. Clones were screened for the ability to replicate and to form foci at permissive (35°C) and nonpermissive (41°C) temperatures in Microtest plate wells using a Titer-tec semiautomatic dilutor system (Flow Laboratories, Bonn) to facilitate screening. Out of 440 clones examined, 8 were found to be temperature sensitive for focus formation. None of the 440 clones exhibited temperature sensitivity for replication of progeny; rather, the titers produced at 41°C were consistently higher by a factor of 2 or 3 than those produced at 35°C . The eight temperature-sensitive clones observed were serially cloned three times, after which their properties in various assays for transformation were examined. Table 1 shows a summary of results with some of the mutants,

showing their relative abilities to form foci at permissive and nonpermissive temperatures.

Growth-related parameters of transformation. The ability of transformed cells to form colonies in a suspension medium of soft agar or methyl cellulose is a measure of anchorage-independent growth capacity, a property not exhibited by normal fibroblast cells (23, 36). Table 2 shows the results of a test for the ability of several temperature-sensitive mutants to induce anchorage-independent growth in infected cells at permissive and nonpermissive temperatures. Prague strain Rous sarcoma virus (subgroup A) wild-type-infected cells formed colonies with about the same efficiency at either 35 or 41°C , whereas normal cells failed to do so at both temperatures. Significantly, the group of *ts* mutants, GI251, GI252, and GI253, designated partial transformation-defective (PTD) mutants, produced colonies like the wild-type virus at both temperatures, in either a soft-agar or methyl cellulose suspension culture. The conventional TD *ts* mutants, GI201 and GI202, as well as GI203, GI204, and GI205 (data not shown), failed to form colonies at the nonpermissive temperature.

The ability of a virus to induce anchorage-independent growth is usually considered a useful measure of the oncogenicity of a virus strain (35). Therefore, several temperature-sensitive mutants were tested directly for their ability to induce tumor formation in chickens, which have a body temperature of 42°C . The birds were each inoculated with two dilutions, one in each wing web. The results of the experiment are summarized in Table 3, which shows that although 60% of the animals formed tumors with *ts* GI251, none formed tumors with *ts* GI252, and only 10% formed tumors with *ts* GI253 and with *ts* GI202, even though the doses

TABLE 1. Focus-forming titers of different mutants^a

Virus	Focus titer (FFU/ml)		Focus-forming efficiency ($41^\circ\text{C}/35^\circ\text{C}$)
	35°C	41°C	
WT PrA	8×10^5	8×10^5	1
<i>ts</i> GI201	8×10^5	$<10^1$	$<10^{-4}$
<i>ts</i> GI202	1×10^6	1×10^2	1×10^{-4}
<i>ts</i> GI251	2×10^6	$<10^1$	$<10^{-5}$
<i>ts</i> GI252	7×10^5	$<10^1$	$<10^{-4}$
<i>ts</i> GI253	8×10^5	$<10^1$	$<10^{-4}$

^a Cloned virus prepared in small stocks at 35°C was titrated in a focus test performed in duplicate at 35 and 41°C . The number of foci appearing was counted on day 8 after plating. FFU, Focus-forming units; WT PrA, wild-type Rous sarcoma virus, Prague strain, subgroup A.

TABLE 2. Efficiency of colony formation in soft-agar and methyl cellulose suspension cultures

Virus	Fraction of inoculated cells forming colonies ^a in:					
	Soft agar			Soft methyl cellulose		
	35°C	41°C	Efficiency ^b (41°C/35°C)	35°C	41°C	Efficiency (41°C/35°C)
None	<0.001	<0.001		<0.0001	<0.0001	
WT PrA ^c	0.09	0.08	<u>0.9</u>	ND ^d	ND	
<i>ts</i> GI201	0.1	<0.001	<u><0.01</u>	0.02	<0.0001	<u><0.005</u>
<i>ts</i> GI202	0.3	<0.001	<u><0.003</u>	0.02	<0.0001	<u><0.005</u>
<i>ts</i> GI251	0.04	0.1	<u>2.5</u>	0.08	0.2	<u>2.5</u>
<i>ts</i> GI252	0.3	0.2	<u>0.7</u>	0.02	0.002	<u>0.1</u>
<i>ts</i> GI253	0.2	0.2	<u>1.0</u>	0.07	0.2	<u>2.9</u>

^a Suspension culture media were prepared as previously described (8, 31, 46). For the soft-agar colony test, 10³ cells infected at a multiplicity of infection of approximately 2 focus-forming units (FFU)/cell were plated on a 35-mm plate. For the soft-methyl cellulose colony test, 10⁴ cells, also infected at 2 FFU/cell, were used on 50-mm plates.

^b The efficiency of colony formation expresses the ratio of the number of colonies obtained at the nonpermissive temperature to that obtained at the permissive temperature. The values are underlined to stress their derivative nature.

^c WT PrA, Wild-type Rous sarcoma virus, Prague strain, subgroup A.

^d ND, Not done.

used with the mutant viruses were 100-fold greater than those needed to produce tumors in 100% of the animals with the wild-type parent. Hence, a significant degree of defectiveness for tumor induction was observed with all mutants tested; thus the focus test, according to which all mutants studied were temperature sensitive, was a better measure of oncogenicity in this case than the suspension culture test, which had indicated that PTD mutants were as capable of inducing anchorage-independent growth at the nonpermissive temperature as the wild-type virus.

Another measure of growth characteristic for transformed cells, but not normal cells, is the absence of, or increased, saturation density (39). Table 4 shows that after 96 h, normal cells cultured in medium containing 5% calf serum attain a maximum density much lower than that of mutant-transformed cells at the permissive temperature. This density, about 1.5×10^5 cells/cm², remains relatively constant with normal cells for several days more in culture, after which the cells detach spontaneously as a monolayer. Transformed cells, on the other hand, continue to increase in density until medium is exhausted or cells spontaneously detach. It is apparent from these results (Table 4) that whereas *ts* GI202-infected cells grow like transformed cells at 35°C and like normal cells at 41°C, *ts* GI253-infected cells behave like the wild-type virus-infected cells at both temperatures. Likewise, an experiment (Table 4) con-

TABLE 3. *In vivo* tumor formation in chickens (body temperature, 42°C)

Virus	Dose (FFU) ^b	Tumor-positive animals/total animals inoculated ^a after:			
		14 days	21 days	28 days	35 days
WT PrA ^c	10 ²	8/10	10/10	3/3 ^d	3/3 ^d
	10 ³	10/10	10/10	2/2	2/2 ^d
<i>ts</i> GI202	10 ³	0/9	0/9	0/9	0/9
	10 ⁴	1/9	1/9	1/9	1/9
<i>ts</i> GI251	10 ³	0/10	0/10	0/10	0/10
	10 ⁴	5/10	6/10	4/8 ^d	4/8 ^d
<i>ts</i> GI252	10 ³	0/9	0/9	0/8 ^e	0/8 ^e
	10 ⁴	0/9	0/9	0/8 ^e	0/8 ^e
<i>ts</i> GI253	10 ³	0/12	0/12	0/12	0/12
	10 ⁴	1/12	1/12	1/12	1/12

^a Groups of 9 to 12 3-week-old white leghorn chickens from a leukemia-free flock were inoculated in the wing webs, each with 0.05 ml of virus diluted to contain the indicated dose. The right wing webs received a 10-fold-lower dose than the left, so that the titration could be done at two dilutions with a minimal number of animals. Independent titration of inocula in separate wings of a single animal has been demonstrated (Bauer, unpublished observation). Examinations for tumor formation were made biweekly.

^b FFU, Focus-forming units.

^c WT PrA, Wild-type Rous sarcoma virus, Prague strain, subgroup A.

^d Selected animals were sacrificed after 21 days because large necrotic tumors had developed, rendering the animals moribund.

^e One animal died of causes unrelated to virus inoculation.

TABLE 4. Cell densities observed under various cultural conditions

Virus	Cells counted/cm ² ($\times 10^{-5}$) after 96 h ^a								
	GM + 5% CS ^b			GM - 5% CS ^c			GM agar overlay + 5% CS ^d		
	35°C	41°C	41°C/35°C ^e	35°C	41°C	41°C/35°C ^e	35°C	41°C	41°C/35°C ^e
None	1.7	1.2	<u>0.7</u>	0.4	0.6	<u>1.5</u>	1.1	1.2	<u>1.1</u>
<i>ts</i> GI201	ND ^f	ND		ND	ND		4.0	1.3	<u>0.3</u>
<i>ts</i> GI202	3.0	1.6	<u>0.5</u>	1.3	0.7	<u>0.5</u>	ND	ND	
<i>ts</i> GI251	ND	ND		ND	ND		1.7	4.4	<u>2.6</u>
<i>ts</i> GI253	3.0	3.7	<u>1.2</u>	1.4	1.6	<u>1.1</u>	3.7	6.0	<u>1.6</u>
WT PrA	3.0	3.1	1.0	1.6	1.7	1.0	3.0	3.7	1.2

^a Chicken embryo cells were infected with the various viruses at a multiplicity of infection of approximately 2 focus-forming units/cell, and after 4 days of incubation at 35°C the cells were seeded into duplicate 30-mm plates at a density of 0.5×10^5 cells/cm² in media and at temperatures as indicated. After 96 h, without medium change during the days since seeding, liquid medium culture supernatants were collected and pooled with cells suspended by trypsin treatment (0.25% trypsin for 10 min at room temperature) from the corresponding plate. Cells that had been overlayed with agar-containing media were collected after agar had been gently aspirated using suction through a Pasteur pipette, followed by trypsin treatment to suspend the cells. The cell numbers were determined by an electronic cell counter, and the results are expressed as the cell number per square centimeter of plate surface area.

^b Growth medium (GM) was Dulbecco-modified Eagle medium containing 10% tryptose phosphate broth and 5% calf serum (CS).

^c GM was made for footnote *b* except that CS was absent.

^d GM was made as for footnote *b* except that 24 h after seeding, plates received GM containing a final concentration of 0.5% agar (Difco Corp.).

^e The ratio of the cell number per square centimeter observed at 41°C to that observed at 35°C is presented to summarize the differences between TD and PTD mutant-infected cells and between normal cells and wild-type Rous sarcoma virus strain Prague, subgroup A (WT PrA)-infected cells. These derivative values are underlined.

^f ND, Not done.

ducted without serum in the medium showed that the characteristic inhibition of growth expected with normal cells (13) was seen with *ts* GI202 only at the nonpermissive temperature, and not at all with *ts* GI253. Finally, Table 4 shows an experiment in which an agar overlay, inhibitory to normal cell growth, perhaps owing to the high concentration of anionic groups contained in the crude agar, was used to demonstrate that whereas *ts* GI201-infected cell growth is temperature sensitive under these inhibitory conditions, *ts* GI251- and GI253-infected cells exhibited no growth inhibition at the nonpermissive temperature. *ts* GI201, *ts* GI202, *ts* GI203, *ts* GI204, and *ts* GI205 behaved alike in such experiments (data not shown), whereas the PTD mutants behaved like the wild type.

Figure 1 shows a growth kinetic experiment similar to that described in Table 4, which clearly shows the temperature independence of *ts* GI251-infected cells for growth in liquid medium on a solid substrate and the temperature sensitivity of *ts* GI201-infected cells.

Concanavalin A agglutinability and hexose transport as measures of transformed cell altered membrane functions. Lectin agglutinability of cells infected by the various mutants, at permissive and nonpermissive temperatures, was studied using concanavalin A. Table 5 shows that according to this criterion of altered membrane function in transformed cells, presumably resulting from increased mobility of the lectin binding sites (32), the PTD and TD mutants do not differ. A definite temperature sensitivity was apparent for all mutants.

Greatly increased transport of certain hexoses and amino acids is rather uniquely characteristic of transformed fibroblasts, since normal cells, under any state of growth, never attain transport levels equivalent to those of transformed cells (11, 43). Therefore, the mutants were examined for changes in the 2-deoxyglucose transport rate, which might occur after a shift of infected cells from the nonpermissive to the permissive temperature. Figure 2 shows that the increase in rate of 2-deoxyglucose transport seen with *ts* GI201 is precisely re-

FIG. 1. Growth curves with *ts* GI201 (□)- and *ts* GI251 (△)-infected cells, as well as with normal cells (○), is shown at 35°C (open symbols) and 41°C (closed symbols). Cells were infected with a multiplicity of infection of 2 focus-forming units/cell at time 0 and plated after absorption for 30 min at room temperature in suspension on 35-mm plates at an initial density of 3.5×10^5 cells/plate in liquid medium. Cell numbers per plate were determined at intervals as shown.

TABLE 5. Agglutination of mutant-infected cells with concanavalin A^a

Virus mutant	Growth temp (°C)	Degree of agglutination at concn-avalin A concn (μg/ml) ^b of:				
		0	62.5	125	250	500
None	35	—	—	—	—	+
	41	—	—	—	—	+
<i>ts</i> GI201	35	—	++	++	+++	++++
	41	—	—	—	—	+
<i>ts</i> GI202	35	—	+++	+++	++++	++++
	41	—	—	—	—	+
<i>ts</i> GI251	35	—	+++	++++	++++	++++
	41	—	—	—	—	—
<i>ts</i> GI252	35	—	+++	+++	++++	++++
	41	—	—	—	+	+
<i>ts</i> GI253	35	—	+++	++++	++++	++++
	41	—	—	—	—	—

^a The procedure used is described in Materials and Methods.

^b Agglutination was scored microscopically as follows: —, 90 to 100%; +, 70 to 90%; ++, 50 to 70%; +++, 30 to 50%; +++, 0 to 30% single cells.

flected in a similar increased rate seen with *ts* GI251 after shift to the permissive temperature. Table 6 shows the normalized values obtained with several mutant-infected cell types, normal cells, and wild-type virus-infected cells. A significant temperature sensitivity was seen with all mutants for this transport function (data for

ts GI203, *ts* GI204, and *ts* GI205 not shown). The values obtained for the three PTD mutants at the nonpermissive temperature were, regularly, slightly higher than those seen with the TD mutants or normal cells. This is perhaps explainable in that PTD-infected cells are not subject, at the nonpermissive temperature, to the growth inhibition that, even though the cultures were still subconfluent, may already have begun to retard mutant-infected or normal cell growth. That the state of cell growth influences hexose transport rates to some degree (two- to threefold) is recognized (24, 43). The very large differential between uptake by normal phenotype and transformed phenotype cells is probably attributable to the compound influences of transformation and density inhibition of normal cell growth.

Examination of expression of LETS protein in mutant-infected cells. Transformed cells exhibit reduced amounts or a total absence of a 250,000-dalton glycoprotein, the so-called LETS glycoprotein (12, 14, 34, 37, 45). In the present study the level of LETS glycoprotein was found to be temperature sensitive in cells infected with *ts* GI202, *ts* GI251, *ts* GI252, and *ts* GI253 as detected either by lactoperoxidase-catalyzed cell surface iodination (Fig. 3) or Coomassie blue staining of total cell protein (Fig. 4). Interestingly, cells infected by *ts* GI201 retained a considerable amount of LETS glycoprotein

FIG. 2. Uptake of 2-[³H]deoxyglucose by *ts* GI201 (□)- and *ts* GI251 (△)-infected cells at various intervals after a shift from the nonpermissive to the permissive temperature is shown. Chicken embryo cells were infected as described in footnote a, Table 6, transferred into 30-mm dishes, and maintained for 18 h at 41°C. At intervals, plates were shifted to 35°C; at 36 h after transfer all plates were washed, and 2-[³H]deoxyglucose uptake was determined as described in Table 6. The results are expressed as counts per minute per 10⁶ cells.

TABLE 6. 2-Deoxyglucose uptake by normal and virus-infected cells

Virus	2-Deoxyglucose uptake (cpm/10 ⁶ cells) ^a		
	35°C	41°C	41°C/35°C ^b
None	1,700	1,700	<u>1.0</u>
WT PrA ^c	28,000	25,000	<u>0.9</u>
<i>ts</i> GI201	19,000	1,400	<u>0.07</u>
<i>ts</i> GI202	23,000	1,800	<u>0.08</u>
<i>ts</i> GI251	26,000	2,100	<u>0.08</u>
<i>ts</i> GI252	28,000	2,400	<u>0.09</u>
<i>ts</i> GI253	29,000	3,600	<u>0.12</u>

^a Chicken embryo cells were infected at a multiplicity of approximately 2 focus-forming units/cell and, after 3 days of incubation at 35°C, were transferred to 30-mm plates at the temperatures indicated, with an initial density of 0.5×10^6 cells/cm². Two days later, when the cells were nearly confluent with approximately 1.2×10^6 cells/cm², the plates were gently washed twice with glucose-free Hanks balanced salt solution (H minus) at 41°C, incubated with 0.5 μ Ci of 2-[³H]deoxyglucose-supplemented H minus solution for 10 min at 41°C, and washed three times with ice-cold H minus solution. The cells were suspended using trypsin (0.05% trypsin for 10 min at room temperature), a sample was counted with an electronic cell counter, and 80% of the total was lysed with 1% Triton X-100 followed by liquid scintillation counting in a water-compatible mixture. The results obtained are expressed as counts per minute observed per 10⁶ cells present in the sample.

^b The ratio of the counts per minute uptake measured with cells grown at 41°C to that observed with cells grown at 35°C is presented to illustrate the differences observed with the different mutant-infected and wild-type virus-infected cells. The values are underlined to stress their derivative nature.

^c WT PrA, Wild-type Rous sarcoma virus, Prague strain, subgroup A.

even when grown at 35°C (50% of the level observed at 41°C), although according to other parameters, as shown above, *ts* GI201-infected cells at 35°C are fully transformed. This result would suggest that *ts* GI201 is nonconditionally defective for an activity that is responsible for the loss of LETS glycoprotein in transformed cells.

Measures of plasminogen activator protease with mutant-infected cells. The finding that loss of LETS glycoprotein was temperature sensitive with all mutants tested suggested that expression of plasminogen activator protease (40) or other protease activities (7, 44) might be defective at the nonpermissive temperature, since proteases have been proposed as one possible mechanism by which LETS protein

might be lost during transformation (15, 18). Experiments were first performed using a focus test modified to include opaque casein in the overlay as a substrate for protease activities, especially plasmin (10). It was found that whereas TD mutant-infected cells failed to produce clear areas or plaques in the overlay at the nonpermissive temperature, the cells infected with PTD mutants did, though somewhat later than wild-type virus-infected cells (data not shown). At the permissive temperature, all mutants tested, as well as wild-type virus, could induce plaques over cells, which were later apparent as foci of transformed cells. The delayed positive plaque formation with PTD mutant-infected cells at the nonpermissive temperature suggested some protease activity, though probably less than that induced by wild-type virus.

A second type of caseinolytic test was performed based on the suspension culture plaque test of Balduzzi and Murphy (3). Figure 5 shows the results with *ts* GI202- and *ts* GI253-infected cells in suspension culture at 35 and 41°C. Cleared areas or plaques are seen with *ts* GI202 only at the permissive temperature, whereas with *ts* GI253 plaques are numerous at both temperatures, being of even larger diameter at the nonpermissive temperature. These tests had been incubated for only 3 days, so the ability of *ts* GI253 to form colonies in suspension at the nonpermissive temperature played no major role. Single transformed cells are apparently capable of activating sufficient plasmin to produce a plaque. The plaques observed in this test with wild-type virus-infected cells (data not shown) were similar to those seen with cells infected with PTD mutants; hence, according to this assay, the PTD mutants, in contrast to TD mutants, were not defective at the nonpermissive temperature for a plasminogen activator protease.

Quantitative measurements of plasminogen activator were performed using the fibrinolytic assay of Unkeless et al. (41). This fibrinolytic activity has been identified as arising from the action of urokinase-like protease (plasminogen activator; 40) on the zymogen plasminogen, resulting in activation of plasmin, the protease actually measured with the insoluble [¹²⁵I]fibrin test under the conditions used. To meet the requirements of a test for temperature sensitivity, it was necessary to obtain harvests of plasminogen activator from cells grown at either 35 or 41°C and to be able to make comparisons of their activities. Hence, separate harvest fluids were taken from cells at these temperatures and reacted on cell-free plates containing insoluble [¹²⁵I]fibrin at a single temper-

FIG. 3. Iodinated cell surface proteins of mutant-virus-infected cells grown at 35 and 41°C. The LETS band is prominent in cells infected with all mutants at 41°C and with normal cells, but is significantly reduced with mutants at 35°C and wild-type virus-infected cells. Cells were labeled using the lactoperoxidase system when nearing confluence. Gel tracks contain equal amounts of cell protein. (a-e) Cells grown at 41°C: a, *ts* GI201; b, *ts* GI202; c, *ts* GI251; d, *ts* GI252; e, *ts* GI253. (f-j) Cells, in the same order, grown at 35°C. (k) Normal cells. (l) Wild-type-infected cells grown at 35°C. The k and l LETS bands are displaced relative to others because these samples were subjected to electrophoresis on a separate slab gel.

FIG. 4. Total cellular proteins of mutant-virus-infected cells grown at 35 and 41°C, as detected by Coomassie blue stain. The tracks are labeled as in Fig. 3. Tracks h and j received marker protein of 130,000 (*E. coli* β -galactosidase) and 68,000 (bovine serum albumin) molecular weight, respectively. The band corresponding to the major iodinated protein (LETS; molecular weight, 250,000) is also indicated.

FIG. 5. Suspension culture system containing casein is presented to indicate the plasminogen activator expression of *ts* GI202-infected cells (a and b) and *ts* GI253-infected cells (c and d) at permissive (a and c) and nonpermissive (b and d) temperatures.

ature, 37°C, as preliminary tests had indicated that mutant-infected, cell-derived plasminogen activator was no more temperature sensitive than such plasminogen activator derived from wild-type virus-infected cells.

Based on the experiments with the caseinolytic assay, a method was used to distinguish between cell-bound and supernatant activator. This was possible because harvest fluids obtained from well-washed cells, incubated in a medium lacking serum as plasminogen source, could only contain plasminogen activator. Such plasminogen activator was mixed with chicken serum directly on the insoluble [125 I]fibrin plate, so that the final measurement was actually plasmin-digested [125 I]fibrin. On the other hand, cell-bound plasminogen activator could be measured by obtaining harvest fluids from cells incubated in the presence of chicken serum as a plasminogen source. Of course, this assay of cell-bound activity must include also the supernatant plasminogen activator, which will have simultaneously activated plasmino-

gen during the incubation; however, the actual problem with the mutants studied has been to recognize cell-bound plasminogen activator under conditions where supernatant plasminogen activator is absent. Furthermore, supernatant plasminogen activator, perhaps because its half-life is shorter than that of plasmin during the 12-h incubation on cells, always gives a reaction of lower activity on the insoluble [125 I]fibrin plates than is seen with the cell-bound activator test.

Results of a typical experiment using these procedures to measure cell-bound (including supernatant) plasminogen activator, compared with those measuring supernatant plasminogen activator alone, are shown in Table 7. *ts* GI201 is of particular interest because it exhibits almost no detectable supernatant plasminogen activator at either temperature, and the cell-bound activity is also extremely low. *ts* GI202 is simply temperature sensitive for both types of plasminogen activator. The PTD mutants, on the other hand, although strikingly

TABLE 7. *Temperature dependence of plasminogen activator protease expression by virus-infected cells*

Virus	[¹²⁵ I]fibrin solubilized/harvest unit ^a			
	Cell-bound plasminogen activator ^b		Supernatant plasminogen activator ^c	
	35°C ^d	41°C	35°C	41°C
None	630	260	330	380
WT PrA ^e	31,800	27,600	13,500	10,900
ts GI201	2,200	900	690	360
ts GI202	24,000	460	11,100	640
ts GI251	29,100	24,800	15,100	660
ts GI252	27,200	21,800	13,000	550
ts GI253	27,900	23,600	12,500	400

^a Thirty-millimeter (7.06-cm²) plates prepared with 8,000 cpm of insoluble [¹²⁵I]fibrin and 10 µg of cold fibrin per cm², according to the method of Unkless et al. (41), were used to measure the proteolytic activity of two types of plasmin-containing preparations. One milliliter of harvest fluid was incubated on the fibrin plates at 37°C. At intervals, a sample was taken and counted in an aqueous mixture in a liquid scintillation counter. The values shown are for 8-h digestion of the fibrin plates corrected to reflect the counts per minute ¹²⁵I solubilized per plate per harvest unit. One harvest unit was defined as 1 ml of harvest fluid obtained after 12 h of incubation of the appropriate medium at the indicated temperature with 10⁶ cells. Cell counts were performed on plates from which harvest fluids had been obtained, and, based on the results, the fluid sample was diluted with unincubated medium to normalize for cell density.

^b For measurement of cell-bound plasminogen activator, harvest fluids were prepared as described above using Dulbecco-modified Eagle medium supplemented with 2.5% chicken serum as a source of plasminogen. Fluids were frozen at -80°C.

^c For measurement of supernatant plasminogen activator, harvest fluids were obtained using Dulbecco-modified Eagle medium without supplement. Fluids were frozen at -80°C. At the time of assay for proteolytic activity on [¹²⁵I]fibrin plates, chicken serum was added to a final concentration of 2.5% to the fluids as a source of plasminogen. Controls done without this source of plasminogen showed only a background activity (approximately 600 cpm/plate per harvest unit), which was subtracted individually from the values presented.

^d Chicken embryo cells were infected at a multiplicity of approximately 2 focus-forming units/cell and, after 3 days of incubation at 35°C, were transferred to 30-mm plates at the temperatures indicated. Two days later, cells were washed and prepared as described above for harvest fluid harvests.

^e WT PrA, Wild-type Rous sarcoma virus, Prague strain, subgroup A.

temperature sensitive for expression of plasminogen activator in harvested supernatants, display virtually undiminished activity of the cell-bound type. Hence, the finding that foci and colonies of PTD mutant-infected cells exhibited temperature-independent caseinolysis may be understood. The casein-containing media also contained serum as a matter of course. This serum made plasminogen available to the cell-bound plasminogen activator, and the resulting plasmin was responsible for caseinolysis.

DISCUSSION

Table 8 presents a summary of the response of cells infected with the various mutants in separate parameters of transformed behavior. For purposes of this discussion it is of central importance to distinguish between the parameters of cell behavior that can be measured and the functions of the virus-coded transformation protein that indirectly, after an unknown number of intervening processes, induces the phenotypic parameters we recognize. The TD mutants, like most previously studied temperature-sensitive mutants of Rous sarcoma virus, induce consistently temperature-sensitive behavior in the host cells. In contrast, the results with the PTD mutants delineate a dissociation between certain parameters of transformation. PTD mutant-infected cells behave at the non-permissive temperature like wild-type virus-infected cells according to three measures of altered social behavior: loss of anchorage, serum, and density-dependent growth control. Concomitant with, and perhaps causally related to, this altered growth control, the PTD mutants induce in their host cells, at the non-permissive temperature, expression of plasminogen activator. This plasminogen activator is indeed not really similar to that found with wild-type virus-transformed cells, since it cannot be detected free in the supernatant but only with procedures that expose plasminogen to the cell surface. This mutant-induced plasminogen activator is, however, in terms of enzymatic activity, comparable to that of the wild-type virus-infected cells, and, most significantly, the cell-bound activity found with the PTD mutant system is lacking with all other TD mutants at the nonpermissive temperature.

In reporting the existence of mutants like those in the PTD category, the question of whether these mutants may not be simply "leaky" in the conventional genetic sense (i.e., fractionally functional owing perhaps to a merely statistical inactivation of the altered

TABLE 8. Summary of parameters and responses observed with different mutants at 41°C

Virus	Category	Focus formation	Tumor formation in vivo	Anchorage-, density-, serum-independent growth	Concanavalin A agglutination	Increased hexose uptake	Disappearance of LETS	Bound plasminogen activator	Free plasminogen activator
None	Uninfected	—	—	—	—	—	—	—	—
WT PrA ^a	Wild type	+	+	+	+	+	+	+	+
<i>ts</i> GI201	TD mutants	—	—	—	—	—	—	—	—
<i>ts</i> GI202		—	—	—	—	—	—	—	—
<i>ts</i> GI203		—	ND ^b	—	ND	—	ND	ND	ND
<i>ts</i> GI204		—	ND	—	ND	—	ND	ND	ND
<i>ts</i> GI205		—	ND	—	ND	—	ND	ND	ND
<i>ts</i> GI251	PTD mutants	—	—	+	—	—	—	+	—
<i>ts</i> GI252		—	—	+	—	—	—	+	—
<i>ts</i> GI253		—	—	+	—	—	—	+	—

^a WT PrA, Wild-type Rous sarcoma virus, Prague strain, subgroup A.^b ND, Not done.

protein at the nonpermissive temperature) is immediately encountered. Admittedly, the PTD mutants might be loosely described as leaky according to some parameters of transformation, although according to other parameters they are not more leaky than many other conventional TD mutants. The questions of sensitivity of measurement with the various parameters and of linearity of the assays become problematic for arriving at a conclusion. The concept of "leakiness" resolves into the first of two hypothetical models that may be constructed to explain the existence of PTD as well as TD mutants. (i) One may assume that as a result of the nature of the lesion in certain mutants, a small fraction of the altered transformation protein retains function. Then, if one assumes that this functional protein acts in the same way (with a single active site) on several discrete cellular sites, some of which have higher thresholds than others in order to exert an effect on the total cellular phenotype, one could expect to obtain results like those found with PTD mutants. TD mutants would simply be mutants in which the altered protein retained no function. (ii) A virus-coded transformation protein may not be limited to a single type of function in its interaction with the cellular machinery. If several transformation functions, presumably carried out by a single protein molecule with multiple active sites, have independent effects on the host cell, one may assume that under certain conditions a mutation in this protein would lame one functional site without injury to the other(s). This would also result in a phenotypic dissociation of various parameters measuring the indirect effects of the transformation functions. So a PTD mu-

tant would be a mutant in which a particular function is selectively destroyed, and a TD mutant would not necessarily be a multiple mutant but one that might also have suffered a lesion, which would alter the conformation of the entire protein.

A dissociation of transformation parameters has been reported with lines of mouse 3T3 cells infected with a thermosensitive mutant polyoma virus (25); however, in this case, though transformed growth properties were temperature sensitive, agglutination by concanavalin A was enhanced at nonpermissive as well as permissive temperatures. Hence, these cell lines, assuming this dissociation is directly brought about by a mutation in the viral genome, exhibit not the same properties, but rather properties complementary to those shown by cells infected with the PTD mutants discussed above.

Extensive analysis of the whole range of transformation parameters exhibited by a variety of random clones of mouse 3T3 cells infected by simian virus 40 has been reported by Risser and Pollack (31). In this work the virus was not mutagenized; rather, the variability in properties detected among the different clones is assumed to derive from the nature of the virus-cell interaction. This approach led to the recognition of distinct categories in the alteration of host growth control after infection; it was possible to identify clones that showed anchorage-dependent growth but retained serum- and density-dependent growth properties.

Dissociation of transformation parameters like that seen between the different simian virus 40-infected clones has been recognized with cells infected with two different avian sarcoma

virus *ts* mutants. *ts* LA334-infected cells exhibit a degree of anchorage-independent growth, although density-independent growth was not noted (9, 47). *ts* LA25-infected cells, although almost completely anchorage dependent, show a striking density- (22, 47) and serum-independent growth pattern (Friis and Weber, unpublished observations).

The involvement of a protease activity, now recognized as a urokinase-like plasminogen activator (41) acting to produce plasmin in serum-containing media (30), in determining the morphological (27) and many of the growth-related properties of transformed cells (26) has been further emphasized by the work of Pollack et al. (29) and Shin et al. (35), which has shown strong correlation among anchorage-independent growth, plasminogen activator protease, and ability of cells to induce tumors in vivo in "nude" mice.

Jones et al. (19) have further shown that tumors resulting from inoculation of low-level plasminogen activator-producing cell lines are invariably made up of high-level plasminogen activator-producing cells.

The results reported above with the PTD mutants do not fundamentally contradict the existence of a correlation between plasminogen activator production and oncogenicity. The PTD mutants seem to be a sort of intermediate category in which the dissociation of transformation parameters is placed differently. The demonstrated anchorage-independent growth with the PTD mutant-infected cells is paralleled by protease activity, as the casein overlay plaque test showed (Fig. 5). Furthermore, experiments in our laboratory have shown that anchorage-independent growth of transformed cells requires the presence of plasminogen in the soft-agar suspension culture medium (Becker, unpublished observation). The fact that in vivo tumor formation is not efficient with the PTD mutants may indeed reflect an important functional difference between cell-bound and supernatant plasminogen activator in contributing to tumorigenicity.

The presence of LETS glycoprotein on the surfaces of PTD mutant-infected cells at the nonpermissive temperature is an important finding, because it argues against the hypothesis that LETS may play a regulatory role in establishing density inhibition of growth (15, 16). Our findings are, however, consistent with the report that restoration of LETS glycoprotein to transformed cells results in increased adhesion to substrate and more normal cell morphology but fails to impose density-dependent growth regulation (48), since PTD mutants

at the nonpermissive temperature do exhibit normal or near-normal morphology and relatively high adhesion to substrate (Friis and Weber, unpublished observations) under conditions approximating transformed cell growth.

The TD mutant *ts* GI201, which is unusual in being non-conditionally defective for cell-bound and supernatant plasminogen activator protease, brought useful insight into the question of the mechanism by which LETS glycoprotein is removed from transformed cells. *ts* GI201, which is transformed according to almost all parameters at the permissive temperature, shows only partial removal of LETS glycoprotein. Since the PTD mutant-infected cells at the nonpermissive temperature, under conditions where significant cell-bound plasminogen activator is expressed and in medium containing high plasmin activities, nonetheless exhibit LETS glycoprotein in normal amounts, one can speculate that a different protease, one that is not plasmin and is perhaps not even involved in plasminogen activation, is responsible for LETS removal.

The study of the transformation events occurring after avian sarcoma virus infection is very complex because of the variety of parameters to be monitored and the unknown relationships of the parameters to each other. Studies such as that reported above may help to clarify the relationships of the parameters, for example, the possible dependence on particular protease activities for loss of the LETS glycoprotein. It is to be hoped that examination of mutants such as these PTD mutants and others of a complementary nature, i.e., mutants defective for inducing growth parameters of transformation but like the wild-type virus for inducing certain biochemical changes in infected cells (such as increased hexose transport), could eventually lead to recognition of distinct transformation functions directly coded for by the sarcoma viral genome.

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