MOLECULAR AND CELLULAR BIOLOGY, Nov. 1983, p. 2099–2109
0270-7306/83/112099-11$02.00/0

Vol. 3, No. 11

Dissociation Between Transformed and Differentiated Phenotype in Rat Thyroid Epithelial Cells After Transformation with a Temperature-Sensitive Mutant of the Kirsten Murine Sarcoma Virus

GIULIA COLLETTA,[1] ANTONIO PINTO,[1] PIER PAOLO DI FIORE,[1] ALFREDO FUSCO,[1] MARIA FERRENTINO,[1] VITTORIO E. AVVEDIMENTO,[1] NOBUO TSUCHIDA,[2] AND GIANCARLO VECCHIO[1]*

Centro di Endocrinologia e Oncologia Sperimentale del C.N.R., Istituto di Patologia Generale, II Facoltà di Medicina e Chirurgia, Università degli Studi di Napoli, Italy[1]; and Central Institute for Microbiological Research, 1976 Yaho, Kunitachi-Shi, Tokyo, Japan[2]

Received 15 April 1983/Accepted 23 August 1983

Differentiated rat thyroid epithelial cells, infected in vitro with a temperature-sensitive mutant of the Kirsten murine sarcoma virus, expressed at the permissive temperature (33°C) some phenotypic properties typical of transformed cells, including morphological features, colony formation in agar, and induction of tumors in newborn animals. Specific functional markers of these differentiated cells, i.e., synthesis/secretion of thyroglobulin, synthesis of thyroglobulin mRNA and iodide uptake, were blocked during growth at 33°C. Normal morphology, failure to grow in agar, and the requirement of hormones for optimal growth were all restored after shifting to the temperature nonpermissive for transformation (39°C), though the typical differentiated functions remained blocked. Infection with a leukemia helper virus clone (Moloney or Kirsten murine leukemia virus) did not lead to the loss of the differentiated phenotype of rat epithelial thyroid cells, thus demonstrating that the loss of the differentiated phenotype is caused by the sarcoma virus component. These results indicate that the expression of some of the phenotypic properties of transformed differentiated rat thyroid epithelial cells is under the direct control of the p21 thermosensitive activity, whereas the block in the expression of two typical differentiation markers of thyroid epithelial cells is irreversible and probably controlled by different mechanisms.

Of the numerous functional, biochemical, and morphological changes induced in virally transformed cells, inhibition of the expression of the differentiated phenotype is one of the most striking (26).

It is a general and well-documented behavior of RNA and DNA tumor viruses to modify the normal pattern of the differentiated functions of a cell after infection. Infection with Rous sarcoma virus prevents both the synthesis of melanin and the assembly of melanosomes in melanoblasts (10), inhibits the incorporation of collagen into the extracellular matrix in fibroblasts (1, 6, 30, 33, 38), prevents the synthesis of myosin and the fusion of myoblasts (17, 27), and reduces the amount and size of proteoglycans in chondroblasts (36). Avian erythroblastosis virus blocks the differentiation program of immature erythrocytes in culture (8). Among DNA tumor viruses, simian virus 40 transforms human placental cells which then synthesize low levels of human chorionic gonadotropin (12). Similar effects on the production of α-fetoprotein, albumin, and transferrin were observed in simian virus 40-infected rat fetal liver cells (13).

We have recently demonstrated the transforming potential in vitro of Kirsten murine sarcoma virus (KiMSV) in epithelial cells of a clear endodermal origin, such as rat thyroid cells (20, 21). Two different cell lines (FRT-L and T-79) derived from normal rat thyroid gland when infected with two strains of the KiMSV, the KiMSV (Moloney murine leukemia virus; MoMuLV) or the KiMSV (Kirsten Murine Leukemia virus; KiMuLV), change morphology, begin to proliferate rapidly, show a high colony-forming efficiency when grown in agar, and form tumors when transplanted into syngeneic rats. The tumors morphologically resemble undifferentiated adenocarcinomas. Moreover, differentiated thyroid cells transformed by KiMSV lose two of their most specific differentiation functions, i.e., thyroglobulin (TG) secretion and iodide uptake.

In line with previous work performed with other differentiated systems, we infected the cloned population of rat thyroid cells (FRT-L) with a KiMSV mutant temperature sensitive for transformation to study the correlation between transformation and differentiation. It is already known that KiMSV exercises its oncogenic action by means of a single transformation-specific protein, a 21,000-dalton protein (p21 *ras*) coded for by the viral genome (42). The viral gene encoding this protein is derived from a family of cellular *onc* genes (*ras* family) which are broadly conserved evolutionarily and transcribed frequently in a wide variety of normal cells (18, 40). The KiMSV *ts* mutant used in this study (42) produces the p21 *ras* protein required for cellular transformation, but the protein is significantly more thermolabile than the wild-type p21, suggesting that it is structurally altered. KiMSV *ts* infection of FRT-L cells in culture was used here to determine whether the p21 transforming protein also affects the expression of the differentiated functions in this cell system.

We report here that the KiMSV mutant, like the wild-type KiMSV (MoMuLV) and another strain of the KiMSV (KiMSV KiMuLV), inhibits the expression of two differentiated functions typical of the thyroid gland when the cells are grown at the permissive temperature (33°C). Several properties of KiMSV *ts*-infected cells characteristic of transformed cells, such as morphological changes and ability to grow in agar, appear at the permissive temperature for transformation (33°C) and disappear at the non-permissive temperature (39°C). By contrast, functions that are specific differentiation markers of the original cells, i.e., TG synthesis/secretion and iodide uptake, are irreversibly lost at both permissive (33°C) and nonpermissive (39°C) temperatures. Our data also suggest that, at least for TG synthesis, the irreversible block occurs at the site of the TG gene transcription.

MATERIALS AND METHODS

Cells and viruses. FRT-L cells, derived from Fischer rat thyroid glands as described by Ambesi-Impiombato et al. (4), were grown in mF12 medium plus 6H, which is modified Ham F12 medium containing 5% calf serum (GIBCO Laboratories, Grand Island, N.Y.) plus six growth factors (6H: insulin, hydrocortisone, transferrin, thyrotropin, somatostatin, and the tripeptide glycyl-hystidyl-lysine).

The 424 cell line has properties identical to those of the FRT-L line but grows in 0.5% calf serum plus the six factors. The wild-type cell line was obtained by transforming the FRT-L cells with KiMSV *wt* (MoMuLV) (20, 21). BALB 3T3, NIH 3T3, NRK (normal rat kidney), NRK58967 (40), and C-127 cells (42) were all grown in Dulbecco modified Eagle medium containing 10% fetal calf serum (GIBCO). The KiMSV *wt* (MoMuLV) and the KiMSV *ts* (MoMuLV) viruses were obtained from C-127 cells infected with these two virus strains (42).

Infection of cells and virus titration. FRT-L cells were infected at 33°C with the KiMSV *ts* mutant essentially as described for infection of these cells with the KiMSV (KiMuLV) virus (21).

FRT-L cells were plated at 3×10^5 cells per dish (60 mm; Falcon Plastics, Cockeysville, Md.) and were infected the next day with 1 ml of undiluted fresh supernatants from C127 cells infected with the 731 KiMSV *ts* (MoMuLV) virus in the presence of Polybrene (2 μg/ml final concentration) and incubated for 1 h at 37°C in an atmosphere of 5% CO_2 in air. After that time, 4 ml of fresh medium containing Polybrene (2 μg/ml) was added to the dishes. The mutant virus preparation contained 10^4 focus-forming units per ml, which had been titrated on normal rat kidney cells. After two passages at 33°C, FRT-L cells transformed by the *ts* virus were cloned. All clones obtained were kept in vitro for a few passages and then were stored frozen in liquid nitrogen to prevent phenotypic alterations. TS is the uncloned population; A-6, A-18, A-1, A-16, A-19, A-4, and A-2 are some of the clones studied in detail.

Transforming virus produced by the infected thyroid cells was titrated onto either SC-1 or BALB 3T3 cells (20).

The reverse transcriptase assay was performed as described previously (14).

The soft agar colony assay was performed according to Macpherson and Montagnier (31).

Tumor induction. Newborn Fischer rats (3 to 4 h old) were injected subcutaneously with 10^6 cells in a final volume of 0.1 ml of tissue culture medium, and rats were palpated in the inoculated region twice each week. Tumor excision, fixation, and staining procedures were performed as described previously (20).

The iodide uptake assay was performed as described previously (20).

Cell labeling, TG immunoprecipitation, and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Cell lines were grown until nearly confluent in the usual medium in 60-mm dishes, washed with phosphate-buffered saline, and incubated overnight in 2 ml of methionine-free medium containing 2% dialyzed fetal calf serum and 200 μCi of [^{35}S]methionine per ml (>600 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) (Amersham Corp.). The medium was then analyzed by immunoprecipitation. Samples of 500,000 cpm were mixed for 1 h at 37°C with 5 to 10 μl of anti-rat TG antibody purified by affinity chromatography and preabsorbed with an excess of unlabeled rat purified TG (2). The *Staphylococcus aureus* immunoprecipitation technique was used as described previously (3). For direct immunoprecipitation, the samples were left overnight at 4°C; then the pellets were washed five times with 100 mM Tris (pH 7.4)–150 mM NaCl–5 mM EDTA–1% Triton X-100–1% sodium deoxycholate and dissolved in 25 μl of 100 mM Tris-hydrochloride (pH 6.8)–2% mercaptoethanol–2% SDS–20% glycerol–0.002% bromophenol blue.

Discontinuous slab gel electrophoresis of proteins was performed as described by Laemmli (29).

Radioimmunological assays of rat TG were performed according to published procedures (28).

DNA extraction and hybridization. High-molecular-weight DNA was prepared from confluent cell mono-

layers by the procedure of Gross-Bellard et al. (25). Nick translation of the cloned DNA was performed as described previously (32). Nitrocellulose filters (Millipore Corp., Bedford, Mass.) were soaked in water for 2 h in 20× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate) and dried under light before use. Samples (10 μl) of single-stranded DNA at various concentrations were then spotted on the filter, and the paper was dried again under the light. The filters were soaked for 1 min in 0.1 M NaOH–1.5 M NaCl, for 1 min in neutralizing buffer (0.2 M Tris-hydrochloride [pH 7.0]–2× SSC), and for 1 min in 2× SSC; they were then dried in vacuo at 80°C for 2 h. Before hybridization, the filters were pretreated for 24 h at 42°C with prehybridization buffer (5× Denhardt solution–50% formamide–5× SSC–1% glycine–250 μg of denatured calf thymus DNA per ml) (44). The filters were then hybridized for 24 h at 42°C in plastic bags containing (per ml) 1×10^6 to 2×10^6 cpm (10^8 cpm/μg) of a nick-translated DNA probe representative of a portion of the rat TG gene (16) in hybridization buffer (50% formamide–1× Denhardt solution–6× SSC–100 μg of denatured calf thymus DNA per ml). After hybridization, the filters were washed twice in 0.1% SDS–2× SSC at 25°C for 30 min, twice in 0.1% SDS–0.1× SSC at 58°C for 30 min; once at 25°C in 2× SSC for 20 min. The filters were then dried and exposed at −70°C to Kodak X-ray film, with the use of intensifying screens.

Cytoplasmic RNA extraction and hybridization. Cytoplasmic RNA was purified from cultured cells by previously reported procedures (9). Briefly, cells were collected, washed three times with cold phosphate-buffered saline, and pelleted at 1,000 × *g* for 5 min at 4°C. The washed cells were suspended in 1 ml of ice-cold buffer (0.4 M NaCl–0.01 M Tris [pH 7.9]–0.001 M EDTA) for 2×10^7 cells. Nonidet P-40 was added to a final concentration of 0.5%. Nuclei were pelleted by centrifugation at 1,500 × *g* for 5 min at 4°C. The supernatants were brought to 0.5% in SDS and treated for 5 min at 37°C with 500 μg of proteinase K per ml, and then the RNA was extracted twice with phenol-chloroform and ethanol precipitated. Dot-blot experiments were performed essentially as described for DNA blots, except that the alkali and neutralization treatments of filter paper were omitted.

RESULTS

Properties of the FRT-L cells transformed by the *ts* KiMSV. FRT-L rat thyroid epithelial cells were infected with the KiMSV *ts* strain of KiMSV (MoMuLV) and grown at the permissive (33°C) and nonpermissive (39°C) temperatures. FRT-L cells grow in islets that adhere to the plate and exhibit a typical epithelial morphology (21). Figure 1A shows KiMSV *ts*-infected FRT-L cells (uncloned) grown at 33°C. The cells are somewhat spindle shaped and grow in a disordered way, a phenotype reminiscent of the same cells transformed by another strain of KiMSV (21) or by the wild-type KiMSV (not shown). Figure 1B shows the same cells after shifting to the nonpermissive temperature of 39°C and after 5 days of growth at this temperature. The cells become morphologically similar to the uninfected cells and more adherent to the plate. A similar pattern of transformation and morphological reversion was observed with other clones (A-18, A-19, A-16, A-1, A-4, A-2; data not shown) of *ts*-infected cells isolated from the original uncloned population transformed by the *ts* virus (Fig. 1C and D). Table 1 lists some of the properties exhibited by the TS population and by various clones isolated from it. These properties are compared with those of uninfected FRT-L or of FRT-L cells infected with the *wt* KiMSV. Virus production was tested in the culture medium by the reverse transcriptase assay and by focus formation on BALB 3T3 cells. With the latter technique, only the transforming component of the Kirsten complex (KiMSV-MoMuLV) can be detected and measured. Virus production was high as measured in the reverse transcriptase assay in the uncloned TS cells at both permissive and nonpermissive temperatures (Table 1). Uninfected FRT-L cells showed no virus production. Clone A6 is representative of several clones (A-19, A-16, A-4, A-2) isolated after transformation that were also positive for virus production in the reverse transcriptase assay. The A18 clone behaves as a transformed clone (see below) and does not produce virus; thus, it represents a nonproducer clone, infected only by the defective component of the Kirsten complex. Clone A6 consistently produced high levels of transforming virus as measured by the focus-forming assay in fibroblasts grown at 37°C. The absence of transforming activity found in the supernatant media from the A-18 clone is in agreement with the results obtained with the reverse transcriptase assay.

Transformed cells grown at 33°C were inoculated subcutaneously in newborn Fischer rats. In our conditions, they were expected to behave as malignant, since we have observed that the subcutaneous temperature of the rat does not exceed 34°C (a temperature which is still permissive for transformation). In fact, all clones were able to induce tumor growth, and the neoplasms resembled carcinomas histologically (data not shown). These results are in agreement with those obtained with adult rats injected with cells transformed by two wild-type strains of KiMSV (21, 45).

When assayed for their growth capacity in semisolid media, all of the *ts*-induced clones showed high colony-forming efficiency at 33°C. At 39°C, almost no colonies formed, and the few that did were much smaller than those growing at the permissive temperature.

Effects of temperature shift on growth and hormone requirements of KiMSV *ts*-infected FRT-L cells. Normal FRT-L cells require the addition of six growth factors (4) to the culture medium for optimal growth. Uninfected FRT-L

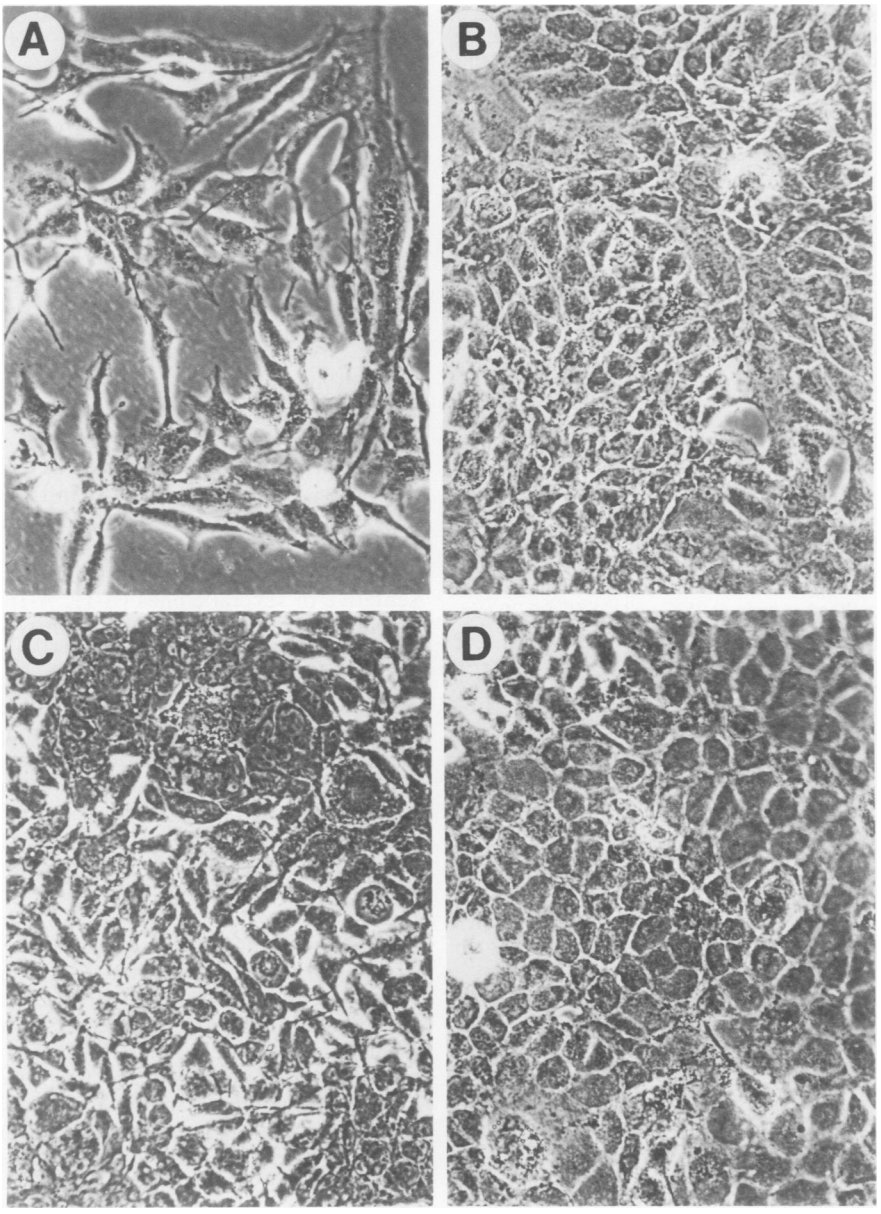

FIG. 1. FRT-L cells transformed by KiMSV *ts* virus grown at permissive and nonpermissive temperatures. (A) FRT-L-TS grown at 33°C. (B) FRT-L-TS grown at 33°C and then shifted to 39°C for 5 days. (C) Cloned population A-6 grown at 33°C. (D) Cloned population A-6 grown at 33°C and then shifted to 39°C for 5 days. Cells were propagated in vitro for 5 to 7 days, fixed with 95% ethanol–40% formaldehyde (9:1), and then photographed. Magnification, ×341.

cells in these growth conditions had a doubling time of 58 h and reached a saturation density of 1.5×10^6 cells per 60-mm plate (Table 2). By contrast, cells infected with the KiMSV *ts* virus and grown at 33°C no longer required these growth factors. This property might be due to the fact that cells transformed in vitro by sarcoma viruses are able to produce their own growth factors in the culture medium (15). Furthermore, all of the infected clones showed an increased growth rate at the permissive temperature (Table 2). The addition of the growth factors had no effect on either saturation density or doubling time at the permissive temperature, but, interestingly, both cloned and uncloned transformed FRT-L cells growing at the nonpermissive tem-

TABLE 1. Characteristics of FRT-L clones infected with KiMSV *ts*

Cells	Growth temp (°C)	Virus production		Tumor incidences[c]	Colony efficiency in agar[d]
		Reverse transcriptase[a]	FFU/ml[b]		
FRT-L (uninfected)	37	6.7	0	0/10	0
TS	33	521	2,200	8/8	58
	39	520	800		3
A-18	33	12.3	0	6/6	83
	39	1.7	0		5
A-6	33	941.5	27,400	8/8	90
	39	415	18,800		4
A-1	33	NT[e]	NT	3/3	82
	39	NT	NT		1
FRT-L infected with KiMSV *wt* (MoMuLV)	37	221	NT	NT	49.2

[a] Expressed as picomoles of [^{3}H]dTTP incorporated per 10^6 cells.
[b] Expressed as focus-forming units per milliliter of medium as titrated at 37°C on BALB 3T3 cells.
[c] Only cells grown in culture at 33°C were tested for tumorigenicity in vivo by subcutaneous injection into Fischer newborn rats (10^6 cells per rat). Tumors appeared between 13 and 20 days after injection.
[d] Calculated by the formula [(number of colonies formed)/(number of plated cells)] × 100.
[e] NT, Not tested.

perature reacquired, in part, their dependence on growth factors. All of the infected populations grown at 39°C in the presence of the six hormones exhibited a doubling time comparable to that of control FRT-L cells. When maintained at the nonpermissive temperature without the addition of hormones, all of the clones studied showed a clear decrease of their growth rate and a four- to fivefold decrease in saturation density as compared with the same infected cells maintained in the presence of the hormones.

Effect of temperature shift on TG production and iodide uptake. Cells were either grown continuously at 33°C or grown at 33°C and shifted to 39°C, and the analysis was performed 5 days after the shift to ensure that all cells had undergone at least one replication cycle. The cells were labeled with [^{35}S]methionine overnight, and the culture supernatants were immunoprecipitated with a purified antibody against rat TG.

Figure 2 shows the results of immunoprecipitation and gel electrophoresis obtained with supernatants from control FRT-L cells and the A6 clone grown at the two temperatures. Identical results were obtained with all other KiMSV *ts*-infected clones (data not shown).

The results obtained with the uninfected FRT-L cells are shown in Fig. 2A.

Immunoprecipitation was performed with a purified antibody against rat TG (3). As previously demonstrated (5), these cells produce in the culture medium high levels of TG. The major band present in lane 1 is TG, as demonstrated by its comigration with purified authentic 12S TG (lane 3) and by displacement of this band when the culture supernatant had been immunoprecipitated with the same antibody preabsorbed with cold purified TG (lane 2).

Figure 2B shows a gel in which two tech-

TABLE 2. Growth properties of 371 KiMSV (MoMuLV) transformed cell lines

Cell line	Growth conditions[a]	Doubling time (h)	Saturation density[b]
FRT-L	37°C + 6H	58	1.5×10^6
	− 6H	ND[c]	ND
TS	33°C + 6H	26.4	3×10^6
	− 6H	26.4	3×10^6
TS	39°C + 6H	67.2	1.2×10^6
	− 6H	88.8	0.44×10^6
A6	33°C + 6H	28.8	2×10^6
	− 6H	28.8	2×10^6
A6	39°C + 6H	52.8	1.8×10^6
	− 6H	84	0.46×10^6
A18	33°C + 6H	24	2×10^6
	−6H	24	2×10^6
A18	39°C + 6H	57.6	2.1×10^6
	−6H	91.2	0.5×10^6

[a] 6H = the six growth factors described in the text; + and − indicate the presence or absence of these factors.
[b] Number of cells per 60-mm plate.
[c] ND, Not determinable.

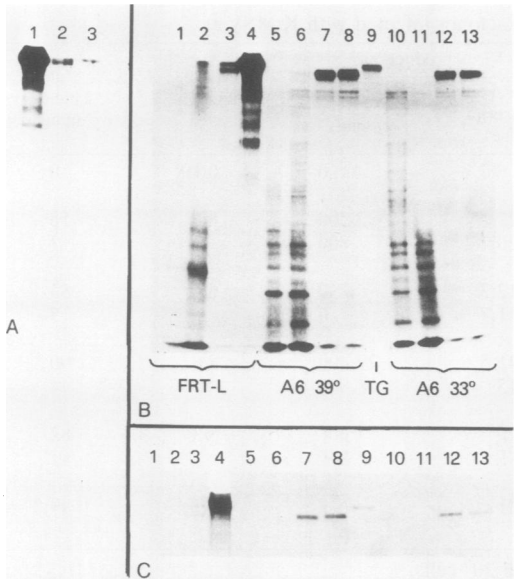

FIG. 2. SDS-polyacrylamide gel analysis of immunoprecipitates obtained from supernatants of FRT-L transformed cells. Cells were labeled with [^{35}S]methionine, and the supernatants were analyzed for the presence of TG. (A) Supernatants of FRT-L control cells, lanes 1 and 2; lane 3: rat 330,000-dalton 12S TG. (B) Lanes 1, 2, 5, 6, 10, and 11 show the results of direct immunoprecipitation; lanes 3, 4, 7, 8, 12, and 13 show the results obtained with culture media immunoprecipitated with the *S. aureus* technique. Lane 9 shows the migration of authentic rat 330,000-dalton 12S TG. Cells from the A-6 clone were either labeled at 33°C or shifted to 39°C for 5 days and then labeled as described in the text. Slab gel electrophoresis was performed with 6% polyacrylamide. Panel C is the same gel autoradiogram represented in panel B, but exposed for a shorter period of time.

niques were used: the *S. aureus* precipitation (used also in the experiment shown in Fig. 2A) and the direct immunoprecipitation. The *S. aureus* technique was used in lanes 3-4, 7-8, and 12-13. In lanes 3-4 are the immunoprecipitates obtained with uninfected FRT-L with and without displacement, respectively.

The band present in the high-molecular-weight region of the gel comigrates with the control rat TG (330,000 daltons) in lane 9. This is more clearly represented in Fig. 2C, in which a shorter time of exposure to the X-ray film of the same gel is shown. In lanes 7-8 and 12-13 are shown the immunoprecipitation data obtained with the A6 clone grown at permissive and nonpermissive temperatures; no bands migrating exactly as TG are present either at the permissive or at the nonpermissive temperature. But a protein in the high-molecular-weight region of the gel was always observed, whether or not the preabsorbed antibody was used (Fig. 2B and C). Thus, this protein which also migrates slightly faster than 12S TG probably represents a protein nonspecifically bound to the *S. aureus* protein. Clearer results were obtained with the direct immunoprecipitation, as shown in lanes 1-2, 5-6, and 10-11. In this assay, FRT-L uninfected cells showed a band near the top of the gel (lane 2); this band disappeared when the immunoprecipitation was done with the anti-TG antibody preabsorbed with purified unlabeled TG (lane 1), thus showing that it represents authentic TG; the other bands present in lane 2 probably represent degraded TG. The same direct immunoprecipitation with the normal (lane 6) and preabsorbed antibody (lane 5) was done with supernatants from the A6 clone grown at either the nonpermissive or the permissive temperature. The gel revealed no clear band in the molecular weight region corresponding to the 12S TG subunit (lane 9). Thus, the immunoprecipitation data either with the *Staphylococcus* technique or with direct immunoprecipitation demonstrate that the secretion of TG into the culture medium is blocked after transformation and is not restored at the nonpermissive temperature. Identical results were obtained by using cell lysates instead of culture supernatants for the immunoprecipitation, indicating that not only TG secretion but also synthesis was blocked in the *ts*-transformed cells (data not shown).

The results concerning TG secretion were confirmed in radioimmunoassays (Table 3). The supernatant from uninfected FRT-L cells contained high levels of TG, whereas KiMSV *ts*-infected cells grown at 33°C contained undetectable or extremely low amounts of TG in the culture supernatants. After a 5-day shift to growth at 39°C, none of the infected clones tested reacquired the ability to secrete TG into the medium. In Table 3 are reported the data obtained with the uncloned *ts* population as well as with six of the several clones obtained.

We also tested the ability of the infected cells grown at the two temperatures to concentrate radioactive iodide added to the culture medium. The cells transformed by the *ts* virus lost this property irreversibly, even when tested up to 5 days after the temperature shift to 39°C (Table 3). The data also indicate that the effect on the differentiated phenotype is dependent on the transforming component of the virus complex, as the same cells infected only with the leukemic viruses (MoMuLV or KiMuLV) retained the capacity to concentrate iodide and to secrete TG (Table 3).

Effect of temperature shift on TG mRNA. Cytoplasmic RNA from the various infected or uninfected cell lines was hybridized to in vitro

nick-translated cloned TG DNA representative of a portion of the rat TG gene (16). Figure 3A shows the results of the dot-blot hybridizations performed with RNA from uninfected FRT-L, from the various clones of FRT-L cells infected with KiMSV *ts* at permissive and nonpermissive temperatures, from FRT-L cells infected with the KiMSV *wt* (MoMuLV) (46), and from two lines of FRT-L cells infected with the nontransforming KiMuLV helper. RNA specific for the TG gene was detected in the uninfected or KiMuLV-infected FRT-L cells, whereas RNA from FRT-L cells transformed by either the *wt* or *ts* viruses and at both 33 and 39°C did not hybridize to the same DNA clone.

Presence of TG-specific DNA in the chromosomal DNA of transformed cell lines. The molecular clone of rat TG DNA described above was used to test for the presence of sequences homologous to this clone in the chromosomal DNA of the various KiMSV-transformed lines. Dot-blot hybridizations indicated the presence of DNA homologous to the TG cloned DNA in all lines tested: uninfected FRT-L and FRT-L cells infected with *wt* as well as with *ts* viruses (see Fig. 3B). Thus, at least the portion of the TG gene homologous to the cloned TG DNA is present in the chromosomal DNA of the various transformed lines.

TABLE 3. Differentiated thyroid functions in FRT-L cells infected with KiMSV *ts*

Cells	Growth temp (°C)	Iodide uptake[a] (%)	TG production[b]
424	33	9.2	NT
	37	10.9	NT
	39	(14)	NT
FRT-L	33	NT	394
	37	19.7	842.5
	39	NT	170.2
TS	33	0.5	2
	39	(0.35) 0.6	ND
A-18	33	0.5	ND
	39	(1.6) 0.5	2.5
A-6	33	0.6	ND
	39	(0.5) 0.5	1.1
A-1	33	NT	ND
	39	0.6	ND
A-2	33	NT	ND
	39	NT	ND
A-4	33	NT	2.1
	39	NT	ND
A-16	33	NT	ND
	39	NT	ND
A-19	33	NT	ND
	39	NT	1.5
FRT fibroblasts[c]	37	0.75	NT
NRK fibroblasts[d]	37	0.5	ND
FRT-L MoMuLV[e]	37	10.0	283.3
FRT-L KiMuLV[f]	37	9.5	41.6

[a] Expressed as percentage of the total ^{125}I added, uptake by 10^6 cells. The values obtained with cells at 39°C were measured after 5 days of temperature shift. Values in parentheses are those obtained after a 24-h shift. NT, Not tested.

[b] Expressed as nanograms of TG produced per milliliter by 10^6 cells as determined by radioimmunoassay. The values at 39°C were obtained after 5 days of temperature shift. ND, Not detectable.

[c] FRT, Fischer rat thyroid.

[d] NRK, Normal rat kidney.

[e] FRT-L cells infected only with MoMuLV.

[f] FRT-L cells infected only with KiMuLV.

DISCUSSION

Carcinomas are the most common neoplasms of humans, and the biology of carcinoma cells differs considerably from that of sarcoma cells (19). Identification of changes in transformed epithelial cells may provide a tool to study the disturbance of regulation of epithelial cell growth and differentiation.

The epithelial rat thyroid cells transformed by the Kirsten sarcoma virus (20, 21) represent a unique system for these studies. Transformation of cells by tumor viruses results in (i) a decrease in anchorage and density-dependent growth, (ii) changes in cellular morphology and, generally, (iii) a modification of the specific differentiated cell properties. In several instances, the differentiated cells infected in vitro by different types of retroviruses lose their differentiated features (6, 10, 22, 34, 36). In the case of Abelson virus-transformed B lymphocytes, several clonal populations of transformed cells have been isolated that retain their ability to synthesize one of the specific differentiation products of these cell lines, i.e., immunoglobulin μ or κ chains (43).

Much work has been done with retroviruses that are temperature sensitive in their transforming ability in an effort to better understand the interaction of viral replication with the phenotypic program of the cell that results in a loss of differentiated cell properties and in the expression of transformation markers. In some cases, the differentiated functions of the cells are reacquired after the shift to the temperature nonpermissive for transformation. This is the pattern observed by Graf et al. (23) with avian erythroblasts transformed by a *ts* mutant of avian erythroblastosis virus in which it appears that a

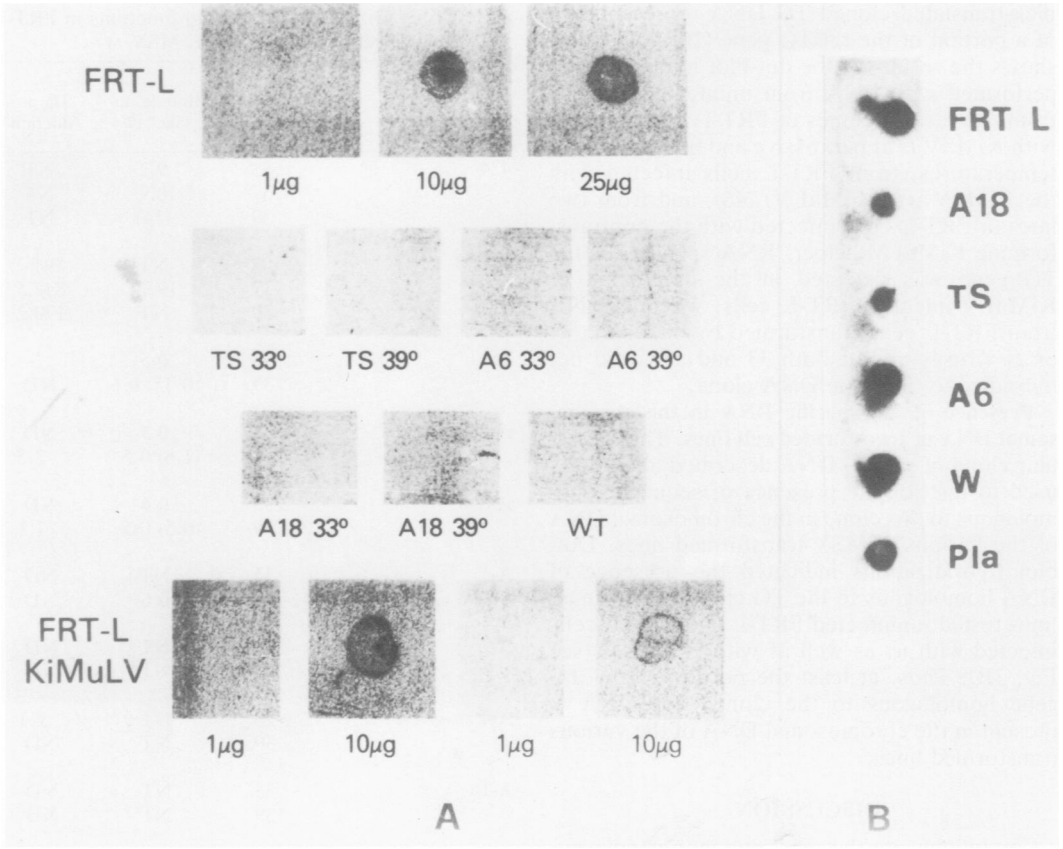

FIG. 3. Dot-blot analysis of cytoplasmic RNA and chromosomal DNA from uninfected and virus-infected FRT-L cells. (A) Cytoplasmic RNA was extracted from the uninfected, KiMSV *wt*, KiMSV *ts*, or KiMuLV-infected FRT-L cells, and dot-blot hybridizations were performed by using the TG-specific DNA probe as described in the text. In the case of all KiMSV-transformed cells, 25 μg of RNA was used. The bottom row shows the results obtained with two different lines of KiMuLV-infected FRT-L cells. (B) High-molecular-weight DNA was extracted as described in the text and hybridized by dot blot to the TG-specific DNA probe. The amount of the DNA used was 10 μg. Pla indicates a positive control of hybridization obtained by hybridizing the ^{32}P-labeled TG recombinant plasmid to unlabeled pBR322 (100 ng).

virus-encoded product must be continuously expressed to maintain the block in the differentiation program of this specific cell lineage and, therefore, also to maintain the leukemic state of the cells. In other cases, the loss of the differentiating potential of the cells is independent of the expression of the transforming gene after infection with a retrovirus. The infection of limb bud chondroblasts with *ts* mutants of Rous sarcoma virus, for instance, markedly inhibits chondrogenesis at both permissive and nonpermissive temperatures (24). Dissociation among transformation parameters has also been observed by Weber and Friis, using a particular type of *ts* mutant of Rous sarcoma virus and chick embryo fibroblasts (47). The virus effect on differentiated cells in culture might also be more complex, involving alterations and differential expression of the properties analyzed. For example, in BALB 3T3 fibroblasts infected with KiMSV, the relative rate of in vitro procollagen synthesis is clearly regulated by the transforming protein, whereas the latter protein is not required for the relative increase in the proportions of type III procollagen observed after transformation, as such proportions remain unchanged after the cells are shifted to growth at the nonpermissive temperature (7). In the system described here of rat thyroid cells infected with a *ts* strain of KiMSV, the transforming product seems to interfere with some cell parameters, such as growth control, but the most specific properties of these cells, i.e., TG production and iodide uptake, are irreversibly lost at both the permissive and nonpermissive temperatures.

As shown by immunoprecipitation and radio-

immunoassay, the KiMSV *ts*-infected FRT-L cells do not reacquire the ability to produce TG in the culture supernatants when shifted to growth at the nonpermissive temperature. The effect of the virus on the expression of the differentiated phenotype is clearly dependent on the transforming virus present in the Kirsten complex, since FRT-L cells infected only with the helper virus component of the complex (either KiMuLV or MoMuLV) secrete TG and concentrate iodide at levels comparable to those of uninfected FRT-L cells.

The RNA blot hybridization studies show that RNA specific for TG is not present in the cytoplasm of KiMSV *ts*-infected FRT-L cells at the permissive or nonpermissive temperature. The presence of DNA that hybridizes to a portion of the rat TG gene (16) in the chromosomal DNAs of all of the cloned lines infected by the *ts* KiMSV excludes the possibility that a complete deletion of the rat TG gene accounts for the absence of TG mRNA in the transformed lines. However, more detailed studies now in progress in our laboratory show that the structure of the TG gene is not altered after infection with the transforming virus. This has been controlled by hybridization experiments done with other cDNA clones, representative of the whole coding region of the TG gene (A. Fusco and P. P. Di Fiore, unpublished data).

The results reported here are compatible with the hypothesis of either a transcription or a maturation block of the TG mRNA in cells infected by the *ts* KiMSV.

Some properties of *ts* virus-infected rat thyroid cells, such as cell morphology and growth properties, are thermosensitive: the drastic morphological changes typical of the transformed lines disappear completely after only 24 h of growth at the nonpermissive temperature. It is interesting to note that the efficiency of colony formation in agar in the case of both KiMSV *ts*-infected or KiMSV *wt*-infected FRT-L cells (see Table 1) is very high. This is at variance with the common observation that transformed epithelial cells usually display no growth capacity in agar (19). However, there are cases described in the literature of epithelial cells transformed in vitro by oncogenic viruses which show low levels of colony formation in agar. These systems include cells transformed by adenovirus (37), mink epithelial cells transformed by an endogenous retrovirus (39), and neuroretinal chick embryo cells transformed by Schmidt-Ruppin Rous sarcoma virus (11).

Presumably, the effect of the *ts* KiMSV on the morphology and agar growth of FRT-L cells is dependent on the virus transforming product, the p21 kinase protein that is thermosensitive, as shown previously for KiMSV *ts*-transformed fibroblasts (42). Normal growth rate and the requirement for hormones are also restored after the temperature shift.

Kirsten sarcoma virus-transformed rat and mouse fibroblasts are known to secrete a growth factor into the culture supernatants which induces growth to high density at low serum concentrations (35). Preliminary evidence obtained in our laboratory indicates that a similar factor is produced by the Kirsten-transformed FRT-L cells (M. Ferrentino et al., unpublished data). A block in the production of this factor after the temperature shift to 39°C might account for the changes in growth properties and the reacquired hormonal dependence of the FRT-L cells. Alternatively, transformed FRT-L cells might lose one or more of the hormone receptors, for example, the receptor for the thyrotropic hormone, which is present on the plasma membranes of uninfected FRT-L cells (41, 48). This receptor might reappear after the temperature shift and might therefore be identified as a typical differentiated property of thyroid cells which is under the direct control of the p21 transforming protein. Further experiments are needed to discriminate fully between these two possibilities.

The data presented emphasize the complexity of the effect of KiMSV on FRT-L cells. The changes in epithelial morphology and growth properties require the continuous expression of the viral transforming gene function, as demonstrated by the suppression of these malignant properties at the nonpermissive temperature. The irreversible suppression of TG synthesis and iodide uptake could be interpreted in the light of three different hypotheses.

(i) A residual p21 transforming activity might be present even at the nonpermissive temperature.

(ii) The dissociation in the behavior of transformation versus differentiation markers could be ascribed to a pleiotropic effect of the p21 protein. It is reasonable to imagine that the two effects, transformation and block of differentiation, are under the control of two different domains of the p21 protein, only one of which (the one controlling the transformation phenomenon) is temperature sensitive.

(iii) The loss of the differentiated phenotype might be caused by events not necessarily related to the oncogene expression but dependent on the virus cell interactions that can cause irreversible alterations in the cellular genome structure or in its expression.

To discriminate between these hypotheses, experiments are in progress to determine whether epithelial rat cells retain or lose their differentiated phenotype after infection at 39°C.

Further studies are in progress in our labora-

tory to analyze whether the structure of the TG gene has been modified by the transformation event(s) in such a way as to render the infected cells incapable of synthesizing the TG molecule.

ACKNOWLEDGMENTS

We are grateful to E. M. Scolnick for providing us with the two C127 fibroblast cell lines which continuously produce the KiMSV *wt* (MoMuLV) and the *ts* KiMSV, respectively; to S. Raiti, Director of the National Hormone and Pituitary Program, for providing us with the bovine thyrotropic hormone; to F. S. Ambesi-Impiombato and D. Tramontano for continuously making available to us the FRT-L cell line; to R. di Lauro for giving us the rat TG clone; to G. Tajana for the histological studies; to L. Diamond of the Wistar Institute, Philadelphia, Pa., for help during the course of the work; to Marina Hoffman and the Editorial Services Department of the Wistar Institute; to P. Galli and M. D'Esposito for art work; and to G. Salvatore for the continuous encouragement given to our work on the transformed thyroid cell lines. Radioimmunological assays were kindly performed by Lily Chu in the laboratory of A. Schneider at Michael Reese Hospital, Chicago, Ill.

This work was supported by the Progetto Finalizzato Controllo della Crescita Neoplastica of the Consiglio Nazionale delle Ricerche C.N.R. no. 82.00431.96.

LITERATURE CITED

1. **Adams, S. L., M. E. Sobel, B. H. Haward, K. Olden, K. M. Yamada, B. de Crombrugghe, and I. Pastan.** 1977. Levels of translatable mRNA for cell surface protein, collagen precursors, and two membrane proteins are alterated in Rous Sarcoma virus transformed chick embryo fibroblasts. Proc. Natl. Acad. Sci. U.S.A. **74**:3399–3403.
2. **Alvino, C. G., V. Tassi, and G. Ambrosio.** 1978. Purification and characterization of rabbit antibody against rat 19S thyroglobulin by affinity chromatography. Bull. Mol. Biol. Med. **3**:130–139.
3. **Alvino, C. G., V. Tassi, B. M. Patterson, and R. Di Lauro.** 1982. *In vitro* synthesis of 300.000 M rat thyroglobulin subunit. FEBS Lett. **137**:307–313.
4. **Ambesi-Impiombato, F. S., L. A. M. Parks, and H. G. Coon.** 1980. Culture of hormone-dependent functional epithelial cells from rat thyroids. Proc. Natl. Acad. Sci. U.S.A. **77**:3455–3459.
5. **Ambesi-Impiombato, F. S., R. Picone, and D. Tramontano.** 1982. Influence of hormones and serum on growth and differentiation of the thyroid cell strain FRT-L. Cold Spring Harbor Conf. Cell Proliferation **9**:483–492.
6. **Arbogast, B. W., M. Yoshimura, N. A. Kefalides, H. Holtzer, and A. Kaji.** 1977. Failure of cultured chick embryo fibroblasts to incorporate collagen into their extracellular matrix when transformed by Rous sarcoma virus. J. Biol. Chem. **252**:8863–8868.
7. **Bateman, J. F., and B. Peterkofsky.** 1981. Mechanism of Kirsten Murine Sarcoma virus transformation induced changes in the collagen phenotype and synthetic rate of BALB 3T3 cells. Proc. Natl. Acad. Sci. U.S.A. **78**:6028–6032.
8. **Beug, H., A. Von Kirchbach, G. Doberlein, J. F. Conscience, and T. Graf.** 1979. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell **18**:375–390.
9. **Bilello, J. A., G. Colletta, G. Warnecke, G. Koch, D. Frisby, I. B. Pragnell, and W. Ostertag.** 1980. Analysis of the expression of spleen focus forming virus related RNA and gp55, a Friend and Rauscher virus specific protein. Virology **107**:331–344.
10. **Boettiger, D., K. Roby, J. Brumbaugh, J. Biehl, and H. Holtzer.** 1977. Transformation of chicken embryo retinal melanoblasts by a temperature-sensitive mutant of Rous Sarcoma Virus. Cell **11**:881–890.
11. **Calothy, G., F. Paisier, G. Dambrine, and B. Pessac.** 1978. A transformation defective mutant of Rous sarcoma virus inducing chick embryo neuroretinal cell proliferation. Virology **89**:75–84.
12. **Chou, J. Y.** 1978. Human placental cells transformed by a ts A mutant of simian virus 40: a model system for the study of placental functions. Proc. Natl. Acad. Sci. U.S.A. **75**:1409–1413.
13. **Chou, J. Y., and S. E. Schleger-Haueter.** 1981. Study of liver differentiation in vitro. J. Cell Biol. **89**:216–222.
14. **Colletta, G., P. P. Di Fiore, M. Ferrentino, C. Pietropaolo, M. C. Turco, and G.Vecchio.** 1980. Enhancement of viral gene expression in Friend erythroleukemic cells by 12-0-tetradecanoyl-phorbol-13-acetate. Cancer Res. **40**:3369–3373.
15. **De Larco, J. E., and G. J. Todaro.** 1978. Growth factors from murine sarcoma virus transformed cells. Proc. Natl. Acad. Sci. U.S.A. **75**:4001–4005.
16. **Di Lauro, R., S. Obici, A. Acquaviva, and C. Alvino.** 1982. Construction of recombinant plasmids containing rat thyroglobulin mRNA sequences. Gene (Amst) **19**:117–125.
17. **Easton, T., and E. Reich.** 1972. Muscle differentiation in cell culture. Effects of nucleoside inhibitors and Rous sarcoma virus. J. Biol. Chem. **247**:6420–6431.
18. **Ellis, R. W., D. De Feo, T. Y. Shih, M. A. Gonda, H. A. Young, N. Tsuchida, D. R. Lowy, and E. M. Scolnick.** 1981. The p21 *src* genes of Harvey and Kirsten sarcoma virus originate from divergent members of a family of normal verbrate genes. Nature (London) **292**:506–511.
19. **Franks, L. M.** 1979. What is a cancer cell? Phenotypic markers in *in vitro* carcinogenesis in epithelial system, p. 1–314. *In* L. M. Franks and C. B. Wigley (ed.), Neoplastic transformation of differentiated epithelial cell systems *in vitro*. Academic Press, Inc., New York.
20. **Fusco, A., A. Pinto, F. S. Ambesi-Impiombato, G. Vecchio, and N. Tsuchida.** 1981. Transformation of rat thyroid epithelial cells by Kirsten murine sarcoma virus. Int. J. Cancer **28**:655–662.
21. **Fusco, A., A. Pinto, D. Tramontano, G. Tajana, G. Vecchio, and N. Tsuchida.** 1982. Block in the expression of differentiation markers of rat thyroid epithelial cells by transformation with Kirsten murine sarcoma virus. Cancer Res. **42**:618–626.
22. **Gazzolo, L., C. Moscovici, M. G. Moscovici, and J. Samarut.** 1979. Response of hemopoietic cells to avian acute leukemia viruses: effects on the differentiation of the target cells. Cell **16**:627–638.
23. **Graf, T., N. Ade, and H. Beug.** 1978. Temperature-sensitive mutant of avian erythroblastosis virus suggests a block of differentiation as mechanism of leukemogenesis. Nature (London) **275**:496–501.
24. **Gross, J., and D. B. Rifkin.** 1979. The effect of avian retroviruses on limb bud chondrogenesis *in vitro*. Cell **18**:707–718.
25. **Gross-Bellard, M., P. Oudet, and P. Chambon.** 1973. Isolation of high molecular weight DNA from mammalian cells. Eur. J. Biochem. **36**:32–38.
26. **Hanafusa, H.** 1975. Avian RNA tumor viruses, p. 49–90. *In* F. B. Becker (ed.), Cancer—a comprehensive treatise, vol. 2. Plenum Publishing Corp., New York.
27. **Holtzer, H., J. Biehl, G. Yeoh, R. Meganathan, and A. Kaji.** 1975. Effect of oncogenic virus on muscle differentiation. Proc. Natl. Acad. Sci. U.S.A. **72**:4051–4055.
28. **Ikekubo, K., R. Pervos, and A. B. Schneider.** 1980. Clearance of normal and tumor-related thyroglobulin from the circulation of rats: role of the terminal sialic acid residues. Metabolism **29**:673–681.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **277**:680–685.
30. **Levison, W., R. S. Bhatnagar, and T. Lin.** 1975. Loss of ability to synthesize collagen in fibroblasts transformed by Rous sarcoma virus. J. Natl. Cancer Inst. **55**:807–810.
31. **Macpherson, I., and L. Montagnier.** 1964. Agar suspension culture for the selective assay of cells transformed by

polyoma virus. Virology **23**:291–294.
32. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage 7 Lambda. Proc. Natl. Acad. Sci. U.S.A. **72**:1184–1188.
33. **Olden, K., and K. M. Yamada.** 1979. Mechanism of the decrease in the major cell surface protein of chick embryo fibroblasts after transformation. Cell **11**:954–969.
34. **Okayama, M., M. Yoshimura, M. Muto, J. Chi, S. Roth, and A. Kaji.** 1977. Transformation of chicken chondrocytes by Rous sarcoma virus. Cancer Res. **37**:712–717.
35. **Ozanne, B., R. J. Fulton, and P. L. Kaplan.** 1980. Kirsten murine sarcoma virus transformed cell lines and a spontaneously transformed rat cell line produce transforming factors. J. Cell Physiol. **105**:163–180.
36. **Pacifici, M. O., K. Boettiger, K. Roby, and H. Holtzer.** 1977. Transformation of chondroblasts by Rous sarcoma virus and synthesis of the sulfated proteoglycan matrix. Cell **11**:891–899.
37. **Paraskeva, C., and P. H. Gallimore.** 1980. Tumorigenicity and *in vitro* characteristics of rat liver epithelial cells and their adenovirus transformed derivatives. Int. J. Cancer **25**:631–639.
38. **Parker, I., and W. Fitschen.** 1980. Protocollagen mRNA metabolism during the fibroblasts cell cycle and its synthesis in transformed cells. Nucleic Acids Res. **8**:2823–2833.
39. **Rappi, V. R., and G. Y. Todaro.** 1980. Generation of oncogenic mouse type C viruses: *in vitro* selection of carcinoma-inducing variants. Proc. Natl. Acad. Sci. U.S.A. **77**:624–628.
40. **Roy-Burman, P., and V. Klement.** 1975. Derivation of mouse sarcoma virus Kirsten by acquisition of genes from heterologous host. J. Gen. Virol. **28**:193–198.
41. **Salvatore, G., J. B. Stanbury, and J. E. Rall.** 1980. Inherited defects of thyroid hormone biosynthesis, p. 443–487. *In* M. de Visscher (ed.), The thyroid gland. Raven Press, New York.
42. **Shih, T. Y., M. O. Weeks, H. A. Young, and E. M. Scolnick.** 1970. p21 of Kirsten murine sarcoma virus is thermolabile in a viral mutant temperature sensitive for the maintenance of transformation. J. Virol. **31**:546–556.
43. **Siden, E. J., D. Baltimore, D. Clark, and N. E. Rosemberg.** 1979. Immunoglobulin synthesis by lymphoid cells transformed *in vitro* by Abelson murine leukemia virus. Cell **16**:389–396.
44. **Varshavsky, A.** 1981. Phorbol ester dramatically increases incidence of methotrexate resistant mouse cells. Possible mechanism and relevance to tumor promotion. Cell **25**:561–572.
45. **Vecchio, G., L. Nitsch, G. Tajana, A. Pinto, and A. Fusco.** 1981. Experimental thyroid carcinogenesis by RNA tumor viruses, p. 11–21. *In* M. Andreoli, F. Monaco, and J. Robbins (ed.), Advances in thyroid neoplasia. Field Educational, Rome.
46. **Vecchio, G., A. Pinto, G. Colletta, P. P. Di Fiore, A. Fusco, M. Ferrentino, M. Grieco, and N. Tsuchida.** 1982. Differentiation in retrovirus transformed cells. Establishment of a new system of epithelial origin, p. 501–507. *In* R. F. Revoltella, G. M. Pontieri, C. Basilico, R. C. Gallo, G. Rovera, and J. H. Subak-Sharpe (ed.), Expression of differentiated functions in cancer cells. Raven Press, New York.
47. **Weber, M. J., and R. R. Friis.** 1979. Dissociation of transformation parameters using temperature conditional mutants of Rous sarcoma virus. Cell **16**:25–32.
48. **Yavin, E., Z. Yavin, M. D. Schneider, and L. D. Kohn.** 1981. Monoclonal antibodies to the thyrotropin receptor. Implication for receptor structure and the action of autoantibodies in Graves' disease. Proc. Natl. Acad. Sci. U.S.A. **78**:3180–3184.