Stimulation of Growth Rate of Chondrocytes by Rous Sarcoma Virus Is Not Coordinated with Other Expressions of the *src* Gene Phenotype

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Infection and transformation of chondrocytes by Rous sarcoma viruses (RSVs) (Schmidt-Ruppin, Prague) stimulated the rate of cell growth. In contrast, several transformation-defective (*td*) mutants (*tdPRA*, *tdNY105*, *tdNY106*, *tdNY107*, and *tdNY108*) retaining various sizes of the *src* gene did not stimulate cell growth, indicating that the stimulation of growth of chondrocytes is due to the function of the *src* gene. With the use of various T (transformation)-class temperature-sensitive (*ts*) mutants of RSV, growth stimulation of chondrocytes by the *src* gene was examined. It was found that there are two types of T-class *ts* mutants with regard to the stimulatory effect on the growth of chondrocytes. One type (*tsNY68*) stimulates cell growth at both permissive (36°C) and nonpermissive (41.5°C) temperature, as does the wild type of RSV. Another type (*ts GI201* [clone 9]) stimulates cell growth only at the permissive temperature. Chondrocytes infected with either of these two types of T-class *ts* mutants showed *ts* properties in other transformation markers, such as uptake of 2-deoxy-D-glucose, change of cell morphology, and focus formation. These data indicate that the effect of the *src* gene on cell growth does not occur coordinately with other transformation markers.

There have been numerous studies on the action of the *src* gene of Rous sarcoma virus (RSV) (4, 12, 34). Under culture conditions normally used, fibroblasts transformed by RSV do not show a higher growth rate compared with uninfected cells (12, 18, 22), even though anchorage-independent cell growth in agar medium and higher saturation density are observed. Recently, however, it is reported that RSV stimulates growth of differentiated cells such as neural retina cells under normal culture conditions (6, 7, 26). Other differentiated cells such as chicken chondrocytes (24, 25), iris epithelial cells (10), pigment retina cells (5), myoblasts (14, 19), and neural crest cells (15) can be transformed by RSV. Since most of these differentiated cells show a relatively slow growth rate, the effect of *src* gene on cell growth may be observed much more distinctly than with fibroblasts.

In this communication, we report that some of the T-class temperature-sensitive (*ts*) mutants of RSV have a stimulatory effect on the growth rate of chicken chondrocytes at both permissive and nonpermissive temperatures, indicating that the thermosensitive lesion in the *src* gene does not necessarily lead to thermosensitive stimulation of cell growth. On the other hand, other T-class mutants had a *ts* growth

stimulatory effect. None of the transformation-defective (*td*) mutants which have various sized deletions in the *src* gene stimulated cell growth. These data indicate that the stimulatory effect of RSV on the growth of chondrocytes is due to the *src* gene, but the effect is not necessarily observed coordinately with other transformation markers.

MATERIALS AND METHODS

Preparation of cells. Chondrocytes were prepared from vertebral cartilage of leukemia-free 11-day-old chicken embryos (Specific Pathogen Free Avian Supply Co., Storrs, Conn.) as described previously (24, 25). Briefly, vertebral cartilages were treated with 0.25% (wt/vol) trypsin and 0.05% (wt/vol) collagenase at 36°C for 2 h and cultured at 36°C for 6 days in F10 medium (pH 7.0) containing 10% (vol/vol) fetal calf serum, 10% (wt/vol) tryptose phosphate broth, anti-PLO (60 µg/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml). The nonadherent cells in the primary culture were collected on day 6 and treated with 0.25% (wt/vol) trypsin at 36°C for 15 min and plated on new culture dishes. Chondrocytes thus prepared were more than 98% pure by microscopic inspection of the culture. Fibroblasts were prepared also from 11-day-old chicken embryos and cultured in the same F10 medium described above except that it contained 5% (vol/vol) fetal calf serum. Fibroblasts thus obtained were trypsinized (0.25%, wt/vol) and used for experiments.

Viruses. All RSVs are of subgroup A except as otherwise mentioned. Wild-type Schmidt-Ruppin (SR-RSV), a T-class *ts* mutant of SR-RSV (*ts*NY68 [18]), and *td* mutants of SR-RSV (*td*NY105, *td*NY106, *td*NY107, and *td*NY108 [17]) were generous gifts from H. Hanafusa of Rockefeller University. R. R. Friis, (Institut für Virologie, Giessen, Germany) kindly provided the wild-type Prague strain of RSV (PR-RSV) and T-class temperature-sensitive mutants of PR-RSV (*ts*GI201, *ts*GI202) (2). RSV *ts*GI201 was further cloned in our laboratory, and clones 9 and 4 were used in this communication. A *td* mutant of PR-RSV (*td*PRA) (35) was obtained from W. S. Mason (Institute for Cancer Research). SR strain (wild type) belonging to subgroup D (SR-RSV-D) was obtained from H. Hanafusa. A clone of SR-RSV-D obtained from W. S. Mason was found to be a *ts* mutant for cell transformation called *ts*PH2001. PA101 was generously supplied by G. Calothy (Institute Curie-Biologie, Orsay, France) (7). This virus stock is a mixture of subgroup A and D (personal communication).

All viruses except PA101 were obtained from virus-infected fibroblast culture media containing 5% (vol/vol) fetal calf serum, 1% (vol/vol) dimethyl sulfoxide, 2 μ g of polybrene per ml, and other components as described above. Mock virus was obtained from the culture medium of uninfected normal fibroblasts. PA101 was harvested from the culture of neural retina cells which were infected with this virus and cultured in F10 medium without dimethyl sulfoxide and polybrene.

Virus infection. Chicken chondrocytes (20×10^4 cells per 60-mm-diameter dish) were plated. After cells attached to the substrate, cells were infected at 36°C for 1.5 h with viruses (20 focus-forming units per cell). The multiplicity of infection with the *td* viruses was the equivalent of approximately 20 focus-forming units of *ts*NY68 as measured by the reverse transcriptase assays. After the infection, the cultures were given 5 ml of fresh medium containing 5% (vol/vol) fetal calf serum, 1% (vol/vol) dimethyl sulfoxide, and 2 μ g of polybrene per ml. Medium was changed every 2 days. More than 95% of the cells were transformed, judging from the morphology of the infected chondrocytes. That these transformed cells are indeed derived from chondrocytes was proven by raising the temperature to the nonpermissive temperature, which restored typical chondrocyte morphology.

Cell growth measurement. Cell number per dish was measured with a haemocytometer after cells were harvested with 0.25% (wt/vol) trypsin.

Measurement of reverse transcriptase. To measure both the titer of *td* viruses and virus production of wild-type RSV-infected cells, reverse transcriptase activity was measured by the method of Kacian and Spiegelman (16) with modifications. Culture media (10 ml) containing virus was centrifuged at 25,000 rpm in a Beckman SW27.1 rotor at 4°C for 60 min. Pelleted viruses were suspended in 50 μ l of Tris-hydrochloride buffer (1 mM Tris-hydrochloride, 10 mM NaCl, pH 7.4), and an aliquot (25 μ l) of each sample was added to 25 μ l of reaction mixture containing 100 mM Tris-hydrochloride, 13 mM MgCl₂, 50 mM NaCl, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, deoxythymidine-[methyl-³H]-5'-triphosphate (dTTP) (200 μ Ci/

ml), 10 mM dithiothreitol, and 0.3% Nonidet P-40. The reaction was performed at 37°C for 15 min. A 35- μ l aliquot was taken, and trichloroacetic acid-insoluble radioactivity was measured with a liquid scintillation counter (Packard model 3375).

Chemicals. F10 medium, fetal bovine serum, trypsin, and anti-PPL0 were purchased from GIBCO Laboratories, Grand Island, N.Y. Polybrene was purchased from Aldrich Chemical Co. dATP (Sigma), dGTP (Sigma), dCTP (Sigma), [³H]TTP (86 Ci/mole) (ICN), and Nonidet P-40 (BDH Chemicals) were used for the assay of reverse transcriptase.

RESULTS

Stimulation of growth of chondrocytes by *ts*NY68. Data shown in Fig. 1 indicate that the growth of chondrocytes is significantly stimulated by infection with wild-type SR-RSV as well as its *ts* mutant (*ts*NY68) at the permissive temperature (36°C). At 8 days after infection of the chondrocytes, the number of cells in cultures transformed by these viruses was at least three times higher than that of control mock-infected cells. The rate of growth for the transformed cells was approximately five times higher on day 6 than that of mock-infected cultures. A surprising result was obtained when similar experiments were conducted at the nonpermissive temperature (41.5°C) at which the *src* gene product(s) of *ts*NY68 is supposed to be inactive (18). As shown in Fig. 1b, a similar stimulatory effect of this virus was observed even at 41.5°C. In fact, the stimulatory effect of *ts*NY68 at 41.5°C was identical to that of the wild type belonging to the same strain. These experiments suggest that the growth-stimulatory effect of *ts*NY68 was not influenced by the *ts* lesion in the *src* gene of this virus.

Stimulation of DNA synthesis by RSV. Stimulation of growth of chondrocytes by RSV was further confirmed by increased incorporation of [³H]thymidine (TdR) into DNA at the permissive temperature. In the experiment shown in Fig. 2a, *ts*NY68-infected chondrocytes were labeled continuously with [³H]TdR at the permissive temperature (36°C). On day 6 after infection, the total labeled DNA of transformed chondrocytes was 2.5-fold higher than that of uninfected chondrocytes. The kinetics of incorporation of [³H]TdR into DNA gave results somewhat different from those of the cell counts. The difference was due perhaps to the slight toxic effect of [³H]TdR on chondrocytes.

The stimulatory effect on DNA synthesis was further supported by autoradiography of [³H]TdR-labeled chondrocytes. At 60 h after infection at 36°C, a significant difference of labeling indexes of *ts*NY68-transformed and uninfected chondrocytes could be observed (Fig. 2b). We conclude that, in parallel with the

FIG. 1. Lack of temperature response of the growth-stimulatory effect of RSV tsNY68. Chondrocytes (20×10^4 cells per 60-mm-diameter dish) were plated and incubated at 36°C for 20 h, and cells were infected with virus. Cell number per dish was determined at the indicated time. The plotted value shows the mean value of duplicate cultures. The floating cells were also counted and added to total cell number per dish. Symbols: ▲, normal chondrocytes infected with mock virus; ●, chondrocytes infected with tsNY68; ■, Chondrocytes infected with SR-RSV (wild type). (a) Cells were cultured at 36°C . (b) Cells were cultured at 41.5°C .

FIG. 2. Stimulation of [^3H]TdR incorporation into chondrocytes by tsNY68. Chondrocytes (20×10^4 cells per 60-mm-diameter dish) were plated, cultured at 36°C for 18 h, infected with tsNY68 (●), or mock virus (▲) at 36°C for 2 h and cultured in F10 medium. (a) Chondrocytes were continuously labeled with $2 \mu\text{Ci}$ of [^3H]TdR per ml of F10 medium (5 ml of medium per dish). On days 0.4, 2, and 6 after virus infection, the incorporation of [^3H]TdR into a trichloroacetic acid-insoluble fraction in chondrocytes was examined. Each plot is an average of duplicate samples, and its deviation was within 3.5% of each value. (b) At the indicated time, cultures were labeled with $10 \mu\text{Ci}$ of [^3H]TdR in 2 ml of Eagle minimal essential medium at 36°C for 1 h, and the labeling index, i.e., the percentage of the number of labeled nuclei to that of total nuclei, was determined by autoradiography as described previously (19). Each plot shows an average and its deviation in duplicate samples.

growth stimulation, the *src* gene product appears to stimulate DNA synthesis of chondrocytes.

Effect of serum concentrations on growth of tsNY68-infected chondrocytes. It has been shown that transformed cells in general can grow with lower concentrations of serum than the normal counterparts (3). It is therefore impor-

tant to examine whether the stimulatory effect of tsNY68 is dependent on an appropriate serum concentration. Data presented in Fig. 3 show indeed that the stimulation is largely dependent on the presence of serum. However, at all concentrations of serum tested, significant stimulation of growth could be observed at 41.5°C as well as at 36°C .

FIG. 3. Serum effect on growth of *tsNY68*-infected chondrocytes. Chondrocytes were infected with *tsNY68* or mock virus and cultured at 36°C. On day 6 chondrocytes (20×10^4 cells per 60-mm-diameter dish) were replated, and all cultures were incubated at 41.5°C for 1 day. Culture media were changed with F10 media containing various concentrations of fetal calf serum (0, 0.2, 1.0, 5.0, and 10.0% [vol/vol]). Some cultures were shifted down to 36°C and the others were kept at 41.5°C at this point. Both 36 and 41.5°C cultures were incubated for an additional 4 days at 36 and 41.5°C, respectively, and cell number per dish was determined on day 5 after replating. The cell number is plotted against serum concentrations. Symbols: ●, *tsNY68*-transformed chondrocytes cultures at 36°C; ○, *tsNY68*-infected chondrocytes cultured at 41.5°C; ▲, uninfected chondrocytes at 36°C; △, uninfected chondrocytes at 41.5°C.

Effect of various *ts* mutants of RSV on cell growth. In addition to *tsNY68*, we examined the effect of other *ts* mutants on the rate of growth of chondrocytes at logarithmic phase. As shown in Table 1, *tsGI201* (clone 9) had a significant temperature-dependent effect. At the permissive temperature, chondrocytes infected with *tsGI201* (clone 9) grew approximately 2.5 times faster than uninfected cells, whereas no stimulatory effect was observed at the nonper-

missive temperature. Similar temperature-dependent growth stimulation was observed with RSV *tsLA24*. In contrast, *tsGI202* and *tsGI201* (clone 4) stimulated growth approximately two-fold even at 41.5°C, although stimulation was higher at 36°C. The extents of growth stimulation by these mutants at 41.5°C were similar to that by wild-type virus (PR-RSV).

We also examined the growth stimulatory effect of RSVs belonging to subgroup D, *tsPH2001* and PA101, which is a partial *td* virus because of its differential expression of transformation markers at a permissive temperature (7). During week 1 after infection with virus, the cell growth stimulation effect by these viruses could not be detected even at the permissive temperature (36°C) due to cytopathic effect of these viruses (11). On the other hand, after 1 week growth stimulation was noticeable, and after prolonged culture (2 to 3 weeks) stimulation was very distinct. As shown in Table 2, PA101-infected chondrocytes appear to exhibit *ts* growth, whereas chondrocytes transformed by the wild type of SR-RSV (subgroup D) did not. A moderate temperature-dependent stimulation of growth was observed with RSV *tsPH2001*.

Effect of the temperature shifts on the growth curve of chondrocytes infected with RSV *tsGI201* (clone 9). Since *tsGI201* (clone 9) exhibited the most clear-cut temperature-dependent growth effect, we examined the effect of temperature shifts on the growth curve of chondrocytes infected with this mutant RSV. As shown in Fig. 4, the growth of chondrocytes, upon shift from the permissive to the nonpermissive temperature, slowed down. From these data one can conclude that the growth change occurs within 72 h of temperature shifts. However, in a separate study (data not shown), the effect was noticeable within 48 h of the temperature shifts. These data add support to the notion that the *ts* lesion of the *src* gene of *tsGI201* (clone 9) influences cell growth.

Lack of effect of various *td* mutants of RSV on growth of chondrocytes. The experiments described above strongly suggest that the stimulatory effect on cell growth is the effect of the *src* gene. To confirm this point we examined various *td* mutants (*tdNY105* through *tdNY108*, and *tdPRA*) as described in Table 3. It was suggested that these *td* mutants have various lengths of deletions from the *src* gene (17). For example, *tdNY105* has the smallest remaining portion of the *src* gene, and *tdNY108* has the largest length of *src* gene. Although it is possible that, after long-term cultivation, *td* mutants having small deletions from the *src* gene can partially express cell transformation (13, 33), this did not occur in the experiment shown in Table

TABLE 1. *Effect of mutants of subgroup A RSV on growth of chondrocytes^a*

Viruses	36°C		41.5°C	
	Cell no. per dish ($\times 10^{-4}$)	Ratio (in- fected/unin- fected)	Cell no. per dish ($\times 10^{-4}$)	Ratio (in- fected/unin- fected)
Uninfected	56.3 $\pm$ 0.3	1.0	49.5 $\pm$ 5.7	1.0
<i>tsGI201</i> (clone 4)	204.4 $\pm$ 6.9	3.6	107.7 $\pm$ 1.3	2.2
<i>tsGI201</i> (clone 9)	161.0 $\pm$ 8.9	2.9	45.1 $\pm$ 3.1	0.9
<i>tsGI202</i>	130.4 $\pm$ 3.0	2.3	91.5 $\pm$ 4.7	1.9
<i>tsLA24</i>	126.5 $\pm$ 4.1	2.2	40.7 $\pm$ 2.8	0.8
PR-RSV	95.3 $\pm$ 1.3	1.7	106.7 $\pm$ 5.3	2.2

^a Chondrocytes (20×10^4 cells) were plated in each 60-mm diameter dish, cultured at 36°C for 1 day, infected with virus at 36°C for 2 h, and cultured at 36°C or 41.5°C for 5 additional days. Cell number per dish was determined on day 6. Each value shows the mean value in duplicates.

TABLE 2. *Effect of subgroup D on growth of chondrocytes^a*

Viruses	Temp (°C)	Cell no. per dish		
		Day 1	Day 7	Ratio (day 7/ day 1)
PA101	36	27.5 $\pm$ 0.8	329.5 $\pm$ 14.4	12.0
	41.5	18.0 $\pm$ 0.8	120.2 $\pm$ 11.2	6.7
<i>tsPH2001</i>	36	20.1 $\pm$ 0.9	331.0 $\pm$ 18.0	16.5
	41.5	18.7 $\pm$ 1.0	178.5 $\pm$ 10	9.5
SR-RSV-D (wild type)	36	22.6 $\pm$ 0.7	392.0 $\pm$ 2.0	17.3
	41.5	23.2 $\pm$ 0.5	315.0 $\pm$ 3.0	13.6

^a Chondrocytes were plated and infected with viruses belonging to subgroup D as described in the text. After a 12-day cultivation at 36°C, cells were replated and cultured at 36°C for additional 12 days. Some of the cultures were shifted to 41.5°C, and cells were cultured at both 36 and 41.5°C for an additional 2 days. Both cultures were dispersed with trypsin, and cells (20×10^4 cells per 60-mm diameter dish) were plated at 36 and 41.5°C, respectively. Media were changed every day. On day 7 (total 33 days after infection), cell number per dish was determined. Uninfected chondrocytes cultured for a prolonged period, became sensitive to 41.5°C, and did not grow at all.

3. During 28 days of cultivation, none of them stimulated growth of chondrocytes at 36°C significantly. There were also no differences in the cell morphology of *td* mutant-infected and normal uninfected chondrocytes (data not shown). The titer of the *td* viruses in the culture media was 30% of that of *tsNY68*-infected cultures.

Morphological change of chondrocytes infected with various *ts* mutants of RSV. Morphological changes induced by various mutants used in the preceding section are indicated in Fig. 5. In general, very little morphological change is observed at 41.5°C with any of the mutants used. However, at 36°C the morphology of the transformed chondrocytes varied depending on the mutants used. In the morphology changes induced by PA101 or *tsGI201* (clone 9), cells look "stretched out" without changing the

basic shape, whereas *tsNY68* induces typical spindle-like transformed morphology described earlier (25). In addition, *tsGI201* (clone 4) hardly induced morphological change even at 36°C. As indicated in Table 1 and Fig. 1, stimulation of growth rate occurred even in the absence of "transformed" morphology (*tsGI201* [clone 4] at 36°C or *tsNY68* at 41.5°C).

Focus formation by chondrocytes infected with various *ts* mutants. As discussed in the preceding section, at 36°C all mutants used in this study induced morphological change. It is therefore of interest to examine whether the foci formed by transformed chondrocytes have different morphology. As shown in Fig. 6, one can observe focus formation with the chondrocytes with all three *ts* mutants. The focus looked essentially the same with PA101 and *tsGI201* (clone 9), but *tsNY68* produces a plaque in the center of focus. The formation of "plaque" within the foci could be observed even at an earlier stage, suggesting that under our focus assay conditions, *tsNY68* may make chondrocytes detach from the substratum. Although there is no clear-cut difference between the foci of PA101 and *tsGI201* (clone 9), there are subtle differences about the shape of these foci.

An important question is whether or not one would observe focus formation at 41.5°C because some mutants stimulate growth even at this temperature. It is clear from Table 4 that none of the mutants formed visible foci with chondrocytes. These results show clearly that stimulation of the rate of growth does not contribute to formation of foci. The reason for reduction of foci by the wild type at 41.5°C remains obscure.

[2-³H]deoxy-D-glucose uptake of chondrocytes infected with *ts* mutants of RSV. Table 5 shows that uptake of [2-³H]deoxy-D-glucose by chondrocytes infected with RSV *ts* mutants is significantly higher than that of normal chondrocytes at 36°C but not at 41.5°C. In contrast, wild type-infected chondrocytes had

FIG. 4. Kinetics of cell growth of chondrocytes infected with *tsGI201* (clone 9) after temperature shifts. Chondrocytes in suspension were infected with *tsGI201* (clone 9) on ice for 1.5 h, plated (20×10^4 cells per 60-mm-diameter dish), and cultured at 36 and 41.5°C. (a) Cultures were shifted from 41.5 to 36°C at the points of arrows. Symbols: ●, shifted down to 36°C; ○, kept at 41.5°C. (b) Cultures were shifted from 36 to 41.5°C at the points of arrows. ●, kept at 36°C; ○, shifted up to 41.5°C.

TABLE 3. Lack of effect of *td* mutants on growth of chondrocytes^a

Viruses	Cell no. per dish ($\times 10^{-4}$)	Reverse transcriptase activity (cpm/ 10^6 cells)
Uninfected	70.0 ± 0.9	0
<i>tdNY105</i>	82.2 ± 1.9	Not measured
<i>tdNY106</i>	76.6 ± 7.3	550
<i>tdNY107</i>	83.3 ± 0.1	470
<i>tdNY108</i>	86.0 ± 1.0	400
<i>tdPRA</i>	63.0 ± 3.3	550
<i>tsNY68</i>	221.3 ± 4.0	1,450

^a Chondrocytes (50×10^4 cells per 100-mm dish) were plated and infected with virus. After 9 days of cultivation at 36°C, cells were dispersed with 0.25% (wt/vol) trypsin, plated (50×10^4 cells per dish), and cultured at 36°C for an additional 4 days. These cultures were dispersed with 0.25% (wt/vol) trypsin, and cells (20×10^4 cells) were plated on 35-mm dish. Cell number per dish was determined on day 3, 7, and 15 after the last trypsinization. The data presented in this table were the mean value of duplicate samples on day 15. To make sure that the virus infection took place, reverse transcriptase activity was measured in culture medium which was incubated for 24 h from day 14 to 15. The activity is expressed as [^3H]TTP incorporated into a trichloroacetic acid-insoluble fraction per 10^6 cells as described in the text. The values presented in this table were already subtracted with the background radioactivity of uninfected cells (300 cpm) from the experimental values of each virus-infected cells. The background value represents physical trapping of radioisotope in the acid-precipitable material and does not represent the virus production by uninfected cells.

significant increase of rate of [$2\text{-}^3\text{H}$]deoxy-D-glucose uptake both at 36 and 41.5°C. Comparison of Table 5 and Fig. 5 reveals that increased rate of glucose uptake is correlated with change of morphology. When cells look normal as those infected with *tsNY68* at 41.5°C, the rate of [$2\text{-}^3\text{H}$]deoxy-D-glucose uptake was similar to that of normal cells. Furthermore, when stimulation of the rate of glucose uptake is less at 36°C (as with *tsGI201* [clone 9], PA101), less extensive morphology change is observed. One exception to this generalization was chondrocytes infected with *tsGI201* (clone 4), which showed no morphology change even at 36°C, while higher levels of sugar uptake (about three times higher than normal chondrocytes) were observed. In addition, the plasminogen activator of these cells was about 60-fold higher than the normal counterpart (data not shown). The change of rate of sugar uptake was not correlated with the growth rate stimulation. For example, RSV *tsNY68* does not stimulate glucose uptake while it stimulates growth at 41.5°C.

DISCUSSION

Cell growth pattern in in vitro culture has two separate elements: rate of growth and final level of cell density. The latter characteristic has been studied in detail and appears to be partly related to a local depletion of nutrient (32) or inhibition of cellular mobility upon cellular contact (1). For this phenomenon, terms such as "contact inhi-

FIG. 5. Morphology of chondrocytes infected with various *ts* mutants of RSV. Chondrocytes infected with RSV belonging to subgroup A were cultured at 36 or 41.5°C for 6 days, and photos were taken. Chondrocytes infected with RSV belonging to subgroup D PA101 and *ts*PH2001 were cultured at 36°C for 12 days, replated, cultured at 36 or 41.5°C for 4 days and photos were taken. A, B, C, G, H, I: 36°C cultures. D, E, F, J, K, L: 41.5°C cultures. A, D: uninfected; B, E: *ts*NY68 infected; C, F: *ts*GI201 (clone 4) infected; G, J: *ts*GI201 (clone 9) infected; H, K: PA101-infected; I, L: *ts*PH2001 infected.

FIG. 6. Morphology of foci formed by chondrocytes. Chondrocytes (4×10^5 cells per 35-mm diameter dish) were plated and cultured at 36°C for about 20 h. Cells were then infected with RSV at 36°C for 2 h, and covered with 3 ml (per dish) of F10 medium containing 0.7% (wt/vol) Noble agar, 10% (vol/vol) fetal calf serum, 0.5% (vol/vol) dimethyl sulfoxide, 5% (wt/vol) tryptose phosphate broth, and $0.7 \mu\text{g}$ of fungizone per ml. Cells were cultured at 36°C for first 3 days, and some of them were shifted up to 41.5°C . Agar media (2 ml/dish) were placed on top of the already existing agar every other day. On day 12, cultures were fixed with 4% (vol/vol) formaldehyde overnight and dipped in water for several hours, the agar layer was removed gently and stained with Diff-Quik solution II (Harleco), and the picture was taken. Photo shows that foci appeared at 36°C as described in Table 4; no foci were observed at 41.5°C , except for the wild type of RSV. A white bar in A shows 0.5 mm. (A) uninfected; (B) ts NY68; (C) ts GI201 (clone 9); (D) PA101; (E) wild-type Schmidt-Ruppin strain (subgroup A); (F) wild-type Prague strain (subgroup A).

bition" or "density-dependent inhibition" are often used (32). Cellular transformation usually causes loss of density-dependent inhibition of

cell growth. (12). However, this loss of density-dependent inhibition of growth is usually coordinated with other transformation markers, but

not always so, depending on the virus used (2, 36, 37). It has been difficult to study the effect of cellular transformation by RSV on the first element of cell growth, the rate of growth at the logarithmic phase, because fibroblasts which are used mostly for these studies are not influenced by RSV in this regard even though MC29RNA tumor virus appears to stimulate the growth rate of chicken fibroblasts (28).

Differentiated cells such as chondrocytes, neuroretina cells (6, 7, 26), or neural crest cells (15) offer an unique opportunity to study the effect of transformation on the "rate" of growth because infection with RSV apparently stimulates the rate of cell growth significantly. On the basis of data obtained with chicken chondrocytes, we can conclude that (i) stimulation of cell growth rate is a function of the *src* gene of RSV (even a partial deletion of the *src* gene would abolish

the stimulatory function of the *src* gene); and (ii) *ts* mutation of the *src* gene would result in two kinds of phenotypes: one in which the growth rate is coordinately *ts* with other transformation markers or one in which the growth rate stimulation is expressed even in the absence of expression of other transformation markers. The example of the former mutant is RSV *ts* GI201 (clone 9), and that of the latter is RSV *ts* NY68. Chondrocytes infected with *ts* NY68 grew faster than normal chondrocytes even at 41.5°C, whereas other transformation markers disappeared. Another example of stimulation of growth not coordinated with other expression of transformation markers is the chondrocytes transformed with RSV *ts* GI201 (clone 4). These chondrocytes had a cell morphology like normal even at the permissive temperature (36°C), while strong stimulation of growth was observed even at 41.5°C as well as at 36°C. On the other hand, expression of other transformation markers, such as plasminogen activator and 2-deoxy-D-glucose uptake are clearly *ts* (data not shown).

As to the possible mechanism of expression of markers of transformation, two possibilities exist: (i) the *src* gene product has only one possible biological action or an enzymatic activity which might lead to multitudes of responses observed with transformed cells; (ii) the *src* gene product(s) itself is multifunctional, leading to various expressions (7, 36). If we assume that the possibility (i) is correct, we would postulate that, depending on the level of the single biological activity (enzyme activity) of the *src* gene product, various transformation markers are expressed partially or completely. In other words, we would postulate that the single *src* activity of all the *ts* RSVs would be reduced at the nonpermissive temperature, but the extent of

TABLE 4. Focus formation by virus-infected chondrocytes^a

Virus	RSV subgroup	No. of foci per 2 dishes		
		36°C	41.5°C	Ratio (41.5°C/36°C)
SR <i>ts</i> NY68	A	673	0	<0.001
PR <i>ts</i> GI201 (clone 9)	A	104	0	<0.01
SR <i>ts</i> PH2001	D	192	0	<0.005
SR PA101	A, D	446	0	<0.002
SR-RSV wt	A	774	168	0.22
PR-RSV wt	A	375	128	0.34
SR-RSV wt	D	67	38	0.57

^a After an appropriate dilution of virus, focus formation assay was performed and foci were counted with a microscope after fixing cultures as described in Fig. 6. wt, Wild type of RSV. The values represent the number of foci per 0.1 ml of culture fluid after a 10³ dilution. When there were no foci at a 10³ dilution, no foci were seen even with a 10² dilution.

TABLE 5. Uptake of [2-³H]-deoxy-D-glucose^a

Viruses	Subgroup	36°C		41.5°C	
		cpm/100 µg of protein	Ratio (infected/uninfected)	cpm/100 µg of protein	Ratio (infected/uninfected)
Uninfected		3,970 ± 70	1.0	3,330 ± 10	1.0
SR <i>ts</i> NY68	A	11,350 ± 150	2.9	3,960 ± 110	1.2
PR <i>ts</i> GI201 (clone 9)	A	7,040 ± 10	1.8	3,570 ± 60	1.1
SR PA101	A, D	7,390 ± 20	1.9	3,290 ± 100	1.0
SR <i>ts</i> PH2001	D	10,870 ± 900	2.7	3,700 ± 140	1.1
SR-RSV wt	A	10,360 ± 610	2.6	9,800 ± 580	2.9
PR-RSV wt	A	10,000 ± 420	2.5	8,960 ± 330	2.7

^a Chondrocytes (20 × 10⁴ cells per dish) were plated, infected with viruses as described in Fig. 1, and cultured at 36°C for 7 days. A total of 20 × 10⁴ cells per 35-mm-diameter dish were replated and cultured at 36°C and 41.5°C for 3 days. Cultures were then incubated with [2-³H]-deoxy-D-glucose at 36°C, and the uptake level was determined (23). Protein was determined by the Lowry method (21). wt, Wild type.

reduction varies depending on the mutant. For example, at 41.5°C the *src* gene product of *ts*NY68 would retain a certain portion of the single biological activity which is large enough to stimulate the growth rate but not enough to maintain transformed morphology. The *src* gene product of *ts*GI201 (clone 9) would lose its single biological activity at 41.5°C so completely that all the transformation markers were not expressed. If we assume, on the other hand, that the second possibility is correct, we would postulate that only one of the multiple biological activities of the *src* gene product is responsible for stimulation of growth. With *ts*NY68, the activity responsible for growth stimulation would not be *ts*, whereas with *ts*GI201 (clone 9) it would be. The possible multiple activities of the *src* gene product may be due to presence of multiple *src* gene products. As a matter of fact, it has been reported that there may exist more than one *src* gene protein. In addition to the well-known *src* gene product with a molecular weight of 60,000, a protein of 52,000 molecular weight has been found and the primary sequence of this protein is apparently related to the 60,000-molecular-weight *src* protein (30). It is conceivable that one of the processed products is concerned with cell growth stimulation, whereas other transformation markers are related to the 60,000 protein. The fact that some of the transformation markers expressed at the nonpermissive temperature vary (36) depending on the mutants of RSV may support the possibility of a multifunctional *src* protein.

The only known enzymatic activity of the *src* gene product so far reported is the protein kinase (8, 9, 20, 27, 29, 31). Consistent with the possibility (ii) as discussed above, the protein kinase activity of *ts*NY68 has been reported to be clearly *ts* (8, 20), suggesting the possibility that the protein kinase activity of the *src* gene product has nothing to do with the growth stimulation of the host. On the other hand, consistent with the possibility (i) as discussed above, other laboratories reported that, even at the nonpermissive temperature, a significant amount of protein kinase activity of *ts*NY68 remains (27, 31), suggesting the possibility that a residual activity of the *src* gene protein kinase may be enough to stimulate the growth of chondrocytes. Although we are unable to determine which one of the above two possibilities is correct, experiments are currently in progress determining the kinase activity of the *src* gene product in chondrocytes infected with various mutants.

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