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Temperature-Dependent Transformation of Cells Infected with a Mutant of Bryan Rous Sarcoma Virus

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Chick embryo cells infected with a mutant (Ta) of the Bryan high-titer strain of Rous sarcoma virus (RSV-BH) are morphologically transformed at 36 C but appear similar to uninfected cells at 41 C. When cells infected with RSV-BH-Ta are switched from 41 to 36 C, morphological changes characteristic of transformation are observable within 10 min. The transformation is reversible; cells shifted from 36 to 41 C have been observed to lose their transformed morphology within 1 hr. The transformation after a shift in temperature is unaffected by inhibition of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or protein synthesis, demonstrating that the proteins involved in the morphological change are already present. Transformed cells infected with RSV-BH or RSV-BH-Ta take up hexose and synthesize hyaluronic acid at higher rates than uninfected cells or RSV-BH-Ta-infected cells grown at 41 C. However, inhibition of either protein or RNA synthesis, but not DNA synthesis, prevented the induction of increased hexose uptake and hyaluronic acid synthesis after a shift of RSV-BH-Ta-infected cells from 41 to 36 C. Therefore, these biochemical changes are secondary to a more basic change responsible for morphological transformation.

Several investigators have described the isolation of avian sarcoma virus mutants with temperature-sensitive properties. Some mutants can go through full cycles of reproduction at both high (41 C) and low (36 C) temperatures, but the infected cells become transformed only at the low temperature (6, 11, 13, 19). Other mutants are temperature dependent for virus reproduction as well as transformation (21). All of these mutants were derived from either the Schmidt-Ruppin or B77 strains of avian sarcoma virus. Transformation by these viruses is recognized by the increased refractility or rounding of cells, or both, morphological changes which often accompany malignant transformation in other systems.

Transformation of chick embryo fibroblasts by the Bryan high-titer strain of Rous sarcoma virus (RSV-BH) is morphologically unique, consisting of a change from elongated to polygonal or rounded shape, and the appearance of large numbers of vacuoles. Single transformed cells can be identified against a background of nontransformed cells (15). Both lethal and temperature-dependent mutations in RSV-BH have been found after exposure of newly infected cells to 5-bromodeoxyuridine (4, 5). One mutant isolate, RSV-BH-Ta, was selected for its ability to reproduce at 41 and 36 C and to transform cells only at the lower temperature. Several features of cells infected with RSV-BH-Ta were examined and are described here.

MATERIALS AND METHODS

Cell cultures. Chick embryo cells were prepared from 10-day-old embryos and were replated at intervals as described previously (15). Growth medium consisted of Eagle minimal essential medium (MEM) supplemented with dextrose (2 g/liter final concentration), sodium pyruvate (5 mM), 10% tryptose phosphate broth (Difco), 5% fetal bovine serum, penicillin (50 μg/ml), streptomycin (50 μg/ml), and tylosine (50 μg/ml). Cultures were maintained in humidified, CO_2-atmosphere incubators. Cells in 50-mm plastic petri dishes were used before reaching confluency.

Virus. RSV-BH is the Bryan high-titer strain of RSV_0 mixed with a nontransforming Rous-associated virus (RAV_1). Virus mutants were obtained after treatment of newly infected cells with 5-bromodeoxyuridine (5). Mutants were selected for ability to reproduce at both 41 and 36 C, and all contain RAV_1. The mutants of the transforming particles may have mutations in other regions of the virus genome, but if the products of these mutant regions are required for virus reproduction they are complemented by

RAV_1 (5). The mutant RSV-BH-Ta described herein was propagated in chick embryo cells by infecting cells at a high multiplicity of virus to cell, maintaining the growing cells at 39 C through two passages at 3-day intervals, and collecting the cell culture fluids. New cells were infected monthly and were used in transformation experiments within 1 to 6 weeks after infection. During this time, practically all cells assumed the morphology of transformed cells upon incubation at 36 C.

The focus formation method (20) was used for assays of Rous sarcoma virus, and infectious virus is counted in focus-forming units (FFU). Focus agar medium consisted of the above growth medium with 3% calf serum substituting for fetal bovine serum, and with the addition of 1% beef embryo extract and 0.8% agar. When RSV-BH-Ta was being assayed, cultures were incubated for 2 days at 41 C before being shifted to 36 C for an additional 5 days. Neutral red (0.01% in Eagle MEM) was layered onto the nutrient agar the day before microscope scanning for foci.

The assay method for the growth of infected cells in colonies suspended in soft agar has been described (2).

Antimetabolites. Cytosine arabinoside (NIH-CCNSC) at 10^{-4} M reduced incorporation of deoxythymidine-^{3}H into deoxyribonucleic acid (DNA) to less than 10% within 30 min. Actinomycin D (2 μg/ml; Merck, Inc.) reduced incorporation of uridine-^{3}H into ribonucleic acid (RNA) to less than 5% within 30 min. Cycloheximide (10 μg/ml; Calbiochem) and puromycin (50 μg/ml; Calbiochem) reduced incorporation of leucine-^{14}C into protein to less than 2% within 30 min. When cytosine arabinoside was used, tryptose phosphate broth was eliminated from the medium.

Hyaluronic acid synthesis. Hyaluronic acid content was determined by quantitative electrophoresis in polyacrylamide-agarose mixed gels (J. P. Bader, D. A. Ray, and T. L. Steck, Biochim. Biophys. Acta, *in press*). Cell culture fluids were applied directly to the gels after treatment for 15 min with 0.1 N NaOH. Cellular cytoplasms were separated from nuclei after treatment with 0.5% Nonidet P-40 (J. P. Bader et al., *in press*). These cytoplasmic extracts were treated with Pronase (50 μg/ml; 37 C; 30 min), then 0.1 N NaOH (15 min; room temperature). The samples were dialyzed, lyophilized to dryness, then resuspended in the electrophoresis buffer at one-fifth the original volume. Nonconcentrated cytoplasmic samples taken directly after treatment with Pronase and NaOH gave comparable results, as determined by viewing of stained gels, but amounts of hyaluronate were too low for quantitation.

Hexose uptake. The rate of hexose uptake was determined by the incorporation of deoxyglucose-^{3}H (9). Culture fluids were removed, and the cells were rinsed once with Dulbecco's phosphate-buffered saline (PBS). Deoxyglucose-^{3}H (2 μCi in 2 ml of PBS) was added, and all cultures were incubated at 39 C for 15 min. (Under these conditions deoxyglucose-^{3}H is incorporated at a linear rate for at least 30 min.) Cold PBS was added to the cultures, and the cells were rinsed four times with cold PBS. Nonidet P-40 (0.5% in PBS) was added, and after 15 min at room temperature the cytoplasmic fluids were analyzed for radioactivity and protein (12).

RESULTS

Reproduction of RSV-BH-Ta. The mutant, RSV-BH-Ta, was selected for its ability to reproduce without restriction over the temperature range 36 to 41 C. Chick embryo cells were exposed to a high multiplicity of RSV-BH-Ta, the cells were incubated, and the amount of progeny virus appearing in the cell culture fluids the next day was determined (Table 1). Reproduction occurred at all temperatures. Inhibition of the appearance of progeny virus by cytosine arabinoside demonstrated that the usual reproductive cycle was responsible for the appearance of virus, rather than merely the persistence and release of superficially adsorbed virions.

Focus formation and growth of colonies in agar. When cells were infected with RSV-BH-Ta at either high (>2 FFU/cell) or low (<1 FFU/cell) multiplicity and cultures were maintained in liquid growth medium, no morphological transformation was observed at 41 C (Fig. 1). Similar cultures incubated at 36 C became transformed to about the same extent as wild-type RSV-BH, at high and low multiplicities of infection.

The development of foci of transformed cells under agar was also examined. Cells were infected with dilutions of RSV-BH or RSV-BH-Ta, then were overlaid with focus agar medium and incubated at 41 or 36 C (after 2 initial days at 41 C). Seven days later, the number of recognizable foci appearing at 36 C exceeded by 100-fold the number at 41 C (Table 2).

The cells comprising the foci at 36 C were deeply stained with neutral red, a characteristic of RSV-BH-transformed cells. Foci induced by

TABLE 1. *Reproduction of RSV-BH-Ta at various temperatures*[a]

Cells infected with	FFU × 10^{-2} per ml			
	36 C	37 C	39 C	41 C
RSV-BH	140[b]	150	180	260
RSV-BH-Ta	240	340	360	420
RSV-BH-Ta + ara-C	2.3	2.1	0.7	1.6

[a] Chick embryo cells were exposed to about 3 focus-forming units (FFU) per RSV-BH or RSV-BH-Ta cell. Growth medium or medium containing cytosine arabinoside (ara-C, 10^{-4} M) was added, and 24 hr later culture fluids were removed for subsequent assay.

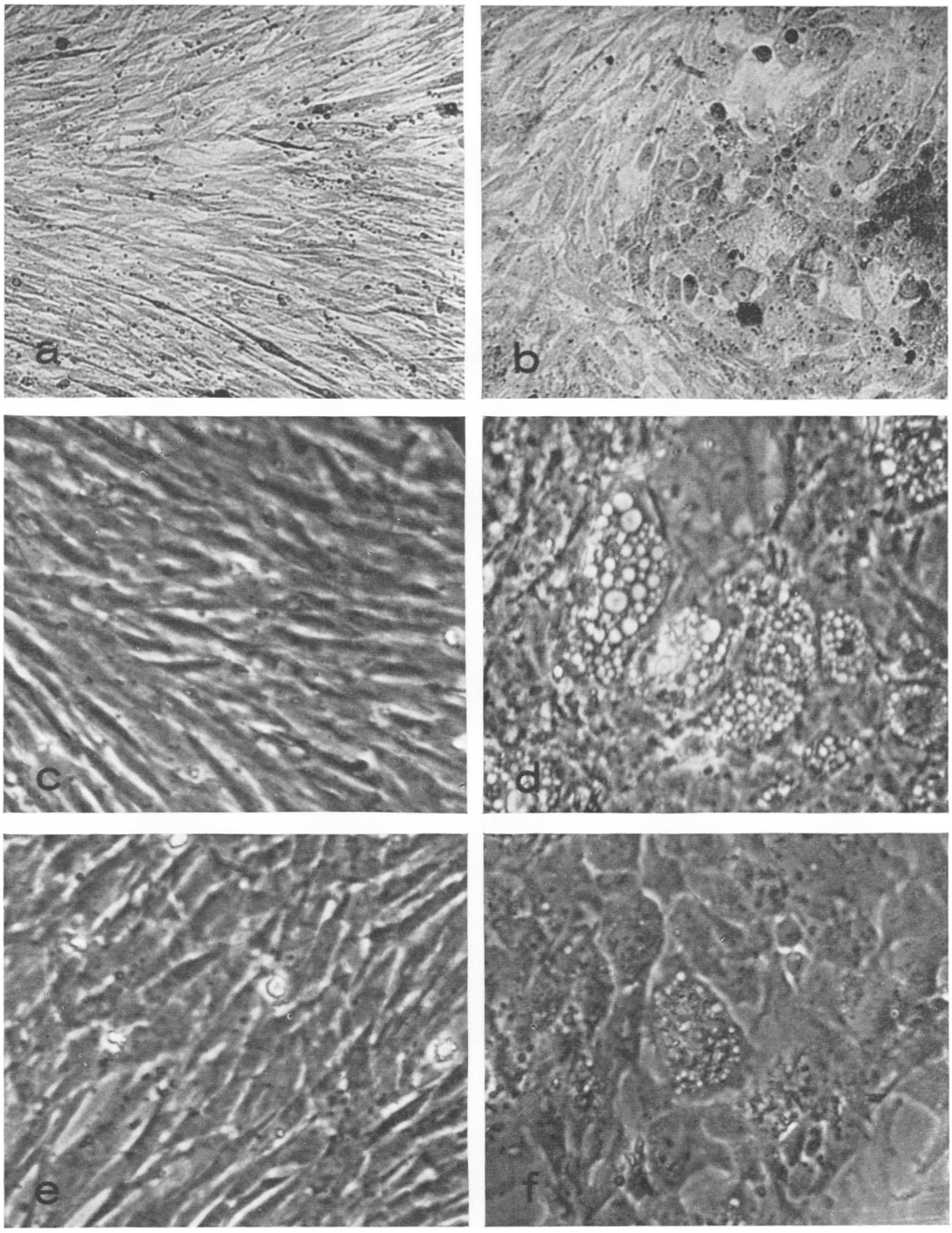

FIG. 1. *Effects of temperature on morphology of cells infected with RSV-BH-Ta. Chick embryo cells were infected with RSV-BH or RSV-BH-Ta and transferred several times. Light microscopy (×160) of RSV-BH-Ta infected cells at 41 C (a) and at 36 C (b). Phase-contrast micrography (×256) of noninfected chick embryo cells at 41 (c), RSV-BH-infected cells at 41 C (d), RSV-BH-Ta-infected cells at 41 C (e), and at 36 C (f).*

RSV-BH-Ta at 41 C usually could be identified only as bare areas or "plaques" in the cellular monolayer, a phenomenon often found when foci of RSV-BH-transformed cells develop under nutrient agar. Such plaque formation is probably due to the loss of adherence of transformed cells to the substratum, since transformed cells are found piled up at the periphery of the plaque.

TABLE 2. *Focus formation and suspended colony formation of infected cells at high and low temperatures*

Cells infected with	Dilution of virus	No. of foci[a]		No. of colonies[b]	
		36 C	41 C	36 C	41 C
RSV-BH	10^{-2}	Confluent	Confluent	171	390
	10^{-3}	504	726	6	19
	10^{-4}	42	68	0	0
RSV-BH-Ta	10^{-1}	Confluent	(66)[c]	Confluent	700
	10^{-2}	Confluent	(16)	370	57
	10^{-3}	540	(3)	24	7
	10^{-4}	48	0	0	0

[a] Sparse monolayers were exposed to various dilutions of RSV-BH or RSV-BH-Ta, then overlaid with focus agar medium. Six days later, neutral red (0.01%) was added, and foci were observed with a microscope the next day.

[b] Colonies of cells growing suspended in nutrient agar. Cells were dispersed with trypsin, exposed to virus, then mixed with soft nutrient agar and layered into 50-mm petri dishes. Ten (41 C) and 12 (36 C) days later colonies were counted with the aid of a microscope.

[c] Numbers in parentheses represent atypical foci composed of lightly stained cells, in contrast to the darkly stained cells of RSV-BH foci.

However, deeply stained cells were rarely found in the foci of RSV-BH-Ta-infected cells developing at 41 C.

In the same experiment, infected cells were suspended in soft nutrient agar, and the ability of these cells to form colonies (2) was examined. A temperature restriction on the growth of RSV-BH-Ta-infected cells in suspension was found, compared with cells infected with the wild type (Table 2). However, some colonies were formed at 41 C even after infection at low multiplicity; the number at 41 C was about one-fifth the number at 36 C. The generally lower efficiency of colony formation at 36 C, as shown by the number of colonies developing after infection of cells with RSV-BH, indicates that the number of colonies developing from RSV-BH-Ta-infected cells was about 10-fold greater at 36 than 41 C. The growth at 41 C of RSV-BH-Ta-infected cells into colonies suspended in agar, or into plaques in a monolayer, may be due to either a partial reversion for the transformation phenotype in some virions, or to a general "leakiness" of RSV-BH-Ta.

Cells infected with RSV-BH-Ta at high multiplicity could be grown and subcultured at 41 C for several weeks while maintaining essentially a normal appearance. At any time, these cultures could be shifted to 36 C, and morphological changes soon became apparent. All of the following studies were done with cultures infected at high multiplicity and in which practically all cells were carrying transforming virus.

Time course of transformation and reversal. Cells infected with RSV-BH-Ta were grown at 41 C, then shifted to 36 C and examined with a microscope at periodic intervals for morphological changes. By using standard light microscopy we detected some rounding of the cells by 1 hr after changing temperature. Within 3 to 4 hr, cell shapes and intercellular associations were clearly different from cells kept at 41 C, and by 12 hr the cultures were indistinguishable from cultures infected with wild-type RSV-BH.

Cytoplasmic vacuolization is a characteristic feature of cells transformed by RSV-BH or RSV-BH-Ta, and is rarely found in uninfected chick embryo cells or RSV-BH-Ta-infected cells kept at 41 C. This vacuolization is more readily detectable by using phase-contrast microscopy than standard light microscopy. Examination of RSV-BH-Ta-infected cultures by phase-contrast microscopy revealed newly formed vacuoles in 1 to 5% of the cells within 10 min after shifting from 41 to 36 C (Table 3, Fig. 2). By 20 to 30 min, the vacuolization was extensive; vacuoles were found throughout the cytoplasm of many cells, and as many as half the cells contained vacuoles. Vacuolization often was found to precede a change in cell shape.

The transformation was reversible. Transformed cells maintained at 36 C lost their vacuoles and became elongated after being shifted to 41 C. This reversal of vacuolization was less readily detectable than the appearance of vacuoles after a shift from 41 to 36 C (Table 3). Both the general cell morphology of the transformed cells, as seen in the light microscope, and the degree of vacuolization, as assessed by phase-contrast microscopy, took considerably longer to revert to normal with increased temperature than to be transformed after a temperature shift down. Also,

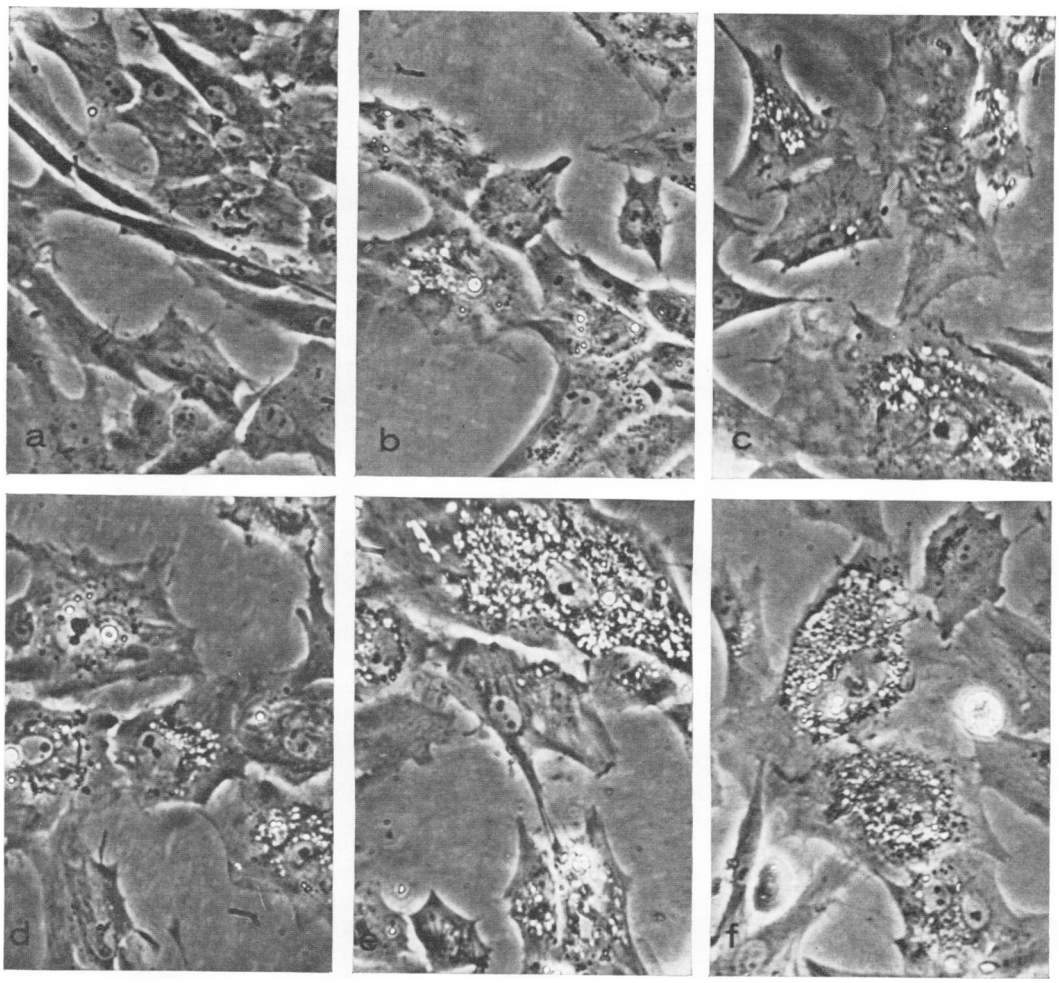

FIG. 2. *Effects of temperature shift on morphology of RSV-BH-Ta infected cells. Cells grown at 41 C (a) were shifted to 36 C and observed by phase-contrast micrography after 10 min (b), 15 min (c), 20 min (d), 25 min (e), and 30 min (f). Note the increase in perinuclear vacuolization with time at 36 C.*

when cells were grown for 2 or more days at 36 C, the reversal to the nontransformed state at 41 C took several hours longer than if the cells had originally been maintained for less than 12 hr at 36 C.

Effects of antimetabolites. The rapidity of the morphological changes occurring in shifted cultures indicated that the degree of complexity of metabolic reactions was small. The specific metabolic requirements for transformation to occur in "shifted-down" cultures was examined by using cytosine arabinoside to inhibit DNA synthesis, actinomycin D to inhibit RNA synthesis, and cycloheximide or puromycin to inhibit protein synthesis. None of these compounds prevented the appearance of vacuoles or gross transformation in RSV-BH-Ta-infected cultures when added simultaneously with a shift of the cultures from 41 to 36 C (Table 4; Fig. 3). No differences were observed either in the rate of morphological changes or in the number of cells exhibiting these changes during the first 6 hr after treatment and temperature change. Also, preincubation of cells with antimetabolites at the higher temperature for up to 6 hr before shifting to the lower temperature had little effect on the morphological change occurring during the 2 hr after temperature shift.

These results demonstrated that transcriptional and translational requirements for morphological transformation had been fulfilled at 41 C. A slight decrease in the extent of vacuolization was noticed in cultures treated with cycloheximide or puromycin for longer than 8 hr (Fig. 3), although

TABLE 3. *Time course of appearance of and reversal of transformation*[a]

Time after shift	Transformation	
	41 → 36 C	36 → 41 C
0	0	++++
10 min	+	++++
20 min	++	++++
30 min	++	++++
1 hr	++	+++
3 hr	+++	+
6 hr	+++	+
12 hr	++++	0

[a] Cultures were placed at 41 or 36 C 1 day after a cell transfer. The following day, cultures were shifted to the opposite temperature. At periodic intervals, cultures were removed from the incubators along with cultures which had not been shifted, and each was observed by both light and phase-contrast microscopy. The degree of transformation was related to that of RSV-BH infected cultures. Symbols: 0, no detectable vacuolization; +, detectable vacuolization in less than 10% of the cells; ++, vacuoles easily recognizable in 5–20% of the cells; +++, vacuolization of 20 to 50% of the cells; ++++, vacuolization of greater than 50% of the cells.

the cells never completely reverted to normal. This phenomenon was observed in cells infected with either mutant or wild-type virus, suggesting that new proteins eventually were needed for the maintenance of the transformed state.

The antimetabolites, likewise, had little effect on reversal, i.e., the loss of transformation upon shifting cultures from 36 to 41 C (Table 4). In fact, cultures could be shifted up and down at the two temperatures several times at 2-hr intervals, resulting in the appearance or loss of transformation, and the antimetabolites had no effect on these morphological changes.

Uptake of hexose. The rate of uptake of hexose is increased in cells after transformation by RNA tumor viruses (8, 17). The uptake of deoxyglucose-^{3}H into chick embryo cells and into cells infected with RSV-BH or RSV-BH-Ta was examined after these cells had been cultured at 36 or 41 C. The rate of deoxyglucose uptake was greater in cells transformed by RSV-BH than noninfected cells (Table 5), and the growth temperature had little influence on the uptake. Cells infected with RSV-BH-Ta responded as transformed cells after growth at 36 C, and as normal cells after growth at 41 C (Table 5), consistent with the morphological observations reported above.

It was possible that the uptake of hexose was itself temperature sensitive. Temperature-sensitive cells grown at the two temperatures were incubated with deoxyglucose-^{3}H at 36 and 41 C (Table 6). Uptake was dependent only on the temperature of growth of cultures and not on the temperature during the short incubation with deoxyglucose. This result indicated that the alteration in deoxyglucose uptake accompanying transformation was not due to a temperature sensitivity of molecules interacting directly with deoxyglucose to affect its uptake.

Reduction of the temperature of RSV-BH-Ta-infected cells grown at 41 C resulted in an expected increase in deoxyglucose uptake after a short delay (Fig. 4). However, both actinomycin D and the protein inhibitors, puromycin and cycloheximide, prevented this increase in deoxyglucose uptake, indicating that both new RNA and new protein were required for the increased uptake to occur. In the same cultures, morphological changes were unaffected by these antimetabolites. Cytosine arabinoside had no effect on the increase in hexose uptake found after the cells were shifted to 36 C.

Cultures grown at 36 C showed relatively high levels of hexose uptake which remained high during the 6 hours following a shift to 41 C. Actinomycin D had little influence on the rates of hexose uptake over this interval. However, cycloheximide, whether added to shifted cultures or those held at 36 C, reduced hexose uptake levels to those commensurate with cultures grown at 41 C. These results demonstrate that the reversibility of the rate of hexose uptake is a more gradual process than the reversal of transformation, and further suggest that the proteins involved

TABLE 4. *Effects of antimetabolites on changes in cellular morphology*[a]

Treatment	Transformation			
	41 C	41→ 36 C	36 C	36→ 41 C
None	0	++++	++++	+
Cytosine arabinoside	0	++++	++++	+
Actinomycin D	0	++++	++++	+
Cycloheximide	0	+++	+++	0
Puromycin	0	+++	+++	0

[a] Cultures infected with RSV-BH-Ta were grown at 41 or 36 C. Antimetabolites were added; some cultures were shifted to the higher or lower temperature; and the degree of transformation (0 to ++++; see footnote *a* in Table 3) was recorded 12 hr later. No delay in the appearance or reversal of transformation was detected in drug-treated cultures observed at earlier times.

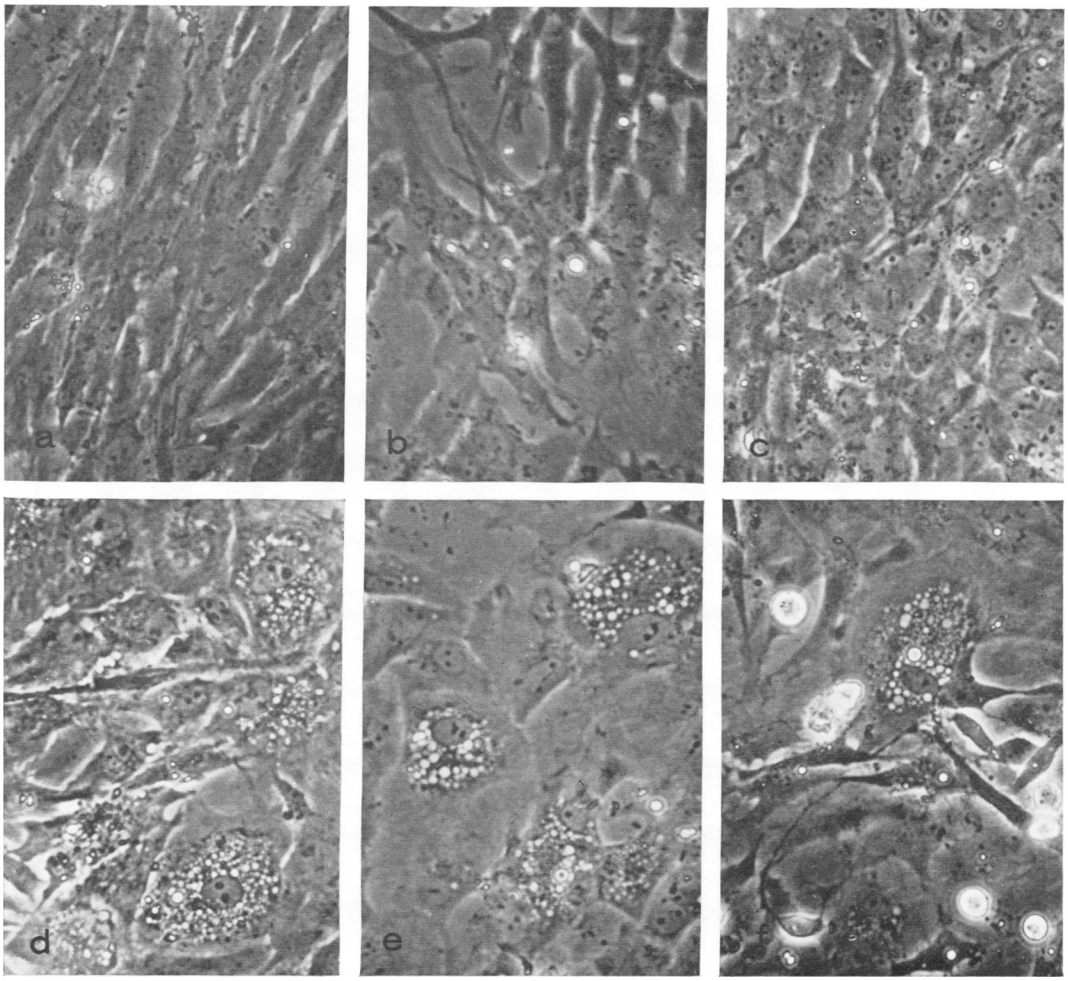

FIG. 3. *Effects of cycloheximide and puromycin on transformation of RSV-BH-Ta-infected cells. Cycloheximide (10 μg/ml) or puromycin (50 μg/ml) was added to cultures before shifting from 41 to 36 C. At intervals, these cultures were compared with other cultures held at 41 C. Phase-contrast micrographs were taken 12 hr after the temperature change: (a) 41 C, (b) 41 C plus cycloheximide, (c) 41 C plus puromycin; (d) shifted to 36 C, (e) 36 C plus cycloheximide, (f) 36 C plus puromycin. Vacuolization is seen only in those cultures shifted to 36 C. The isolated refractile bodies seen in all cultures were extracellular.*

in maintaining high levels of hexose uptake in transformed cells are relatively unstable.

Increased hyaluronic acid synthesis. Another characteristic of cells transformed by avian sarcoma viruses is the increased synthesis of hyaluronic acid (7, 10, 18). Cells transformed by RSV-BH not only produce more hyaluronate than normal cells but more hyaluronate is found associated with cells (Table 7). As anticipated, RSV-BH-Ta cells contained more hyaluronate at 36 than 41 C.

The effect of temperature shift on synthesis of hyaluronate was examined. When the temperature-sensitive cells were treated with actinomycin D or cycloheximide at the time of the temperature change, the induction of hyaluronate synthesis found in untreated cultures was inhibited (Table 8). Increases of hyaluronate, both intracellularly and in cell culture fluids, was prevented by actinomycin D and cycloheximide, but not by cytosine arabinoside, demonstrating that RNA and protein synthesis but not DNA synthesis, were required. The morphological changes observed at 36 C, therefore, were not dependent on increased hyaluronate synthesis.

TABLE 5. *Effects of growth temperature on deoxyglucose uptake*[a]

Cells infected with	Deoxyglucose uptake[b]	
	36 C	41 C
Noninfected	62	60
RSV-BH	183	176
RSV-BH-Ta	156	68

[a] Cells were placed at 36 or 41 C the day after transfer. The following day, the growth medium was removed, and deoxyglucose-^{3}H (1.0 μCi/ml) in hexose-free medium was added. After incubation for 15 min at 39 C, intracellular radioactivity was measured.

[b] ^{3}H counts per minute per microgram of protein.

TABLE 6. *Effect of growth temperature versus temperature during incubation with deoxyglucose-^{3}H*[a]

Temp of cell growth (C)	Deoxyglucose-^{3}H uptake[b]	
	36 C	41 C
36	233	239
41	123	135

[a] Cells infected with RSV-BH-Ta were incubated overnight at 36 or 41 C. Deoxyglucose-^{3}H uptake during a 15-min incubation period at 36 or 41 C was measured.

[b] ^{3}H counts per minute per microgram of protein.

DISCUSSION

The mutant of Rous sarcoma virus described in this paper provides hope for a biochemical definition of malignancy and for this purpose may have advantages over those mutants previously described (6, 11, 13, 19, 21). The morphological transformation induced by the Bryan high-titer strain of RSV is characteristic of the virus, is rarely seen in uninfected cultures, and is unambiguous with respect to other physiological changes which may affect the morphology of the cell. The mutant, RSV-BH-Ta, is capable of full cycles of reproduction at temperatures in which transformation of infected cells is inapparent. This observation demonstrates that, as in the case of some other avian sarcoma viruses (6, 11, 13, 19), the functional product of the gene responsible for transformation is not required for virus reproduction; i.e., the gene responsible for transformation is gratuitous.

The production of fully infectious virus at 41 C shows that the synthesis and packaging of viral

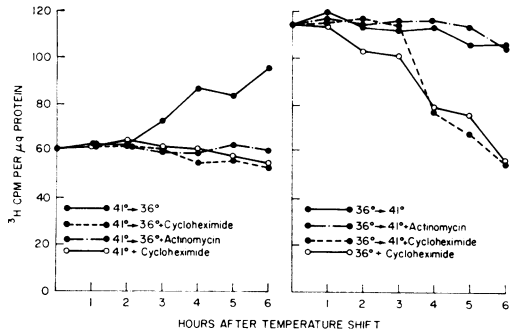

FIG. 4. *Effects of temperature shifts on hexose uptake. Cultures grown at 41 or 36 C for 1 day were shifted to the opposite temperature at hourly intervals before the removal of the cell culture medium and the addition of deoxyglucose-^{3}H (2.0 μCi/plate). All cultures were incubated at 39 C for 15 min before determination of intracellular radioactivity. Left, Cultures grown at 41 C. Note the increase in rate of hexose uptake in cultures shifted to 36 C beginning at 3 hr. Cycloheximide (10 μg/ml) and actinomycin D (2 μg/ml) prevented this increase. Right, Cultures grown at 36 C. No rapid effect of an increase in temperature was seen; however, ability to take up hexose decreased in cultures containing cycloheximide.*

TABLE 7. *Effect of growth temperature on cell-associated hyaluronic acid*[a]

Cells infected with	Hyaluronic acid content[b]	
	36 C	41 C
Noninfected	0.16	0.14
RSV-BH	0.86	0.78
RSV-BH-Ta	0.90	0.22

[a] Cells were placed at 36 or 41 C overnight. Culture fluids were removed, and cells rinsed and suspended with trypsin. Cells were counted, and cytoplasmic fractions were analyzed for hyaluronic acid content by quantitative electrophoresis in polyacrylamide-agarose mixed gels.

[b] Picograms of hyaluronate per cell.

RNA into virions continues at this high temperature. The temperature-sensitive molecule, therefore, is a gene product rather than something interfering with transcription of the viral genome. This was demonstrated in another way through the use of metabolic inhibitors. Inhibition of DNA synthesis, RNA synthesis, or protein synthesis had no effect on the change in cellular morphology after a shift of RSV-BH-Ta-infected cells from 41 to 36 C. On the other hand, both actinomycin D and cycloheximide interfere rapidly with production of oncornaviruses (1, 3); virus production virtually ceases within a few hours of the addition of either antimetabolite. Nonethe-

TABLE 8. *Effect of antimetabolites on induction of hyaluronate synthesis in RSV-BH-Ta-infected cells*[a]

Treatment	Incubation temp (C)	Hyaluronate content[b] in		Total hyaluronate content
		Cells	Fluids	
None	41	0.55	1.9	2.5
Cycloheximide	41	0.50	1.6	2.1
None	36	3.2	3.9	7.1
Cycloheximide	36	0.40	1.2	1.6
Actinomycin D	36	0.45	1.7	2.2
Cytosine arabinoside	36	3.0	4.3	7.3

[a] Cells infected with RSV-BH-Ta were grown at 41 C. Fluids were replaced with fresh growth medium, antimetabolites were added, and cultures were incubated for 6 hr at 36 or 41 C. Culture fluids were analyzed directly by electrophoresis in acrylamide-agarose gels. Cytoplasmic fractions were processed for hyaluronate determinations as described in Materials and Methods.

[b] Micrograms per culture.

less, pretreatment of cells with antimetabolite for as long as 6 hr before shifting from 41 to 36 C did not prevent transformation by RSV-BH-Ta, demonstrating that virus production is not correlated with transformation even at the transforming temperature.

Thus, it seems likely that the temperature-sensitive molecule is a protein, perhaps an enzyme involved in synthesis of other cellular components or participating directly in the structural organization of the cell, or both. The partial loss of the transformed morphology upon continued exposure to cycloheximide or puromycin further suggests that proteins are involved in the maintenance of the transformed state.

It is possible that more than one virus gene is responsible for the changes observed during transformation with RSV-BH, and that the products of these genes are both temperature sensitive in cells infected with RSV-BH-Ta. Complementation of at least two mutant classes of the Schmidt-Ruppin strain of RSV has been observed (H. Hanafusa, *personal communication*). Possible complementation among mutants of RSV-BH (5) remains to be analyzed.

Inhibitors of nucleic acid or protein synthesis failed to prevent reversion to the nontransformed state upon a shift from 36 to 41 C. This indicates that the "transformation molecule" does not produce its effect by interfering with the synthesis of a specific nucleic acid or protein. Otherwise, in the absence of this nucleic acid or protein, reversal to nontransformed morphology could not occur.

Other investigators have found that inhibition of protein synthesis is sufficient to prevent morphological transformation in their temperature-dependent avian sarcoma systems (6, 11). Clearly, the transformation they observe requires the induction of other proteins. Since actinomycin D did not prevent transformation in these systems, an interesting, but complex, mechanism involving translational control is suggested.

A multitude of biochemical changes has been reported to occur in malignant cells, and each laboratory has its favorite potential cancer molecule. We have examined the increase in hyaluronic acid synthesis and the increase in hexose uptake which accompany the transformation of cells by RSV-BH. These capacities are both induced upon a shift of RSV-BH-Ta-infected cells from 41 to 36 C. The induction was shown to be at the level of transcription for both hyaluronic acid synthesis and hexose uptake, as the inhibition of induction by both cycloheximide and actinomycin demonstrate. Therefore, the change in morphology cannot be attributed to increased hyaluronate synthesis or molecules responsible for increased hexose uptake. The outcome of these experiments suggests that we should look elsewhere for the primary temperature-sensitive molecule.

In another study, levels of cyclic adenosine monophosphate (AMP) were shown to be correlated with transformation of chick embryo cells by RSV-BH and RSV-BH-Ta (16). Also, transformation of RSV-BH-Ta-infected cells shifted from 41 to 36 C could be partially prevented by the addition of exogenous dibutyryl cyclic AMP and theophylline, an inhibitor of phosphodiesterase. Experiments to define more clearly the role of cyclic AMP in transformation by RSV-BH are in progress.

The induction of increased hexose uptake and hyaluronate synthesis as described for this temperature-dependent system provides an excellent system for the analysis of such induction in eukaryotic cells. It is possible that analyses of temperature-dependent regulation of hexose uptake or hyaluronate synthesis will lead to a molecular definition of some regulatory processes in vertebrate cells.

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