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Cell Biology

Surface ruffles as markers for studies of cell transformation by Rous sarcoma virus

(scanning electron microscopy/chick embryo fibroblasts/protein synthesis)

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ABSTRACT **Confluent chick embryo fibroblasts infected with the Ts68 mutant of Rous sarcoma virus were examined by scanning electron microscopy at the permissive (36°) and nonpermissive (41°) temperatures for transformation. Infected cells shifted from 41° to 36° undergo a change in shape from elongated to rounded. This process is preceded by the appearance of surface ruffles on the cell. These surface ruffles are not observed on cells maintained at 41°, appear as early as 0.5 hr after a shift to 36°, and are common on cells maintained at 36°. By 3.5 hr after the shift from 41° to 36°, cultures appear fully transformed by the criteria of cell roundedness and the presence of surface ruffles. This surface alteration of cells is the earliest event of those so far reported during the transformation process and is not dependent upon protein synthesis and extracellular plasminogen during the period of temperature shift.**

Transformation of cultured cells by RNA tumor viruses is accompanied by changes in morphology and other biochemical functions. When chick embryo fibroblasts are infected with Kawai and Hanafusa's Ts68 mutant of the Schmidt-Ruppin strain (subgroup A) of Rous sarcoma virus (RSV), the expression of the transformation phenotype of infected cells is dependent upon temperature, i.e., permissive at 36° and nonpermissive at 41°, whereas other viral related functions are reported to be the same (1). Alterations in several transformation-related biochemical functions in Ts68-infected cells have been reported during the time course of a temperature shift from 41° to 36°. In brief, there was an increase in glucose uptake from 4 to 6 hr; loss of membrane-associated protein of molecular weight 45,000 from 3 to 6 hr (2); secretion of proteases from 8 to 20 hr (3); and reduction in a surface protein of 250,000 daltons (LETS or Z protein) after 20 hr (4).

However, the most remarkable observation has been the rapid change in cell morphology from a flattened and elongated to a more rounded, refractile shape during temperature shift. This significant change can be detected by use of the light microscope as early as 1.5–6 hr (1, 4). These observations strongly suggest that there are important early cell structural alterations that occur prior to the changes in other biochemical functions. If the transformation is composed of a series of cascade events, the current available information would imply that perhaps the metabolic changes, for example, glucose uptake, are the result of earlier alterations in cell structures. However, the changes in morphology are difficult to define by light microscopy. Since such changes take place very early after temperature shift, it is likely that they may be more closely linked to the primary expression of transformation than are other changes. Therefore, it is important to characterize further these morphological changes by a more sensitive technique, such as scanning electron microscopy. It is also important to establish whether these alterations are dependent upon cellular processes such as protein synthesis. The characterization of such an early event may provide grounds for studies of causal relationships among the various transformation-related morphological and biochemical changes.

Abbreviation: RSV, Rous sarcoma virus.

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MATERIALS AND METHODS

Cell culture and virus infection were performed as described (3). Primary chick cells and virus were generous gifts of Dr. P. W. Robbins. *Confluent* cultures of secondary chick embryo fibroblasts were prepared and fixed on 18-mm glass coverslips for scanning electron microscopy by a slight variation of the procedure of Porter *et al.* (5). Cultures were not washed prior to fixation to avoid dislodging poorly attached cells. The medium was sucked off and a glutaraldehyde solution, 2.5% in 0.05 M cacodylate and half-strength phosphate-buffered saline at pH 7.2, was added gently to the culture dish containing the coverslip for 20 min. Cultures were then washed in 0.1 M cacodylate, postfixed for 20 min in 2% osmium tetroxide buffered with 0.1 M cacodylate at pH 7.2, and finally washed with distilled water. The cells were then subjected to sequential dehydration by transfer through a graded series of water/acetone and then acetone/amyl acetate mixtures, and finally dried at the critical point of CO_2 in a critical point dryer. Specimens were stored in a desiccator under vacuum until coated with a 200–300 Å film of gold. The preparations were viewed in a JSM-U3 scanning electron microscope at 25 kV. Transmission electron micrographs were made by the method of Lenk and Penman (6).

RESULTS

Ts68-infected chick embryo fibroblasts maintained at 41° (Fig. 1A) have a flattened, elongated shape and a smooth surface, and are essentially identical in appearance in uninfected cells. As early as 30 min to 1 hr after a shift in temperature from 41° to 36°, some of the cells assume a rounded or partially rounded shape with regions of the membrane drawn together into ribbons or flower-like shapes, i.e., surface ruffles (Fig. 1B). In order to determine whether cells undergo a morphological transition as a function of time, we fixed cultures at various times after the temperature shift for examination in the scanning electron microscope. Many low magnification photographs showing a total of 600 cells were taken at each time point. Cells that were flattened and with-

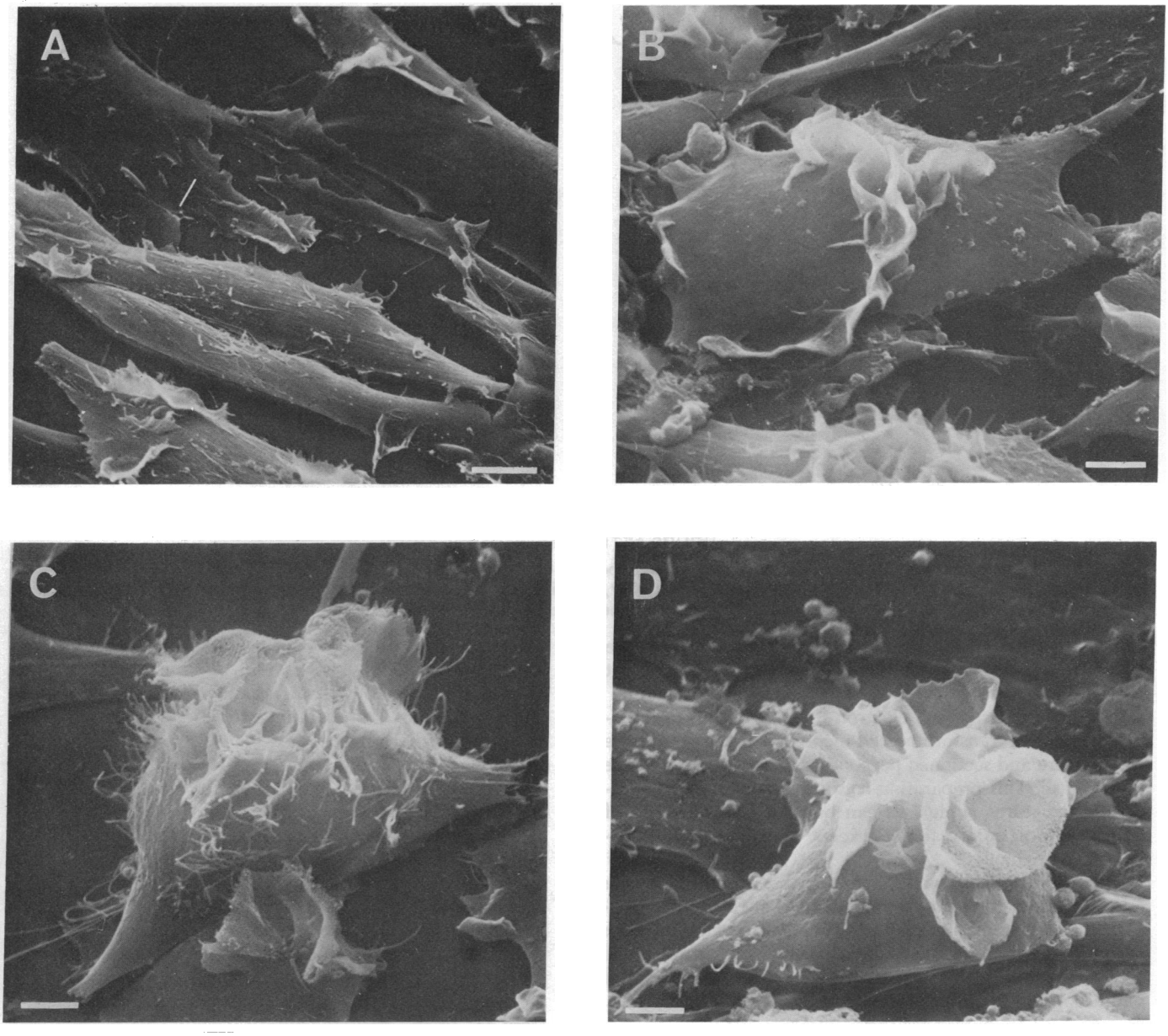

FIG. 1. Scanning electron micrographs of Ts68-infected chick embryo fibroblasts. Cells were maintained at 41° for 48 hr and fixed either before or at various times after a shift to the permissive temperature. (A) Surface of cells maintained at 41°; bar = 5 μm. (B) Cells fixed 1 hr after shift from 41° to 36°; bar = 3 μm. (C and D) Cells fixed 2 hr after shift from 41° to 36°; bar = 3 μm.

out surface ruffles (Fig. 1A) were scored as untransformed. Cells that were still partially flattened, but had ruffles or flowers (Fig. 1B), and rounded cells with ruffles (Fig. 1C and D) were scored as transformed cells. Fig. 2 plots the percentage of all cells that are transformed, i.e., with surface ruffles, as a function of time after temperature shift. By this criterion, transformation seems to reach a plateau at about 2 hr after the shift. Fig. 3 plots for the ruffled cells the relative proportions of rounded and partially flattened as a function of time after temperature shift. The most common variety of transformed cell from 0.5 to 2.0 hr after the temperature shift is the partially flattened type. After 2 hr the rounded, transformed variety begins to predominate. By 3.5 hr these cultures have the same ratio of flat to rounded cells as those maintained at 36°.

Fig. 4 is a highly magnified view of a surface ruffle on a cell 2 hr after shift to 36°. A striking feature of these structures is the complexity of folding. Another point of interest is the cross bridge between folds visible in the lower left corner of this figure.

The application of cycloheximide, an inhibitor of protein synthesis, at the beginning of the temperature shift had no effect on the rounding of cells or the appearance of surface ruffles. Fig. 4 is actually a picture of a cell incubated with 5 μg/ml of cycloheximide from the time of the shift from 41° to 36° until fixation 2 hr later. In a control experiment we have shown that under these conditions the level of incorporation of radioactive leucine into protein in the presence of cycloheximide is only 1% of that occurring in its absence. Therefore, it is likely that all proteins necessary for the expression of surface ruffles and cell rounding, including the temperature-sensitive protein, are already present at 41° and that the temperature-sensitive process does not depend on protein synthesis. We did find, however, that cycloheximide itself does have an effect on the surface morphology of these cells. Cells treated with 5 μg/ml of cycloheximide for

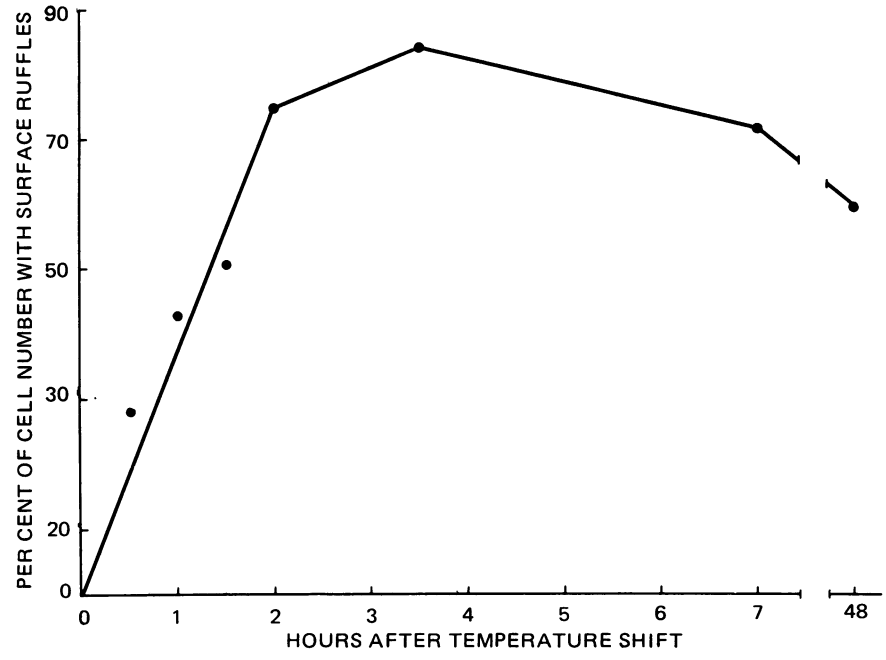

FIG. 2. The time course of appearance of the ruffled phenotype after shift of Ts68-infected chick embryo fibroblasts from 41° to 36°. Infected cells were maintained at 41° for 48 hr, then shifted to 36°, and fixed at various times afterward. A count of a total of 600 cells was made from the scanning electron micrographs. Ordinate gives percent of cells that show surface ruffles.

2 hr showed the appearance of spikes protruding from the surface in the region where there are no ruffles (lower right corner of Fig. 4).

Fig. 5 is a transmission electron micrograph (6) of a surface ruffle on a transformed cell incubated at the permissive temperature and sectioned perpendicular to the plane of the culture dish. This preparation confirms the existence of ruffle-like structures, which, as can be seen are composed of normal cytoplasmic materials except that they do not contain large organelles.

The appearance of surface ruffles and rounding of cells was also found to occur when cells were incubated in plasminogen-free serum. This observation confirms the previous report (3) that the morphological changes in transformed cells as judged by the light microscope are independent of plasminogen.

DISCUSSION

Transformation of fibroblasts in culture by virus or chemical carcinogen is one of the most extensively investigated model systems used in an effort to understand the process of malignancy *in vivo*. Despite many years of research, the mechanism of transformation of chick embryo fibroblasts by RSV, in particular the interaction of a viral gene product with the host, remains almost completely obscure.

In this report, we have confirmed and extended the observation that a major difference in morphology between normal and RSV-transformed chick embryo fibroblasts is the presence of surface ruffles on the transformed cells and the absence of such structures on normal cells in dense culture. Our micrographs of Ts68-transformed cells appear similar to those taken by Boyde and Weiss (7) and Stone, Smith, and Joklik (8) of cells transformed by the avian sarcoma virus B77 and the Prague strains of RSV, respectively, and to those of RSV-3T3 cells by Albrecht-Bühler (personal communication), but differ significantly from those observed by Hale, Winkelhake, and Weber (9) of cells infected with wild-type RSV (Schmidt-Ruppin strain). Probably the differences in appearance of the structures seen in our electron micrographs and those of Hale *et al.* result from the long fixation period used by them, which we have found leads to partially degraded preparations. The existence of surface ruffles has also been confirmed by transmission electron microscopy. The material appearing in the ruffles is indistinguishable from normal cytoplasmic components except that they do not contain large organelles.

We have described that the earliest event after a shift of Ts68-infected chick embryo fibroblasts from 41° to 36°, as revealed by scanning electron microscopy, is the appearance of surface ruffles on the cell. It is striking that this event starts as early as 30 min, and that by the end of 2 hr more than 70% of the cells bear ruffles. That this event is not an artifact of temperature change is supported by the fact that mock-infected cells do not give rise to ruffles when shifted from 41° to 36°. Also, the appearance of Ts68-infected cells kept at 36° and wild-type RSV-transformed cells kept at

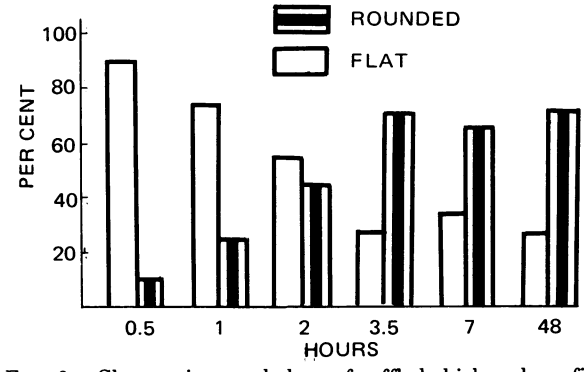

FIG. 3. Changes in morphology of ruffled chick embryo fibroblasts after a shift from 41° to 36°. The experiment is the same as in Fig. 2. At each time point transformed cells (those showing surface ruffles) were counted. *Solid bars* show the percentage of transformed cells that were rounded. *Open bars* show the percentage of transformed cells that were flattened.

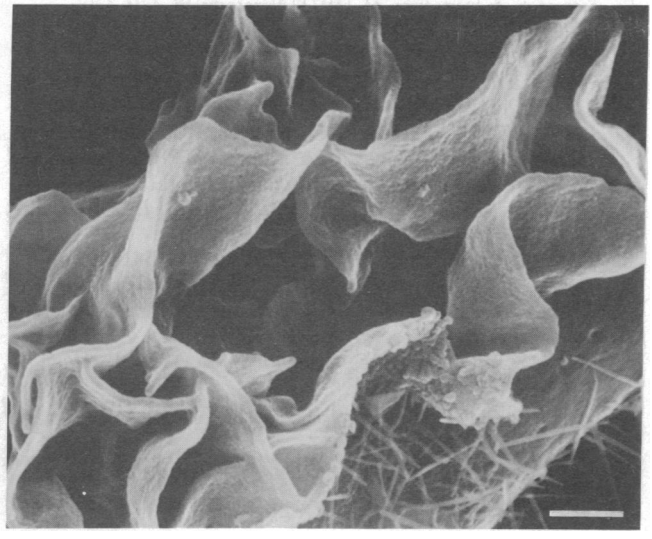

FIG. 4. A high magnification of a surface ruffle formed in the presence of an inhibitor of protein synthesis. Ts68-infected chick embryo fibroblasts were maintained at 41° for 48 hr. The temperature was then shifted to 36° with the addition of cycloheximide at a concentration of 5 μg/ml. The cells were fixed after 2 hr of incubation for scanning electron microscopy; bar = 1 μm.

both 36° and 41° is indistinguishable from that of Ts68-infected cells shifted from 41° to 36°.

We have not as yet unambiguously determined whether the appearance of the surface ruffles on transformed cells is cell-cycle-dependent. However, in an exponentially growing culture of transformed cells, where less than 40% of the cells are in S phase (as measured by labeled nuclei, data not shown), more than 80% of the cells bear ruffles. Thus, we conclude that the development of surface ruffles is not restricted to the S phase.

A further property of the Ts68-infected cells after a shift to 36° is that after ruffling takes place there is change of shape from flat to round. This observation implies that flat cells with surface ruffles give rise to rounded cells with surface ruffles and that surface ruffling rather than cell rounding is the first indication that cells are undergoing transformation.

Whether the conversion from the flat to rounded variety of transformed cell is cell-cycle-dependent has not yet been unambiguously determined. However, if the conversion from flat to rounded cells were cell-cycle-dependent, we would not expect to see a change in the ratio of the flat to rounded varieties from 8:1 to 3:7 within the first 3 hr after the temperature shift. This argument is further supported by observations with the light microscope. Therefore, it appears that the morphological changes associated with transformation in a temperature shift can be dissected into two events, first, the appearance of surface ruffles and then the rounding up of cells. The latter event may well be a direct consequence of the former.

We were rather surprised to find that this surface alteration is not dependent upon protein synthesis. Whereas increase in glucose transport after a temperature shift is dependent on protein synthesis, the appearance of surface ruffles is not. It appears, therefore, that a temperature-sensitive protein, preexisting in Ts68-infected cells held at 41°, is the initiator of the surface changes described here upon a shift to 36°.

At the present moment ruffles have been observed only on cells transformed by some strains of Rous sarcoma virus.

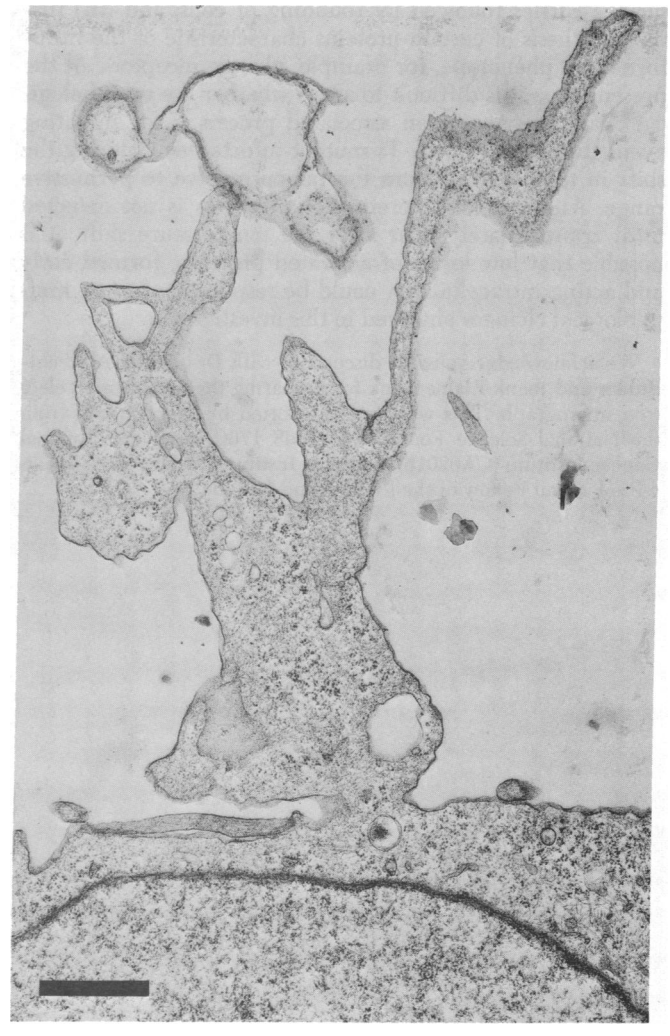

FIG. 5. Transmission electron micrograph of a chick embryo fibroblast containing a surface ruffle. The micrograph was made of a culture of chick embryo fibroblast infected with Ts68 RSV and maintained at 36°; bar = 1 μm.

Whether this phenomenon may be attributed to a "transforming protein" coded by such viruses or to other factors requires further investigation.

In view of the briefness of time and the lack of requirement of protein synthesis, the appearance of ruffles must represent a rearrangement of preexisting membrane structures. Recently Pollack *et al.* (10) have shown that the cable-like structures, which are normally present in untransformed cells and which may be revealed by an immunofluorescent technique involving anti-actin and anti-myosin, are absent or reduced in simian virus 40-transformed murine fibroblasts. Even though similar work on RSV-transformed chick cells has not been reported, we would predict that such structures would also be absent in transformed chick cells. With these reservations our data are compatible with the belief (10) that there is a redistribution and reorganization of actin and myosin-containing filaments in the transformed cell.

In summary, our view of the transformation process after infection with the Ts mutant would seem to involve the following sequential steps: (*i*) synthesis of an essential viral coded protein characteristic of transformed cells, i.e., "transformation protein"; (*ii*) reaction of this preformed temperature-sensitive protein at the permissive temperature to yield

surface ruffles followed by rounding of cells; and (*iii*) further synthesis of certain proteins characteristic of the transformation phenotype, for example, glucose receptors. At the present time it is difficult to assess whether the morphological change *per se* or an associated process is the initiating event that occurs in the Ts mutant-infected cells during the shift in temperature from the nonpermissive to permissive range. Although the secretion of proteases is not detected until approximately 8 hr after the temperature shift, it is possible that low levels of activated protease, formed early and acting intracellularly, could be responsible for the morphological changes observed in this investigation.

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