Localization of mitochondria in living cells with rhodamine 123

(fluorescence microscopy/Rous sarcoma virus/colchicine)

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ABSTRACT   The laser dye rhodamine 123 is shown to be a specific probe for the localization of mitochondria in living cells. By virtue of its selectivity for mitochondria and its fluorescent properties, the detectability of mitochondria stained with rhodamine 123 is significantly improved over that provided by conventional light microscopic techniques. With the use of rhodamine 123, it is possible to detect alterations in mitochondrial distribution following transformation by Rous sarcoma virus and changes in the shape and organization of mitochondria induced by colchicine treatment.

Since the classical investigations of the mitochondrion some 90 years ago, much work has been carried out on the structure and function of this complex organelle. Extensive biochemical studies of mitochondria have proved that they play a cardinal role in the generation of energy essential for the survival and proliferation of eukaryotic cells (1–4). As intracellular organelles, mitochondria show remarkable plasticity, mobility, and morphological heterogeneity (5–13). Mitochondrial morphology is influenced by the metabolic state of the cell, cell cycle, cellular development and differentiation, and by pathological states (14–20). In addition, both morphological and functional changes in mitochondria have been shown to occur in conjunction with neoplastic transformation (21).

Although isolated mitochondria and mitochondria in fixed cells have been extensively investigated, much less attention has been paid to mitochondria in living cells. Previous investigations of mitochondria in living cells have been hampered by the lack of techniques allowing high-resolution visualization of these organelles. Use of Janus Green B, a dye relatively specific for mitochondria, aids somewhat in their recognition but also causes mitochondrial distortion and cytotoxic effects (5, 22). Rhodamine compounds have been used as histological stains (23, 24), and a number of fluorescent probes have been utilized in investigations of the energy state of isolated mitochondria (25–27). The compound rhodamine 6G has been reported to act as a potent inhibitor of oxidative phosphorylation and to block the adenine nucleotide translocase in isolated rat liver mitochondria (28). It has also been proposed that the phenotypic expression of rhodamine 6G resistance in mutants of the yeast *Saccharomyces cerevisiae* may be controlled by nuclear and mitochondrial (29) or cytoplasmic (30) genes.

Walsh *et al.* (31) have described the staining of mitochondria-like structures and lysosomes in cultured cells by serum proteins conjugated with rhodamine B isothiocyanate. In subsequent studies, when mixed isomers of rhodamine B isothiocyanate were used instead of pure rhodamine B isothiocyanate, only mitochondria were stained. The major component re-
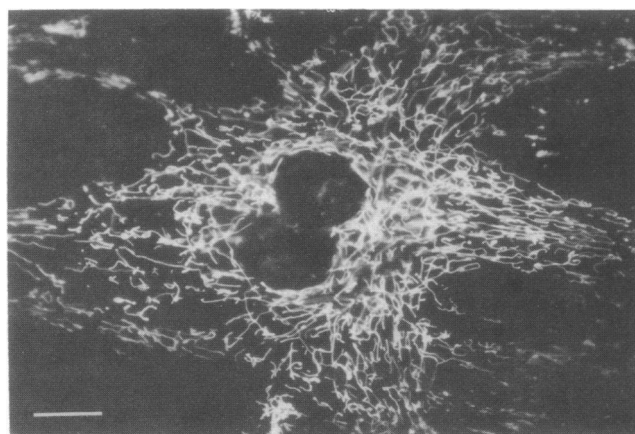
FIG. 1.   Mitochondria stained by rhodamine 123 in live gerbil fibroma cell. Bar represents 25 μm.

sponsible for mitochondrion-specific staining in such a preparation has been characterized as rhodamine 3B (unpublished data). This finding prompted us to screen various rhodamine compounds for mitochondrion-specific staining. The results reported here describe the use of the laser dye rhodamine 123 as a specific fluorescent probe for mitochondria in living cells. Rhodamine 123 stains mitochondria directly (without passage through endocytic vesicles and lysosomes) and provides low-background high-resolution fluorescent images of mitochondria without apparent cytotoxic effects.

MATERIALS AND METHODS

The purified laser dye rhodamine 123 (Eastman) was dissolved in double-distilled water at a concentration of 1 mg/ml and subsequently diluted to 10 μg/ml in Dulbecco's modified Eagle's medium (GIBCO). Cultured cells grown on 12-mm round glass coverslips (Rochester Scientific, Rochester, NY) were incubated with rhodamine 123 (10 μg/ml) for 30 min in a 10% CO_2 incubator at 37°C. Coverslips were then rinsed through three 5-ml changes of medium (5 min per rinse) and mounted in medium supplemented with 10% fetal calf serum (GIBCO) on a live-cell observation chamber prepared from a piece of silicon rubber (0.7 mm thick) punched with 10-mm holes and pressed onto a standard 25 × 75 mm microscope slide. Stained cells were examined by epifluorescent illumination at either 546 nm (rhodamine excitation) or 485 nm (fluorescein excitation) on a Zeiss photomicroscope III equipped with a Zeiss Planapochromat objective lens (×40). Photographs were made by using Kodak Tri-X (ASA 400) or Kodak Ektachrome 400 (ASA 400) film with the automatic exposure control of the microscope set at ASA 6300 for 546-nm excitation and ASA 1600 for 485-nm excitation. Tri-X film was developed for 10
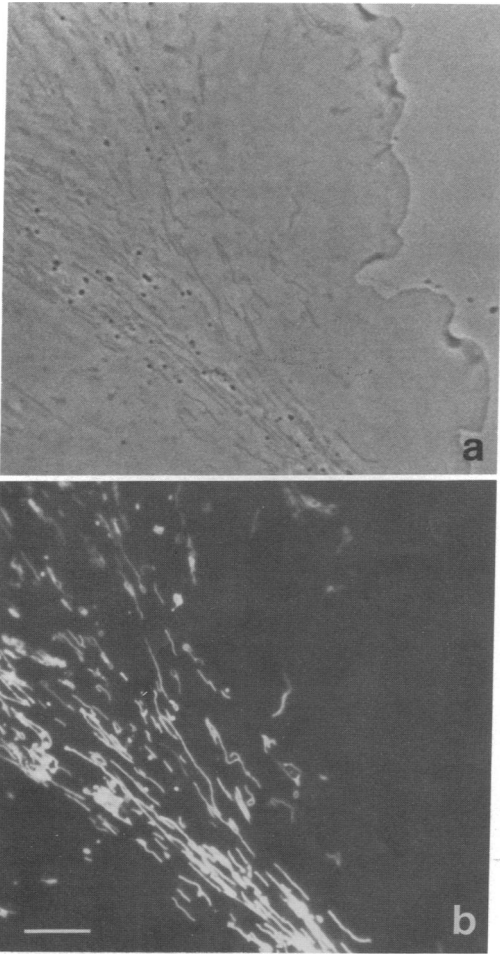
FIG. 2.   (a) Phase-contrast micrograph of a portion of a live gerbil fibroma cell showing phase-dense mitochondria. (b) Fluorescence micrograph of the same field showing fluorescent rhodamine 123-stained mitochondria. Bar represents 10 μm.

min in Kodak HC 110 (dilution B) and Ektachrome 400 was developed with Kodak E-6 processing.

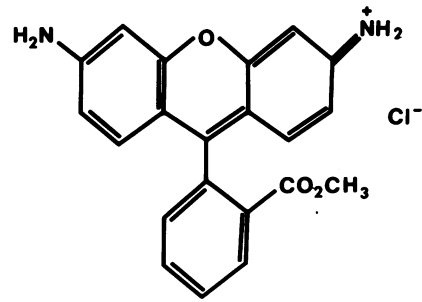Various dye concentrations, times and temperatures of

FIG. 3.   Chemical structure of rhodamine 123: methyl *o*-(6-amino-3'-imino-3H-xanthen-9-yl)benzoate monohydrochloride (33).

staining, solubilizing media, and cell types were tested to determine optimal conditions for rhodamine 123 staining. In addition, changes in mitochondrial organization in Rat 1 cells transformed by a temperature-sensitive mutant of Rous sarcoma virus (Ts-B77-Rat 1, developed by J. Wyke and provided by R. O. Hynes) and IMR-33 gerbil fibroma cells (CCL 146, American Type Culture Collection) treated with colchicine are described.

RESULTS

Living gerbil fibroma cells (IMR-33) were used in the initial investigations of the mitochondrial staining by rhodamine 123 because of their flattened morphology. The cytoplasmic structures that stained (Fig. 1) appeared to be typical of mitochondria. To substantiate this observation, mitochondria identifiable by phase-contrast optics were compared with rhodamine 123-stainable structures (Fig. 2). The two images are essentially superimposable. In a few areas the images do not exactly coincide, probably as a result of mitochondrial movement during the interval (15–30 sec) between the fluorescent and phase-contrast photographs. In fact, the movements of rhodamine 123-stained mitochondria which can be traced in such photographs reflect the dynamic aspect of these organelles in the living cell. In addition, fluorescently labeled structures could be isolated from rhodamine 123-stained cells by standard fractionation procedures used for mitochondrial isolation, further indicating that rhodamine 123-stained structures in
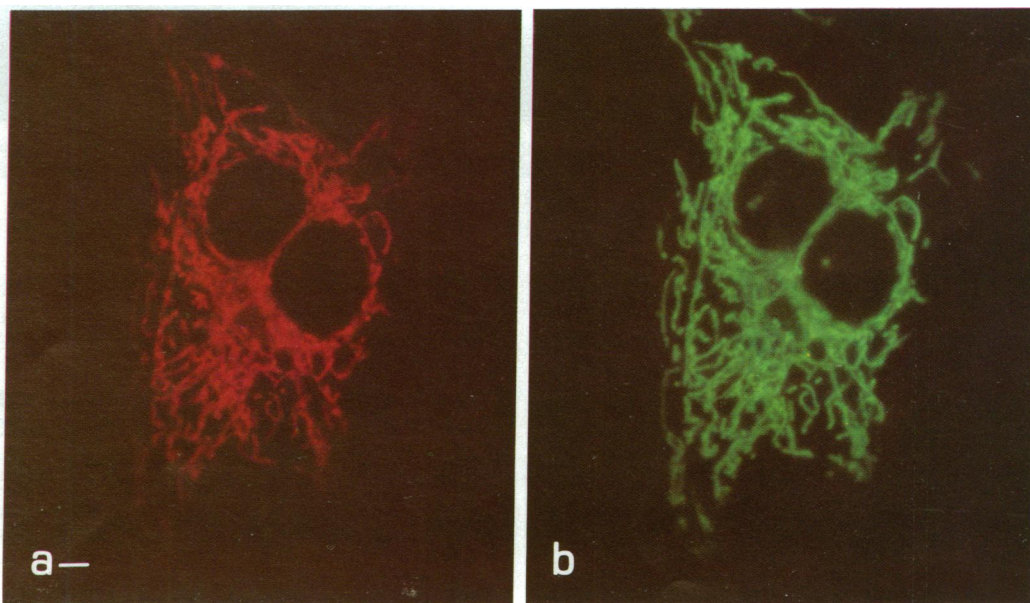

FIG. 4.   Fluorescence micrographs of a gerbil fibroma cell stained with rhodamine 123 and illuminated at 546 nm (*a*) or 485 nm (*b*). Bar represents 25 μm.
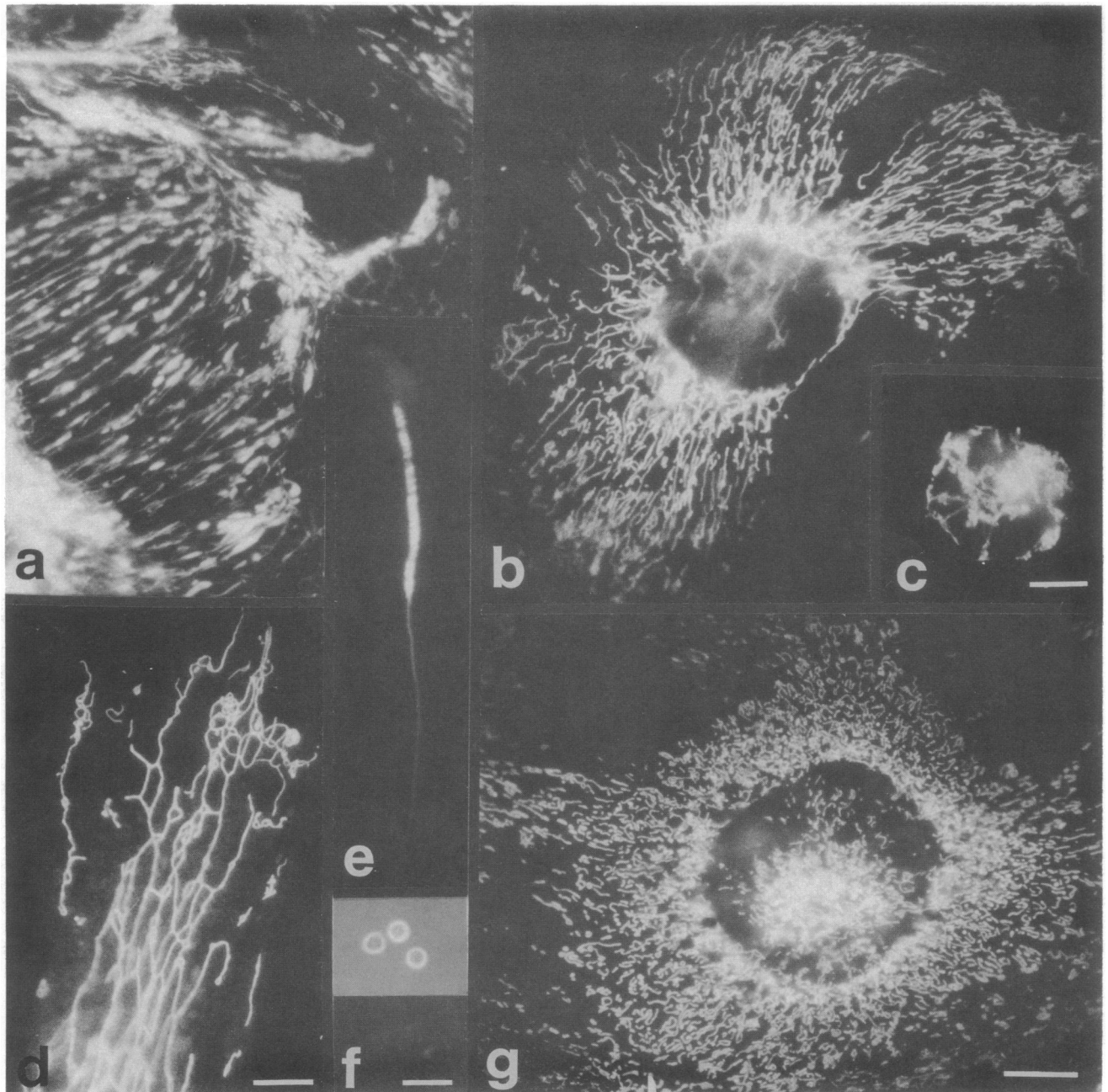
FIG. 5. Living cells stained with rhodamine 123: (a) rat cardiac muscle; (b) Pt Kl marsupial kidney; (c) mouse B lymphocyte; (d) mouse 3T6; (e) mouse sperm, (f) human erythrocytes (phase-contrast above and rhodamine 123-treated but unstained below); (g) rat embryo fibroblast. Bar represents: 15 μm in a, b, e, and g; 10 μm in c; 8 μm in d; 10 μm in f.

living cells are mitochondria. Furthermore, the antibiotic valinomycin which has been shown to act as a potassium ionophore in biological membranes and to induce mitochondrial swelling (4, 32) also caused the swelling of rhodamine 123-stained structures and the release of rhodamine 123 fluorescence into the cytoplasm. The above results indicate that it is highly unlikely that the cytoplasmic structures stained by rhodamine 123 represent new organelles or organelles other than mitochondria.

Rhodamine 123 is an unusual rhodamine derivative because when it is excited at a wavelength of 485 nm it produces a green fluorescence similar to that typically associated with fluorescein compounds, in addition to emitting red fluorescence when excited at the standard rhodamine excitation wavelength of 546

nm. The chemical structure of rhodamine 123 is presented in Fig. 3 (33). An example of mitochondria stained with rhodamine 123 and excited at both 546 and 485 nm is presented in the color photographs of Fig. 4.

Mitochondria were stained by rhodamine 123 at concentrations of 0.1, 1.0, 10, 100, and 1000 μg/ml (30 min, 37°C). The intensity of staining at 100 and 1000 μg/ml did not appear significantly greater than that at 10 μg/ml. Although some toxic effects were observed at the higher concentrations (100 and 1000 μg/ml), there was no apparent cytotoxicity in cells treated at 10 μg/ml or less. In addition, the growth rates of cells treated with rhodamine 123 at 10 μg/ml for 30 min and then returned to standard culture medium and of cells grown continuously in the presence of rhodamine 123 at 5 μg/ml did not differ

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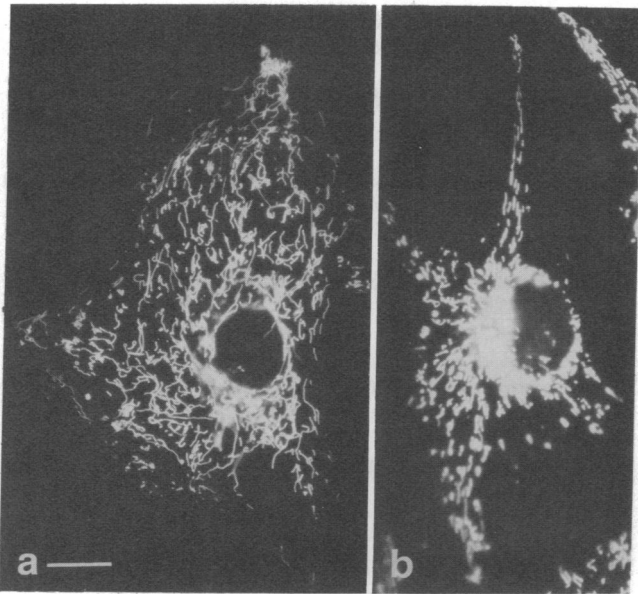
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FIG. 6. Live Rat-1 fibroblasts transformed with a temperature-sensitive mutant of Rous sarcoma virus and stained with rhodamine 123 at a nonpermissive temperature (*a*) or 30 min after shifting to a permissive temperature (*b*). Bar represents 25 μm.

FIG. 7. Live gerbil fibroma cell treated with colchicine (10 μg/ml, 16 hr) and stained with rhodamine 123. Compare with normal mitochondrial distribution shown in Figs. 1, 2, 4, and 5. Bar represents 20 μm.

significantly from those of untreated controls over a 96-hr period. In cells stained with rhodamine 123 at concentrations below 10 μg/ml, mitochondria were clearly visible but the intensity of staining was somewhat diminished.

A wide variety of established cell lines and primary cell cultures have been stained with rhodamine 123. Some of these results are presented in Fig. 5 which shows: (*a*) rat cardiac muscle cell with characteristic large, globular mitochondria, (*b*) Pt K1 (marsupial kidney) cell with filamentous mitochondria radiating from the perinuclear region, (*c*) mouse B lymphocyte, (*d*) mouse 3T6 cell demonstrating an interconnecting mitochondrial network, (*e*) mouse sperm showing mitochondria characteristically located in the midpiece, (*f*) human erythrocytes which contain no mitochondria and are unstained by rhodamine 123, and (*g*) rat embryo fibroblast with abundant small mitochondria of various shapes, some of which lie beneath the nucleus. Rhodamine 123 staining has also been used to monitor mitochondrial distribution under a number of experimental conditions. Two examples are described here.

(*i*) Rat fibroblasts infected with Rous sarcoma virus with a temperature-sensitive mutation in the *src* gene (Ts-B77-Rat 1) were shifted from a nonpermissive temperature (39°C) to a permissive temperature (34°C); they showed a rapid redistribution of mitochondria to the perinuclear region that could be detected by rhodamine 123 staining (Fig. 6). This event occurred as early as 30–60 min after the temperature shift.

(*ii*) The disruption of microtubules by colchicine treatment (10 μg/ml, 16 hr) resulted in the distortion of mitochondrial shape and disorganization of mitochondrial distribution in gerbil fibroma (IMR-33) cells (Fig. 7). Such an effect was not detectable in cells treated with lumicolchicine (10 μg/ml, 16 hr).

DISCUSSION

The molecular basis of specific interaction between rhodamine 123 and mitochondria remains to be determined. No staining of the plasma membrane or nuclear envelope is detected, and apparently membranes of lysosomes, endoplasmic reticulum, or Golgi complex are not stained. Therefore, rhodamine 123 does not react nonspecifically with biological membranes. A
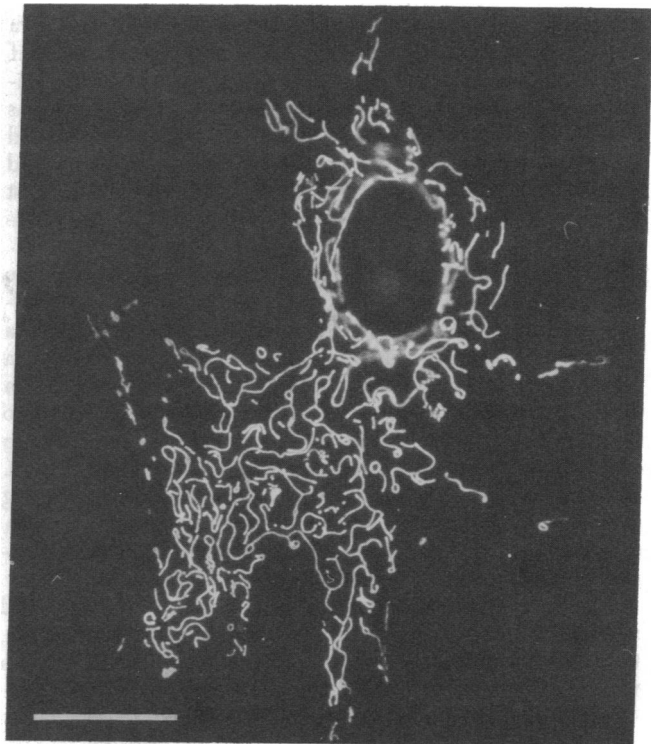
number of lipophilic fluorescent probes utilized in studies of isolated mitochondria interact with the mitochondrial membrane and show changes in their fluorescent characteristics based on the energy state of the mitochondria (25–27). Rhodamine 6G, which is positively charged at pH 7, has been shown to inhibit oxidative phosphorylation and block adenine nucleotide translocation in isolated rat liver mitochondria, whereas the related compound rhodamine B, which is uncharged at physiological pH, does not influence energy-linked mitochondrial functions (28). Rhodamine 123 also is positively charged at physiological pH and therefore may behave similarly to rhodamine 6G. However, rhodamine 123 does not appear to have the cytotoxic effects of rhodamine 6G. In screening a number of rhodamine compounds it has become apparent that only those that are positively charged at physiological pH—namely, 123, 6G, and 3B—are able to stain mitochondria specifically whereas uncharged rhodamines (such as B, 19, 110, and 116) and the negatively charged compound fluorescein do not. These results suggest that an attraction of cationic rhodamine molecules by the relatively high negative electric potential across the mitochondrial membrane may be the basis for the selective staining of mitochondria by rhodamine 123 in living cells.

After colchicine treatment, mitochondria are relatively difficult to visualize by phase-contrast microscopy, and alterations in mitochondrial shape and distribution are difficult to detect. However, by the use of rhodamine 123 it is clear that colchicine results in distortion in both the shape and distribution of mitochondria (Fig. 7), probably by effecting the depolymerization of microtubules. Electron microscopic studies of neuronal axons by Smith and coworkers (34, 35) have revealed the existence of crossbridges between microtubules and adjacent mitochondria which may be involved in the maintenance of mitochondrial distribution and their migration. A disorgani-

zation and randomization of mitochondrial distribution as a result of microtubule depolymerization might be predicted if microtubules play an essential role in the cytoplasmic distribution and movement of mitochondria (36). The observations by Heggeness *et al.* (37), made by the use of antisera directed against cytochrome *c* oxidase for mitochondrial localization and tubulin for microtubule localization in fixed cells, also suggest a structural relationship between these two cytoplasmic components.

Mitochondrial staining by rhodamine 123 also allows the visualization of a relatively early event in Rous sarcoma virus-induced transformation. The clustering of mitochondria to a perinuclear region is observed in cells transformed with a temperature-sensitive mutant in the *src* gene of Rous sarcoma virus as early as 30-60 min after a shift from nonpermissive to permissive temperature (Fig. 6). This event may be related to transformation-associated changes in the cellular cytoskeleton. However, we have thus far been unable to detect accompanying alterations in cytoskeletal systems including microfilaments, microtubules, and 10-nm filament as detected by indirect immunofluorescence in such temperature-shift experiments.

Alternatively, if one adopts the view that the distribution and movement of mitochondria are not dictated by the cytoskeleton, then the redistribution of mitochondria in Rous sarcoma virus-transformed cells may reflect a rapid change in a non-cytoskeletal element that is normally involved in the regulation of mitochondrial distribution. Finally, it is also possible that the transformation-associated change in mitochondrial distribution observed here may reflect other biochemical and functional changes that may occur in the mitochondria of transformed cells (21). In accordance with such a possibility is the report that Rous sarcoma virus RNA and Rous sarcoma virus associated proteins are present in the mitochondria of cells infected with Rous sarcoma virus (38).

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