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Persistence of the Viral Genome in Adenovirus Type 12-infected Hamster Cells

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Induction of T antigen by adenovirus type 12 was studied in growing and growth-inhibited cultures of the Nil-2 line of Syrian hamster cells. At a viral input multiplicity of 10, neoantigen was present in 100% of the cells by 24 hr. T antigen gradually disappeared in descendants of these cells so that 2 weeks after infection only 1% gave specific immunofluorescence. When cellular replication was prevented by addition of fluorodeoxyuridine, T antigen persisted in all cells for the 2-week period. Upon infection of growing cultures with purified ^{3}H-labeled adenovirus type 12 and autoradiographic analysis of the cells at various times thereafter, a gradual reduction in labeled nuclear loci was noted which paralleled the decrease in T antigen-containing cells. In nongrowing cultures, no change in labeled loci was noted. Correlation of T antigen and labeled loci revealed that fluorescent cells contained, on the average, about 10 times more silver grains than nonfluorescent cells. All of 92 preselected fluorescent cells showed labeled loci, whereas, of 100 nonfluorescent cells, 18 were free of silver grains. The implications of these findings are discussed.

Cells infected by oncogenic viruses either produce viral progeny and lyse or may become transformed and continue to grow. The transformed cells show altered properties and synthesize virus-specific T antigens (1, 4, 6, 8). Recent observations indicate additional alternatives. It was demonstrated that growth of a large proportion of adenovirus type 12-infected hamster cells was discontinued due to virus-induced chromosomal aberrations (12). In simian virus 40-infected cultures, the number of neoantigen-producing cells was significantly higher soon after infection than shortly before colonies of transformed cells became detectable (2, 3, 7). Oxman and Black (7) suggested that, in addition to integration of the viral genome into host cell deoxyribonucleic acid (DNA), there might also exist a nonintegrated association of viral and cellular DNA. With the use of purified ^{3}H-labeled adenovirus type 12 and autoradiography, such a relationship was indeed demonstrable (10). The label preparations provided an important tool for the detection and localization of viral genomes within infected hamster cells. The specificity of the results was established by appropriate controls, i.e., neutralization of virus by antiserum, infection of nonsusceptible cells, and addition of "cold" thymidine during viral adsorption (500 μg) and 2 μg thereafter until harvest of infected cells (10, 11). The present study was undertaken to obtain further information on the persistence of the viral genome in hamster cells after abortive infection with purified ^{3}H-labeled adenovirus type 12. T antigen synthesis and labeled nuclear loci were taken as evidence for the persistent presence of viral genomes in growing and in growth-inhibited cells. In addition, labeled loci were correlated with T antigen-specific fluorescence in individual cells.

MATERIALS AND METHODS

Virus and cells. Origin and preparation of adenovirus type 12 stock virus and origin and maintenance of the Nil-2 line of Syrian hamster cells, human KB-cells, and primary human embryonic kidney (HEK) cells have been described (10, 11).

Labeling and autoradiography. Labeling of the virus and methods employed for autoradiography have been reported (9, 10, 13). The ^{3}H-thymidine had a specific activity of 11 c/mM. For autoradiography, exposure time to the NTB-3 emulsion was 16 days.

Viral purification. The virus was propagated in approximately 5×10^8 KB cells (10). The cells were harvested 3 days after infection, and the virus was purified according to the method of Green and Piña (5) except that cesiumchloride was used instead of rubidiumchloride. After the virus had been subjected twice to isopycnic banding in CsCl, 0.2-ml fractions were collected through a puncture in the bottom of the centrifuge tube. For determination of radioactivity, a 10-μliter amount was removed from each fraction. The virus-containing fractions were dialyzed

for 24 hr against two changes of 800 volumes each of 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 8.1) at 4 C with continuous stirring. After dialysis, 10 μliters of the culture was removed for titration in HEK cells.

Infection of cells. The procedure for infection of Nil-2 cells has been described (12). During the adsorption period, 500 μg of "cold" thymidine was present. Thereafter, unattached virus was removed and fresh medium, containing 2 μg of cold thymidine, was added.

Growth inhibition of infected cells: ^{3}H-adenovirus type 12 was adsorbed to Nil-2 cells in the presence of 6×10^{-5} M fluorodeoxyuridine (FUDR; Hoffman-La Roche, Inc., Nutley, N.J.). Thereafter, medium containing the same amount of FUDR and 10% dialyzed fetal calf serum (FCS) was added. At 24 hr after infection, fresh medium was added containing nondialyzed fetal calf serum and no FUDR.

Immunofluorescence. For immunofluorescence studies, infected Nil cells and uninfected controls were grown on slides in large petri dishes (diameter, 14 cm) each containing five slides. At various time intervals, slides were removed aseptically, washed briefly in phosphate-buffered saline (PBS) and distilled water, and fixed in acetone for 5 min at room temperature. The preparations were stained for 1 hr at 37 C with fluorescein isothiocyanate-conjugated hamster antibodies to adenovirus type 12 T antigen (purchased from Flow Laboratories, Rockville, Md.) diluted 1:5 in distilled water. The slides were then washed twice each in PBS and distilled water. Cover slips were affixed by a mounting medium consisting of an equal mixture of glycerol and PBS. Cells were examined in a Zeiss Ultraphot microscope. Locations of fluorescent cells were noted on the stage scale and the cells were photographed. After selection of a sufficient number

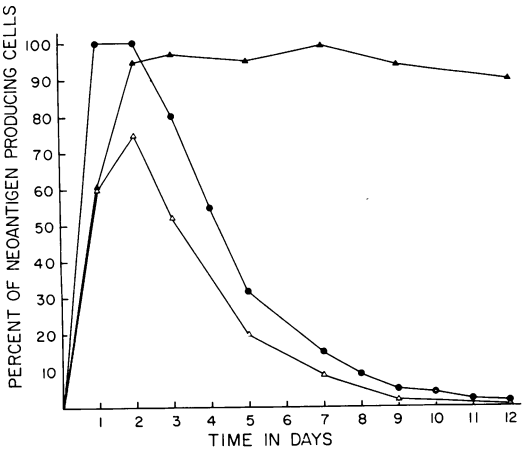

FIG. 1. *T antigen synthesis in growing and growth-inhibited cells. Symbols:* ▲, *FUDR-treated cells (input multiplicity of 5);* △, *growing cells (input multiplicity of 5);* ●, *growing cells (input multiplicity of 10).*

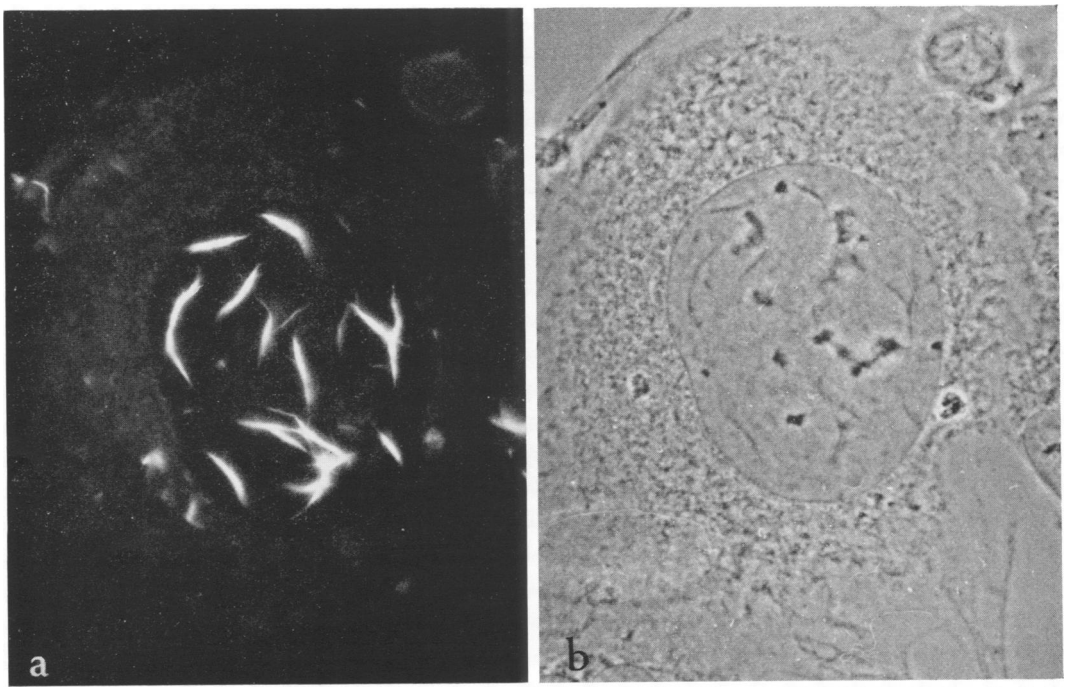

FIG. 2. *T antigen in an individual cell as revealed by (a) immunofluorescence and (b) phase-contrast microscopy. Note correspondence between fluorescent structures and dark filaments.* ×2,800.

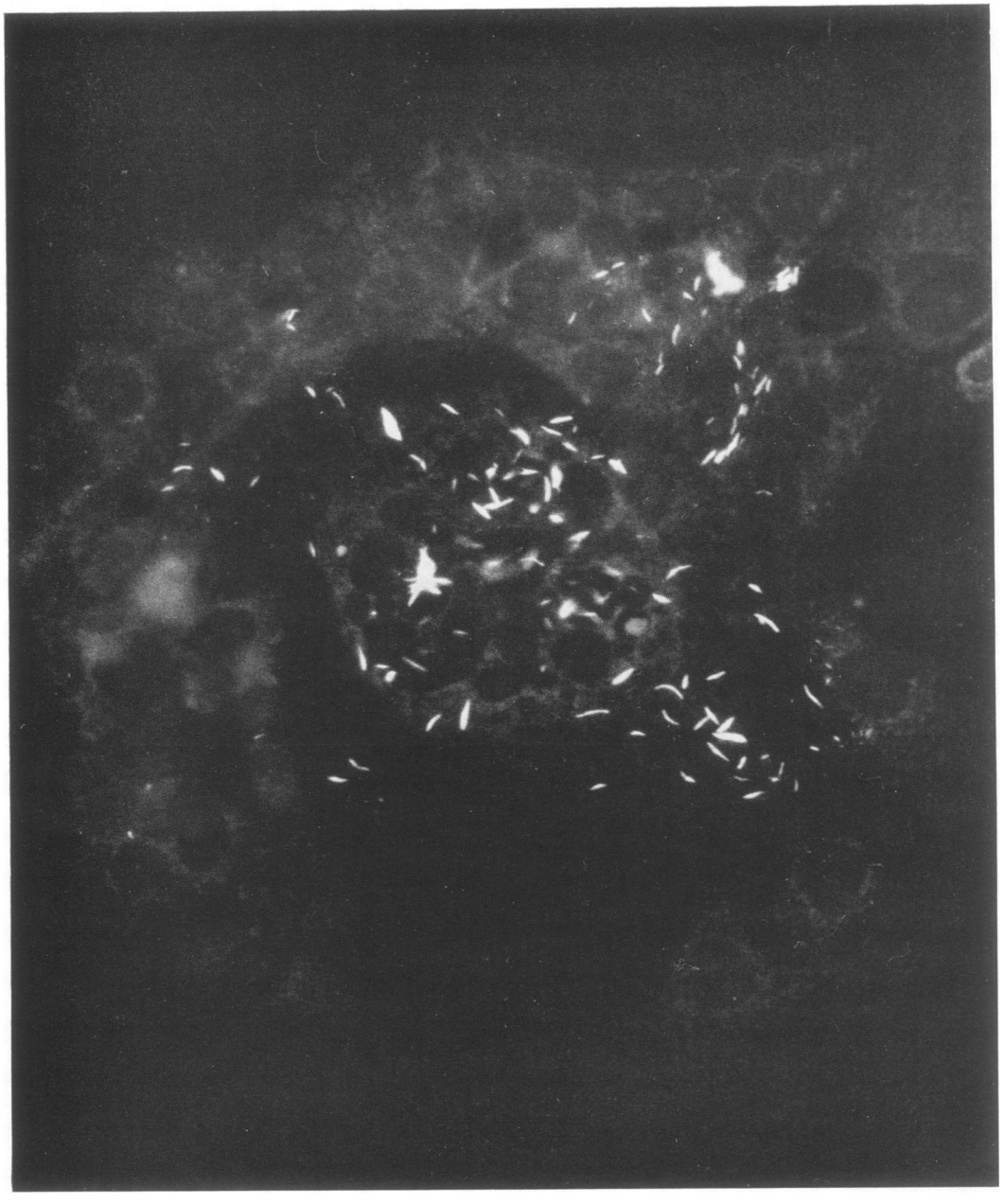

FIG. 3. *Island of growing cells 7 days after infection with adenovirus type 12. Giant cell formation in the centra part of the island with strong immunofluorescence. Most peripheral cells are unstained.* ×*1,500.*

of cells, the cover slips were gently removed by immersion of the slides in distilled water. After air-drying, the slides were processed for autoradiography as described. After development, the cells were stained with Giemsa (9). The previously photographed cells were relocated and scored for presence of labeled loci and were photographed again.

RESULTS

Immunofluorescence studies. Nil cells, infected at input multiplicities of 5 and 10, were examined for presence of adenovirus type 12 T antigen specific immunofluorescence over periods of up to 2 weeks. The percentages of T antigen-pro-

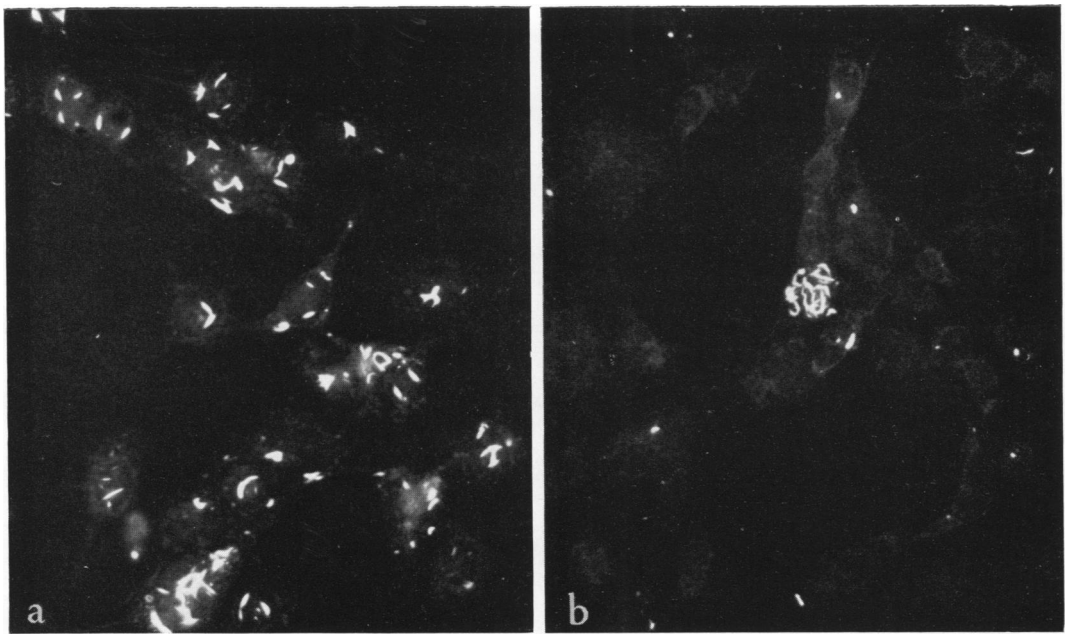

FIG. 4. *T antigen in (a) FUDR-treated and (b) growing cells 7 days after infection with adenovirus type 12 ×1,500.*

ducing cells were determined at intervals of 1 or 2 days. At an input multiplicity of 10 (Fig. 1), all of the 1,000 cells counted 1 and 2 days after infection revealed T antigen. If any nonfluorescent cells were present, they amounted to well below 0.1%. From the third day on, the percentages of fluorescent cells declined. The cultures started to grow rapidly and were passaged every 3 or 4 days. T antigen-producing cells decreased with each division by a factor of about 2 (Fig. 1). The presence of some nondividing, T antigen-containing cells (12) and fluctuations in growth rates during the observation period were presumably responsible for irregularities in the curve.

The fluorescence elicited in the hamster cells was very brilliant and of rod or needle shape. It was limited on the whole to the nucleus, but some cells revealed, in addition, fluorescent dots in the cytoplasm. In many cells the fluorescent rods corresponded to dark filaments seen in the nucleus by phase-contrast microscopy (Fig. 2). In growing cultures, the central cells of islands usually showed fluorescence and frequently giant cell formation (Fig. 3), whereas only few cells at the periphery revealed T antigen.

Upon inhibition of cellular growth by FUDR, the fluorescent cells persisted for at least 2 weeks (Fig. 1). Figure 4 demonstrates representative areas of growth-inhibited and growing cultures 7 days after infection.

TABLE 1. *Labeled loci in growing and FUDR-treated Nil cells infected with ^{3}H-adenovirus type 12 at an input multiplicity of 1 (100 cells counted)*

Infection of cells with	Days after infection				
	1	3	5	12	14
^{3}H-adenovirus 12....	685	413	198	40	15
^{3}H-adenovirus 12 + FUDR.....	582	625	760	794	754

Autoradiographic studies. Preparations of ^{3}H-labeled adenovirus type 12 of high purity were used for infection of Nil cells. Infectivity, induction of T antigen synthesis, and electron microscopic demonstration of viral particles corresponded to the peak of radioactivity. The infectivity titers ranged in different preparations from 10^7 to 10^9 $TCID_{50}$/ml. At various times after infection, preparations were processed for autoradiography and the number of labeled loci per nucleus was determined. The decline in labeled loci in growing cultures corresponded to some extent to the reduction of T antigen-producing cells (Table 1). In growth-inhibited cultures, the nuclear grains persisted. The slight increase during the observation period was probably due to fusion of labeled cells.

Correlation of labeled loci and virus-specific

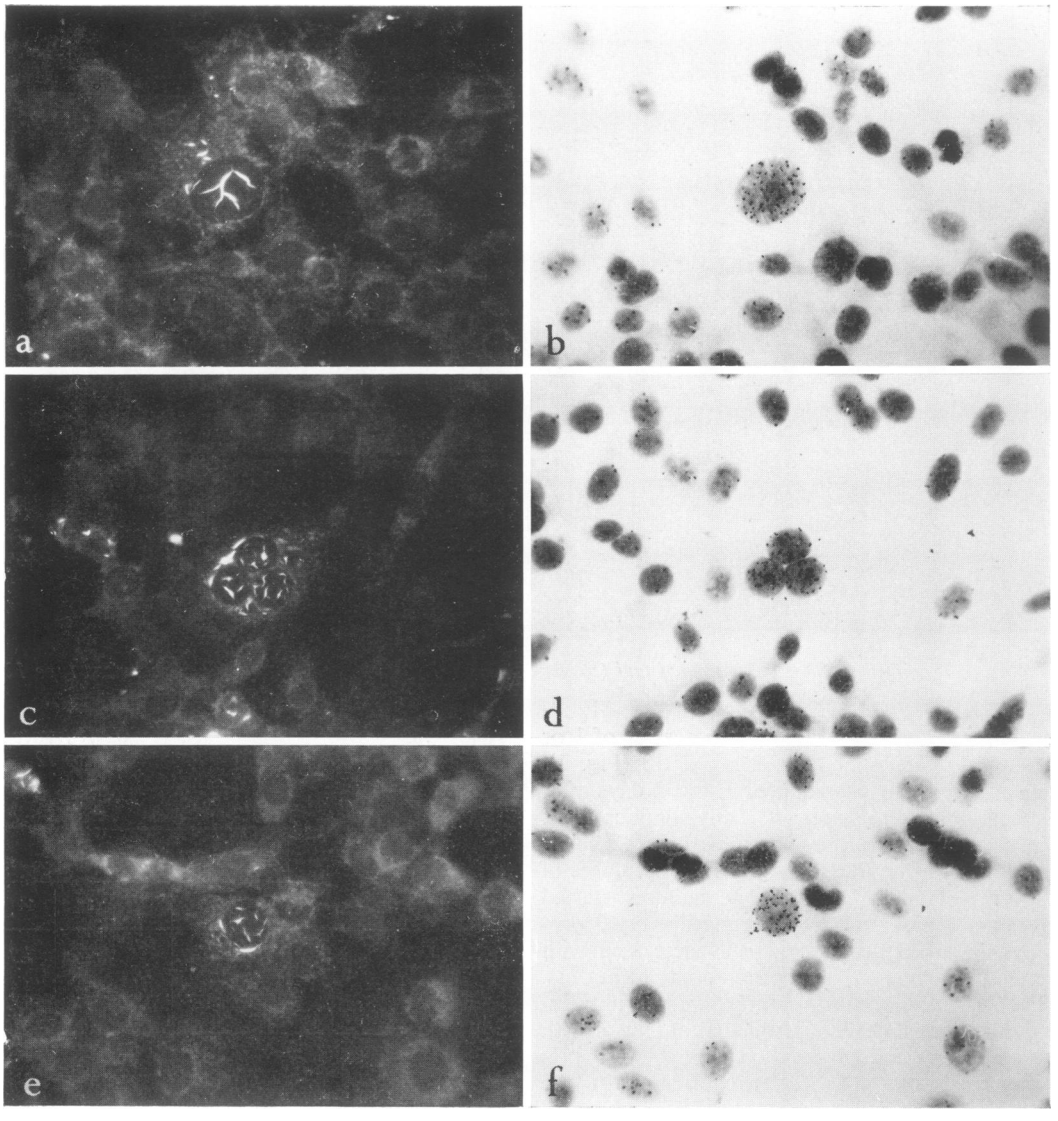

FIG. 5. *Comparison of individual cells infected by immunofluorescence and autoradiography 7 days after infection of the cultures with ^3H-labeled adenovirus type 12. Nuclei with T antigens contain numerous labeled loci. ×1,000.*

fluorescence in individual cells. Preselected immunofluorescent cells were processed for autoradiography 7 days after infection. After exposure for 16 days, 92 cells out of 100 were relocated. They contained nearly 10 times more labeled loci per nucleus than did 100 nonfluorescent cells. The count of labeled loci in fluorescent cells was 29.9 and in nonfluorescent cells it was 3.05. None of the fluorescent cells was unlabeled, whereas 18% of the nonfluorescent cells were free of grains. However, a few nonfluorescent cells revealed between 20 and 30 labeled loci. The label was restricted almost exclusively to the nucleus (Fig. 5) and at high magnification often revealed clustering of the grains (Fig. 6).

DISCUSSION

The results demonstrated that dividing cells lose their T antigen almost completely within 2 weeks. Since 100% of these cells revealed specific fluorescence 24 and 48 hr after infection, the nonfluorescent progeny must originate from fluorescent cells, although a large proportion of these were incapable of dividing (12). The per-

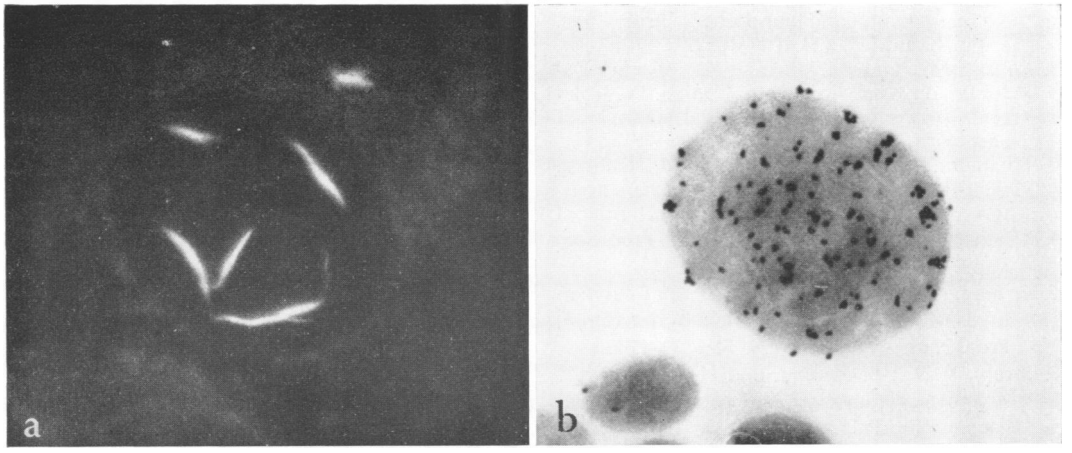

FIG. 6. *Individual cell infected with ^{3}H-labeled adenovirus type 12. (a) Virus-specific T antigen and (b) labeled loci. Note frequent clustering of grains.*

sistence of the latter type of cells and the asynchronous growth of the cell population obscure to some extent the exact slope of the regression. Nevertheless, the results suggest approximately a 50% reduction in the number of T antigen-containing cells with each division. Interpretation of these data is still speculative. Oxman and Black (7) suggested that a nonintegrated state of the viral genome might be responsible for similar findings in simian virus 40-infected cells. Results obtained upon infection of HEK cells with ^{3}H-labeled adenovirus type 12 (10) were compatible with this interpretation. The presence of isochromatid labeling of chromosomes in hamster cells infected with ^{3}H-labeled adenovirus type 12 indicates that, in addition, "silent" integration occurs (zur Hausen, *in preparation*). It is conceivable that both events may be involved in a single infected cell.

In growing cells infected with ^{3}H-labeled adenovirus type 12, reduction of labeled loci paralleled the regression of T antigen-synthesizing cells. This clearly indicated, in addition to other results (12), that adenovirus type 12-infected Nil-2 cells can divide. In FUDR-treated cells, the label persisted over a period of at least 2 weeks in the nuclei of infected cells. Since these cells did not replicate, the label could not represent free thymidine from breakdown products of viral genomes. It is suggested that the label represents the intact viral genomes in the nuclei of infected cells.

The finding of labeled nuclear loci correlated well with the presence of virus-specific T antigens. None of the 92 preselected fluorescent cells was found unlabeled 7 days after infection, whereas 18% of the nonfluorescent cells were free of silver grains. The average number of labeled loci in fluorescent cells was 10 times higher than in nonfluorescent cells. These results strongly indicate that at least parts of the viral genome have to be present for induction of T antigen synthesis.

The presence of labeled loci in nonfluorescent cells poses a problem of interpretation. Although the grain counts were generally low, up to 30 labeled loci were noted in a few cells. These results might be due either to defective viral particles, which are incapable of inducing T antigen synthesis, or to cellular repression of virus-specific functions.

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