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Cytoplasmic DNA Synthesis Induced by RNA Tumor Viruses

(mouse embryo fibroblasts/murine sarcoma virus/Rauscher murine leukemia virus/autoradiography)

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ABSTRACT Infection of mouse embryo cells with two strains of murine sarcoma virus and one strain of murine leukemia virus was followed rapidly by synthesis of DNA in the cytoplasm. Persistently infected cells have not shown such synthesis, and ultraviolet-irradiated virus did not induce DNA synthesis. This new DNA presumably represents an intermediate in the virus replication cycle specified by the virion DNA polymerase(s). Failure to observe cytoplasmic DNA synthesis in persistently infected cells suggests, in keeping with the results of inhibitor experiments, that the new "viral" DNA becomes associated with cellular DNA in some form of stable interaction.

Extensive studies of DNA synthesis in eukaryocytes by light and electron microscopic autoradiography have shown that such synthesis is largely restricted to the nucleus (1). Some DNA synthesis is also detectable in mitochondria (2); however, with this exception, cytoplasmic DNA synthesis in normal cells has never been observed. We present evidence here that infection with RNA tumor viruses leads to the synthesis of nonmitochondrial cytoplasmic DNA.

MATERIALS AND METHODS

Virus

The Harvey strain of murine sarcoma virus, H-MSV, the Rauscher pseudotype of MSV, MSV(RLV), and the Rauscher strain of murine leukemia virus, RLV, were passaged in mouse-embryo fibroblasts. Preparations used in this work were passed through 0.45-μm filters and found to be free of bacterial and mycoplasmal contamination.

Procedure

4×10^5 cells were plated in 5 ml of Eagle's minimal essential medium (MEM) containing 10% fetal-bovine serum. After 3 days the medium was changed to MEM containing 2% fetal-bovine serum. After an additional 24 hr the medium was removed and cells were infected with 3.5×10^3 focus-forming units of H-MSV in 0.4 ml for 1 hr at 37°C. The spent medium was then returned to the dish and, at specified times, 0.1 ml of [^{3}H]thymidine (14.1 Ci/mmol, 0.5 mCi/ml) was added.

Autoradiography

After incubation with [^{3}H]thymidine, samples in duplicate were fixed by treatment for 1 hr with 2.5% glutaraldehyde in S-collidine buffer (pH 7.4) followed by treatment for 1 hr with 1% osmic acid. Each sample was then treated with cold 5% trichloroacetic acid for 30 min. Samples were then washed and dehydrated with acetone; wash solutions were monitored for release of radioactivity. By the final wash, no soluble radioactivity was recovered. After embedding in an Epon-araldite mixture, sections 1 μm thick were mounted on glass slides for light-microscopic autoradiography. These were either dipped in warmed Nuclear Track Emulsion or covered by autoradiographic stripping film, AR-10 (Kodak) and exposed for 3 weeks. For light-microscopic autoradiography, two different methods were used for calculation of results: (*a*) the planimeter method: the total nuclear area was subtracted from the total cell area, both of which were determined by a planimeter; or (*b*) the Monte Carlo method: a transparent sheet (10 × 10 cm) was marked with 100 randomly chosen points. This sheet was placed over the photomicrographs. The number of random points that fell on the image of the cytoplasm was divided by 100 and multiplied by the total area (100 cm^2) of the transparent sheet. This gives an estimate of the cytoplasmic area. The areas calculated by these methods were in fair agreement, differing by not more than 15%. The grain densities in the cytoplasm of infected and control cells were expressed as grains/cm^2.

RESULTS

Attempts to demonstrate induction of DNA synthesis in mouse embryo cells infected with murine sarcoma or leukemia viruses have been equivocal in numerous trials in our laboratory. Table 1 records one result in which some stimulation is seen at 4–10 hr after infection, but this has not been a general finding. Since synthesis of new virus-specific DNA need not be substantial in relation to cellular DNA synthesis, it was felt that a cytologic method would hold greater promise.

TABLE 1. *Incorporation of [^{3}H]thymidine into DNA of BALB/3T3 cells infected with H-MSV*

Hours after infection	cpm/μl	
	Uninfected	H-MSV infected
0–4	573	456
4–10	416	749
10–20	315	329

For determination of total DNA synthesis, labeled cells were washed with Earle's balanced salt solution and disrupted with a Dounce homogenizer into 1 ml of water. A 0.1-ml aliquot was then precipitated with 5 ml of 6% cold trichloroacetic acid. The precipitate was then collected on a Millipore filter, washed with cold trichloroacetic acid, and counted in a Beckman LS 250 liquid counter. Values are cpm/μl.

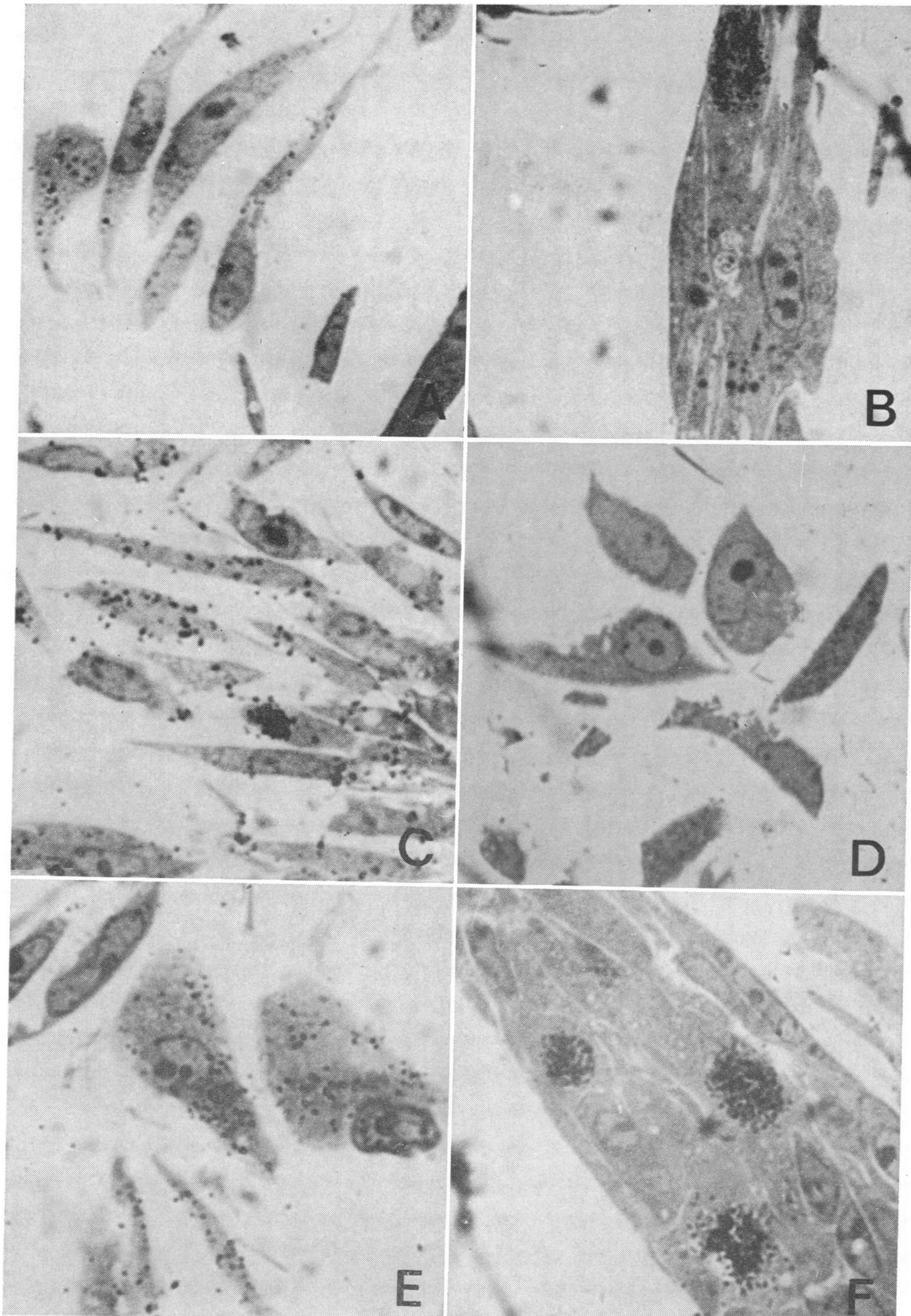

FIG. 1. Light microscope autoradiograph of cells infected with H-MSV and control cells pulse-labeled with [^{3}H]thymidine. *A*, infected cells pulsed 0–4 hr after infection. *B*, control cells pulsed for 7 hr. *C*, infected cells pulsed 6–10 hr after infection. *D*, cells infected with irradiated virus, pulsed 32–38 hr after infection. *E*, infected cells pulsed 32–38 hr after infection. *F*, control cells pulsed 32–38 hr after mock "infection" with buffer.

All the H-MSV-infected cells show considerable cytoplasmic labeling, whereas uninfected cells or cells treated with UV-inactivated virus show no cytoplasmic labeling. Labeled nuclei can be seen in both infected and control preparations.

Examination by autoradiography of a number of cell lines from several species chronically infected with C-type viruses has not revealed DNA synthesis except in the nucleus. In contrast, cells infected with the three viruses studied showed cytoplasmic DNA synthesis within a short time after infection. This could clearly be seen by both light and electron microscope autoradiography. Cells infected with H-MSV have been studied in the greatest detail, and the data pre-

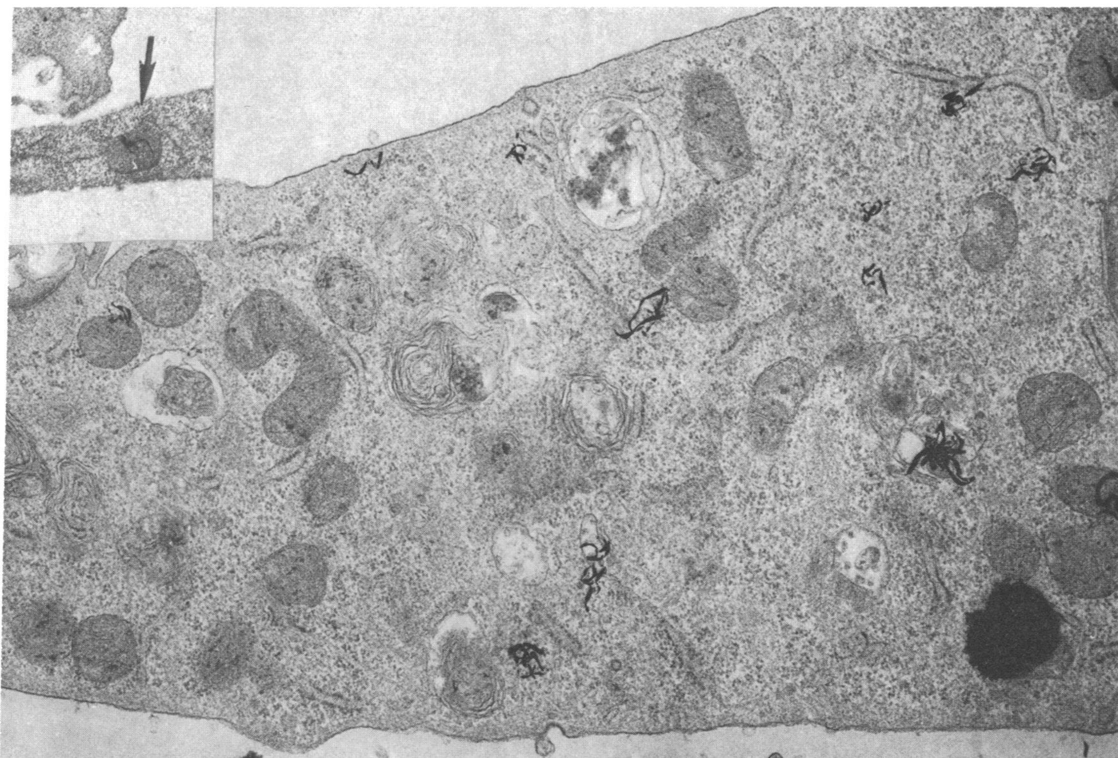

FIG. 2. Electron microscope autoradiography of cells infected with H-MSV and irradiated H-MSV. A number of photopositive grains is observed in the cytoplasmic matrix of this H-MSV-infected cell. In contrast, cells infected with irradiated virus showed very few cytoplasmic grains. The arrow (insert) shows a labeled mitochondrion. Heavy labeling of a nucleus is shown in the lower right corner.

sented concern only these cells; however, no apparent differences were noticed between these cells and those infected with MSV(RLV) or RLV. As shown in Fig. 1*A*, incorporation of [^{3}H]thymidine in the cytoplasm became evident as early as 4 hr after infection with H-MSV, as detected by light-microscope autoradiography. The density of cytoplasmic grains was 8 times as high as that of uninfected cells and cells treated with UV-inactivated H-MSV. 4–10 hr after infection, the grain counts of infected cells were 16 times as high as those of the control cells (Fig. 1, *C* and *D;* Table 2). Cytoplasmic labeling did not increase beyond the level attained after 10 hr even after prolonged incubation for more than 48 hr (Fig. 1, *E* and *F*).

TABLE 2. *DNA synthesis in the cytoplasm of MSV-infected cells detected by light microscope autoradiography*

Labeling, no. of hours after infection	Uninfected BALB/3T3	UV-heated MSV	MSV
0–4	~0.05*	~0.25	1.94
4–10	~0.25	—	4.14
10–20	0.9	0.43	—
20–25.5	—	~0.25	2.91
25.5–31.5	0.54	~0.25	2.22
31.5–51.5	~0.25	~0.25	3.47

Cells were washed with Earle's BSS and fixed in glutaraldehyde (1% in 0.01 M Tris buffer, pH 7.4). After pelleting, the packed cells were examined for DNA synthesis by autoradiography. UV treatment was for 1.5 hr at 60 erg/mm^2. Values are grain counts per cm^2 of cell cytoplasm.

* Background value.

By electron microscopic examination (Fig. 2) it was found that the number of grains on the mitochondria or intracytoplasmic vesicles (phagosomes or pinocytotic vesicles) was similar in both control and infected cells. In control cells, the number of grains outside mitochondria or cytoplasmic vesicles was considerably lower than that on these organelles. By contrast, we found a large number of grains in the cytoplasmic matrix of infected cells, in addition to the normal extent of labeling on mitochondria and cytoplasmic vesicles.

DISCUSSION

The discovery of an RNA-dependent DNA polymerase in RNA tumor viruses (3, 4) has provided strong circumstantial evidence in favor of the concept of a DNA intermediate (5) in the synthesis of these viruses. However, direct demonstration of the hypothetical DNA intermediate during intracellular virus replication has not yet been reported, and indirect methods still provide the most compelling evidence in favor of the existence of such an intermediate (6). Attempts to demonstrate induction of DNA synthesis by such viruses under standard conditions of infectivity have been equivocal in our laboratory (Hatanaka, unpublished data), although some positive results have been reported (7). The autoradiographic technique has, however, provided clear evidence that new DNA synthesis does occur rapidly after virus infection and because of its localization we speculate that this represents a true intermediate in the replication cycle. This speculation may be verified by *in situ* application of nucleic acid hybridization techniques, and other experiments that

are in progress. The finding that chronically infected cells no longer show evidence of cytoplasmic DNA synthesis is entirely consistent with results obtained with inhibitors of DNA synthesis, which prevent virus replication only early in the replication cycle (8, 9). Whether the "viral" DNA is actually integrated into the cell genome, thus becoming stabilized, or remains extranuclear has yet to be established.

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