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Cellular Integrity Is Required for Inhibition of Initiation of Cellular DNA Synthesis by Reovirus Type 3

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Synchronized HeLa cells, primed for entry into the synthesis phase by amethopterin, were prevented from initiating DNA synthesis 9 h after infection with reovirus type 3. However, nuclei isolated from synchronized cells infected with reovirus for 9 or 16 h demonstrated a restored ability to synthesize DNA. The addition of enucleated cytoplasmic extracts from infected or uninfected cells did not affect this restored capacity for synthesis. The addition of ribonucleotide triphosphates to nuclei isolated from infected cells stimulated additional DNA synthesis, suggesting that these nuclei were competent to initiate new rounds of DNA replication. Permeabilization of infected cells did not restore the ability of these cells to synthesize DNA. Nucleoids isolated from intact or permeabilized cells, infected for 9 or 16 h displayed an increased rate of sedimentation when compared with nucleoids isolated from uninfected cells. Nucleoids isolated from the nuclei of infected cells demonstrated a rate of sedimentation similiar to that of nucleoids isolated from the nuclei of uninfected cells. The inhibition of initiation of cellular DNA synthesis by reovirus type 3 appears not to have been due to a permanent alteration of the replication complex, but this inhibition could be reversed by the removal of that complex from factors unique to the structural or metabolic integrity of the infected cell.

Reovirus type 3 has been shown to inhibit cellular DNA replication in transformed cells although it has little or no effect on DNA replication in normal cells (5). This inhibition has been found to be selective for the replicative but not the transcriptive function of cellular DNA and specific for the initiation step of DNA replication (4, 6, 15).

The inhibition of cellular DNA synthesis in reovirus-infected L-929 mouse fibroblasts can be detected 8 h after infection and is 80% complete by 12 h after infection (4, 6, 13). Synchronized L cells fail to enter the DNA synthesis phase (S phase) of the cell cycle when infected with reovirus 8 h before the initiation of the S phase (4).

It has been shown (23) that the S1 double-stranded RNA segment is responsible for the capacity of reovirus type 3 to inhibit cellular DNA synthesis in mouse L cells. It has been suggested that this inhibition may be mediated through an interaction at the cell surface by the infecting virus particle, since the S1 gene codes for the viral hemagglutinin (27). It has also been suggested that the S1 gene product may exert an intracellular effect that is responsible for the inhibition of cellular DNA synthesis.

The inhibition of initiation of DNA synthesis by reovirus type 3 has been shown to be unrelated to a modification of precursor pools, DNA polymerase activity, thymidine kinase activity, or template integrity (6, 24). This inhibition cannot be caused by noninfectious particles or top component particles (14, 20). However, when infectious subviral particles are used, an inhibition of cellular DNA synthesis does occur, but the degree of inhibition is significantly reduced when compared with that obtained with intact virions (3).

The inhibition of DNA synthesis in infected cells is accompanied by a reduction in active chromatin initiation sites (15). By using ultrastructural analysis of cells infected with reovirus, it was discovered that as DNA synthesis declined, the normal decompaction of chromatin associated with DNA synthesis took place but there was no recondensation (2). It was suggested that a virus product may damage chromatin, preventing recondensation. It has been reported that proteins of the μ and σ classes are present in the nuclei of infected cells 6 to 8 h after infection (25). In addition, the σ class protein σ 3 appears to nonspecifically bind both native and denatured cellular DNA. Although reovirus replicates in the cytoplasm of infected cells (12, 26), it has been suggested that the presence of viral proteins in the nuclei of these infected cells may be related to a requirement for a nuclear product for successful virus replication. Removal of the nucleus inhibits virus replication by 90% (7).

The effect of removing the nuclei from reovirus-infected cells on the maintenance of virus-mediated inhibition of cellular DNA synthesis and on the characteristics of nucleoids isolated from these nuclei was examined in this study. In addition, the effect of permeabilization of reovirus-infected cells on the inhibition of DNA synthesis and nucleoid sedimentation was investigated.

MATERIALS AND METHODS

Cells and growth media. Suspension cultures of HeLa S3 cells were cultivated in Spinner-modified minimal essential medium (MEM; KC Biologicals, Inc.) supplemented with 5% fetal bovine serum (KC Biologicals, Inc.) at 37°C.

Virus. Reovirus type 3 strain Dearing was propagated in HeLa cell suspension cultures. The cells were concentrated to 10^7 cells per ml in MEM-Spinner medium without serum and infected with 10 to 20 PFU per cell. After adsorption for 1 h at 37°C, the infected cell suspension was diluted to 10^6 cells per ml in MEM-Spinner medium containing 5% fetal bovine serum. When the cell viability, measured by trypan blue dye exclusion, decreased to 80%, the cells were chilled in an ice bath and centrifuged for 10 min at 1,200 × *g*. The cell pellets were frozen at −20°C.

Virus purification. All experiments were conducted with virus purified from infected cells by the method of Duncan et al. (5).

Virus titration. Reovirus type 3 stocks were titrated by a modification of the procedure of Shaw and Cox (24). The

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modification consisted of an additional 24 h of incubation before overlaying with neutral red.

Detection of viral antigens. Intracellular viral antigens were detected by indirect immunofluorescence. At 9 h after infection of amethopterin-synchronized cells, 5×10^6 cells were removed from the Spinner culture and pelleted by centrifugation at 1,200 × *g* for 10 min. The medium was decanted, and the cell pellet was suspended in 0.1 ml of phosphate-buffered saline at pH 7.4. The cell suspension was spread onto microscope slides and allowed to air dry. The cells were fixed with 70% ethanol for 90 s, and the ethanol was removed by rinsing the slides with distilled water for 45 s. The cells were then overlaid with anti-reovirus antiserum and incubated in a moist chamber for 20 min. The slides were washed for 60 s with distilled water, and the cells were overlaid with fluorescein-conjugated goat anti-rabbit gamma globulin (Cappel Laboratories; 1:20 dilution in phosphate-buffered saline) for 20 min in a moist chamber. The slides were washed with distilled water, dried, and observed with a Nikon inverted microscope (Diaphot-TMD-EF) in the epifluorescence mode.

Cell viability and permeability. Cell viability was determined by the trypan blue dye exclusion method (17). The degree of permeabilization of cells was monitored by the inability of the permeabilized cells to exclude the trypan blue dye.

Synchronization of HeLa cells and determination of their mitotic index. The mitotic index of HeLa cells synchronized with amethopterin was determined by using a modification of the procedure of Hsu (17). HeLa cells were suspended in fresh MEM-Spinner medium supplemented with 5% fetal bovine serum at a concentration of 10^6 cells per ml. After 4 h, the cells were treated with amethopterin (1 μM) and adenosine (50 μM). After an additional 16 h, the cells were released into the S phase by the addition of thymidine (2 mg/liter) to the culture. At various times, 2×10^6 cells were removed from the culture and pelleted at 200 × *g* for 3 min. The supernatant was decanted, and the cells were suspended in 5 ml of hypotonic buffer (one part MEM, two parts KCl [0.075 M]). After 7 min, the cells were pelleted at 200 × *g* for 3 min, the supernatant was decanted, and 5 ml of fixative (three parts methyl alcohol, one part glacial acetic acid) was added. The pellet was left undisturbed for 20 min, after which the cells were suspended and pelleted at 200 × *g* for 3 min. The supernatant was decanted, and 5 ml of fresh fixative was added. This process was repeated three times, after which the final volume of fixative was reduced to 1 ml, and the cells were suspended. The cell suspensions were spread onto microscope slides and allowed to air dry. The cells were stained with Giemsa stain for 10 min and observed by phase microscopy. The percentage of cells in mitosis, the mitotic index, was then calculated.

DNA synthesis in whole cells. DNA synthesis in synchronized HeLa cells was measured by a modification of the method of Hershey et al. (16). Cells were suspended in fresh MEM-Spinner medium supplemented with 5% fetal bovine serum, at a concentration of 10^6 cells per ml. After 4 h, the cells were treated with amethopterin (1 μM) and adenosine (50 μM). The culture was divided equally 7 h later. One culture was infected with reovirus at a multiplicity of infection (MOI) of 200 PFU per cell, and the second culture was mock infected with saline sodium citrate, pH 7.4. After 9 h, the cultures were released by the addition of thymidine (2 mg/liter). At selected times, three 1-ml samples were removed from each culture and placed into test tubes. Tritium-labeled thymidine (ICN Pharmaceuticals, Inc.; 77 Ci/mmol) was added to each tube to give a final concentration of 2.5 μCi/ml. The tubes were incubated at 37°C in a shaking water bath for 15 min, after which the labeling was stopped by placing the tubes in an ice bath for 10 min, and the amount of incorporated radioactivity was determined.

DNA synthesis in isolated nuclei. DNA synthesis in nuclei isolated from synchronized HeLa cells was measured by modifications of the procedures of Friedman et al. (8, 9), Hershey et al. (16), Krokan et al. (18, 19), Reinhard et al. (22), Gautschi et al. (10), and Brun and Weissbach (1). HeLa cells were treated with amethopterin and adenosine as described above. The culture was either immediately divided equally or divided 7 h later, depending on the experiment as described below. In both cases one of the cultures was infected with reovirus at an MOI of 200 PFU per cell, and the other culture was mock infected with saline sodium citrate, pH 7.4. At the end of the 16-h synchronization period, the cells were harvested by centrifugation at 800 × *g* for 5 min at 4°C. The pellet was suspended in buffer A (9 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.9, 5 mM 2-mercaptoethanol, 1 mM EGTA, and 4.5 mM $MgCl_2$) at a concentration of 2×10^6 cells per ml. The suspension was centrifuged at 800 × *g* for 5 min at 4°C, and the pellet was suspended in buffer A at a concentration of 3.3×10^7 cells per ml for 10 min at 0°C. The swelled cells were then lysed with a Dounce homogenizer, and the resulting homogenate was centrifuged at 800 × g for 5 min at 0°C. The supernatant was removed and used immediately or stored at −70°C. (The supernatant will be referred to below as the enucleated cytoplasmic extract.) The pellet was suspended in buffer A at a concentration of 2×10^7 nuclei per ml, distributed to test tubes at a concentration of 4×10^6 nuclei per tube, and held at 0°C while the cell-free DNA synthesis reagents were added. To each tube containing nuclei, 0.4 ml of the specified enucleated cytoplasmic extract was added. In addition, 0.4 ml of assay mixture (40 mM HEPES, pH 7.8, 80 mM KCl, 4 mM 2-mercaptoethanol, 1.5% [wt/vol] dextran [10,500 M_w], 225 mM sucrose, 1 mM EGTA, 4 mM $MgCl_2$, 4 mM ATP, and 0.3 mM each of dATP, dCTP, dGTP, and dTTP) was added to each tube. Methyl-tritiated dTTP (ICN; 60 Ci/mmol) was added to each tube to a final concentration of 2.5 μCi/ml. The tubes were transferred to a shaking water bath at 37°C. At the indicated times, the tubes were transferred to an ice bath, and the amount of incorporated radioactivity was determined.

DNA synthesis in permeabilized cells. DNA synthesis in permeabilized HeLa cells was measured by a modification of the procedure previously described for measuring DNA synthesis in isolated nuclei. Permeabilization was monitored by the loss of the ability of permeabilized cells to exclude trypan blue dye. Cells were synchronized, processed, and infected as described for DNA synthesis measurement in isolated nuclei. After 16 h, the cells were permeabilized by swelling in buffer A at a concentration of 3.3×10^7 cells per ml for 10 min at 0°C. After permeabilization, the cells were distributed to test tubes at a concentration of 4×10^6 permeabilized cells per tube. The cell-free DNA synthesis reagents were added, and the cells were incubated as previously described for the incubation of isolated nuclei.

Measurement of incorporated radioactivity. The contents of each assay tube were layered onto Gelman 25-mm type E fiberglass filters wetted with cold 0.85% NaCl. The filters were washed three times with cold 0.85% NaCl (2 ml per wash), and the acid-insoluble materials were precipitated by three washes of a solution containing 10% trichloroacetic acid and 1% sodium pyrophosphate (2 ml per wash). The

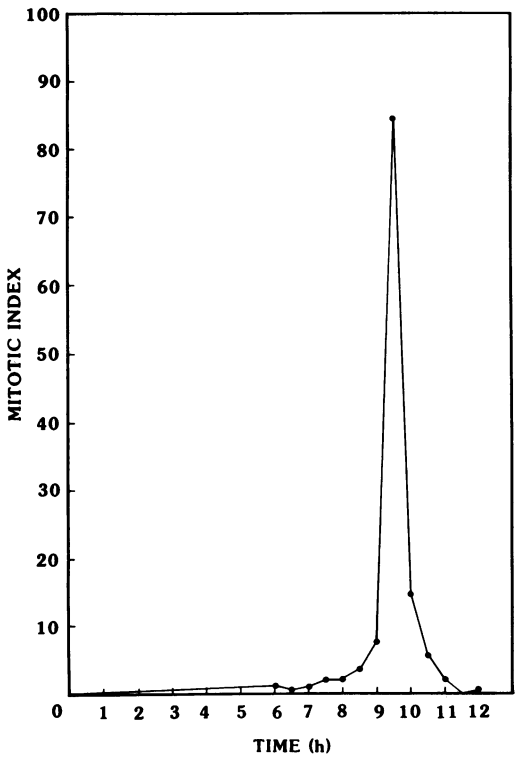


FIG. 1. Mitotic index of HeLa cells synchronized by treatment with amethopterin. HeLa cells were suspended in fresh medium, incubated for 4 h, and then treated with amethopterin and adenosine and incubated for an additional 16 h. At this time, time zero, the synchronized cultures were released by the addition of thymidine. Samples were removed at the indicated times, and the percentage of cells undergoing mitosis was determined. These results represent the average of two experiments.

filters were placed into scintillation vials and dried for 1 h at 37°C. Beckman Cocktail D liquid scintillation fluid (6 ml; 5 g of diphenyloxazole, 100 g of naphthalene, 10 ml of water, and dioxane added to 1 liter) was added to each vial, and the radioactivity was determined by using a Beckman LS8100 liquid scintillation counter.

Stimulation of initiation sites in subcellular systems. At various times during the incubation of either permeabilized cells or isolated nuclei in the indicated assay mixtures, three ribonucleotide triphosphates (rNTP's) were added to stimulate additional DNA synthesis through the production of RNA primers (1). The three rNTP's added were rCTP, rGTP, and rUTP, each to a final concentration of 0.5 mM.

Isolation and sedimentation of nucleoids. Nucleoids were isolated by the procedure described by Mattern and Painter (21). HeLa cells were treated with amethopterin (1 μM) and adenosine (50 μM) as previously described except that [^{3}H]thymidine (1 μCi/ml) was added to the culture when the cells were suspended in fresh medium. At 7 h after the addition of amethopterin and adenosine, the culture was divided equally. One culture was infected with reovirus at an MOI of 200 PFU per cell, and the second culture was mock infected with saline sodium citrate, pH 7.4. After 9 h, 2×10^6 whole cells or 2×10^6 permeabilized cells, prepared as described for DNA synthesis in permeabilized cells, or 2×10^6 nuclei, prepared as described for DNA synthesis in isolated nuclei, were pelleted by centrifugation at $800 \times g$ for 10 min at 4°C. The pellet was suspended in 0.5 ml of buffer (0.15 M NaCl, 0.015 M sodium citrate [pH 8.0]) and layered onto an 18-ml neutral sucrose gradient (15 to 30% in 1.9 M NaCl, 0.01 M EDTA, 0.01 M Tris-hydrochloride [pH 8.0]) onto which 0.5 ml of lysis buffer (0.1% Triton X-100, 0.02 M EDTA, 0.01 M Tris-hydrochloride [pH 8.0]) had been previously layered. The gradient was incubated for 30 min at 25°C and centrifuged in an SW27 rotor at 10,000 rpm for 90 min at 20°C. The gradients were then fractionated from the top, and the radioactivity was determined in each fraction by liquid scintillation counting.

RESULTS

Synchronization of HeLa S3 cells with amethopterin. To study the ability of reovirus-infected cells to initiate DNA synthesis, it was important to establish a method of cell synchronization with respect to the S phase of the cell cycle. HeLa cells were treated with amethopterin and adenosine for 16 h, a treatment commonly used to synchronize HeLa cells by preventing entry into the S phase (10). After 16 h, the cells were released into the S phase by the addition of thymidine to the culture, and the mitotic index was determined at selected times after release. It was apparent that the amethopterin-treated cells were highly synchronized,

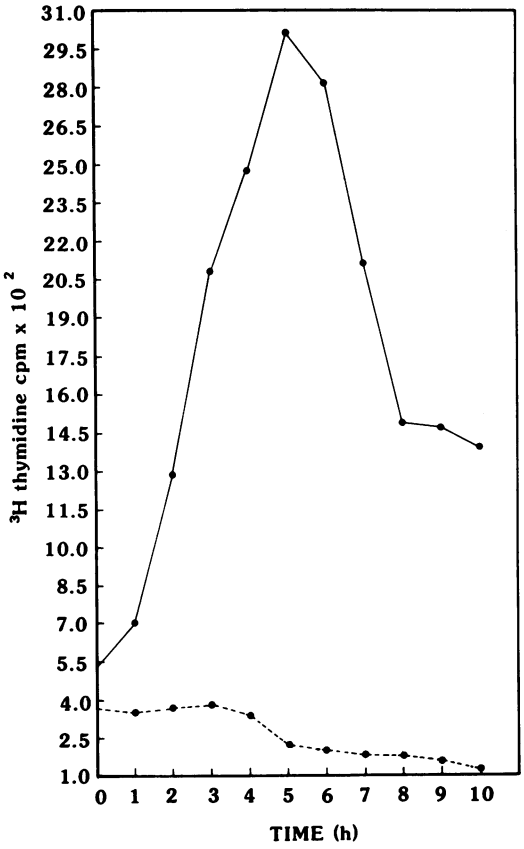


FIG. 2. DNA synthesis in synchronized HeLa cells infected for 9 h. HeLa cells were suspended in fresh medium and incubated for 4 h. Amethopterin and adenosine were added, and 7 h later, one-half of the culture was infected with reovirus at 200 PFU per cell (●---●), and the second half was mock infected (●—●). After 9 h, the cultures were released by the addition of thymidine. At the indicated times, 10^6 cells were removed and pulse-labeled for 15 min with [*methyl*-^{3}H]thymidine. Trichloroacetic acid-insoluble counts per 10^6 cells are shown. These results represent the average of two experiments.

TABLE 1. Replication of reovirus type 3 in amethopterin-treated and untreated HeLa cells

Time of infection (h)	Amethopterin treatment	Intracellular virus (PFU per cell ± SD)	% Reduction in infectious virus
9	No	35.15 ± 3.4	
9[a]	Yes	23.20 ± 2.0	34
16	No	162.50 ± 10.7	
16[b]	Yes	64.49 ± 6.1	60

[a] Cells were infected 7 h after amethopterin treatment.
[b] Cells were infected immediately after amethopterin treatment.

with ca. 85% undergoing mitosis 9.5 h after entry into the S phase (Fig. 1).

Effect of reovirus on DNA synthesis in synchronized HeLa cells. It was reported by Cox and Shaw that synchronized L cells infected with reovirus for 8 h failed to initiate DNA synthesis (4). HeLa cells infected with reovirus and synchronized with amethopterin were examined to determine whether a similar inhibition pattern occurred. Amethopterin-treated HeLa cells were infected at an MOI of 200 PFU per cell 9 h before release into the S phase. The cells infected with reovirus for 9 h failed to initiate DNA synthesis (Fig. 2).

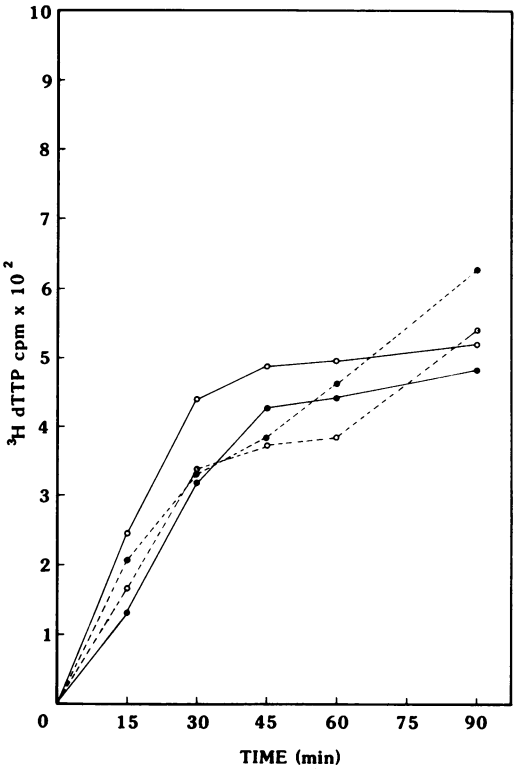


FIG. 3. DNA synthesis in nuclei isolated from synchronized HeLa cells infected for 9 h. HeLa cells were synchronized and infected for 9 h as described in the legend to Fig. 2. The nuclei were isolated and placed in a cell-free DNA-synthesizing system. The nuclei from uninfected cells were incubated in the presence of enucleated cytoplasmic extracts from uninfected cells (●—●) or infected cells (○—○). The nuclei from infected cells were incubated in the presence of enucleated cytoplasmic extracts from uninfected cells (●---●) or infected cells (○---○). Incorporation of [*methyl*-^{3}H]dTTP into acid-insoluble counts per 4 × 10^6 nuclei is shown. These results represent the average of two experiments.

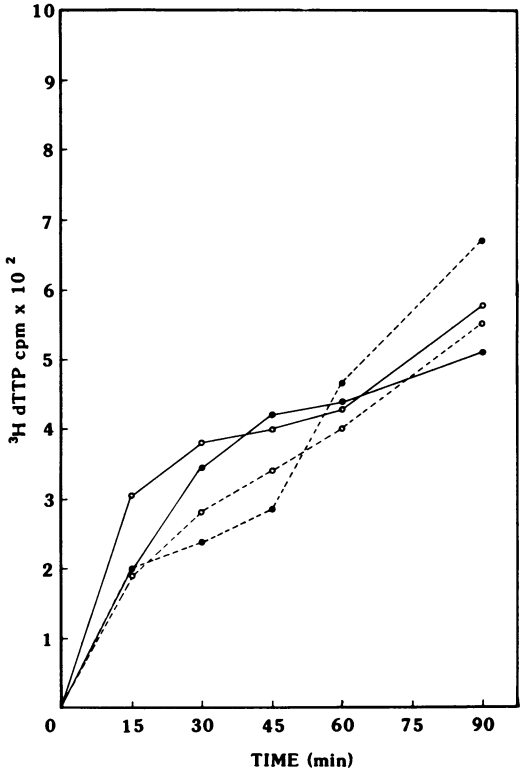


FIG. 4. DNA synthesis in nuclei isolated from synchronized HeLa cells infected for 16 h. HeLa cells were suspended in fresh medium and incubated for 4 h. Amethopterin and adenosine were added, one-half of the culture was infected with reovirus at 200 PFU per cell, and the second half was mock infected. After 16 h, the nuclei were isolated and placed in a cell-free DNA-synthesizing system. The nuclei from uninfected cells were incubated in the presence of enucleated cytoplasmic extracts from uninfected cells (●—●) or infected cells (○—○). The nuclei from infected cells were incubated in the presence of enucleated cytoplasmic extracts from uninfected cells (●---●) or infected cells (○---○). Incorporation of [*methyl*-^{3}H]dTTP into acid-insoluble counts per 4 × 10^6 nuclei is shown. These results represent the average of two experiments.

Effect of amethopterin on reovirus replication. Since amethopterin-synchronized cells were infected with reovirus while these cells were exposed to amethopterin, it was of interest whether reovirus replication was modified by this treatment.

Amethopterin-treated cells were exposed to 200 PFU of reovirus per cell either at the time of amethopterin treatment or 7 h later. At 16 h after amethopterin treatment, both intracellular virus infectivity and viral antigens were measured. More than 95% of cells infected for 9 or 16 h (0 to 7 h after amethopterin treatment) showed the presence of viral antigens as determined by indirect immunofluorescence staining (data not shown). However, amethopterin caused a time-dependent reduction in the production of intracellular infectious reovirus particles (Table 1). Thus, although amethopterin-treated cells supported viral antigen production, amethopterin caused a measurable inhibition of the production of infectious virus but no apparent effect on the virus-induced inhibition of cellular DNA synthesis.

Effect of reovirus on DNA synthesis in nuclei isolated from synchronized HeLa cells. To determine whether the inhibition of initiation of DNA synthesis in cells infected with reovirus was due to a permanent alteration of the DNA replication complex or to reversible interactions between the

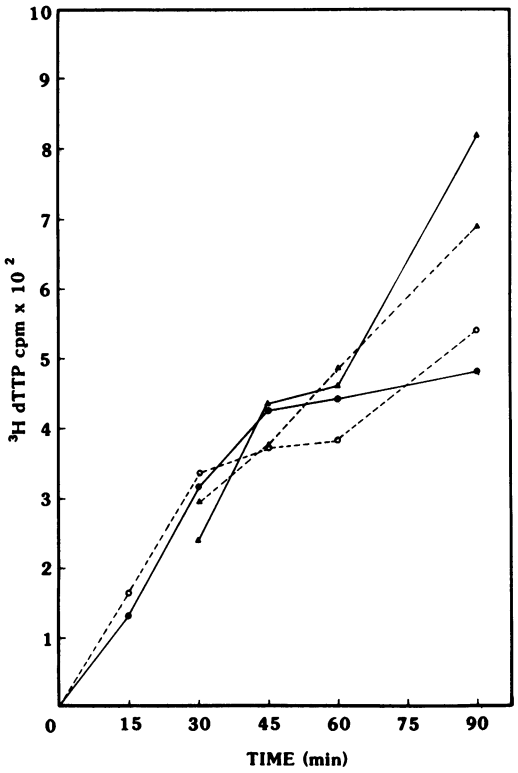


FIG. 5. Stimulation of DNA synthesis in nuclei isolated from synchronized HeLa cells infected for 9 h. HeLa cells were synchronized and infected for 9 h as described in the legend to Fig. 2. The nuclei were isolated and placed in a cell-free DNA-synthesizing system. The nuclei from uninfected cells were incubated in the presence of enucleated cytoplasmic extracts from uninfected cells, with (▲—▲) or without (●—●) the addition of rNTP's after 30 min of incubation. The nuclei from infected cells were incubated in the presence of enucleated cytoplasmic extracts from infected cells, with (△---△) or without (○---○) the addition of rNTP's after 30 min of incubation. Incorporation of [*methyl*-^{3}H]dTTP into acid-insoluble counts per 4 × 10^6 nuclei is shown. These results represent the average of two experiments.

virus or virus products and the nuclear complex, we isolated nuclei from synchronized, infected cells and measured their ability to regain the capacity to synthesize DNA. Synchronized cultures of cells were infected with 200 PFU per cell for 9 or 16 h (Fig. 2), at which times the cells were unable to synthesize DNA. The nuclei from infected and uninfected cells were then isolated and transferred to in vitro DNA-synthesizing systems. A restored ability to synthesize DNA was observed in nuclei isolated from infected cells (Fig. 3 and 4). In addition to a restored ability to synthesize DNA, nuclei isolated from infected cells were also able to initiate new DNA synthesis. This was demonstrated by the ability of rNTP's to stimulate new DNA synthesis in the isolated nuclei (Fig. 5), reported to be the result of the generation of Okazaki fragments and the initiation of new replicons (1).

The addition of enucleated cytoplasmic extracts from either infected or uninfected cells did not affect the rate of DNA synthesis observed in nuclei isolated from these cells (Fig. 3 and 4).

Effect of reovirus on DNA synthesis in synchronized permeabilized HeLa cells. Since the inhibition of the initiation of DNA synthesis by reovirus did not appear to be due to a permanent alteration of the replication complex, an attempt was made to relate reovirus-mediated inhibition of cellular DNA synthesis to the structural and possibly metabolic integrity of the infected cell. To preserve unique interactions between the virus or viral products and cellular components but allow for the passage of nucleotides into the cytoplasm and the nucleus, cells were permeabilized by swelling in a hypotonic buffer. Synchronized cultures of cells were permeabilized after infection with 200 PFU per cell for 9 or 16 h. After permeabilization, molecules that were excluded by the intact cells, such as trypan blue and nucleotide triphosphates, were able to enter both the cytoplasm and the nuclei of these cells. The increased permeability, the presence of nucleotide triphosphates, and the addition of rNTP's were all insufficient to restore the ability of permeabilized, infected cells to initiate DNA synthesis (Fig. 6 and 7). Thus, the maintenance of some aspect of cellular integrity prevented reversal of the virus-mediated inhibition of DNA synthesis. The addition of enucleated cytoplasmic extracts from infected cells to permeabilized uninfected cells resulted in an increase in the rate of DNA synthesis in these cells (Fig. 7).

Reovirus antigens associated with infected HeLa cells and isolated nuclei. Reovirus antigens are known to accumulate in infected cells in perinuclear inclusions (26). Since removal of the nucleus from an infected cell restores its ability to

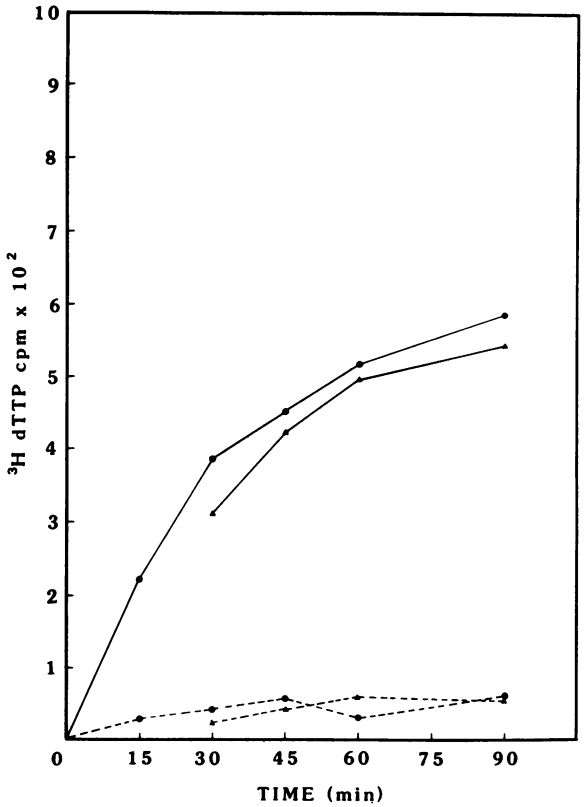


FIG. 6. DNA synthesis in permeabilized, synchronized HeLa cells, infected for 9 h. HeLa cells were synchronized and infected for 9 h as described in the legend to Fig. 2. The cells were then permeabilized. Uninfected, permeabilized cells were incubated directly in the assay mixture (●—●) or in the presence of rNTP's after 30 min of incubation (▲—▲). Infected, permeabilized cells were incubated directly in assay mixture (●---●) or in the presence of rNTP's after 30 min of incubation (▲---▲). Incorporation of [*methyl*-^{3}H]dTTP into acid-insoluble counts per 4 × 10^6 permeabilized cells is shown. These results represent the average of two experiments.

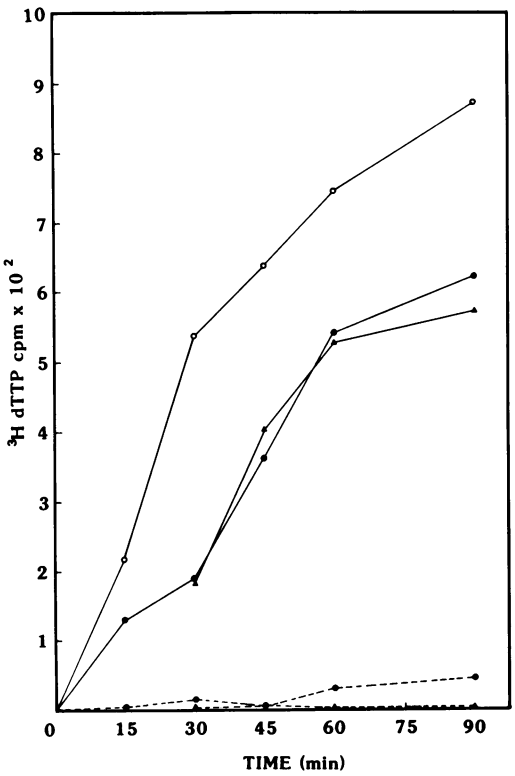


FIG. 7. DNA synthesis in permeabilized, synchronized HeLa cells, infected for 16 h. HeLa cells were synchronized and infected for 16 h as decribed in the legend to Fig. 4. The cells were then permeabilized. Uninfected, permeabilized cells were incubated directly in the assay mixture (●—●), in the presence of rNTP's after 30 min of incubation (▲—▲), or in the presence of enucleated cytoplasmic extracts from infected cells (○—○). Infected, permeabilized cells were incubated directly in the assay mixture (●---●) or in the presence of rNTP's after 30 min of incubation (▲---▲). Incorporation of [*methyl*-^{3}H]dTTP into acid-insoluble counts per 4×10^6 permeabilized cells is shown. These results represent the average of two experiments.

synthesize DNA, the distribution of viral antigens associated with infected cells and isolated nuclei was of interest. HeLa cells were treated with amethopterin and infected with 200 PFU per cell. After 9 h, samples were removed and analyzed for the presence of viral antigens by indirect immunofluorescence. More than 95% of the infected cells displayed viral antigens (Fig. 8A), and nonspecific fluorescence was observed at very low levels in uninfected cells (Fig. 8B). When the nuclei were isolated from infected cells and subjected to indirect immunofluorescence, more than 95% retained accumulations of viral antigens (Fig. 9A). These nuclei also retained large amounts of associated cellular material (Fig. 9C and D). Nonspecific fluorescence occurred at very low levels in nuclei isolated from uninfected cells (Fig. 9B). The nuclei isolated from infected cells retained accumulations of cytoplasmic components and viral antigens (Fig. 9A and C), yet they displayed a restored ability to synthesize and initiate DNA synthesis.

Effect of reovirus on nucleoid sedimentation. In an attempt to further define the nature of the reversibility of reovirus inhibition of DNA synthesis associated with the removal of the nucleus, the effect on nucleoid sedimentation after this removal was examined.

Nucleoids isolated from synchronized, infected cells showed an increased rate of sedimentation when compared with nucleoids isolated from synchronized, uninfected cells (Fig. 10). These infected cells were also unable to initiate DNA synthesis (Fig. 2).

Nucleoids isolated from synchronized, infected and permeabilized cells retained the increased rate of sedimentation over uninfected, permeabilized cells also seen with the

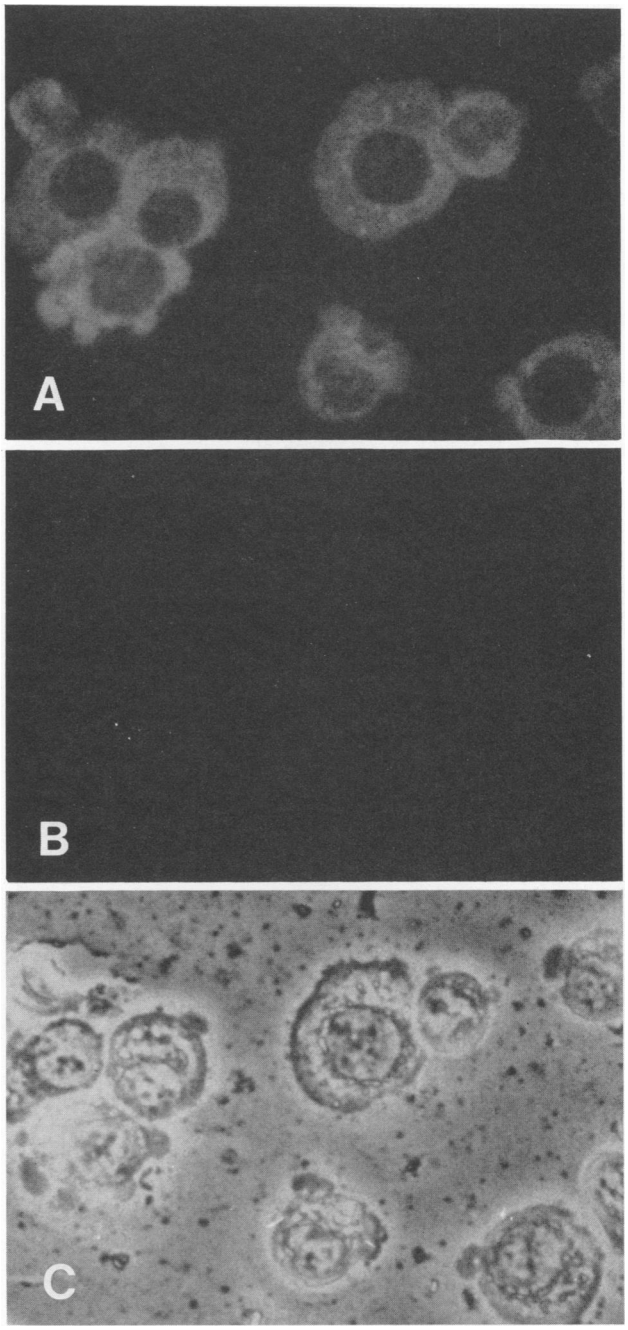
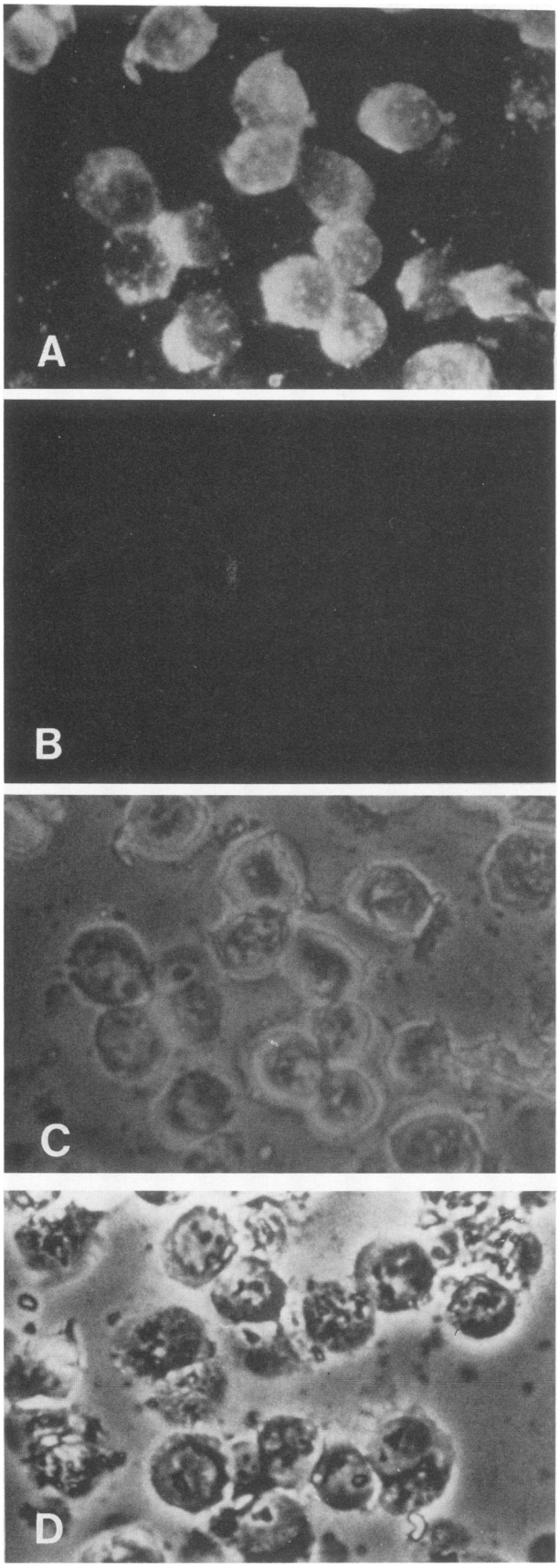


FIG. 8. Immunofluorescence and phase microscopy of reovirus-infected and uninfected, synchronized HeLa cells. HeLa cells were synchronized and infected for 9 h as described in the legend to Fig. 2. Samples were then removed and subjected to indirect immunofluorescence staining or phase microscopy. (A) Indirect immunofluorescence of reovirus-infected HeLa cells; (B) indirect immunofluorescence of mock-infected HeLa cells; (C) phase microscopy of reovirus-infected HeLa cells.



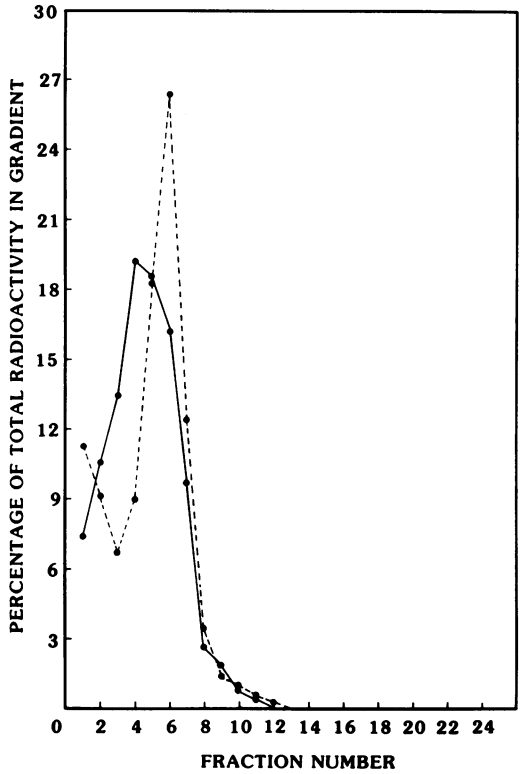


FIG. 10. Sedimentation profiles of nucleoids isolated from infected and uninfected HeLa cells. HeLa cells were synchronized and infected for 9 h as described in the legend to Fig. 2. The cells were then layered onto linear neutral sucrose gradients. Nucleoids were isolated from infected cells (●---●) or mock-infected cells (●—●). Sedimentation is from left to right. These results represent the average of three experiments.

nucleoids isolated from intact infected cells (Fig. 11). These permeabilized, infected cells were also unable to initiate DNA synthesis (Fig. 6).

Nucleoids isolated from the nuclei of synchronized, infected cells regained a rate of sedimentation similar to the rate of sedimentation present in nucleoids isolated from the nuclei of synchronized, uninfected cells (Fig. 12). These nuclei were also able to synthesize and initiate DNA synthesis (Fig. 3 and 5).

DISCUSSION

HeLa cells synchronized by amethopterin at a stage in the cell cycle before entry into the S phase were prevented from initiating DNA synthesis 9 h after reovirus infection. The interval of time after infection that was required to com-

FIG. 9. Immunofluorescence and phase microscopy of nuclei isolated from reovirus-infected and uninfected, synchronized HeLa cells. HeLa cells were synchronized and infected for 9 h as described in the legend to Fig. 2. The nuclei were then isolated and subjected to indirect immunofluorescence staining or phase microscopy. (A) Indirect immunofluorescence of nuclei isolated from reovirus-infected HeLa cells; (B) indirect immunofluorescence of nuclei isolated from mock-infected HeLa cells; (C) phase microscopy of the nuclei isolated from reovirus-infected HeLa cells; (D) phase microscopy of the nuclei isolated from mock-infected HeLa cells.

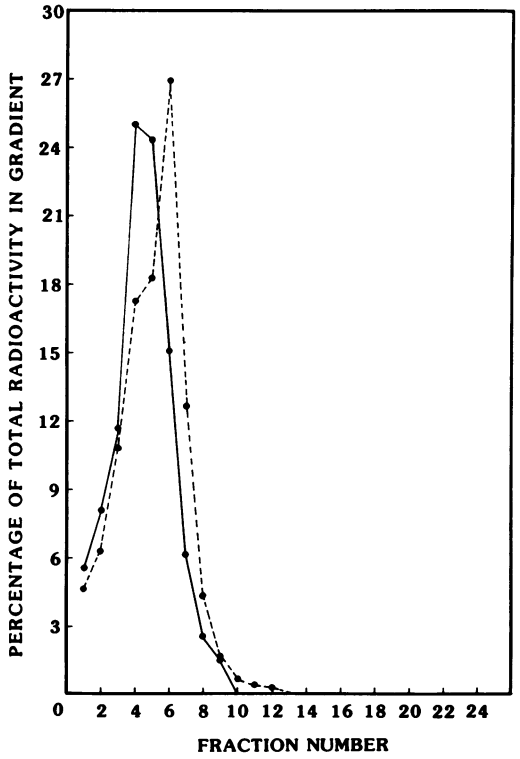


FIG. 11. Sedimentation profiles of nucleoids isolated from permeabilized, infected and uninfected HeLa cells. HeLa cells were synchronized and infected for 9 h as described in the legend to Fig. 2. The cells were then permeabilized and layered onto linear neutral sucrose gradients. Nucleoids were isolated from infected, permeabilized cells (●---●) or mock infected, permeabilized cells (●—●). Sedimentation is from left to right. These results represent the average of three experiments.

pletely inhibit the initiation of cellular DNA synthesis in HeLa cells was in agreement with the time interval observed in other cell systems (4, 6, 13).

When nuclei were isolated from synchronized cells infected with reovirus for 9 or 16 h and placed in a cell-free DNA-synthesizing system, a complete restoration of the capacity for DNA synthesis of the nuclei was observed. The isolated nuclei demonstrated the ability to synthesize DNA at levels equal to that of nuclei isolated from uninfected cells. In addition, these nuclei were also able to initiate new DNA synthesis as shown by rNTP-mediated stimulation of additional DNA synthesis in the isolated nuclei. This suggests that the nuclei are able to synthesize RNA primers, an ability which is unclear in reovirus-infected whole cells. The procedure used to isolate the nuclei was gentle and resulted in the nuclei being contaminated with considerable cytoplasmic components and viral antigens. In spite of the incomplete removal of these components, a complete restoration of the capacity for DNA synthesis of the nuclei isolated from reovirus-infected cells was achieved.

This restoration of the capacity for DNA synthesis may be due to removal of the replication complex from an inhibitory environment present in the infected cell or to increased accessibility to precursors. Damage by reovirus infection to the components necessary for the initiation and maintenance of cellular DNA synthesis appears to be reversible. The restoration of the capacity for DNA synthesis of nuclei isolated from reovirus-infected cells appears to be unrelated to the virus replication cycle. Restoration is possible 9 h after infection, a time corresponding to the achievement of the complete inhibition of initiation of host cell DNA synthesis and production of low levels of infectious virus. Restoration is also possible 7 h later, 16 h after infection, a time when host cell DNA and protein synthesis are inhibited (11) and large amounts of infectious intracellular virus are present. This provides further evidence that reovirus-mediated inhibition of initiation of cellular-DNA synthesis is specific and not the result of a generalized destruction of the host cell.

The addition of enucleated cytoplasmic extracts from infected or uninfected cells to the cell-free DNA-synthesizing system containing nuclei isolated from infected or uninfected cells had no effect on the capacity of these nuclei to synthesize DNA. It is possible that the absence of any effect by these cytoplasmic extracts was due to a disruption of the structural or metabolic integrity of the infected cell, which may result in a loss of the cellular site of action. It should be noted that isolation of the nuclei from infected cells does not result in removal of the accumulations of viral antigens normally associated with the perinuclear region of intact, infected cells and that the isolated nuclei already contain considerable cytoplasmic material.

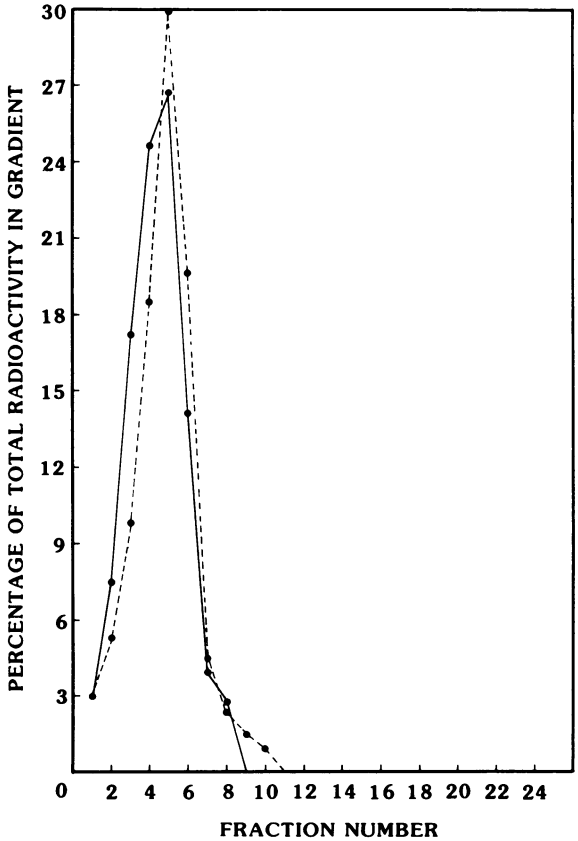


FIG. 12. Sedimentation profiles of nucleoids isolated from the nuclei of infected and uninfected HeLa cells. HeLa cells were synchronized and infected for 9 h as described in the legend to Fig. 2. The nuclei were then isolated and layered onto linear neutral sucrose gradients. Nucleoids were isolated from the nuclei of infected cells (●---●) or from the nuclei of mock infected cells (●—●). Sedimentation is from left to right. These data represent the average of three experiments.

Permeabilization of reovirus-infected cells did not restore the capacity for DNA synthesis of these cells. It appears that permeabilization is an insufficient treatment to remove or alter the inhibitory factor(s) associated with reovirus-infected cells. The addition of enucleated cytoplasmic extracts from infected cells to uninfected, permeabilized cells did not cause an inhibition of cellular DNA synthesis but stimulated DNA synthesis in these cells. Reovirus inhibition of cellular DNA synthesis may cause an accumulation of certain precursors of DNA synthesis. These precursors, when added as cytoplasmic extracts from infected cells to permeabilized, uninfected cells, could produce the stimulation of DNA synthesis seen in these cells.

Nucleoids isolated from both reovirus-infected whole cells and permeabilized cells display an increased rate of sedimentation when compared with nucleoids isolated from uninfected whole cells and permeabilized cells and are unable to initiate DNA synthesis. This altered sedimentation is most likely due to an alteration in the DNA supercoiling present in these cells (21). Although the procedure used (21) is designed to measure alterations in DNA supercoiling, the possibility exists that reovirus may be increasing the nucleoid sedimentation rate by some other unknown mechanism. This mechanism would have to result in an alteration of nucleoid sedimentation yet allow for the reversibility of replicative activity.

In contrast, when the sedimentation of nucleoids isolated from the nuclei of reovirus-infected cells was compared with that of uninfected cells, similar sedimentation profiles were seen, indicating similar degrees of DNA supercoiling. The removal of the nucleus from a reovirus-infected cell restores its ability to synthesize and initiate DNA synthesis and is associated with a shift in DNA supercoiling such that it resembles that found in nuclei isolated from uninfected cells. This suggests that reovirus is preventing the initiation of cellular DNA synthesis by acting directly or indirectly in a manner which prevents normal levels of DNA supercoiling. Whether the altered supercoiling is the cause of the inhibition of initiation or the result of the inhibition is not known. These findings are in apparent conflict with a report (2) that reovirus prevents recondensation of chromatin.

In summary, it appears that the inhibition of initiation of DNA synthesis by reovirus type 3 is not due to a permanent alteration of the replication complex but is reversible by the removal of that complex from factors unique to the structural or metabolic integrity of the infected cell and that this removal is associated with a return to normal levels of DNA supercoiling.

Cellular DNA synthesis in eucaryotic cells is highly regulated, and a disruption of the regulation may be responsible for, or the result of, transformation of normal cells to cancerous cells. It can be postulated that events associated with reovirus replication uniquely affect cellular factors present in transformed cells but absent or present in a modified state in normal cells. These virus-specific modifications appear to be selective for the factors which directly affect the initiation sequence of transformed-cell DNA synthesis. If so, reovirus may be useful in analyzing the differences in the control of DNA function in normal and transformed cells.

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