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Synthesis of Viral DNA in the Cytoplasm of Duck Embryo Fibroblasts and in Enucleated Cells after Infection by Avian Sarcoma Virus

(RNA tumor virus/cell enucleation/nucleic acid hybridization)

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ABSTRACT **Two lines of evidence indicate that synthesis of viral DNA occurs in the cytoplasm of duck embryo fibroblasts infected with avian sarcoma virus: (*i*) viral DNA is detected first in the cytoplasmic fraction of infected cells and subsequently in the nuclear fraction; and (*ii*) viral DNA is synthesized at a normal rate in cells infected after enucleation with cytochalasin B. The presence of viral DNA in the cytoplasmic fraction is not a consequence of leakage of newly synthesized viral DNA from the nucleus, since it remains in nuclear fractions late after infection when integration of viral DNA into the host genome is inhibited by ethidium bromide.**

After infection of susceptible cells by RNA tumor viruses, RNA of the input virus is transcribed into viral DNA, which is subsequently integrated into the host cell genome as provirus (1). The biochemistry of viral DNA synthesis, integration, and transcription is largely unknown. Studies with inhibitors (2, 3) and direct measurement with molecular hybridization techniques (4, 5) indicate that viral DNA synthesis occurs early after infection, but the intracellular site of its synthesis has not been unambiguously established (6–8).

The present study was undertaken to establish the intracellular site of viral DNA synthesis by direct measurement in cytoplasmic extracts of infected cells and in infected enucleated cells. We find that early after infection viral DNA can be detected exclusively in the cytoplasmic fraction of the cell and that it later appears in the nucleus, where it eventually becomes covalently linked to the host genome (4). These results are supported by the observation that synthesis of viral DNA occurs at a normal rate in cells infected after enucleation by cytochalasin B.

METHODS AND MATERIALS

Cells and Viruses. Pekin duck embryo fibroblast cells are susceptible to avian sarcoma viruses (ASV) of subgroup C (e.g., B77 strain of ASV) and are permissive for replication of ASV at normal rates, but they are devoid of any detectable endogenous ASV-specific sequences (4). Primary cells were prepared from embryonated eggs (12–14 days old; obtained from Reichardt Duck Farm, Petaluma, Calif.) and were propagated in Medium 199 (GIBCO) supplemented with 10% (v/v) tryptose phosphate broth, 0.1% (w/v) sodium bicarbonate, 5% (v/v) calf serum, and 1.5% (v/v) heat-inactivated chicken serum. B77 strain of ASV was grown in chick embryo fibroblast cells either in Falcon petri dishes or roller bottles (Belco). To prepare virus for infection of small numbers of enucleated cells, we clarified medium from virus-producing cells at 7500 rpm (Sorvall RC-3) for 10 min and centrifuged at 19,000 rpm for 2 hr at 4° (Spinco 19 rotor). The viral pellet was resuspended in 1–5% of the starting volume of medium, and briefly sonicated in a Bransonic bath sonicator. Infection of intact or enucleated cells was carried out as described in the legends. Virus titers were determined by focus formation on chicken cell monolayers; titers were 2 to 10 $\times$ 10^6 focus-forming units (FFU)/ml for medium harvested from virus-producing cells and 1 to 5 $\times$ 10^8 FFU/ml for concentrated virus. The efficiency of plating B77 on duck cells is about 40% relative to chicken cells.

Cell Fractionation. Cells were scraped into medium 199, collected by centrifugation, washed twice, and allowed to swell for 5 min at 0° in a hypotonic buffer containing 10 mM NaCl, 1 mM Tris·HCl (pH 7.4), 0.15 mM $MgCl_2$. Deoxycholate and NP-40 were added to give a final concentration of 1.0% and 0.5% (w/v), respectively, and the cells were broken by five strokes in a glass Dounce homogenizer. The disrupted cells were centrifuged at 800 $\times$ g for 10 min and the supernatant (cytoplasm) was separated from pelleted nuclei. Alternatively, the cells were swollen for 5 min NaCl–Tris·HCl–$MgCl_2$ buffer containing 0.5% NP-40, broken, and centrifuged as above. The two methods gave similar results.

Cell Enucleation. Primary cultures of duck embryo fibroblasts were trypsinized and seeded at a density of about 2 $\times$ $10^5/cm^2$ on plastic disks cut from 75-cm^2 T-flasks (Falcon). Before use, the disks were gently rubbed with fine sandpaper and exposed to concentrated sulfuric acid for 10 min to augment cell adhesion; they were then washed with distilled water and sterilized by treatment with 70% ethanol overnight. After 1 day of growth, secondary cultures were placed in centrifuge tubes filled with warmed medium containing 10 or 15 μg/ml of cytochalasin B (Aldrich). Two disks cut to fit into 50-ml plastic centrifuge tubes (Sorvall) were placed back-to-back in each of four tubes and centrifuged for 40 min at 11,000 rpm between 35 and 39° in warmed HB-4 rotor in a Sorvall RC-2B centrifuge. About 10^7 cells could be processed in each centrifugation. After centrifugation, cells

Abbreviations: ASV, avian sarcoma virus; cDNA, single-stranded DNA complementary to the viral genome; srDNA, slowly reassociating fraction of the double-stranded DNA product of ASV polymerase; FFU, focus-forming units; C_0t, product of concentration of nucleotide sequences of DNA and time of incubation.

TABLE 1. *Evaluation of enucleation procedure*

Radioisotope	Period of labeling	Cl_3CCOOH-precipitable cpm	
		Control cells	Enucleated cells
(*a*) [^{14}C]Thymidine and	16 hr before enucleation	10.5×10^3	2.6×10^2
[^{3}H]aminoacids		3.7×10^2 ($^{14}C/^3H = 28.5$)	2.6×10^2 ($^{14}C/^3H = 1$) (% enucleation = 96)
(*b*) [^{3}H]Thymidine	0–4 hr after enucleation	1.5×10^5	2.1×10^3 (% of control = 1.3)
(*c*) [^{3}H]Aminoacids	0–8 hr after enucleation	3.4×10^3	1.2×10^3 (% of control = 35)

10^6 duck embryo fibroblasts were plated on plastic disks and enucleated as described in *Methods and Materials* or kept as controls. A minimum of two disks in each set were (*a*) labeled before enucleation with 1 μCi/ml of [^{14}C]thymidine and 1 μCi/ml of [^{3}H]aminoacids; (*b*) labeled for 4 hr after enucleation with 500 μCi/ml of [^{3}H]thymidine; or (*c*) labeled in 2-hr pulses during the 8 hr after enucleation with 50 μCi/ml of [^{3}H]aminoacids. Incorporation of isotope into acid-precipitable products was determined after lysis of the cells with 0.5% sodium dodecyl sulfate and precipitation with 5% trichloroacetic acid. Averaged results are presented without correction for loss of cells during the enucleation; this loss averaged about 50%, as noted in *Methods and Materials*.

were placed in fresh medium for 30–45 min, until normal cytoplasmic morphology was restored. The extent of enucleation was invariably between 91 and 99%, as evaluated by direct observation of stained disks, by acid-precipitation of cells labeled with [^{3}H]thymidine and [^{14}C]aminoacids, and by incorporation of labeled thymidine (Table 1). Loss of cells during enucleation averaged about 50%, as determined in a Coulter counter after trypsinization. Cell viability was assessed principally by the ability to incorporate labeled amino acids into acid-precipitable molecules; 30–60% of the expected rate of incorporation was observed for 8 hr after centrifugation (Table 1).

DNA Extraction. DNA from whole cells, cytoplasmic and nuclear fractions, or enucleated cells was extracted as described (9). DNA was sheared at 50,000 lbs/inch^2 in an Aminco high pressure cell (9) or boiled for 20 min in 0.3 M NaOH to reduce its size to 300–400 nucleotides (ref. 10, and unpublished data of authors). The DNA was precipitated with ethanol, collected by centrifugation at 8500 $\times$ *g*, and taken up in a small volume of 3 mM EDTA. When possible, DNA concentrations were determined by absorbance at 260 nm or by the diphenylamine method; the concentrations were not measurable in preparations from enucleated cells. About 5–6% of the total DNA was recovered in the cytoplasmic fraction from intact cells. This amount is substantially more than that expected to be found in the cytoplasm. Analysis of the cytoplasmic DNA in a neutral sucrose gradient showed that at least 60–70% of it is much smaller (<4–5 S) than nuclear DNA (>30 S). This result suggests either that very low-molecular-weight DNA leaked from the nucleus during cell fractionation or that DNA from broken nuclei was degraded in the cytoplasmic extract.

Test for Integration. Integration of viral DNA into cell DNA was tested by network formation, the principle and details of which have been described (4). Very briefly, the procedure consists of the following steps: (*a*) isolation of high-molecular-weight DNA; (*b*) denaturation of DNA by boiling for 5 min; (*c*) annealing at 68° in 0.6 M NaCl to a C_0t of about 5 moles-sec/liter; and (*d*) centrifugation at 40,000 rpm in a Spinco type 40 rotor for 15 min at 4°. The supernatant fraction and the DNA that sediments under these conditions (i.e., DNA covalently linked to reiterated host DNA) were sheared and assayed for virus-specific sequences.

Preparation of Hybridization Reagents. Labeled virus-specific DNA (specific activity 1.5×10^4 cpm/ng) was synthesized *in vitro* by the DNA polymerase of detergent-activated ASV as described (9). Single-stranded DNA complementary to the viral genome (cDNA) was prepared in the presence of 100 μg/ml of actinomycin D (Calbiochem) and labeled with [^{3}H]thymidine (Schwartz/Mann, specific activity 57 Ci/mole). Under these conditions, the entire ASV genome is transcribed, although all sequences are not equally represented in the product (11). Double-stranded DNA was similarly labeled in the absence of actinomycin D, and the slowly reassociating fraction (srDNA, $C_0t_{1/2}$ 1×10^{-2} mole-sec/liter) was obtained as described (9). ^{125}I-labeled 70S RNA was prepared by modification of the method described by Commerford (12). Specific activities of 30 to 50 $\times 10^6$ cpm/μg were obtained, and pancreatic ribonuclease sensitivity of the RNA was greater than 95% under the conditions used to assay hybridizations.

Assay for ASV-Specific DNA. Virus-specific DNA was detected either (*a*) by measuring the ability of unlabeled cell DNA to affect the reassociation of ^{3}H-labeled, slowly reassociating double-stranded DNA (srDNA) or (*b*) by measuring the capacity of a sample of DNA to anneal labeled cDNA or 70S RNA. For the first method, 2–3 ng/ml of srDNA was incubated at 68° in 0.4 M phosphate buffer (pH 6.8) in a volume of 50–100 μl. At appropriate times, aliquots were removed and analyzed on hydroxyapatite (Bio-Rad) for the extent of reassociation. C_0t (concentration in moles/liter $\times$ time in seconds) values for the radioactive DNA were computed and plotted against percent reassociated DNA. Reduction in $C_0t_{1/2}$ caused by the cell DNA is proportional to the amount of virus-specific DNA, and copy numbers per cell or genome were computed by the arithmetic given by Gelb *et al.* (13). Copy numbers for cytoplasmic DNA were based on the total number of cells used to extract DNA. For the second method, unlabeled DNA from enucleated or control cells was incubated at 68° for 60–70 hr in a reaction mixture

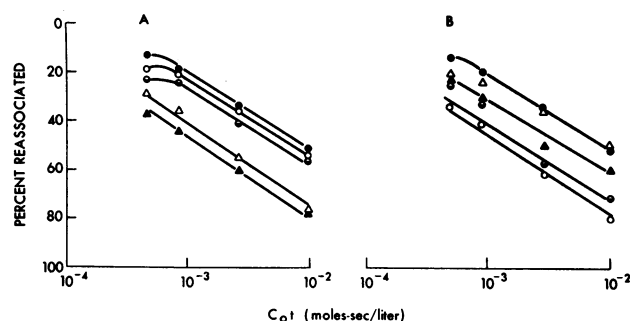

FIG. 1. Synthesis of viral DNA. Duck cells (6–7 × 10^6 cells/100-mm plate) were infected with ASV-B77 in the presence of 2 μg/ml of polybrene at a multiplicity of infection of 3 to 4. At indicated times, cells were harvested and fractionated into cytoplasm and nuclei. DNA was isolated from both fractions and incubated with 2.4 ng/ml of ^{3}H-labeled B77 srDNA (15,000 cpm/ng) at 68° in 0.4 M phosphate buffer in 50 μl. At various times, samples were removed and analyzed for the extent of reassociation on hydroxyapatite. (A) The cytoplasmic DNA was prepared from 1.0 × 10^8, 1.2 × 10^8, 1.0 × 10^8, and 1.0 × 10^8 cells infected for 3 (△), 6 (▲), 9.5 (◒), and 24 (○) hr, respectively. (B) The corresponding concentrations of nuclear DNA are 2.08, 2.6, 2.4, and 2.9 mg/ml. Normal duck DNA (92.4 mg/ml) was used as a control (●).

of 30 μl containing 0.6 M NaCl and either 1000 cpm of ^{125}I-labeled 70S RNA or 1000 cpm of [^{3}H]cDNA. Hybridization of 70S RNA was determined by digestion with 50 μg/ml of pancreatic ribonuclease in 0.30 M NaCl–0.030 M Na_3citrate at 37° for 45 min; annealing of cDNA was assayed by digestion with the single-strand-specific S1 nuclease from *Aspergilla oryzae* (14). The amount of virus-specific DNA detected in these assays was determined by comparing the results to annealings under the same conditions using varying amounts of XC cell DNA. XC cells contain about 20 copies of ASV DNA (4), and unpublished results show that annealing of up to 30–40% of cDNA or 70S RNA is approximately linear with amounts of cell DNA containing from 0.1–1 ng of viral DNA per assay.

RESULTS

Site of Viral DNA Synthesis in Intact Cells. Duck cells were infected with B77 virus, and at various times after infection cells were collected and fractionated into cytoplasm and nuclei. DNA was extracted from both fractions and assayed for virus-specific sequences. Cytoplasmic DNA prepared from cells infected for 3 or 6 hr markedly accelerates the reassociation of slowly-reassociating B77 DNA, but nuclear DNA from these cells has a negligible or small effect upon the rate of reannealing (Fig. 1). Computation of the number of copies of ASV-specific DNA per cell reveals that 0.4–1.0 copy is present during this period (Table 2). All of the viral DNA is confined to the cytoplasm 3 hr after infection; 3 hr later, over 80% remains in the cytoplasm. These results suggest that viral DNA is synthesized in the cytoplasm. Between 6 and 9 hr after infection, however, viral DNA appears to be transported to the nucleus, since nuclear DNA isolated 9 hr after infection has a 5-fold greater effect on the reassociation kinetics than does cytoplasmic DNA (Fig. 1 and Table 2). By 24 hr after infection, very little of the 0.8–1.5 copies of viral DNA synthesized per cell is found in the cytoplasm (Fig. 1 and Table 2).

TABLE 2. *Synthesis of viral DNA*

Hr after infection	No. of copies/cell					
	Cytoplasm		Nucleus		% Nuclear	
	I	II	I	II	I	II
3.0	0.60	0.40	0.00	0.00	0	0
6.0	0.80	0.40	0.18	0.05	18	12
9.5	0.10	0.05	0.80	0.30	90	86
24.0	0.05	0.05	1.50	0.70	97	93

Conditions of infection and fractionation of cells were as given in the legend to Fig. 1. Copy numbers have been calculated from the data in Fig. 1 (Exp. I); Exp. II was done exactly as Exp. I, except that multiplicity of infection was about 2.

Since four to six copies per cell of ASV-specific DNA are found in fully transformed cultures (4), we asked whether the relatively low copy numbers observed in the experiment shown in Table 2 could be due to low multiplicity of infection. The data presented in Table 3 substantiate this possibility. There is a direct proportionality between the multiplicity of infection and number of copies of viral DNA synthesized per cell. As many as three copies per cell were made in a culture infected for 8 hr with 8 FFU of B77 per cell.

We have excluded the possibility that we detected viral DNA in the cytoplasm as a consequence of nuclear breakage during fractionation by observing that pulse-labeled 45S RNA is confined to the nuclear fraction when analyzed by polyacrylamide gel electrophoresis (data not shown). It could be argued, however, that minor damage to the nuclear membrane during cell fractionation might permit unintegrated viral DNA, but not other molecules such as 45S RNA, to escape into the cytoplasmic fraction. Ethidium bromide, at a concentration of 0.5–1.0 μg/ml, which does not affect cell growth or morphology for 48 hr, markedly inhibits integration of viral DNA into the host cell genome, without inhibiting the synthesis of viral DNA or its movement to the nucleus (R.R.G., J.M.B., and H.E.V., manuscript in preparation). The data presented in Table 4 demonstrate that unintegrated viral DNA can be detected only in the nucleus 40 hr after infection in the presence of ethidium bromide. This finding provides strong evidence against leakage of viral DNA into the cytoplasm during cell fractionation.

Synthesis of Viral DNA in Enucleated Cells. To test the validity of the conclusion that viral DNA is synthesized in the cytoplasm of infected cells, we asked whether duck cells enucleated by treatment with cytochalasin B and centrif-

TABLE 3. *Effect of multiplicity of infection on copy numbers*

Multiplicity of infection (FFU per cell)	Copy number/cell
8.0	2.70
2.0	0.90
0.4	0.14

Duck cells were infected with ASV-B77 for 8 hr at different multiplicities, as indicated. Total cell DNA was extracted and assayed for virus-specific sequences with 2.43 ng/ml of ^{3}H-labeled B77 srDNA. Copy numbers have been calculated from the reduction in $C_0t_{1/2}$ caused by cell DNA. FFU were determined by assay on chick cell monolayers.

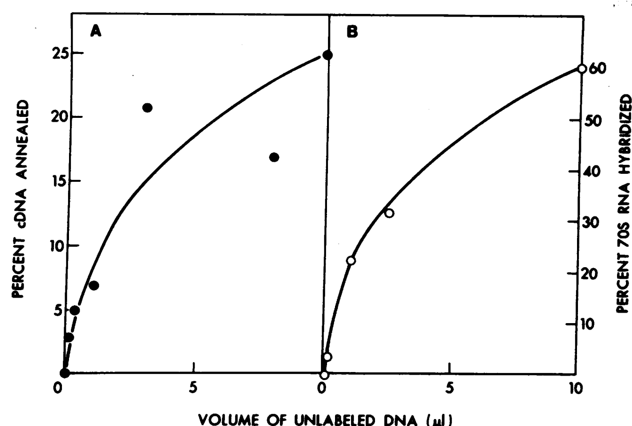

FIG. 2. Detection of both strands of viral DNA in infected, enucleated cells. Ten plastic disks of duck embryo fibroblasts were enucleated in the presence of 15 μg/ml of cytochalasin B and infected for 7 hr with concentrated B77, as described in the *text*. DNA was extracted from the cultures, in the presence of 200 μg of calf thymus DNA as carrier, and suspended in a final volume of 40 μl. Samples were then hybridized with either (A) B77 [^{3}H]cDNA or (B) ^{125}I-labeled 70S RNA and assayed. The percent hybridized is plotted against the volume of DNA solution used in each annealing reaction.

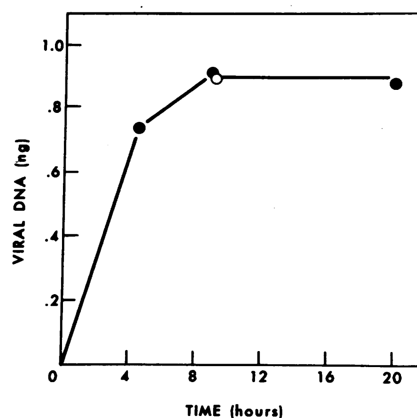

FIG. 3 Kinetics of viral DNA synthesis in enucleated cells. Twenty-four plastic disks of enucleated duck cells (●) and 8 disks of control cells (O) were infected with B77 virus for 4, 9, or 20 hr. Samples were processed as in the experiment shown in Fig. 3, and the amount of viral DNA synthesized per culture (8 disks) was determined from annealings with B77 [^{3}H]cDNA and plotted against time. Similar results were obtained when the hybridizations were performed with ^{125}I-labeled 70S RNA (data not shown).

ugation (15) would support production of viral DNA. Plastic disks (35 × 25 mm) containing 1 to 2 × 10^{6} cells per disk were enucleated, returned to normal medium for 30–45 min, and then infected with 0.2–0.4 ml of concentrated B77 virus containing 4 μg/ml of polybrene. Control cells, plated on similar disks or on petri dishes, were also infected. The multiplicities of infection were about 20–40 FFU per cell; these high levels were used to optimize detection of viral DNA synthesis in small numbers of cells. After 1–2 hr, the cells were returned to a normal volume of fresh medium and harvested 3–20 hr later for extraction of DNA and hybridization with cDNA and 70S RNA. In Fig. 2, we show the annealing of cDNA and 70S RNA to increasing amounts of DNA extracted from 10 disks infected for 7 hr. By comparing these results with the results of the hybridization of cDNA to varying amounts of XC cell DNA (see *Methods and Materials*), we calculate that a total of 1.3 ng of viral DNA was synthesized in the 10^{7} cells remaining after enucleation. This corresponds to about 3.5 copies per cell. Control cells infected at a 2.5-fold higher multiplicity of infection with the same virus preparation acquired about 5.5 copies per cell in the same period of time; this result indicates that viral DNA synthesis was at least as efficient in cultures of cells over 95% enucleated as in intact cells. Fig. 2 also demonstrates that both strands of viral DNA were made in the enucleated cells (since both 70S RNA and cDNA annealed to the product), and that a substantial fraction of the genome (at least 60%) was present in newly synthesized DNA.

The kinetics of synthesis of viral DNA in enucleated cells are presented in Fig. 3. Most of the synthesis occurred within the first 4 hr, and there was no accumulation of viral DNA after 9 hr of infection. The amount of viral DNA made in a parallel control culture on plastic disks was the same as that made in the enucleated cells (about 0.9 ng per 8 disks); since about 50% of the cells were lost during enucleation, the multiplicity of infection was doubled in the enucleated cultures, and about twice as much viral DNA was made per cell. These experiments demonstrate that the cytoplasm of enucleated cells is sufficient to sustain the early events in the replication of ASV, including the synthesis of both strands of viral DNA.

DISCUSSION

In this report we provide direct evidence for the synthesis of ASV-specific DNA in the cytoplasm of infected duck cells and in cells infected after enucleation. We detect viral DNA in the cytoplasm of intact cells about 3 hr after infection, and it accumulates these until about 6 hr after infection. Thereafter, viral DNA moves into the nucleus, and by 9–10 hr, virtually all the virus-specific DNA is found in the nucleus. Although 10–20% of the viral DNA appears in the nucleus as early as 6 hr after infection, it is not yet integrated into the cell genome (unpublished data). Likewise, at least 60% of the viral DNA is unintegrated at 9.5 hr (4), but it is found principally in the nucleus.

In the presence of low concentrations of ethidium bromide, normal quantities of viral DNA are made and transported to the nucleus, but very little viral DNA is integrated into the cell genome. Similar results have been obtained with cyclo-

TABLE 4. *Effect of ethidium bromide on integration of viral DNA*

	Copy number/cell		%
	Cytoplasm	Nucleus	Integration
Control	0.05	1.2	100
Ethidium bromide	0.10	1.2	15

Duck cells were treated with 1.0 μg/ml of ethidium bromide for 8 hr, then infected with ASV-B77 in the presence of ethidium bromide and 2 μg/ml of polybrene. After 40 hr of incubation at 38°, cells were fractionated into cytoplasm and nuclei, and DNA was extracted from both sources. Nuclear DNA was tested for integration by network formation. DNAs from cytoplasm and nuclei and from nuclear supernatant and network fractions were then assayed for virus-specific DNA.

hexamide, which depresses production of viral DNA and completely blocks integration (data not shown). In both cases unintegrated DNA is accumulated in the nucleus. Although the very low-molecular-weight cell DNA (4–5 S) present in our cytoplasmic extracts may have leaked from nuclei (as noted in *Methods and Materials*), we have documented elsewhere that newly synthesized viral DNA is considerably larger (6×10^6 daltons) and that its size is unaffected by treatment of cells with ethidium bromide (16, 17). These results argue against the possibility that viral DNA was synthesized in the nucleus and released into the cytoplasm during cell fractionation.

Experiments with enucleated cells strongly support the notion that viral DNA is synthesized in the cytoplasm. Moreover, these experiments demonstrate that induction of nuclear functions after infection is not required for the early events in the replication of ASV, since enucleated cells permit viral DNA synthesis to proceed at a rate at least as great as that observed in parallel, nonenucleated cells. It is possible, however, that the cell plays some active role in these early events, since viral DNA synthesis was observed only in enucleated cells that retained the capacity to synthesize protein at greater than 30% of the normal rate. Cells enucleated by a different procedure than described here incorporated amino acids into protein at less than 5% the normal rate; viral DNA synthesis could not be detected in these cells.

Our results are in agreement with those of Hatanaka *et al.* (6), who have detected cytoplasmic viral DNA synthesis by autoradiography of mouse cells infected with murine sarcoma virus. By contrast, Dales and Hanafusa (7) have observed a rapid transfer of the viral genome into the nucleus. They have taken this to mean that viral DNA synthesis occurs in the nucleus. There are at least three possible explanations for this discrepancy: (*i*) the 70S RNA detected in the nucleus might not participate in viral DNA synthesis; (*ii*) the 70S RNA template might be active in the cytoplasm but be preserved in a subviral structure or in an RNA·DNA hybrid molecule; or (*iii*) the viral DNA made in the cytoplasm might not participate in viral replication but be superseded by viral DNA synthesized in the nucleus 9 hr after infection. The sequential appearance and disappearance of viral DNA from cytoplasm, however, argue strongly against this last possibility.

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