Cytoplasmic Synthesis of an Arginine-Rich Nuclear Component During Infection with an Influenza Virus

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A component relatively rich in arginine which was induced by infection with an influenza virus was synthesized in the cytoplasm of the infected cell and migrated to the nucleus. This conclusion was drawn from the grain distribution in autoradiograms and the displacement of ^3H -arginine in isolated cytoplasmic and nuclear fractions after a short pulse and subsequent chase.

During infection with fowl plaque virus, an arginine-rich component appears in the nucleus of the host cell (1). This component can readily be seen in autoradiographs when the cells are fixed with acid ethanol. After this treatment normal cells carry a uniform distribution of grains over the whole cell, whereas a heavily labeled nucleus shows up prominently when influenza-specific material is produced. A minimal pulse length of 10 min is needed until the nuclear area is covered with the grain density characteristic for infected cells. This finding did not resolve the question of whether the arginine-rich nuclear material is synthesized in the cell nucleus or whether it is produced in the cytoplasm and transferred to the nucleus thereafter, where it accumulates during the pulse of 10 min. Experiments employing short pulses with subsequent chase periods are described here which strongly suggest that the arginine-rich component is synthesized in the cytoplasm and that it migrates to the nucleus.

MATERIALS AND METHODS

About 5×10^4 primary chick embryo fibroblasts were seeded into small plastic petri dishes which contained three cover slips. The following day the cultures were infected with fowl plaque virus (Rostock strain) at a multiplicity of at least 100 plaque-forming units per cell. Baby hamster kidney (BHK) cells were infected with a strain of fowl plaque virus adapted to grow in this cell line by McPherson (*personal communication*). After an adsorption period of 30 min, these cells and a series of noninfected control cells were washed and incubated with 2 ml of minimal medium (3). Three hours after infection, 20 to 100 μCi of ^3H -arginine per ml (specific activity, 500 mCi/mM) was added. The pulse was stopped by addition of ice-cold

phosphate-buffered saline after 1, 2, or 5 min. The cells were washed twice with ice-cold phosphate-buffered saline and fixed with cold 80% ethanol in 0.25 N HCl for 30 min at room temperature. The fixed cells were treated for another 30 min with 0.25 N HCl and washed successively with cold 6% trichloroacetic acid and 70, 80, and 96% ethanol; they were finally air-dried.

In a parallel series, the pulse was terminated by diluting the label with Eagle's medium containing a 10-fold concentration of arginine. The cells were washed twice and incubated further with the same medium. All of these manipulations were carried out at 37 C. The fixation procedure was the same as described above.

The air-dried cover slips were mounted on slides, covered with Kodak NTB 2 emulsion, and exposed at 4 C. Exposure periods varied from 1 to 8 weeks and were chosen empirically by developing one of the three cover slips of the same petri dish after different periods. Preparations with low grain densities were selected for determination of grain numbers; higher concentrations were suitable for direct inspection. Some preparations, particularly when grains were to be enumerated, were stained through the emulsion with 0.0025% Toluidine Blue in McIlvaine citrate buffer at pH 6.

In a different approach, incorporation of ^3H -arginine was measured after separation of nuclei and cytoplasm. Monolayers of primary chick embryo fibroblasts on 90-mm petri dishes were infected and labeled with 2.5 μCi of ^3H -arginine per ml as described for the autoradiographs. Instead of fixing the cells, the monolayers were covered with 4 ml of hypotonic buffer (6), left in the cold for 15 min, and scraped off the glass in this buffer. The cells were disrupted with a Dounce homogenizer, and the nuclei were spun down and washed once with the same buffer. The nuclear pellet was extracted with 2 ml of 80% ethanol in 0.25 N HCl for 30 min at room temperature and centrifuged for 10 min at $2,200 \times g$. The supernatant was removed, and the pellet was washed with 96% ethanol, dried

FIG. 1. (a) BHK-cells infected with fowl plaque virus strain "McPherson." The cells received a 1-min pulse with 20 μ Ci of 3 H-arginine per ml and were fixed immediately. (b) Infected BHK-cells. A chase period of 10 min with cold arginine followed the 1-min pulse. (c) Uninfected BHK-cells. The conditions for pulse and chase were the same as for b. (d) Secondary chick embryo fibroblasts infected with fowl plaque virus strain "Rostock." 3 H-arginine (20 mCi/ml) was applied for 2 min, followed by a chase of 10 min with cold arginine. (e) Infected secondary chick embryo fibroblasts. The cells were fixed immediately after the 2-min pulse. Cells in a to c were exposed for 8 weeks at 4 C. The preparations were not stained; merely the grain patterns were photographed. Cells in d and e were exposed for 7 weeks and were stained with Toluidine Blue.

with ether, and hydrolyzed with 1 ml of 0.2 N NaOH. The cytoplasmic fractions were precipitated by addition of an equal volume of 12% trichloroacetic acid; the sediment was washed once with 6% trichloroacetic acid and extracted as described above for the nuclei. The supernatants and the hydrolyzed sediments were transferred to counting vials, and 10 ml of Bray's scintillation fluid was added.

RESULTS

Upon microscopic inspection of the autoradiographic preparations labeled briefly for 1 or 2 min, the nuclei of infected cells carried only few grains which were less densely spaced than the grains of the cytoplasm (Fig. 1a). There was hardly any difference in the appearance of infected cells and controls. After a pulse length of 5 min, infected cells and controls had a rather uniform distribution of grains, but some cells in the infected preparations showed heavily labeled nuclei.

When, however, a chase period of 10 min or longer followed the short pulses, all infected cells consistently had a heavy nuclear grain count (Fig. 1b) and presented the pictures which had been found previously (1) when a pulse length

TABLE 1. Average percentage of grains over the nuclei of chick embryo fibroblasts^a

Length of pulse (min)	Length of chase (min)	Per cent nuclear grains in ^b	
		Controls	Infected cells
1	0	5.5	6.8
1	10	19.5	44.5
1	60	26.4	40.0

^a After 3 hr of infection, 50 μ Ci of ³H-arginine/ml was added. The time of exposure of the autoradiograms was 8 days. Fifty cells were evaluated.

^b Proportion of grains in the nucleus was listed as the percentage of grain counts of the whole cell.

TABLE 2. Average percentage of grains over the nuclei of BHK-cells^a

Length of pulse (min)	Length of chase (min)	Per cent nuclear grains in	
		Controls	Infected cells
1	0	5.0	5.6
1	5	16.0	32.5
1	10	24.0	40.0

^a After 3 hr of infection, 20 μ Ci of ³H-arginine per ml was added. The time of exposure of the autoradiograms was 19 days. Twenty-five cells were evaluated and their nuclear grains were listed as in Table 1.

TABLE 3. Incorporation of ³H-arginine into nuclear and cytoplasmic fractions^a

Fraction	Counts per minute ^b			
	Controls		Infected cells	
	Pulse (2 min)	Chase (20 min)	Pulse (2 min)	Chase (20 min)
Hydrochloric acid-ethanol soluble				
Nuclear	0.99	2.16	2.50	5.33
Cytoplasmic	9.30	8.50	14.49	9.57
Insoluble residue				
Nuclear	6.27	7.14	13.18	19.64
Cytoplasmic	48.70	48.53	105.34	65.66
Total counts per minute	65.26	66.33	135.51	100.20

^a A 2-min pulse of 2.5 μ Ci/ml was applied 3 hr postinfection. A 20-min chase period followed in a parallel series. Nuclear and cytoplasmic fractions were prepared immediately and treated with hydrochloride-ethanol. Radioactivity levels in soluble extracts and insoluble residues were determined separately.

^b Values to be multiplied by 10³.

of 10 or more min had been applied. Concomitantly, control cells showed a uniform distribution of grains (Fig. 1c).

To quantitate this translocation of labeled material from the cytoplasm to the nucleus, the relative number of grains over nucleus and cytoplasm was determined in stained preparations. About 7% of the grains were found over the nucleus in control cells when the length of the pulse was 1 min only. The percentage of grains in the nucleus of infected cells was 8 to 12. If the cells were incubated for at least 10 min or longer with an excess of cold arginine after they had been in contact with the isotope for 1 min, the nuclear grain concentration increased to about 20% in the controls and 40% in infected cells. Intermediate values were obtained after a chase period of 5 min. Essentially the same results were obtained with chick embryo fibroblasts (Table 1) and BHK cells (Table 2).

Total incorporation of ³H-arginine into infected cells was higher than in the control series, which corresponds to previous observations (1). This was true for the material soluble in acid ethanol and the insoluble residue both in the nuclear pellet and the cytoplasmic fraction. When infected cells were incubated with an excess of cold arginine after a pulse of 2 min, the radioactivity in the acid-insoluble fraction of the

nuclei had increased considerably. The counts of the cytoplasmic acid precipitates had decreased concomitantly after the chase (Table 3). In accordance with the experimental results presented by Robbins and Borun (7), migration of newly synthesized histones to the nucleus could be observed in normal fibroblasts. No attempts have been made to prepare "clean" nuclei or to determine their degree of leakiness.

DISCUSSION

The results of these experiments are apt to explain the autoradiographic pictures described in a previous communication which had shown prominently labeled nuclei of all infected cells, if incubation with ^3H -arginine lasted for at least 10 min. They warrant the conclusion that this component is synthesized in the cytoplasm and then transferred to the nucleus, where it accumulates.

This scheme was reproducible in two independent experimental approaches. In one series of experiments the nuclei were separated from the cytoplasm after short pulses and subsequent chase periods. A transport of acid-precipitable material could be traced from the cytoplasmic to the nuclear fractions.

Since it is difficult in this type of investigation to get all cells disrupted and since one risks damage and leakiness of nuclei (9), our topographic examinations of the synthesis of the arginine-rich component were mainly based on autoradiographic techniques. The autoradiograms obtained show that, during short pulses of 1 or 2 min, the cytoplasm of infected and normal cells readily incorporates the radioactive amino acid, whereas the nuclei hardly contain any label. The few grains found in the nuclear area are probably due to radioactivity in the cytoplasmic sections covering the nucleus. This means that major synthetic events cannot take place in the nucleus during multiplication of an influenza virus.

A large part of the material which incorporates the radioactive precursor in the cytoplasm can be chased to the nucleus where about 40% of all the grains in the whole cell accumulate. This grain density is sufficient for the nuclei of infected cells to become outlined and discernible in autoradiographs. The fact that some cellular material, which is also insoluble in hydrochloride-ethanol, follows the same pathway of synthesis only causes an even distribution of grains all over the cell.

Our findings are consistent with experimental results obtained by Taylor et al. (8) in an acrylamide gel analysis of influenza-infected cells.

These authors identified three structural components of an influenza A virus and presented evidence that two of them are synthesized in the cytoplasm and migrate to the nucleus. Our results are also in accord with the well established observations that subunits of deoxyribonucleic acid viruses are synthesized in the cytoplasm and transferred to the nucleus where they are assembled (4, 5, 9, 10).

Since some hydrochloride-ethanol-insoluble cellular material is also transferred to the nucleus, the possibility cannot be rigorously excluded that the arginine-rich material of infected cells is coded by the cell genome. Besides the reasons which were discussed before and which indicate that it represents a virus-specific component (1), it seems improbable that the synthesis of this particular cellular material would be stimulated and would escape the general inhibition of cellular functions after the viral infection. In view of the nuclear localization of the nucleocapsid of influenza viruses with fluorescent antibodies (2), it is tempting to assume that we are dealing with this antigen. There is no definite proof, however, that the arginine-rich material is identical with the viral inner component or any other of the structural entities known so far.

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