SYNTHESIS OF POLYOMA DNA BY ISOLATED NUCLEI[1]

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Summary Nuclei from polyoma or mock infected baby mouse kidney, 3T3 or 3T6 cells were incubated under conditions suitable for DNA synthesis. The labeled DNA thus formed by the infected nuclei was by several criteria indistinguishable from the replicative intermediate formed in intact cells as first described by Bourgeaux et al. (1).

Nuclei isolated from mammalian cells show a limited capacity to incorporate radioactive deoxynucleoside triphosphates into DNA and, at least under certain circumstances, this incorporation appears to be a continuation of DNA synthesis initiated in intact cells (2,3). We now report studies of DNA synthesis in nuclei isolated from polyoma infected cells.

Materials and Methods Primary mouse kidney cells, 3T3 or 3T6 cells were grown as monolayers in Dulbecco's medium. The Glasgow large plaque polyoma variant, purified as described (4, 5), was used for infection at a calculated multiplicity of 50 plaque forming units per cell. Viral DNA was obtained from purified virus after sodium dodecyl-SO_4-pronase digestion, precipitation at M NaCl (6) and two successive phenol extractions (7).

The preparation of nuclei was performed at $+2^{\circ}$. Cell layers from 10 - 20 Petri dishes (9 cm) were overlayed with 4 ml of hypotonic Hepes[2] (20 mM Hepes, pH 8.1, 1 mM $MgCl_2$, 1 mM DTT[2], 0.5 mM $CaCl_2$). After 5 minutes the buffer was decanted, 1 ml of fresh hypotonic Hepes was added, the cells were scraped off and homogenized by 5 strokes with a Dounce homogenizer. One tenth volume of 1.5 M NaCl was added and the nuclei were sedimented by centrifugation for 10 minutes at 200 g. The pellet was sus-

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2. Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; DTT, dithiothreitol

pended in 2 to 5 volumes of isotonic Hepes (50 mM Hepes, pH 8.1, 110 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT and 0.5 mM $CaCl_2$) and used immediately.

The final reaction mixture contained in a volume of 0.125 ml: 90 mM NaCl, 40 mM Hepes, pH 8.1, 8 mM $MgCl_2$, 8 mM phosphoenol pyruvate, 5 mM ATP, 0.8 mM DTT, 0.4 mM $CaCl_2$, 0.4 mM each of dATP, dCTP and dGTP, 0.04 mM ^{3}H-dTTP (200 cpm/pmole), and 2 μg of pyruvate kinase. After incubation at 25° for 30 minutes, the reaction was stopped by the addition of 0.5 ml of 50 mM Hepes, pH 8.1 - 10 mM EDTA, followed by 0.1 ml of 7% sodium dodecyl-SO_4. After 30 minutes at 25°, 0.2 ml of 5 M NaCl was added as described by Hirt (6). The solution was kept at 4° for 12-18 hours and then centrifuged at 20,000 g for 30 minutes. The resulting Hirt supernatant contained viral and other small molecular weight DNA while the precipitate contained the bulk of the cellular DNA. Aliquots of the supernatants were used directly for sucrose gradient centrifugation or - after precipitation with 5% $CHCl_3COOH$ - 0.1 M pyrophosphate - for the determination of total "viral" DNA synthesis. The Hirt precipitates were dissolved in 1 ml of 0.3 M NaOH and used for the determination of total cellular DNA synthesis.

Results and Discussion The experiments by Friedman and Mueller (2) on DNA synthesis in a nuclear system from HeLa cells served as a starting point to our studies. In an attempt to obtain a better defined product, we studied the synthesis of viral DNA by nuclei from cells, productively infected in tissue culture with polyoma virus.

Contact inhibited kidney cells or growing 3T3 cells were found to have maximal viral and cellular DNA synthesis 25-30 hours after polyoma infection . Cells at this stage were used as the source of nuclei for our experiments.

After virus infection, nuclei from both cell types incorporated about four times more radioactivity from ^{3}H-dTTP into DNA from Hirt supernatants (= "viral" DNA) than mock infected controls (Table I). Virus infection

TABLE I. Effect of polyoma infection on DNA synthesis by isolated cell nuclei. The different experiments were done as described in the experimental part with nuclei containing between 0.13 and 0.19 mg of DNA.

Source of nuclei	cpm/mg of DNA in	
	"viral" DNA	cellular DNA
mouse kidney, polyoma infected	17,000	10,000
mouse kidney, mock infected	4,800	2,600
3T3, polyoma infected	25,000	33,000
3T3, mock infected	6,000	25,000

similarly stimulated the cellular DNA synthesis of nuclei from resting kidney cells but not from growing 3T3 cells.

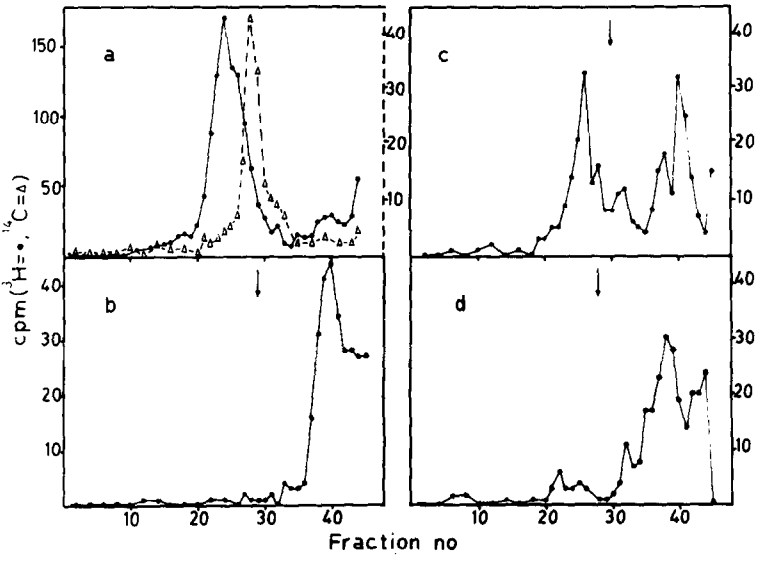

Fig. 1. Neutral sucrose gradient centrifugation of "viral" DNA formed by mouse kidney or 3T3 cell nuclei. Aliquots (0.2 ml) of Hirt supernatants, together with reference ^{14}C-polyoma DNA, were layered onto linear 5-20% sucrose gradients (M NaCl, 10 mM Tris HCl, pH 7.6, 10 mM EDTA) and centrifuged at 4° in a SW 50.1 rotor for 4 hours at 40,000 rpm. Fractions were precipitated with 1 ml of 5% $CHCl_3$ COOH - 0.1 M pyrophosphate, and counted on glass filters. The counts for the supercoiled ^{14}C-polyoma DNA (≈ 20 s) are given only in panel a; in panels b to d the positions of the reference peaks are indicated by arrows. Direction of sedimentation is to the left. Panel a: polyoma infected 3T3 nuclei; panel b: mock infected 3T3 nuclei; panel c: polyoma infected kidney nuclei; panel d: mock infected kidney nuclei.

The "viral" DNA was analyzed further by neutral sucrose gradient centrifugation (Fig. 1). The nuclei from both types of virus infected cells produced radioactive DNA which sedimented as a broad peak (about 25 s) ahead of reference supercoiled polyoma DNA (Figs. 1a and 1c). This peak was absent from the mock infected controls (Figs. 1b and 1d) which contained a slower moving radioactive peak around 10 s. A radioactive 10 s peak was also observed in the experiment with nuclei from infected kidney cells (Fig. 1c).

Radioactive DNA in the two main peaks from infected kidney nuclei was used for hybridization experiments with polyoma and mouse DNA (Table II). The material from the 25 s peak behaved like authentic polyoma DNA while the material from the 10 s peak appeared to contain a mixture of mouse and polyoma DNA.

TABLE II. Characterization of ^{3}H-DNA formed by polyoma infected kidney nuclei with DNA-DNA hybridization. The experiments were done as described by Benjamin (9).

Labelled DNA in solution	Total cpm incubated	DNA on filter	Per cent hybrid
25 s peak	102 (146)*	none	0.4 (0)*
25 s peak	102 (146)*	polyoma (0.5 μg)	36 (32)*
25 s peak	102 (146)*	mouse (5 μg)	3 (0.7)*
10 s peak	114	none	0.5
10 s peak	114	polyoma (0.5 μg)	8
10 s peak	114	mouse (5 μg)	8
Control polyoma	722 (653)*	none	1.1 (0.3)*
Control polyoma	722 (653)*	polyoma (0.5 μg)	39 (31)*
Control polyoma	722 (653)*	mouse (5 μg)	1.7 (1.8)*

* Duplicate experiment.

From these experiments it seems clear that the DNA sedimenting at 25 s is of viral origin. A DNA with similar properties was earlier described to occur in intact cells after infection with polyoma (1) or SV 40 (8) and characterized as a replicative intermediate in the synthesis of viral DNA.

For further characterization, DNA synthesized by nuclei from infected 3T3 cells was centrifuged in an alkaline sucrose gradient (Fig. 2a). For comparison, Fig. 2b presents a similar run of the replicative intermediate of polyoma DNA which was isolated from infected 3T3 cells by chromatography on benzoylated-naphtoylated DEAE-cellulose (8). In both cases the supercoiled form of polyoma DNA, sedimenting around 40 s, was absent.

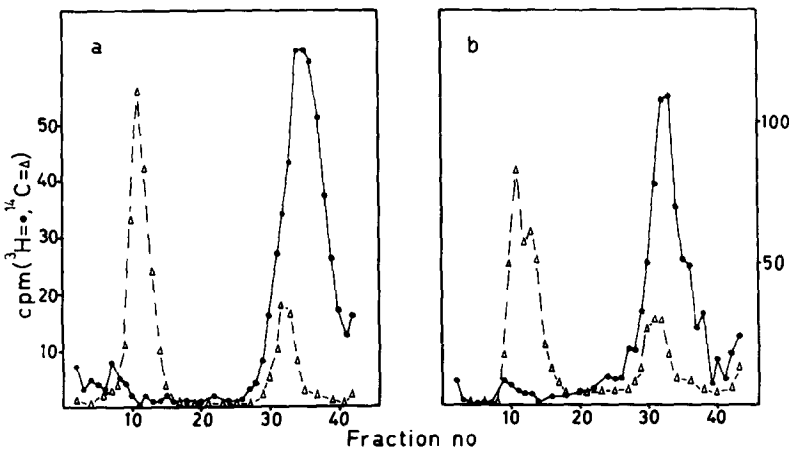

Fig. 2. Alkaline sucrose gradient centrifugation of (a) "viral" DNA formed by 3T3 nuclei and (b) purified replicative intermediate formed in intact cells. The experiments were performed with linear gradients (5-20% sucrose, 0.3 M NaOH, 0.7 M NaCl, 10 mM EDTA) as described for Fig. 1. Authentic ^{14}C-labeled polyoma DNA was included as a reference.

Instead the material sedimented as a broad peak slightly behind the reference single stranded polyoma DNA.

Finally, in experiments not detailed here we found that the bulk of the radioactive DNA made by infected 3T6[3] nuclei was eluted from benzoylated-

3. 3T6 cells rather than 3T3 cells were used in recent experiments because of the relative ease with which larger amounts of nuclei can be obtained.

naphtoylated DEAE cellulose columns under the same chromatographic conditions as authentic replicative intermediate.

In conclusion, it appears that the polyoma DNA formed in the nuclear system shows properties similar to the replicative intermediate of polyoma or SV 40 DNA found in intact cells (1,8). Our results do not conclusively distinguish between extensive repair synthesis or replication. Experiments in which bromo-dUTP was substituted for dTTP during the incubation of nuclei and ^{3}H-dATP was used as the source of label demonstrated an increase in the buoyant density of the product DNA from 1.705 to 1.720 g x cm^{-3} during centrifugation in neutral CsCl. The DNA was then broken into smaller pieces (5-7 s) by sonication. These pieces banded at a density of 1.745, showing that almost half of all thymines now were substituted by bromouracil (10). These results strongly suggest that the nuclear system was capable of continuing the replication of DNA which had been initiated in the intact cell. This system thus may be useful for a further analysis of the details of polyoma DNA replication and for more general studies on DNA synthesis in mammalian cells.

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