NUCLEAR COLUMNS: LABELING AND RELEASE OF VIRUS SPECIFIC RNA BY NUCLEI OF DNA-VIRUS (SV 40) INFECTED CELLS

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SUMMARY

Isolated nuclei of simian virus-40 (SV 40) infected cells were immobilized in columns. The columns were continuously perfused with a medium containing labeled RNA precursors. The effluent was submitted to sucrose gradient centrifugation and RNA was isolated from the 15-40 S zone. The RNA hybridized to about 10 per cent with SV 40 DNA, while the RNA of controls did not hybridize.

The results show that RNA complementary to DNA is labeled and continuously released into the effluent during 30 min of perfusion. The data further indicate that the RNA is labeled by transcription of DNA and not by unspecific extension of RNA chains.

A new methodology has been developed for the study of the kinetics of metabolic processes in nuclei (1). Isolated nuclei are immobilized in a column (nuclear column) which can, within the course of an experiment, be perfused with different media.

Experiments with rat liver nuclei have revealed that labeled RNA is continuously released when the column is perfused with a solution containing the four labeled nucleoside triphosphates (1). The products found in the effluent fractions are labeled ribonucleoproteins with a buoyant density of 1.40 - 1.43 g/cm^3 in CsCl (2).

In the present experiments we aimed to investigate whether DNA-specific RNA is formed and released in the continuous flow system and whether labeling occurs by transcription or by unspecific addition of nucleotides to RNA chains (3).

The use of nuclei of SV 40 infected cells appeared to be

advantageous for the following reasons: SV 40 DNA can be purified extensively for hybridization experiments. The DNA can be freed of cellular DNA and it does not contain clusters of homopolydeoxyribonucleotides. Furthermore it is known that transcription of viral DNA occurs in the nuclei of infected cells (4). That virus-specific RNA is formed by incubation of nuclei isolated from virus-infected cells has been demonstrated by others for Adenovirus 2 (5,6).

Methods. African green monkey kidney cells (CV-1) were grown to confluence in roller bottles in Eagle's basal medium(BME) supplemented with 10 per cent fetal bovine serum. Virus (Simian Virus 40, strain Rh 911, moi: 6.6 pfu/cell) was allowed to adsorb for 2 hours after which BME containing 2 per cent fetal bovine serum was added to the cells and incubation continued at 37°C for 30 hours. The cells were dispersed by trypsin, washed and lysed by incubation with the nonionic detergent NP 40 (0.25 M sucrose, 0.003 M $MgCl_2$, 0.01 M Tris.HCl (pH 7.4), 0.5 per cent NP 40) at 0°C for 5 minutes. The nuclei were sedimented and washed once with lysing buffer.

The nuclear columns were prepared as previously described (1). The pelleted nuclei were adsorbed onto the surface of membrane filter pieces and in this state transferred into microcolumns. One µl of the bed volume contained about 1-3 µg DNA. The columns were immersed in a water bath and the incubation medium was pumped through at a constant rate.

SV 40 DNA was purified as described elsewhere (7). For the isolation of RNA, ethanol and SDS were added to the pooled fractions of the sucrose gradient and the precipitated RNA was submitted to phenol extraction and DNAse treatment as described by Scherrer (8) except that the extraction procedure was carried out at 25°C.

The RNA was hybridized with excess of DNA at 37°C for 18 hours. Aliquots of 2 µg of alkali-denatured SV 40 DNA (form I) were immobilized on Millipore filters. The hybridization mixture (0.6 ml) contained 50 per cent formamide, 0.45 M NaCl, 0.045 M Na-citrate, 0.5 per cent SDS and ^{3}H-RNA (9). Two filters with SV 40 DNA and one filter with 0.5 µg E.coli DNA were incubated with the hybridization mixture. After incubation each filter was washed with 50 ml SSC (0.15 M NaCl, 0.015 M Na-citrate) containing 0.5 per cent SDS and then further incubated with 10 ml hybridization buffer containing 100 µg pancreatic ribonuclease and 100 U T-1 ribonuclease for 2 hours at 37°C. Filters were washed again extensively with hybridization buffer and SSC, dried and the radioactivity determined by liquid scintillation counting.

Results and discussion. The present methodology has been designed in order to study metabolic processes in isolated nuclei in a continuous flow system. The obvious advantage of the system over the conventional methods of incubation of nuclei in test tubes is that the conditions of incubation can be repeatedly changed during the course of one experiment. The nuclei can be exposed to different incubation media and the kinetics of formation and release of products can be continuously followed. In experiments with prelabeled nuclei it has been possible to elaborate conditions which allow the release of ribonucleoproteins to be reversibly switched on and off (10).

When unlabeled nuclei are immobilized in a column, perfusion with a solution of radioactive nucleosidetriphosphates leads to formation and release of labeled RNA (1,2). The rate of this reaction is influenced by temperature, flow rate, concentration of reactants and inhibitors and by other variables (10,11).

The results of an experiment with nuclei of CV-1 cells is

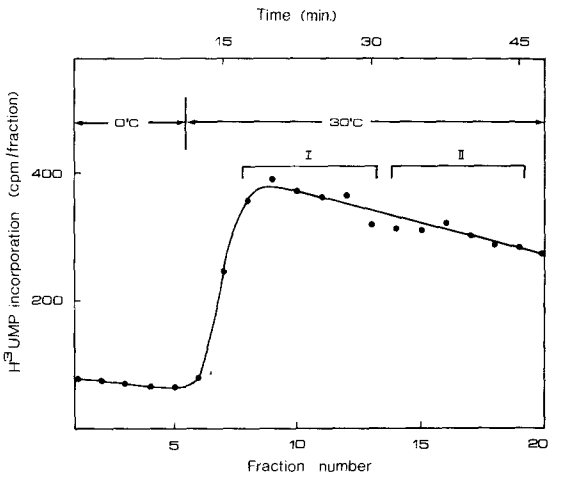

Fig. 1. Time course of RNA formation by monkey kidney cell nuclei. Column: 10 µl volume; rate of flow: 7.5 µl/min. Medium: 25 µM ^{3}H-UTP (75 µCi/ml), 1.25 mM ATP, CTP, GTP; 125 mM sucrose, 50 mM Tris.HCl (pH 8.1), 5 mM $MgCl_2$, 6 mM KF, 5 mM dithiothreitol; dialyzed ribonuclease inhibitor from rat liver cytosol (2). The column was equilibrated with the incubation medium by perfusion at 0°C. The reaction was started by raising the temperature of the column to 30°C. The effluent fractions were collected on filter paper disks. The disks were repeatedly washed with trichloroacetic acid, ethanol and ether (2) and the radioactivity was determined by liquid scintillation counting.

shown in Fig.1. A column containing nuclei of cells infected for 30 hours with SV 40, was first equilibrated with the incubation medium by perfusion at 0°C for 10 minutes. The reaction was then started by raising the temperature of the column to 30°C which lead to an immediate appearance of acid-insoluble labeled material in the effluent. Release of the radioactive product continued at a slightly decreasing rate for 30 minutes.

To determine whether the labeled product resulted from a DNA dependent transcription process we analyzed the RNA in the effluent for its capacity to hybridize with SV 40 DNA. As it was of further interest to see whether SV 40 specific RNA is released over the whole incubation period, in some experiments the effluent was collected for 2 periods of 15 minutes each following the temperature shift (Pool I,II). The effluent of the column

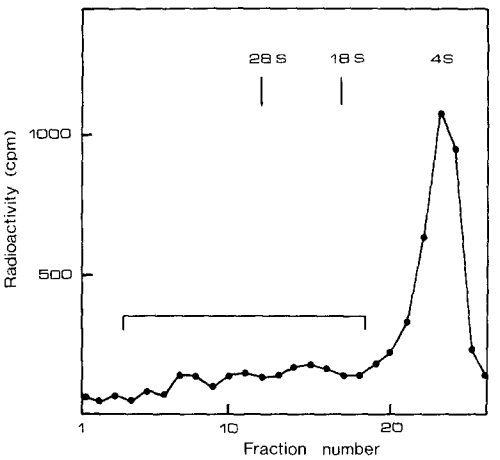

Fig. 2. Sucrose gradient centrifugation of effluent I of a column with nuclei from SV 40 infected cells. Columns of 60 µl volume with nuclei of SV 40 infected cells and with nuclei of controls (uninfected cells) were prepared. Incubation medium as in Fig.1 except that ^{3}H-UTP was 17 µM (250 µCi/ml). Rate of flow: 40 µl/min. After the temperature shift to 30°C the effluent was separately pooled over 2 subsequent periods of 15 min each (Pool I, II) as indicated in Fig.1. After addition of rat liver r-RNA the effluents were layered on top of a 10 - 40 % (w/w) sucrose gradient in 10 mM triethanolamin.HCl (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$ and centrifuged at 9 x 10^5 g for 16 h in the Spinco SW 65-Ti rotor at 2°C. Aliquots of the fractions were placed on filter paper disks and the radioactivity of the acid-insoluble material was determined. Fractions 4 - 18 were pooled and the RNA was extracted for the hybridization experiments.

with nuclei of virus-infected cells and the effluent of the control columns with nuclei of uninfected cells were separately centrifuged on sucrose gradients.

Fig.2 shows the sedimentation pattern of the acid-insoluble material of Pool I from an experiment with nuclei of virus-infected cells. There is a peak in the area of 4 S and a broad zone of material with values up to 40 S. Experiments with columns or rat liver nuclei have already shown that the peak in the area of 4 S consists of free RNA while the material with a higher sedimentation rate consists mainly of ribonucleoprotein particles with a buoyant density of 1.40 - 1.43 in CsCl (2). As viral RNA was expected to be present in this zone, the material

Table 1. Hybridization of SV 40 DNA with RNA from the nuclear column effluents

Exp. No.	RNA sample	input RNA (cpm)	cpm bound to SV 40 DNA	cpm bound to E.coli DNA
1	SV 40 infected pool I	5790	511	16
	SV 40 infected pool II	4770	342	41
	uninfected pool I	5300	21	27
	uninfected pool II	3620	44	19
2	SV 40 infected pool I+II	3380	448	45
	uninfected pool I+II	2200	37	105
3	SV 40 infected pool I	1850	242	19
	SV 40 infected pool II	1640	178	34
	uninfected pool I	2320	56	47

sedimenting between 15 S and 40 S was pooled and the RNA was isolated for hybridization. About 1/3 of the total acid-insoluble radioactive material released was found in this zone.

Table 1 shows the results of the hybridization. About 10 per cent of the labeled RNA obtained from the SV 40 nuclear column effluent hybridizes with the virus DNA, while radioactivity found in the controls is only slightly above background. The RNA from the effluent collected during the second period of perfusion hybridizes to about the same extent as the RNA collected during the first 15 minutes. Thus, release of hybridizable radioactive material remains fairly constant throughout the perfusion.

Any nucleotides attached by chain extension after the DNA-dependent formation of RNA are expected to be split off during

hybridization and washing procedures. The results thus indicate that the hybridizing product released is labeled in a DNA-dependent reaction. This is supported by the fact that in the liver nuclear system the formation of labeled ribonucleoproteins is inhibited by actinomycin D (10,11).

The size of the hybridizing RNA, estimated from sucrose gradient centrifugation, is within a range expected for viral RNA in the late phase of infection. The extent of hybridization is remarkably high in comparison to the rates obtained with RNA isolated from prelabeled SV 40 infected cells (12). Apparently, the conditions in the continuous flow system favour the labeling and release of DNA-like ribonucleic acid.

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