ISOLATION AND PARTIAL CHARACTERIZATION OF A NUCLEAR RNA POLYMERASE – SV40 DNA COMPLEX

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1. Introduction

Several laboratories have reported the isolation of nucleoprotein complexes from polyoma (py) or simian virus 40 (SV40) infected cells [1–5]. A role for these complexes in viral transcription has not yet been demonstrated. Girard et al. [6] have recently described late SV40 transcriptional intermediates constituted by nascent viral mRNA molecules hydrogen-bound to the DNA templates; the method of isolation of these molecules should prevent the RNA polymerase activity in their preparation.

The anionic detergent sarkosyl has been shown to cause a several-fold enhancement of endogenous RNA polymerase activity in mouse nuclei. This stimulatory effect was ascribed to at least two factors: an increased initial activity of RNA polymerase II and a prolongation of the total polymerase reaction [7]. Sarkosyl causes the release of nearly all the proteins from cellular DNA [7], but apparently does not affect RNA polymerase that has already initiated RNA synthesis [8].

The effect of sarkosyl on cellular transcriptional activity [7–9] and the efficient extraction of viral DNA with this detergent [10] allowed us to isolate a SV40 transcription complex from late infected monkey cells.

The SV40 DNA-RNA polymerase complex we describe here has an endogenous transcriptional activity, which is highly sensitive to α-amanitin, a specific inhibitor of nucleoplasmic polymerase B or II [11]; this should prove of great interest in the study of the regulation of viral gene expression.

2. Materials and methods

African Green Monkey Kidney cells CV1 were grown in glass rollers or in Greiner plastic Petri dishes in Dulbecco's modified Eagle's medium, supplemented with 10% foetal calf serum. The cells were infected with the large plaque type of SV40 at a multiplicity of 30 to 50 plaque-forming units per cell. Mock infected cells (control) were treated in the same way as infected cells except that the virus was absent.

For the preparation of the SV40 transcription complex, growing cells were infected when they had just reached confluency. After 24–43 hr, the cells were washed twice with ice-cold phosphate buffered saline (PBS). Nuclei were prepared according to Shmookler et al. [9] and were resuspended in Re buffer (50 mM Tris–HCl pH 7.9, 50 mM KCl, 0.5 mM dithioerythritol); one volume of a 0.5% sarkosyl, 0.2 M NaCl solution was added and the mixture was centrifuged at 30 000 *g* for 30 min at 2°C. The supernatant (sarkosyl supernatant) was carefully separated from the chromatin pellet and either assayed directly for endogenous RNA polymerase activity or subjected to further purification (concentration at 102 000 *g* for 60 min or sucrose gradient centrifugation). This activity was stable for at least 24 hr when the sarkosyl supernatant was kept at 2°C (not shown).

In vitro RNA polymerase assays were carried out in duplicate. The final reaction volume of 0.25 ml was 76 mM Tris–HCl pH 7.9, 80 mM NaCl, 28 mM KCl, 6 mM NaF, 1.8 mM dithioerythritol, 46 mM ammonium sulfate, 1.8 mM $MnCl_2$, 0.2% sarkosyl, 0.6 mM each of GTP, CTP and ATP, 1.2–1.8 μM [^{3}H] UTP (4.4–

6.6 μCi per assay), unless otherwise indicated. The reaction was carried out for 60 min at 26°C and stopped by addition of 3 ml of ice-cold 5% trichloroacetic acid (TCA) containing 0.01 M pyrophosphate (PP_i). The precipitates were collected on Whatman GF/C filters, washed six times with 5% TCA (0.01 M PP_i), dried and counted by liquid scintillation spectrometry. Assays at zero time incubation were used to determine background levels of incorporation; these ranged between 100–200 cpm and were substracted from all data shown.

SV40 DNA was extracted by the Hirt procedure [12] from cells infected at low multiplicity (0.01 PFU/cell). Form I DNA was then purified by isopycnic centrifugation in CsCl gradients in the presence of ethidium bromide [13].

To obtain purified CV1 DNA, the cells were lysed with 0.6% sodium dodecyl sulfate (SDS), 10 mM EDTA, 10 mM Tris, pH 7.5; DNA was deproteinized with cold phenol, then extracted with chloroform: isoamyl alcohol (24 : 1) and dialyzed against 0.1 × SSC at 2°C. DNA from sarkosyl supernatants was purified in a similar way except that dialysis was replaced by ethanol precipitation. DNA concentrations were determined by Burton's method [14].

Labelled DNA (0.2 ml) was analyzed by sedimentation through 4 ml alkaline cesium chloride density gradient (ρ = 1.5; pH = 12.5–13.0) at 90 000 *g* for 150 min at 20°C. Fractions were collected directly into vials containing 5 ml of toluene based scintillation liquid, Triton X-100 and water (10 : 5 : 1, respectively), mixed and counted.

SV40 cRNA was synthesized in a volume of 0.5 ml containing 44 μg of purified SV40 form I DNA, 50 μg *E. coli* RNA polymerase obtained by the method of Humphries et al. [15], adjusted to 40 mM Tris–HCl pH 7.9, 130 mM KCl, 10 mM $MgCl_2$, 0.1 mM dithioerythritol, 0.1 mM each of ATP, CTP and GTP, 10 mM [^{3}H] UTP (100 μCi). After incubation for 40 min at 36°C, the product was purified according to Lindstrom and Dulbecco [16].

RNA synthesized in vitro was purified after DNase digestion by the SDS-hot phenol method [7]. Carrier *E. coli* tRNA and non-radioactive UTP were then added and the RNA was precipitated several times with ethanol.

DNA-RNA hybridizations were carried out in 1 ml of 5 × SSC, 0.05% SDS, for 48 hr at 65°C (optimal conditions), using excess DNA. The DNA was alkali denaturated and irreversibly fixed to Sartorius cellulose nitrate filters (SM 11306). After incubation the filters were washed 3 times with 2 × SSC, then treated with preboiled RNase (20 μg/ml in 2 × SSC, 30 min at 20°C), washed 5 times as above, dried and counted in toluene based scintillation liquid.

α-Amanitin was purchased from Boehringer, and DNase (electrophoretically purified) from Worthington Biochemical. Rifamycin AF/013 was a gift from Dr L. Thiry.

3. Results and discussion

Permissive monkey kidney cells (CV1) were infected with SV40 at high multiplicity. The cellular nuclei were isolated, lysed with sarkosyl, the chromatin separated by centrifugation, and the supernatant fraction was assayed for RNA polymerase activity as described in Methods. Table 1A shows that the sarkosyl supernatants from infected cultures displayed an active endogenous RNA synthesis as determined by [^{3}H]-UMP incorporation into TCA precipitable material. Transcription was not observed in similar extracts from mock (non-infected) cells, indicating that viral infection was responsible for this activity. The sarkosyl supernatant prepared 42 hr after infection was more active that that obtained 24 hr after infection, suggesting that the number of templates active in transcription increased with the number of viral copies. In the presence of α-amanitin, a specific inhibitor of nucleoplasmic RNA polymerase B or II [11], the activity of sarkosyl extracts from infected cells was reduced to the level of mock infected cells. Since the concentration of RNA polymerase in the sarkosyl supernatant fraction is not known, it was interesting to study the inhibitory effect of α-amanitin over a wide range of concentrations. Table 1 B shows that the transcription activity of the complex was strongly inhibited even at very low drug concentration. This suggests that the observed RNA synthesis was performed by RNA polymerase II or B, which is the enzyme responsible for SV40 transcription in nuclei of infected monkey cells [17].

In order to rule out a non-specific binding of polymerase molecules to DNA during the isolation procedure, a reconstruction experiment was performed. DNA

Table 1
Effect of infection and of α-amanitin on endogenous RNA polymerase activity in sarkosyl supernatant fractions

Sarkosyl supernatant	A [^{3}H] UMP incorporated (cpm) Without α-amanitin	With α-amanitin
Mock	43	2
Infected (24 hr)	2207	39
Infected (42 hr)	7101	53

(A) Sarkosyl supernatants from mock and infected (24 hr and 42 hr p.i.) cells were prepared from purified nuclei and assayed (1.8, 2.1, and 4.0 μg DNA per assay respectively) for endogenous RNA polymerase activity (Methods). When needed, α-amanitin (2 μg/ml final) was preincubated for 5 min at 0°C before the addition of the radioactive reaction mixture. The assays were done in duplicate and the average of the results is given.

α-Aminitin concentration (μg/ml)	B [^{3}H] UMP incorporated cpm	Percent
0	17 166	100.0
4×10^{-3}	2420	14.1
4×10^{-2}	376	2.2
4×10^{-1}	202	1.2
4×10^{0}	148	0.9
4×10^{1}	161	0.9

(B) Aliquots (0.2 ml) of sarkosyl supernatant fraction from infected cells (same as in table 3) were preincubated for 10 min at 0°C with various final concentrations of α-amanitin. RNA synthesis was then assayed in duplicate (see Methods) and the average of the results is given.

from an active sarkosyl supernatant of an infected culture (containing the molecules susceptible to be bound to the enzyme) was purified and then incubated with nuclei of uninfected cells. Sarkosyl extracts were prepared and RNA polymerase activity was assayed in the same way for these mock* infected and normally infected nuclei. Table 2 shows that transcription activity was associated with the sarkosyl supernatant of infected cells (line 4), whereas the reconstructed (mock*) supernatant had no activity (line 3). As a control, the results shown in lines 1 and 2 indicate that the nuclei used in this experiment were capable of active synthesis of RNA (corresponding to 5630 and 6114 cpm per μg DNA for mock and infected nuclei respectively). This experiment suggests that the transcription activity we observed in sarkosyl supernatants is not an artefact due to the isolation method. Since this kind of reconstruction experiment cannot duplicate exactly the conditions prevailing in the cell at the time of extraction, we give in table 3 better evidence that the complex contained RNA polymerase that had initiated RNA synthesis in vivo. The rifamycin derivative AF/013, which is known to inhibit initiation but not elongation of RNA synthesis [18], had no effect on the transcription activity of the complex even at very high concentrations (200 μg/ml). At 246 mM ammonium sulfate (actually more than 350 mM in salt, see Methods), a concentration which prevents the binding of RNA polymerase to DNA [18–20] the RNA synthesis in the sarkosyl supernatant is not affected. At higher ammonium sulfate concentrations (446 mM), about 40 percent inhibition is observed,

Table 2
Reconstruction experiment

Conditions	[^{3}H]UMP incorporated (cpm)
Uninfected nuclei	2.3×10^6
Infected nuclei	3.6×10^6
Mock*, sarkosyl supernatant	9.6×10^2
Infected, sarkosyl supernatant	2.8×10^5

The DNA (18 μg) contained in the sarkosyl supernatant from about 10^8 infected nuclei (36 hr p.i.) was purified (see Methods) and resuspended in 2 ml of Re buffer. This DNA was added to the same amount of uninfected nuclei. After 15 min of incubation at 4°C, sarkosyl supernatant was prepared (mock* supernatant) and assayed for RNA polymerase activity as described in Methods. Simultaneously, the same amount of infected nuclei (36 hr p.i.) were incubated with 2 ml of Re buffer; sarkosyl supernatant was obtained and assayed as above. An aliquot (0.1 ml) of the uninfected and infected nuclei in this experiment was assayed for total RNA synthesis (line 1 and 2). The measures were done in duplicate and the average of the results is indicated. The values given in this table correspond to about 10^8 cells.

probably due to the release of initiated enzyme molecules from the template [7,21].

From these results, together with the fact that all the assays are made in the presence of sarkosyl, which is known to remove nearly all the proteins from DNA except RNA polymerase that had initiated transcription [7,8], we may conclude that the active complex we isolated had initiated RNA synthesis in vivo, during viral development.

The best proof that the complex is virus-specific was obtained through molecular hybridization experiments. The RNA synthesized in the sarkosyl extract was purified (see Methods) and hybridized to a saturating amount of DNA immobilized upon nitrocellulose filters. As shown in table 4, approximately the same extent of hybridization was found for the RNA synthesized in sarkosyl supernatants prepared 24 hr after infection and 43 hr after infection, and for the SV40 cRNA control (73.6, 83.0 and 84.2 percent of the input respectively). Furthermore, very low levels of hybridization with cellular (CV1) DNA were observed for these RNA's (0.07, 0.11 and 0.51 percent of the input respectively). Thus, the RNA made in the sarkosyl supernatant of infected cultures behaves similarly to SV40 cRNA, suggesting that only viral DNA sequences are transcriptionally active in the complex we have isolated. Table 4 also shows that in the case of RNA synthesized in the chromatin pellet isolated 24 hr and 43 hr p.i., only 1.2 and 4.2 percent

Table 3
Effect of rifamycin AF/013 and salt on the endogenous RNA polymerase activity in sarkosyl supernatant fraction from infected cells

Incubation conditions		[^{3}H]UMP incorporated cpm	Percent
Standard		17 166	100.0
Dimethylformamide		17 003	99.1
Rifamycin AF/013 (μg/ml)	16	16 015	93.3
	80	17 190	100.1
	200	17 579	102.4
Ammonium sulfate (mM)	146	20 784	121.1
	246	16 657	97.0
	446	10 286	59.9

Sarkosyl supernatant was prepared from purified nuclei from infected (42 hr p.i.) cells (see Methods). Duplicate aliquots of this supernatant were preincubated for 10 min at 0°C with no inhibitor (standard), rifamycin AF/013 or ammonium sulfate (final concentrations are given), then assayed for endogenous RNA polymerase activity as described (Methods). The rifamycin AF/013 was dissolved in dimethylformamide; as control, the same volume of this solvent (10 μl) was added instead of the inhibitor. Under similar conditions this drug inhibits more than 99% of the activity of *E. coli* RNA polymerase that has not initiated RNA synthesis (not shown).

Table 4
Hybridization properties of [^{3}H] RNA synthesized in sarkosyl supernatant and chromatin pellet

Source of RNA	[^{3}H] RNA input (cpm)	DNA on filter	[^{3}H] RNA hybridized (cpm) 2 μg DNA	4 μg DNA	% of input
SV40 cRNA	36 185	SV40	29 675	31 281	84.2
		CV1	181	191	0.51
Sarkosyl supernatant (24 hr p.i.)	22 755	SV40	15 626	17 848	73.6
		CV1	12	18	0.07
Sarkosyl supernatant (43 hr p.i.)	82 690	SV40	70 875	66 463	83.0
		CV1	80	94	0.11
Chromatin (24 hr p.i.)	212 631	SV40	2251	2963	1.23
		CV1	463	740	0.35*
Chromatin (43 hr p.i.)	159 050	SV40	6211	7255	4.23
		CV1	457	689	0.43*
Chromatin (mock)	179 967	SV40	94	130	0.06
		CV1	335	462	0.26*

CV1 cells were infected and fractionated into sarkosyl supernatant and chromatin pellet 24 and 43 hr p.i. Concentrated sarkosyl supernatants as well as chromatin fractions were resuspended in Re buffer and incubated for 60 min at 30°C for RNA polymerase reaction as indicated in Methods, except that 6 μM [^{3}H] UTP (22 μCi) were used in the case of sarkosyl supernatant. The purified RNA products, as well as the SV40-cRNA, were hybridized in duplicate with saturating amounts of DNA (2 μg and 4 μg). The percentage of hybridization of the input was calculated on the average of the results, except for * where only the value obtained with 4 μg DNA was used, since with 2 μg DNA the saturating conditions were not reached.
The background of hybridization (0.04 to 0.06 percent of the input) was obtained in a parallel experiment in which calf thymus DNA was immobilized on the filters.

of the input, respectively, was able to hybridize with SV40 DNA. This comparatively low extent of hybridization may be due to a contamination of the pellet with SV40 transcription complex which has not been totally extracted by sarkosyl; it may also reflect the occurrence of a closer association between SV40 DNA molecules and the chromatin of the infected cells (covalent integration for instance). The low proportion of hybridization (0.26% to 0.43% of the input) observed between RNA synthesized in vitro in the chromatin pellet and cellular DNA, could mean that, under our conditions, essentially non-repetitive sequences are transcribed by the endogenous polymerase.

In order to further characterize the SV40 complex, parallel sarkosyl supernatants obtained 42 hr after infection, from [^{3}H] thymidine labelled (24 to 42 hr p.i.) and unlabelled cultures, were analyzed by sedimentation through linear sucrose density gradients. Fig.1 shows that the majority of both the labelled DNA and the endogenous RNA polymerase activity have a sedimentation constant of more than 80 S. The fractions of [^{3}H] DNA corresponding to the highest values of polymerase activity were pooled and analyzed by sedimentation through an alkaline CsCl density gradient (inset, fig.1). It can be seen that this DNA behaves essentially as SV40 DNA form I (53 S). The same result was obtained with DNA extracted from the sarkosyl supernatant (not shown), indicating that the majority of the DNA extracted with sarkosyl is viral DNA.

It is very likely that most of the nuclear SV40 DNA transcribing activity is present in the sarkosyl supernatant, since the amount of viral DNA present in the supernatant represent 70 to 90 per cent of the amount of viral DNA extracted with the Hirt method [12] (not shown). In addition, hybridization experiments have established that the total virus specific

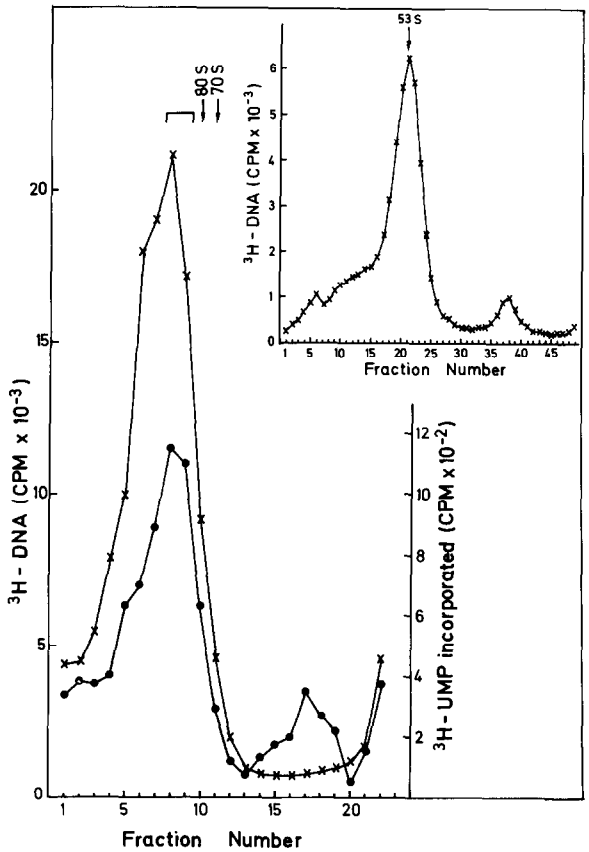

Fig.1. Sedimentation pattern of [^{3}H] DNA and RNA polymerase activity in sarkosyl supernatant of infected cells. Sarkosyl supernatants were prepared (42 hr p.i.) from unlabelled or [^{3}H] thymidine labelled (24 to 42 hr p.i.) infected cultures (see Methods). Samples (0.45 ml) were sedimented for 100 min at 38 000 rev/min in a Spinco SW50.1 rotor at 2°C through 4.8 ml neutral sucrose density gradients (5–20% wt/vol in Re buffer). Fractions were collected and assayed for endogenous RNA polymerase activity (•–•) or [^{3}H] DNA (x–x). The arrows indicate the positions of 80 S MS_2 phage and 70 S *E. coli* ribosomes run in parallel gradients. Fractions 8 and 9 of the [^{3}H] thymidine labelled gradient were pooled and analyzed (inset figure) by sedimentation through an alkaline CsCl density gradient (see Methods). Purified [^{14}C]-SV40 DNA (53 S) was used as internal sedimentation marker.

[^{3}H] RNA synthesized at 43 hr p.i. in the sarkosyl supernatant represents about 96 percent of the total SV40 specific [^{3}H] RNA synthesized in the same preparation of infected nuclei, lysed with sarkosyl as above, but not fractionated (not shown).

We conclude that the nucleoprotein complex present in sarkosyl supernatants from SV40 infected monkey cells exhibits the properties expected for an active virus specific transcription complex, since:

1) it is found only after infection;
2) its polymerase molecules have initiated RNA synthesis in vivo and are highly sensitive to inhibition by α-amanitin;
3) the RNA product contains exclusively SV40 sequences.

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