SARKOSYL ACTIVATION OF RNA POLYMERASE ACTIVITY IN MITOTIC MOUSE CELLS

Patricio GARIGLIO, Janice BUSS and Melvin H. GREEN
Department of Biology, University of California, San Diego
La Jolla, California 92037, USA

Received 21 May 1974

1. Introduction

The transition from the G2 phase of the eukaryotic cell cycle to the mitotic phase produces dramatic morphological alterations, among which are the condensation of chromatin to form chromosomes, and the disappearance of the nuclear membrane and nucleoli. These changes are accompanied by a nearly complete inhibition of cellular RNA synthesis [1]. Recent work by Simmons et al. [2] indicates that inhibitors of protein synthesis did not prevent the resumption of RNA synthesis when mitotic cells entered the G_1 phase, suggesting that RNA polymerase is present but not active during mitosis.

In recent studies on the regulation of polyoma virus transcription, we observed that an anionic detergent, Sarkosyl, caused a several-fold stimulation of endogenous RNA polymerase activity in mouse chromatin [3]. Nearly all of the increased activity was due to the α-amantin-sensitive RNA polymerase, [4–6], i.e., form II or B [7,4]. It was postulated that the Sarkosyl effect is due primarily to the release of one or more proteins which repress RNA polymerase II. Except for RNA polymerase, Sarkosyl caused the release of essentially all the proteins from chromatin (unpublished data).

Based on these observations, it was questioned whether Sarkosyl could unmask the RNA polymerase activity from metaphase-arrested cells, in which the condensation of DNA is maximal. The experiments presented here provide evidence that RNA polymerase forms I and II remain associated with the chromosomes of mouse cells undergoing mitosis.

2. Materials and methods

Balb/C 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. To arrest cells in metaphase, subconfluent cultures were treated for 19 hr with 0.4 μg/ml. colcemid. The extent of mitosis was visually assayed by determining the percentage of cells which were bright and rounded by phase contrast microscopy. RNA synthesis was assayed by labeling cultures for 90 min with 5′-[^{3}H]uridine (16 μCi/0.2 μg/ml) and determining the amount of acid precipitable [^{3}H]RNA, optical density at 260 nm, and DNA concentration [8] of separate aliquots.

In vitro RNA polymerase assays were carried out as follows. Growing and colcemid-arrested cells were harvested by centrifugation and washed with Tris-buffered saline. The cells were resuspended in HR buffer (25 mM Tris–HCl, pH 7.9, 0.5 mM dithiothreitol, 1.0 mM $MgCl_2$, 0.4 mM $CaCl_2$; ref. [9]), homogenized with a loose fitting Dounce homogeniser, and adjusted to 0.09 M KCl, 0.2 M sucrose. Duplicate aliquots of the whole cell homogenates were treated with Sarkosyl (0.4%) plus Triton X-100 (0.1%) in TE buffer (0.01 M Tris–HCl, pH 7.9, 0.2 mM EDTA). A modification of the reaction mixture of Roeder and Rutter [10] was used in a final vol of 0.25 ml containing 56 mM Tris–HCl pH 7.9, 8 mM KCl, 6 mM NaF, 1.6 mM dithiothreitol, 46 mM ammonium sulfate, 1.8 mM $MnCl_2$, 0.6 mM each of GTP, CTP, and ATP, 3 μM UTP, and 0.6 μM [^{3}H]UTP (2.2 μCi per assay). Polymerase reactions were carried out for the indicated periods of time at 22°C.

 North-Holland Publishing Company – Amsterdam

Reactions were stopped by the addition of 2 ml of ice-cold 0.1% trichloroacetic acid (TCA), followed by vigorous pipetting and an additional 1 ml of cold 10% TCA containing 0.02 M pyrophosphate. After 30 min on ice, the precipitates were collected on Whatman GF/C filters, washed three times with cold 5% TCA containing 0.01 M pyrophosphate and once with 95% ethanol, then dried and counted by liquid scintillation spectrometry. Assays with zero time incubation were used to determine background levels of incorporation; samples were left on ice and TCA was added immediately after addition of the radioactive reaction mixture. The backgrounds ranged from 100–300 cpm and have been subtracted from all data except those in table 3A.

Native calf thymus DNA (grade A) was obtained from Calbiochem and where applicable, was denatured by boiling for 10 min. α-Amanitin was also purchased from Calbiochem. Rifamycin AF/013 was generously donated by Drs. Silvestri, Lancini and Cricchio of GruppoLepetit in Milan, Italy.

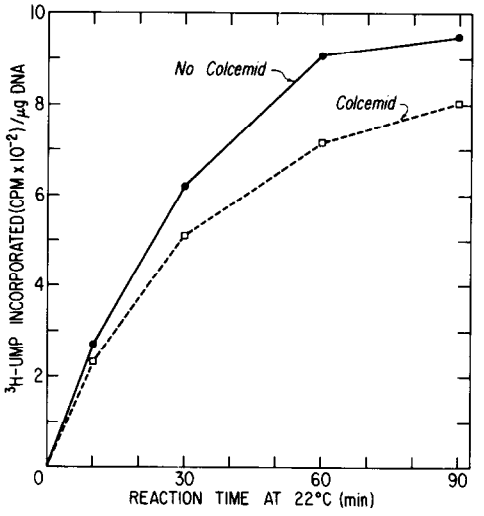

Fig. 1. Kinetics of RNA synthesis by Sarkosyl extracts of growing vs mitotic mouse cells. Growing and colcemid-arrested 3T3 cells were lysed by Sarkosyl treatment, then assayed for endogenous RNA polymerase activity as described in Materials and methods. Activities were normalized per input amounts of DNA, which was 10.2 μg from the colcemid-arrested cells and 12.2 μg from the control cells.

3. Results and discussion

Mouse 3T3 cells were arrested in metaphase by treatment with colcemid (0.4 μg/ml). Treated and control cultures were examined for the appearance of mitotic cells after 19 hr, and parallel plates were labeled for 90 min with [^{3}H]uridine. In the experiment described in table 1, 80–90% of the colcemid-treated cells were observed to be in mitosis, and the incorporation of [^{3}H]uridine into RNA was reduced to 6% of that in growing cells. This result is in accordance with previously published data [1].

Parallel unlabeled cultures of growing and colcemid-treated cells were scraped off the plates and lysed by the addition of Sarkosyl. These extracts were used immediately to assay the kinetics of incorporation of [^{3}H]UTP into RNA. As seen in fig. 1, the Sarkosyl extract from mitotic cells had 80–85% of the RNA polymerase activity found in growing cells. The activities have been normalized to the amounts of RNA present, and are very close to the specific activity found by Sarkosyl addition to purified nuclei (unpublished data). In whole cell extracts of growing and metaphase-arrested cells prepared without Sarkosyl, the RNA synthesized during the first 10 min of the reaction was rapidly and completely

Table 1
RNA synthesis in growing vs mitotic mouse cells

Conditions	Mitotic cells (%)	[^{3}H]Uridine incorporated		
		CPM	CPM/OD_{260}	CPM/μg DNA
No colcemid	< 5	98 488	24 622	1 244 (100%)
Colcemid	80–90	5 676	2 447	76 (6.1%)

Assays and conditions were as described in Materials and methods.

Table 2
Effect of α-amanitin and Rifamycin AF/013 on RNA polymerase activity in extracts of growing vs mitotic mouse cells

Additions		[^{3}H]UMP incorporated			
		No colcemid		Plus colcemid	
		(cpm)	(%)	(cpm)	(%)
None		11 099	100	7266	100
α-Amanitin:	1 μg/ml	2947	27	2140	30
	10 μg/ml	2855	26	2121	29
Rifamycin AF/013:	12 μg/ml	11 256	101	6942	96
	120 μg/ml	10 335	93	6918	95

Aliquots of the same cell homogenates used in the experiment described in fig. 1 were preincubated with the indicated concentrations of α-amanitin or Rifamycin AF/013 for 5 min at 0°C, then assayed for endogenous RNA polymerase activity. Reactions were carried out for 60 min at 22°C.

hydrolyzed upon subsequent incubation (data not shown). Sarkosyl thus not only permits the efficient recovery of RNA polymerase activity from mitotic cells, but apparently is also a potent inhibitor of RNase in mouse cell extracts.

It is possible that the small reduction of total activity in mitotic cell extracts was due to the specific loss of RNA polymerase I or III. The levels of both of these forms are thought to fluctuate markedly during the development of embryonic cells [6,11]. Using α-amanitin to inhibit RNA polymerase II we found the same fraction (26–30%) of drug-resistant activity in the mitotic and growing cell extracts (table 2). Since the α-amanitin resistant activity may be due to both RNA polymerase forms I and III [6], we cannot be certain that the ratio of forms I to III remained constant following colcemid arrest. However, it is clear that the small decrease in activity found in extracts from mitotic cells was not due to the preferential loss of RNA polymerase II.

We next addressed the question as to whether the Sarkosyl-activated RNA polymerase was localized on the chromosomes in mitotic cells. As seen in table 2, Rifamycin AF/013 caused almost no inhibition over a 10-fold concentration range. Since this drug inhibits the known forms of uninitiated eukaryotic RNA polymerase [12], it appears that the activity found in mitotic cell extracts is present in an initiated state. Furthermore, there was no stimulation of endogenous activity by the addition of calf thymus DNA to the Sarkosyl extracts (data not shown).

The strongest support for the above conclusion is provided by the differential effect of Sarkosyl on uninitiated as compared with initiated RNA polymerase. DNA-dependent RNA polymerase was partially purified from HeLa cells as described by Kedinger et al. [13]. In table 3A, it may be seen that 0.5% Sarkosyl, as well as Rifamycin AF/013, caused the nearly complete inactivation of uninitiated HeLa RNA polymerase. Little inhibition was caused by dimethylformamide (the solvent for Rifamycin) at a concentration present in the Rifamycin analyses.

To examine the effect of Sarkosyl on initiated RNA polymerase, the activity was compared with that found when Rifamycin AF/013 was added after starting the reaction (table 3B). Under the conditions described, approximately 65% of the initiated (i.e., rifamycin insensitive) polymerase activity was resistant to Sarkosyl with native calf thymus DNA as a template. With denatured DNA as a template, only about 25% of the initiated polymerase was resistant to Sarkosyl. In this experiment it was also found that with no inhibitors added, there was a 2.5-fold increase in RNA synthesized between 60 and 210 min of the reaction with native DNA as a template (data not shown). With rifamycin or Sarkosyl present, the increase in newly synthesized RNA during that time interval was only 1.3-fold. This result indicates that Sarkosyl and Rifamycin AF/013 were equally active in blocking reinitiation by HeLa RNA polymerase.

We thus conclude that RNA polymerase becomes resistant to Sarkosyl after initiating RNA synthesis on double-stranded DNA. Further studies are necessary in order to establish the precise step in transcription at which resistance occurs. Based on these and the above results we infer that there is nearly as much initiated RNA polymerase in mitotic cell chromosomes as found in growing cells.

The resistance of initiated RNA polymerase to Sarkosyl NL-30 may seem surprising in that the structure of this anionic detergent ($CH_3(CH_2)_7CON(CH_3)CH_2COONa$) superficially resembles that of sodium dodecyl sulfate (SDS), which inactivates initiated mouse RNA polymerase [3]. The similarity in the action of Sarkosyl and Rifamycin AF/013 may be based on the 8 carbon hydrophobic chain common

Table 3
A. Effect of Sarkosyl on uninitiated HeLa RNA polymerase activity

	[^{3}H]UMP incorporated (cpm)
No additions	18 898
Dimethylformamide	16 230
Rifamycin AF/013	1 254
Sarkosyl	459

B. Effect of Sarkosyl on initiated HeLa RNA polymerase activity

Template	Incubation conditions	[^{3}H]UMP incorporated (cpm)	Percent
Native calf DNA	Rifamycin, 60 min	855	100
	Sarkosyl, 60 min	556	65
	Rifamycin, 210 min	1113	100
	Sarkosyl, 210 min	743	67
Denatured calf DNA	Rifamycin, 60 min	876	100
	Sarkosyl, 60 min	193	22
	Rifamycin, 210 min	1149	100
	Sarkosyl, 210 min	309	27

A. DNA-dependent RNA polymerase (P-40 faction) was purified from HeLa cells according to the procedure of Kedinger, et al. [13]. The enzyme was mixed with native calf thymus DNA (65 μg/ml) for 10 min at 0°C in MS [30] buffer [13]. Duplicate aliquots were then incubated for 5 min at 0°C either with 1% dimethylformamide, Rifamycin AF/013 (L00 μg/ml) in 1% dimethylformamide, or 0.25% Sarkosyl in a final vol of 0.2 ml. The nucleoside triphosphate-containing reaction mixture (0.05 ml) was then added, the samples were incubated for 30 min at 26°C, and RNA synthesis was measured as in Materials and methods. The data is shown subtracting zero time backgrounds.
B. HeLa RNA polymerase and native or heat-denatured calf thymus DNA were mixed as in part A, non-radioactive reaction mixture was added, and RNA synthesis was allowed to proceed for 10 min at 26°C. The samples were then cooled to 0°C, and to separate aliquots were added either 80 μg/ml Rifamycin AF/013 or 0.25% Sarkosyl. Assays were then incubated at 26°C for either or 210 min, and RNA synthesis was analyzed as in Materials and methods.

to each. As Chambon and his coworkers point out, the nature of this side-chain plays an important role in the activity of Rifamycin derivatives on eukaryotic RNA polymerases [12]. It may be that the more hydrophobic chain in SDS makes this detergent more readily accessible than Sarkosyl to RNA polymerase which has initiated synthesis on a native DNA template.

Further studies with purified chromosomes and chromatin are needed in order to understand how RNA polymerase is repressed within these structures. Since RNA polymerase appears to be in the initiated state on the chromosomes of mitotic cells, it is conceivable that nascent RNA chains as well as proteins play a role in making DNA unavailable for transcription. The Sarkosyl extraction method affords the opportunity to study RNA polymerases which were present in a repressed state during various stages of viral or cell growth and development.

Acknowledgements

This research was supported by the National Science Foundation (Grant GB-36653).

References

[1] Mitchison, J. M. (1971) The Biology of the Cell Cycle (Cambridge University Press, London) 116–117.
[2] Simmons, T., Heywood, P. and Hodge, L. (1973) J. Cell. Biol. 150–164.
[3] Shmookler, R. J., Buss, J. and Green, M. H. (1974) Virology 57, 122–127.
[4] Kedinger, C., Gniazdowski, M., Mandel, J. L., Gissinger, F. and Chambon, P. (1970) Biochem. Biophys. Res. Commun. 38, 165–171.
[5] Jacob, S. T., Sajdel, E. M. and Munro, H. N. (1970) Nature 225, 60–62.
[6] Lindell, T. J., Weinberg, F., Morris, P. W., Roeder, R. G. and Rutter, W. J. (1970) Science 170, 447–449.
[7] Roeder, R. G. and Rutter, W. J. (1969) Nature 224, 234–237.
[8] Burton, K. (1955) J. Biochem. 61, 473–483.
[9] Winnacker, E. I., Magnusson, G. and Reichard, P. (1971) Biochem. Biophys. Res. Commun. 44, 952–957.
[10] Roeder, R. G. and Rutter, W. J. (1970) Proc. Natl. Acad. Sci. U.S., 65, 675–682.
[11] Kohl, D. M., Norman, J. and Brooks, S. (1973) Cell Differentiation 2, 21–32.
[12] Meilhac, M., Tysper, Z. and Chambon, P. (1972) Eur. J. Biochem. 28, 291–300.
[13] Kedinger, C., Gissinger, F., Gniazdowski, M., Mandel, J. L. and Chambon, P. (1972) Eur. J. Biochem. 28, 269–276.