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Mercurated nucleotides: assessment of a new tool to study RNA synthesis and processing in isolated nuclei

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ABSTRACT

Mercurated pyrimidine nucleotides have been used to study RNA synthesis and processing in isolated nuclei from mouse L cells. 5-mercuridine triphosphate (5-Hg-UTP) or 5-Hg-CTP are accepted as substrates by the purified RNA polymerases (I+III) and (II) from mouse cells, respectively, as well as by the enzymes still bound to the nuclear chromatin. In nuclei, RNA synthesis in the presence of Hg-UTP is reduced to 60-70% of a control. 30-60% of RNA labeled in vitro with (^{3}H)UTP in isolated nuclei is not retained on sulfhydryl sepharose columns. Sucrose gradient analysis reveals a size distribution of the non-bound RNA similar to non-mercurated control RNA. Hg-RNA is found in a single peak from 4-10S. Chase experiments indicate that this RNA is the original transcript. It is argued that Hg-nucleotides may cause premature chain termination. Methylation of RNA in vitro by S-adenosyl methionine ((^{3}H)SAM) is reduced to 75% of controls in the presence of Hg-UTP. Only 6% of the methyl groups appear in Hg-RNA. Polyadenylation is reduced as well. 15% of poly(A)(+)RNA are found in control assays whereas only 1% of Hg-RNA carries a poly(A) end added in vitro. These results limit the use of mercurated nucleotides for studies of nuclear RNA synthesis and processing.

INTRODUCTION

Mercurated pyrimidine nucleoside triphosphates seemed to offer almost ideal possibilities to study certain aspects of RNA transcription and metabolism in vitro. They can be easily synthesized and are stable under conditions of RNA synthesis usually applied (1). They are accepted as substrates with surprisingly high efficiency by RNA polymerase from E.coli using either DNA (1) or isolated chromatin (2,3) as template. Even the endogenous RNA polymerase(s) in isolated nuclei is able to incorporate the mercurated triphosphates with about 65% efficiency (3). The mercurated RNA can be separated from the bulk of non-mercurated RNA via affinity chromatography on sulfhydryl

12 mM) for 30 min at 25°. Nucleoside triphosphates were present at 0.3 mM including Hg-UTP. The labeled triphosphate (^{3}H)CTP or (^{3}H)GTP) was added at 50-200 µCi/ml (5-20 µM).

Preparation of RNA: The method has been described in full detail elsewhere (8). Briefly, it is based on the method developed by Kirby (9) using anionic detergents which inhibit RNAases effectively. The method is highly reliable with a deviation of less than ± 5% of extracted total cpm in parallel experiments. After extraction the RNA is treated with DNAase (Boehringer Nr. 15469, tested to be RNAase-free by incubation with (^{3}H)poly(U) and (^{3}H)rRNA (8)) and after that with proteinase K (E.Merck, Darmstadt).

Comment: The A_{260} absorption pattern which we obtain upon collection of the sucrose gradients is perfectly reproducible (8) when no Hg-nucleotides are used. With Hg-UTP or Hg-CTP present, however, this profile clearly shows breakdown of RNA, e.g. loss of 28S and 18S rRNA (data not shown). This breakdown is caused by the presence of the metal compounds and cannot be eliminated by applying other methods of RNA preparation e.g. the hot phenol/SDS method.

Preparation of sulfhydryl sepharose: SH-sepharose (10) was prepared from Sepharose 4B (Pharmacia) following the procedure given by Dale et al. (4). The sulfhydryl content was determined spectrophotometrically using 5,5'-dithiobis (2-nitrobenzoic acid) (11). It varied from 1 to 3 µmol SH/ml of packed gel.

Preparation of mercurated uridine triphosphate: Mercuration of UTP was achieved according to Dale et al. (1, 4). To 2 ml of a 0.02 M UTP solution in 0.1 M Na-acetate, pH 6.0, were added 2 ml of a 0.1 M solution of mercuriacetate in the same buffer. The mixture was warmed to 50° for 3 hr. After cooling to room temperature the mixture was applied to a Sephadex G-10 column (1.5 x 45 cm). Elution was with 0.02 M Tris.HCl, pH 7.0. The flow through was loaded onto a DEAE cellulose column (2 x 7 cm) and after washing eluted with a linear gradient of LiCl (0 to 0.4 M) in the above Tris buffer. The triphosphate peak was used for affinity chromatography on SH-sepharose. After application the SH-sepharose column (1.5 x 10 cm) was washed with the above

Tris buffer containing 0.1 M NaCl. All non-mercurated UTP came off in this step. The Hg-UTP was then eluted with the Tris-NaCl buffer containing 0.2 M ß-mercaptoethanol. This peak was immediately diluted twice and loaded onto another DEAE cellulose column, thereby minimizing contact time with high concentrations of the thiol compound. Elution of Hg-UTP was achieved by a 0.4 M LiCl salt step. After a reduction in volume by rotary evaporation the Hg-UTP was precipitated with acetone. Overall yield in Hg-UTP varied from 30 to 50% when related to the original amount of UTP. The substance was checked spectrophotometrically and by chromatography on PEI cellulose thin layer plates (1, 3). No unmercurated UTP could be detected. Hg-UTP was stored as 0.01 M aqueous solution at -20°. The same procedure was used for Hg-CTP preparation.

Separation of mercurated RNA: The sample containing the mercurated RNA in a low ionic strength buffer was made 0.2% in SDS and applied to a 5 ml column of SH-sepharose in SH-sepharose loading buffer (see table 1 for buffer compositions). After elution of the non-bound material and extensive washing with the same buffer, the mercurated RNA was detached from the column with SH-sepharose elution buffer and ethanol precipitated immediately.

Methylation of RNA: (8) Methylation of RNA was achieved by adding 20 µCi/ml of (^{3}H)S-adenosyl methionine (Amersham) to the incubation assay which also contained 0.3 mM of all four triphosphates including Hg-UTP. Incubation was for 30 min at 25°.

Preparation of poly(A)-containing RNA: (8) The RNA sample was adjusted to 0.7 M NaCl and 20% (v/v) formamide and applied to a poly(U) sepharose (Pharmacia) column (0.7 ml) equilibrated with 0.05 M Tris.HCl, pH 7.5, 0.7 M NaCl, 0.01 M EDTA, 5 µg/ml polyvinyl sulfate, 20% (v/v) formamide. After binding and washing with the same buffer the poly(A)(+)RNA was eluted from the column with 0.01 M K.PO_4, pH 7.5, 0.01 M EDTA, 5 µg/ml polyvinyl sulfate, 90% (v/v) formamide. After dialysis the poly(A)(-)RNA and the poly(A)(+)RNA were precipitated by ethanol from the flow through and the eluate, respectively.

Sucrose gradient analysis of RNA: RNA was analysed on 10-60% (w/v) sucrose gradients (12, 8). The gradients (17 ml) were spun

for 19 hr and 25.000 rpm in a Beckman SW 27.1 rotor at 21°. The gradients were collected using an LKB fraction collector in combination with an LKB Uvicord II 254 nm monitoring system.

RESULTS AND DISCUSSION

Incorporation kinetics of the ribonucleoside triphosphates in the presence of Hg-UTP: Nuclei from L cells synthesize RNA for 60 min and longer (fig. 1 A)(8) when incubated with all four ribonucleoside triphosphates. When UTP is replaced by Hg-UTP, however, incorporation rates drop to about 60% of control values and incorporation nearly stops after 30-40 min. This situation is comparable to transcription in mouse myeloma nuclei although in this case no plateau is reached within 60 min (2). The labeling kinetics is dependent on the nucleotide used as tracer (fig. 1 B). The control was done with (^{3}H)CTP as tracer. In the presence of Hg-UTP there was about 75% of control incorporation but only 50% using (^{3}H)GTP. The extremely low incorporation of

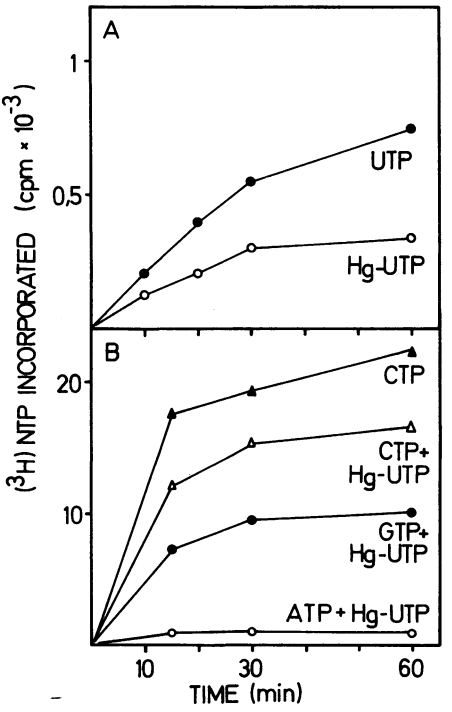

Figure 1: Incorporation kinetics of the ribonucleoside triphosphates in the presence of Hg-UTP.

A 3 x 10^6 nuclei/200 µl were incubated in incubation buffer at 25°. All NTP were present at 0.3 mM.

(^{3}H)UTP was added as 10 µCi/assay.

-●- control with UTP

-o- as before, but Hg-UTP replacing UTP

B 2 x 10^6 nuclei/200 µl were incubated as before. All NTP except the tritiated tracer were present at 0.3 mM. The tracer was added as 5 µCi/assay ≙ approx. 1 µM.

-▲- control with (^{3}H)CTP and UTP, ATP, GTP

-△- (^{3}H)CTP and Hg-UTP, ATP, GTP

-●- (^{3}H)GTP and Hg-UTP, ATP, CTP

-o- (^{3}H)ATP and Hg-UTP, GTP, CTP.

(^{3}H)ATP may be explained by the presence of an intranuclear pool of unlabeled ATP (which amounts to about 10 µM, H. Land, unpublished observation). In addition, Hg-UTP interferes with the polyadenylation reaction, thereby diminishing the incorporation of ATP even further (see fig. 6 and table 2). In later experiments either (^{3}H)CTP or (^{3}H)GTP, respectively, were used. I have not tested whether an increase in ionic strength would eliminate the plateau in the Hg-UTP-containing reactions. The low ionic strength conditions were chosen to allow not only RNA synthesis but also the various RNA processing reactions like 3' poly(A) addition (13) as well as 5' end capping and methylation (14).

Isolation of Hg-RNA on SH-sepharose columns: One critical step during the isolation of Hg-RNA is the separation from the bulk RNA on SH-sepharose columns (fig. 2). This separation has to be complete if any conclusions are to be drawn from the properties, sequence complexity and modification of the Hg-RNA.

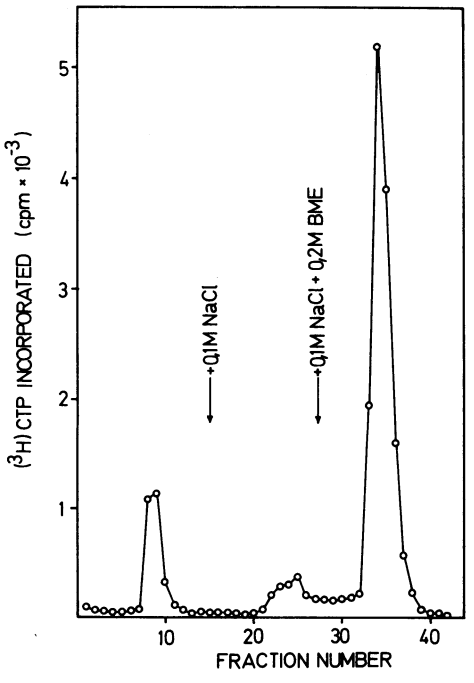

Figure 2: Elution profile of Hg-RNA on SH-sepharose. Calf thymus DNA was transcribed in vitro by RNA polymerase from E.coli using (^{3}H) CTP as tracer and including Hg-UTP in the reaction. The RNA was isolated as described and applied to an SH-sepharose column (2 x 10^{4}cpm total). The buffers used are given in table 1. Recovery of cpm was 95%.

We have found the procedure outlined in table 1 to give optimum results with respect to the purity of the Hg-RNA.

Total RNA should be loaded to the SH-sepharose column in low ionic strength buffer containing SDS. The low ionic strength keeps secondary and tertiary structure of the RNA to a minimum and allows maximum access of the SH-groups to all mercurated sequences. The SDS eliminates unspecific binding of DNA and non-mercurated RNA.

In addition, one has to make sure that the RNA is no longer bound to protein or template DNA or both (table 1). Whereas it seems to be the minor problem to get rid of all non-mercurated RNA it is probably impossible to get all Hg-RNA to bind to the column. Unspecific RNA aggregation was not detectable when the washing procedure used by Crouse et al. (3) including DMSO (dimethylsulfoxide) treatment was applied. On the other hand, raising the ionic strength to 0.1 M NaCl during the washing step always caused the release of a few percent of

Table 1 Separation of Hg-RNA on SH-sepharose columns.

Loading buffer: 0.01 M Tris.HCl, pH 7.4, 0.2% SDS
Wash buffer: 0.01 M Tris.HCl, pH 7.4, 0.1 M NaCl, 0.2% SDS
Elution buffer: 0.01 M Tris.HCl, pH 7.4, 0.1 M NaCl, 0.2 M ß-mercaptoethanol, 0.2% SDS.

RNA sample		Percent of cpm in fraction Flow-through	Wash	Eluate
Control RNA (Hg-free)		> 98	< 1	< 1
Hg-RNA (AT)*		0.1	2.2	97.7
Hg-RNA (CT)**		2 - 15	7 - 14	71 - 91
Hg-RNA (CT) + DNAase treatment only		17	~ 0	83
Hg-RNA (CT) no DNAase treatment		38	~ 0	62
Hg-RNA (nuclei)***	average	40	60	
	range	30 - 60	70 - 40	

*transcribed from poly(dA-dT)
**transcribed from calf thymus DNA } using E.coli RNA polymerase
***transcribed in vitro in nuclei by endogenous RNA polymerases

mercurated RNA. This material could be rebound after decreasing the ionic strength. Our interpretation would be that short mercurated sequences at the 3' end of RNA chains may be hidden within the RNA coil after shifting up the ionic strength. It is interesting in this respect (fig. 3) that the flow-through RNA contains a large proportion of long RNA chains. From experiments with (^{203}Hg)UTP it becomes obvious that the RNA which does not bind to the SH-sepharose column does indeed contain mercurated sequences (data not shown). What remains puzzling, however, is the broad range of non-binding RNA which varies from 30 to 60% for RNA transcribed in nuclei. The amount of (^{3}H) counts in this fraction is clearly higher than could be expected from the (^{203}Hg) counts. It was not possible to find experimental conditions neither for the transcription reaction nor for the RNA isolation under which the amount of flow-through RNA would be constant within a few percent.

In later experiments with RNA transcribed in isolated nuclei we have omitted the intermediate washing step but made the elu-

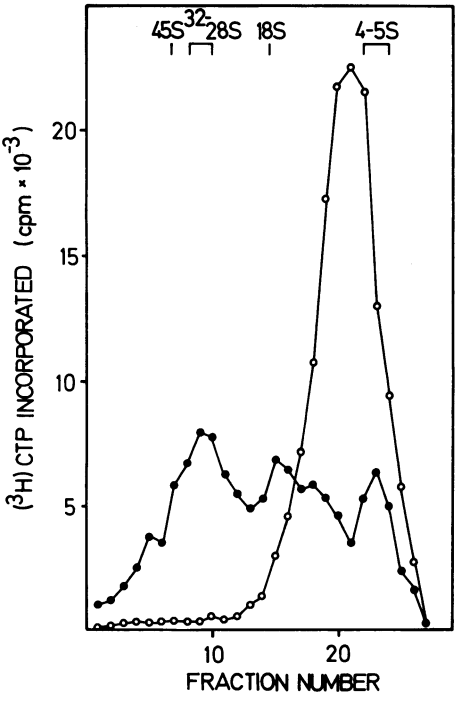

Figure 3: Sucrose gradient analysis of in vitro synthesized RNA. 70 x 10^{6} nuclei/1.2 ml were incubated as described for 60 min at 25° using (^{3}H)CTP (100 µCi/assay ≙ 4 µM) and ATP, GTP and Hg-UTP (0.3 mM each). RNA was isolated and the Hg-RNA separated by SH-sepharose chromatography (5 ml column). Hg-RNA and presumably non-mercurated flow-through RNA were analyzed on 10-60% sucrose gradients.

- ● - flow-through RNA
- o - Hg-RNA

tion buffer 0.1 M in NaCl and 0.2 M in ß-mercaptoethanol. After loading, the column was always washed very extensively with at least ten column volumes.

Sucrose gradient analysis of Hg-RNA: When the non-bound and the mercurated RNA fractions, respectively, are analysed on sucrose gradients the pattern of fig. 3 is obtained. The RNA which does not attach to the SH-sepharose exhibits a broad size spectrum from 4S up to 45S. The Hg-RNA, however, sediments as a rather homogeneous peak from 4S to about 10S.
Whether this RNA is a degradation product of longer precursor RNA can be decided by a pulse/chase experiment (fig. 4). In this experiment (^{3}H)CTP served as radioactive tracer and Hg-UTP as mercurated substrate. RNA was isolated either after 5 min or after 5 min followed by a period of 25 min during which the Hg-UTP was diluted by a tenfold excess of UTP, respectively. In both cases non-mercurated and mercurated RNA were separated and analyzed separately on gradients. The Hg-RNA labeled for 30 min under identical conditions was included as a control in fig. 4 B. As is evident from the gradients mercurated RNA arises as a class of short RNA chains and not by trimming or breakdown of larger precursors. Not all of the low molecular weight RNA, however, is mercurated (fig. 4 A). Practically no RNA longer than 18S is retained on the SH-sepharose column although considerable label flows into this class of RNA during the chase period (fig. 4 A).

From this experiment it cannot be decided, however, whether the relative homogeneity of the Hg-RNA is an experimental artifact; e.g. if Hg-UTP would cause premature chain termination - possibly in combination with a general slowdown of the RNA polymerizing reaction - we would expect a peak similar to that of figs. 3, 4 and 6 for the mercurated RNA.

Response of isolated RNA polymerases to Hg-UTP: Another possibility would be a differential response of the three RNA polymerases to Hg-UTP. Therefore RNA polymerase (I + III) and (II), respectively, were prepared from mouse ascites cells (15). The isolated enzymes were tested with and without Hg-UTP on poly(dA-dT), on denatured mouse DNA and on native mouse DNA (fig. 5). The E.coli RNA polymerase was included for comparison.

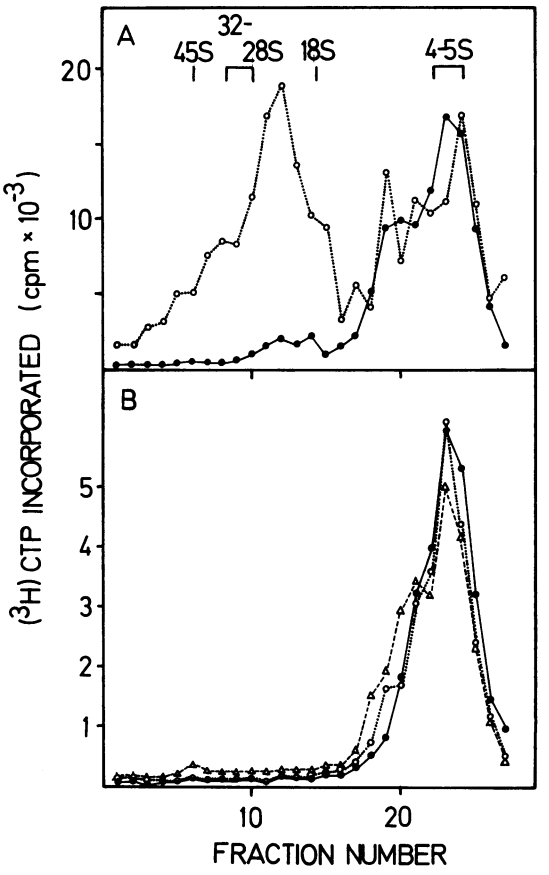

Figure 4: Labeling kinetics and fate of mercurated RNA.

50 x 10^6 nuclei/1 ml were incubated at 25° as described. (^{3}H)CTP (62 µCi/assay ≙ 3 µM) and ATP, GTP and Hg-UTP (each 0.3 mM) were present. After isolation, Hg-RNA (B) and presumably non-mercurated RNA (A) were analyzed on 10-60% sucrose gradients.

- ● - 5 min incubation; --Δ-- 30 min incubation

..o.. 5 min incubation followed by a 25 min chase in the presence of 3 mM UTP.

RNA polymerases (I + III) seem to be less active on native DNA than enzyme (II) when incubated with Hg-UTP. This effect, however, is not very dramatic and could certainly not explain the size distribution of the mercurated RNA. We would expect RNA of this size if RNA polymerase (III) would be responsible for its synthesis. In this case synthesis of Hg-RNA should be

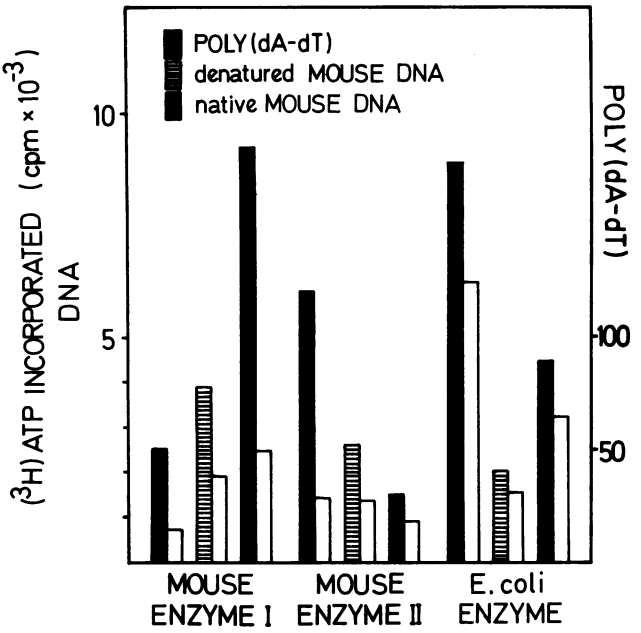

Figure 5: Response of purified RNA polymerases to Hg-UTP
Assays (0.2 ml) contained: UTP (black or shaded columns) or Hg-UTP (open columns), respectively, and CTP, GTP (0.3 mM each) as well as (^{3}H)ATP (2 /uCi/assay ≙ 15 /uM) as tracer.
Poly(dA-dT) (5 /ug/assay) and denatured or native mouse DNA (10 /ug/assay) were used as templates.
E.coli RNA polymerase (7.2 /ug/assay) was prepared according to Burgess (16) through the high salt glycerol gradient.
RNA polymerases (I + III) (230 /ug/assay) and (II)(20 /ug/assay) of mouse ascites cells were isolated using the method of Sugden and Keller (15) up to the glycerol gradient step.
Incubation was for 30 min at 37° in incubation buffer.

sensitive to high doses of α-amanitin. The data of table 2 indicate a higher proportion of α-amanitin resistant (at 10 /ug/ml) RNA synthesis in the presence of Hg-UTP. But using 150 /ug/ml at which concentration RNA polymerase (III) should be totally inactive, there is still about 50% of incorportated label, probably due to RNA polymerase (I). Since it has been shown that mRNA sequences are among the Hg-RNA transcripts (2, 3) it seems that all three RNA polymerases are contributing to the mercurated RNA class. It is, therefore, more likely that an artifact like premature chain termination is responsible for the sedi-

Table 2: RNA synthesis and modification in the presence of mercurated UTP in isolated nuclei

Control assays were run with unmodified UTP.
In all other assays Hg-UTP replaced UTP. In these cases Hg-RNA was separated via SH-sepharose from non-mercurated RNA.
Values are given as percent cpm incorporated or isolated as compared to controls.

Effect of α-amanitin					
cpm (^{3}H)NTP incorporated	control RNA		Hg-RNA		
	no α-amanitin	+ α-amanitin*	no α-amanitin	+ α-amanitin*	
	100%	50%	60% (100%)	46% (76,5%	
Methylation with (^{3}H)SAM					
cpm (^{3}H)SAM incorporated	control RNA		Hg-RNA	Hg-free RNA	
	100%		4,5% (6 ± 0.25%)	70,5% (94%)	
Polyadenylation					
Percent of poly(A)(+) RNA in total labeled RNA	(^{3}H)CTP (3 µM, limited substrate)		control RNA 14.5%	Hg-RNA 1%	Hg-free RNA 6,3%
				50% of control	
	(^{3}H)ATP (30 µM, non-limited substrate)		32%	3%	20%
				66% of control	

* 10 µg/ml of α-amanitin

mentation pattern of Hg-RNA. This is supported by the fact that the (^{3}H) labeled RNA which is not bound to SH-sepharose does contain some (^{203}Hg) but shows a "normal" size distribution. The assumption is that this class of RNA comprises all molecules whose transcription had almost been completed in vivo and which only get added a short mercurated sequence at their 3' end in vitro. As mentioned above, this short stretch, however, might become hidden within the RNA coil. If premature chain termination does occur in such a case it would practically not affect the length of the molecule.

To carry speculation even further, one might assume that the Hg-RNA chains arise by initiation in vitro - or at least from RNA having just been initiated - a conclusion suggested by the experiment of fig. 4. In such a case (almost) the whole length of the molecule is mercurated and binding to SH-sepharose assured. In vitro initiation would also allow an explanation of the varying percentage of RNA attaching to those columns: the observed range would be a reflection of the varying capacity of individual nuclear preparations to initiate RNA synthesis de novo (compare fig. 3 and 6 B).

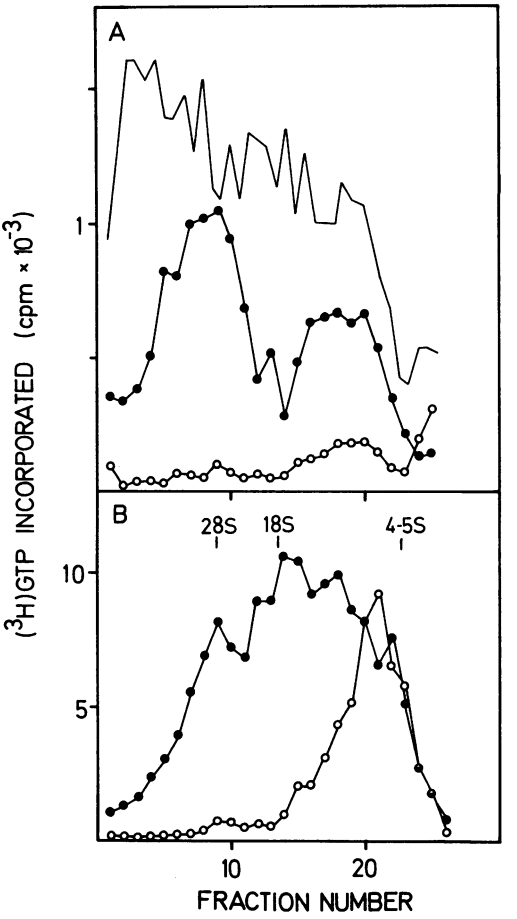

Figure 6: Polyadenylation of Hg-RNA

10^8 nuclei/ml were incubated at 25° for 30 min as described. (^{3}H)GTP (200 µCi/assay ≙ 15 µM) and ATP, CTP and Hg-UTP (0.3 mM each) were present. Hg-RNA and presumably non-mercurated RNA were prepared via SH-sepharose chromatography. Both classes of RNA were subjected to poly(U) sepharose binding to separate the poly(A)-containing RNA from the poly(A)(-)RNA. All four fractions were analyzed on 10-60% sucrose gradients.
A poly(A)(+)RNA: the thin line is a reference taken from a similar experiment without Hg-UTP. No denaturing conditions were used. Formation of aggregates, therefore, is not excluded.
B poly(A)(-)RNA
-●- non-mercurated RNA; -o- Hg-RNA.

Methylation and polyadenylation in the presence of Hg-UTP:

From these results it could be expected that steps in RNA processing like polyadenylation or various methylations are severely affected by mercuration.

These reactions either require an intact i.e. full length RNA chain or might be inhibited by the presence of mercury.

RNA methylation in the presence of Hg-UTP is reduced to 75% of a control. But only 6% of all methyl groups are found in the mercurated RNA (table 2).

Very similar results are obtained for polyadenylation. Only 1 to 3% of the total labeled RNA is found as poly(A)(+)RNA in the mercurated RNA fraction (table 2). Gradient analysis reveals a relatively short length for the poly(A)(+)Hg-RNA (fig. 6 A). These data certainly exclude the use of Hg-UTP in the analysis of the temporal sequence of processing steps.

It should be mentioned that one possible way to circumvent some of the difficulties in the use of Hg-UTP may be the dilution of the mercurated nucleotide by unmercurated UTP.

Taken together, the results presented here indicate that mercurated nucleotides although of considerable value for some purposes e.g. as a probe in hybridization studies are only of limited use when the mechanisms of RNA transcription and processing are studied in isolated nuclei.

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