Sequential Sepharose chromatographic isolation of polysomes and polysomal RNAs depleted in nuclear RNA from *Xenopus*

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ABSTRACT

Analyses of sequence complexities, stage specific gene expression, and mRNA sequence divergence require polysomal RNA preparations relatively free of nuclear RNA contamination. Conventional procedures for the isolation of uncontaminated polysomal RNAs which rely on sucrose density centrifugations are laborious and unsuitable for large scale isolations. We describe here a method using sequential Sepharose chromatography for isolating polysomes and polysomal RNAs depleted for nuclear RNA. Polysomes and polysomal RNAs isolated from livers of *Xenopus* stimulated to produce vitellogenin were capable of directing protein synthesis *in vitro* and showed little evidence of degradation. The polysomal RNAs contained less than 0.5% of nuclear RNA.

INTRODUCTION

The isolation of polysomal RNAs uncontaminated with nuclear RNAs is of critical importance for the accurate measurement of a variety of significant aspects of cellular functions at the molecular level. The contamination of presumptive mRNA populations with nuclear RNAs can result in serious overestimation of mRNA sequence complexity, since HrRNAs have between four to ten times the sequence complexity of polysomal RNAs¹⁻⁴. Analysis of stage-specific mRNA expression also requires uncontaminated mRNAs because the nucleus contains transcripts processed as polysomal RNA at several stages⁵⁻⁶. Determination of rates of mRNA evolution requires uncontaminated preparations since mRNAs can be highly conserved⁷ while HrRNAs are much less conserved and diverge as rapidly as do total single copy sequences⁸. Because HrRNAs contain repetitive sequence transcripts and mRNA populations are largely single copy sequence transcripts¹⁻⁹, polysomal RNA populations contaminated with nuclear RNA could yield misleading information concerning the

amounts of message transcribed from repetitive and single copy DNA.

Since some nuclear lysis is inevitable during polysomal preparation and large nuclear RNPs accompany polysomes during their isolation^{1-2,10}, conventionally prepared polysomes are often contaminated with nuclear RNP. Sucrose density gradient fractionation before and after mRNA release by puromycin (Figure 1a) has been shown to be effective in isolating polysomal RNA containing 0.2% pulse-labeled nuclear RNA¹⁻². We have developed a procedure based on a similar principle, which was made simpler and more rapid by the substitution of Sepharose (molecular sieve) column chromatography (Figure 1b) for sucrose density gradient centrifugation.

It is well known that the isolation of undegraded polysomes from amphibia is difficult to achieve. To demonstrate the efficacy of this method, we chose tissue from *Xenopus laevis*. Furthermore, we used livers from *Xenopus* stimulated to produce vitellogenin. Since vitellogenin synthesizing polysomes are large¹¹⁻¹², any degradation would be more readily detectable. In the first steps of the procedure we recovered large polysomes

Figure 1. Procedures for the isolation of polysomal RNAs depleted for nuclear RNAs. A conventional procedure based on sucrose density gradient centrifugation (a) is contrasted with the procedure based on sequential Sepharose chromatography (b).

from livers of Xenopus producing vitellogenin. The polysomal RNAs isolated by this method appeared to be intact, were capable of directing protein synthesis in vitro, and had, at most, 0.5% nuclear RNA contamination.

MATERIALS AND METHODS

Animals

Xenopus laevis were obtained from the South African Reptile Farm or were laboratory bred. "Vitellogenic" livers from estradiol-17 β injected Xenopus were the kind gift of H. Whatley and L.D. Smith. Vitellogenin synthesis was induced by intramuscular injection of 1 mg estradiol-17 β (Sigma) in 0.2 ml soybean oil emulsion on days 0 and 7. On day 14, the livers were removed.

Buffers

All solutions were prepared using glass distilled water, treated with 0.02% diethylpyrocarbonate (Sigma), and autoclaved. All glassware was autoclaved. Resuspension (RS) buffer was 200 mM Trizma-base, pH 8.5, 50 mM KCl, 40 mM MgCl₂, 25 mM EGTA (Sigma), 1 mM dithiothreitol, and 10% glycerol. Column I buffer was RS buffer with the Trizma-base changed to 10 mM at pH 7.8. Column II buffer was 10 mM Trizma-base, pH 7.6, 100 mM NaCl, 25 mM EGTA and 10 mM EDTA.

Column Chromatography

Columns were constructed from glass tubing coated with 0.1% bovine serum albumin and then autoclaved. Sepharose 2B (Pharmacia) Columns I and II were of similar size (0.8 x 20 cm) and were reusable. The average load volumes were 2.5 ml and the average fraction volumes were 0.46 ml. All columns were run at 4°C.

Sequential Sepharose Chromatographic Isolation of Polysomes and Polysomal RNAs

Immediately after dissection, tissues were frozen in liquid nitrogen and stored at -55°C. Frozen livers were ground to a fine powder in the presence of dry ice in a Waring blender. The dry ice was sublimed by overnight storage at -55°C. All subsequent operations were at 4°C. The tissue powder was suspended in 2.5 volumes (w/v) RS buffer and gently homogenized with three strokes of a loose-fitting Dounce pestle. Twenty-five percent Triton X-100 (Sigma) was added to 0.5% and the mixture

Figure 2. Absorbance profiles from a typical sequential Sepharose isolation. Liver tissue powder from a vitellogenic female was suspended and centrifuged as described in Materials and Methods. The postnuclear supernatant (12,100 x g) was passed over Sepharose column I (a). The bracketed fractions were pooled, released with 100 mM EDTA and then passed over Sepharose Column II (b). Absorbances (260 nm) were determined with a Beckman Model 25K spectrophotometer. The bracketed fractions were pooled for RNA extraction.

was centrifuged at 1085 x g for 10 min. Without disturbing the lipid layer, the supernatant was removed and 25% Triton was added to a final concentration of 2.5%. Following a 10 min incubation, it was centrifuged at 12,100 x g for 10 min. This supernatant was passed over a Sepharose 2B column (Column I). Absorbance at 260 nm was monitored until the exclusion peak was eluted. Without waiting for the column to completely develop, the

exclusion peak fractions were pooled (indicated by brackets in Figure 2a), made 100 mM EDTA, incubated 10 min, and passed over a second Sepharose 2B column (Column II). Fractions retained by the column were pooled (indicated by brackets in Figure 2b) and precipitated by the addition of 0.1 volume of 2.75 M sodium acetate, pH 5.95, and 2 volumes of 100% ethanol.

RESULTS AND DISCUSSION

Preparation of Postnuclear Supernatants

Maintenance of stored tissues and tissue powders at -55°C and freeze-grinding were used to reduce nuclease activity. Polysomes showed no evidence of degradation whether isolated from tissues stored for more than one year at -55°C or from freeze-ground tissue powders stored for over one month at -55°C .

To reduce nuclear lysis, freeze-ground tissue powders were suspended in RS buffer and homogenized in the absence of detergent in a Dounce homogenizer. The high magnesium ion concentration (40 mM) of the buffers was selected to compensate for any chelation by EGTA (25 mM), while avoiding magnesium induced aggregation¹³. The RS buffer was intended to maintain polysome integrity, reduce endogenous ribonuclease activity, and avoid extensive nuclear lysis. Unfortunately, commonly used ribonuclease inhibitors such as heparin and diethylpyrocarbonate disrupt nuclei¹³. However, buffers having high pH, high ionic strength, and EGTA¹⁴ appeared to prevent polysome degradation during preparation of postnuclear supernatants from *Xenopus* liver (Figure 3a).

Isolation of High Molecular Weight Polysomes

The combination of RS and Column I buffers and Sepharose column chromatography yielded preparations more enriched for high molecular weight polysomes (Figure 3b) than was the postnuclear supernatant prior to chromatography (Figure 3a). Previous work had shown that Sepharose can be used in the isolation of polysomes from *E. coli*¹⁵ and mouse tissue culture cells¹⁶.

While the preparations were enriched for high molecular weight polysomes, significant amounts of monosomes were also recovered (Figure 3b; Table I). Control experiments indicated that this phenomenon was not due either to low available magnesium (increased magnesium concentrations led to aggregation) or to runoff¹⁷ (postnuclear supernatants from a non-induced

Figure 3. Enrichment for high molecular weight polysomes following Sepharose 2B chromatography. Polysomes from a postnuclear supernatant (a) from the liver of a vitellogenic female and from the ascending side of the exclusion peak (b) following Sepharose chromatography of the same postnuclear supernatant are shown. Polysomes were analyzed on 15-60% linear sucrose density gradients prepared in 40 mM Trizma-base, pH 8.5, 20 mM KCl and 10 mM $MgCl_2$. Centrifugation was for 45 min at 45,000 rpm at 4°C in an SW 50.1 rotor in a Beckman Model L Ultracentrifuge. Gradients were collected by upward displacement with 60% sucrose containing 45 μg uracil/ml through the flow cell of an ISCO Model UA-5 recording monitor. Monosome positions are indicated by the letter "M".

Table I. Monosome Loss After Sepharose Chromatography

Sex	Induced	Percent as Monsomes ¹		
		PNS ²	Column I Exclusion	Monosome Loss After Chromatography (%)
Female	NO	67	20	70.0
Female	YES	42	9	78.6
Male	YES	51	7	86.0

¹Percent as monosomes was calculated by comparing the weights of traced monosome peaks (M) to the weights of traced monosome + polysome peaks (M + P) from sucrose density gradient centrifugations. $[(M)/(M + P) \times 100]$

²PNS=postnuclear supernatant (12,100 x g)

female contained 72% monosomes in the absence, and 59% monosomes in the presence of cycloheximide). Since there was a substantial reduction in the percentage as monosomes following chromatographic fractionation (Table I), we concluded that the monosomes in the exclusion peak fractions represented the leading edge of the monosome peak, most of which would have been retained during passage over Sepharose 2B¹⁵. In the vitellogenic liver a tissue actively involved in a major protein synthetic event, we observed fewer monosomes in the post nuclear supernatant, and relatively few monosomes in exclusion peak fractions (Table I).

Several tests were made to ensure that the high molecular weight materials were indeed large polysomes and not the result of polysomal aggregation or due to non-polysomal materials. Polysomes from pooled exclusion peak fractions (Figure 4a) that were digested with proteinase K showed only minor changes in sedimentation behavior (Figure 4b). Proteinase K disrupts protein-mediated aggregates and improves the resolution of non-aggregated polysomes by digesting nascent protein chains¹⁸.

Figure 4. Enzymatic digestions or EDTA release of Sepharose isolated polysomes. Pooled exclusion peak fractions (a) from the liver of a vitellogenic female were digested with 200 μ g proteinase K/ml for 15 min at room temperature (b), 200 μ g pancreatic RNase/ml for 10 min at 37°C (c), or released with 50 mM EDTA (d) or 100 mM EDTA (e) for 10 min at 4°C. Sucrose density analysis was as described in Figure 3.

Polysomes from pooled exclusion peak fractions were sensitive to RNase digestion (Figure 4c). EDTA release of pooled exclusion peak fractions led to virtually complete release of ribosomes (Figure 4d,e).

Vitellogenin Synthesis and the Appearance of High Molecular Weight Polysomes

Enrichment for high molecular weight polysomes following Sepharose 2B chromatography is especially apparent in a vitellogenic Xenopus liver, since polysomes synthesizing vitellogenin are very large (estimated at greater than 30-mers¹¹⁻¹²) as a result of the large size of the mRNA¹⁹ coding for the vitellogenin monomer (200,000 daltons²⁰). Polysome profiles of fractions taken from the ascending side of exclusion peaks from Xenopus livers are shown in Figure 5. Profiles from vitellogenic females (Figure 5d) and males (Figure 5c) show marked enrichment for high molecular weight polysomes. Fewer high molecular weight polysomes were observed in non-induced females with normal vitellogenin synthesis (Figure 5b). The non-induced male, which should not synthesize vitellogenin²¹, exhibited virtually no high molecular weight polysomes (Figure 5a) as well as less intermediate molecular weight polysomes.

Vitellogenin is synthesized on membrane bound polysomes²². Sepharose column chromatography apparently also can be used in the isolation of membrane-bound polysomes. By using less than 0.3% Triton X-100 during the preparation of the postnuclear supernatant (1085 x g), an exceptionally high molecular weight peak was observed from preparations derived from livers synthesizing vitellogenin (Figures 6b-d). In contrast, a very small peak was observed in preparations from non-induced males (Figure 6a).

Isolation of Polysomal RNAs

Either puromycin²³ or EDTA^{10,24} may be used to release mRNA from polysomes. Following Column I chromatography, the polysomes (exclusion peak fractions) are in a buffer to which only KCl (to 0.5 M) and puromycin (to 1 mM) must be added to initiate puromycin release. However, EDTA release, which involves less incubation time (10 compared to 60 min), also led to more complete conversion of polysomes to monosomes and subunits in our hands. Presumably because of the high concentration of magnesium

Figure 5. Vitellogenin synthesis and enrichment for high molecular weight polysomes. Fractions from the ascending side of exclusion peaks (Column I) from livers of normal males (a), normal females (b), vitellogenic males (c), and vitellogenic females (d) are shown. Sucrose density analysis was as described in Figure 3.

In our buffers, 100 mM EDTA (Figure 4e) was more effective in mRNA release than was 50 mM EDTA (Figure 4d).

Some aggregation may occur during the time required (about 2 hr) to complete Column II chromatography of EDTA released polysomal materials if Column II buffers that lack EDTA are used. Analysis of EDTA released polysomal materials in sucrose density gradients in the absence (Figure 7a) or the presence of EDTA (Figure 7b) indicated that aggregation started to occur in the absence of EDTA during the 3 hr centrifugation, despite centrifugal force.

Although salt concentration²⁵, temperature, or specific cations²⁶ can cause artifacts in Sepharose chromatography of

Figure 6. Vitellogenin synthesis and the appearance of membrane bound polysomes. Exclusion peak fractions were prepared as usual, except that less than 0.3% Triton X-100 was used and the postnuclear supernatant was prepared by centrifugation at $1085 \times g$ only, from normal males (a), normal females (b), vitellogenic males (c), and vitellogenic females (d). Fractions from the ascending side of exclusion peaks were analysed by sucrose density as described in Figure 3, except that centrifugation was for 30 min. Presumptive membrane bound polysomes are indicated by arrows.

extracted RNA, they are probably not as critical to our procedure, since puromycin or EDTA released polysomal RNAs are present as RNPs rather than as deproteinized RNA²⁴. However, we found that the use of column buffers of pH's >7.8 or prewashing of the Sepharose with alkali led to nearly complete retention of

Figure 7. Aggregation of ribosomal subunits in the absence of EDTA. Pooled Column I exclusion peak fractions were released with 100 mM EDTA and then analyzed on 0-32% linear-log sucrose density gradients prepared in 10 mM Trizma-base, pH 8.5, 10 mM KCl, 10 mM $MgCl_2$, 0.1 mM EGTA in the absence (a) or the presence (b) of 10 mM EDTA. Centrifugation conditions were as described in Figure 3, except that centrifugation time was for 3 hr.

polysomes by the matrix.

Analysis of Polysomal RNAs

During the time and column fractionations required for the isolation of polysomal RNAs, no further degradation occurred than was already apparent in RNAs from the postnuclear supernatant. Gel electrophoresis of polysomal RNAs extracted from pooled Column II fractions before (Figure 8a) and after heating (Figure 8b) were essentially similar to those of postnuclear supernatant RNAs (data not shown).

It was not our intention to isolate RNAs suitable for translation by this procedure, since a single Sepharose fractionation has already been used to isolate RNAs adequate for this purpose¹⁵. However, to demonstrate the presence of messenger sequences on the polysomes and in their polysomal RNAs, we analyzed their ability to support in vitro protein synthesis. The time course of incorporation of 3H -leucine into TCA-

Figure 8. Gel electrophoresis of polysomal RNAs isolated by sequential Sepharose chromatography. RNA was extracted from pooled Column II fractions by the method of Lovett and Leaver²⁷. Gel electrophoresis was carried out as described by Loening²⁸. Approximately 0.5 A_{260} units in sterile water were loaded in a final volume of 50 μ l. Samples were adjusted to approximately 10% sucrose prior to loading. Gels were run for 2 hr at 6 mamp/gel, soaked for 30 min in glass distilled water, and then scanned using a Gilson linear gel scanner at 260 nm. Samples were either loaded as described (a) or heated to 60°C for 45 sec just prior to the addition of sucrose and loading (b).

precipitable protein using a wheat germ cell- free translation system indicated a linear rate for 20 min for polysomes and at least 50 min for polysomal RNAs (Figure 9). Since vitellogenin mRNA is a relatively poor template in the wheat germ translation system (D. Shapiro, personal communication), we anticipated a rather low level of incorporation. In contrast, RNAs isolated by sequential Sepharose column chromatography from a different organism (corn) were more than 200 times more active as templates for protein synthesis (35 pmoles leucine incorporated by 50 min).

RNAs isolated by sequential Sepharose column chromatography from livers removed from 7-8 year old *Ambystoma mexicanum* were also capable of acting as templates for the synthesis of cDNA using reverse transcriptase, the average size of such cDNAs being 400 nucleotides.

Figure 9. Time course of incorporation of ^3H -leucine into TCA insoluble protein by polysomes and polysomal RNAs isolated by Sepharose chromatography. Pooled exclusion peak fractions (polysomes; $\text{---}\bullet\text{---}$) were centrifuged into a 50% sucrose cushion (prepared in RS buffer containing 2 mM dithiothreitol) at 45,000 rpm for 40 min at 4°C . The polysomes were resuspended in column I buffer. Polysomal RNAs ($\text{---}\blacktriangle\text{---}$) were precipitated in the presence of potassium acetate instead of sodium acetate for this experiment and resuspended in sterile water just prior to analysis. A slight modification of a wheat germ cell-free translation system²⁹ (substitution of potassium acetate for potassium chloride) was used to determine template activity. ^3H -leucine (105 Ci/mmol; Amersham) was used to monitor incorporation. Data were analyzed by spotting 5 μl of a 50 μl reaction mixture on paper discs prior to TCA precipitation³⁰. Data points are the average of two readings. The ordinates represent total counts in the 50 μl reaction mixture. Each 10,000 cpm is equivalent to one pmole leucine incorporated.

Extent of Nuclear RNA Contamination

Our aim was the rapid isolation of polysomal RNAs relatively free of nuclear RNA. In order to measure the extent of nuclear RNA contamination, the RNA of a female *Xenopus* stimulated to increased vitellogenin synthesis was labeled for 45 min, which was estimated to label nuclear RNA without significant labeling of polysomal mRNA (D. Shapiro, personal communication). The liver tissue powder was separated into two parts, half of which had the nuclei purposefully lysed, and half of which was treated

as usual. The amount of contaminating nuclear RNA was determined by following the distribution of the pulse labeled RNA through both fractionations. The nuclear lysis experiment was intended to demonstrate the potential final contamination by nuclear RNA under conditions where maximum initial nuclear RNA contamination would occur. Probable final nuclear RNA contamination under normal conditions was estimated using the usual procedure. However, since so few cpm were excluded from Column I in the usual experiment (Table II), it was necessary to quantitate the final contamination under normal circumstances by multiplying the percentage pulse labeled RNA excluded from Column I in the usual experiment by the percentage of pulse labeled RNA retained by Column II in the nuclear lysis experiment.

Although the amount of nuclear RNA contamination of the polysomal RNA following vigorous homogenization was relatively high, the contamination with nuclear RNA was about 0.5% when the usual procedure was followed (Table II).

These estimates of nuclear RNA contamination represent an upper limit for HnRNA contamination. If less than 100% of the nuclei were lysed following vigorous homogenization, then the amount of contamination would be correspondingly smaller. If, during the labeling time, rRNA precursors were labeled, then the amount of HnRNA contamination would be reduced. If the labeling time were too long, and some polysomal RNA were labeled, then this, too, would lower the amount of actual contamination of mRNA with HnRNA sequences. For example, within 10 min, only nuclear RNA is labeled in sea urchin embryos³¹. When we repeated the above experiment using sea urchin embryos, the final estimation of nuclear RNA contamination was in the same range or smaller, about 0.2% of nuclear RNA.

Summary

Sequential Sepharose chromatography for the isolation of polysomal RNAs depleted in nuclear RNA is economical, reproducible, rapid, and does not require access to specialized equipment such as ultracentrifuges. This procedure eliminates the buffer changing step between the isolation of polysomes and the release of mRNA required by the conventional procedure¹⁻² (Figure 1b), since the first Sepharose column can be equilibrated with a buffer appropriate for mRNA release and the next fractionation. An average preparation requires about 5 hr

Table II. Distribution of Pulse-Labeled RNA during Sequential Sepharose Chromatographic Isolation of Polysomal RNA

Animal	Experiment ²	Column I Exclusion		Column II Retained	
		CPM	Percent ¹	CPM	Percent ¹
<u>Xenopus</u>	Nuclear Lysis	5918	53.8	1342	12.2
	Usual	231	2.1 ³	52 ⁴	0.47 ⁴
Sea urchin	Nuclear Lysis	3807	24.5	1321	8.5
	Usual	451	2.9 ³	34 ⁴	0.22 ⁴

¹ Total counts were determined from the TCA precipitable counts fractionated on Column I in the Nuclear Lysis experiment.

² A 120 g female Xenopus was injected intraperitoneally with 4 mg estradiol-17 β and 24 hr later with 1.5 mC ³H-uridine (28 C/mmol; 1 mC/ml; ICN). After a 45 min label (and 5 min anaesthesia time) the liver was removed and frozen in liquid N₂. The freeze ground liver powder was separated into two equal aliquots. One was homogenized vigorously with a Polytron homogenizer to lyse nuclei. The other aliquot was fractionated in the usual manner. Aliquots taken at various points in the procedure and 50 μ g horse DNA (carrier) were pipetted into 2 ml cold 10% TCA and chilled 30 min. TCA precipitates were collected on millipore filters and washed using cold 5% TCA. Filters were dried and counted in 3 ml Aquasol. Pulse-labeled sea urchin (Lytechinus pictus) embryos (gift of A. Aronson) were incubated in artificial sea water at 18°C for 12 hr post-fertilization. After a 10 min label at 18°C with 0.5 mC ³H-uridine (58 C/mmol; Amersham) followed by brief centrifugations twice through sea water made 20 mM in acetic acid, final pH 4.5, they were suspended in 1.5 M dextrose and recentrifuged. Following resuspension in RS buffer, they were frozen in an ethylene-glycol/dry ice bath, and stored at -55°C. Aliquots taken during the isolation were counted in 1 ml 0.12 M sodium phosphate buffer and 2 ml Aquasol. Some fractions were pooled and chromatographed on Sephadex G-100 to obtain more accurate estimates of incorporated counts.

³By comparison of the numbers of counts in the exclusion peak (Column I) from the Nuclear Lysis with the Usual experiment.

⁴Calculated by multiplying the Usual column I exclusion by the percent that was chromatographed on the Nuclear Lysis column II (22.7% of exclusion, Xenopus; 34.7% of exclusion, sea urchin).

between tissue powder suspension and RNA extraction, while methods employing sucrose density centrifugation require between 20 and 33 hr. This method should be useful for the isolation of

polysomal RNAs, although some parameters such as the RS buffer may need to be altered to meet the requirements of different organisms.

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