Yeast histone mRNA is polyadenylated

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ABSTRACT

Histone mRNA from *S. cerevisiae* has been identified and partially purified. The RNA is quantitatively retained on oligo (dT) cellulose or poly(U) sepharose as assayed by *in vitro* translation or hybridization of radiolabeled cloned yeast histone sequences to RNA immobilized on DBM paper. Retention of yeast histone mRNA on either of these chromatographic systems is most likely the result of polyadenylation since, when primed with oligo (dT), the RNA is an extremely good template for reverse transcriptase, as determined by hybrid arrest translation or by hybridization to *D. melanogaster* histone DNA sequences.

INTRODUCTION

Histone mRNA has been identified in a number of different higher eucaryotes. In general it has been found that - as was originally demonstrated by Adesnik and Darnell for Hela cells (1) - most of these histone mRNAs do not contain poly(A) (for review see Kedes (2)).

A notable exception is the histone mRNA of *Triturus* and *Xenopus* oocytes. Ruderman and Pardue have shown that in these cells, histone mRNA is almost exclusively polyadenylated (3,4). They have further shown that, upon fertilization, the poly(A)⁺ histone mRNA of *Xenopus* oocytes is replaced by poly(A)⁻ RNA (4). This latter finding has led to the suggestion that polyadenylation may play a role in the storage of histone mRNA during oogenesis (5). Although amphibian oocytes represent the most dramatic example of polyadenylated histone mRNA, there is evidence that in other organisms - such as Hela cells (6), sea urchins (4) and clams (7) - a small fraction of some histone mRNAs may be polyadenylated.

In contrast to higher eucaryotes, little information exists on the histone mRNAs of lower eucaryotes. In this paper we report the first identification and partial purification of yeast histone mRNA. Using *in vitro* translation, we have identified an 8.7-10S fraction of RNA which is enriched for

histone mRNA. Surprisingly, this fraction is almost totally ($\geq 90\%$) retained on oligo(dT) cellulose or poly(U) sepharose. We have further demonstrated that retention on either of these chromatographic systems is most likely a consequence of polyadenylation by showing that yeast histone mRNA, in the presence of an oligo(dT) primer, can serve as a template for reverse transcriptase as assayed by either hybrid arrest translation (8) or by hybridization to histone gene sequences in *Drosophila melanogaster* genomic DNA. This observation represents the second reported case (the first being the aforementioned histone mRNAs from amphibian oocytes) in which histone mRNA exists predominantly in a polyadenylated form.

METHODS AND MATERIALS

Yeast Strains RNA for preparative gels was isolated from strain 3430-3C (gal4, suc, mal, his, tryp2, met8, ade5, tyr7) which was obtained from D. Hawthorne. Cells from this strain were grown in (per liter) 10 gm yeast extract, 20 gm peptone, 20 gm galactose and 24 ml ethanol. All other RNA was isolated from strain A364A (ade1,2, ura1, his7, lys2, tyr1) which was obtained from L. Hartwell. This strain was grown in modified YM-1 medium (9).

Bacterial Plasmids Plasmid TRT-1 which contains the yeast histone genes H2A and H2B has been described (10). The Dm histone clone was obtained from D. Miller. It is a single histone repeat subcloned from the original histone clone cDm 500 described by Lifton et al (11). Plasmid DNA was isolated by standard procedures (12).

RNA Extraction Cells were routinely grown to $2-4 \times 10^7$ cells/ml. RNA was extracted from 3430-3C as follows: five minutes prior to harvesting, cycloheximide was added at a concentration of 0.1 mg/ml cells and the cells were allowed to continue shaking for 10 minutes. The cells were then collected by centrifugation, resuspended in 0.05 M Tris pH 6.8, 2 mM EDTA, and 0.05 mg/ml cycloheximide, and broken by vortexing in the presence of 0.45-0.5 mm glass beads. A 1/10 volume of 10% SDS was added, followed by addition of 1/10 volume of 2 M NaOAc pH 5.2 and then the suspension was extracted with H_2O saturated phenol. After a 10 minute centrifugation, the aqueous phase was re-extracted with phenol-chloroform-isoamyl alcohol (50-50-1) prior to ethanol precipitation of the RNA. RNA from A364A was isolated as previously described (9).

Polyacrylamide Gel Electrophoretic Fractionation of RNA Preparative polyacrylamide gel fractionation of RNA was carried out using an apparatus designed by Hazen which continuously elutes RNA from the bottom of the gel

(13). A gel of 2% acrylamide, 0.5% agarose was poured to a height of 10 cm with a cross-sectional area of 46.5 cm². Between 2-20 mg of RNA was routinely loaded in 40 mM Tris pH 7.8, 20 mM NaAc, 2 mM EDTA, 70% DMSO, and 10% glycerol. Electrophoresis was performed at room temperature at a constant voltage of 40V for 24 hrs. Two-milliliter fractions were collected and EtOH precipitated in the presence of 20 µg of purified yeast tRNA. The fractions were then redissolved in sterile H₂O and stored at -80°C.

In vitro translation. Wheat germ extract was prepared according to published procedures (14). Each batch of wheat germ was optimized for KOAc and MgOAc using total yeast RNA. Translation was carried out over a 10 fold concentration range to ensure that, for each RNA preparation, synthesis was proportional to RNA concentration. All translations utilized 15 µCi each of ³(H) lysine (60-80 Ci/mMOL) and ³(H) leucine (> 110 Ci/mMOL) which was purchased from New England Nuclear.

Protein Gels 1D Triton acid urea and 2D acid urea - Triton acid urea gels were run as described by Spiker (15) except that the gels were not pre-electrophoresed. Sample preparation was as follows. 2-5 µg of ribonuclease was added to each in vitro translation sample and the samples were incubated at 37°C for 10 min. The ribonuclease step was found to be essential for quantitative recovery of the histones since reconstruction experiments showed that histone recovery decreased with increasing RNA concentration. After ribonuclease treatment, 7X the sample volume of 20% TCA was added, the sample was centrifuged in a microfuge and the TCA was decanted. The pellet was washed once with 0.2% HCl in acetone, then once with acetone. The samples were dried and then resuspended in 10 µl of 8 M urea, 10 mM in NH₄ CO₃, and 0.4% DTT. Once samples were in solution, 10 µl of 10% acetic acid, 25% glycerol was added. After electrophoresis the gels were stained to reveal the positions of marker proteins, then subjected to fluorography at -80°C (16) using Kodak XR5 X-ray film.

Other procedures Southern hybridization (17) and Northern hybridization (18) were performed according to published procedures. Autoradiograms were exposed to Kodak XR5 X-ray film at -70°C using DuPont Cronex Lightening Plus intensifying screens. cDNA synthesis (9) and nick translation (19) were also performed according to published procedures. In both cases, the label used was ³²P d(CTP) from New England Nuclear. AMV reverse transcriptase was a gift of Dr. J. Beard.

RESULTS

Identification and Partial Purification of Histone mRNA We initially attempted to identify yeast histone mRNA by in vitro translation of total yeast RNA. The results of this experiment are shown in Fig. 1. Lane A shows partially purified yeast histones electrophoresed on a 1-D Triton acid urea gel; Lanes C and D are calf thymus and chicken erythrocyte histones, respectively. As reported by Mardian and Isenberg (20) yeast histones display quite distinct mobilities on this gel system when compared to histones isolated from higher eucaryotes. Also of note is the absence of H1 which has not yet been identified in yeast (20). Lane B shows the in vitro translation products of total yeast RNA. The lines indicate very faint bands which appear to comigrate with the yeast histone markers. Analysis of these

Figure 1 - Identification of Yeast Histone mRNA by in vitro Translation of Total RNA. Total yeast RNA was translated in a wheat germ extract using [3 H] leucine and lysine and the protein products were electrophoresed on a Triton acid urea gel (see Methods and Materials for details).

Lane A: Partially purified yeast histones.

Lane B: Autoradiogram of in vitro translation products of total yeast RNA. Lines indicate bands which appear to comigrate with yeast histone markers.

Lane C: Calf thymus histones.

Lane D: Chicken erythrocyte histones.

products on 2-D gels confirmed that these bands are indeed histones (data not shown). The majority of the other bands on this gel are ribosomal proteins as determined by coelectrophoresis with purified ribosomal proteins (data not shown). Since the in vitro translations were performed with a mixture of ^3H leucine and lysine and since the percentage of these amino acids is roughly equivalent in histones and ribosomal proteins (21,22), the band intensities should reflect the relative concentrations of the two types of proteins. We have previously estimated that the concentration of an individual ribosomal protein mRNA is approximately 0.2% of total mRNA (23). Because the intensity of any one of the four histone bands is comparable to any one of the ribosomal protein bands, we estimate that the concentrations of histone and ribosomal protein mRNAs are also comparable. Therefore, yeast histone mRNAs are not an abundant mRNA species.

To enrich for histone mRNA, total yeast RNA was fractionated by electrophoresis on a continuous elution agarose-acrylamide preparative gel (13). The optical density profile is shown in Fig. 2A and the in vitro translation products of selected fractions in Fig. 2B. As is evident from Fig. 2B, histone mRNA appears to be enriched in the 8.7-10S region of the gel. To confirm that this region was in fact enriched for histone mRNA, the 8.7-10S fractions were pooled, translated in vitro, and the products were electrophoresized on a 2D acid urea -Triton acid urea gel (14). Fig. 3A shows partially purified yeast histones and Fig. 3B, the in vitro translation products of the pooled 8.7-10S region. The four histones are clearly present in these products. Comparison of the intensity of the bands in the enriched fraction with their intensities after in vitro translation of total RNA suggested that we had enriched approximately 10 fold for histone mRNA (data not shown).

Poly(U) Sepharose Fractionation Since we assumed that the bulk of yeast histone mRNA, like histone mRNAs isolated from a variety of sources, would not be polyadenylated (2), we attempted further purification by fractionation of the 8.7-10S RNA on poly(U) sepharose. As shown in Table I, approximately 60% of the starting 260 nm absorbing material was retained by poly(U) sepharose, and this fraction contained 90% of the poly(A) containing RNA as monitored by ^3H poly(U) hybridization (24). Also shown in Table I is the template activity of both the poly(U) sepharose bound and unbound RNA fractions for in vitro translation. Both fractions showed equivalent stimulation per μg m of RNA, and the translation was comparable to that of the starting material. Equal cpms of the in vitro translation products were then electrophore-

Figure 2 - Partial Purification of Yeast Histone mRNA. Total yeast RNA was fractionated by electrophoresis on a continuous elution preparative gel (see Methods and Materials for details). The optical density of the RNA was monitored during elution and the eluted RNA was collected into 2 milliliter fractions. Following precipitation, the RNA of every other fraction was translated *in vitro* with ^3H leucine and lysine.

(A) The optical density profile of the eluted RNA plotted against fraction number.

(B) Autoradiogram of the *in vitro* translation products of fractionated RNA. The *in vitro* translation products of fractions, indicated by the numbers shown above the gel lanes, are displayed on a Triton acid urea gel. The position of the histones was determined by comigration with purified histone markers (not shown). The histones could be detected predominantly in fractions 28-34 which correspond to RNAs of approximately 8.7-10S in size. Equal cpm's (50,000) were loaded onto each lane.

Total: *in vitro* translation products of unfractionated RNA.

Endo: endogenous *in vitro* translation products of wheat germ extract.

Figure 3 - Two-dimensional Electrophoresis of the *in vitro* Translation Products of 8.7-10S RNA. The RNA fractions most enriched for histone mRNA (i.e., fractions 28-34 (Figure 2B)) were pooled, precipitated, then translated *in vitro*. The protein products were electrophoresed on a two dimensional gel which consisted of acid urea in the first dimension and Triton acid urea in the second dimension. The direction of electrophoresis in the first dimension is from left to right.

(A) Yeast histone markers.

(B) Autoradiograms of $^3\text{[H]}$ leucine and lysine *in vitro* translation products of pooled mRNA fractions 28-34 (8.7-10S RNA). Approximately 100,000 cpm were loaded on the gel.

TABLE I: Properties of Histone MRNA

RNA	Fraction of RNA	CPMS of $^3\text{(H)}$ poly (U) hybridized	CPMS of $^3\text{(H)}$ leucine and lysine incorporated in <i>in vitro</i> translation	CPMS of $^{32}\text{(P)}$ dCTP Incorporated into cDNA
8.7 - 10 S		2,438 ¹	10,015 ¹	+primer 3700 ¹ -primer 212
bound	.58	2,296	10,944	+primer 4112 -primer 192
Not bound	.42	342	9,036	+primer 538 -primer 206

¹CPMS are normalized for RNA concentration

sed on the 2D gel system shown in Fig. 3. Fig. 4B shows the *in vitro* translation products of the bound or poly(A)⁺ fraction: the four histones are clearly visible. At an identical exposure time, the gel of the poly(A)⁻ fraction showed greatly reduced amounts of the histones. Exposure of this gel for a 10 time longer period produced the autoradiogram shown in Fig. 4A. At this exposure time histones H2B and H3 are visible, although at reduced intensities as compared to the poly(A)⁺ gel. H4 is not visible and H2B is obscured by a non-histone protein (indicated by arrow).

The difference in the intensities of the four histone proteins in the translation of poly(A)⁺ and poly(A)⁻ fractions suggested that histone mRNAs were greater than 10 fold enriched in the poly(A)⁺ fraction. This conclusion rests on the assumption that translation in the wheat germ system is quantitative. To circumvent this problem, we determined the fraction of H2A and H2B mRNA in poly(A)⁺ and poly(A)⁻ RNA directly by hybridization to a clone (TRT-1) which contains the yeast histone H2A and H2B genes (10). Total yeast RNA was fractionated by 3 successive cycles of binding to oligo (dT) cellulose. As monitored by ³H poly(U) hybridization, the unbound poly(A)⁻ RNA con-

Figure 4 - Two-dimensional Electrophoresis of the *in vitro* Translation Products of 8.7-10S poly(A)⁺ and poly(A)⁻ RNA.

(A) Autoradiogram of the *in vitro* translation products of poly(A)⁻ RNA.

(B) Autoradiogram of the *in vitro* translation products of poly(A)⁺ RNA. The gel is the same as that described in the legend to Figure 3. Histones were identified on the basis of comigration with histone markers. Approximately 100,000 cpm were loaded onto each gel.

tained approximately 10% of the poly(A) present in unfractionated RNA. Equal amounts of poly(A)⁻ RNA, total RNA and 1/10 this amount of poly(A)⁺ RNA were then electrophoresed on methyl mercury gels, the RNA was transferred to 3BM paper, (Northern gel, (17)), and hybridized to nick translated TRT-1 DNA. As shown in Fig. 5, virtually all of the H2A and H2B mRNA was found in poly(A)⁺ RNA, thereby corroborating the results seen by *in vitro* translation.

cDNA template activity of histone mRNAs To determine whether retention of histone mRNAs on poly(U) sepharose or oligo (dT) cellulose was due to polyadenylation we asked whether these mRNAs could act as an oligo (dT) primer dependent template for reverse transcriptase. When the poly(A)⁺ and poly(A)⁻ RNAs from the 8.7-10S fraction were tested for priming activity, only the bound fraction stimulated cDNA synthesis, and this stimulation was totally dependent upon the addition of exogenous oligo (dT) primer. (Table I.)

Histone cDNA synthesis was assayed in two ways. Since histones are highly conserved, it was expected that yeast histone cDNA would cross hybridize to histone genes of other organisms. Therefore, the first assay consisted of hybridizing radiolabelled 8.7-10S cDNA to Eco RI cut *Drosophila* genomic DNA

Figure 5 - Hybridization of Yeast Histone Plasmid to poly(A)⁺, poly(A)⁻ and Total RNA. The autoradiogram shows the hybridization of ³²P labelled TRT-1 DNA to poly(A)⁺, poly(A)⁻ and total RNA. Five µg of total and poly (A)⁻ RNA were used. The amount of poly(A)⁺ RNA used was 0.5 µg. The identification of specific RNA bands was determined by hybridization of TRT-1 subclones which contain the individual genes (data not shown). Protein 1 RNA is the product of a non-histone gene present on TRT-1 (10).

which had been electrophoresed on a 1% agarose gel, then transferred to Millipore paper (17). The cDNA hybridized to a single 5 kb band (Figure 6, "8.7-10S cDNA"). This band corresponds to the 5kb Drosophila histone gene repeat (11), as shown by the fact that rehybridization of the same gel, (after melting off the cDNA cpms), with radiolabelled cloned Drosophila histone DNA labels the identical band (Figure 6, "DM").

While these data argue that yeast histone mRNA can act as a template for cDNA, they say nothing about the template efficiency, since the hybridization signal depends both on the amount of histone cDNA and the homology of yeast and Drosophila histone sequences. To obtain a more quantitative estimate of the template efficiency, histone cDNA was also analyzed by hybrid arrest translation as described by Hastie and Held, (8). cDNA synthesized from 8.7-10S poly(A)⁺ RNA was hybridized to 8.7-10S poly(A)⁺ RNA. At various times after initiation of hybridization aliquots were removed, translated.

DM
Histone
8.7-10S
cDNA

Figure 6 - Hybridization of 8.7-10S cDNA to EcoR] cut Drosophila melanogaster genomic DNA. (See text for details).

in vitro, and the products analyzed on 1-D Triton acid urea gels. As can be seen in Fig. 7A, the amount of translatable histone mRNA decreased with increasing time of hybridization, and this decrease could be eliminated by heating the samples prior to in vitro translation. As a control, 8.7-10S RNA was incubated without cDNA (Fig. 7B) or with cDNA synthesized from Xenopus oocyte RNA (data not shown). Under these conditions no decrease in translational capacity was observed.

The rate at which histone mRNA translational capacity disappears should be a semiquantitative measure of the amount of histone cDNA present in total cDNA (8). This can be simply measured by comparing the rate of translational arrest of the histone mRNAs to other mRNAs present in the population at concentrations roughly equivalent to the histone mRNAs. If there is much less histone cDNA compared to the cDNAs for these mRNAs then the rate of histone translational arrest will be much less than the corresponding rate for these other mRNAs. In Fig. 7A we have indicated by arrows three proteins

Figure 7 - Hybrid arrest translation

(A) Hybridization of 8.7-10S cDNA to 8.7-10S RNA. 0, 30', 60', 100', and 300' indicate times at which samples were removed from the hybridization reaction and translated in vitro. 60 Δ , 100 Δ and 300 Δ , indicate samples which were heat denatured prior to in vitro translation.

(B) Effect of incubating 8.7-10S RNA under the hybridization conditions without homologous cDNA.

whose concentrations appear roughly equivalent to the histones. As can be seen, the loss of histone mRNA translation capacity parallels the loss of translational capacity of these mRNAs. Therefore the cDNA template activity of the four histone mRNAs appears comparable to that of the mRNA for these three proteins. Furthermore, by quantitating the intensities of the histone bands and utilizing the equations derived by Hastie and Held (8) we estimate that the concentration of any histone cDNA is roughly 1-2% of the total 8.7-10S cDNA. This number is in good agreement with our estimates of histone mRNA concentration by *in vitro* translation of the 8.7-10S poly(A)⁺ RNA (0.1-0.2%, 20 fold enriched). Therefore, we conclude that histone mRNA is an extremely good template for cDNA synthesis, when primed with oligo(dT)

DISCUSSION

By *in vitro* translation we have shown that $\geq 90\%$ of all four yeast histone mRNAs are found in poly(A)⁺ RNA. In the case of the H2A and H2B mRNAs we have directly demonstrated that *in vitro* translation quantitatively reflects the distribution of these mRNAs in poly(A)⁺ and poly(A)⁻ RNA since hybridization of cloned sequences for the H2A and H2B genes to RNA immobilized on DBM paper reveals that $\geq 90\%$ of the hybridization is to mRNA present in poly(A)⁺ RNA. Moreover, these data argue that the poly(A) tract cannot be less than 5-30 nucleotides since poly(A) tracts shorter than this are not retained on oligo(dT) cellulose (25).

Consistent with our interpretation that yeast histone mRNA is polyadenylated is the observation that the RNA is an extremely good template for cDNA when used with an oligo(dT) primer. Since we have not directly isolated the poly(A) tracts, it could be argued that these tracts are not added posttranscriptionally, but instead, reside within the primary mRNA sequences. We feel this is unlikely since the DNA sequence of the yeast H2B gene has been determined and there are no poly(A) tracts within the sequence (Wallace, Hereford and Grunstein, manuscript in preparation).

The function of the poly(A) on yeast histone mRNA -- and the reason for the lack of adenylation of most metazoan histone mRNAs -- is obscure. The only other reported case where histone mRNA is predominantly adenylated is in amphibian oocytes (3,4), a cell type in which the metabolism of mRNA is unusual. Yeast and amphibian oocytes have little in common; this consideration, therefore, renders even more obscure the function of poly(A) on yeast histone mRNA or its absence from most histone mRNAs. Further studies will be required to understand the biological significance of these observations.

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