ADENYLIC ACID-RICH SEQUENCES IN MESSENGER RNA FROM YEAST POLYSOMES

Jennifer REED and Erhard WINTERSBERGER
Physiologisch-Chemisches Institut, Universität Würzburg, Koellikerstrasse 2, D-87 Würzburg, Germany

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1. Introduction

Sequences, 150–250 nucleotides long, which contain over 90% adenylic acid (poly(A)) have been found in the messenger RNA (mRNA) and the heterogeneous nuclear RNA of mammalian cells as well as in viral RNA and virus-specific mRNA [1–9]. The function of these post-transcriptionally added sequences is at present unknown. Similar segments of poly(A) appear to be absent from the mRNA of bacterial polysomes [10]. In this context it is interesting to note, however, that according to a recent report poly(A) sequences are present in the mRNA of a comparatively simple organism, the cellular slime mold *Dictyostelium discoideum* [11]. Going still further down the complexity of eukaryotic cells, we wish to report here that poly(A) sequences, 40–60 nucleotides long, can also be found in polysomal mRNA from the most primitive unicellular eukaryotic, *Saccharomyces cerevisiae*. This indicates that whatever the role of poly(A) sequences, it must have to do with some specific property common to all eukaryotic mRNA's. A preliminary account of this work was presented at the 8th. FEBS Meeting, Amsterdam, 1972 [12].

2. Methods and materials

2.1. *Preparation and labelling of yeast spheroplasts*

Yeast cells (strain D273-10B, α, p^+) were grown at 28°C to a density of 2×10^7 cells/ml in a medium containing 0.3% yeast extract, 0.5% peptone and 1% glucose. Cells were then harvested and spheroplasts prepared according to the method of Hutchison and Hartwell [13]. Washed spheroplasts were suspended (2×10^8/ml) in a medium containing 1 M sorbitol, 0.1% yeast extract, 0.2% peptone and 1% glucose and conditioned for 1 hr at 28°C. Label (either 5 μCi/ml [^{3}H]adenine or [^{3}H]uridine, or 0.25 μCi/ml [^{14}C]-adenine) was added and incubation continued for 5 min at 28°C. At the end of the pulse labelling period, cycloheximide was added to a final conc. of 200 μg/ml and spheroplasts rapidly cooled and harvested by centrifugation.

2.2. *Preparation of polysomal RNA*

0.5 to 1.0×10^{10} labelled spheroplasts were suspended in 10 ml of 0.015 M KCl–0.01 M Tris-HCl (pH 7.5)–0.01 M $MgCl_2$ containing 50 μg/ml polyvinylsulfate and completely lysed by the addition of deoxycholate to a final conc. of 0.5%. After 3 min at 0°C, Brij 58 was added again to a final conc. of 0.5%. After another 5 min at 0°C, the lysate was centrifuged for 15 min to remove most of the chromatin material and debris. The turbid and slightly yellow supernatant was layered onto 25 ml gradients of 10–40% sucrose made up in the same buffer and centrifuged for 4 hr at 25 000 rpm in the SW 25.1 rotor of the Spinco centrifuge. After centrifugation, gradients were pumped through the flow cell of a Gilford spectrophotometer to record automatically the optical density at 260 nm and collected in about 25 fractions. Acid insoluble radioactivity was determined on 50 μl aliquots of each fraction.

Alternatively, polysomes were isolated from the lysate by a simplified procedure involving centrifugation for 90 min at 105 000 *g* through a cushion of 20% sucrose. Polysomes were dissociated in a buffer consisting of 0.015 M KCl–0.01 M Tris-HCl (pH 7.5)–0.03 M EDTA and 50 μg/ml polyvinylsulfate, and the ribonucleoproteins separated by centrifugation for 18 hr at 21 000 rpm in 25 ml gradients of 10–40%

sucrose (in 0.015 M KCl–0.01 M Tris-HCl (pH 7.5) containing 50 μg/ml polyvinylsulfate).

Polysomal RNA was extracted either directly from polysomal suspensions or from fractions of the ribonucleoprotein separated by sucrose gradients. Deproteinization was carried out using the method described by Adesnik and Darnell [14], which involves extraction with chloroform/isoamylalcohol in the presence of sodium dodecyl sulfate. Separation of the isolated polysomal RNA in sucrose gradients was done by layering approx. 1 mg of RNA dissolved in 1 ml of 0.1 M NaCl–0.01 M Tris-HCl (pH 7.5)–1 mM EDTA–0.05% SDS onto 25 ml of a 10–30% sucrose gradient, (made up in the same buffer), and centrifuging for 16 hr at 20°C and 25 000 rpm in the SW 25.1 rotor. After centrifugation, the optical density of the gradients was recorded automatically and acid precipitable radioactivity determined on 50 μl aliquots of the ca. 25 fractions collected.

2.3. *Isolation and characterization of poly(A)*

The presence of poly(A) in the sample was assayed by digesting the RNA dissolved in 0.3 M NaCl–0.03 M sodium citrate (pH 7.0) with a mixture of pancreatic (2–5 μg/ml) and T1 (2–5 U/ml) ribonucleases and determining the acid precipitable radioactivity remaining after the process had reached completion. Digested or non-digested samples were analyzed by electrophoresis in 10% polyacrylamide gels prepared according to the method of Loening [15], using diacrylate as the cross-linking agent. After electrophoresis the gels were scanned at 260 nm, frozen, sliced and the slices dissolved in 10% piperidine at 60° for 1 hr. 10 ml Bray's scintillator were then added and the samples counted in a Nuclear Chicago Mark I scintillation counter

2.4 *Materials*

All radiochemicals were obtained from Radiochemical Centre, Amersham. The specific activities were: [^{3}H]adenine: 17 Ci/mmol; [^{3}H]uridine: 5 Ci/mmol; [^{14}C]adenine: 287 mCi/mmol. Glusulase, the snail gut enzyme used for the preparation of yeast spheroplasts, was from Endo Laboratories, Garden City, N.Y.; pancreatic ribonuclease was obtained from Boehringer, Mannheim and ribonuclease T1 from Worthington Biochemical Corp. All other chemicals and reagents used were of analytical grade quality.

3. Results

Table 1 shows the acid precipitable counts remaining in total polysomal RNA after short-term and long-term labelling. Digestion resistant sequences are present only in the pulse-labelled RNA, and are essentially absent from RNA labelled under long-term conditions. Thus the polyadenylic acid containing sequences present in yeast polysomes are associated only with the rapidly labelled RNA fraction, presumably messenger. Two control experiments provide good evidence that the

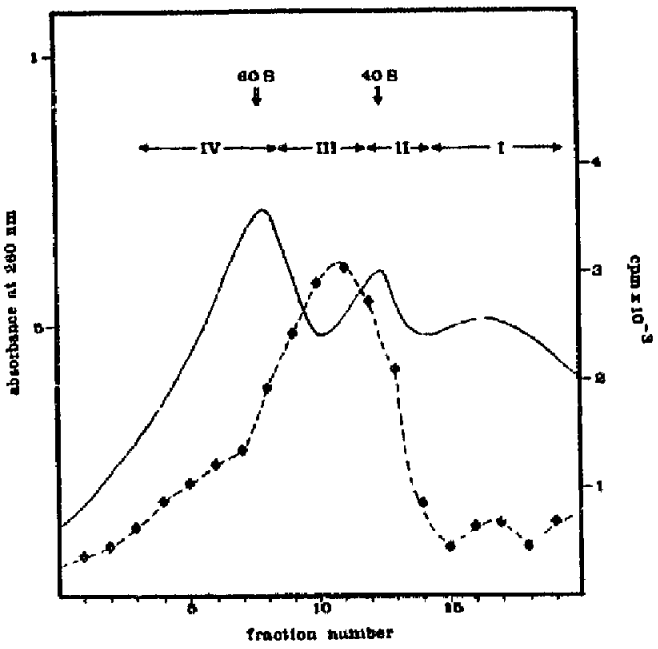

Fig. 1. Sucrose gradient sedimentation of yeast polysomes after dissociation with EDTA. About 10^{10} spheroplasts were pulse labelled for 5 min with 250 μCi [^{3}H]adenine as described by Hutchison and Hartwell [13]. Polysomes were isolated, treated with EDTA and centrifuged through a 10–40% sucrose gradient as outlined in Methods and materials. After centrifugation the contents of the centrifuge tube were pumped through the flow cell of a Gilford Spectrophotometer to record automatically the absorbance at 260 nm (——) and then collected in fractions of about 1 ml each. A 50 μl aliquot of each fraction was used to determine acid insoluble radioactivity (•—•—•). Fractions corresponding to the four arbitrarily chosen size classes, (I: 5–20 S; II: 20–40 S; III: 40–60 S; IV: > 60 S), were pooled prior to the isolation of RNA according to the procedure of Adesnik and Darnell [14]. Complete digestion with a mixture of pancreatic and T1 ribonucleases yielded the following percentage of resistance for the four size classes: I: 20%; II: 7%; III: IV: 5%. Sedimentation was from right to left.

Table 1
Ribonuclease resistance of short and long term labelled polysomal RNA.

	0 min	2 min	5 min	10 min	20 min	40 min	70 min
Short term	7238	1588	774	470	378	238	269
Long term	7801	34	18	18	31		

Polysomal RNA was prepared from yeast spheroplasts pulse labelled for 5 min or steady state labelled throughout the growing period of the cells and digested as described in Methods and materials. The numbers given are cpm of acid precipitable radioactivity in 100 µl aliquots taken from the digestion mixture at the times indicated.

ribonuclease resistant material obtained from polysomal RNA consists of polyadenylic acid: first, exposure of the short-term digestate to 0.1 M KOH at 30°C for 24 hr results in the total loss of acid precipitable material, which shows that the ribonuclease-resistant fraction is a polyribonucleotide; second, when RNA

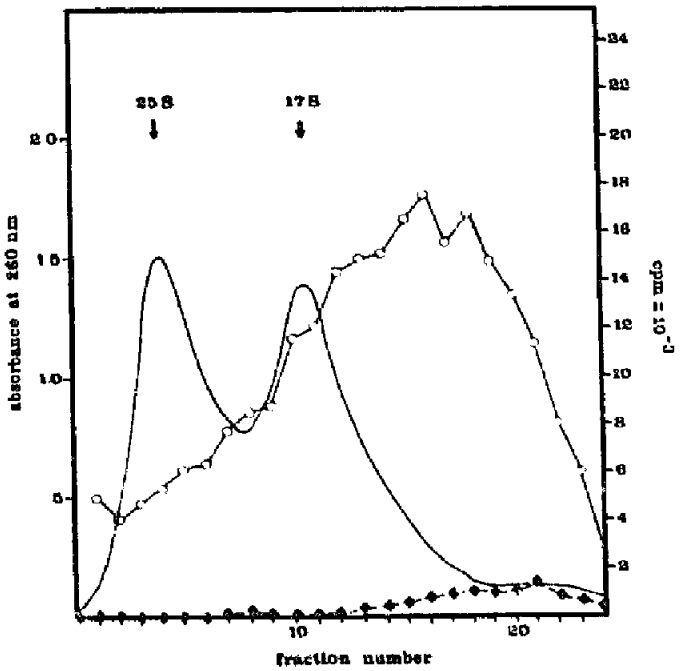

Fig. 2. Sucrose gradient sedimentation of deproteinized yeast polysomal RNA. Polysomes were extracted with chloroform/isoamylalcohol as described in Methods and materials and the purified RNA centrifuged on a 10–30% sucrose gradient. The contents of the centrifuge tube were then pumped through the flow cell of a Gilford Spectrophotometer to record the optical density at 260 nm (——) and then collected in fractions of about 1 ml each. 50 µl aliquots of each fraction were used to determine the acid precipitable radioactivity (o—o—o). Another 50 µl aliquot of each fraction was diluted 5-fold with 0.3 M NaCl–0.03 M sodium citrate (pH 7.0) and a mixture of pancreatic and T1 ribonucleases added to a final conc. of 10 µg/ml and 10 U/ml, respectively. Digestion was carried out for 30 min at 35°C after which the remaining acid precipitable radioactivity was determined (•—•—•).

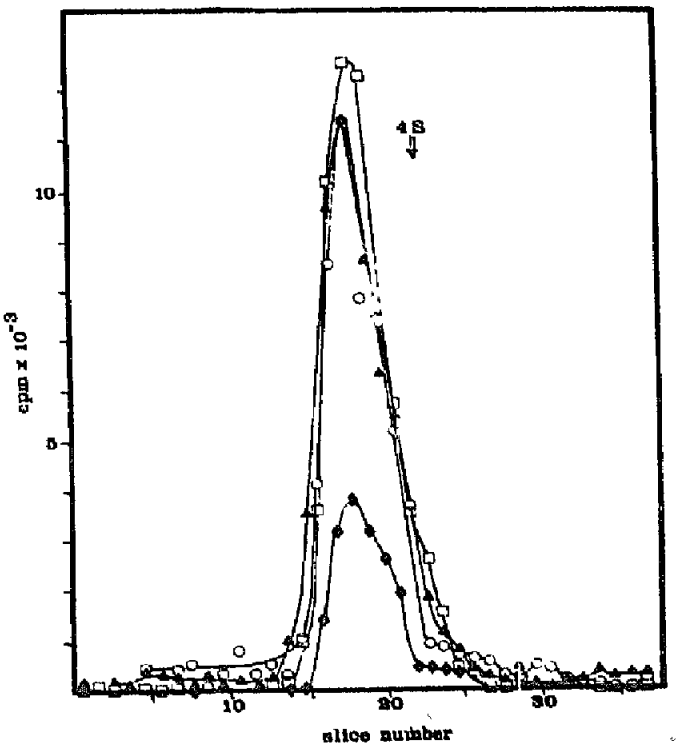

Fig. 3. Polyacrylamide gel electrophoresis of the ribonuclease resistant fraction of the [^{3}H]adenine pulse-labelled mRNA. The four fractions (I–IV) of mRNA obtained from the experiment of fig. 1 were digested with ribonuclease as described in the text. Electrophoresis of the digests was in 10% diacrylate cross-linked polyacrylamide gels (0.4 × 8.5 cm) at 5 mA/gel for 1 hr. After electrophoresis, gels were sliced, the slices dissolved in 0.5 ml 10% piperidine and treated for 1 hr at 60°C. After addition of 10 ml Bray's solution, the samples were counted. (o—o—o I; ▲—▲—▲ II; □—□—□ III; ♦—♦—♦ IV). Yeast tRNA served as the 4 S marker; its position in the gel was determined by scanning the gel at 260 nm. Migration was from right to left.

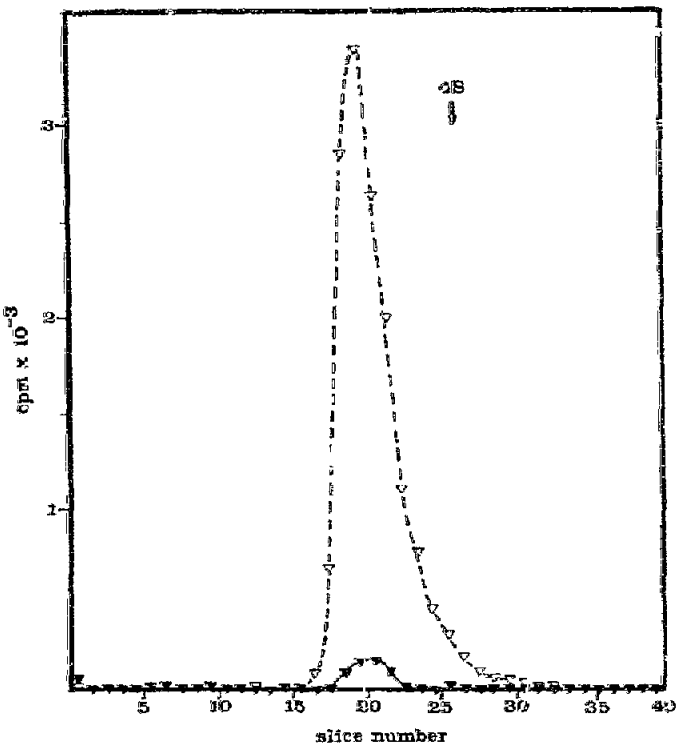

Fig. 4. Polyacrylamide gel electrophoresis of the ribonuclease resistant fraction of long-term and short-term labelled yeast polysomal RNA. Cultures were labelled and digestion carried out as described in the text. The ribonuclease resistant fractions were placed on 10% polyacrylamide gels and electrophoresed at 5 mA/gel for 1 hr. Radioactivity was assayed as described for fig. 3. Yeast tRNA was used as marker. Migration was from right to left. (▼—▼—▼) long-term labelled; (▽—▽—▽) short-term labelled.

labelled with [^{3}H]uridine was digested, little or no counts were detectable in the acid precipitable material, which therefore must be composed primarily of adenylic acid.

If ribonucleoprotein is released from short-term labelled polysomes and separated on a sucrose gradient (fig. 1, polyadenylic acid sequences are found to be associated with the high specific activity RNA throughout the range from 5–60 S. The per cent of the resistant material decreases roughly with increasing size, which suggests that the actual length of the poly(A) sequences remains roughly constant regardless of the size of the messenger with which it is associated. If short-term labelled total polysomal RNA is deproteinized before separation on a sucrose gradient the distribution of digestion resistant material is as shown in fig. 2. Again, the poly(A) is associated with messenger throughout a wide range of sizes, and the possibility that this association is mediated through bound protein is eliminated.

The size of the poly(A) sequences was determined by electrophoresis in 10% polyacrylamide gels, using yeast transfer RNA (ca. 80 nucleotides) as a marker. Fig. 3 shows the results for digestion resistant material from the four size classes of fig. 1. The size of the poly(A) segments is seen to be independent of that of the messenger with which it is associated. The rate of migration relative to the marker yields a size estimate of about 55–60 nucleotides. Discrepancies between the size of a poly(A) segment and its expected mobility on polyacrylamide gels and sucrose gradients have been noted, however [6], and were also observed in our work, as poly(A) from yeast was found to sediment on sucrose gradients considerably slower than 4 S RNA (not shown). The actual size of the segment can by the methods applied therefore merely be bracketed as being between 40 and 60 nucleotides.

In order to get a rough estimate of the homogeneity of poly(A), a double label experiment was carried out. Cells were labelled with both [^{3}H]uridine and [^{14}C]-adenine, polysomal RNA prepared and the ribonuclease resistant fragment analyzed on polyacrylamide gels. As judged from the extent of contamination of uridine counts in the poly(A) region, the latter consists of over 90% adenylic acid.

If long-term labelled RNA is digested (table 1) and analysed on 10% polyacrylamide gels the results are as shown in fig. 4. The long-term digest is compared with a total polysomal digest from short-term labelled material. Clearly there is essentially no poly(A) associated with ribosomal RNA; the very low peak which appears on the gel is probably due to a small percentage of label incorporated in messenger under these conditions. In agreement with this observation, it is found that where 70% of pulse-labelled RNA is bound to millipore filters and 60% to poly(U)-Sepharose columns, steady-state labelled material, in contrast, is retained very poorly, (18% and 20%, respectively). Further, digestion with RNAase of the portions of the pulse-labelled RNA retained and excluded on poly(U)-Sepharose and analysis of the digestion products on acrylamide gels showed that only that part of the RNA which is retained on the column contains the typical poly(A) material, which is absent from RNA

which is excluded. These findings, apart from providing further evidence for the presence of poly(A) sequences in yeast mRNA, will be useful for studies involving the isolation and characterization of this mRNA.

To show that the poly(A) present in yeast polysomes is an integral part of the messenger, the RNA released from polysomes by EDTA treatment was separated according to size on sucrose gradients both before and after removal of protein, as shown in figs. 1 and 2. As subsequent digestion of the separated fractions in both cases reveals the presence of poly(A) in ribonucleoprotein sedimenting throughout the range from 5–60 S, and in purified RNA sedimenting up to at least 17 S (that from heavier messenger is not present in large enough concentrations to be observed under these sampling conditions), it is clear that the vast majority of poly(A) associated with yeast polysomal RNA is released from the messenger only after enzymatic hydrolysis.

4. Conclusions

These experiments indicate that polyadenylic acid segments are associated with the rapidly-labelled RNA of yeast. The segments seem to be covalently linked to the messenger, and approx. 40–60 nucleotides in length. This size is significantly lower than the 150–250 nucleotides reported for animal cells. It is of interest, though, that the poly(A) sequences found in the mRNA of *D. discoideum* are also shorter than those of animal cells [11] and this may be of significance with regard to the function of poly(A).

While this paper was in preparation, results essentially identical to ours were reported by McLaughlin et al. [16].

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