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Structure of Polyadenylic Acid in the Ribonucleic Acid of *Saccharomyces cerevisiae*

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Investigations of the structure of polyadenylic acid [poly(A)] in yeast have shown that there are two classes of poly(A) distinguished by size and kinetics of synthesis. Each class is found directly on the 3′ end of messenger RNA. One class contains poly(A) molecules ranging from 60 to less than 20 nucleotides long. The longest molecules in this poly(A) class are the first to become labeled when cells are exposed to [^{3}H]adenine. Label then appears in progressively smaller molecules. The second class of poly(A) is about 20 nucleotides long. The length homogeneity of this class and the presence in nuclear DNA of many copies of a polythymidylate sequence which is the same length suggests that this poly(A) is synthesized by transcription from DNA.

Yeast, like other eucaryotes, contains polyadenylate sequences in its messenger RNA (mRNA) (10, 17, 22). In *Saccharomyces cerevisiae* these polyadenylate sequences have an average length of about 40 nucleotides (10) and appear to shorten as turnover of mRNA occurs in the cytoplasm (unpublished data).

The presence of template-independent polyadenylic acid [poly(A)] polymerase activity in mammalian cells (6) and yeast (12; S. Sogin, personal communication), and RNA labeling experiments in the presence of cordycepin or actinomycin D (3), constitute the major evidence that these poly(A) sequences are synthesized by post-transcriptional polymerization of adenylate residues onto the 3′ ends of mRNA. However Jacobson et al. (15) have presented strong evidence that a portion of the poly(A) in mRNA of the cellular slime mold *Dictyostelium discoideum* is transcribed from nuclear DNA.

During our investigations of the turnover of poly(A) in the mRNA of *S. cerevisiae* we have observed after brief labeling times a class of short poly(A) that is very homogeneous in length (11; N. E. Hynes, Ph.D. thesis, University of Pittsburgh, Pittsburgh, Pa., 1975). This is in contrast to the overall length distribution of poly(A) which ranges from approximately 60 to less than 20 nucleotides in length. Kinetics of labeling suggest a unique synthetic origin for this short homogeneous poly(A) class. These results, in addition to the homogeneity of their length, suggested to us a transcriptional origin for this poly(A). Here, we describe experiments which further characterize poly(A) in yeast mRNA and show that the short homogeneous poly(A) class is on the 3′ end of mRNA just as the bulk of the poly(A) has been shown to be (17). We directly show that the DNA of *S. cerevisiae* contains a polythymidylate [poly(T)] sequence which appears to have the correct length to serve as a template for the synthesis of the short homogeneous poly(A) class. In addition, we confirm the earlier observations of Mol et al. (18) by showing that sufficient poly(T) is present in the nuclear DNA of a haploid cell to account for approximately 700 of these poly(T) sequences.

MATERIALS AND METHODS

Cell growth and spheroplast preparation. *S. cerevisiae* S288c was grown at 30°C in minimal medium containing 7 g of yeast nitrogen base without amino acids (Difco) and 20 g of glucose per liter of deionized water. Spheroplasts were prepared according to the method of Hutchinson and Hartwell (13) from cells grown to a density of 2×10^7/ml with the enzyme preparation Glusulase (Endo Laboratories).

Preparation of ^{3}H-labeled poly(A). Cultures of spheroplasts (40 ml each) derived from a total of 1.6×10^9 cells were incubated for 2 h. [2-^{3}H]adenine (15 ci/mmol, Amersham) was added to a final concentration of 37.5 μCi/ml for 2 or 15 min. At this concentration of isotope, sufficient adenine is in the medium to sustain isotope uptake for at least 15 min. Labeling was stopped by quickly pouring the cultures over 1.25 volumes of frozen crushed sorbitol followed by gentle stirring. The spheroplasts were harvested and washed free of culture fluid by layering the chilled culture over cold 2 M sorbitol and centrifuging at $1,600 \times g$ for 10 min in a swinging bucket rotor of a model PR2 International Refrigerated Centrifuge. The culture fluid and 2 M sorbitol layer were removed by aspiration and discarded.

Spheroplast pellets were lysed in 8 ml of sodium dodecyl sulfate (SDS) buffer (0.1 M NaCl, 20 mM EDTA, 50 mM Tris, pH 7.4, and 0.5% SDS) and deproteinized by the hot SDS-phenol-chloroform method of Penman (21). Nucleic acids were precipitated from the aqueous phase by adding 2 volumes of cold 95% ethanol and held overnight at −10°C, and the precipitate was recovered by centrifugation at 22,000 × *g* for 1 h in an RCII-B Sorvall centrifuge set at −15°C.

Poly(A) was obtained from each sample by the methods of Groner et al. (10) except that RNase A and T_1 RNase were used at fourfold higher concentrations of 12 μg/ml and 20 U/ml, respectively. Poly(A) was recovered from the nuclease digest by binding to and elution from oligodeoxythymidylic acid [oligo(dT)]-cellulose and was sized by electrophoresis through 14% polyacrylamide as described previously (10).

Analysis of the 2',3'-AMP and adenosine content of size classes of poly(A). Poly(A) was recovered from 1-mm gel slices by shaking for 3 days in 0.5 ml of a buffered salt solution containing 0.1 M NaCl, 0.005 M EDTA, 0.05 M Tris-hydrochloride, pH 7.5, and 0.2% SDS. Quantitative elution of the RNA was facilitated by several freeze-thaw cycles. Pooled fractions of eluate were combined with 100 μg of commercial poly(A), and 2.5 volumes of ice-cold 95% ethanol were added. The solution was mixed and held overnight at −10°C. Precipitate was recovered by centrifugation at 22,000 × *g* for 1 h. The precipitate was dissolved in 1 ml of 0.3 N KOH and incubated for 36°C for 16 h. The pH was brought to 8.5 to 9.0 by careful addition of a small quantity of 3 N HCl. A 200-μg portion of adenosine was then added to provide an absorbance marker for the nucleoside.

Adenosine and 2',3'-AMP in the hydrolysate were separated by chromatography over Dowex-1-formate by modification of published procedures (5). The sample was slowly passed into a column (0.9 by 10 cm) of Dowex-1-formate in water. The column was then washed at a rate of 110 ml/min first with 90 ml of water to elute the adenosine and then with 90 ml of 1 N formic acid to elute adenylic acid. Figure 1 presents the column profile for a sample fractionation. Approximately 60% of the tritium in [2-^{3}H]adenine is exchanged with the hydrogens in water during the course of alkaline hydrolysis. A control experiment demonstrated that this rate of exchange is the same when the labeled starting material is adenosine, 2',3'-AMP, or poly(A). Adenosine has a weak affinity for Dowex-1-formate and thus it elutes slightly after the tritium water in the column volume. However, there is a slight overlap of the adenosine peak and the ^3H_2O peak. To insure that the isotope content measured in the adenosine region contained no ^3H_2O, samples were pooled and processed by three cycles of freeze-drying and dissolving in water. Greater than 95% of the adenosine is recovered from this procedure. Recovery of isotope from the polyacrylamide gel slices through the eluate of the Dowex-1-formate was at least 70%. Additional evidence that the isotope eluting in the adenosine region is [^{3}H]adenosine was obtained by chromatography with the appropriate nucleoside and nucleotide standards on thin layers of cellulose in isopropanol-water-concentrated HCl (65:17.6:17.2, vol/vol) (5).

Isolation of [^{32}P]poly(T) from yeast DNA. Cells were grown at 30°C for 16 h to midlog phase in 500 ml of phosphate-depleted minimal medium containing 20 mCi of $^{32}P_i$ (Amersham). Stock solutions of yeast nitrogen base for the minimal medium were depleted of phosphate by treatment with magnesium sulfate at alkaline pH and removal of the magnesium sulfate precipitate by filtration. The pH was returned to approximately 6.0 by addition of HCl. To avoid limiting phosphate during labeling, sodium phosphate was added to the medium to a final concentration of 0.4 mM. Nucleic acids were extracted from spheroplasts as described above and dissolved in 0.1× SSC (0.015 M NaCl–0.015 M sodium citrate). A 400-μg amount of carrier calf thymus DNA was added and the solution was brought to 0.3 N KOH and held for 20 h at 37°C. After the pH was increased to approximately 7 by addition of HCl, the solution was brought to 0.5× SSC, and 0.54 volumes of cold isopropanol was added. The precipitate recovered by centrifugation at 22,000 × *g* for 1 h was dissolved in 0.5 ml of water. Depurination of DNA was carried out as described by Burton (4) and modified by Birnboim et al. (2). After ethanol precipitation the material from the diphenylamine-formic acid treatment was dissolved in 0.4 ml of binding buffer (0.12 M NaCl, 0.01 M sodium phosphate, pH 7.0, and 0.5% SDS). Poly(A)-Sepharose was prepared by incubating 1 g (dry weight) of Affigel-10 (BioRad Laboratories) with 1 mg of poly(A) (Biogenic Research Corp.) according to instructions provided by the manufacturer. Poly(A), which had not reacted with the Affigel 10, was removed by extensively washing the gel with 0.1 M phosphate buffer (pH 7.0)–1 M

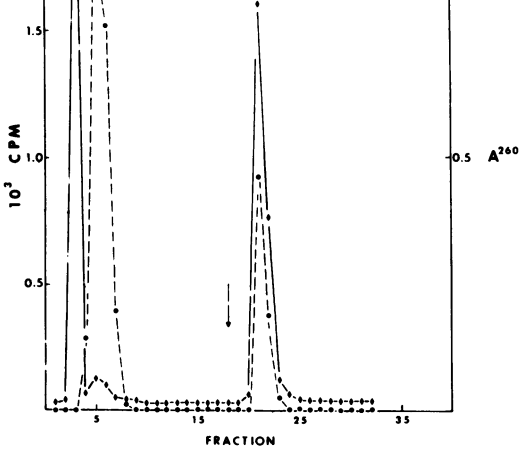

FIG. 1. *Fractionation of an alkaline hydrolysate of [^{3}H]poly(A) on Dowex-1-formate. The arrow designates when the elution solution was changed from water to 1 N formic acid.* (●) *Absorbance at 260 nm;* (◆) *counts per minute.*

NaCl followed by binding buffer. The capacity and specificity of the resin to bind polyuridylic acid [poly(U)] was checked with [^{3}H]poly(U) and yeast [^{3}H]rRNA. Chromatography was carried out at room temperature in a column containing a bed volume of 3.5 ml of poly(A)-Sepharose. The 0.4-ml sample was run into the bed and held for 30 min. The column was washed with 36 ml of binding buffer followed by 20 ml of a low-ionic-strength solution containing 0.01 M Tris-hydrochloride (pH 7.6)-0.2% SDS. Fractions of 4 ml each were collected and their ^{32}P content was monitored as Cerenkov radiation in glass vials in a scintillation spectrometer.

Analysis of deoxyribonucleotide composition. A 500-μg portion of calf thymus DNA, which had been boiled for 10 min to destroy phosphomonoesterase activity, was added to each pooled sample. The samples were then brought to 0.3 M sodium acetate. Two volumes of 95% ethanol was added, while mixing, and the samples were held overnight at −10°C. Precipitates collected by centrifugation were resuspended in 0.3 ml of water containing 0.01 M $MgSO_4$. A 25-μg amount of DNase I (Sigma type DN-EP) was added to each, and the samples were incubated at 37°C for 1 h. The reactions were brought to 50 mM Tris-hydrochloride (pH 8.6), and 10 μg of snake venom phosphodiesterase (Boehringer-Mannheim) was added. The incubation was continued for an additional 80 min. During the reaction the pH was maintained at 8.3 to 8.5 by periodic addition of small amounts of NaOH. Digestion was terminated by bringing each sample to pH 4.0 with acetic acid.

High-voltage paper electrophoresis was carried out on 3MM paper in pyridine acetate buffer (pH 3.5) as described by Sebring and Salzman (23) for 130 min with a 3,500-V drop across the paper. After treating the wet paper with ammonium hydroxide vapors for 30 min and drying overnight at 50°C, the deoxyribonucleoside monophosphate spots were visualized under UV light, and 2-cm strips were cut out and assayed for ^{32}P.

Hybridization of [^{3}H]poly(A) to sheared denatured DNA. DNA was prepared from cells grown to late exponential phase in minimal medium. Washed cell pellets suspended in a solution of 0.05 M Tris-hydrochloride (pH 7.6), 0.1 M NaCl, and 0.01 M EDTA were opened by shaking with glass beads in a Braun homogenizer cooled with CO_2. The homogenate was brought to 0.5% SDS and deproteinized by extraction with phenol-chloroform (21). DNA, recovered by precipitation from the aqueous phase with two volumes of ethanol, was dissolved in the above solution and rapidly passed 20 times through a 24-gauge needle to insure that the DNA was sufficiently sheared to reduce the consequences of rapid renaturation caused by cross-links (8). RNA was hydrolyzed by incubating in 0.3 N NaOH at 37°C for 16 h. After neutralization, the preparation was treated with protease (Subtilisin BPN', type VII, Sigma) for 30 min at 37°C. SDS was added and the sample again was deproteinized by extraction with phenol chloroform. The DNA, recovered by ethanol precipitation, was dialyzed against three changes of 0.1× SSC-2 mM EDTA for 20 h at 4°C and stored frozen in small portions. DNA mass was determined both by spectrophotometry at an absorbancy at 260 nm (A_{260}) by using an extinction coefficient of 1 A_{260} cm = 50 μg/ml and diphenylamine (24) with calf thymus DNA as a standard. Values were used only when there was agreement between the two methods. Nuclear DNA was prepared by sedimenting nucleic acid extracts of spheroplasts to equilibrium in CsCl. The band at a density of 1.700 g/cm^3 was resedimented to equilibrium in CsCl, dialyzed against 0.1× SSC-2 mM EDTA, and sheared as described above.

Nucleic acid hybridization was carried out essentially as described by Jacobson et al. (15). Reaction mixtures of 1 ml containing 0.36 N Na^+, 10 mM sodium phosphate (pH 7.0), and 1 μg of DNA that was boiled for 3 min in 0.8 ml of water were incubated at 45°C for 16 h in the presence of various amounts of [2,8-^{3}H]-poly(A) (New England Nuclear; specific activity, 8.75 μCi/μg). Radioactive poly(A) as hybrid was assayed as a T_2 RNase-resistant form using digestion conditions established to maximize the stability of a hybrid containing [^{3}H]poly(A) and oligo(dT)$_{12-18}$ (Collaborative Research). The hybridization reaction was allowed to come to room temperature and was brought to a 2-ml volume containing 0.3 M Na^+ and 25 mM sodium phosphate (pH 6.0). The temperature was lowered to 12°C, 10 units of T_2 RNase (Sigma, grade V) was added, and incubation was continued at 12°C for 3 h. Digestion was terminated by adding cold trichloroacetic acid and pyrophosphate to final concentrations of 5% and 0.12 M, respectively. A 100-μg portion of bovine serum albumin was added as carrier. Precipitates were collected on glass fiber filters and processed, and the radioactivity retained on each filter was determined by scintillation spectrometry (14).

RESULTS

The structure of poly(A) in yeast mRNA. In our initial studies of poly(A) in the mRNA of yeast (10) we demonstrated that longer sequences of poly(A) were preferentially labeled when cells were pulse-labeled with [^{3}H]adenine (10). During subsequent investigations the existence of a short homogeneous subpopulation of poly(A) became evident at intermediate labeling times (11; Hynes, Ph.D. thesis, 1975). Figure 2 presents the results of an electrophoretic analysis in 14% polyacrylamide gels of poly(A) obtained from spheroplasts labeled with [^{3}H]adenine for 2 min (A) or 15 min (B). These results confirm our earlier observation that the longer species of poly(A) are preferentially labeled during a short pulse with [^{3}H]adenine. In addition, cells labeled for 15 min show a predominant sharp peak of rapidly migrating radioactivity. We have previously shown by electrophoretic analysis under denaturing conditions that the large poly(A), preferentially labeled during a pulse, has a length of approximately 60 nucleotides and that the average poly(A) length is approximately 40 nucleotides (10). Comparison of the electrophoretic mobility of the rapidly

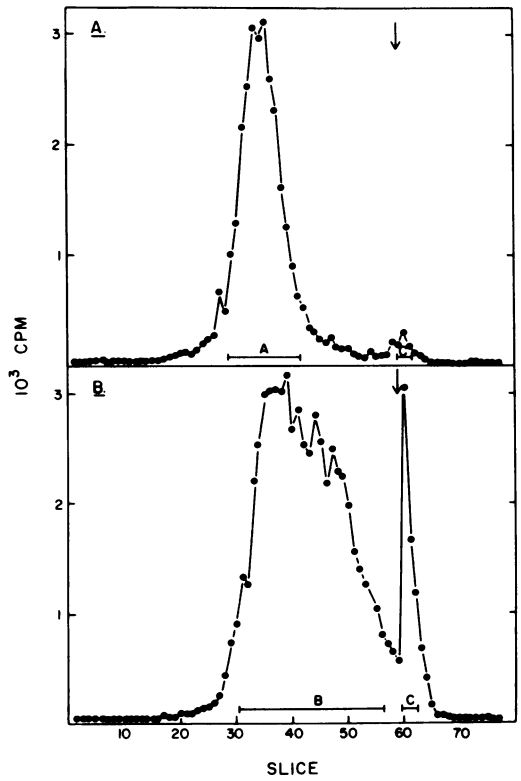

FIG. 2. *Electrophoresis in 14% polyacrylamide gels of poly(A) obtained from cells labeled for 2 min (A) or 15 min (B) with [^{3}H]adenine. Poly(A) was prepared and electrophoresis was carried out as described in the text. Samples of 25 μl each from the 0.5-ml eluate of each gel slice were assayed for radioactivity content. The arrow designates the position of the bromophenol blue marker. Fractions pooled from each gel for further analysis are indicated by bars lettered A through C. At least 70% of the labeled RNA was recovered from the gel slices.*

migrating homogeneous poly(A) population in Fig. 2 with the mobility of the bulk poly(A) suggests it has a length of approximately 20 nucleotides. To confirm that this rapidly migrating material is indeed poly(A), composition analysis of alkaline hydrolysates were carried out by chromatography over Dowex-formate (5). Greater than 95% of the isotope in this rapidly migrating species prepared from spheroplasts labeled with $^{32}P_i$ cochromatographed with authentic AMP (data not shown). The kinetics of labeling poly(A)$_{20}$ are quite different from those labeling the bulk poly(A). [To avoid repetitive bulky nomenclature we have designated the short homogeneous class of poly(A) as poly(A)$_{20}$.] After 2 min of labeling, poly(A)$_{20}$ is just discernible (Fig. 2A). As the labeling time is extended the proportion of the isotope found in the poly(A)$_{20}$ peak increases. In addition, the major class of poly(A) becomes broader and eventually overlaps the poly(A)$_{20}$ peak (Fig. 3).

Previous investigations have demonstrated that short poly(A) sequences exist internally within an RNA transcript (15, 20). This is in contrast to the bulk of the poly(A) which is found directly on the 3′ end of mRNA (3). To determine whether poly(A)$_{20}$ is internal or on the 3′ end of yeast mRNA, we measured the ratio of AMP to adenosine in alkaline hydrolysates of pooled fractions of poly(A) eluted from the gels shown in Fig. 2. Table 1 presents the results of this analysis. The ratio of AMP to adenosine of approximately 20 obtained from hydrolysates of the short homogeneous poly(A) agrees with the length predicted from its electrophoretic mobility. Because this ratio is independent of the time spheroplasts are exposed to label, at least up to a time nearly equivalent to the half-life of polyadenylated mRNA, we conclude that poly(A)$_{20}$ in polyribosomes is present on the 3′ end of mRNA. Ratios of AMP to adenosine of 60 for the major peak of pulse-labeled poly(A) and 45 for the broad peak of poly(A) labeled for 15 min confirm our previous estimate of their size.

Lodish et al. (15) have proposed a model for the 3′ end of cellular slime mold mRNA in which a short poly(A)$_{25}$ sequence is internal and separated from a longer 3′ terminal poly(A) by a short sequence which is cleaved by pancreatic RNase A but not by T_1 RNase. To determine whether this structure exists in yeast poly(A)-containing RNA we conducted an electrophoretic study of poly(A)-rich sequences in RNase A or T_1 digests. RNA was extracted from three identical cultures of spheroplasts labeled for 60 min with [^{3}H]adenine. Figure 3 presents an electrophoretic analysis of poly(A) released from this RNA by RNase A and/or T_1 RNase. Poly(A)-rich sequences obtained from the RNase A digest are similar in quantity and in electrophoretic mobility to the poly(A) obtained by digestion with both RNase A and T_1 (compare A and B, Fig. 3). Furthermore, the majority of the molecules which migrate as poly(A)$_{20}$ in fraction 29 of Fig. 3A also appear to be present in the T_1 RNase digest shown in Fig. 3C. This population of molecules, in fractions 29 and 30 of Fig. 3C, is very likely to be poly(A)$_{20}$ since no smaller homogeneous population of poly(A) is apparent in Fig. 3A which would be able to shift to a lower mobility on omission of RNase A from the digestion. Thus, it appears from these data that the majority of the poly(A)$_{20}$ in yeast is not linked to a larger poly(A) by an RNase T_1-insensitive sequence. The shift to lower mobility ap-

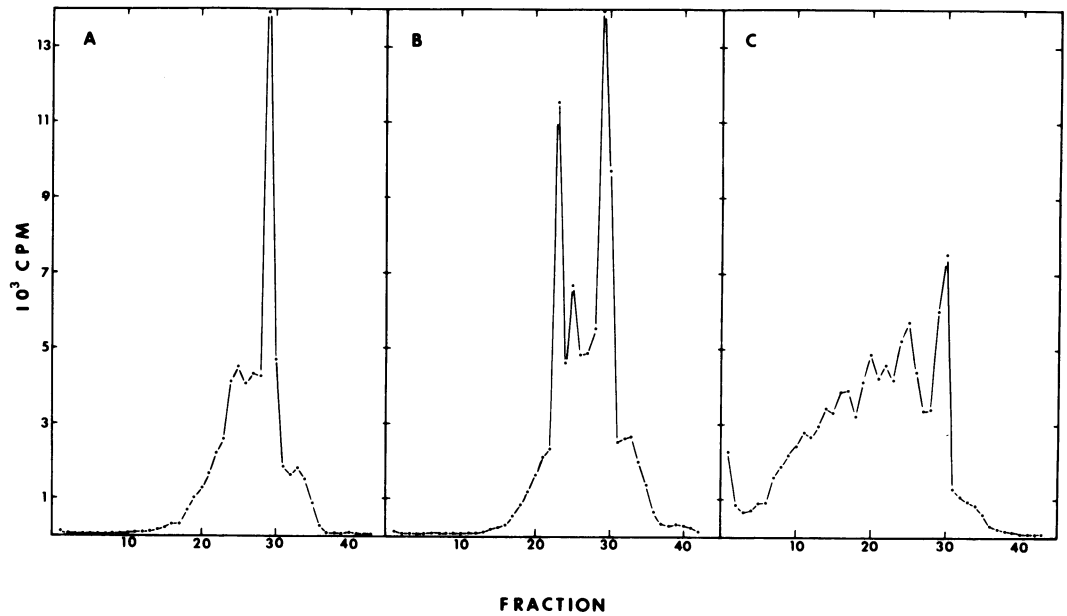

FIG. 3. *Electrophoresis in polyacrylamide gels of polyadenylate-rich oligonucleotides released from RNA by digestion with RNase A and/or RNase T_1. Poly(A)-rich oligonucleotides were prepared from spheroplasts labeled for 60 min with [^{3}H]adenine by the methods described in the text for the preparation of poly(A), except that only RNase A (B) or T_1 RNase (C) were used for two of the three samples. Electrophoresis was carried out as described in the text.*

TABLE 1. *Analysis of the AMP and adenosine content of alkaline hydrolysates of yeast poly(A)*[a]

Pooled poly(A) fraction	Analysis of:		2',3'-AMP-adenosine ratio
	AMP	Adenosine	
Experiment 1			
A	22,197	373	59
C	5,284	251	21
Experiment 2			
B	297,897	6,674	45
C	24,178	1,336	18

[a] The fractions indicated in Fig. 1 were pooled and hydrolyzed, and the counts per minute in 2',3'-AMP and adenosine in each pool was determined as described in the text.

parent for some of the larger poly(A)-rich sequences in Fig. 3C may be the cause of the decrease in the amount of the poly(A)$_{20}$ peak. Since we have shown that all poly(A)$_{20}$ sequences appear to be located directly on the 3' end of mRNA (Table 1), this suggests that some poly(A)$_{20}$ is adjacent to sequences on their 5' end which are devoid of accessible G residues.

Fractionation of poly(A)-containing RNA by controlled elution from immobilized poly(U) or oligo(dT) has been used to probe mRNA structure (7) and function (9, 19). The rationale of this approach is that RNA containing a short poly(A) sequence will be more readily released from the immobilized nucleic acid than RNA containing a long poly(A) sequence. To provide further information about the relationship between the two classes of yeast poly(A) or their involvement in the secondary structure of yeast mRNA, poly(A)-containing RNA or the poly(A) released from mRNA by nuclease digestion was bound to oligo(dT)-cellulose in the presence of 0.1 M NaCl and was slowly eluted with a shallow gradient of decreasing ionic strength. Figure 4 presents an electrophoretic analysis of the poly(A) present in four fractions pooled from the poly(A)-containing RNA released by an immediate drop from 0.1 to 0.04 M salt and at progressively lower salt concentrations (a–d), and of an equivalent elution of the free poly(A) (e–h). Compositional analysis of alkaline hydrolysates of each of these fractions confirms that they contain only AMP residues (data not shown). Two conclusions are apparent from these data. First, and most importantly, it is evident from a comparison of panels e through h that the elution of free poly(A) from oligo(dT)-cellulose does not fractionate it according to length. Fractionation occurs only, if at all, for a very minor class of the shortest molecules. Second, since there is no sizing of the poly(A)-containing RNA by this elution procedure, the data show that when poly(A) sequences are contained

within mRNA, a portion of these sequences may not be available for as strong an interaction with oligo(dT)-cellulose as when they are free (cf. panels b and c with f and g, Fig. 4). Whether this is of any in vivo functional significance for mRNA is unknown although this view is encouraged because there are poly(U)-rich sequences in yeast RNA (B. Groner, Ph.D. thesis, University of Pittsburgh, Pittsburgh, Pa., 1975) and because the effect observed in Fig. 4 is even more dramatic when slowly turning-over poly(A)-containing RNA species are preferentially labeled.

A search for poly(T) in yeast DNA. The homogeneity of size of the poly(A)$_{20}$ suggests that its synthesis might be under the direction of a template. To directly determine whether yeast DNA contains polydeoxythymidylate tracts of sufficient length to code for poly(A)$_{20}$ we prepared a diphenylamine formic acid digest of yeast DNA from cells labeled for 15 h with $^{32}P_i$ and chromatographed it on poly(A)-Sepharose in 0.1 M salt. Figure 5 presents the elution profile of the poly(A)-Sepharose column. The great majority of labeled material passes directly through the column. However, a small amount of material appears to bind and is eluted from the column by low-ionic-strength buffer. Fractions eluted off of the poly(A)-Sepharose column with high salt buffer (pool A) and low salt buffer (pool C) were recovered for further analysis. Because label continued to leach off the column after extensive washing with high salt buffer, the tailing end of the high salt eluate was also recovered for analysis (pool B).

Figure 6 presents an electrophoretic analysis of the labeled material recovered from the poly(A)-Sepharose column. The material which

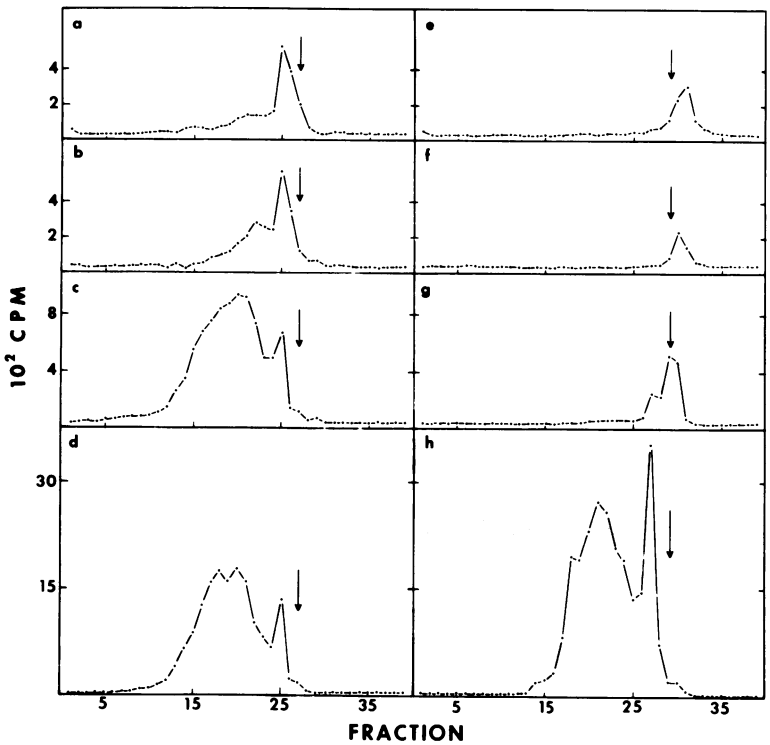

FIG. 4. *Electrophoresis in polyacrylamide gels of poly(A) in fractions eluted from a column of oligo(dT)-cellulose with a gradient of decreasing salt concentration. (a-d) RNA obtained from 2 × 10⁸ to 5 × 10⁸ spheroplasts labeled with [³H]adenine was applied at room temperature to a 2-ml column of oligo(dT)-cellulose in 0.1 M NaCl. The nonbound RNA was eluted with 14 ml of application buffer. The poly(A)-containing RNA was eluted at room temperature with 80 ml of linear gradient starting with 0.04 M NaCl, 5 mM EDTA, 50 mM Tris-hydrochloride (pH 7.6), and 0.2% SDS and ending with 0.2% SDS in water. The elution rate was 0.5 ml/min. Fractions of poly(A)-containing RNA eluting at progressively lower salt concentrations were pooled, and the poly(A) was isolated and subjected to compositional and electrophoretic analysis as described in the text. (e-h) The procedure was identical to that described above except that the RNA was subjected to digestion with RNases A and T₁ before oligo(dT)-cellulose chromatography. The arrow in each panel designates the position of migration of the bromophenol blue dye marker. Note the changes in the scale of the ordinate for each pair of panels.*

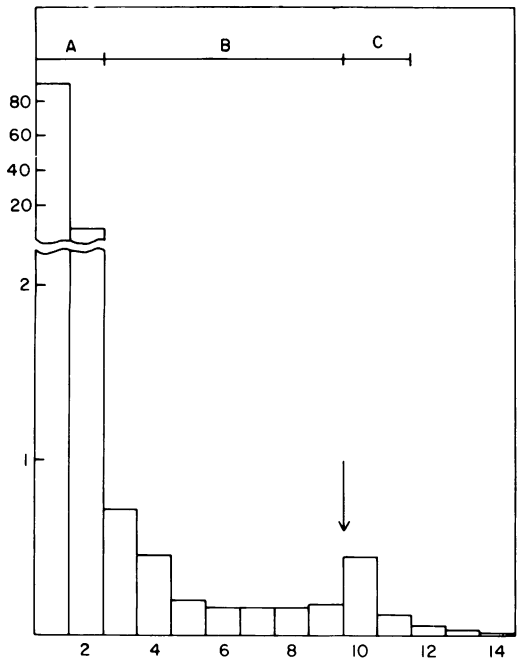

FIG. 5. *Poly(A)-Sepharose chromatography of the diphenylamine-formic acid hydrolysate of a ^{32}P-labeled DNA preparation from yeast. DNA was prepared, hydrolyzed in diphenylamine formic acid, and chromatographed on poly(A)-Sepharose as described in the text. Fractions (4 ml) were collected and their ^{32}P content was monitored as Cerenkov radiation. The arrow designates when the low-ionic-strength buffer wash was started. The bars designate the fractions which were pooled for further analysis.*

is recovered from the column in the low-salt eluate contains one electrophoretic species which penetrates the gel (Figure 6C). This material is sensitive to digestion by DNase and contains thymidylic acid (TMP) residues (Fig. 7, panel IV). This poly(T) tract has an electrophoretic mobility which is similar to the electrophoretic mobility of $poly(A)_{20}$.

Figure 6A presents the electrophoretic analysis of material which does not bind to poly(A)-Sepharose. The broad peak migrating from fractions 28 to 37 is composed of polydeoxypyrimidine tracts containing dCMP and TMP (Fig. 7, panel II). dAMP and dGMP are not present, which attests to the effectiveness of the diphenylamine-formic acid hydrolysis.

Each of the pooled fractions from the poly(A)-Sepharose column contains material which does not effectively penetrate into 14% polyacrylamide gels. This material, in addition to the peak of material migrating from fractions 12 through 17 in Fig. 6B, is not sensitive to DNase hydrolysis and does not move from the origin during high-voltage electrophoresis in pyridine acetate (Fig. 7, I and III). Because phosphomannen is a major constituent of the yeast cell wall (1) and because soluble forms of phosphomannen are very likely to copurify with DNA, we tested the pooled fractions I through IV from the gels shown in Fig. 6 for binding to concanavalin A. The results in Table 2 show that the label in pools I and III from the electrophoretic analysis binds to concanavalin A-Sepharose and is eluted by *α-methyl* mannose. The label in pools II and IV, which is in polydeoxypyrimidines, does not bind to concanavalin A. Since we find little of the phosphomannen-like material in aqueous extracts of cells opened by physical methods, we conclude that production of spheroplasts with the crude enzyme preparation glusulase liberates this polysaccharide.

Two variations in the results were observed from repetitions of the preceding analysis. The proportion of ^{32}P label in polysaccharide which contaminates the formic acid diphenylamine digest is quite variable and can render the elution profile of the poly(A)-Sepharose column noninformative. More significantly, the poly(T) which is recovered from the diphenylamine-formic acid digest by poly(A)-Sepharose frequently contains a heterogeneous population of smaller molecules. This suggests that the poly(A)-Sepharose has a limited and variable capacity to bind short poly(T) tracts.

Titration of poly(T) content in yeast DNA by hybridization to saturation with [^{3}H]-poly(A). Accurate calculation of the amount of poly(T) in yeast DNA from the recoveries of [^{32}P]poly(T) in the foregoing analysis is not possible because of the presence of isotope in polysaccharide and because of the unknown efficiency with which poly(A)-Sepharose binds short poly(T) tracts. Thus we sought to confirm the earlier estimate of poly(T) content of Mol et al. (18) by hybridizing 1-μg samples of sheared, denatured DNA to excess [^{3}H]poly(A). Table 3 summarizes the results obtained when saturation of the DNA with poly(A) was reached. The analysis indicates that in the nuclear DNA of a single cell approximately 14,000 deoxyribonucleotides are present in poly(T) sequences that are long enough to hybridize to [^{3}H]poly(A) and render it resistant to RNase T_2. This is equivalent to a maximum of 700 sequences of $poly(T)_{20}$ per haploid equivalent to DNA.

DISCUSSION

The experiments reported in this paper demonstrate that there are two classes of poly(A) in

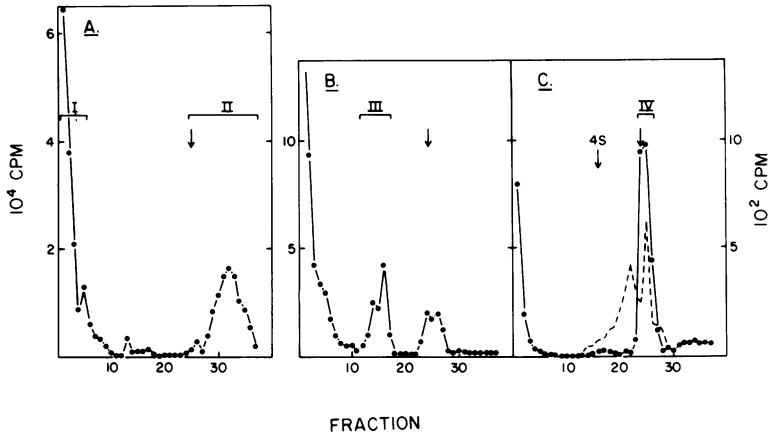

FIG. 6. *Electrophoresis in polyacrylamide gels of pooled samples from the poly(A)-Sepharose column. 14% polyacrylamide gels were prepared as previously described (10). The three pooled fractions of eluant from the poly(A)-Sepharose column were brought to 0.3 M sodium acetate. An 80-μg portion of tRNA and 2 volumes of 95% ethanol were added while mixing, and the solution was held overnight at −10°C. The precipitate was collected by centrifugation and dissolved in Tris-acetate buffer (0.02 M sodium acetate, 0.03 M Trizma base [Sigma], 0.001 M EDTA, 0.2% SDS, pH 7.8). Electrophoresis was carried for 3.25 h at 5 mA/gel, and the gels were sliced as previously described (10). Consecutive pairs of gel slices were placed in vials with 0.5 ml of water, capped, and shaken for 3 days to elute the labeled material. ^{32}P was monitored as Cerenkov radiation. The brackets indicate those fractions which were pooled for subsequent analysis. Recoveries of label from the gel slices were 90% for samples II and IV, 50% for sample III, and 22% for sample I. A-C correspond to the three pooled fractions designated A-C in Fig. 2. The dashed line in panel C is the electrophoretic profile of yeast poly(A) seen in a separate gel. The arrow in each of the panels designates the position of the bromophenol blue marker.*

the RNA of the bakers yeast *S. cerevisiae*. The major class of poly(A) is heterogeneous in size ranging from approximately 60 nucleotides to less than 20 nucleotides in length. Since our methods for the separation of poly(A) from other nuclease-resistant products in a digest are unable to recover very small poly(A) we cannot place a lower limit on the poly(A) size of this class. The second class of poly(A) is very homogeneous in size and is approximately 20 nucleotides in length. A qualitatively similar situation exists in the cellular slime mold *D. discoideum* (15). However, there appear to be fundamental differences in the way poly(A) is integrated into mRNA in these two lower eucaryotes. It has been proposed that each polyadenylated mRNA in *D. discoideum* contains two poly(A) sequences—one directly on the 3′ end of mRNA and the other on the 5′ side of the first and separated from it by a short RNase A-sensitive sequence (15). We have no evidence for such a structure in yeast. Measurements of AMP-to-adenine ratios in alkaline hydrolysates of both classes of poly(A) obtained from spheroplasts pulse-labeled with [^{3}H]adenine or labeled for a time equal to the half-life of mRNA indicate that they both are present on the 3′ end of mRNA. Furthermore, studies of poly(A)-rich oligonucleotides released by either RNase A or T_1 digestion and of the controlled elution of poly(A)-containing RNA from oligo(dT)-cellulose do not contradict this conclusion.

Studies of poly(A) polymerase activities purified from yeast suggest the presence of two enzymes (12). One of these is reported to synthesize poly(A) only by extension of a preexisting short poly(A) sequence (12). Whether the $poly(A)_{20}$ class we describe serves as a primer for the subsequent addition of AMP residues in vivo is not addressed by the present work. However, the kinetics of labeling poly(A) in nuclei and polyribosomes are not consistent with a simple precursor-product relationship between the two classes of poly(A) (unpublished data). Thus, since we find no free poly(A) in yeast and since this poly(A) class is present in a petite mutant which is devoid of mitochondrial DNA (unpublished data), there appears to be a subpopulation of cytoplasmic mRNA which carries only the short homogeneous class of poly(A). The distribution of counts between the two poly(A) classes obtained from cells labeled for 1 h with [^{3}H]-adenine (Fig. 3A) suggests that the RNA molecules carrying this short homogeneous poly(A) class constitute approximately 30% of the total number of poly(A)-containing RNA molecules in the cell.

The homogeneous length distribution of the

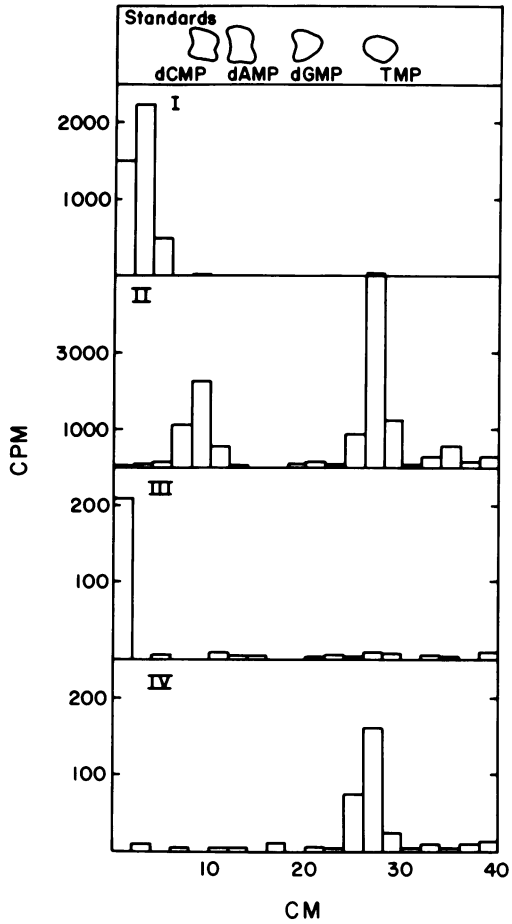

FIG. 7. *High-voltage electrophoretic analysis of nuclease digests of ^{32}P-labeled material eluted from the gels in Fig. 6. The top panel shows a typical set of spots which arise from the deoxyribonucleotide digestion products of the carrier calf thymus DNA. The four lower panels (I-IV) are the electropherograms for each of the pooled fractions designated in Fig. 6.*

small poly(A) class suggests that it may have a transcriptional origin. Our isolation of poly(T) of the appropriate size from polydeoxyribopyrimidine runs derived from yeast DNA indirectly supports this possibility. Hybridization of denatured DNA to saturation with poly(A) suggests that there is sufficient poly(T) to accommodate a maximum of approximately 700 sequences long enough to code for poly(A)$_{20}$. This confirms the earlier work of Mol et al. (18). However, the recent elegant studies of Valenzuela et al. (25) demonstrate that there are extensive runs of thymidylic acid adjacent to the 5S RNA and rRNA genes of *S. cerevisiae*. Thus, many of the poly(T) sequences we detect by saturation hybridization are present in these genes. Further

TABLE 2. *Percent of ^{32}P-labeled materials eluted from the gels shown in Fig. 3 which specifically bind to concanavalin A*[a]

Sample	%
I	92
II	5
III	98
IV	7

[a] A portion of the samples eluted from the gel slices indicated in Fig. 3 and prepared in 0.01 M sodium phosphate buffer (pH 6.7)-0.2 M NaCl was loaded on columns (8 by 25 mm) of concanavalin A-Sepharose equilibrated with the same solution. The columns were then washed with 10 ml of the application solution followed by 10 ml of the application solution containing 0.1 M *α-methyl* mannose. Samples of 1 ml were collected and monitored for radioactivity. The amounts of radioactivity in samples I, II, III, and IV were 2,000, 3,800, 480, and 250 cpm, respectively. Recovery of the radioactivity applied to the columns was 95% or greater.

TABLE 3. *Hybridization of denatured yeast DNA to saturation with [^{3}H]poly(A)*[a]

Source of DNA	[^{3}H]poly(A) (ng)	poly(A) in hybrid (cpm)[b]	Deoxynucleotides in hybrid/haploid equivalent of DNA
Total DNA	42	2,670	
	56	2,600	
	70	2,770	
	84	2,496	
	112	2,780	
	140	2,490	
Average		2,634	13,400[c]
Nuclear DNA	14	2,920	
	23	2,860	
Average		2,890	14,500[c]

[a] Conditions for hybridization and T_2 RNase treatment are described in Materials and Methods.

[b] These values are corrected for trichloroacetic acid-precipitable isotope present after digestion at 12°C when denatured DNA was added just prior to the addition of the RNase T_2. For reactions containing the largest amounts of poly(A) this value represents 20% of the gross cpm hybridized.

[c] These values are derived by using a molecular weight of 9×10^9 for a haploid equivalent of DNA and an isotope counting efficiency measured to be 26.5%, i.e.,

$$13{,}400 = \left(\frac{2634 \text{ cpm}}{0.265 \text{ cpm/dpm}}\right) \cdot \left(\frac{9 \times 10^9 \ \mu\text{g}}{\text{haploid equivalent of DNA}}\right) \Big/ \left(\frac{2.2 \times 10^6 \text{ dpm}}{\mu\text{Ci}}\right)\left(\frac{8.76 \ \mu\text{Ci}}{\mu\text{g}}\right)\left(\frac{347.2 \ \mu\text{g}}{\mu\text{mol}}\right)$$

studies of DNA fragments in a restriction enzyme digest of yeast DNA will be needed to determine the extent and distribution of poly(T) sequences in regions other than the 5S and rRNA genes.

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LITERATURE CITED

1. **Ballou, C. E.** 1976. Structure and biosynthesis of the mannon component of the yeast cell envelope. Adv. Microbiol. Physiol. **14**:93–158.
2. **Birnboim, H. C., R. E. J. Mitchel, and N. A. Straus.** 1973. Analysis of long pyrimidine polynucleotides in HeLa cell nuclear DNA: absence of polydeoxythymidylate. Proc. Natl. Acad. Sci. U. S. A. **70**:2189–2192.
3. **Brawerman, G.** 1976. Characteristics and significance of the polyadenylate sequence in mammalian messenger RNA. Prog. Nucleic Acid Res. Mol. Biol. **17**:117–147.
4. **Burton, K.** 1967. Preparation of apurinic acid and of oligodeoxyribonucleotides with formic acid and diphenylamine. Methods Enzymol. **12**:222–224.
5. **Edmonds, M., and M. G. Caramela.** 1969. The isolation and characterization of adenosine monophosphate rich polynucleotides synthesized by Ehrlich ascites cells. J. Biol. Chem. **244**:1314–1324.
6. **Edmonds, M., and M. A. Winters.** 1974. Polyadenylate polymerases. Prog. Nucleic Acid Res. Mol. Biol. **17**: 147–179.
7. **Firtel, R. A., K. Kindle, and M. P. Huxley.** 1976. Structural organization and processing of the genetic transcript in the cellular slime mold, *D. discoideum*. Fed. Proc. **35**:13–22.
8. **Forte, M. A., and W. L. Fangman.** 1976. Naturally occurring cross-links in yeast chromosomal DNA. Cell **8**:425–431.
9. **Gielen, J., H. Aviv, and P. Leder.** 1974. Characteristics of rabbit globin mRNA purification by oligo(dT)-cellulose chromatography. Arch. Biochem. Biophys. **163**: 146–154.
10. **Groner, B., N. Hynes, and S. Phillips.** 1974. Length heterogeneity in the polyadenylic acid region of yeast messenger RNA. Biochemistry **13**:5378–5383.
11. **Groner, B., and S. L. Phillips.** 1975. Polyadenylate metabolism in the nucleic and cytoplasm of *Saccharomyces cerevisiae*. J. Biol. Chem. **250**:5640–5646.
12. **Haff, L., and E. Keller.** 1975. Polyadenylate polymerases from yeast. J. Biol. Chem. **250**:1838–1846.
13. **Hutchison, H. T., and L. Hartwell.** 1967. Macromolecule synthesis in yeast spheroplasts. J. Bacteriol. **94**: 1697–1705.
14. **Hynes, N. E., and S. L. Phillips.** 1976. Turnover of polyadenylate-containing ribonucleic acid in *Saccharomyces cerevisiae*. J. Bacteriol. **125**:595–600.
15. **Jacobson, A., R. A. Fintel, and H. F. Lodish.** 1974. Transcription of polydeoxythymidylate sequences in the genome of the cellular slime mold, *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. U. S. A. **71**:1607–1611.
16. **Leech, R. M., et al.** 1968. The determination of base ratios of very small samples of ribonucleic acid using thin layer chromatography. J. Chromatogr. **34**:364–369.
17. **McLaughlin, C. S., J. R. Warner, M. Edmonds, H. Nakazato, and M. Vaughan.** 1973. Polyadenylic acid sequences in yeast messenger RNA. J. Biol. Chem. **248**: 1466–1471.
18. **Mol, J. N. M., R. A. Flavell, and P. Borst.** 1976. The presence of $(dA.dT)_{20-25}$ tracts in the DNA of primitive eucaryotes. Prog. Nucleic Acid Res. Mol. Biol. **3**:2367–2377.
19. **Morrison, M. R., J. Gorski, and J. B. Lingrel.** 1972. The separation of mouse reticulocyte 9S RNA into fractions of different biological activity by hybridization to poly(U)-cellulose. Biochem. Biophys. Res. Commun. **49**:775–781.
20. **Nakazato, H., M. Edmonds, and D. W. Kopp.** 1974. Differential metabolism of large and small poly(A) sequences in the heterogeneous nuclear RNA of HeLa Cells. Proc. Natl. Acad. Sci. U. S. A. **71**:200–204.
21. **Penman, S.** 1969. Preparation of purified nuclei and nucleoli from mammalian cells, p. 35–48. *In* K. Habel and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press Inc., New York.
22. **Reed, J., and E. Wintersberger.** 1973. Adenylic acid rich sequences in messenger RNA from yeast polysomes. FEBS Lett. **32**:213–217.
23. **Sebring, E. D., and N. P. Salzman.** 1964. An improved procedure for measuring the distribution of $^{32}PO_4^-$ among the nucleotides of ribonucleic acid. Anal. Biochem. **8**:126–129.
24. **Shatkin, A. J.** 1969. Colorimetric reactions for DNA, RNA, and protein determinations, p. 231–237. *In* K. Habel and N. Salzman (ed.), Fundamental techniques in virology. Academic Press, New York.
25. **Valenzuela, P., et al.** 1977. Ribosomal RNA genes of *Saccharomyces cerevisiae* II physical map and nucleotide sequence of the 5S ribosomal RNA gene and adjacent intergenic regions. J. Biol. Chem. **252**:8126–8135.