Detection, Sizing, and Quantitation of Polyadenylated Ribonucleic Acid in the Nanogram–Picogram Range†

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ABSTRACT: A method is described for using very high specific activity [^{3}H]poly(deoxythymidylate) [[^{3}H]poly(dT)] to detect, size, and quantitate subnanogram amounts of nonradioactive polyadenylated RNA. Short (~100 nucleotides long) [^{3}H]-poly(dT) is hybridized to the poly(adenylate) [poly(A)] tracts in polyadenylated RNAs. The RNA may then be sized and quantitated by sucrose gradient analysis. The addition of the small [^{3}H]poly(dT) molecules does not significantly alter the *s* values of RNAs. The amount of [^{3}H]poly(dT) hybridized to polyadenylated RNA increases linearly with the amount of RNA. A room temperature hydroxylapatite (HA) method has also been developed to detect and quantitate poly(A)-containing RNA after hybridization to radioactive poly(dT). S-1 nuclease (S-1) analysis can also be used to measure the poly(A) content of polyadenylated RNA to less than nanogram RNA amounts. For both the S-1 and HA approaches, the amount of [^{3}H]poly(dT) hybridized increases with the amount of RNA and the methods can detect to as little as 10^{-12} g of polyadenylated RNA with [^{3}H]poly(dT). Greater sensitivity is possible with higher specific activity poly(dT). The approaches presented here significantly extend the uses of radioactive homopolymers to detect, quantitate, and characterize RNAs containing complementary homopolymer tracts.

Poly(adenylate) [poly(A)][1] tracts in RNA may be detected by utilizing the ability of poly(A) to hybridize with poly(uridylate)[poly(U)] or poly(deoxythymidylate)[poly(dT)]. A number of approaches have been developed for the detection and quantitation of poly(A) which depend on annealing radioactive poly(U) or poly(dT) to the poly(A) tract (Gillespie et al., 1972; Fan & Baltimore, 1973; Kaufman & Gross, 1974; Sawin et al., 1977). The amount of poly(dT) or poly(U) hybridized to the RNA is then a measure of the poly(A) content of the RNA. A similar but slightly different poly(A) detection method employs the hybridization of oligo(deoxythymidylate)[oligo(dT)] to RNA fractions of a sucrose gradient. If poly(A) is present, an oligo(dT)–poly(A) complex is formed which may then be detected by monitoring cDNA synthesis by exogenously added reverse transcriptase (Strickland & Hellman, 1977). Another variation involves hybridizing iodinated poly(U) to RNA and then treating with bisulfite which deiodinates nonhybridized poly(U) but not poly(U) hybridized to poly(A) (Sawin et al., 1977). The above methods were also used to size the poly(A)-containing RNA by first sedimenting the RNA into a sucrose gradient and then detecting poly(A) in each gradient fraction by individually hybridizing aliquots of each fraction with radioactive poly(U) or poly(dT) (Rosbash & Ford, 1974; Sawin et al., 1977; Schlom et al., 1973). Radioactive homopolymers have also been used to detect homopolymer tracts present in higher organism DNAs (Shenkin & Burdon, 1972; Bishop et al., 1974).

The poly(A) detection sensitivity of the published methods ranges from 0.7 ng of AMV RNA detectable by a reverse transcriptase method (Strickland & Hellman, 1977) to 1–10 ng of various RNAs as assayed by hybridization with radioactive poly(U) or poly(dT) (Fan & Baltimore, 1973; Gillespie et al., 1972). None of the published methods demonstrated a proportional linear relationship between the amount of poly(A) present and the amount of radioactivity detected as being associated with the poly(A).

The data presented here provide a solid base from which to use [^{3}H]poly(dT) as a powerful tool for the detection, sizing, and quantitation of nanogram quantities of poly(A)-containing RNAs. Three methods [the sucrose gradient, HA, and S-1 nuclease (S-1) methods] are described which are useful for detecting and quantitating the reaction of [^{3}H]poly(dT) with poly(A). Calibration curves constructed for each method are linear over at least 100-fold concentration range. Each method is useful for a different aspect of detecting poly(A). The poly(A) detection sensitivity of these methods is very high. By use of [^{3}H]poly(dT) of 3.4×10^8 dpm per microgram specific radioactivity, the sensitivity of detection of poly(A)-containing RNA ranges from 1 to 50 pg depending on the RNA and the method of assay. The limit of detection of pure poly(A) is ~0.1 pg. The sucrose gradient assay for poly(A) has been greatly improved by hybridizing the [^{3}H]poly(dT) to the RNA before sedimenting the RNA into a sucrose gradient. The gradient can then be fractionated directly into scintillation vials and assayed immediately for radioactivity. The [^{3}H]poly(dT) attached to the RNA does not measurably alter the *s* value of the RNA. This method can be done with as little as 0.1–10 ng of RNA and greatly facilitates the characterization of extremely small amounts of poly(A)-containing RNAs.

Materials and Methods

Cells and Maintenance. Moloney murine leukemia virus (M-MuLV) infected 3T3 cells, clone 1, were a gift from M. Goulian. Feline leukemia virus (FeLV) (Theilen strain) infected feline lymphoblastoid cells, termed FL-74 (Theilen et al., 1969), were obtained from T. Kawakami. 3T3 and FL-74 cells were grown as reported elsewhere (Van Beveren & Goulian, 1979; Theilen et al., 1969).

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; PB, phosphate buffer; $NaDodSO_4$, sodium dodecyl sulfate; Sarcosyl, sodium *N*-lauroylsarcosinate; poly(dT), poly(deoxythymidylate); poly(U), poly(uridylate); poly(A), poly(adenylate); poly(C), poly(cytidylate); poly(G), poly(guanylate); EDTA, ethylenediaminetetraacetic acid; HA, hydroxylapatite.

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Isolation and Purification of RNA. Murine embryonic α-fetoprotein (α-FP) mRNA was the gift of T. Tamaoki, Semliki Forest Virus (SFV) [^{3}H]RNA was the gift of I. Kennedy, and encephalomyocarditis (EMC) virus RNA was the gift of D. Rowlands. Polio virus was grown and RNA isolated as described (Kacian & Myers, 1976). Avian myeloblastosis virus (AMV) was isolated from the serum of infected chickens as described by Myers et al. (1977).

Poly(A)-containing viral RNA was isolated from disrupted virus on oligo(dT)–cellulose (OTC) (Collaborative Research; T-3) by a modification of the method of Smith et al. (1976). Concentrated virus in 0.1 M NaCl, 10 mM Tris, pH 7.5, and 1 mM EDTA (TNE) was lysed on ice with 1% Sarcosyl (K and K Laboratories) in the presence of 200 μg/mL proteinase K (E. Merck) for 15 min. The lysate was transferred to 37 °C for 15 min and then cooled to room temperature. The lysate was made 6 M in NaSCN from a stock of 8 M NaSCN, shaken gently at room temperature for 5 min, and diluted to 1 M NaSCN with OTCB (0.5 M NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, and 1% Sarcosyl). The lysate was then dripped through a 3-mL bed of OTC in a sterile plastic pipet at a flow rate of 1 mL/min. The column was then washed with 30 mL of each of the following buffers: (1) 1 M NaSCN and OTCB; (2) 4 M NaCl and 10 mM Tris, pH 7.5; (3) OTCB; (4) OTCB without Sarcosyl. Poly(A)-containing RNA was eluted with sterile glass-distilled water in 0.5-mL aliquots. The concentration of the RNA was calculated from the absorbance at 260 nm (25 units = 1 mg of RNA). Nonradioactive RNA was aliquoted and frozen at −70 °C. Radioactive RNA was aliquoted and stored at 4 °C. RNA isolated in this manner routinely had an A_{260}/A_{280} absorbance ratio greater than 2.0.

Reverse Transcriptase Synthesis of [^{3}H]Poly(dT). AMV reverse transcriptase was supplied from the Logistics Program of NCI. [^{3}H]Poly(dT) was synthesized in a 50-μL reaction mix in 0.4-mL Eppendorf conical polypropylene tubes. Reagents were added in the following order at room temperature, with the exception of reverse transcriptase: 29.75 μL of water, 2 μL of 0.1 M sodium pyrophosphate, 4 μL of 1 mM [^{3}H]dTTP (50 Ci/mmol, 50 mCi/mL) (New England Nuclear), 2 μL of 1 M KCl, 4 μL of 0.1 M $MgCl_2$, 2.5 μL of 1 M Tris, pH 8.3, 2.5 μL of 0.1 M dithiothreitol,1 μL of 0.5% Nonidet P-40, 1.25 μL of 2 mg/mL poly(A)–oligo-$(dT)_{12-18}$ (Miles), and 1 μL (6127 units/mL) of reverse transcriptase. The reaction mixture was then briefly vortexed to mix and placed at 40 °C for 20 min. The reaction was stopped with 100 μL of 0.1 M EDTA, pH 7.5, and 2 μL of 20% $NaDodSO_4$ and shaken once with 10:90 phenol–chloroform. The aqueous layer was put over a 10-mL bed of Sephadex G-100 (Pharmacia) in TNE and 0.1% Sarcosyl. The excluded peak was made 30 μg with purified glycogen and 0.5 M in NaCl and precipitated in ethanol overnight at −20 °C. The precipitate was collected by centrifugation, suspended in 200 μL of water, made 0.4 M in NaOH, and incubated at 60 °C, 1 h. This mixture was neutralized by passage over a new Sephadex G-100 column as above. The eluting poly(dT) in the excluded volume was again ethanol precipitated, collected by centrifugation, and resuspended in a convenient volume of 0.01 M each of NaCl, Tris, pH 7.5, and EDTA, pH 7.5. The poly(dT) was stored at 4 °C.

The average length of the [^{3}H]poly(dT) as measured by denaturing gel electrophoresis was 150–170 bases. After 12 months of storage, the average length was 70 bases long (data not shown).

Hybridization Conditions. Samples of RNA for hybridization with [^{3}H]poly(dT) were standardly annealed in 0.5 M NaCl and 0.03% Sarcosyl at room temperature in 10–20 μL. Sarcosyl or $NaDodSO_4$ (0.01–0.03%) was found to inhibit nonspecific sticking of the poly(dT) to surfaces. MS-2 bacteriophage RNA does not anneal to poly(dT) (Gillespie et al., 1972) and may be used as a carrier if desired. Unless noted, [^{3}H]poly(dT) was present in the reactions in at least a five- to sixfold excess relative to the expected amount of poly(A) in the RNA. The measured $C_0t_{1/2}$ of the [^{3}H]poly(dT)–poly(A) reaction under these conditions was $\sim 1 \times 10^{-6}$ mol s L^{-1}. Hybridization times were typically 30 min unless otherwise specified. For layering on sucrose gradients, reactions were then made up to 100 μL with 0.5 M NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, and 0.03% Sarcosyl. Reactions were diluted to 2 mL with 0.1 M PB and 0.02% $NaDodSO_4$ for hydroxylapatite (HA) analysis. For S-1 nuclease analysis, the reactions were diluted to 1 mL with 0.3 M NaCl, 0.05 M NaOAc, pH 4.5, and 0.003 M $ZnSO_4$.

Sucrose Gradient Analysis. Sucrose solutions were in 0.1 M NaCl, 10 mM Tris, pH 7.5, and 1 mM EDTA. Linear 5–20% (w/v) gradients were poured and centrifuged in a Beckman SW50.1 rotor, 45 000 rpm, 20 °C, for various times depending on the size of the RNA. Gradients were stopped without breaking to minimize mixing. Sucrose was RNase free (Schwarz/Mann), and all stock buffers were autoclaved prior to use. Gradients were fractionated from below directly into scintillation vials. To each vial was added 1 mL of water and 6 mL of Aquasol-2 (New England Nuclear) prior to counting in a liquid scintillation counter. For purposes of quantitation, a peak is defined by all material between the lowest levels of [^{3}H]poly(dT) on either side of the peak.

Hydroxylapatite Analysis. RNA and [^{3}H]poly(dT) were hybridized at room temperature in 10–30 μL of 0.5 M NaCl or 1 M NaCl and 0.02% $NaDodSO_4$ for varying times. The reaction mixture was diluted with 1 mL of 0.1 M PB (phosphate buffer composed of equimolar amounts of NaH_2PO_4 and Na_2HPO_4) and 0.02% $NaDodSO_4$. The sample was passed rapidly over a bed of 0.5–1 mL of HA (HTP DNA Grade, Bio-Rad) equilibrated with 0.1 M PB and 0.02% $NaDodSO_4$ at room temperature. A 3-mL wash of the same buffer eluted the unbound [^{3}H]poly(dT). Bound poly(dT)–RNA complexes were eluted with 3-mL washes of 0.3 M PB. The HA was then dissolved in 2 mL of 6 N HCl, and all the fractions were assayed for radioactivity.

S-1 Nuclease Analysis. Reaction mixtures were diluted to 1 mL with S-1 nuclease buffer consisting of 0.3 mL of NaCl, 0.003 M $ZnSO_4$, and 0.05 M NaOAc, pH 4.5. To this was added 2 μg of *Escherichia coli* DNA and 50 units of S-1 nuclease (Calbiochem). The mixture was incubated at room temperature (20–24 °C) for 1 h and then assayed for acid-precipitable radioactivity. Both the acid-precipitable and acid-soluble fractions were collected and assayed for radioactivity.

Analysis of Hybridization Results. All quantitations of poly(A) results have been corrected for the self-reactive fraction of [^{3}H]poly(dT); the data presented have had the self-reactive fraction subtracted. One milligram of poly(A) represented 30 A_{260} units, and 1 mg of biological RNA represented 25 A_{260} units. The specific radioactivity of the [^{3}H]poly(dT) was 3.4×10^8 dpm/μg. Data presented as counts per minute represent a tritium counting efficiency of 36%.

Results

Conditions for Hybridizing [^{3}H]Poly(dT) to Poly(A) RNA. The salt concentration used for these reactions, 0.5 M NaCl, yielded close to optimum rates of hybridization at room tem-

Table I: Rates of Hybridization of [^{3}H]Poly(dT) with Poly(A)

	[poly(dT)]/[poly(A)]	[NaCl] (M)	$C_0t_{1/2}$ (mol s L^{-1}) (based on excess nucleic acid)
excess poly(A) + [^{3}H]poly(dT)	0.059	0.1	1×10^{-5}
	0.059	0.5	6×10^{-7}
	0.059	1	2×10^{-7}
excess [^{3}H]poly(dT) + 28 nucleotide long poly(A)	9	0.5	8×10^{-7}
excess [^{3}H]poly(dT) + poly(A) greater than 280 nucleotides	9	0.5	1×10^{-6}
excess poly(dT) + polio RNA	10[a]	0.5	1×10^{-6}

[a] Poly(dT) excess was calculated over the amount of poly(A) in polio RNA. Hybridizations were performed and analyzed as described under Materials and Methods.

Table II: Characterization of [^{3}H]Poly(dT)[a]

[^{3}H]-poly(dT) prepn	assay method	% reaction with excess poly(A)	% reaction with an excess of other RNAs	% reaction when no RNA is added
1	HA	84		
1	HA		polio 84	
1	HA		MS-2 2.4	
1	HA			2.3
2	HA	91.6		
2	HA			3.6
3	HA	98		
3	HA			1.3
3	S-1	92		
3	S-1			2.3

[a] Newly made preparations 1, 2, and 3 of [^{3}H]poly(dT) were sized respectively at 4 S in alkaline sucrose. Reactions were assayed by HA or S-1 nuclease as described under Materials and Methods. [^{3}H]Poly(dT) preparation 3 was self-reacted and passed twice over HA in 0.1 M PB at room temperature in order to reduce the amount of apparent self-reaction. Before passage over the HA, 6–7% self-reaction was observed.

perature as measured by hydroxylapatite. Data regarding rates of hybridization of poly(dT) to poly(A) are summarized in Table I.

The [^{3}H]poly(dT) is used to detect the presence of a small amount of poly(A) attached to a large complex RNA molecule. The kinetics of reaction of [^{3}H]poly(dT) with the poly(A) of such an RNA molecule was examined by measuring the rate of hybridization of [^{3}H]poly(dT) with polio type 1 RNA under conditions of a 10-fold excess of [^{3}H]poly(dT) relative to the amount of poly(A) present in the polio virus RNA. Polio virus contains ~0.6–1% poly(A). This is equivalent to a sequence consisting of ~50–85 adenine residues/average molecule (Yogo & Wimmer, 1972; Hruby & Roberts, 1976). In 0.5 M NaCl, the [^{3}H]poly(dT) $C_0t_{1/2}$ [based on poly(A) content] of this reaction is $\sim 1 \times 10^{-6}$ mol s L^{-1} or about the same as that seen for excess poly(dT) and poly(A) (Table I).

Characterization of [^{3}H]Poly(dT). [^{3}H]Poly(dT) produced as described under Material and Methods was examined for its ability to react with poly(A), its ability to react with non-poly(A)-containing RNA, its self-hybridization characteristics, its ability to bind hydroxylapatite immediately after being denatured, and its size by alkaline sucrose sedimentation analysis. Table II summarizes these data. Different [^{3}H]-poly(dT) preparations self-reacted to an extent of 2–3% and

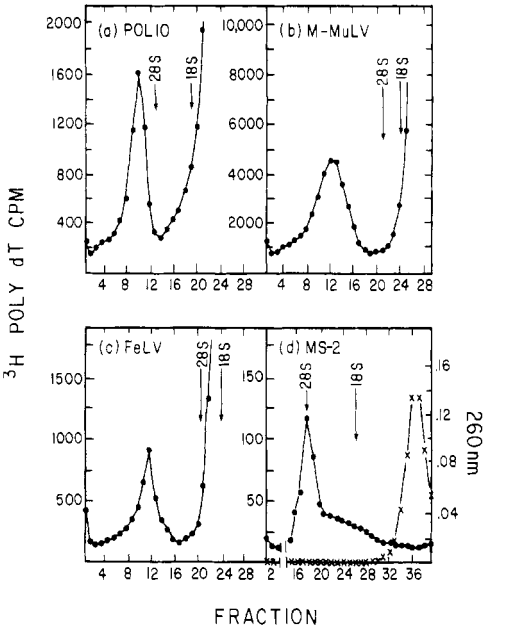

FIGURE 1: Detection of different RNAs with [^{3}H]poly(dT). (a) 6 ng of polio virus RNA was annealed with 1.73 ng of [^{3}H]poly(dT) in 20 μL of 0.5 M HCl and 0.03% Sarcosyl at room temperature, 20 min. The reaction mixture was made 100 μL with 0.5 M NaCl, 0.03% Sarcosyl, 10 mM Tris, pH 7.5, and 1 mM EDTA and layered onto a 5–20% (w/v) linear sucrose gradient made in 0.1 M NaCl, 10 mM Tris, pH 7.5, and 1 mM EDTA. Centrifugation was in a Beckman L5-50 ultracentrifuge with the Beckman SW50.1 rotor: 45000, 100 min, 20 °C, slow acceleration, and no brake. Gradients were fractionated from below directly into scintillation vials to which were added 1 mL of water and 6 mL of ACS (Fisher). Vials were counted in a liquid scintillation counter. (b) 10.5 ng of M-MuLV RNA was annealed with 1.73 ng of [^{3}H]poly(dT), centrifuged, and assayed as in (a) except that centrifugation was for 45 min. (c) 1.9 ng of FeLV RNA was annealed with 1.73 ng of [^{3}H]poly(dT) and treated as in (b). (d) 22 μg of MS-2 RNA was hybridized with 0.35 ng of [^{3}H]poly(dT) in 100 μL of 0.5 M NaCl and 0.03 Sarcosyl for 20 min at room temperature. The reaction mixture was centrifuged as in (a). The gradient was fractionated from below, 1 mL of water was added to each fraction, and the absorbance at 260 nm was read for the fractions. 6 mL of Beta Phase (WestChem Chemical Co.) was then added to each sample,, and the vials were counted in a liquid scintillation counter: (●) A_{260}; (×) [^{3}H]poly(dT) cpm.

reacted to greater than 80% with an excess of poly(A) and polio RNA. No significant reaction of the [^{3}H]poly(dT) with an excess of RNA from the bacteriophage MS-2 could be detected.

Sizing of Poly(A)-Containing RNA: Sucrose Gradient Analysis. The hybridization of [^{3}H]poly(dT) with the poly(A) portion of nonradioactive RNA permits the detection of nanogram amounts of these RNAs. The size of these poly(A)-containing RNAs labeled with [^{3}H]poly(dT) can be determined by sucrose gradient analysis. Figure 1 shows the result of one typical experiment detecting poly(A)-containing RNA with [^{3}H]poly(dT). Polio virus RNA, FeLV RNA, and M-MuLV RNA were hybridized to [^{3}H]poly(dT) in separate reactions and layered onto 5–20% sucrose gradients. Each RNA sedimented at its expected *s* value. Less than 20 ng of RNA was used for each of these experiments, an amount undetectable by standard spectrophotometric means. More than 20 μg of MS-2 bacteriophage RNA which contains no poly(A) was also reacted with [^{3}H]poly(dT) and analyzed both spectrophotometrically and as described above. The [^{3}H]-poly(dT) did not exhibit any binding to the MS-2 RNA which was detected in the gradient by its absorbance at 260 nm.

It was expected that the amount of [^{3}H]poly(dT) actually hybridized to the poly(A)-containing RNA would not be

Table III: Comparison of [^{3}H]Poly(dT)-Determined and Published s Values of RNAs[a]

RNA	reported value (S)	[^{3}H]-poly(dT)-determined value (S)	internally labeled value (S)
M-MuLV	60-70[b]	60	60
FeLV	58[c]	58-60	58-60
AMV	65[d]	58-63	ND[e]
SFV	45[f]	46	46
polio	35[g]	33-34	ND
α-FP mRNA	18[h]	18	ND

[a] [^{3}H]Poly(dT)-determined and internally labeled s values are relative to 18 and 28S rRNA markers. [b] Lai & Duesberg (1972). [c] Brian et al. (1975). [d] Erickson (1969). [e] ND = not determined. [f] Sonnabend et al. (1967). [g] Bishop et al. (1965). [h] Tamaoki et al. (1976).

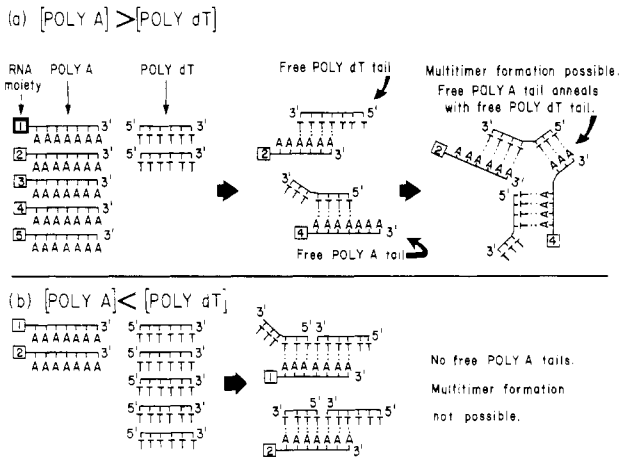

FIGURE 2: Hypothetical interaction of poly(A) and poly(dT). Hybridization of poly(A) with poly(dT) in (a) poly(A) excess and poly(dT) excess.

enough to markedly change the s value of the RNA. Smaller RNAs would be expected to reflect more change in s value. The smallest poly(A)-containing RNA checked was murine α-fetoprotein (α-FP) mRNA. When newly isolated, this RNA was sized at 18 S by polyacrylamide gel electrophoresis (Tamaoki et al., 1976). This α-FP mRNA also contains the largest fraction of poly(A), ~5%. This mRNA was repeatedly sized at 18–18.5 S with [^{3}H]poly(dT), indicating that saturation of as much as 5% of the total molecule with [^{3}H]-poly(dT) does not appreciably change the s value. A summary of [^{3}H]poly(dT)-determined s values for a variety of poly-(A)-containing RNAs is presented in Table III. These data show a good correlation between [^{3}H]poly(dT)-determined sizes and those reported by others using conventional methods.

When using the [^{3}H]poly(dT) method to size and quantitate poly(A)-containing RNA, it is important to hybridize the RNA to an excess of [^{3}H]poly(dT), relative to the amount of poly(A) present in the RNA. Good results are obtained with a 6–10-fold excess of poly(dT). When the amount of polio poly(A) was in excess of the [^{3}H]poly(dT), some of the polio RNA sedimented more rapidly than the main peak. Most of the polio RNA is present in the main monomer peak which has the s value expected for intact polio RNA. However, additional peaks which appear to be dimers and trimers of polio RNA are also seen (data not shown).

The reason for these multimer peaks may be explained by a scheme such as that presented in Figure 2a. This figure portrays a case of poly(A) excess relative to poly(dT). In this case, poly(dT) does not saturate all the poly(A) available and,

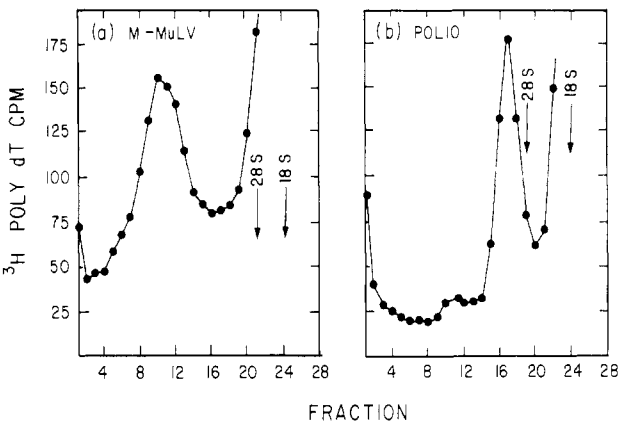

FIGURE 3: Sedimentation profiles of subnanogram amounts of [^{3}H]poly(dT)-labeled RNAs. (a) 0.3 ng of M-MuLV RNA were annealed to 1.73 ng of [^{3}H]poly(dT), sedimented, and assayed as described for Figure 1b. (b) 0.5 ng of polio virus RNA was annealed to 0.26 ng of [^{3}H]poly(dT) in 20 μL of 0.5 M NaCl at room temperature, 30 min and then layered onto a 5–20% (w/v) linear sucrose gradient and centrifuged in the SW50.1 rotor: 45 000 rpm, 85 min, and 5 °C. The gradient was assayed as in Figure 1a.

consequently, poly(A) tails may exist. These may hybridize with poly(dT) already partially hybridized to the poly(A) region of a different molecule of RNA, with the result that a molecule of poly(dT) links two or more RNA molecules to form multimers. Poly(dT) at an excess will completely saturate available poly(A), leaving no free, unhybridized poly(A) tails. Figure 2b presents a schematic view of the poly(dT) excess situation. Sucrose gradient analysis at high salt concentration and at low temperature may also encourage the formation of multimer peaks and also enhance the aggregation of RNA (Parrish, 1972).

The [^{3}H]poly(dT) can be used to detect subnanogram quantities of poly(A)-containing RNA. Figure 3 presents the sedimentation profiles obtained by using 0.3 ng of M-MuLV RNA and 0.5 ng of polio RNA. The high molecular weight RNA peaks are readily apparent. The polio RNA peak contains ~500–600 cpm while the M-MuLV peak has ~1000 cpm. The peak regions could be further separated from the nonhybridized [^{3}H]poly(dT) by using a longer gradient.

It is at times useful to eliminate the nonhybridized [^{3}H]-poly(dT) before analyzing the sample on a sucrose gradient. This can be accomplished by first passing the sample over HA (Materials and Methods) in 0.1 M PB and 0.02% $NaDodSO_4$. Under these conditions, the single-strand [^{3}H]poly(dT) will pass through the column while the [^{3}H]poly(dT) hybridized to the RNA will adsorb to the HA. The adsorbed fraction can be recovered by high salt elution and analyzed on a sucrose gradient.

[^{3}H]Poly(dT) may also be used to detect denatured RNA. Glyoxal denatures RNA and DNA by forming adducts with the guanine residues, preventing G-C base pair formation and thereby effectively denaturing the molecules (McMaster & Carmichael, 1977). [^{3}H]Poly(dT) may be used in conjunction with this method to label denatured polyadenylated RNA because glyoxylation does not affect the poly(A) sequence of the RNA (data not shown).

Breakdown of a particular RNA preparation may also be rapidly assessed by using [^{3}H]poly(dT) by observing the profile of radioactivity on a sucrose gradient. One preparation of polio RNA which yielded 18S (alkaline) cDNA when transcribed with reverse transcriptase using oligo(dT)$_{12-18}$ as the primer (Kacian & Myers, 1976) was shown to be 34 S in sucrose by using [^{3}H]poly(dT) (data not shown). A different preparation

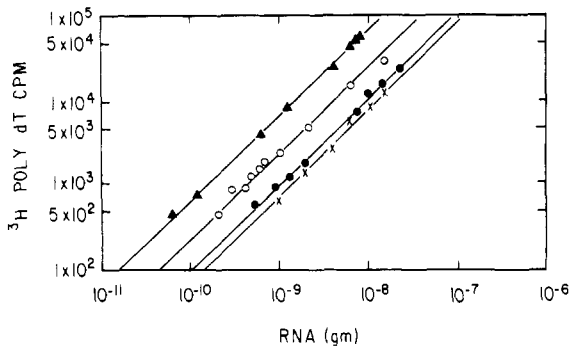

FIGURE 4: Standard curves for quantitation of specific [^{3}H]poly-(dT)-labeled RNAs. The amount of [^{3}H]poly(dT) radioactivity in the gradient peak is plotted vs. the amount of RNA in the specific reaction: (▲) α-FP mRNA; (○) M-MuLV RNA; (●) FeLV RNA; (×) polio virus RNA.

which yielded 4–5S cDNA when transcribed in the same manner by reverse transcriptase showed no detectable RNA larger than the [^{3}H]poly(dT) itself. This RNA was not heated before reacting with the [^{3}H]poly(dT). The absence of any peak indicated that the RNA was badly degraded.

The poly(dT) method may also be used to determine whether a poly(A)-containing RNA is completely intact; that is, none of its phosphodiester bonds have been broken. A situation often occurs where only a few random nicks (i.e., breaking of phosphodiester bonds) of the RNA have occurred. Unless denatured, this RNA will remain intact and will give the proper *s* value when sized with [^{3}H]poly(dT). The nicks can be detected, however, by first denaturing the RNA and then reacting it with the [^{3}H]poly(dT) and sizing it. The resulting sedimentation profile will show RNA with a lower *s* value than expected.

Quantitation of RNA Containing Poly(A) by [^{3}H]Poly(dT) Hybridization. If the proper calibration curves are available, the amount of [^{3}H]poly(dT) present in a particular sucrose gradient peak can serve as a measure of the quantity of total RNA present in that peak. Construction of such a calibration curve involves determining how much [^{3}H]poly(dT) will hybridize with differing amounts of a particular poly(A)-containing RNA. Figure 4 presents such a calibration curve for a variety of poly(A)-containing RNAs. Varying amounts of each RNA were hybridized to an excess of [^{3}H]poly(dT) relative to the poly(A) present in the RNA. The reaction mixture was then subjected to sucrose gradient analysis, and the amount of [^{3}H]poly(dT) (counts per minute) in each peak was determined. The radioactivity in the peaks was plotted against the respective RNA concentrations. The results of such calibrations are depicted in Figure 4.

It is apparent that there is an essentially linear relationship between the amount of RNA in the reaction and the amount of [^{3}H]poly(dT) hybridized to it. This relationship remains linear over a range where amounts of RNA differ by 100-fold. It is also clear that subnanogram amounts of poly(A)-containing RNA can be quantitatively assayed by this method (see also Figure 3). The results presented in Figure 4 show an internal consistency which speaks well for the accuracy and reproducibility of the method. The slope of each of the lines is equal to ~1.02. This indicates that an increase in the quantity of a specific poly(A)-containing RNA will result in an essentially proportional increase in the quantity of [^{3}H]-poly(dT) hybridized to the RNA.

Hydroxylapatite and S-1 Nuclease Methods of Quantitating RNA Containing Poly(A). The hydroxylapatite (HA) and S-1 methods of detecting poly(A)–[^{3}H]poly(dT) hybrid-

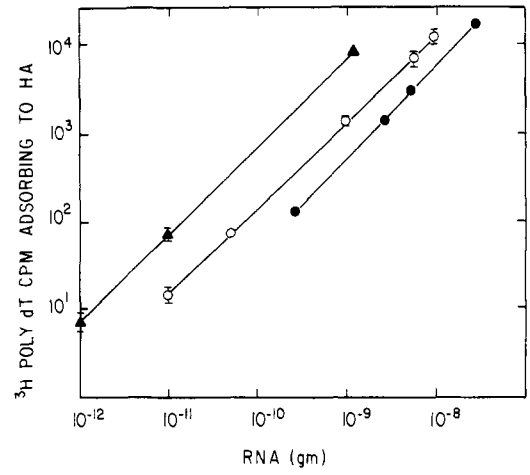

FIGURE 5: HA and S-1 nuclease standard curves for quantitation of polio and α-FP RNAs. The cpm at each point has been corrected for the amount of self-reaction of the [^{3}H]poly(dT). Assays were performed as described under Materials and Methods: (▲) α-FP mRNA assayed by HA; (○) polio RNA assayed by HA; (●) polio RNA assayed by S-1 nuclease.

ization add a useful dimension to the quantitation of poly-(A)-containing RNAs. These methods can be carried out more rapidly than sucrose gradients and have a much greater potential sensitivity.

Figure 5 shows a calibration curve constructed by hybridizing different amounts of α-FP and polio RNA to excess [^{3}H]poly(dT) and then analyzing the reaction mixtures by the HA method. The amount of [^{3}H]poly(dT) adsorbed to the HA is then plotted vs. the respective RNA concentration. These data show that there is an essentially linear relationship between the quantity of RNA and the amount of radioactive poly(dT) adsorbing to the HA. This linearity extends over a range where amounts of RNA differ by 1000-fold. The slope of the polio and α-FP lines is 0.98. These slopes indicate that an increase in the quantity of RNA will result in a proportional increase in the amount of radioactivity hybridized to the RNA.

It is apparent that the HA method is much more sensitive in detecting small amounts of poly(A)-containing RNA than the sucrose gradient approach. The most striking case is shown by the α-FP standard curve which ranges from 1 pg to 1 ng. In this case a total of 4×10^{-13} g of [^{3}H]poly(dT) (~40 cpm) was reacted in 0.01 mL of 1 M NaCl with 1×10^{-12} g of α-FP mRNA which contains $\sim 5 \times 10^{-14}$ g of poly(A). Because the concentration of [^{3}H]poly(dT) was so low, the time of incubation at room temperature was increased to 7 h. About 20% of the input counts per minute adsorbed to the HA in these experiments. This is equivalent to ~7–8 cpm. The 2σ background of the counting system used was 16 ± 0.9 cpm (four different background vials, each counted for 45 min and the data averaged). The data point presented in Figure 5 for 1 pg of α-FP RNA is an average of four different determinations. Each of the four separate determinations for the 1-pg point was counted for 65 min. The individual counts per minute values ranged from 21.9 cpm (1423 total counts) to 24.5 cpm (1593 total counts). After the background (16 ± 0.9 cpm) was subtracted, these samples had 2σ standard deviations counts per minute values of 5.9 ± 1.2 and 8.5 ± 1.2 cpm. The average 2σ standard deviation value was 7 ± 1.2 cpm for the four determinations. A similar analysis was done for the 0.01-ng values for the polio RNA. The data point presented in Figure 5 for 0.01 ng of polio RNA is an average of six separate determinations. The range of values obtained for average points is shown by the solid lines above and below each point.

Table IV: Quantitation of Poly(A) by HA and S-1 Nuclease[a]

RNA	assay method	large [^{3}H]poly(dT) [ng of [^{3}H]-poly(dT) per ng of RNA in hybrid]	small [^{3}H]poly(dT) [ng of [^{3}H]-poly(dT) per ng of RNA in hybrid]
long poly(A)	S-1	0.88	0.93
(greater than	S-1	1.0	
280 nucleotides)	HA	2.1	1.6
	HA	1.8	
	HA	1.9	
28 nucleotide	S-1	0.99	0.84
long poly(A)	S-1	0.98	
	HA	2.6	1.6
	HA	2.4	
	HA	2.4	
polio type 2	S-1	0.006	
	S-1	0.006	
	HA	0.012	0.0085
	HA	0.013	0.011
	HA	0.013	

[a] The HA and S-1 nuclease methods are described under Materials and Methods. Large [^{3}H]poly(dT) (preparation no. 3 in Table I) reacted with an excess of poly(A) and assayed by HA and S-1 nuclease gave respectively 98 and 93.7% hybridization. Large [^{3}H]poly(dT) reacted by itself and assayed by HA and S-1 nuclease gave respectively 1.3 and 2.4% hybridization. Small [^{3}H]-poly(dT) was the result of preparation no. 2 (Table I) aging. Small [^{3}H]poly(dT) reacted with an excess of poly(A) and assayed by HA and S-1 nuclease gave 40 and 26% hybridization, respectively. The self-reaction of the small [^{3}H]poly(dT) was 3.6 and 5.0% as measured by HA andS-1 nuclease, respectively. All reactions with large [^{3}H]poly(dT) were done at [^{3}H]poly(dT)/poly(A) ratios of 4–7:1. Reactions with the small [^{3}H]poly(dT) were done at [^{3}H]poly(dT)/poly(A) ratios of 12–15:1. The large and small [^{3}H]poly(dT) molecules were respectively 150–200 and 30–40 nucleotides as measured by alkaline sucrose sedimentation.

Figure 5 also presents a polio RNA calibration curve done by assaying with S-1 nuclease in a manner similar to that described by Fan & Baltimore (1973). There exists an essentially linear relationship between the amount of RNA and the quantity of [^{3}H]poly(dT) hybridized to the RNA. The slope of the S-1 nuclease line in Figure 5 is 1.03. The sensitivity of poly(A) detection of this method should be similar to that of the HA method.

Quantitation of Poly(A) Present in RNA. The poly(A) present in an RNA preparation can be quantitated by saturating the poly(A) with [^{3}H]poly(dT) and then determining the amount of poly(dT) hybridized to the RNA. The most accurate quantitation values are obtained by the S-1 nuclease method. Table IV compares the S-1 nuclease and HA methods. Known amounts of short (28 nucleotides long on average) and long (greater than 300 nucleotides long) poly(A) were reacted with an excess of large (150–200 nucleotides long) or small (30–40 nucleotides long) [^{3}H]poly(dT). These mixtures were then assayed by S-1 nuclease and HA. The mass of [^{3}H]poly(dT) hybridized was calculated from the specific radioactivity of the [^{3}H]poly(dT), and the mass ratio of [^{3}H]poly(dT) to poly(A) in the poly(A)–poly(dT) hybrid was determined. The ratios obtained with the S-1 nuclease method were close to the ideal value of 1. These ratios ranged from 0.84 to 1. In contrast, the ratios obtained with the HA method ranged from 1.6 to 2.6. This high ratio indicates that extensive regions of single-strand poly(dT) are present in the poly(A)-poly(dT) hybrid. These regions are destroyed in the S-1 nuclease assay.

It is clear that the S-1 nuclease method is the preferred method for quantitating poly(A). Table IV also presents the [^{3}H]poly(dT)/RNA ratios for poly(A)–poly(dT) hybrids formed when [^{3}H]poly(dT) was reacted with polio type 1 RNA. The S-1 nuclease data indicate that 0.6% of the polio RNA is poly(A). The HA method yields about twice this value. If it is assumed that all of the poly(A) present in this RNA preparation is attached to the polio RNA, it can be calculated that each polio RNA contains on the average 45–50 A residues. This value is comparable to those obtained by other workers for picornavirus RNA (Yogo & Wimmer, 1972; Hruby & Roberts, 1976).

Discussion

The specific radioactivity of the [^{3}H]poly(dT) limits the sensitivity of detection of free poly(A) and poly(A)-containing RNA. The [^{3}H]poly(dT) used here had a specific radioactivity of 3.4×10^8 dpm/μg. At a 36% counting efficiency, this corresponds to $\sim 1 \times 10^8$ cpm/μg. Higher specific radioactivity poly(dT) can be produced by synthesizing the poly(dT) with [^{32}P]dTTP or by ^{32}P end labeling poly(dT) with polynucleotide kinase (Richardson, 1971). Radioactive poly(dT) of a defined average size can also be produced by including an appropriate quantity of dideoxythymidine triphosphate in the poly(dT) synthesis mixture.

The [^{3}H]poly(dT) is useable for a period of 6–12 months; the chain length becomes shorter with time. Even [^{3}H]poly-(dT) 30–40 nucleotides long on average can be used to detect and quantitate poly(A), although when tested only ~40% of it hybridizes to an excess of poly(A). This small [^{3}H]poly(dT) has an advantage in that it does not promote multimer formation when reacted with an excess of polio RNA (data not shown). If quantitating RNA with such small [^{3}H]poly(dT), it is important to use at least a sixfold excess of the reactable poly(dT). A 6-fold excess of reactable small poly(dT) is equivalent to a 15-fold excess of the total population of small poly(dT).

The hybridization of [^{3}H]poly(dT) to poly(A) occurs very rapidly at room temperature in 0.5 M NaCl even at very low concentrations. The RNA attached to the poly(A) tract does not appear to greatly affect the rate of reaction of poly(dT) with the poly(A). Similar rates of hybridization are seen for the reaction of an excess of poly(dT) with long ($C_0t_{1/2} = 1 \times 10^{-6}$ mol s L^{-1}) and short ($C_0t_{1/2} = 8 \times 10^{-7}$ mol s L^{-1}) poly(A) (Table I). The kinetics are approximately pseudo first order in form. The poly(A) attached to polio RNA reacts at about the same rate ($C_0t_{1/2} = 1 \times 10^{-6}$ mol s L^{-1}; this assumes a polio RNA content of 0.6%).

The hybridization of radioactive homopolymers has been used by others (Rosbash & Ford, 1974; Sawin et al., 1977) to size poly(A)-containing RNA by first sedimenting the RNA into a sucrose gradient, then fractionating the gradient, and then hybridizing each fraction to the homopolymer. The sucrose method described here is much easier since it involves only one hybridization reaction. Since the single-strand [^{3}H]poly(dT) is small, nonhybridized [^{3}H]poly(dT) does not migrate far into the gradient while the hybridized [^{3}H]poly-(dT) sediments with the RNA to which it is attached.

Very small quantities of RNA can be sized and quantitated by using this procedure. A 0.3-ng amount of sedimented polio RNA resulted in a clearly visible peak containing ~500–600 cpm. Polio contains ~0.6–1% of its RNA as poly(A). With this same design, a peak containing 100–200 cpm is readily detectable. This would be equivalent to detecting ~0.05–0.1 ng of polio RNA. A key to detecting even smaller amounts by this method is the ability to separate the RNA peak from the unreacted [^{3}H]poly(dT) in order to have no overlap between these two classes. This can be done in several ways.

One may use a longer tube for the gradient analysis, or the hybridized [^{3}H]poly(dT)–RNA may be separated from the nonhybridized [^{3}H]poly(dT) before the gradient analysis with HA. Higher specific radioactivity poly(dT) may also be used. A 10-fold increase would result in a 10-fold greater detection sensitivity.

The sucrose gradient, HA, and S-1 nuclease methods can all be used to quantitate poly(A)-containing RNA. Standard curves constructed using purified RNAs are shown in Figures 4 and 5. The slopes of these curves demonstrate that, over a 100–1000-fold range in RNA concentration, the quantity of [^{3}H]poly(dT) hybridizing to an RNA increases proportionally with the amount of RNA. The linear and proportional character of these curves show that the [^{3}H]poly(dT)–poly(A) hybridization reaction is behaving according to theory.

A standardization curve should be constructed for each preparation of [^{3}H]poly(dT) and stock of purified RNA. When sucrose gradient or HA assays are used, the amount of [^{3}H]poly(dT) hybridized to a particular RNA can vary, depending on the size of the [^{3}H]poly(dT) and the integrity of the RNA. Table IV presents data regarding the effect of [^{3}H]poly(dT) molecular weight on the amount of poly(dT) hybridized to polio RNA. Large [^{3}H]poly(dT) (~150–200 nucleotides long) and short [^{3}H]poly(dT) (~30–40 nucleotides long) were hybridized to large poly(A) (greater than 300 nucleotides long) and small poly(A) (average of 28 nucleotides long). Since some poly(A)-containing RNAs have longer poly(A) tracts than others, this study was designed to find out whether the size of the poly(dT) or the poly(A) would greatly influence the values obtained in poly(A) quantitation. The HA data in Table IV show that the [^{3}H]poly(dT)–poly(A) hybrids contain 1.6–2.6 times more [^{3}H]poly(dT) than poly(A). The ratio is higher for the large [^{3}H]poly(dT)–poly(A) combinations and lowest for the small [^{3}H]poly(dT) combinations. Therefore, small [^{3}H]poly(dT) will give fewer counts per minute per nanogram of RNA than will larger poly(dT) when measured by HA or by sucrose gradient analysis.

When the S-1 nuclease assay is used, the poly(dT)–poly(A) ratio is not greatly affected by the poly(A) size or the [^{3}H]-poly(dT) size. The nuclease will remove any single-strand [^{3}H]poly(dT) in the hybrid and may well destroy one strand of a two poly(dT)–one poly(A) triple-strand hybrid; RNase destroys one poly(U) strand of a two poly(U)–one poly(A) hybrid (Bishop et al., 1974). The poly(dT)–poly(A) ratios obtained with the S-1 nuclease method are close to 1 and range from 0.84 to 1.0. This indicates that S-1 nuclease is the method of choice for precise quantitation of poly(A).

The S-1 nuclease and HA methods provide a measure of the total amount of poly(A) present in an RNA preparation while the sucrose gradient method measures the poly(A) present in a particular size class of RNA. The sucrose gradient and HA methods should therefore give comparable counts per minute per nanogram of RNA values if all of the poly(A) present in an RNA preparation is attached to RNA of the proper size class. A lower sucrose gradient value may indicate that breakdown of the RNA has occurred. This situation should also be reflected in the sedimentation profile of the [^{3}H]poly(dT)-labeled RNA. A lower value could also indicate the presence of free poly(A) or single-strand DNA. Higher organism DNAs contain ~0.5% poly(dT)–poly(dA) sequences (Shenkin & Burdon, 1972; Bishop et al., 1974). Care should be taken to remove DNA before assaying for poly(A).

The techniques presented here should prove useful for situations in which it is desirable to characterize much less than microgram quantities of polyadenylated RNA. We have routinely used the sucrose gradient method to size nanogram amounts of polyadenylated RNAs prior to reverse transcription and in vitro translation, as well as specific polyadenylated RNAs annealed to [^{3}H]poly(dT) as sedimentation markers in sucrose gradients. This labeling method has also been used to size α-FP mRNA on neutral polyacrylamide gels and should be applicable to a wide range of gel techniques. In addition to characterizing purified RNA, we have developed a method for reacting [^{3}H]poly(dT) with detergent-disrupted retroviral particles in order to quantitate and detect the presence of retrovirus RNA and viral cores. This method can detect the presence of low levels of virus in tissue culture media. The HA method is routinely used to obtain a rapid estimate of poly(adenylate) content of purified RNA. Other radioactive homopolymers can be used in a similar manner and will be useful in specific situations. Gillespie et al. (1972) reported the use of [^{3}H]poly(C) to detect poly(guanylate) tracts present in polio virus RNA. We have used [^{3}H]poly(G) to size EMC RNA (data not shown) which possesses an internal poly(cytidylate) tract.

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References

Bishop, J. M., Summers, D. F., & Levintow, L. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 1273.

Bishop, J. O., Rosbash, M., & Evans, D. (1974) *J. Mol. Biol. 85*, 75.

Brian, D. A., Thomason, A. R., Rottman, F. M., & Velicer, L. F. (1975) *J. Virol. 16*, 535.

Erickson, R. L. (1969) *Virology 37*, 124.

Fan, H., & Baltimore, D. (1973) *J. Mol. Biol. 80*, 93.

Gillespie, D., Marshall, S., & Gallo, R. C. (1972) *Nature (London) 236*, 227.

Hruby, D. E., & Roberts, W. K. (1976) *J. Virol. 19*, 325.

Kacian, D., & Myers, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2191.

Kaufman, S. J., & Gross, K. W. (1974) *Biochim. Biophys. Acta 353*, 133.

Lai, M. M. C., & Duesberg, P. H. (1972) *Nature (London) 235*, 383.

McMaster, G., & Carmichael, G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4835.

Myers, J., Spiegelman, S., & Kacian, D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2191.

Parrish, J. H. (1972) *Principles and Practice of Experiments with Nucleic Acids*, p 139, Longman Group Limited, London.

Richardson, C. C (1971) *Proced. Nucleic Acid Res. 2*, 815.

Rosbash, M., & Ford, P. J. (1974) *J. Mol. Biol. 85*, 87.

Sawin, V., Greenman, B., & Scherberg, N. (1977) *Anal. Biochem. 82*, 141.

Schlom, J., Colcher, D., Spiegelman, S., Gillespie, S., & Gillespie, D. (1973) *Science (Washington, D.C.) 179*, 696.

Shenkin, A., & Burdon, R. H. (1972) *FEBS Lett. 22*, 157.

Smith, R. G., Donehower, L., Gallo, R. C., & Gillespie, D. H. (1976) *J. Virol. 17*, 287.

Sonnabend, J. A., Martin, E. M., & Mecs, E. (1967) *Nature (London) 213*, 365.

Strickland, J. E., & Hellman, A. (1977) *Anal. Biochem. 82*, 342.
Tamaoki, T., Miura, K., Lin, T., & Banks, P. (1976) *Onco-Developmental Gene Expression* (Fishman, W. H., & Sell, S., Eds.) pp 115–122, Acadamic Press, New York.
Theilen, G. H., Kawakami, T. G., Rush, J. D., & Munn, R. J. (1969) *Nature (London) 222*, 589.
Van Beveren, C., & Goulian, M. (1979) *Cancer Res. 39*, 2532.
Yogo, Y., & Wimmer, E. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1877.

Multistep Mechanism of Codon Recognition by Transfer Ribonucleic Acid†

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ABSTRACT: The mechanism of codon recognition by tRNA is investigated in the system $tRNA^{Phe}$ + UUC by temperature-jump measurements using the Wye base fluorescence as a label. In 0.4 M Na^+ and 5 mM Mg^{2+} a two-step reaction is observed and described quantitatively; UUC is shown to bind preferentially to one of two conformations on the anticodon loop. In 0.1 M Na^+ and 10 mM Mg^{2+} an additional relaxation effect is observed, which indicates a codon-induced conformation change leading to an association of tRNA molecules. The codon-induced tRNA association is demonstrated independently by equilibrium sedimentation. The present results suggest a more active role of tRNA during translation than anticipated.

The main step in the translation of a genetic message is the recognition of codons by adaptor molecules, the tRNAs. Compared to the codons, the adaptors are large and complex structures consisting of ~80 nucleotide residues and yet using only 3 of them for *direct* interaction with the codon. There are several reasons for the rather high complexity of tRNA molecules. tRNAs have to provide recognition sites during numerous biological activities in which they participate (Clark, 1977). The following question arises, however. (1) Is tRNA a passive structure, which exposes a variety of sites for interaction with different components of the cell, or (2) does tRNA participate more actively in the decoding process? In the second case the whole tRNA structure could modulate the interaction between codon and anticodon triplets and, conversely, the interaction between codon and anticodon could trigger some response in the remote parts of the molecule.

In recent years much conflicting evidence has been presented concerning different conformations of tRNAs and their function during translation. Two structural transitions are mainly under discussion.

The anticodon loop of tRNA may convert from the 3′-stacked conformation, found in crystals [for review, cf. Rich & RajBhandary (1976) and Clark (1977)], to the 5′-stacked conformation. This transition, proposed by Fuller & Hodgson (1967), may be important during protein biosynthesis (Woese, 1970). Recently, a relaxation process observed in $tRNA^{Phe}$ has been assigned to the conversion between the 3′- and 5′-stacked conformations (Urbanke & Maass, 1978).

The second transition is the unfolding of the TΨC loop induced by the codon–anticodon interaction (Ofengang & Henes, 1969; Shimuzu et al., 1969). The TΨC loop is usually hidden due to tertiary interactions (Rich & RajBhandary, 1976). According to oligonucleotide binding data, the TΨCG sequence becomes exposed upon complex formation between codon and anticodon (Möller et al., 1979). Evidence for a codon-induced rearrangement of tRNA structure has also been obtained from chemical modification experiments (Wagner & Garrett, 1979). Other measurements using different techniques did not give any evidence for codon-induced unfolding of tRNA (Yoon et al., 1975; Geerdes et al., 1978; Grosjean et al., 1976; Davanloo et al., 1979; Geerdes, 1979).

In the present investigation the binding of UUC to its cognate $tRNA^{Phe}$ is studied by temperature-jump spectroscopy and equilibrium centrifugation. The fluorescence of the Wye base, a native label located adjacent to the 3′ side of the anticodon triplet (RajBhandary & Chang, 1968), is used to follow the reaction. The results indicate a four-step mechanism of codon recognition, involving two conformational changes and dimerization of tRNA. It shows that tRNA is not a passive adaptor but actively participates in the process of codon reading.

Materials and Methods

$tRNA^{Phe}$ from yeast and ApApA were purchased from Boehringer, Mannheim. The tRNA specific acceptor activity was 1.1 nmol/A_{260} unit. Highly pure $tRNA^{Phe}$ samples (specific activity of 1.5 nmol/A_{260} unit) were kindly given by Drs. M. Sprinzl and H. Faulhammer from Max-Planck-Institut für experimentelle Medizin, Göttingen. The codon oligonucleotide UpUpC was synthesized as described by Sprinzl et al. (1976). $tRNA^{Phe}$ samples were dissolved in buffer AB (10 mM Tris–cacodylate, 400 mM $NaClO_4$, and 5 mM $Mg(ClO_4)_2$, pH 7.1) or in buffer ABT (50 mM Tris–cacodylate, 100 mM $NaClO_4$, and 10 mM $Mg(ClO_4)_2$, pH 7.2) and annealed at 65 °C for 5 min as described by Grosjean et al. (1976). For measurements of Mg^{2+} concentration (c_{Mg}) dependence, the c_{Mg} was varied in ABT buffer as indicated. For c_{Mg} = 0, 0.5 mM Na_3EDTA was added instead of Mg^{2+}–salt. The $tRNA^{Phe}$ concentration (c_{tRNA}) was calculated on the basis of its acceptor activity. The concentrations of UpUpC (c_{UUC})

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