Length of the Polyadenylated Sequence in Cytoplasmic Ribonucleic Acid Isolated from Fetal Calf Myoblasts Differentiating in Vitro†

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ABSTRACT: Poly(adenylic acid) [poly(A)] containing cytoplasmic ribonucleic acid (RNA) in differentiating fetal calf myoblasts cultivated in vitro was examined by hybridization with radioactive poly(uridylic acid). The size distribution of the poly(A)-containing RNA after sucrose-gradient centrifugation was similar in cells before and after differentiation. There was no apparent correlation between the length of the poly(A) segment and the change in stability of messenger RNA which occurs on differentiation, nor with the polysomal or nonpolysomal localization of the RNA in the cytoplasm. The average length of the poly(A) segments in cytoplasmic RNA in the steady state was found to be dependent on the size of the RNA: the longer the RNA, the longer the average length of the poly(A) sequence. In contrast, in pulse-labeled RNA, the length of poly(A) is similar in all size classes of RNA.

The existence of poly(adenylic acid) [poly(A)][1] associated with messenger RNA and heterogeneous nuclear RNA in eukaryotic cells has been well established [see Brawerman (1974)]. Biological roles of poly(A) have remained unclear, although it has been suggested for eukaryotic cells that poly(A) may have a role in the nucleus in the biogenesis of messenger RNA (Darnell et al., 1971a; Sheiness et al., 1973) or in the cytoplasm in the translation (Johnston & Bose, 1972; Slater & Slater, 1974) or stability of messenger RNA (Perry & Kelley, 1973; Sheiness et al., 1975; Marbaix et al., 1975).

During the differentiation of fetal calf myoblasts in vitro, it has been shown that the half-life of classes of messenger RNA [i.e., poly(A)-containing RNA] increases considerably upon differentiation of the cells (Buckingham et al., 1976). This raises a question as to the mechanism by which messenger RNA is stabilized in the differentiated cells; it is possible that the poly(A) sequences of the messengers play a role in this process. In this study, poly(A) chain length was examined in various size classes of cytoplasmic poly(A)-containing RNA in undifferentiated and differentiated myoblasts in vitro, using a technique of radioactive poly(U) hybridization with poly(A). No apparent change took place during differentiation, but the length of the poly(A) sequence was found to vary according to the size of the messengers examined.

Experimental Procedures

Cell Culture and Radioactive Labeling of Cells. Primary cultures of fetal calf myoblasts were prepared as described by Buckingham et al. (1974, 1976). Myoblasts usually began to fuse between the second and the third day after plating. The cell fusion (i.e., morphological differentiation) continued to increase for about 2 days and then leveled off. When comparisons were made between undifferentiated, dividing myoblasts and differentiated, fused myotubes, plates of the second-day and sixth-day cultures, respectively, were used. The growing, undifferentiated cell population contained less than 5% of multinucleated cells. In the fused, differentiated cell population, 50–60% of nuclei were usually found in myotubes. In some experiments, cells were labeled with [^{3}H]adenosine (the Radiochemical Centre, Amersham, 26 Ci/mmol) for 3 h in fresh medium at a final concentration of 10 μCi/mL.

Cell Fractionation and Extraction of RNA. All glassware was dry-sterilized at 180 °C for 30 min, and solutions were autoclaved. Cells in monolayers were lysed in a buffer containing 0.25 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.005 M $MgCl_2$, and 0.5% (v/v) Nonidet P40 (Shell Chemical Co.). Nuclei and cell debris were removed by centrifugation at 1000*g* for 5 min at 4 °C. Cytoplasmic RNA was purified from the supernatant by repeated extractions with an equal volume of a chloroform–phenol mixture (1:1 v/v) containing 1% (v/v) isoamyl alcohol (Perry et al., 1972) after addition of 0.5% (v/v) sodium dodecyl sulfate and 0.005 M EDTA. RNA in the final aqueous phase was either processed for oligo(dT)–cellulose column chromatography (see below) after removing the phenol remaining in the aqueous phase with ether or precipitated with 2 volumes of ethanol in 0.1 M NaCl at −20 °C overnight.

For the preparation of polysomal RNA, the medium was changed 2 h beforehand, and the cultures were subsequently treated with 50 μg/mL cycloheximide (Sigma Chemical Co.) for 3 min, before they were lysed in 0.2 mL per plate of polysome buffer (0.25 M NaCl, 0.01 M Tris-HCl, pH 7.5, 0.005 M $MgCl_2$, and 50 μg/mL cycloheximide) and 0.5% (v/v) Nonidet P40 by homogenization in a Potter–Elvehjem-type glass–Teflon homogenizer. The resulting homogenate was centrifuged at 10000*g* for 10 min. Polysomes in the supernatant were analyzed on sucrose (ribonuclease-free sucrose from Schwarz/Mann Co.) gradients (10–40% w/v) in the polysome buffer at 40 000 rpm (Beckman SW 56 rotor) for 30 min at 4 °C. Fractions were collected with an Isco gradient fractionator, and the optical density at 254 nm was recorded. Aliquots were taken from each fraction and mixed with 0.5 mL of 2 × SSC and 0.1% (w/v) sodium dodecyl sulfate in preparation for [^{3}H]poly(U) hybridization as de-

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[1] Abbreviations used: poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); EDTA, ethylenediaminetetraacetate; oligo(dT), oligo(thymidylic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; tRNA, transfer ribonucleic acid; rRNA, ribosomal RNA; SSC, 0.3 M NaCl and 0.03 M trisodium citrate; NETS, 0.05 M NaCl, 0.01 M EDTA, 0.02 M Tris-HCl, pH 7.5, and 0.2% sodium dodecyl sulfate.

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scribed below. For the analysis of RNA and poly(A), fractions were pooled and precipitated with 2 volumes of ethanol at −20 °C. The pellets were dissolved in a buffer containing 0.02 M Tris-HCl, pH 7.5, 0.005 M EDTA, and 1% (w/v) sodium dodecyl sulfate. RNA was extracted in the same way as described above.

Separation of Poly(A)-Containing RNA by Oligo(dT)–Cellulose Column Chromatography. Poly(A)-containing RNA was separated by oligo(dT)–cellulose column chromatography (Aviv & Leder, 1972). The column was equilibrated at room temperature with a binding buffer containing 0.5 M NaCl, 0.02 M Tris-HCl, pH 7.5, and 0.5% (w/v) sodium dodecyl sulfate. RNA in 2–5 mL of binding buffer was applied to the column, followed by a wash with 3 mL of binding buffer before elution with 1 mL of eluting buffer containing 0.01 M Tris-HCl, pH 7.5, and 0.2% (w/v) sodium dodecyl sulfate. The recovery of poly(A)-containing RNA as measured by [^{3}H]poly(U) hybridization was usually about 80%.

Formamide–Sucrose-Gradient Centrifugation. Formamide (Merck Co.) was used after deionization with an ion-exchange resin. For the analysis of poly(A)-containing RNA, centrifugation on 80% formamide-containing sucrose gradients was employed throughout this study unless otherwise noted in order to avoid possible aggregation of poly(A)-containing RNA. RNA precipitates were dissolved in 50 μL of a solution containing 80% formamide, 0.01 M Tris-HCl, pH 7.5, 0.002 M EDTA, and 0.2% (w/v) sodium dodecyl sulfate and heated at 60 °C for 1 min, followed by quick chilling in an ice bath. The RNA solutions were layered on top of 5–20% (w/v) sucrose gradients containing 80% (v/v) formamide, in the same buffer. The gradients were centrifuged for 18 h at 49000 rpm at 20 °C in a Beckman SW 56 rotor. Fractions were collected from the top using an Isco gradient fractionator.

[^{3}H]Poly(U) Hybridization. Tritiated poly(U) was prepared according to the procedure described by Bishop et al. (1974). Purified polynucleotide phosphorylase of *Escherichia coli* was kindly given by Dr. Grunberg-Manago. [^{3}H]Poly(U) samples of various specific activities (1×10^5 to 1.6×10^7 cpm/μg) were prepared. In most experiments, a preparation of 0.71×10^6 cpm/μg was used. The poly(U)preparation usually contained 0.3–0.5% of pancreatic ribonuclease resistant material under standard assay conditions of hybridization (see below). The size of the [^{3}H]poly(U) was found to be mostly (more than 95%) longer than 250 nucleotides as determined by polyacrylamide gel electrophoresis. Hybridization was carried out essentially as described by Bishop et al. (1974).

Determination of Poly(A) Chain Length. RNA obtained as an ethanol precipitate was dissolved in 0.2 mL of a buffer solution containing 0.3 M NaCl and 0.01 M Tris-HCl, pH 7.5; 1 μg of pancreatic ribonuclease A (Sigma, type III-A) and one unit of ribonuclease T1 (Sankyo Co., Japan) were added. After incubation at 37 °C for 20 min, the protein was removed by phenol–chloroform extraction. In experiments where nonlabeled RNA was treated by the enzymes, poly(A) segments were precipitated from the aqueous phase by adding *E. coli* tRNA (20 μg as a carrier) and 0.5 mL of ethanol. However, in experiments where RNA was labeled in culture, the aqueous phase was adjusted to 0.5 M NaCl, 0.01 M Tris-HCl, pH 7.5, and 0.5% (w/v) sodium dodecyl sulfate and the poly(A) RNA separated on an oligo(dT)–cellulose column (5 × 5 mm). Thus, contamination by non-poly(A), ribonuclease-resistant fragments in the poly(A) preparation, which could obscure the analysis of poly(A) chain length on polyacrylamide gel electrophoresis, was minimized. This extra step of oligo(dT)–cellulose column chromatography used for labeled RNA did not result in selection for particular size classes of poly(A).

The poly(A) segments thus obtained were dissolved in 30 μL of electrophoresis buffer (see below) and mixed with 5 μL of 60% (w/v) sucrose–bromphenol blue and ^{14}C-labeled molecular weight markers, 5.8 S, 5 S, and 4 S. A 10% polyacrylamide gel (7 × 90 mm) was employed for the analysis of poly(A) chain length. The gels were composed of 9.5% (w/v) acrylamide, 0.5% (w/v) bis(acrylamide), 0.6% (v/v) *N,N,N′,N′*-tetramethylethylenediamine, and 0.04% (w/v) ammonium persulfate in a buffer containing 0.09 M Tris-HCl, pH 8.3, 0.09 M boric acid, 0.0025 M EDTA, and 0.2% (w/v) sodium dodecyl sulfate (Peacock & Dingman, 1967). Electrophoresis was performed for about 2 h at 5 mA/tube and 130 V. After the run, the gels were frozen with dry ice and cut into 1-mm slices. Three slices were pooled for each fraction, and poly(A) was extracted with 0.5 mL of 2 × SSC, overnight, at room temperature. Poly(A) content was assayed by the [^{3}H]poly(U) hybridization technique, using extracts from parallel gel slices not containing poly(A) as a blank. The efficiencies of elution and hybridization with poly(A) markers of different lengths (see below) were checked. No significant differences were found in the 16–200-nucleotide size range. Following elution, some (usually over 50%) of the ^{14}C-labeled marker RNAs survived the poly(A) assay procedure involving ribonuclease digestion in 2 × SSC, thus serving as internal molecular weight markers in each gel.

In fact, under the nondenaturing gel conditions employed, the RNA markers migrate anomalously, and their apparent molecular weights under these circumstances were calibrated against poly(A) samples of defined length obtained from Miles Laboratories. The calibration of these fractions by Miles was based on calculations of chain lengths from sedimentation velocity data by extrapolation of empirical formulas obtained by Eisenberg & Felsenfeld (1967). The lengths of these markers were verified by analysis of their hydrolysis products on urea–acrylamide gels after 5′ labeling with [γ-^{32}P]ATP. A 10-μg sample of each oligonucleotide was hydrolyzed in 10 μL of deionized formamide at 100 °C for variable times (4 min–4 h) according to length (Stanley & Vassilenko, 1978). After lyophilization, the hydrolysis products were incubated for 30 min at 37 °C in a 10-μL volume containing [γ-^{32}P]ATP (3 Ci/mmol, The Radiochemical Centre, Amersham) (final concentration 5 μM, 0.3 Ci/μmol), 2 units of T_4 polynucleotide kinase (a kind gift of G. Keith), and buffer as described by Stanley & Vassilenko (1978). The labeled hydrolysis products were separated on 20% acrylamide–urea gels using the urea–borate buffer system (4.5 mM Tris, 45 mM H_3BO_4, 1 mM EDTA, and 8 M urea, pH 8.3) (Donis-Keller et al., 1977). After autoradiography, the oligonucleotide lengths were estimated from the banding pattern of their partial digests. The poly(A) standards (220, 160, 120, 54, and 16 nucleotides in length) were electrophoresed, eluted, and hybridized to [^{3}H]poly(U), in the presence of the ^{14}C-labeled RNA markers, as described. Under these conditions, 5.8 S (150 nucleotides) has an apparent length of 76 nucleotides, 5 S (120 nucleotides) of 48 nucleotides, and 4 S (80 nucleotides) of 26 nucleotides. All calculations of nucleotide length (see, e.g., Table I) were therefore based on these values.

Preparations of duck and rabbit globin messenger RNAs, used for verifying the poly(A) estimations, were kindly given to us by Drs. K. Scherrer and J. Lewis, respectively.

Results

Validity of the Method of Analysis of Poly(A). Several methods have been reported for the analysis of poly(A)-con-

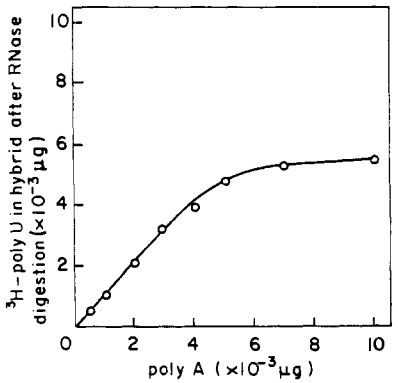

FIGURE 1: Stoichiometry of [^{3}H]poly(U) and poly(A) hybridization. Various amounts of poly(A) (Sigma Chemical Co.) were incubated with a fixed amount of [^{3}H]poly(U) (0.0056 µg; 3700 cpm) in 0.5 mL of 2 × SSC solution at 45 °C for 15 min. The mixture was then treated with pancreatic ribonuclease (10 µg/mL) in 2 × SSC solution at 0 °C for 15 min. The radioactivity in the ribonuclease-resistant hybrid was measured and converted to the weight of poly(U).

taining RNA by hybridization with radioactive poly(U) to detect unlabeled poly(A) (Bishop et al., 1974; Gillespie et al., 1972; Kwan & Brawerman, 1972; Fraser & Loening, 1973; Hunt, 1973). We employed the method described by Bishop et al. (1974) since this is a method which is well characterized stoichiometrically. We have confirmed that a one to one ribonuclease-resistant hybrid between poly(A) and radioactive poly(U) was formed (Figure 1), contrary to what some authors have reported (Nokin et al., 1975), under similar experimental conditions.

To see further how the method works on poly(A)-containing RNA, rabbit reticulocyte polysomal RNA was separated by sucrose-gradient centrifugation, and poly(A)-containing RNA was detected by the [^{3}H]poly(U) hybridization technique (Figure 2A). Poly(A)-containing RNA was present almost exclusively in the 9S position as a sharp peak as expected for globin messenger RNA. No radioactivity was detected in 4S, 18S, and 28S RNAs, which were present in large amounts. Using 18S and 28S RNAs purified by sucrose-gradient centrifugation followed by oligo(dT)–cellulose column chromatography, it was found that these RNAs contained less than 0.0008% of segments which appeared to form ribonuclease-resistant hybrids with radioactive poly(U). *E. coli* tRNA, used as a carrier for the precipitation of poly(A)-containing RNA or poly(A) in some experiments, contained less than 0.0006% of poly(A). Thus, formation of ribonuclease-resistant hybrids between radioactive poly(U) and rRNA or tRNA is negligible.

In order to evaluate the method for the analysis of poly(A) chain length (by [^{3}H]poly(U) hybridization) in steady-state RNA preparations, 9S RNA was obtained from sucrose-gradient fractions and digested with pancreatic ribonuclease A and ribonuclease T1 in the presence of 0.3 M NaCl. The digests were fractionated by polyacrylamide gel electrophoresis, and poly(A) segments were assayed with [^{3}H]poly(U) after elution from gel slices. Relative to 5.8S, 5S, and 4S RNAs used as internal molecular weight markers, rabbit reticulocyte 9S RNA contained poly(A) segments ranging from 150 to less than 30 nucleotides with an average size of about 70 nucleotides. However, in this gel system, the standard small molecular weight RNA markers do not migrate at the same rate as poly(A) fragments of corresponding length. It was found that 5.8S (150 nucleotides), 5S (120 nucleotides), and 4S (80 nucleotides) RNAs migrate in the same position as that of poly(A) standards corresponding to 76, 48, and 26 nucleotides in length, respectively. When recalibrated against poly(A) sequences of defined length, the average size of this sequence

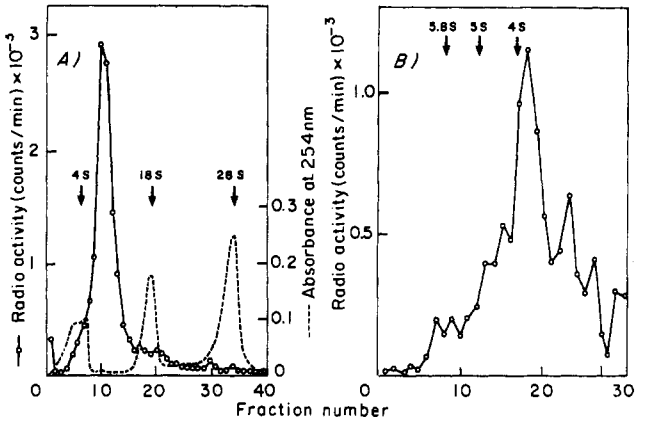

FIGURE 2: Distribution of poly(A)-containing RNA and the poly(A) segments of rabbit reticulocyte. (A) Polysomal RNA prepared from reticulocytes of anemic rabbits was fractionated by centrifugation on 5–20% sucrose gradients, and [^{3}H]poly(U) hybridization was performed as described in Figure 1. The solid and broken lines indicate radioactivity and optical density, respectively. Positions of 4S, 18S, and 28S RNAs are indicated by arrows. (B) Poly(A)-containing RNA was collected from the gradient shown in (A). The RNA was digested with ribonucleases A and T1, and the poly(A) segments were analyzed by electrophoresis on 10% (w/v) polyacrylamide gels. [^{3}H]Poly(U) hybridization was performed as described under Experimental Procedures. Arrows (from left to right) indicate positions of ^{14}C-labeled 5.8S, 5S, and 4S RNAs added as molecular weight markers in the same gels.

in the globin 9S preparation shown in Figure 2B is about 25 nucleotides, with a size range of about 10–70 residues. The experiment was repeated with another preparation of rabbit globin RNA, and the range of poly(A) lengths obtained was again similar, with a slightly higher average length of 30–35 nucleotides. A preparation of globin 10S messenger RNA prepared from duck reticulocytes had an average poly(A) length of 50–55 nucleotides. In the experiments reported here, the radioactive small RNAs were used for convenience as internal markers in the gels, and their nucleotide length corrected to that of the corresponding poly(A) sequence.

Distribution on Formamide–Sucrose-Gradient Centrifugation of Poly(A)-Containing RNA of Undifferentiated and Differentiated Calf Myoblasts. In some systems (Greenberg & Perry, 1972; Lee et al., 1971; Adesnik et al., 1972), more than 70% of RNA with properties of messenger RNA has been reported to contain poly(A), while other estimates are lower (40–70%) (Sheldon et al., 1972; Sullivan & Roberts, 1973; Milcarek et al., 1974; Nemur et al., 1974; Rosen et al., 1975a; Greenberg, 1976). In the present system, it has been shown that template activity for translation of RNA lacking poly(A) or containing a short (<20) poly(A) tract is very low except for histone messenger RNA and part of the mRNA fraction coding for actin. This is not due to an inhibitory effect of rRNA, as shown by comparison between total and poly(A+) RNA translation. In preparations of RNA from fused cultures, about 10% of the coding capacity is in the fraction not retained on oligo(dT)–cellulose. This figure is slightly higher with RNA from unfused cultures where histone mRNAs are present (Buckingham et al., 1975; Whalen & Gros, 1977). Thus, most messenger RNA in this system apparently contains a sequence of poly(A). Poly(A)-containing RNA from differentiated and undifferentiated cells was partially purified by column chromatography on oligo(dT)–cellulose and then fractionated by centrifugation on formamide–sucrose gradients. [^{3}H]Poly(U) hybridization was performed on each fraction to localize poly(A)-containing RNA (Figure 3A,B). The use of affinity chromatography did not bring about any measurable change in the pattern of poly(A) distribution on the gradient.

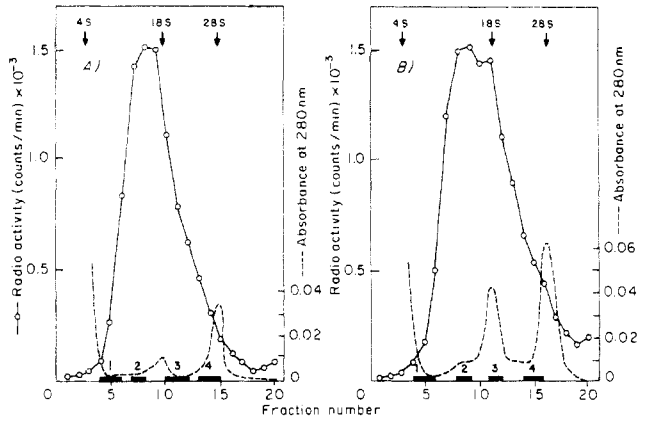

FIGURE 3: Distribution on formamide–sucrose gradient of cytoplasmic poly(A)-containing RNA of undifferentiated (A) and differentiated (B) fetal calf myoblasts. Cytoplasmic poly(A)-containing RNA was partially purified by oligo(dT)–cellulose column chromatography and analyzed by centrifugation on 5–20% (w/v) sucrose gradients in 80% (v/v) formamide. Small aliquots of each fraction were assayed for the presence of poly(A) by [^{3}H]poly(U) hybridization as described in Figure 1. The solid line indicates radioactivity, and the broken line indicates contaminating ribosomal RNA and tRNA, added as carriers, which are internal markers for the estimation of sedimentation coefficients in each gradient. Positions of 4S, 18S, and 28S RNAs are shown by arrows.

It therefore seemed unlikely that special size classes of poly(A)-containing RNA were selected by the chromatography. As is seen in Figure 3A,B, there is no marked difference in the distribution of poly(A)-containing RNA between different stages of myogenesis. Messenger RNA for myosin heavy chain which has been described in muscle cells as sedimenting at about 26 S [e.g., see Morris et al. (1973)] was not clearly detected by the present technique, although more material is present in this region in the differentiated cells. It is possible that this messenger species, unlike most muscle mRNAs (Whalen & Gros, 1978), is mainly present in the unbound fraction after oligo(dT)–cellulose chromatography (Benoff & Nadal-Ginard, 1979). Poly(A)-containing RNA smaller than 8–9 S was present in a very small amount, suggesting that there is little degradation of the RNA during extraction. For determination of whether poly(A)-containing RNA sedimenting in each fraction is actually different or whether aggregation is a problem, recentrifugation of five different pooled fractions of the first centrifugation was performed (Figure 4). RNA of each fraction, precipitated with carrier *E. coli* tRNA, was heated in formamide-containing buffer prior to the second centrifugation in order to minimize possible aggregation of the RNA. RNA of each fraction sedimented in almost the same position on the gradient as after the first centrifugation, relative to 4S, 18S, and 28S RNAs added as molecular weight markers. It should be noted that poly(A)-containing RNA larger than 25 S is in fact present in the cytoplasm of the cells and that heavier RNA seen in the first centrifugation is not simply due to skewed RNA from the more slowly sedimenting RNA present in abundance. It is, therefore, suggested from Figure 3A,B that poly(A)-containing RNA bigger than 25 S represents a relatively large part of total poly(A)-containing RNA, since what is seen in the pattern of the figures is only the poly(A) portion of the molecules, thus biasing the profile in favor of the smaller RNA. The general distribution of poly(A)-containing RNA on sucrose gradients is in good agreement with the results of other investigations on mammalian cells (Singer & Penman, 1973; Darnell et al., 1971b).

Size Distribution of Poly(A) Segments in Different Size Classes of RNA. We next investigated the size of the poly(A) chain in different size classes of RNA in the steady state.

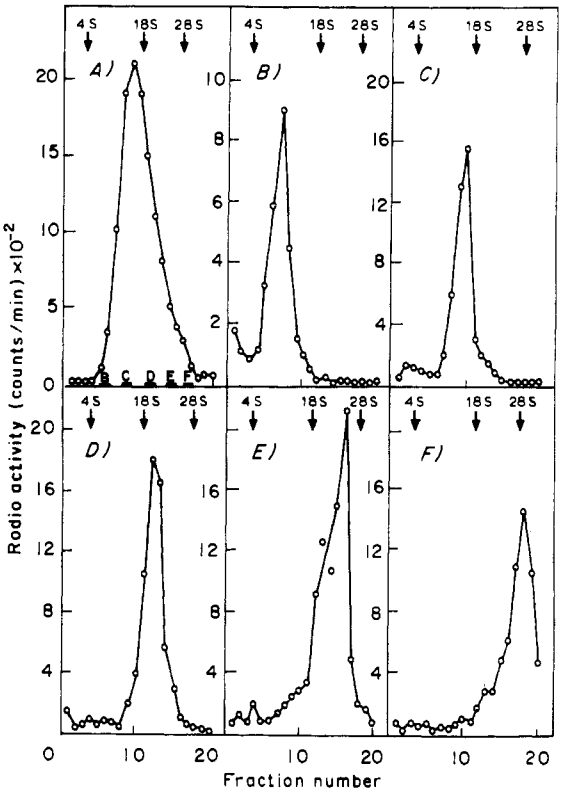

FIGURE 4: Recentrifugation of poly(A)-containing RNA on formamide-containing sucrose gradients. Cytoplasmic poly(A)-containing RNA fractionated on the first gradient (A) was subdivided into five pooled fractions as indicated by bars with letters (B–F) and collected with *E. coli* tRNA as carrier. RNA of each fraction was then analyzed on further gradients (B–F) together with a small quantity of 18S and 28S rRNAs as molecular weight markers, which are indicated by arrows. [^{3}H]Poly(U) hybridization was performed as described under Experimental Procedures.

RNA separated as in Figure 3A,B was subdivided into four pooled fractions, and poly(A) segments were obtained from each fraction. The distribution on polyacrylamide gel electrophoresis of the poly(A) segments hybridizable with [^{3}H]-poly(U) is shown in Figure 5A,B. Steady-state poly(A)-containing RNA was found to have poly(A) segments dependent on the size of RNA [i.e., the longer the RNA, the longer the size of poly(A)]. This was true in both undifferentiated (Figure 5A) and differentiated (Figure 5B) cells. These observations raise questions as to the origin of each RNA class and its poly(A). First, smaller RNA classes may contain substantial amounts of mitochondrial messenger RNA (Ojala & Attardi, 1974; Perlman et al., 1973; Avadhani et al., 1974). Therefore, the patterns of poly(A)-containing RNA from postnuclear and postmitochondrial cytoplasmic extracts prepared without using the detergent Nonidet P40 were compared after formamide–sucrose-gradient centrifugation. The results were identical in both cases (data not shown). This rules out the possibility that small poly(A) segments found in smaller RNA species are derived from mitochondrial messenger RNA. Second, small poly(A) segments may be produced by excess digestion of RNA with ribonucleases, since the quantity of smaller RNA used in the enzymatic digestion was usually much less than that of larger RNA fractions. Patterns of poly(A) segments in smaller RNA species were, therefore, compared after varying times of enzymatic treatment. There was no difference in the patterns among samples incubated for 10, 20, or 40 min (data not shown). Such a possibility is ruled out also by the observation that poly(A) segments of any of the size classes of pulse-labeled RNA are

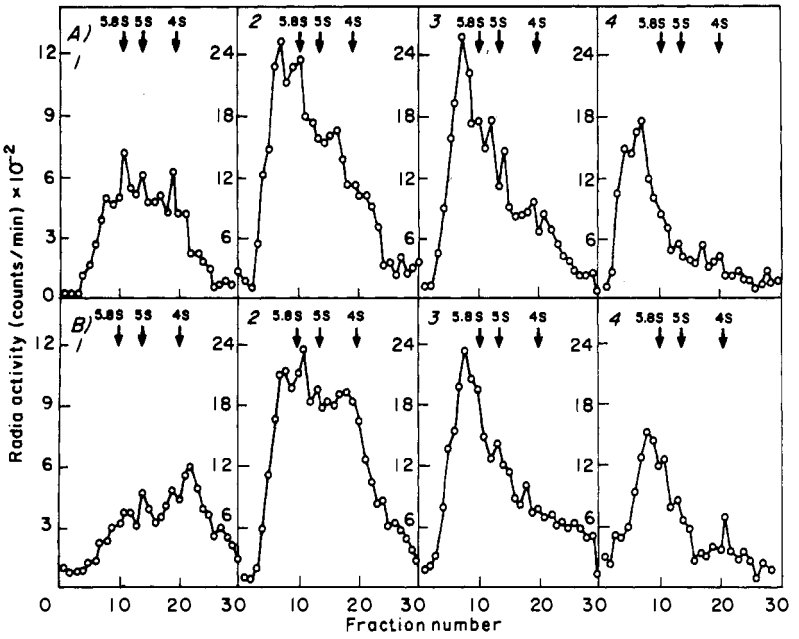

FIGURE 5: Distribution of poly(A) chain length in different size classes of steady-state RNA in undifferentiated (A) and differentiated (B) cells. Poly(A) segments derived from cytoplasmic RNA of different sizes were analyzed by electrophoresis on 10% polyacrylamide gels. [^{3}H]Poly(U) hybridization was performed as described under Experimental Procedures. The numbers of each profile indicate the fractions of the gradient shown in Figure 3 from which the poly(A) segments are derived. Arrows (from right to left) indicate positions of ^{14}C-labeled 4S, 5S, and 5.8S RNAs added as molecular weight markers in each gel.

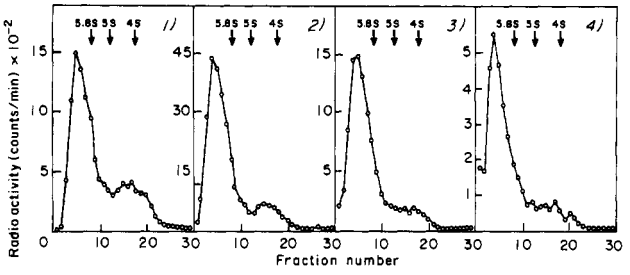

FIGURE 6: Distribution of poly(A) chain length in different size classes of pulse-labeled RNA. Cells were labeled with [^{3}H]adenosine for 3 h. Cytoplasmic poly(A)-containing RNA was fractionated by centrifugation on formamide–sucrose gradients. RNA of different size classes (1:8–11 S, 2:14–17 S, 3:20–22 S, and 4:25–30 S) was pooled, and poly(A) segments of each pooled fraction were processed as described under Experimental Procedures. Arrows (from right to left) indicate the positions of ^{14}C-labeled 4S, 5S, and 5.8S RNAs, added as molecular weight markers.

homogeneous in length after being subjected to the same enzymatic digestion as that in Figure 5A,B (see Figure 6).

The average chain length of poly(A) in each size class of RNA was calculated from Figure 5 according to the following equation. It was assumed that there is a linear relation

$$\text{av chain length of poly(A)} = \frac{\Sigma \text{cpm in each fraction}}{\Sigma \frac{\text{cpm in each fraction}}{\text{chain length of poly(A) in each fraction}}}$$

between the logarithm of the chain length of the polynucleotides and their electrophoretic mobility [see Peacock & Dingman (1968) and experiments not shown here with poly(A) segments of defined length].

For example, if slices corresponding to 40 and 120 nucleotides have 80 and 360 cpm, respectively, an average chain length is 88 = (80 + 360)/(80/40 + 360/120). The result is summarized in Table I. 25–30S RNA, the longest RNA class examined, has an average poly(A) chain length which is about 20 nucleotides longer than that of 8–10S RNA. The difference in average length between fractions is thus not very great but varies consistently with the size of the RNA (Figure 4). The minimum and maximum lengths of poly(A) range from less than 20 nucleotides to 80 nucleotides or more. In both undifferentiated and differentiated cells, an average sedimentation coefficient of poly(A)-containing RNA was about 18 S, i.e., about 2000 nucleotides, using 4S, 18S, and 28S RNAs as internal standards (Noll & Stutz, 1967). The RNA has an average poly(A) chain length of about 45 nucleotides (see Table I). The weight percentage of poly(A)-containing RNA in total cytoplasmic RNA was therefore calculated to be 2–2.5% from the data obtained by the [^{3}H]poly(U) hybridization assay on total cytoplasmic RNA, or RNA fractionated by sucrose-gradient centrifugation. The amount of cytoplasmic RNA was calculated by measuring its optical density at 260 nm, assuming that an RNA solution of 40 μg/mL has an optical density of 1.0. For comparison, it has been reported that the content of poly(A)-containing messenger RNA as a percentage of ribosomal RNA in mammalian cells in culture is 5.5% in HeLa cells (Singer & Penman, 1973) and 1.8–2.7% in mouse 3T3 cells (Johnson et al., 1976), as measured by steady-state labeling of the cells with radioactive precursors.

Table I: Average Size of Poly(A) in RNA of Various Molecular Weights[a]

fraction of RNA[b]	av nucleotide length of poly(A) segments: undifferentiated cells	differentiated cells
1	37 (8–12 S)	33 (7–10 S)
2	49 (14–16 S)	39 (13–15 S)
3	52 (20–23 S)	49 (18–20 S)
4	57 (25–30 S)	53 (25–28 S)

[a] The average length of poly(A) in different size classes of the steady-state population of RNA was calculated from the distribution curves shown in Figure 5A,B by a formula described in the text. The size distribution of RNA is given in parentheses. [b] Numbers are those shown in Figure 3A,B.

Size Distribution of Pulse-Labeled Poly(A) Segments. The poly(A) chain length of cytoplasmic RNA pulse labeled for

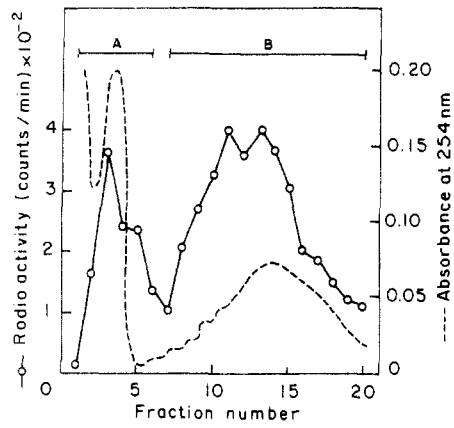

FIGURE 7: Distribution of poly(A)-containing RNA on a polysome gradient. Polysomes in the postmitochondrial fraction of differentiated calf myoblasts were fractionated on a 10–40% (w/v) sucrose gradient. Each fraction was hybridized with [^{3}H]poly(U) in the presence of 0.1% (w/v) sodium dodecyl sulfate as described under Experimental Procedures. The horizontal bars (A and B) show fractions pooled for the analysis of poly(A), shown in Table II.

3 h with [^{3}H]adenosine was examined. After fractionation by centrifugation on formamide–sucrose gradients, the RNA was extracted and digested as in the preceding experiments, before direct sizing of the [^{3}H]poly(A) on gels. As is shown in Figure 6, the distribution of poly(A) segments in different size fractions of RNA is much more homogeneous than in the steady-state population shown in Figure 5. An average chain length in each size class of pulse-labeled RNA is as follows: class 1 (8–11 S), 57; class 2 (14–17 S), 66; class 3 (20–22 S), 67; class 4 (25–30 S), 66. Thus, the average chain length of poly(A) in pulse-labeled RNA is longer and more homogeneous in all size classes than that in the steady-state populations shown in Table I. These values are shorter than those reported in the literature for newly synthesized poly(A) [see, e.g., Sawicki et al. (1977)]. This probably results from the 3-h labeling time and the method of calibrating poly(A) size used here.

It is important to note that one additional step of oligo(dT)–cellulose chromatography introduced after the digestion (see Experimental Procedures) did not result in preferential selection of any size class of RNA. This was verified as follows. Poly(A)-containing RNA was separated by centrifugation on formamide–sucrose gradients, and two size fractions corresponding to 9–12 S and 24–28 S were processed in the same was as the [^{3}H]adenosine-labeled poly(A) RNA, including chromatography on an oligo(dT)–cellulose column after RNAase digestion. Poly(A) chain length was determined as in the other experiments by hybridization with [^{3}H]poly(U) after separation on acrylamide gels. The size patterns obtained with and without the oligo(dT)–cellulose step were similar (data not shown).

Poly(A) in the Polysomal and Nonpolysomal Cytoplasmic Fractions. In a previous paper from this laboratory, it was shown that about 40% of pulse-labeled cytoplasmic poly(A)-containing RNA is present in the nonpolysomal fraction as ribonucleoprotein particles in both undifferentiated and differentiated cells (Buckingham et al., 1974). Messenger ribonucleoprotein particles may be regarded as a pool for functional polysomal messenger RNA (Buckingham et al., 1976). A possibility exists that the polysomal or ribonucleoprotein distribution of messenger RNA could be regulated by the size of the poly(A) segments attached to it, since there have been indications of cytoplasmic polyadenylation and turnover (Slater et al., 1973; Diez & Brawerman, 1974; Brawerman & Diez, 1975; Brawerman, 1976) as well as shortening of

Table II: Percentage Distribution of Poly(A) in Subcellular Fractions[a]

poly(A) location	undifferentiated cells	differentiated cells
polysomal fraction	67, 65	73, 69, 65
nonpolysomal fraction	33, 35	27, 31, 35

[a] Postmitochondrial fractions were separated on polysome gradients, and the presence of poly(A) was assayed by [^{3}H]poly(U) hybridization as described in the test. In all experiments, the distribution of poly(A) was bimodal (see Figure 7). The first peak from the top was regarded as nonpolysomal and the rest as polysomal. Values from individual experiments.

poly(A) segments [see also Adams & Jeffery (1978)]. We therefore examined the poly(A) chain length of RNA in nonpolysomal and polysomal fractions in the steady state. Cultures were treated with cycloheximide just prior to cell lysis to prevent runoff of ribosomes from polysomes. Figure 7 shows the distribution of poly(A) in polysomes separated on sucrose gradients as detected by the [^{3}H]poly(U) hybridization technique. Two major peaks are evident. In addition to the poly(A) distribution over the polysomal region, another peak was reproducibly observed in the monosome region, extending to the very small polysome region. The latter peak may be regarded as ribonucleoprotein particles containing messenger RNA (Perry & Kelley, 1968; Spohr et al., 1970), as described previously (Buckingham et al., 1976). The percentage distribution of poly(A) in the nonpolysomal and polysomal regions in the myoblasts is summarized in Table II. No change in the distribution of poly(A) in the two fractions occurred during the differentiation of the cells, being 30–35% in the nonpolysomal fraction and 65–70% in the polysomal fraction. This is in general agreement with the distribution of pulse-labeled poly(A)-containing RNA in the two compartments (Buckingham et al., 1976). The distribution of poly(A) chain length in the RNA of the two fractions was almost identical (result not shown). Thus, poly(A) chain length does not appear to be related to the sequestration of messenger RNA between the two subcellular compartments.

Discussion

The principal result of this investigation of poly(A) chain length in different classes of messenger RNA during muscle cell differentiation is the correlation between the size class of the messenger RNA and the length of its poly(A) segment. A similar conclusion was inferred by Mondal et al. (1974) from results of [^{3}H]poly(U) hybridization across polysome gradients. In the experiments reported here, messengers sedimenting from 8 to 30 S had poly(A) lengths ranging from about 20 to 80 nucleotides, in proportion to their size in the steady state. In contrast, newly synthesized messenger RNA of all sizes had a similar sequence of longer average length. This corresponds to observations on newly synthesized poly(A)-containing RNA in other cell systems [e.g., Sheiness et al. (1975) and Brawerman (1976)].

Comparison of results in the literature on the poly(A) length of steady-state messenger RNA preparations indicates that considerable discrepancies exist between estimations even for the same species. This is largely due to the different techniques used. The most widely used technique is that adopted here, namely, analysis of the poly(A) segment on gels preceded by nuclease digestion of the rest of the molecule. Under our conditions, this gave poly(A) lengths of 25–35 and 50–55 for 9S rabbit globin and 10S duck globin messenger RNAs, respectively, when the number-average lengths are calibrated

against poly(A) standards. These values are similar to those reported in the literature (Gaskill & Kabat, 1971; Hunt, 1973; Kaufman & Gros, 1974; Bishop et al., 1974) when the steady-state population of messenger RNA is examined and when the data are corrected for the anomalous values given by calibration against 4S and 5S RNAs (Morrison et al., 1973; Kaufman & Gros, 1974; Burness et al., 1975). It should be noted that substantially higher values are obtained for globin messenger RNA (ca. 150 nucleotides) when the poly(A) tract is measured by quantitation of the ADP release after degradation with polynucleotide phosphorylase (Soreq et al., 1974; Williamson et al., 1974). The complete sequence of rabbit globin 9S RNA comprises 589 nucleotides excluding the poly(A) tract (Efstratiadis et al., 1977), but it remains difficult to calculate the length of poly(A) due to inaccuracies in molecular weight estimates which range from 650 (Gaskill & Kabat, 1971) to 710 (Hamlyn & Gould, 1975), although the latter may be an overestimate (Proudfoot, 1977). If the poly(A) lengths reported for other size classes of messenger RNAs are analyzed, bearing in mind the considerations mentioned for globin, then fibroin (16000 nucleotides) has an estimated poly(A) tract of 100–150 (Lizardi et al., 1975); vitellogenin (7000), 75–85 (Jost & Pehling, 1976); myosin (6500), 70–80 (Mondal et al., 1974); ovalbumin (ca. 2000), 44 (Shapiro & Schimke, 1975); the crystallins (900, 1400), 25–30 (Lavers et al., 1974); globin (ca. 650), 25–50 (see above). There would thus seem to be a rough correlation between the size class of the messenger and the length of the poly(A) tract, although more purified messengers and more precise measurements of their poly(A) tracts are required. In particular, the question of the homogeneity of the poly(A) sequence, which may be a factor in the higher values obtained in polynucleotide phosphorylase degradation experiments, requires clarification. It should also be noted that in a number of experiments where poly(A)-containing RNA has been fractionated on the basis of oligo(dT)–cellulose chromatography, the bound and unbound populations have been found to code for similar peptides, although their relative abundance may be different [e.g., see Cabada et al. (1977) and Minty & Gros (1980)]. Morrison et al. (1979), using a combination of oligo(dT)–cellulose and Millipore filter techniques, have fractionated messenger RNAs from neuroblastoma cells into classes containing different poly(A) lengths and also shown that most of the peptides in the translation products of the different fractions are similar. In the case of actin, the messenger coding for β-actin is not bound to oligo(dT)–cellulose whereas those for α- and γ-actin are bound (Hunter & Garrels, 1977). Not only may the same or similar coding sequence have a variable poly(A) tract but also this may change, as observed during sea urchin development (Wilt, 1977). It is thus evident that poly(A) length is not necessarily related to messenger size but may be modified by factors such as the developmental or metabolic state of the cell, or in the case of the actins perhaps for other regulatory reasons which are not yet understood.

During myogenesis, changes take place in the stability and polysomal distribution of messenger RNA (Buckingham et al., 1974, 1976). The small size class of messengers, both before and after fusion, has a relatively short half-life, in contrast to the situation reported for insect (Spralding et al., 1975) and mouse (Meyuhas & Perry, 1979) cell lines where the shorter messenger RNA is enriched for more stable species. The experiments reported here might suggest that for this class of muscle messengers there may be a correlation between a short half-life and a short poly(A) tract. However, the total messenger population undergoes a 2–3-fold increase in half-life at fusion, and no detectable difference in the distribution of poly(A) chain length was observed in the experiments reported here nor was any correlation noticeable between poly(A) length and previously reported differences in stability among different classes of cytoplasmic messenger RNA.

Benoff & Nadal-Ginard (1979) have reported that the myosin heavy chain messenger RNA has a short poly(A) tract after fusion. This species was not separated in our experiments. In general, this size class had a longer poly(A) tract both before and after fusion.

In some cases, reports on a relation between the age of the messenger RNA and the length of the poly(A) tract may be related to the initial shortening of the poly(A) observed for all newly synthesized mRNA classes [see Brawerman (1976)]. In muscle cells, after 3 h labeling, the poly(A) distribution is still predominantly in a mode which differs from that of the steady state. Values obtained, for example, with reticulocytes, where aging is estimated over a 9–12-h labeling period, may still be influenced by initial processing (Merkel et al., 1976; Nokin et al., 1976). Results with adenovirus-infected cells would suggest that the rapidity with which the poly(A) tract is shortened is correlated with the half-life of the messenger RNA (Williamson et al., 1974). If the concept of a limit length of poly(A) is valid (see below), then observations on messenger populations in the steady state will depend on whether certain species attain their limit length of poly(A) before the RNA is degraded. Different size classes of messenger RNA in muscle cells both before and after differentiation appear to attain the same limit lengths of poly(A) in the steady state.

Changes in the polysomal distribution of messengers during myogenesis may be masked when the total population is examined (Buckingham et al., 1976). However, not only was no difference observed in the distribution of poly(A) length among polysomal and nonpolysomal messenger RNAs during differentiation but also the steady-state distribution of poly(A) length was identical in the two cytoplasmic compartments. It has been reported that messenger RNAs with different poly(A) tracts bind to ribosomes with equal efficiency (Bard et al., 1974), and conversely, the RNAs coding for α and β rabbit globins are examples of messengers with similar lengths of poly(A) [e.g., see Nokin et al. (1976)] which bind to ribosomes with different efficiencies (Lodish, 1974). The principal experiments which point to a relation between poly(A) length, translation, and messenger stability are those based on the injection of globin messenger RNA into oocytes (Soreq et al., 1974). It would appear that for functional stability a limit length of poly(A) is necessary (Nudel et al., 1976) and that there is a correlation between the number of times a messenger is translated and its degradation (Huez et al., 1977). Actual degradation of messenger RNA apparently takes place (Marbaix et al., 1975; Huez et al., 1977), although Doel & Carey (1976) have claimed that in the case of the translation of ovalbumin messenger RNA in a reticulocyte lysate, reduction in poly(A) chain length results in reduced efficiency of translation, but no messenger degradation. Some in vivo experiments with inhibitors of protein synthesis suggest that there is an association between translation and shortening of poly(A) chain length (Sheiness et al., 1975), while others do not (Merkel et al., 1976). Adams & Jeffery (1978) have suggested that the turnover rather than the shortening of poly(A) may be linked with the translation cycle.

The studies reported here for muscle cells indicate that a limit length of poly(A), related in general to the size of the

corresponding messenger, is attained in the steady state, apparently as a result of partial degradation of an initial longer poly(A) tract. The factors which determine the limit length for different size classes of RNA may be partly related to structural features influencing the conformational stability of the molecule. In vivo, this will depend not only on the final length of the RNA but also on the proteins associated with its different sequences. Among these, a poly(A) binding protein has been identified (Blobel, 1973; Brawerman, 1976; Schwartz & Darnell, 1976), which confers some resistance to exonuclease attack and points to the importance of the poly(A) protein moiety in preserving a stable messenger RNA configuration. The translation and the metabolic stability of messengers may in some circumstances be associated with modulations of poly(A) length, but there is no indication of this in the experiments on muscle cells described in this report.

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Effect of Transfer Ribonucleic Acid Dimer Formation on Polyphenylalanine Biosynthesis†

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ABSTRACT: *Escherichia coli* $tRNA^{Phe}$ (anticodon GAA) as well as yeast $tRNA^{Phe}$ (anticodon GmAA) forms a strong complex with *E. coli* $tRNA^{Glu}$ (anticodon s^2UUC) through an interaction between their complementary anticodons. This interaction inhibits aminocylation of $tRNA^{Phe}$ but not the formation of a complex with elongation factor Tu. Moreover, at 0 °C, $tRNA^{Glu}$ strongly inhibits the binding of Phe-tRNA to poly(U)-programmed ribosomes via either the enzymic (EF-Tu-promoted) or nonenzymic pathway. At 15 °C, $tRNA^{Glu}$ effectively inhibits polyphenylanine synthesis in the *E. coli* system. The inhibition is reversed at 37 °C, where the Phe-tRNA·$tRNA^{Glu}$ dimer is dissociated. Calculations based upon the *E. coli* intracellular concentrations of tRNAs and the published rates of association and dissociation of the tRNA dimers suggest that this interaction may inhibit protein synthesis in vivo at temperatures below 15 °C.

The translation of mRNA[1] requires the correct codon–anticodon interaction to occur during the binding of AA-tRNA to ribosomes [for a review, see Pongs (1978)]. The discovery that tRNAs bearing complementary anticodons can form tight dimers with dissociation constants as low as 1×10^{-7} M (Eisinger, 1971; Grosjean et al., 1976, 1978; Grosjean & Chantrenne, 1980) raises the question of whether this interaction inhibits the rate of peptide chain elongation. Dimer formation might a priori affect the rate of aminoacylation of the tRNA, the interaction of AA–tRNA with EF-Tu·GTP, or the binding of this ternary complex[2] to ribosomes. Our previous results indicated that ternary complex formation was not inhibited by the interaction of the anticodons (GmAA yeast and GAA *Escherichia coli*) of the Phe-$tRNA^{Phe}$ with the complementary anticodon (s^2UUC) of Glu-$tRNA^{Glu}$ (Yamane et al., 1981). We then examined the effects of dimer formation upon the rates of aminoacylation of $tRNA^{Glu}$ and $tRNA^{Phe}$, upon the rate and extent of binding of Phe-$tRNA^{Phe}$ to ribosomes, and upon the rate of incorporation of Phe-$tRNA^{Phe}$ into polyphenylanine in an *E. coli* in vitro translation system. At 15 °C, the rates of both aminoacylation of $tRNA^{Phe}$ and polyphenylanine synthesis are substantially inhibited by $tRNA^{Glu}$,

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[1] Abbreviations used: mRNA, messenger ribonucleic acid; tRNA, transfer ribonucleic acid; EF-Tu, elongation factor Tu; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; DTT, dithiothreitol; poly(U), poly(uridylic acid); poly(A), poly(adenylic acid).

[2] Throughout the text ternary complex denotes the species AA-tRNA-EF-Tu·GTP.

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