Comparison of the Properties of Cytoplasmic Poly(adenylic acid)-Containing RNA from Polysomal and Nonpolysomal Fractions of Murine Myeloma Cells†

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ABSTRACT: About 40% of the cytoplasmic poly(A)-containing RNA in exponentially growing murine myeloma cells is not found associated with polysomes. This RNA fraction has a size distribution and poly(A) content very similar to that of polysomal poly(A)-containing RNA. In pulse-chase experiments, some, but not all, of the nonpolysomal poly(A)-containing RNA is transferred into polysomes. The kinetics of accumulation of radioactive poly(A)-containing RNA in cytoplasmic fractions are inconsistent with a simple precursor:product relationship between nonpolysomal and polysomal poly(A)-containing RNA; alternative models for this relationship are discussed. A common feature of these models is the existence of translational control mechanisms in these cells.

In a variety of eucaryotic systems polysomal mRNA molecules which contain a sequence of poly(A) have been detected; in two mammalian cell lines, most polysomal mRNA molecules have been shown to contain such a sequence (Greenberg and Perry, 1972; Adesnik et al., 1972). This has provided the basis for convenient preparative methods, and much has been learned about the chemical and metabolic properties of polysomal poly(A)(+)RNA[1] (Greenberg and Perry, 1972; Adesnik et al., 1972; Darnell et al., 1973; Sheiness and Darnell, 1973; Perry et al., 1974). Nonpolysomal poly(A)(+)RNA has been identified in exponentially growing mouse L cells (LaTorre and Perry, 1973) and in nongrowing cultures of mouse fibroblasts (Rudland, 1974). In the latter system, transition from a resting to a growing state results in the transfer of nonpolysomal poly(A)(+)RNA into newly formed polysomes, suggesting that nonpolysomal poly(A)(+)RNA is an inactive (i.e., nontranslating) form of mRNA. Indirect evidence (Schochetman and Perry, 1972; LaTorre and Perry, 1973) suggests that a similar transfer is found when heat-treated L cells are returned to physiological conditions. Transfer of nonpolysomal poly(A)(+)RNA into polysomes in undisturbed cell cultures has not been reported. Thus, the identification of nonpolysomal poly(A)(+)RNA as inactive mRNA in exponentially growing cells is by no means well established. The present experiments were undertaken as a first step in the characterization of the relationship between nonpolysomal and polysomal poly(A)(+)RNA in murine myeloma cells.

Materials and Methods

Cells. The S-194-C1 2 line of γA immunoglobulin secreting murine myeloma cells was obtained from Dr. K. Horibata and grown in modified Eagle's medium containing 10% horse serum as described (Kimmel and Wang, 1972). For radioactive labeling of RNA, exponentially growing cultures were concentrated to 2–4 × 10^6 cells/ml in fresh medium. Labeled uridine or adenosine (New England Nuclear) was added to the concentrations indicated; unlabeled nucleoside was added to give a final concentration of 10^{-5} *M*.

Cell Fractionation. Cytoplasmic extracts were prepared with the nonionic detergent Nonidet P-40 (Shell Chemical Co.) as described (Kimmel and Wang, 1972), and treated with 1% sodium deoxycholate to release membrane-bound polysomes. Cytoplasmic extracts were layered over 5–20% (w/w) linear sucrose gradients prepared in low-salt buffer (5 m*M* NaCl, 5 m*M* KCl, 1.5 m*M* $MgCl_2$, 10 m*M* Tris-HCl (pH 7.4)) and centrifuged at 4° for the times indicated in the figure legends at 26,000 rpm in a Spinco SW 27 rotor (g_{av} = 88,150) or at 35,000 rpm in a Spinco SW 40 rotor (g_{av} = 151,900). Fractions were collected with monitoring of A_{260} through a flow-cell (Gilford Instrumentt).

RNA Isolation. Cytoplasmic extracts were diluted threefold with H_2O, adjusted to 0.1 *M* Tris (pH 9.0) and 1% sodium dodecyl sulfate, and then extracted with phenol as described by Brawerman et al. (1972). The final aqueous phase was adjusted to 1% sodium dodecyl sulfate and 0.1 *M* NaCl and the RNA was precipitated overnight at −20° with 2.5 vol of 95% ethanol. Poly(A)(+)RNA was prepared by chromatography on poly(U)-Sepharose as described (MacLeod, 1975).

Poly(U) Filtration. Poly(U) was coupled to glass fiber filters (Whatman GF/c) as described by Sheldon et al. (1972). Sucrose gradient fractions were adjusted to the salt and detergent concentrations of binding buffer (0.12 *M* NaCl, 0.1 m*M* EDTA, 0.5% sodium dodecyl sulfate, 10 m*M* Tris-HCl (pH 7.4)) and filtered slowly (<1 ml/min) through the poly(U) filters. After washing with binding buffer, filters were dried and counted in a toluene-based scintillation cocktail. As measured with [^{3}H]adenosine-labeled cytoplasmic RNA, poly(U) filters were about 25%

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[1] Abbreviations used are: poly(A)(+)RNA, RNA which contains a sequence of poly(A) as defined by its ability to bind to immobilized poly(U); HnRNA, heterogeneous nuclear RNA; poly(A)(−)RNA, RNA which lacks a sequence of poly(A) as defined by its inability to bind to immobilized poly(U); mlRNA, messenger-like RNA.

Table I: Subcellular Localization of Pulse-Labeled Poly(A)(+)RNA.[a]

Fraction	Radioact. in Poly(A)(−)RNA[b] (% of Total)	Radioact. in Poly(A)(+)RNA[c] (% of Total)
A, large polysomes	24.4	26.9
B, small polysomes	26.8	37.8
C, monoribosomes	18.5	19.3
D, subribosomal supernate	30.1	16.0

[a] Cells (6.4×10^7) were labeled for 3 hr with [5,6-^{3}H]uridine at 50 μCi/ml; cycloheximide (0.3 μg/ml) was present during the last 0.5 hr of labeling. Two-thirds of the cytoplasmic extract was sedimented through a 5–20% sucrose gradient for 1 hr at 26,000 rpm as described under Materials and Methods. RNA was prepared from fractions corresponding to large polysomes, small polysomes, monoribosomes, and subribosomal supernate and assayed by poly(U)–Sepharose chromatography. [b] Calculated from the radioactivity which was not bound to the poly(U)–Sepharose column. A total of 12.9×10^6 cpm was recovered from the four column runs. [c] Calculated from the radioactivity which was bound to and subsequently eluted from the poly(U)–Sepharose column. A total of 0.634×10^6 cpm was recovered from the four column runs.

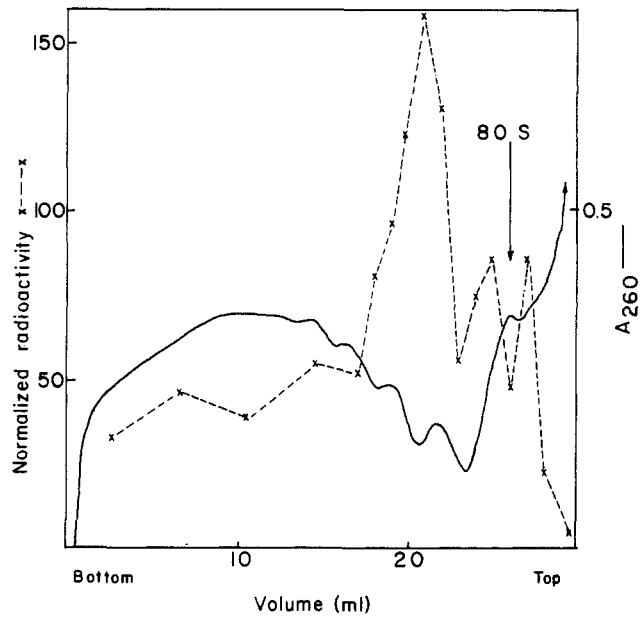

FIGURE 1: Distribution of poly(A)(+)RNA in a polysome gradient. For each fraction of the gradient in the experiment detailed in the legend to Figure 4, the amount of poly(A)(+)RNA labeled in a 2-hr pulse with [2-^{14}C]uridine was divided by the A_{260} of that fraction. This normalization procedure gives a measure of poly(A)(+)RNA per ribosome in the polysomal region of the gradient: (—) A_{260}; (x- - -x) normalized radioactivity in arbitrary units.

less efficient in binding poly(A)(+)RNA than were poly(U)-Sepharose columns.

Polyacrylamide Gel Electrophoresis and Sucrose Gradient Sedimentation. Electrophoresis of RNA through 3% polyacrylamide gels was performed as described (MacLeod, 1975). The frozen gels were cut into 1-mm slices with a Mickle gel slicer (Brinkmann Instruments) and each slice was dissolved as described by Kimmel and Wang (1972). The samples were partially neutralized with 0.5 ml of 0.1 *N* HCl and counted in a toluene–Triton X-100 scintillation cocktail (Triton X-100 and the scintillation cocktail recipe were obtained from Packard Instruments). Sedimentation of RNA through linear sucrose gradients was performed as described (MacLeod, 1975). The bottoms of the tubes were punctured and 0.4-ml fractions were collected, diluted to 1 ml with H_2O, and counted in toluene–Triton X-100.

Poly(A) Analysis. RNA samples were dissolved in RNase buffer (0.3 *M* NaCl, 10 m*M* Tris-HCl (pH 7.5)) and incubated for 1 hr at 37° with 5 μg/ml RNase A and 5 units/ml RNase T_1 (Perry et al., 1974). The digests were then incubated for 30 min at 37° with 0.15 mg/ml self-digested (1 hr at 37°) Pronase in the presence of 0.5% sodium dodecyl sulfate to destroy RNase activity. Undigested RNA was concentrated by precipitation with ethanol and analyzed by sucrose gradient sedimentation (16 hr at 35,000 rpm). One-half milliliter fractions were collected and processed for determination of radioactivity as above. All enzymes were obtained from Sigma Chemical Co.

Results

Existence of Nonpolysomal Poly(A)(+)RNA. To investigate the possible existence of cytoplasmic, nonpolysomal poly(A)(+)RNA in murine myeloma cells, the subcellular distribution of pulse-labeled poly(A)(+)RNA, as defined by its ability to bind to poly(U)–Sepharose, was determined in the experiment detailed in the legend to Table I. RNA was extracted from regions of a polysome gradient corresponding to large polysomes, small polysomes, monoribosomes, and the subribosomal supernate, and was assayed for total radioactivity and radioactivity which bound to poly(U)–Sepharose. All gradient fractions contain radioactive poly(A)(+)RNA, 35% of which is found to sediment no faster than monoribosomes (Table I, fractions C and D). Furthermore, fraction B, which corresponds to small polysomes, is enriched for poly(A)(+)RNA relative to fraction A, the large polysomes, by about 1.3-fold. This enrichment suggests the possibility that the cytoplasmic extract contains poly(A)(+)RNA which is not bound to polysomes but sediments in the region of a sucrose gradient which contains small polysomes.

In order to further analyze the distribution of poly(A)(+)RNA in polysome gradients, poly(U) filtration was used since with this method a number of samples can be analyzed simultaneously. Individual fractions of the polysome gradient shown in Figure 1 were adjusted to 0.12 *M* NaCl, 0.1 m*M* EDTA, and 0.5% sodium dodecyl sulfate and tested for their ability to bind to poly(U) filters. In Figure 1, the poly(U) filter-bound radioactivity in each fraction has been normalized to the ribosomal absorbance of the fraction to provide a measure of poly(A)(+)RNA per ribosome. It can be seen that this measure peaks in the region corresponding to polysomes containing less than four ribosomes. Two possible reasons for this phenomenon have been considered. One possibility is that cytoplasmic extracts contain relatively large, poly(A)(+)RNA containing particles which are nonpolysomal in origin but sediment in these gradients similarly to small polysomes. Data presented below demonstrate that this explanation is at least in part correct. A second possibility is that small polysomes have, on the average, a smaller number of ribosomes per unit length of mRNA than large polysomes. In order to minimize such effects, the experiments have been carried out with cells exposed to cycloheximide for 30 min prior to harvesting. At the concentration used, this reagent has the effect of increasing the fraction of ribosomes in polysomes and increasing the average number of ribosomes per polysome (M. C. MacLeod, unpublished results). This is consistent with its known effects in other systems (Godchaux et al., 1967) where the rate of polypeptide elongation is reduced without

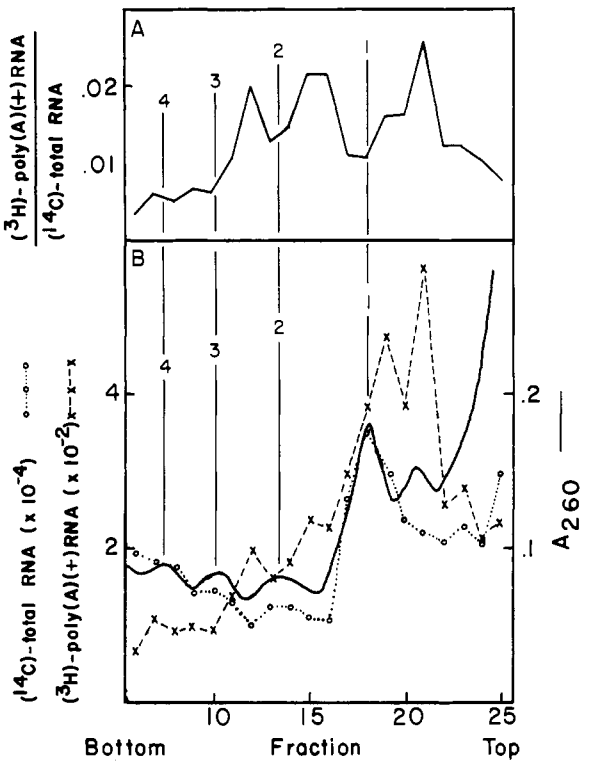

FIGURE 2: Subcellular distribution of 30-min pulse-labeled poly(A)(+)RNA. Cells were labeled for 19 hr with [2-^{14}C]uridine at 41.7 nCi/ml. After resuspension in fresh medium, 2.95 × 10^7 cells were labeled for 30 min with [5,6-^{3}H]uridine at 50 μCi/ml in the presence of 0.3 μg/ml of cycloheximide. The cytoplasmic extract was treated as described in the footnotes to Table I except that centrifugation was for 2 hr. Each 1-ml fraction was assayed for total radioactivity and radioactivity bound to poly(U) filters: (A) ratio of ^{3}H radioactivity bound to poly(U) filters to total ^{14}C radioactivity; (B) (x- - -x) ^{3}H radioactivity bound to poly(U) filters; (O···O) total ^{14}C radioactivity; (—) A_{260}. The vertical lines mark the positions of successive ribosomal absorbance peaks.

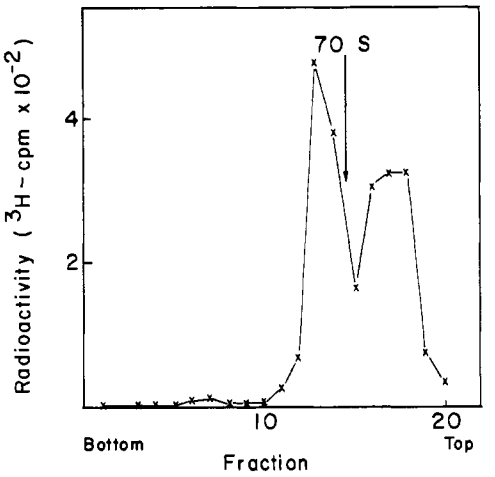

FIGURE 3: Effect of EDTA on small polysomal poly(A)(+)RNA. Cells were labeled for 30 min with [2,8-^{3}H]adenosine (50 μCi/ml) in the presence of cycloheximide. The cytoplasmic extract was centrifuged for 90 min through a 5–20% sucrose gradient and the fractions corresponding to small polysomes (nominally 95–175 S) were pooled and adjusted to 5 m*M* EDTA. One-half of this preparation was centrifuged through a 15–30% sucrose gradient made in a buffer containing 5 m*M* EDTA, 5 m*M* NaCl, 5 m*M* KCl, and 10 m*M* Tris-HCl (pH 7.4) for 3 hr at 26,000 rpm, and fractions of this gradient were assayed for poly(A)(+)RNA as described under Materials and Methods. The arrow at 70 S was positioned by linear extrapolation from the positions of the large (50 S) and small (30 S) ribosomal subunits determined from the absorbance profile.

a decrease in the rate of initiation. Thus, one expects differences in the number of ribosomes per unit length of mRNA to be minimized in cycloheximide-treated cells. In fact, curves similar to that shown in Figure 1 are also obtained in experiments without cycloheximide treatment (M. C. MacLeod, unpublished results).

In order to more precisely localize the putative nonpolysomal poly(A)(+)RNA, the polyribosome gradient shown in Figure 2B was centrifuged for 2 hr to partially separate polysomes containing two, three, and four ribosomes. In this experiment cells were exposed to [^{3}H]uridine for 30 min, a pulse time which preferentially labels nonpolysomal poly(A)(+)RNA (see below). Figure 2B presents the results of analyzing each fraction of the gradient for total ^{14}C radioactivity (dotted line) and ^{3}H radioactivity bound to poly(U) filters (broken line). (More than 98% of the radioactivity in long-term ^{14}C-labeled cytoplasmic RNA is in rRNA and tRNA (M. C. MacLeod, unpublished results). Thus, total ^{14}C radioactivity is coincident with the absorbance profile and can be used as a relative measure of rRNA.) It can be seen that the peaks of poly(U) filter-bound ^{3}H radioactivity in the region corresponding to small polysomes do not coincide with the polysomal peaks, measured either by absorbance or by total ^{14}C radioactivity. This noncoincidence is also clearly seen in Figure 2A, where the poly(U) filter-bound ^{3}H radioactivity has been normalized to the total ^{14}C radioactivity of each fraction, again providing a measure of radioactive poly(A)(+)RNA per ribosome. Thus, at least a portion of the pulse-labeled poly(A)(+)RNA in this region of the gradient (nominal sedimentation coefficients of 95–175 S) is not found associated with polysomes.

Treatment of polysomes with EDTA has been shown to result in the formation of 30S and 50S ribosomal subunits and in the release of mRNA as ribonucleoprotein particles with sedimentation coefficients less than 70 S (Perry and Kelley, 1968). Thus, if all of the poly(A)(+)RNA in the small polysomal region of the gradients described above was in fact polysomal, treatment with EDTA should result in a shift of all the radioactivity from >70 S to <70 S. Therefore, the small polysomal region of a gradient similar to those described above was isolated and treated with 5 m*M* EDTA. This preparation was then centrifuged through a second sucrose gradient containing EDTA and individual fractions were tested for poly(U) filter binding with the results presented in Figure 3. The distribution of radioactive poly(A)(+)RNA is bimodal, with 55% of the radioactivity sedimenting in the 20–70 S region. The remaining 45% of the radioactive poly(A)(+)RNA remains larger than 70 S and is therefore identified as being nonpolysomal in origin.

Size Distribution and Poly(A) Content of Poly(A)(+)RNA Fractions. The finding (Sheldon et al., 1972) that some HnRNA[1] molecules contain a sequence of poly(A) has been suggested by some workers to provide further evidence supporting a precursor:product relationship between HnRNA and mRNA (Darnell et al., 1973; La-Torre and Perry, 1973). The same reasoning suggests that polysomal poly(A)(+)RNA (i.e., mRNA) and nonpolysomal poly(A)(+)RNA may also be closely related metabolically. It was thus of interest to compare the size distributions of polysomal and nonpolysomal poly(A)(+)RNA and of the poly(A) component of these fractions.

In order to compare the size distributions of polysomal

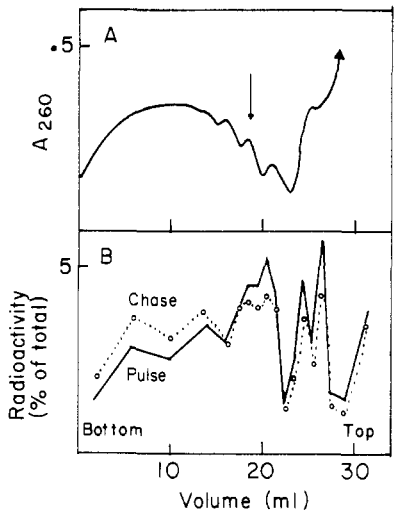

FIGURE 4: Subcellular distribution of poly(A)(+)RNA in a pulse-chase. A single culture containing 4.8×10^7 cells was divided in half. One-half was labeled for 2 hr with [5,6-^{3}H]uridine at 10 μCi/ml, then harvested, resuspended in fresh medium containing 10^{-4} *M* unlabeled uridine, and incubated for a further 2 hr. During this chase, the second half of the original culture was labeled for 2 hr with [2-^{14}C]uridine at 1 μCi/ml. For both labeling periods, the final concentration of uridine was 10^{-5} *M* and the cell density was 2.4×10^6 cells per ml. One-half hour before the end of the labeling period (or the chase period), cycloheximide was added to both cultures at 0.3 μg/ml. The cytoplasmic extracts were mixed and fractionated by sucrose gradient sedimentation at 26,000 rpm for 75 min: (A) absorbance profile. (B) gradient fractions were assayed for poly(A)(+)RNA by poly(U) filtration as described under Materials and Methods; (O···O) ^{3}H radioactivity bound to poly(U) filters; (—) ^{14}C radioactivity bound to poly(U) filters. The arrow in A marks the position of polysomes containing three ribosomes.

and nonpolysomal poly(A)(+)RNA, cells were labeled for 3 hr with [^{3}H]uridine and RNA was extracted from the large polysomal and the subribosomal regions of a sucrose gradient. Poly(A)(+)RNA was prepared from both fractions by chromatography on poly(U)-Sepharose and aliquots were analyzed by sedimentation through sodium dodecyl sulfate containing sucrose gradients and by polyacrylamide gel electrophoresis. Both polysomal and nonpolysomal poly(A)(+)RNA display heterodisperse size distributions which are largely overlapping. By both techniques, nonpolysomal poly(A)(+)RNA is slightly enriched for lower molecular weight species relative to polysomal poly(A)(+)RNA. The size distributions of both RNA fractions are closely similar to the size distribution of total cytoplasmic poly(A)(+)RNA reported in detail previously (MacLeod, 1975).

To compare the size distributions of the poly(A) components of the polysomal and nonpolysomal poly(A)(+)RNA, cells were labeled for 2 hr with a mixture of [^{3}H]adenosine and [^{14}C]uridine, and poly(A)(+)RNA was prepared from large polysomes, small polysomes, and subribosomal particles. Poly(A) was prepared from each of these RNA fractions by digestion at high ionic strength with a mixture of RNase T_1 and RNase A, and the size distributions of these poly(A) preparations were determined by sucrose gradient sedimentation. No [^{14}C]uridine-labeled RNA was recovered in these gradients, confirming the specificity of the digestion procedure. [^{3}H]Adenosine-labeled poly(A) sequences in all three fractions sedimented with a modal sedimentation coefficient of 4 S. A similar sedimentation pattern has been reported for poly(A) sequences obtained from mRNA in sarcoma 180 cells (Lee et al., 1971). Thus, by these two criteria nonpolysomal poly(A)(+)RNA is similar to polysomal poly(A)(+)RNA and the possibility of a close metabolic relationship between the two fractions is left open.

Labeling Kinetics. To investigate the possibility of a metabolic relationship between polysomal and nonpolysomal poly(A)(+)RNA two types of experiments have been done: pulse-chase and continuous labeling. In the first type, a culture of exponentially growing cells was divided in half; one half was pulse labeled for 2 hr before harvesting and the other half was pulse labeled for 2 hr and then chased for 2 hr with a tenfold excess of unlabeled precursor before harvesting. Preliminary experiments demonstrated that under the latter conditions there is a small increase in radioactivity incorporated into cytoplasmic poly(A)(+)RNA (10–15%) during the chase period. Since under the same chase conditions, net incorporation into nuclear RNA ceased within 10 min of chase (C. Kimmel and S. Sessions, manuscript submitted for publication) a large part of this increase must be due to transfer of prelabeled nuclear RNA precursors to the cytoplasm. Figure 4 presents the distribution of radioactive poly(A)(+)RNA in a polysome gradient for both labeling conditions. The profiles are similar, showing coincidence of peaks, but with a small shift toward higher sedimentation coefficients during the chase period. If one arbitrarily selects the position of polysomes containing three ribosomes (arrow in Figure 4A) as a dividing line, then during the chase about 10% of the radioactivity shifts from structures sedimenting slower than this marker into structures sedimenting faster than this marker. Although the magnitude of this shift is rather small, it has consistently been observed in three independent experiments. Since essentially all nonpolysomal poly(A)(+)RNA sediments more slowly than triribosomes while most polysomal poly(A)(+)RNA sediments faster than triribosomes, this finding strongly suggests that a fraction of the nonpolysomal poly(A)(+)RNA is, in fact, a material precursor to polysomal poly(A)(+)RNA. This conclusion is consistent with the results of the continuous labeling experiment presented below.

To investigate the labeling kinetics of cytoplasmic poly(A)(+)RNA, a culture of exponentially growing cells was labeled with [^{14}C]uridine for almost one generation time and then divided into six aliquots. Aliquots were labeled with [^{3}H]uridine for varying periods of time (up to 2 hr), and the distribution of radioactive poly(A)(+)RNA in the polysome gradient prepared from each aliquot of cells was determined. Each gradient was then divided into three regions corresponding to: (I) polysomes containing four or more ribosomes; (II) polysomes containing less than four ribosomes and monoribosomes; and (III) structures sedimenting more slowly than monoribosomes. The total ^{3}H radioactivity in poly(A)(+)RNA was summed for each region of each gradient. The percentage of total cytoplasmic poly(A)(+)RNA radioactivity in each region is plotted as a function of time of labeling in Figure 5. It can be seen that radioactivity first accumulates in regions II and III, that is, in those regions which contain nonpolysomal poly(A)(+) RNA. Only after 15–30 min does a significant amount of radioactivity appear in region I. In order to quantitate this difference, for each gradient the ^{3}H radioactivity in poly(A)(+)RNA in each region was divided by the total ^{14}C radioactivity in poly(A)(+)RNA in the gradient. This procedure normalizes for differences in the number of cells used to prepare each cytoplasmic extract and controls for

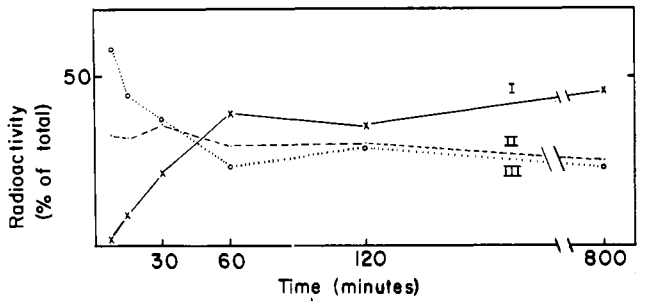

FIGURE 5: Subcellular distribution of poly(A)(+)RNA as a function of labeling time. A culture of cells growing exponentially (generation time = 19 hr) was labeled for 800 min with [2-^{14}C]uridine (5.2 nCi/ml) and then harvested and resuspended in fresh medium. At intervals, aliquots of the culture were mixed with equal volumes of warm medium containing [5,6-^{3}H]uridine to give a final concentration of 50 μCi/ml. Two hours after the first culture was exposed to [^{3}H]uridine, labeling of all cultures was terminated by the addition of ice-cold medium, cytoplasmic extracts were prepared, fractionated as described in the legend to Table I, and assayed for poly(A)(+)RNA as described under Materials and Methods. For each gradient, the amount of ^{3}H radioactivity in poly(A)(+)RNA in regions I, II, and III (see text) was divided by the total amount of ^{3}H radioactivity in the gradient: (x—x) region I; (·· - ··) region II; (O···O) region III.

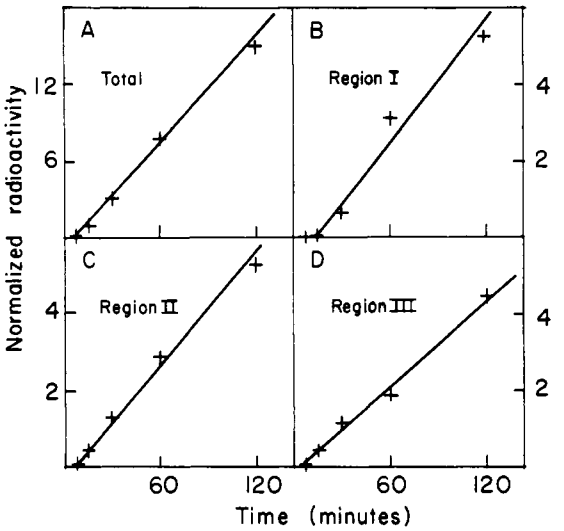

FIGURE 6: Kinetics of incorporation into poly(A)(+)RNA fractions. The ^{3}H radioactivity in poly(A)(+)RNA in regions I, II, and III of the gradients described in the legend to Figure 5 was normalized to the total ^{14}C radioactivity in poly(A)(+)RNA of each gradient: (A) total; (B) region I; (C) region II; (D) region III.

possible differences in poly(U) filter-binding efficiency between the gradients. The normalized data are presented in Figure 6. It can be seen that in each region ^{3}H radioactivity in poly(A)(+)RNA accumulates with apparently linear kinetics after a lag which is approximately 15 min for region I (Figure 6B) and 7.5 min or less for regions II and III (Figures 6C and 6D, respectively). Analysis of these data in terms of various models of the relationship between polysomal and nonpolysomal poly(A)(+)RNA will be reserved for the Discussion section.

Discussion

The data presented above clearly demonstrate the existence of nonpolysomal poly(A)(+)RNA in cytoplasmic extracts of exponentially growing murine myeloma cells. A portion of this RNA is found to sediment more slowly than monoribosomes, and is therefore considered to be nonpolysomal by definition. The existence of nonpolysomal poly(A)(+)RNA in the region of a sucrose gradient which contains small polysomes was inferred from the increased amount of poly(A)(+)RNA per ribosome found in this region compared to the large polysomal region (Figure 1). This was confirmed by the demonstration that almost 50% of the poly(A)(+)RNA in this region retains sedimentation coefficients greater than 70 S in the presence of concentrations of EDTA which disrupt polysomes (Perry and Kelley, 1968). Furthermore, short-term labeled poly(A)(+)RNA does not sediment in coincidence with small polysomes, but shows peaks of radioactivity between the absorbance peaks corresponding to monoribosomes, diribosomes, and triribosomes (Figure 2).

The size distribution of the nonpolysomal poly(A)(+)-RNA and of its poly(A) component is similar to that of polysomal poly(A)(+)RNA. The absence of species with sedimentation coefficients >45 S makes it unlikely that leakage of HnRNA during the preparation of the cytoplasmic extracts contributes significantly to this fraction. Furthermore, attempts to demonstrate nuclear leakage by mixing radioactively labeled nuclear pellets with unlabeled cells gave negative results (Kimmel and Larrabee, 1974; M. C. MacLeod, unpublished results). The failure to find RNA species with sedimentation coefficients of 35 or 70 S suggests that this RNA fraction is not contaminated with RNA species derived from endogenous C-type virus (Fan and Baltimore, 1973; Ihle et al., 1974). Mitochondria are known to produce a number of species of poly(A)(+)RNA (Hirsch et al., 1974). However, based on the recovery of 12S mitochondrial rRNA in the cytoplasmic poly(A)(−)-RNA fraction (MacLeod, 1974), mitochondrial poly(A)(+)RNA should account for less than 5% of the nonpolysomal poly(A)(+)RNA.

The possibility exists that nonpolysomal poly(A)(+)-RNA arises artifactually during the preparation of cytoplasmic extracts by release of polysomal poly(A)(+)-RNA from a particularly labile class of polysomes. Although this possibility cannot be strictly ruled out, three lines of evidence suggest that this is not the case. First, planimeter tracings of typical absorbance profiles show that 80% of the ribosmal absorbance is in polysomes. Thus, the hypothetical labile polysomes would contain a maximum of 20% of the cytoplasmic ribosomes but about 40% of the cytoplasmic poly(A)(+)RNA. This is unlikely since, as discussed above, all messages should be fully loaded with ribosomes due to the use of cycloheximide-treated cells. Second, preparation of polysomes in a buffer of high ionic strength is known to dissociate nonfunctional ribosomes (Zylber and Penman, 1970) and might be expected to increase the breakdown of labile polysomes, and hence the release of poly(A)(+)RNA. Contrary to this expectation, preparation of polysomes in a buffer containing 500 m*M* NaCl did not change the yield of nonpolysomal poly(A)(+)RNA compared to preparation in standard low-salt buffer (5 m*M* NaCl) (M. C. MacLeod, unpublished results). Finally, extracts of cells which were not treated with cycloheximide have a lower average number of ribosomes per polysome, and might therefore be expected to be more labile. Such extracts did not contain more nonpolysomal poly(A)(+)RNA than did extracts of cycloheximide-treated cells (M. C. MacLeod, unpublished results). These data and the mildness of the cell lysis procedure suggest that the nonpolysomal poly(A)(+)RNA is a bona fide cytoplasmic RNA fraction which is functionally distinct from polysomal poly(A)(+)RNA.

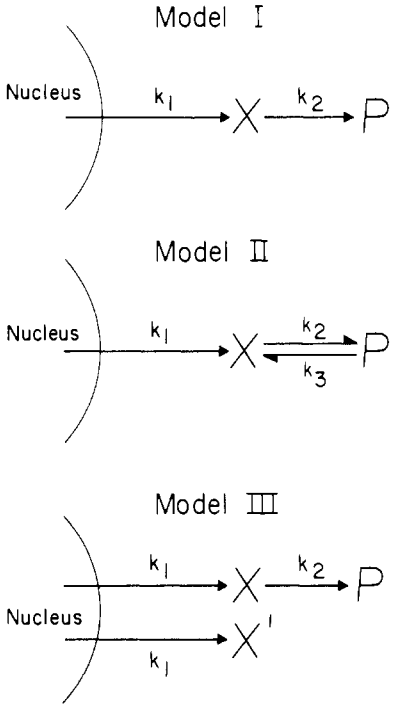

FIGURE 7: Models. In this figure, X represents nonpolysomal poly(A)(+)RNA and P represents polysomal poly(A)(+)RNA. k_1 is the growth rate constant of the cells and k_2 and k_3 are the forward and backward rate constants, respectively, for transfer of poly(A)(+)RNA from fraction X to fraction P. In model II, fraction X contains both newly made and previously translated poly(A)(+)RNA. In model III, fraction X′ is conceived to be inactive poly(A)(+)RNA.

Nonpolysomal RNA fractions with many characteristics similar to polysomal mRNA have been identified in human HeLa cells (Spohr et al., 1970), mouse L cells (Schochetman and Perry, 1972), mouse 3T3 cells (Rudland, 1974), and duck erythroblasts (Spohr et al., 1972), and Scherrer et al. (1970) have suggested the designation messenger-like RNA (mlRNA) for such RNA fractions. The functional significance of mlRNA is unknown. Scherrer and Marcaud (1968) have proposed a theory of "cascade regulation" in which mlRNA is postulated to be permanently inactive mRNA. However, in certain situations it has been possible to demonstrate the transfer of mlRNA into polysomes (Schochetman and Perry, 1972; Rudland, 1974), suggesting the possibility that mlRNA may be a precursor to mRNA. It was thus of interest to compare the kinetics of incorporation of labeled precursor into polysomal and nonpolysomal poly(A)(+)RNA (Figure 6) with the predictions of three relatively simple models for the relationship between these fractions diagrammed in Figure 7. Briefly, in model I mlRNA (designated X in Figure 7) is postulated to be a simple precursor to mRNA (designated P). In model II, the precursor:product relation still holds, but mRNA is postulated to exchange with mlRNA. In model III, part of the mlRNA is a precursor to mRNA, while the rest is inactive (designated X′), that is, not able to enter polysomes. In order to compare the predictions of these models with the experimental results, nonpolysomal poly(A)(+)RNA has been equated with mlRNA and polysomal poly(A)(+)RNA with mRNA. The differential equations implied by these models and their solution in terms of the parameter P^*/A^* = fraction of radioactive cytoplasmic poly(A)(+)-RNA which is polysomal are given in the Appendix. The derivation is based on a number of assumptions which have been justified in detail elsewhere (MacLeod, 1974). Briefly, the assumptions give rise to equations which fit the experimental data for the accumulation of radioactivity in total nuclear RNA, cytoplasmic poly(A)(−)RNA, and cytoplasmic poly(A)(+)RNA.

In order to calculate the value of P^*/A^* as a function of time from the data of Figure 6 it is necessary to estimate the polysomal contribution to the poly(A)(+)RNA in region II, that is the small polysomal region. This has been done by assuming that the incorporation into small polysomal poly(A)(+)RNA follows the same kinetics as the incorporation into large polysomal poly(A)(+)RNA and that the amount of polysomal poly(A)(+)RNA per ribosome is the same in the two regions. By using the ^3H radioactivity in poly(A)(+)RNA divided by the total ^{14}C radioactivity in each region as a measure of poly(A)(+)RNA per ribosome, it is estimated that the polysomal contribution to the ^3H radioactivity in the poly(A)(+)RNA of region II is 0 at 7.5 min, 1/8 at 15 min, 1/4 at 30 min, and 1/2 at times greater than or equal to 60 min. The values of P^*/A^* calculated using these estimates along with the value of P^*/A^* at 800 min calculated from the ^{14}C data are plotted in Figure 8 (crosses) with the theoretical best fit curves generated by the three models (solid lines). It can be seen that although all three models adequately fit the early time points, the theoretical value of P^*/A^* at 800 min calculated from model I (0.974) differs significantly from the experimental value (0.610).[2] A similar analysis of the results of pulse-chase experiments (MacLeod, 1974) was also found to be incompatible with model I. It can be concluded that the relationship between mlRNA and mRNA is more complicated than a simple precursor:product relation. The experimental results do not distinguish between models II and III; in addition, more complicated models would undoubtedly be found to fit the results.

The close similarity between nonpolysomal and polysomal poly(A)(+)RNA in terms of size distribution, poly(A) content, and kinetics of appearance in the cytoplasm strongly suggests that nonpolysomal poly(A)(+)RNA is inactive mRNA, that is, mRNA which is unable to initiate protein synthesis either temporarily (model II) or permanently (model III). Thus, a common feature of these models is the existence of translational control in eucaryotic cells. The molecular basis of translational control in this system and the question of its reversibility remain to be elucidated.

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[2] In formulating the equations in the appendix, rate constants for normal degradation of poly(A)(+)RNA have been left out. This corresponds to assuming that the half-lives of total cytoplasmic poly(A)(+)RNA and polysomal poly(A)(+)RNA are equal. If one assumes that they are not equal, rate constants can be chosen so as to obtain a good fit between the modified equation for model I and the experimental data. However, such solutions lead to a half-life of less than 1 hr for polysomal poly(A)(+)RNA. This is inconsistent with the measured half-lives of polysomal poly(A)(+)RNA in L cells (Greenberg, 1972), HeLa cells (Singer and Penman, 1973), and myeloma cells (Cowan and Milstein, 1974); the minimum half-life found in these studies was 7 hr. Thus, model I cannot be "rescued" by including terms for poly(A)(+)RNA degradation in the equations.

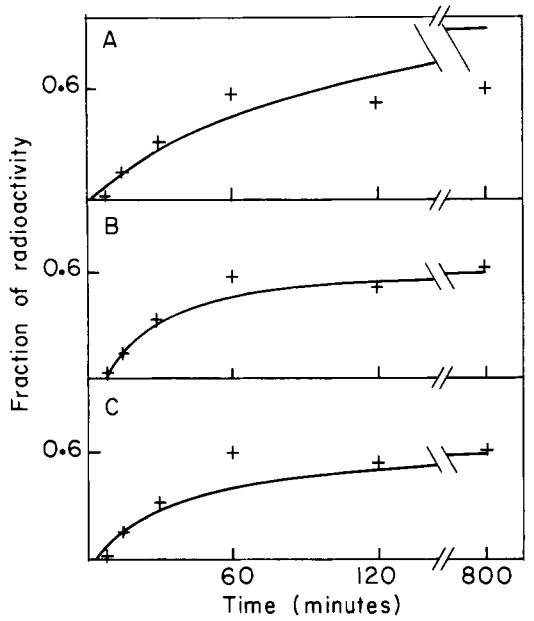

FIGURE 8: Fraction of cytoplasmic poly(A)(+)RNA which is polysomal. The fraction of the radioactivity in total cytoplasmic poly(A)(+)RNA which was polysomal at each time point was calculated as described in the text, and is plotted in each panel (crosses). The solid lines are theoretical, best-fit curves derived (see Appendix) from model I (A), model II (B), and model III (C).

Appendix

In formulating and solving the differential equations implied by the models of Figure 7, the following quantities will be used: A = amount of total cytoplasmic poly(A)(+)RNA at time t; X = amount of nonpolysomal poly(A)(+)RNA at time t; P = amount of polysomal poly(A)(+)RNA at time t; X' = amount of nonpolysomal poly(A)(+)RNA at time t which is "inactive", i.e., not able to enter polysomes; A_0 = amount of total cytoplasmic poly(A)(+)RNA at $t = 0$, and similarly for X_0, P_0, and X'_0; A^* = amount of radioactivity in total cytoplasmic poly(A)(+)RNA at time t, and similarly for X^*, P^*, and X'^*; k_1, k_2, and k_3 are first-order rate constants as defined in Figure 7. By definition, $A = X + P$ and $A^* = X^* + P^*$ for models I and II, and $A = X + P + X'$ and $A^* = X^* + P^* + X'^*$ for model III.

In an exponentially growing culture with growth rate constant k_1, if the amount of A per cell is constant, then the total amount of A per culture is $A = A_0e^{k_1t}$. If radioactive precursor is added to the culture at $t = 0$, then $A^* = sA_0(e^{k_1t} - 1)$, where s is the specific activity of the intracellular precursor to A. For the derivations given below, it is assumed that s is constant and the units of s are chosen so that $s = 1$ (ideal pulse). By inspection, the equations for the rate of change of P^* for the three models of Figure 7 are:

Model I

$$dP^*/dt = k_2X^* = k_2(A^* - P^*)$$

Model II

$$dP^*/dt = k_2X^* - k_3P^* = k_2A^* - (k_2 + k_3)P^*$$

Model III

$$dP^*/dt = k_2X^* = k_2(A^* - P^* - X'^*)$$

(Note that since X' in model III is not postulated to be a precursor to anything, $X'^* = X'_0(e^{k_1t} - 1)$.) These first-order, linear differential equations can be solved using standard Laplace Transforms to yield the following:

Model I

$$\frac{P^*}{A^*} = \frac{[k_2/(k_1 + k_2)](e^{k_1t} - e^{-k_2t}) - (1 - e^{-k_2t})}{e^{k_1t} - 1}$$

Model II

$$\frac{P^*}{A^*} = \frac{\dfrac{k_2e^{k_1t}}{k_1 + k_2 + k_3} + \dfrac{k_1k_2e^{-(k_2 + k_3)t}}{(k_2 + k_3)(k_1 + k_2 + k_3)} - \dfrac{k_2}{k_2 + k_3}}{e^{k_1t} - 1}$$

Model III

$$\frac{P^*}{A^*} = \frac{(A_0 - X'_0)}{A_0} \frac{[k_2/(k_1 + k_2)](e^{k_1t} - e^{-k_2t}) - (1 - e^{-k_2t})}{e^{k_1t} - 1}$$

As t becomes very large, e^{-k_2t} approaches zero and e^{k_1t} becomes much greater than 1. Therefore, at steady state the values of P^*/A^* approach the following limits:

Model I

$$k_2/(k_1 + k_2)$$

Model II

$$k_2/(k_1 + k_2 + k_3)$$

Model III

$$[(A_0 - X'_0)/A_0][k_2/(k_1 + k_2)]$$

If one assumes that the experimental value of 0.61 for P^*/A^* at 800 min is close to steady state, then the rate constant k_2 in model I is determined and in models II and III the ratio of k_2 to the third constant is determined. Reasonable values of k_2 can then be used to calculate P^*/A^* and the resulting curve can be compared with the experimental values. In all cases, the theoretical curves were shifted to the right along the time axis to give a 7.5-min lag. For models II and III this method produced a very good fit to the experimental data (Figure 8), with the following constants: model II, $k_2 = 0.04627$ min^{-1}; $k_3 = 0.03024$ min^{-1}; model III, $k_2 = 0.04627$ min^{-1}; $(A_0 - X'_0)/A_0 = 0.631$. For model I, k_2 determined from the steady state was approximately 0.0009 min^{-1}. This was much too slow to give a good fit to the early time points in Figure 8. The best fit was obtained with $k_2 = 0.0231$ min^{-1}, but this implied a steady-state value for P^*/A^* of 0.974.

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DNA Chain Growth during Replication of Asynchronous L1210 Cells. Alkaline Sedimentation Studies†

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ABSTRACT: The growth of replicating DNA chains was studied in log-phase L1210 cells by an alkaline sucrose gradient sedimentation method which avoids shearing or degradation of the DNA. Three distinct phases of chain growth were identified: (1) rapid joining of short (<4 S) segments to form intermediate size (approximately 25 S) segments; (2) continuous growth from the 25S stage to 50S–70S pieces which may represent completed replicons; and (3) the discontinuous joining of completed replicons to form larger strands that sediment in an aggregate. The first phase was completed within 2–3 min, the second extended over about 15 min, and the third required about 2 hr.

The sequence of chain growth in the replication of nuclear DNA in eukaryotic cells appears to have two points of discontinuity. The first, revealed by alkaline sucrose gradient sedimentation after short pulse labeling (Schandl and Taylor, 1969; 1971; Sato et al., 1970; Nuzzo et al., 1970; Goldstein and Rutman, 1973), may be analogous to the joining of "Okazaki fragments" in bacteria and viruses (Okazaki et al., 1968). This step may involve the replication of all or only part of the DNA in eukaryotic cells. The second, revealed by autoradiography, occurs much later in the course of replication and involves the joining of completed replicons (Huberman and Riggs, 1968; Amaldi et al., 1972; Huberman and Tsai, 1973).

In order to examine the effects of physiological perturbations and drugs on replication (Hori and Lark, 1973; Gautschi et al., 1973), it is desirable to be able to observe the kinetics of chain growth in a quantitative way. With this in mind, we have used alkaline sedimentation to study the sequence of chain growth events from the joining of short strands to the joining of replicons. The procedure used in this study has avoided shear or degradation of the DNA and provides a clearer view of the chain growth sequence than has previously been reported.

Materials and Methods

Cells. L1210 cells (Moore et al., 1966) were grown in suspension culture in RPMI 1630 medium with 20% heated fetal calf serum, plus penicillin and streptomycin. Stock cultures were maintained in static bottles without antibiotics and were used to initiate suspension cultures at weekly intervals. Cultures were periodically tested for PPLO and were free of contamination. Suspension cultures proliferated logarithmically up to $1.5–2.5 \times 10^6$/ml with a doubling time of 12–14 hr; cells harvested for the experiments were at a density of approximately 1.0×10^6/ml.

Alkaline Sedimentation. Cells labeled with [2-^{14}C]thymidine or [*methyl*-^{3}H]thymidine were diluted with 20 volumes of ice-cold PBS (phosphate-buffered saline), centrifuged at 1000 rpm for 5 min at 4°, and resuspended in 0.5–1 ml of cold PBS. Between 0.5 and 1.0×10^6 cells were slowly pipetted onto a 1.0–1.5 ml layer of 0.45 *M* NaOH, 0.55 *M* NaCl, and 0.01 *M* EDTA overlying 30–33 ml of a 5–20% sucrose gradient in 0.1 *M* NaOH, 0.9 *M* NaCl, and

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