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Cellular Titers and Subcellular Distributions of Abundant Polyadenylate-Containing Ribonucleic Acid Species During Early Development in the Frog *Xenopus laevis*

MARK B. DWORKIN* AND JOHN W. B. HERSHEY

Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616

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The distribution of cytoplasmic messenger ribonucleic acids (RNAs) in translationally active polysomes and inactive ribonucleoprotein particles changes during early development. Cellular levels and subcellular distributions have been determined for most messenger RNAs, but little is known about how individual sequences change. In this study, we used hybridization techniques with cloned sequences to measure the titers of 23 mitochondrial and non-mitochondrial polyadenylate-containing [poly(A)$^+$] RNA species during early development in the frog *Xenopus laevis*. These RNA species were some of the most abundant cellular poly(A)$^+$ RNA species in early embryos. The concentrations of most of the non-mitochondrial (cytoplasmic) RNAs remained constant in embryos during the first 10 h of development, although the concentrations of a few species increased. During neurulation, we detected several new poly(A)$^+$ RNA sequences in polysomes, and with one possible exception the accumulation of these sequences was largely the result of new synthesis or de novo polyadenylation and not due to the recruitment of nonpolysomal (free ribonucleoprotein) poly(A)$^+$ RNA. We measured the subcellular distributions of these RNA species in polysomes and free ribonucleoproteins during early development. In gastrulae, non-mitochondrial RNAs were distributed differentially between the two cell fractions; some RNA species were represented more in free ribonucleoproteins, and others were represented less. By the neurula stage this differential distribution in polysomes and free ribonucleoproteins was less pronounced, and we found species almost entirely in polysomes. Some poly(A)$^+$ RNA species transcribed from the mitochondrial genome were localized within the mitochondria and were mapped to discrete fragments of the mitochondrial genome. Much of this poly(A)$^+$ RNA was transcribed from the ribosomal locus. Nonribosomal mitochondrial poly(A)$^+$ RNA species became enriched in polysome-like structures after fertilization, with time courses similar to the time course of mobilization of cytoplasmic poly(A)$^+$ RNA.

Unfertilized eggs of animals contain substantial amounts of messenger-like ribonucleic acid (RNA) that is not associated with ribosomes. Upon fertilization, this maternal messenger RNA (mRNA) is mobilized into polysomes and acts as a template for the large increase in protein synthesis that accompanies fertilization. In sea urchin embryos, the activation process occurs without concomitant RNA synthesis (12). Histone mRNA has been studied in particular, and mobilization rather than new histone mRNA synthesis is responsible for the increase in histone synthesis after activation or fertilization in several animals (28, 30, 38). In frog embryos the level of new RNA synthesis is not substantial during the first few hours after fertilization (7). Nevertheless, qualitative changes in the pattern of protein synthesis during this period have been reported (5, 6). Thus, the formation of polysomes after fertilization may involve specific regulation as well as overall mobilization of maternal mRNA.

To study regulation during embryogenesis we constructed a recombinant deoxyribonucleic acid (DNA) library with double-stranded complementary DNAs (cDNA's) prepared from stage 10 gastrula and stage 41 tadpole polyadenylate-containing [poly(A)$^+$] RNAs from the frog *Xenopus laevis* (18). Several hundred cloned sequences have been characterized by colony hybridization to probes prepared from poly(A)$^+$ RNAs from developmental stages and subcellular fractions (19). We found a very high degree of similarity among the relatively abun-

dant poly(A)$^+$ RNA sequences in eggs, cleaving embryos, and gastrulae, and we also found that a qualitatively new population of poly(A)$^+$ RNA was predominant in embryos at later stages of development. In our analysis of polysomal and nonpolysomal poly(A)$^+$ RNAs, we found that species which were predominantly polysomal at one stage of development were also predominantly polysomal at other stages, if they were present at all. We also described some poly(A)$^+$ RNA species which were predominantly nonpolysomal in gastrulae, and these sequences never become predominantly polysomal in tadpoles. However, we discovered that most of the abundant poly(A)$^+$ RNA species which have been described or studied in detail are transcribed from the mitochondrial genome (M. B. Dworkin, B. Kay, J. W. B. Hershey, and I. B. Dawid, Dev. Biol., in press). It was estimated that the mitochondrial cloned sequences account for about 10% of the total embryonic poly(A)$^+$ RNA in eggs and gastrulae (Dworkin et al., in press).

In this work we studied 19 sequences which were not homologous to mitochondrial DNA and were highly or moderately abundant in embryos. The titers of poly(A)$^+$ RNA species were determined by hybridization techniques that were more accurate than colony hybridization techniques. We determined the RNA levels in cytoplasmic extracts during the first 3 days of development, as well as in polysomal and nonpolysomal subcellular fractions in gastrulae and neurulae. On the basis of this work, we began to describe the kinds of poly(A)$^+$ RNAs that contribute to the large amount of nonpolysomal poly(A)$^+$ RNA in early embryos. We also examined more closely the mitochondrial sequences that were described recently (Dworkin et al., in press). We compared these sequences with the sequences described previously by Rastl and Dawid (25) from ovarian mitochondria and measured the subcellular distributions of these RNAs during the first 10 h of development.

MATERIALS AND METHODS

Preparation of RNA and cDNA. Embryos were harvested at stage 10 (10- to 11-h gastrulae), stage 20 (18- to 22-h neurulae), or stage 37 (2.5-day tadpoles) (24). Unfertilized eggs were squeezed from females. Total cellular RNA was prepared as described previously (18). Cellular lysates were prepared either in buffer A [20 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 7.4, 20°C), 0.3 M KCl, 10 mM $MgCl_2$, 20 μg of polyvinylsulfate per ml] made as previously described (19) but lacking Nonidet P-40 or in buffer B [0.25 M sucrose, 0.03 M tris(hydroxymethyl)aminomethane hydrochloride (pH 7.4, 20°C), 1 mM ethylenediaminetetraacetate, 20 μg of polyvinylsulfate per ml] in a loose-fitting Teflon homogenizer (13). Post-mitochondrial (15,000 × *g*) supernatants either were fractionated into polysomes and a postribosomal (≤70S to 75S) supernatant (free ribonucleoprotein [RNP]) (19) or were suspended in a solution made to contain 0.1 M NaCl, 5 mM ethylenediaminetetraacetate, and 0.5% sodium dodecyl sulfate and extracted with phenol-chloroform. poly(A)$^+$ RNA was isolated by affinity chromatography through oligodeoxythymidylic acid cellulose (3).

[^{32}P]cDNA was synthesized as previously described, except that sodium pyrophosphate was usually omitted from the reaction mixture (18). The specific activity of the [^{32}P]cDNA preparation was about 10^8 dpm/μg. To determine the relative or absolute amounts of total poly(A)$^+$ RNA in various RNA preparations, we used either hybridization with ^{3}H-labeled polyuridylic acid (4) or the ability of an RNA preparation to support cDNA synthesis. Under the conditions which we used (labeled nucleotide at a concentration of 4 μM) (18), [^{32}P]cDNA synthesis was proportional to the input of poly(A)$^+$ RNA template. The amount of poly(A)$^+$ RNA in very small samples of RNA could be estimated from standard curves. From these experiments we determined that ≤5, 35, 65, and 90% of the postnuclear poly(A)$^+$ RNAs from unfertilized eggs, 2- to 3-h morulae, 10- to 11-h gastrulae, and 18- to 22-h neurulae, respectively, were polysomal.

DNA dot hybridizations and quantitations. For each nitrocellulose filter hybridization (DNA dot hybridization), 1 μg of plasmid DNA was denatured in 25 μl of 0.5 N NaOH, neutralized with 25 μl of 0.5 N acetic acid in 7.6× SET [1× SET is 0.15 M NaCl, 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (pH 8.0), plus 0.005 M ethylenediaminetetraacetate], and applied by using suction to a wet (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) nitrocellulose filter (Schleicher & Schuell Co.) over an area of about 10 mm^2. Up to 35 samples and a polydeoxyadenylated pBR322-polydeoxythymidylated pBR322 recombinant plasmid (18) were applied in this manner to papers (6 by 6 cm) on 1-cm^2 squares. After the DNA was applied, the filters were washed with 2× SSC, dried, and baked before hybridization. Hybridization with [^{32}P]cDNA probes (5×10^5 to 20×10^5 dpm in 3 to 5 ml) was performed in small beakers with agitation in a solution containing 50% formamide, 4× SET, 200 μg of salmon sperm DNA per ml, 1× Denhardt solution (15), 50 μg of polyuridylic acid per ml, 0.1% sodium pyrophosphate, and 0.2% sodium dodecyl sulfate (14) for 20 to 24 h at 37°C. The filters were washed in 50% formamide–0.2× SSC several times and then in 2× SSC, dried, placed under Kodak XR film with a Cronex Lightening-Plus (Dupont) intensifying screen, and finally cut up; the radioactivity was measured by Cerenkov or scintillation counting.

Diazobenzyloxymethyl (DBM) paper hybridizations were performed by the method of Stark and Williams (34), using 12 μg of plasmids per filter disk. Several filters containing different cloned DNAs were hybridized together in a small beaker to [^{32}P]cDNA in 1 to 2 ml with agitation. After hybridization and washing as described above, the filters were examined by autoradiography before Cerenkov counting, and the filters with intense, small spots of radioactivity (a

background aberration of DBM filters) were discarded.

The relative amounts of specific RNA species were determined by measuring the percentages of the radioactive probe which hybridized with a particular cloned DNA immobilized on nitrocellulose filters or DBM paper or both. These amounts were converted into percentages of the total poly(A)+ RNA in a preparation by correcting for the efficiency of hybridization, which was 20% with nitrocellulose filters and 50% with DBM paper. The efficiency values were based on the hybridization of sequence C18 [which was calculated to be 2% of the total poly(A)+ RNA of gastrulae] by comparing the hybridization of this sequence with the hybridization of [^{32}P]cDNA prepared from known quantities of vitellogenin mRNA to a homologous clone (18). Consistent with this, 2% of the gastrula-derived clones studied previously contained sequence C18.

Absolute titers are expressed as picograms per egg and were based on constant values of 50 ng of poly(A)+ RNA per egg at all stages up to and including neurula and 100 ng/egg in tadpoles. The amounts of poly(A)+ RNA per mature oocyte and per early embryo have been estimated to be 40 to 85 ng (17, 28, 31). The range of poly(A)+ RNA species measured in the experiments described here was from 2% of the total poly(A)+ RNA (or 1 ng/egg) to 0.02% of the total poly(A)+ RNA (or 10 pg/egg).

Gel electrophoresis, blotting, and hybridization. Alkaline and neutral gel electrophoreses were performed as described by Wahli et al. (37). Plasmid sizes were determined by electrophoresis of molecules restricted with *Hinc*II, which produced a 3,200-base-pair fragment of pBR322 and a second variable fragment consisting of 1,100 base pairs of pBR322 and the *Xenopus* insertion. RNA samples were denatured with glyoxal and separated on 1.2% agarose gels (23). RNA was then transferred either to DBM paper by the method of Alwine et al. (1) or to nitrocellulose by the method of Thomas (35).

Filter strips were incubated with hybridization buffer (the same buffer used for DNA dot hybridizations, except 1% glycine was included for DBM transfers) for several hours or overnight. Excess buffer was squeezed out, and labeled probes were added in 10- to 100-μl volumes (5×10^6 to 20×10^6 dpm/filter, each filter consisting of 2 to 10 lanes of transfer). Labeled probes were prepared by using nick translation of plasmids (26) to about 100×10^6 dpm/μg. Hybridizations were carried out in heat-sealable plastic bags at 38 to 42°C for 18 to 48 h. Filters were washed and prepared as described above for DNA dot hybridizations. After autoradiography, the filters were often washed for 30 min in stripping buffer [5 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 8.0), 0.1 mM ethylenediaminetetraacetate, 0.1% sodium dodecyl sulfate, 0.04% sodium pyrophosphate, 1× Denhardt solution, 25% formamide] at 80°C, rinsed twice in 2× SSC, and dried. This removed 90 to 95% of the radioactivity. These filters could then be reused in subsequent hybridizations.

Mapping. Cloned fragments derived from the mitochondrial genome were mapped onto *Xenopus* mitochondrial DNA cloned into the *Bam*HI site of pBR322 (a gift from Igor Dawid). Mitochondrial DNA was restricted with *Hha*I or *Hin*dIII, separated on 0.9% agarose gels, and transferred to nitrocellulose by the method of Southern (32). Filter strips were then hybridized with nick-translated plasmids containing mitochondrial sequences as described above. After hybridization and autoradiography, the label was removed (see above), and the filters were rehybridized with nick-translated mitochondrial DNA to identify the mitochondrial fragments.

RESULTS

Changes in poly(A)+ RNA titers during early development. We measured 19 cloned sequences derived from non-mitochondrial poly(A)+ RNAs in cellular extracts and subcellular fractions of *Xenopus* embryos. Included among these sequences were the non-mitochondrial poly(A)+ RNA species which were most abundant during these developmental periods, as well as some moderately abundant sequences whose titers were high enough to be measured accurately by the methods used. Post-mitochondrial supernatants prepared in buffer B were used to measure the cytoplasmic titers of these sequences. The validity of this method is discussed below. Plasmid DNA was denatured on nitrocellulose filters in a grid pattern and was hybridized with [^{32}P]cDNA probes prepared from poly(A)+ RNAs from unfertilized eggs, 10- to 11-h gastrulae, and 3-day tadpoles (stage 37). In each case the intensity of the signal was proportional to the concentration of the cloned sequence in the [^{32}P]cDNA probe. We assumed that this accurately reflected the complexity of the RNA. Figure 1 shows the results of such an experiment with 10 clones. The sequences were identified by the nomenclature of Dworkin and Dawid (19). These and other data were quantitated as described above (Fig. 2). Individual sequences varied considerably in concentration compared with each other, but most concentrations did not change appreciably between the egg and gastrula stages. During the first 10 h of development, the concentrations of many sequences remained constant (sequences E23, C4, C11, B20, B10, F19, and F14), whereas the concentrations of sequence G17 and, to a lesser extent, sequences A10, E24 and C20 increased. These sequences were not detected in the 15,000 × *g* pellet fraction (the fraction that was discarded) in either eggs or gastrulae. The concentrations of sequences C12 and D30 also increased during this period, but their absolute concentrations were low, and quantitation was less reliable. After the first day of development, new poly(A)+ RNA species appeared. Several sequences were barely detected or were not detected in eggs and gastrulae but represented

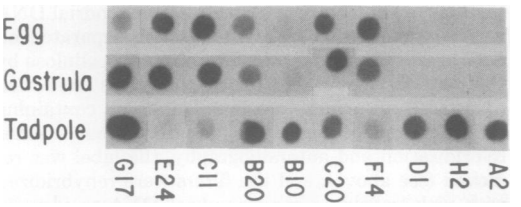

FIG. 1. *DNA dot hybridizations with cytoplasmic probes. DNAs from 10 clones were fixed to nitrocellulose filters in a grid pattern and hybridized to [^{32}P]cDNA probes prepared from poly(A)$^+$ RNAs extracted from a post-mitochondrial 15,000 × g supernatant in buffer B. The cloned DNAs were identified by the nomenclature of Dworkin and Dawid (19). The [^{32}P]cDNA probes were prepared from unfertilized eggs, 10- to 11-h gastrulae, or 3-day tadpoles (stage 37).*

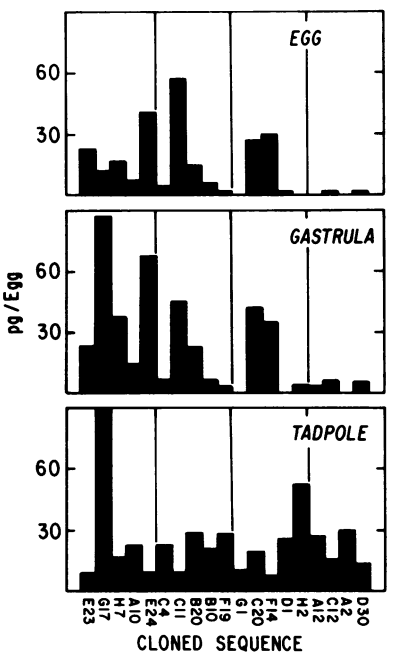

FIG. 2. *Titers of 19 poly(A)$^+$ RNA species during early development. The titers of 19 poly(A)$^+$ RNA species were determined by multiple nitrocellulose DNA dot or DBM paper hybridizations and are displayed graphically for unfertilized eggs, 10- to 11-h gastrulae, and 3-day tadpoles (stage 37). Measurements were made as described in the text.*

prominent poly(A)$^+$ RNA species in tadpoles (sequences F19, G1, D1, H2, A12, C12, A2, and D30). The titers of three sequences decreased substantially by day 3 of development (sequences E24, C11, and F14).

Distribution of sequences in polysomal and nonpolysomal cell fractions in gastrulae and neurulae. To determine to what extent specific RNA sequences were translated at different stages of early development, we fractionated the post-mitochondrial supernatants of cell lysates by sucrose density gradient centrifugation, as described above. Two subcellular fractions were isolated and studied; these were a polysome fraction, which contained material that sedimented faster than 80S monosomes, and a lighter fraction (≤70S to 75S), which contained mRNA's that were not attached to ribosomes. We assumed that the poly(A)$^+$ RNA sequences in the polysome fraction were predominantly the sequences that were actively engaged in protein synthesis. The sequences in the so-called nonpolysomal or postribosomal supernatant fraction were apparently not translated and were very likely complexed with proteins as RNPs, although we did not examine this.

By examining DNA dot hybridization profiles of polysomal and nonpolysomal RNA populations, we could determine whether nonpolysomal RNA reflected polysomal RNA qualitatively or quantitatively or both or whether it was composed of unique RNA sequences. Figure 3 shows an autoradiogram of such hybridization with eight cloned sequences. Of the sequences which we measured, sequence G17 was the most prominent sequence in gastrula polysomes. However, in the gastrula nonpolysomal fraction, several sequences were also abundant (se-

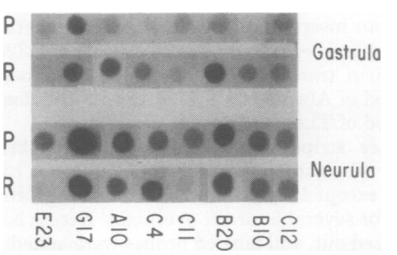

FIG. 3. *DNA dot hybridizations with polysomal (P) and nonpolysomal (R) probes. DNAs from eight clones were fixed to nitrocellulose and hybridized to [^{32}P]cDNA made from polysomal or nonpolysomal poly(A)$^+$ RNA prepared from a post-mitochondrial supernatant in buffer A. The polysomal and nonpolysomal patterns for 10- to 11-h gastrulae and 18- to 22-h neurulae (stage 20) are shown. Similar amounts of [^{32}P]cDNA were used in these hybridizations. However, since poly(A)$^+$ RNA was not equally distributed between polysomal and free RNP fractions [65% of gastrula poly(A)$^+$ RNA was polysomal; 90% of neurula poly(A)$^+$ RNA was polysomal], the gastrula polysome profile must be multiplied by 2 to compare it quantitatively with the gastrula RNP profile; likewise, the neurula polysome prifile must be multiplied by 9 to compare it quantitatively with the neurula RNP profile.*

quences B20, G17, and A10). These and other data are expressed quantitatively in a histogram in Fig. 4, where each bar represents the titer of a sequence in polysomal RNA and nonpolysomal RNA together and the solid portion indicates the polysomal titer alone. Although most sequences were distributed between polysomes and RNPs in gastrulae, the quantitative distributions of all of the sequences were not the same. Some sequences were primarily polysomal (e.g., sequences E23, H7, C11, and C20), whereas others were found substantially in the nonpolysomal fraction (e.g., sequences A10, B20, and, especially, B10). Thus, the relative titers in polysomes and nonpolysomes varied significantly for specific sequences in gastrulae. However, in neurulae none of the sequences was substantially nonpolysomal; i.e., all of these species were found primarily or entirely in the polysomal fraction. Therefore, the relative titers of different RNA species in the two cellular fractions from neurulae were quite similar.

Table 1 shows the percentages of the sequences that were polysomal in gastrulae and neurulae. Table 1 also shows these percentages divided by the mass average percentage of poly(A)$^+$ RNA that was polysomal for each stage (65 and 90% for gastrula and neurula, respectively [see above]); these percentages are normalized and expressed as quotients. Sequences which had a normalized polysomal quotient greater than 1.0 were more concentrated in polysomes than the mass average poly(A)$^+$ RNA; values less than 1.0 indicated sequences which were less concentrated in polysomes than the mass average sequence (relatively more concentrated in free RNPs). These normalized quotients ranged from less than 0.5 (sequences B10 and F19) to more than 1.0 (sequences E23, G17, C20, and F14) in gastrulae. However, in neurulae these quotients were all bunched around 1.0; that is, they were relatively constant. Many quotients which were less than 1.0 in gastrulae were likewise a little less than 1.0 in neurulae. No sequences which we measured were substantially nonpolysomal in neurulae.

Four of the sequences which we studied (sequences A10, C4, B20, and F19) occurred at higher levels in gastrulae when post-mitochondrial supernatants were prepared in buffer A (Fig. 4) than when they were prepared in buffer B (Fig. 2). All of these sequences were relatively enriched in the nonpolysomal fraction in gastrulae (Table 1). This may have reflected the association of these sequences with structures that

TABLE 1. *Normalized subcellular distributions of poly(A)$^+$ RNA sequences*[a]

Sequence	Gastrula		Neurula	
	% Polysomal	Quotient	% Polysomal	Quotient
E23	87	1.3	100	1.1
G17	77	1.2	95	1.1
H7	85	1.3	100	1.1
A10	51	0.78	90	1.0
E24	70	1.1	100	1.1
C4	56	0.86	75	0.83
C11	81	1.2	87	0.97
B20	57	0.88	84	0.93
B10	0	0	79	0.88
F19	30	0.46	84	0.93
C20	78	1.2	78	0.87
F14	79	1.2	92	1.0
H2			93	1.0
A12			100	1.1
C12			87	0.97
A2			100	1.1
D30			73	0.81

[a] The percentage of each sequence in 10- to 11-h gastrulae (stage 10) and 18- to 22-h neurulae (stage 20) that was polysomal in post-mitochondrial supernatants prepared in buffer A is shown. The quotients were obtained by dividing the polysomal percentage by the percentage of total poly(A)$^+$ RNA that was polysomal at each stage (65% for gastrulae, 90% for neurulae).

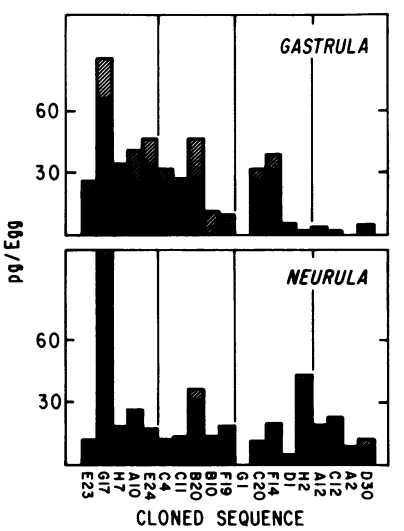

FIG. 4. *Cytoplasmic and polysomal titers of 19 poly(A)$^+$ RNA species during early development. The titers of the 19 poly(A)$^+$ RNA species shown in Fig. 2 were measured in polysomal and nonpolysomal fractions prepared from post-mitochondrial supernatants in buffer A from 10- to 11-h gastrulae and 18- to 22-h neurulae (stage 20), as shown in Fig. 3. The entire bars show the total cytoplasmic titers of the sequences (polysomal and nonpolysomal contributions), and the solid portion of each bar shows the polysomal titer of that sequence.*

pelleted at 15,000 × *g* unless they were disrupted with a high salt concentration (buffer A contained 0.3 M K^+). However, sequence B10, which was entirely nonpolysomal, occurred at comparable levels in both buffer systems.

poly(A)$^+$ RNAs homologous to some cloned sequences. Hybridization of probes to poly(A)$^+$ RNAs separated on gels could be used both to study mRNA sizes (and the presence and sizes of possible precursors) and to confirm quantitations based on the hybridization of [^{32}P]cDNA to cloned insertions. poly(A)$^+$ RNA from unfertilized eggs or gastrulae was fractionated on gels and analyzed by hybridization with nick-translated cloned probes, as described above (Fig. 5). Sequences H7 and A10 produced single bands of about 15S and 14S, respectively, in eggs; each band was the same size in gastrulae. The concentrations of both of these sequences increased during this period (Fig. 2). The concentration of sequence G17 increased more than fivefold during this period (Fig. 2), whereas the concentrations of sequences B20 and C4 remained constant. There was some evidence of high-molecular-weight forms of sequences G17 and C4 in unfractionated egg RNA near the 28S marker, but there was no such evidence for the other sequences. The relative intensities of hybridization of the RNAs homologous to sequences G17, B20, and C4 were consistent with the quantitative data in Fig. 2.

Characterization of mitochondrial cloned sequences. A surprisingly large percentage of the poly(A)$^+$ RNA from unfertilized *Xenopus* eggs and early embryos hybridizes with cloned mitochondrial DNA (Dworkin et al., in press). Based on the frequency of occurrence of cloned sequences homologous to mitochondrial DNA, we estimated that more than 10% of the gastrula poly(A)$^+$ RNA is mitochondrial (Dworkin et al., in press). For further study we chose eight nonhomologous cloned mitochondrial sequences that represented many of the most abundant preneurula poly(A)$^+$ RNA species; these included one representative clone from each of the five homology groups described previously (Dworkin et al., in press); and three additional sequences (D13, F15, and G6) which were nonhomologous to each other and to the members of the five groups. These sequences were en-

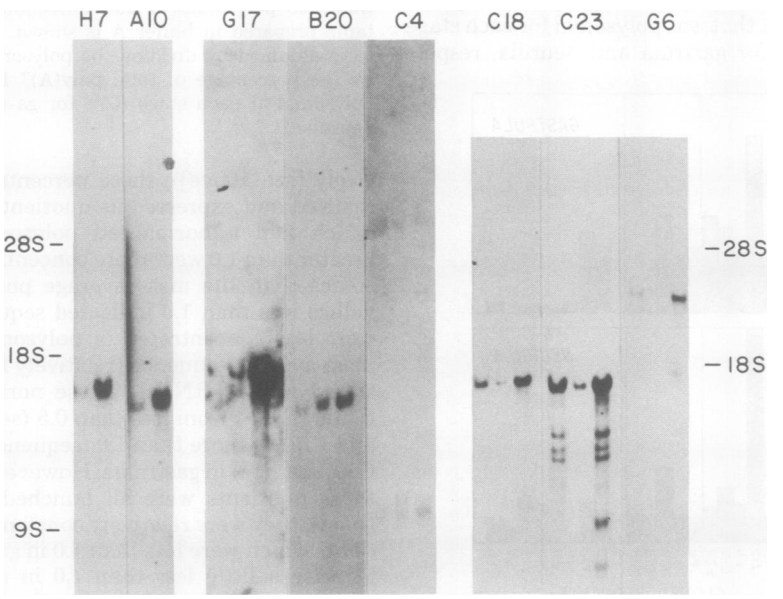

FIG. 5. *Sizes of RNAs that hybridized with cloned sequences. poly(A)$^+$ RNA was prepared from unfractionated unfertilized eggs, unfractionated gastrulae, or the 15,000 × g supernatant (post-mitochondrial supernatant) of eggs prepared in buffer B. The samples were denatured with glyoxal, separated on 1.2% agarose gels, transferred to filters, and hybridized with plasmid DNAs labeled by nick translation. The designations of the clones are shown at the top of the figure. Plasmids H7, A10, G17, B20, and C4 were derived from non-mitochondrial RNA, whereas plasmids C18, C23, and G6 were derived from mitochondrial RNA. The filters containing sequences H7 and A10 were DBM paper filters; the poly(A)$^+$ RNA in the left lane of each pair was from unfractionated eggs (0.4 μg), and that in the right lane was from unfractionated gastrulae (1 μg). The other filters were nitrocellulose filters; the poly(A)$^+$ RNAs in the left, middle, and right lanes of each triplet were from unfractionated eggs (0.4 μg), egg post-mitochondrial supernatants (1 μg), and unfractionated gastrulae (1 μg), respectively.*

riched in mitochondrial cell fractions prepared by the method of Dawid (13) and were absent in post-mitochondrial supernatants (data not shown). The titers in whole lysates of unfertilized eggs and embryos were determined by DNA dot hybridizations to poly(A)+ RNA, as described in the legends to Fig. 1 and 2 for nonmitochondrial sequences. Table 2 shows the percentage of total poly(A)+ RNA that was homologous to each cloned sequence in eggs. Together, these eight sequences represented more than 6% of the total cellular poly(A)+ RNA in eggs. Similar measurements showed comparable levels for these sequences during the first 10 to 12 h of development, through the formation of the gastrula. Soon after neurulation there is an increase in the total cellular poly(A)+ RNA, with the appearance of qualitatively new sequences (19, 31), and mitochondrial poly(A)+ RNA sequences become diluted rapidly (data not shown).

The sizes of the RNAs homologous to the eight cloned mitochondrial sequences were determined by fractionating total poly(A)+ RNA on gels and hybridizing the sequences with cloned probes. Figure 5 shows examples of three mitochondrial sequences (sequences C18, C23, and G6), and Table 2 shows the sizes which were determined for the eight sequences. The mitochondrial poly(A)+ RNA sequences also were mapped on the mitochondrial genome by determining to which restriction fragments of cloned mitochondrial DNA the eight cloned sequences hybridized. The method used is described in the legend to Fig. 6, and the results of this experiment are shown in Table 2. Figure 6A shows the *Hha*I and *Hin*dIII maps of *X. laevis* mitochondrial DNA and the location of the ribosomal locus (25). Figure 6B shows the analyses for sequences C18 and C23, the two sequences which were the most abundant mitochondrial sequences that we observed and which corresponded to RNAs 1,600 bases long (Fig. 5 and Table 2). However, sequence C18 hybridized with *Hin*dIII fragment C, and sequence C23 hybridized with *Hin*dIII fragment B'. Thus, these two cloned sequences mapped in quite different regions of the mitochondrial genome and therefore must have hybridized to different species. Based on the RNA sizes and map positions, we concluded that sequences C23, F15, and H21 were homologous to different portions of the large ribosomal RNA, whereas sequence H26 probably corresponded to the small ribosomal RNA. The four nonribosomal sequences (sequences C18, D13, E20, and G6) mapped to the right of the ribosomal region.

Distribution of mitochondrial poly(A)+ RNA species in polysomal and nonpolysomal cell fractions. We determined the distributions of the mitochondrial sequences in polysomes or polysome-like structures and the postribosomal supernatant by using methods analogous to those used to characterize the nonmitochondrial sequences. Figure 7 shows the results of a set of DNA dot hybridizations. Figure 7A shows the results of hybridizations of [^{32}P]cDNA prepared from nonpolysomal poly(A)+ RNA from unfertilized eggs with the eight cloned DNAs. In eggs almost all of the cellular poly(A)+ RNA was nonpolysomal. Figure 7B and C shows how these sequences were distributed between polysomal and nonpoly-

TABLE 2. *Characterization of mitochondrial poly(A)+ RNA species*[a]

Sequence	% of egg RNA hybridized	Size (no. of bases)	Homologous mitochondrial fragment(s)		Identification[b]
			*Hha*I	*Hin*dIII	
Nonribosomal					
C18	2.0	1,600	A	C	8
E20	1.2	850	A	A,E	6
D13	0.12	2,300	B	A	4
G6	0.10	2,600	B	A	
Ribosomal					
C23	1.6	1,600	D',E	B'	Large rRNA
F15	1.1	1,600	D'	B'	Large rRNA
H21	0.94	1,600	E	B'	Large rRNA
H26	0.12	950	F	A,B'	Small rRNA

[a] Eight cloned sequences which hybridized to cloned *Xenopus* mitochondrial DNA were measured in eggs by multiple DNA dot and DBM paper hybridizations. Cells were lysed in buffer A containing 0.5% Nonidet P-40, which lysed mitochondria, and the sequences were measured in the 15,000 × *g* supernatants. This table shows the percentage of poly(A)+ RNA in eggs that hybridized to each RNA sequence, the sizes of the RNA species which were homologous to the cloned sequences (see Fig. 5), and the *Hha*I and *Hin*dIII mitochondrial DNA fragments to which the sequences mapped (see Fig. 6).

[b] Identification according to the numbering system of Rastl and Dawid (25) or as ribosomal RNA (rRNA).

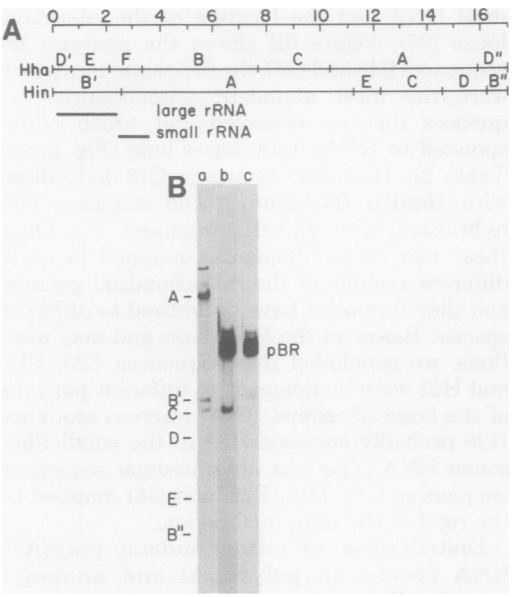

FIG. 6. *Mapping of mitochondrial sequences on the mitochondrial DNA. (A) Map of X. laevis mitochondrial DNA linearized at the BamHI site, showing the HhaI and HindIII fragments and the locations of the large and small ribosomal RNAs. From Rastl and Dawid (25). (B) Cloned mitochondrial DNA was restricted with HhaI or HindIII, and the fragments were separated on 0.9% agarose gels, transferred to nitrocellulose filters, and hybridized to nick-translated probes. This figure shows an autoradiograph of HindIII digests hybridized with mitochondrial DNA (lane a), plasmid C18 (lane b), and plasmid C23 (lane c). The letters on the left refer to the fragments shown on the map. The bands due to pBR322 (and not the mitochondrial insertion) are indicated on the right. rRNA, Ribosomal RNA.*

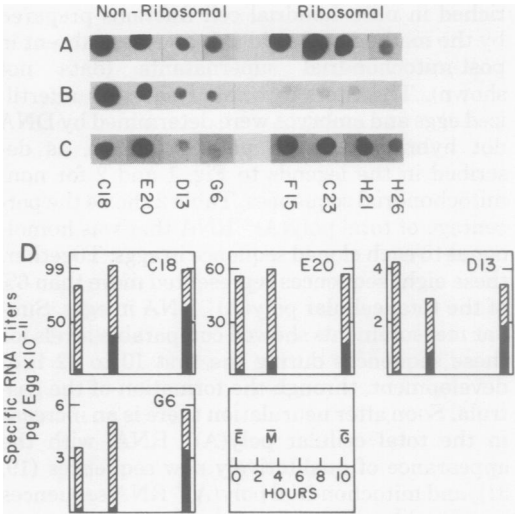

FIG. 7. *Filter hybridizations of mitochondrial sequences with polysomal and nonpolysomal probes. DNAs from the eight clones listed in Table 2 were fixed to nitrocellulose filters and hybridized to [^{32}P]cDNA probes prepared from poly(A)$^+$ RNAs obtained from cellular extracts prepared in buffer B containing 0.5% Nonidet P-40. (A) Postribosomal supernatant of unfertilized eggs. (B) Polysomes of 10-h gastrulae. (C) Nonpolysomal fraction of 10-h gastrulae. (D) Approximate titers of the nonribosomal sequences as a function of development. The entire bars show the total cellular titers of the sequences, and the solid portion of each bar shows the polysomal titer. Titers were determined by multiple nitrocellulose filter and DBM paper hybridizations. Ē, Eggs; M̄, 32- to 64-cell morulae; Ḡ, 10- to 11-h gastrulae.*

somal RNA fractions, respectively, in 10- to 11-h gastrulae. In gastrulae the nonribosomal sequences were enriched in polysomes, whereas the ribosomal sequences were not. Figure 7D shows the titers of the nonribosomal sequences in unfertilized eggs, 32- to 64-cell morulae, and 10- to 11-h gastrulae; the solid portion of each bar indicates the polysomal titer. The embryonic titers of sequences C18, E20, and D13 remained constant during this period, whereas the titer of sequence G6 almost doubled. These mitochondrial poly(A)$^+$ RNA species were enriched in polysomes during the first few hours after fertilization, following patterns similar to the patterns described above for non-mitochondrial, cytoplasmic poly(A)$^+$ RNA species.

DISCUSSION

We describe the use of a recombinant cDNA library to measure levels of several poly(A)$^+$ RNA species during early development. The ability to support [^{32}P]cDNA synthesis was used as a sensitive method for labeling and quantitating poly(A)$^+$ RNA species. Relative and absolute titers were determined as described above. The accuracy of these determinations was limited by several things. First, we only measured poly(A)$^+$ RNA, which is functionally defined as RNA which binds to oligodeoxythymidylic acid cellulose. Second, the accuracy of our measurements depended on the efficiency of [^{32}P]cDNA synthesis. We have observed some species, such as mitochondrial ribosomal RNA, which may be poor templates for [^{32}P]cDNA synthesis (see below). A third problem is that cloned sequences may represent internal regions of poly(A)$^+$ RNA molecules, and these regions may hybridize poorly to [^{32}P]cDNA probes, which tend to correspond to the 3′ end of RNA. The most extreme examples of this are the homologous sequences C18 and D22 (19). These two clones contain *Xenopus* insertions of the same size (560 and 500 nucleotides, respectively), yet sequence C18 hy-

bridizes five times as strongly as sequence D22 to [32P]cDNA probes. However, when poly(A)+ RNA is separated on glyoxal gels, transferred to DBM paper, and hybridized to nick-translated sequence C18 or D22, both probes hybridize equally well to poly(A)+ RNA. Thus, the different degrees of hybridization of these two sequences with [32P]cDNA are misleading. Sequence D22 may represent an internal region of the poly(A)+ RNA species that is poorly represented in the [32P]cDNA probe, whereas sequence C18 may represent the 3' end (36). The latter two limitations are not applicable when labeled, cloned DNAs are hybridized directly to RNAs separated on gels instead of through [32P]cDNA intermediates. In the experiments shown in Fig. 5 we confirmed some of our measurements by using RNAs separated on gels. These data support the data shown in Fig. 2 and 4, but they are limited to a few sequences. We assume that our method was valid, with the caveat that some clones may have represented internal regions and may have been underestimated in our measurements. This could have invalidated some absolute numbers, but the relative developmental and subcellular distributions should still be accurate.

The results described above for individual species are in accord with many of the results described by Sagata et al. (31) for the total (mass average) poly(A)+ RNA of *Xenopus* embryos. These authors described quantitative changes in the mass average poly(A)+ RNA population of *Xenopus* embryos that occurred after about 20 h of development; they also observed an increase in polyadenylation shortly after fertilization. Figure 2 shows the changes that occurred in some abundant poly(A)+ RNA species during the first 10 h of development. Most RNA titers were very similar in eggs and gastrulae; however, the titers of several poly(A)+ RNA species increased during the first 10 h of development (for example, sequences G17, A10, and E24). These increases could have been due to new transcription. However, the data of Sagata and co-workers indicate that instead this may have resulted from polyadenylation of maternal RNA after fertilization. If so, polyadenylation after fertilization occurs differentially among egg RNA species. We also observed the accumulation of new poly(A)+ RNA species during neurulation. Much of this new mRNA differed from preneurula poly(A)+ RNA in sequence content, although many preneurula species continued to be present. We also detected three species whose concentrations significantly decreased during days 2 and 3 of development.

The formation of polysomes after fertilization is largely at the expense of RNPs in sea urchins (12, 28, 29, 39) and probably in *Xenopus* as well (10, 12, 27). By examining the subcellular distributions of RNA species in RNPs and polysomes later in development, we asked whether RNPs continued to be the repository of mRNA (that is, whether new polysomes were still formed at the expense of RNPs). We also could ask whether RNPs consist of a different set of RNA species than polysomes or whether the two subcellular populations contain similar sequences and how the distributions of individual sequences change as the mass average poly(A)+ RNA becomes more concentrated in polysomes as development proceeds. By 10 h after fertilization (gastrula), 35% of the cellular poly(A)+ RNA is still in RNPs. This is not because 35% of all RNA species are entirely RNPs; rather, most sequences are distributed between RNPs and polysomes, with some species more than 65% polysomal and others less than 65% polysomal (Table 1). Although the formation of polysomes before 10 h is largely at the expense of maternal RNPs, the overall concentrations of several species increase in the poly(A)+ RNA population. As mentioned above, this may be the result of new transcription or of differential polyadenylation of preexisting, maternal poly(A)- RNA (8, 11, 31), followed by mobilization. In neurulae the situation is quite different. About 90% of neurula poly(A)+ RNA is polysomal, and all species which we have measured are largely or entirely polysomal at this stage. New polysomal poly(A)+ RNA species in neurulae do not result from shifts in RNA from RNPs to polysomes; rather, new polysomal mRNA is the result of new RNA synthesis or de novo polyadenylation. Thus, with the possible exception of sequence B10, we did not detect the "unmasking" of specific, new sequences that contributed to the accumulation of polysomes after the first few hours of development. This is consistent with kinetic data for mass average poly(A)+ and poly(A)- RNA populations from sea urchins during this period of development; it has been shown that in these populations free RNPs are not stable and therefore are not likely candidates for masked messages (20, 21).

Polysomal quotients are all much closer to the mass average polysomal distribution in neurulae than in gastrulae (Table 1). Furthermore, sequences which have quotients less than 1.0 in gastrulae also have quotients less than 1.0 in neurulae. In contrast to the differential subcellular distributions earlier in development, when protein synthesis is more restricted, the similarity in the subcellular distributions of sequences in neurulae is a predictable consequence of the

relief of competition during a period of increasing cellular protein synthesis (16, 22). According to this model, messages compete for limiting factors during protein synthesis. Messages with higher binding constants (sequences E23, G17, E24, C11, and F14) are represented more highly in polysomes when factors are limiting (when protein synthesis is low, as during the first several hours after fertilization). As competition is relieved, less efficient messages can compete effectively for factors, reducing the differential subcellular distributions of these RNAs. This is precisely what we observed.

Approximately 6 to 7% of the poly(A)$^+$ RNAs isolated from unfertilized eggs and preneurula embryos in *Xenopus* are mitochondrial. These mitochondrial RNAs are the most abundant poly(A)$^+$ RNA species in early embryos. A similar observation has been made recently with sea urchin RNA (29). During day 2 of development, new nuclear poly(A)$^+$ RNA synthesis considerably reduces the relative amount of mitochondrial poly(A)$^+$ RNA. By determining the size of the RNA from which these cloned sequences are derived and by mapping these sequences on the mitochondrial genome, it is possible to compare these species with the ovarian mitochondrial poly(A)$^+$ RNA species described by Rastl and Dawid (25). Three sequences (sequences C18, E20, and D13) correspond to the mitochondrial poly(A)$^+$ RNAs described by Rastl and Dawid (25) (Table 2). A fourth sequence (sequence G6) was not described in ovary mitochondrial poly(A)$^+$ RNA, and this is the only sequence which increases in concentration during the first few hours of development. The other four mitochondrial sequences are probably derived from mitochondrial ribosomal RNA. The appearance of low levels of mitochondrial ribosomal RNA in the poly(A)$^+$ RNA fraction may result from contamination and inefficient priming of cDNA synthesis by the adenylic acid-rich ribosomal RNAs. We detected less than 1 ng of the large mitochondrial ribosomal RNA per egg and only 50 pg of the small mitochondrial ribosomal RNA per egg in these fractions. Chase and Dawid (9) have shown that *Xenopus* eggs contain 13 and 6.9 ng of the large and small mitochondrial ribosomal RNAs, respectively. The isolation of mitochondrial ribosomal RNAs, particularly the large ribosomal RNA, by chromatography through oligodeoxythymidylic acid cellulose has been reported previously in HeLa cells and *Drosophila* (2, 33).

In unfertilized eggs, the mitochondrial sequences are apparently not actively translated since they are found almost entirely in the nonpolysomal fraction. After fertilization the nonribosomal sequences are distributed differentially in polysomal and nonpolysomal cellular fractions. Three of these sequences are predominantly polysomal, whereas one sequence is still more than one-half nonpolysomal by hour 10 of development. Polysomes and nonpolysomal RNPs from mitochondria are not well characterized, and our assignment of these names to subcellular fractions obtained by differential centrifugation could be erroneous. However, the polysome fraction is puromycin sensitive, as expected for true polysomes. The increase in mitochondrial poly(A)$^+$ RNA sequences in the polysomal fraction after fertilization is very similar to the mobilization of nuclear transcripts. That such mobilization apparently occurs both in mitochondria and in the cytoplasm suggests that it is the result of an overall change in cellular physiology.

On the basis of this work, we can begin to describe the kinds of sequences which comprise the large nonpolysomal poly(A)$^+$ RNA pool in *Xenopus* embryos. Most of the RNA species in the nonpolysomal pool are sequences which are also present in polysomes. The subcellular distribution in polysomes and RNPs may result from competition among messages for the cellular protein synthetic machinery, at least after the first few hours of development, or possibly from mRNA masking, the mechanism of which is unknown. Whether an increase in the polysomal titer is strictly dependent upon new RNA synthesis after the first few hours of development and whether the RNP portion of an RNA species can be mobilized as the general protein synthetic rate increases can only be determined by kinetic studies. However, even kinetic studies cannot discriminate unmasking of poly(A)$^+$ RNA from a reduction in competition. A second contribution to nonpolysomal poly(A)$^+$ RNA may be cytoplasmic sequences which are entirely nonpolysomal at one stage of development but are utilized in polysomes at other stages of development. We have observed only one sequence which could fit into this category (sequence B10). On the basis of the few sequences which we have studied, it seems that sequences in this category make up only a small proportion of the nonpolysomal poly(A)$^+$ RNA of *Xenopus* embryos. However, we have examined only a small number of abundant poly(A)$^+$ RNA sequences; less abundant species may behave differently from most of the sequences which we have described and could mimic the behavior of sequence B10.

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