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Structure, Translation, and Metabolism of the Cytoplasmic Copia Ribonucleic Acid of *Drosophila melanogaster*†

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ABSTRACT: We have characterized the copia RNA in the cytoplasm of cultured *Drosophila* cells. Copia RNA was detected and purified by hybridization to DNA of the plasmid cDm1142, which contains the copia sequence. A large fraction (2.2%) of the total cytoplasmic poly(A)$^+$ RNA was found to be copia RNA. Cytoplasmic copia RNA displays all the characteristics expected for a messenger RNA. It possesses a poly(A) tract identical in length with that of total poly(A)$^+$ cytoplasmic RNA. It is associated with polysomes and can be released from this association by treatment with EDTA. When purified copia RNA is added to an mRNA-dependent rabbit reticulocyte lysate, three polypeptides of 51 000, 33 000, and 21 000 daltons are seen. We have not determined if these are different polypeptides or if the two smaller polypeptides are fragments of the 51 000-dalton polypeptide. The half-life of copia cytoplasmic RNA was determined in pulse–chase experiments to be 9.5 h; this is 1.6 times longer than the half-life of the intermediate decay class of total poly(A)$^+$ cytoplasmic RNA. These properties provide strong evidence that copia RNA functions in vivo as a messenger RNA.

A significant fraction of the DNA of metazoans consists of intermediate repeat sequences which are repeated 100–100 000 times in the genome. Although some intermediate repeat DNA codes for structural RNA such as rRNA and tRNA, or messenger RNA such as histone mRNA, the function of most intermediate repeat DNA is unknown.

Three families of repeated DNA with unusual properties have been described in *Drosophila melanogaster*. These families, termed copia, 412, and 297, are dispersed in the *Drosophila* genome. A remarkable feature of the copia, 412, and 297 sequences is that they appear to have the ability to change location in the genome, possibly by recombination between the 0.3-kb[1] repeats located at the 3′ and 5′ ends of the RNA coding regions (Finnegan et al., 1977; Potter et al., 1979; Strobel et al., 1979). The movement of discrete units of DNA within the genome has been postulated to explain a number of genetic phenomena in *Drosophila*, such as high rates of mutation, deletion, and reversion at specific loci (Golubovsky et al., 1977; Green, 1977). The small circular DNAs of *Drosophila*, which are homologous to intermediate repeat DNA, might constitute intermediates in this movement (Stanfield & Lengyel, 1979, 1980).

The copia, 412, and 297 sequences are transcribed in *Drosophila* cultured cells into RNAs of unknown function. By virtue of their binding to oligo(dT)–cellulose, these RNAs are considered to be polyadenylated (Finnegan et al., 1977; Carlson & Brutlag, 1978); whether they are actually messenger RNAs has not been established. Copia RNA is the most abundant and hence the most interesting of these transcripts; it constitutes 3–5% of the cytoplasmic RNA of Echalier Kc_0 cells which binds to oligo(dT)–cellulose (Finnegan et al., 1977).

In order to gain insight into the function of the copia DNA sequences and of mobile DNA sequences in general, it is necessary to characterize the copia cytoplasmic RNA and particularly to determine whether it possesses the properties of a messenger RNA. Using recombinant DNA which contains the copia sequence as a probe, we have examined the cytoplasmic stability, structure, and coding capacity of copia RNA. We demonstrate here that copia cytoplasmic RNA possesses a poly(A) tract, has a cytoplasmic decay rate similar to that of other "middle-abundant" poly(A)$^+$ RNAs, is associated with polysomes, and is released from polysomes by treatment with EDTA. The definitive demonstration that

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[1] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; kb, kilobase (1000 nucleotides); Na-DodSO$_4$, sodium dodecyl sulfate.

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Table I: Steady-State Amount of Poly(A)

expt	no. of cells	poly(A) (μg)	poly(A)/ cell (fg)
1	2.96×10^7	0.074	2.49
2	5.54×10^7	0.157	2.83
3	4.82×10^7	0.132	2.74
4	4.60×10^7	0.149	3.25
			av 2.83 ± 0.30

copia RNA is a messenger RNA is its translation in an in vitro system.

Experimental Procedures

Cell Culture and Labeling. Exponentially growing suspensions cultures of *Drosophila* line 2-L (Lengyel et al., 1975) adapted from Schneider line 2 (Schneider, 1972) were maintained in Dulbecco's modified ME medium containing 2% GIBCO lactalbumin hydrolysate and 10% fetal calf serum (Lengyel et al., 1975). At 25 °C the cells have a doubling time of 24 h. Cells were labeled during exponential growth ($2–6 \times 10^6$/mL). A "chase" of a [^{3}H]uridine pulse label was initiated by the addition of unlabeled uridine and cytidine to a concentration of 1 mM each (Lengyel & Penman, 1977).

Cell Fractionation and RNA Purification. Cells were poured over an equal volume of crushed frozen wash buffer (10 mM Tris, pH 7.4, 100 mM NaCl, and 10 mM magnesium acetate), collected by centrifugation at 1500 rpm in an IEC PR6000 centrifuge for 4 min at 4 °C, and washed once with wash buffer. Cells were resuspended at $^1/_{20}$ of the original volume in lysis buffer (30 mM Tris, pH 7.4, 30 mM NaCl, and 10 mM magnesium acetate) containing 0.5% diethyl pyrocarbonate and lysed by the addition of NP40 to 0.5%. Nuclei were removed by centrifugation over a 6-mL sucrose pad (0.38 M sucrose, 30 mM Tris, pH 7.4, 30 mM NaCl, and 10 mM magnesium acetate) at 4500 rpm for 5 min at 4 °C. The cytoplasmic supernatant was removed and added to an equal volume of 2 × $NaDodSO_4$ buffer (200 mM NaCl, 20 mM Tris, pH 7.4, 2 mM EDTA, and 1% $NaDodSO_4$) containing 500 μg/mL proteinase K, incubated at 37 °C for 3 h, and then precipitated with two volumes of absolute ethanol at −20 °C. Poly(A)$^+$ RNA was obtained by chromatography on oligo(dT)–cellulose (T3, Collaborative Research) as previously described (Anderson & Lengyel, 1979). Poly(A)$^-$ RNA was that fraction which did not bind to oligo(dT)–cellulose. Poly(A)$^-$ cytoplasmic RNA obtained by this technique from sea urchin embryos contains oligo(A) stretches of no longer than six adenylate residues (Nemer et al., 1974).

The amount of poly(A) per cell was determined by hybridization of [^{3}H]poly(U) to cytoplasmic RNA (Milcarek et al., 1974). An average value of 2.9 fg of poly(A) per cell was obtained (Table I). Using this value and assuming that the number average sizes of steady-state mRNA and poly(A) are 2.0 kb and 0.035 kb, respectively (Falkenthal, 1980), we calculate that there are 0.16 pg of poly(A)$^+$ mRNA per *Drosophila* cultured cells. This is 2.6% of the total mass of cytoplasmic RNA [on the basis of 6 pg of cytoplasmic RNA per cell (S. Falkenthal and J. A. Lengyel, unpublished results)].

Sedimentation of Polysomes. Two hours before harvesting, cells were diluted with fresh medium to 2×10^6/mL. Three seconds before harvesting, emetine was added to a concentration of 25 μg/mL in order to prevent elongation of peptide chains and ribosome runoff during the polysomal preparation (Grollman, 1966, 1968). Cells were then poured over crushed frozen wash buffer and swirled in a −20 °C ice–salt bath for 30 s. A cytoplasmic fraction was prepared (see above), brought to 25 mM EGTA and 35 mM magnesium acetate, and layered over a 5–40% sucrose gradient (0.1 M NaCl, 10 mM Tris, pH 7.4, 25 mM EGTA, 35 mM $Mg(OAc)_2$, 1.0 mg/mL heparin, and 25 μg/mL emetine). The gradients were centrifuged 90 min at 40 000 rpm, 4 °C, in a Beckman SW40 rotor and then pumped through an Isco Model UA-5 absorbance monitor.

Preparation of [^{3}H]cRNA. Complementary [^{3}H]RNA ([^{3}H]cRNA) was synthesized by incubating *Escherichia coli* RNA polymerase with 1 μg of purified closed circular cDm1142 DNA in 50 μL of 0.01 M $MgCl_2$, 0.04 M Tris, pH 7.4, 0.07% mercaptoethanol, 400 μM each of ATP and GTP, and 60 μM each of [^{3}H]UTP (9 Ci/mmol) and [^{3}H]CTP (8 Ci/mmol) for 2 h at 37 °C. At the end of the reaction, plasmid DNA was digested with DNase; [^{3}H]cRNA was then purified by phenol extraction followed by Sephadex G-50 chromatography. The specific activity of the [^{3}H]cRNA probe prepared in this way was 2.5×10^7 dpm/μg.

Hybridization to Filter-Bound DNA. Labeled RNA was hybridized to cDm1142 DNA bound to nitrocellulose filters (10 μg/filter) for 20 h at 65 °C in 0.5 mL 2 × TESS buffer (0.3 M NaCl, 0.01 M EDTA, 0.01 M 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid, pH 7.4, 0.2% $NaDodSO_4$), and RNase-resistant counts were quantitated by scintillation counting as described by Gilmore-Hebert & Wall (1978). Since the maximum amount of copia RNA in any hybridization reaction was 15 ng, all hybridizations were in at least 100-fold DNA sequence excess. In each hybridization experiment, a known amount of [^{3}H]cDm1142 cRNA was hybridized to filter-bound cDm1142 DNA to obtain the efficiency of hybridization. This value, which ranged from 60 to 75%, was used to correct all hybridization data to 100% efficiency.

Isolation of Plasmid DNA. The recombinant plasmid cDm1142 (Finnegan et al., 1977), containing *Drosophila melanogaster* DNA, was generously provided by Dr. Michael Young. This plasmid was propagated in HV1 vector systems under P2 biocontainment conditions in accord with the *NIH Guidelines for Research Involving Recombinant DNA Molecules* issued in December 1978. Plasmids were allowed to amplify in log-phase bacteria treated overnight with 100 μg/mL chloramphenicol. The bacteria, harvested and washed by centrifugation, were resuspended in 0.14 M NaCl, 0.2 M Tris, pH 8.0, and 0.02 M EDTA to which 200 μg/mL lysozyme was added. After 20 min of incubation at room temperature, the lysozyme-treated bacteria were diluted 1:1 with ice-cold water and incubated 5 min on ice, 5 min at 65 °C, and finally 48 h at 4 °C. The lysate was cleared by centrifugation for 90 min at 27 000 rpm in an SW27 rotor, and plasmid DNA was prepared from the supernatant. The supernatant was brought to 0.5 M NaCl and 0.5% $NaDodSO_4$, incubated with proteinase K (250 μg/mL) for 3 h at 37° C, and then chromatographed on hydroxyapatite in 8 M urea to remove RNA (Meinke et al., 1974).

Purified DNA from the *Drosophila* actin coding plasmid λDmA2 was a gift from Dr. E. Fyrberg. Purified DNA from the *Drosophila* histone coding plasmid cDm500 was a gift from Dr. K. V. Anderson.

In Vitro Translation of Copia RNA. Cytoplasmic poly(A)$^+$ RNA was hybridized to 2.4-cm-diameter nitrocellulose filters containing cDm1142 DNA for 3 h at 50 °C in 0.2 mL of 65% formamide, 0.4 M NaCl, and 10 mM Pipes, pH 6.4 (Ricciardi et al., 1979).

At the end of the hybridization period, the filter was washed 10 times in 1 × SSC, 0.5% $NaDodSO_4$ at 65 °C, and then

Table II: Percent of Cytoplasmic Poly(A)$^+$ RNA Which Is Copia

time of labeling (min)	fraction of label in copia (%)
30	1.36[a]
60	1.56[a]
120	1.97[a]
180	2.16[a]
steady state	2.24[b]

[a] Cells (3×10^6/mL) were labeled with [^{3}H]uridine (50 μCi/mL). At various times after the addition of label, the cytoplasmic poly(A)$^+$ RNA was isolated. Copia RNA was detected by hybridization to filter-bound cDm1142 DNA (see Experimental Procedures). [b] The fraction of the steady-state cytoplasmic population of poly(A)$^+$ RNA which is copia RNA was determined by in vitro ^{32}P-end labeling of base-hydrolyzed cytoplasmic poly (A)$^+$ RNA and hybridization to cDm1142 (K. V. Anderson and J. A. Lengyel, unpublished results).

3 times in 10 mM Tris, pH 7.9, and 2 mM EDTA at 25 °C, with the final wash in 10 mM Tris, pH 7.9, and 2 mM EDTA at 65 °C. Copia RNA was then eluted by boiling the filter in water for 60 s. The eluted RNA was ethanol precipitated after addition of 10 μg of yeast tRNA and 0.1 volume of 2.0 M potassium acetate, pH 5.1. The RNA was reprecipitated with ethanol from 0.2 M potassium acetate two additional times. The RNA was then resuspended in 0.1 mL of water, lyophilized, resuspended in water, and translated in a micrococcal nuclease treated rabbit reticulocyte lysate (Bethesda Research Labs). Following translation the sample was incubated with 100 μg/mL RNase A at 37 °C for 1 h to digest [^{35}S-*methionyl*]tRNA. Electrophoresis of the translation products in $NaDodSO_4$–polyacrylamide slab gels was performed as described by Laemmli (1970). Fluorography was performed as described by Bonner & Laskey (1974) and Laskey & Mills (1975).

Results

Characteristics of Copia Cytoplasmic RNA. (A) Use of Recombinant Plasmid cDm1142 as a Hybridization Probe for Copia RNA. The availability of a cloned sequence allows the detection and purification of specific gene products from a heterogeneous population of RNA molecules. As a hybridization probe, we used the recombinant plasmid cDm1142 which contains a 12-kb insert of Drosophila embryonic DNA. Copia RNA has been defined as the cytoplasmic poly(A)$^+$ RNA complementary to a 5-kb sequence of this insert. No other region of the insert is homologous to cytoplasmic RNA (Finnegan et al., 1977).

Hybridization of labeled RNA to filter-bound cDm1142 DNA shows that copia RNA is abundant in the cytoplasm of *Drosophila* line 2-L and is present predominantly in the poly(A)$^+$ fraction. Copia RNA sequences constitute 2.2% of the steady-state mass of the poly(A)$^+$ cytoplasmic RNA (Table II) but less than 0.002% of the steady-state mass of poly(A)$^-$ cytoplasmic RNA. With the assumption that there is an amount of poly(A)$^-$ mRNA in the *Drosophila* cultured cells equal to one-half the mass of the poly(A)$^+$ mRNA (Lengyel & Penman, 1977) and that poly(A)$^+$ mRNA is 2.6% of the total mass of cytoplasmic RNA (Experimental Procedures), then copia RNA sequences constitute less than 0.15% of the mass of poly(A)$^-$ mRNA.

The sedimentation behavior of copia RNA was determined by hybridization across a sucrose gradient of ^{3}H-labeled cytoplasmic poly(A)$^+$ RNA with filter-bound cDm1142 DNA (Figure 1). Two species of copia RNA, with modal *s* values of 26 S and 18 S, were observed in the poly(A)$^+$ RNA. These

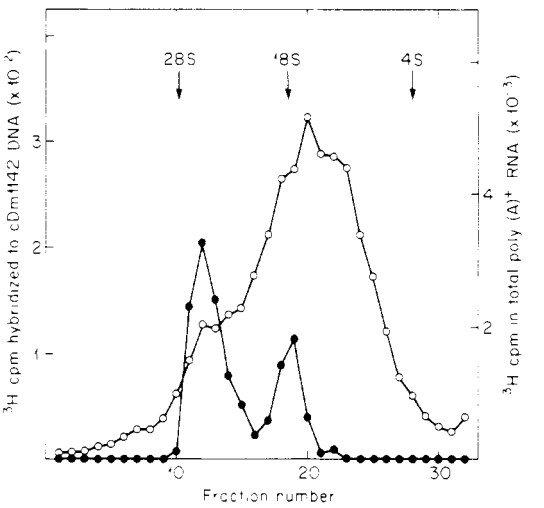

FIGURE 1: Size of copia cytoplasmic RNA determined by sucrose gradient sedimentation. Poly(A)$^+$ RNA, isolated from the cytoplasm of cells labeled for 1 h with [^{3}H]adenosine (50 μCi/mL), was heat denatured (70 °C, 7 min), cooled rapidly on ice, and layered onto a 15–30% sucrose gradient in $NaDodSO_4$ buffer. The SW40 gradient was centrifuged for 17 h at 26 000 rpm and fractionated, and aliquots were hybridized to filter-bound cDm1142 DNA as described under Experimental Procedures. (○) Total ^{3}H cpm; (●) ^{3}H cpm in hybrid. [^{14}C]Uridine-labeled HeLa 28S and 18S rRNA was included in the gradient as marker.

species correspond to the 5.2- and 2.1-kb copia RNAs described by Carlson & Brutlag (1978). The 2.5:1 ratio of cpm in the 5- and 2-kb species indicates that the two copia RNA sequences are present in the cytoplasm in equimolar amounts. The small quantity of copia sequences detected in the poly(A)$^-$ RNA ranges in size from 4 S to ~19 S, with a modal *s* value of 10 S; no discrete size class is apparent (Falkenthal, 1980). These poly(A)$^-$ copia RNA sequences probably constitute degradation products of the poly(A)$^+$ copia RNA.

(B) Poly(A) Tract of Cytoplasmic Copia RNA. As discussed above, the majority of copia RNA is present in the cytoplasmic RNA fraction which binds to oligo(dT)–cellulose. The fact that an RNA molecule binds to oligo(dT)–cellulose, however, is not sufficient evidence for possession of a poly(A) tract. *Drosophila* mitochondrial 14S rRNA, for example, binds to oligo(dT)–cellulose but contains no 3′ poly(A) sequence (Spradling et al., 1977; Lengyel & Penman, 1977). In order to directly demonstrate that copia RNA possesses a poly(A) tract, purified copia RNA from cells labeled for 1 h with [^{3}H]adenosine was digested with ribonucleases A and T1. This ribonuclease resistant material was chromatographed on oligo(dT)–cellulose; material which bound was electrophoresed in a 10% polyacrylamide gel in order to measure the length of the poly(A) tract. The length of the copia RNA poly(A) tract was 90 nucleotides, the same as the modal length of the poly(A) tract of *Drosophila* total cytoplasmic poly(A)$^+$ RNA (Figure 2).

(C) Presence of Copia RNA on Polysomes and Its Release by EDTA. The sedimentation of total mRNA in relation to polysomes was investigated by sedimenting a ^{3}H-labeled cytoplasmic lysate on a sucrose gradient and isolating the poly(A) containing RNA (Figure 3A). After a 1-h labeling period, 74% of the mRNA population sedimented with the polyribosomes, as determined from the distribution in the gradient of [^{3}H]RNA which bound to oligo(dT)–cellulose. The sedimentation of copia RNA with polyribosomes was demonstrated by the hybridization of each fraction of the polysomal gradient to filter-bound cDm1142 DNA (Figure 3A). In five experiments, 80–91% of copia RNA sedimented with polyribosomes,

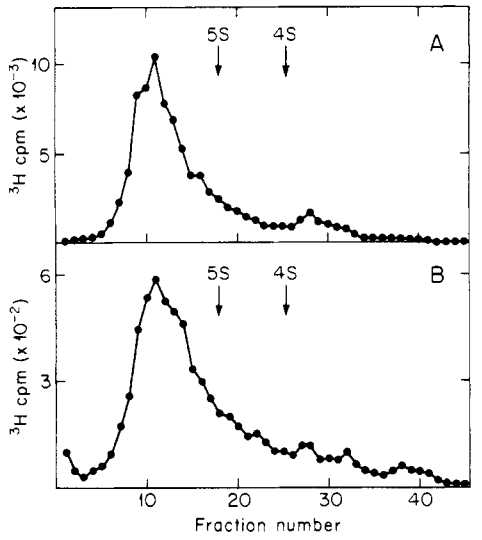

FIGURE 2: Comparison between the poly(A) tracts of copia RNA and total cytoplasmic mRNA. Cells, after concentration to 4 × 10^7/mL and preincubation for 1 h, were labeled for 1 h with [^{3}H]-adenosine (50 μCi/mL). Cytoplasmic poly(A)$^+$ RNA was prepared and a portion was hybridized to filter-bound cDm1142 DNA (see Experimental Procedures). At the end of the hybridization, the filters were washed twice at 65 °C in 2 × SSC and 5 times at 37 °C in 2 × SSC. The hybridized RNA was eluted by immersing the filters in elution buffer (10 mM Tris, pH 7.4, 1 mM EDTA, and 0.2% $NaDodSO_4$) at 100 °C for 10 min and then ethanol precipitated. The poly(A) tracts of the RNA homologous to cDm1142 DNA and of total cytoplasmic poly(A)$^+$ RNA were obtained by digestion of these fractions with DNase I, ribonuclease A, and ribonuclease T1 as described previously (Lengyel & Penman, 1975). The reactions were stopped by the addition of one volume of 2 × $NaDodSO_4$ buffer containing 500 μg/mL proteinase K; the samples were deproteinized as described under Experimental Procedures. Poly(A) tracts from the ribonuclease resistant material were obtained by chromatography on oligo(dT)–cellulose. The RNase resistant RNA which bound to oligo(dT)–cellulose was electrophoresed on 10% polyacrylamide gels as described by Hirsch & Penman (1974). (A) Poly(A) tract of copia RNA; (B) poly(A) tract of total cytoplasmic mRNA. Poly(A) migrates anomalously in polyacrylamide gels. A modal size for the poly(A) tracts in (A) and (B) of 90 nucleotides was calculated assuming that transfer RNA and 5S RNA comigrate with poly(A) tract lengths of 28 and 52 adenylate residues, respectively (Wilt, 1977).

with a peak in the region of tetrasomes.

Both the 2- and 5-kb copia RNA species sediment with tetrasomes. This was demonstrated by an experiment in which cells were labeled for 16 h and the cytoplasmic lysate was sedimented on a polysome gradient. The ribonucleoprotein in the tetrasome region of the gradient was pooled, deproteinized, and displayed on a $NaDodSO_4$–sucrose gradient. The material in the gradient which hybridized with filter-bound cDm1142 showed the same profile (in terms of distribution of label between the 5- and 2-kb species and absence of label in species smaller than 2 kb) as seen in Figure 1 (Falkenthal, 1980). To ensure that the sedimentation of copia RNA in the tetrasome region of the gradient was not due to artifactual aggregation of copia RNA molecules not involved in protein synthesis, the polysomes were displayed in a sucrose gradient containing 500 mM NaCl. High salt causes the dissociation of nonspecific RNA associated with polysomes (Zylber & Penman, 1970). The cosedimentation of copia RNA with polysomes was not altered by the high-salt treatment; the profile was similar to that shown in Figure 3A (Falkenthal, 1980). These experiments suggest that the 5- and 2-kb copia RNAs are genuinely associated with polysomes.

Messenger RNAs which are functionally associated with ribosomes are released from the polysome region of a gradient by EDTA treatment (Perry & Kelley, 1968). The *extent* of

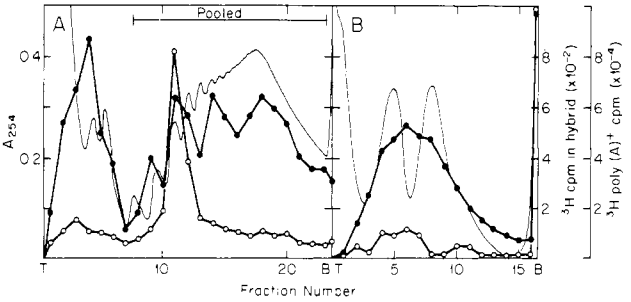

FIGURE 3: Distribution of copia RNA in a polysomal gradient and its release by EDTA. One-hundred milliliters of cells (3 × 10^6/mL) was incubated with [^{3}H]uridine (10 μCi/mL) for 16 h. The cells were harvested, and the cytoplasm was prepared and layered over a 5–40% sucrose gradient (see Experimental Procedures). The gradient was centrifuged 120 min at 25 000 rpm in an SW27 rotor, and fractions were collected into three volumes of 66% 2-propanol at −10 °C. (A) Aliquots of each fraction were removed and deproteinized (see Experimental Procedures). Half of each aliquot was hybridized to filter-bound cDm1142 DNA and cDm500 DNA, and the remainder was bound to oligo(dT)–cellulose. Histone mRNA cosedimented with tetrasomes (data not shown). (B) The remainder of the fractions sedimenting more rapidly than 100 S from (A) were pooled and the pellets were collected by centrifugation, resuspended in 1.0 mL of EDTA release buffer (100 mM NaCl, 10 mM Tris, pH 7.4, 100 mM EDTA, and 0.5 mg/mL heparin), and layered over a 5–40% EDTA sucrose gradient. The gradient was centrifuged 6 h at 36 000 rpm in an SW40 rotor and fractionated. Each fraction was deproteinized and analyzed as in (A). (—) OD_{254}; (○) ^{3}H cpm hybridized to cDm1142 DNA; (●) ^{3}H cpm bound to oligo(dT)–cellulose. Direction of sedimentation is from left to right.

Table III: Release of mRNA and Ribosomes from Polysomes by EDTA Treatment[a]

species	% release
rRNA	65[b]
poly(A)$^+$ RNA	58[c]
copia mRNA	18[d]
actin mRNA	64[d]
histone mRNA	82[d]

[a] Summary of two experiments which were carried out as described in the legend to Figure 3. [b] Determined as the total OD_{254} in species which sediment at less than 80 S (i.e., which do not pellet in the gradient shown in Figure 3B) in the presence of 100 mM EDTA, divided by the total OD_{254} applied to the gradient. [c] Determined as cpm in poly(A)$^+$ RNA [detected by oligo(dT)-cellulose chromatography] which sediments at less than 80 S in presence of 100 mM EDTA, divided by the total poly(A)$^+$ cpm applied to the gradient. [d] Determined as cpm in RNA which sediments at less than 80 S in presence of 100 mM EDTA and which hybridize to a particular filter-bound plasmid DNA, divided by the total cpm applied to the gradient which hybridize to the same plasmid DNA.

this release, particularly for individual mRNA species, has not been published. The extent to which a number of different *Drosophila* mRNA species, including copia RNA, can be released from polysomes by EDTA treatment was examined in the following experiment. Polysomal RNA was labeled with [^{3}H]uridine and displayed on sucrose gradients (Figure 3A). All material sedimenting more rapidly than 100 S was pooled, precipitated, resuspended in buffer containing EDTA, and resedimented in sucrose gradients containing EDTA (Figure 3B). The release of copia RNA, histone mRNA, and actin mRNA was assayed by filter hybridization to appropriate recombinant DNA plasmids (see Experimental Procedures). In the experiment shown in Figure 3B, 9% of copia RNA was released by EDTA treatment. On the average, 18% of copia RNA, 60% of actin mRNA, and 88% of histone mRNA were released from polysomes by EDTA (Table III). For the mRNA population as a whole, 58% of the total polysomal

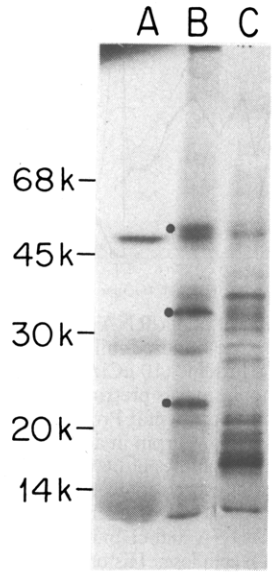

FIGURE 4: Translation of copia RNA in a rabbit reticulocyte lysate. Copia RNA was selected by hybridization to filter-bound cDm1142 as described under Experimental Procedures. This RNA was translated in an mRNA-dependent rabbit reticulocyte lysate containing [^{35}S]methionine, and the translation products were analyzed on a 10–15% gradient polyacrylamide gel (see Experimental Procedures). (A) No added RNA; (B) copia RNA purified by hybridization to filter-bound cDm1142 DNA; (C) total cytoplasmic poly(A)$^+$ RNA. The dots denote the translation products of copia RNA. Molecular-weight markers used (in order of decreasing molecular weight) were bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

poly(A)$^+$ RNA was released by EDTA. The release of only 65% of the polysomal OD_{260} indicates that only two-thirds of the ribosomes in polysomes were dissociated by EDTA treatment. The release of mRNA from polysomes by EDTA, under our conditions, is therefore not quantitative [this may be a function of the irreversible action of emetine (Grollman, 1966) used in preparing the polysomes]. Actin mRNA is released to the same extent as the total mRNA population, histone mRNA to a greater extent, and copia to a lesser extent. The differential EDTA release of mRNAs of different size suggests that there may be a correlation between the size of an mRNA and the extent to which it is released by EDTA treatment. The cosedimentation of copia RNA with polysomes and the release of copia RNA from polysomes by exposure to EDTA indicate that copia RNA is a messenger RNA.

(*D*) *Translation of Copia RNA.* A definitive test for the messenger function of an RNA is its ability to be translated into a polypeptide chain in a cell-free system. Copia RNA was purified from total ^{3}H-labeled poly(A)$^+$ cytoplasmic RNA by hybridization in formamide to filter-bound cDm1142 DNA (see Experimental Procedures). Both size classes of copia RNA, with modal *s* values of 26 S and 18 S, were obtained by this protocol (Falkenthal, 1980). This indicates that full-length 5- and 2-kb copia RNA can be isolated by hybridization to filter-bound cDm1142 DNA.

The purified copia RNA was translated in an mRNA-dependent rabbit reticulocyte lysate; the translation products were analyzed by electrophoresis in 10–15% gradient NaDodSO$_4$–polyacrylamide gels. Three polypeptides specific to copia RNA were seen (indicated by dots in Figure 4, lane B). These had molecular weights of approximately 51 000, 33 000, and 21 000. The other polypeptides seen when copia RNA was added to the reticulocyte lysate (Figure 4, lane B) were also synthesized when no RNA was added to the lysate (Figure 4, lane A); they are thus not copia specific. The questions raised by the translation of purified copia RNA into three distinct polypeptides are addressed under Discussion.

When purified copia RNA was translated in a wheat germ extract, a polypeptide of 20 000 daltons was obtained (Falkenthal, 1980). This is presumably the same as the 21 000-dalton polypeptide obtained in the rabbit reticulocyte lysate; the absence of the higher molecular weight products could be due to the low efficiency with which the wheat-germ system translates large mRNAs.

The translation products obtained when total poly(A)$^+$ cytoplasmic RNA from cultured cells was added to the reticulocyte lysate are shown in Figure 4, lane C. The pattern of polypeptides synthesized is very different from that programmed by the purified copia RNA. Faint bands which comigrate with the copia translation products can be seen, but these are not the major translation products of the total poly(A)$^+$ cytoplasmic RNA. Thus, although copia RNA is the most abundant mRNA in the cultured cells, this abundance is not reflected in the translation products of total poly(A)$^+$ cytoplasmic RNA.

(*E*) *Cytoplasmic Stability of Copia RNA.* The abundance of copia RNA in the cytoplasm (Table II) suggests that copia may be one of the more stable cytoplasmic RNAs in cultured cells. The total poly(A)$^+$ cytoplasmic RNA of *Drosophila* cultured cells has previously been shown to turn over as two components, with half-lives of 1 and 6–7 h (Lengyel & Penman, 1977). The accumulation of label in copia RNA relative to total poly(A)$^+$ cytoplasmic RNA over a period of 3 h (Table II) gives an indication of the stability of copia RNA relative to the two half-life classes of total poly(A)$^+$ cytoplasmic RNA. The percent of poly(A)$^+$ cytoplasmic RNA which is copia increases with time of labeling up to 3 h. This indicates that copia RNA does not belong to the rapidly decaying 1-h half-life class. Copia RNA is a maximal proportion of the total poly(A)$^+$ cytoplasmic RNA at steady state; thus its half-life is at least as long as the 6–7 half-life component of total poly(A)$^+$ cytoplasmic RNA.

We measured the half-life of copia RNA directly in pulse–chase experiments and compared this to the half-life of total poly(A)$^+$ cytoplasmic RNA. Cells were labeled for various periods of time with [^{3}H]uridine (pulse) after which unlabeled uridine and cytidine were added to 1 mM each to terminate rapidly the further incorporation of label (chase) (Lengyel & Penman, 1977). Cessation of labeling was demonstrated by the absence of further incorporation of radioactivity into rRNA, which is stable in exponentially growing cells during the chase period (Figure 5B). That the cessation of labeling is not due to cell death or morbidity is shown by the fact that cells maintain a generation time of 24 h during the chase regime (Figure 5A). A characteristic decay curve of total cytoplasmic poly(A)$^+$ RNA is shown in Figure 5C. This curve is a computer-generated least-squares fit for three components, one of which is stable and two of which decay (see legend to Figure 5C). The rapidly decaying component has a half-life of 1.1 ± 0.3 h, and the intermediate decaying component has a half-life of 5.3 ± 0.6 h. The stable component is the mitochondrial small subunit rRNA which has a high affinity for oligo(dT)–cellulose (Lengyel & Penman, 1977; Spradling et al., 1977). These data, summarized from three experiments in Table IV, are in agreement with previous results (Lengyel & Penman, 1977). The average half-life of the short lifetime kinetic class is 1.3 h and that of the intermediate lifetime kinetic class is 6 h.

The decay kinetics of copia mRNA were measured in the same experiment shown in Figure 5. The amount of labeled

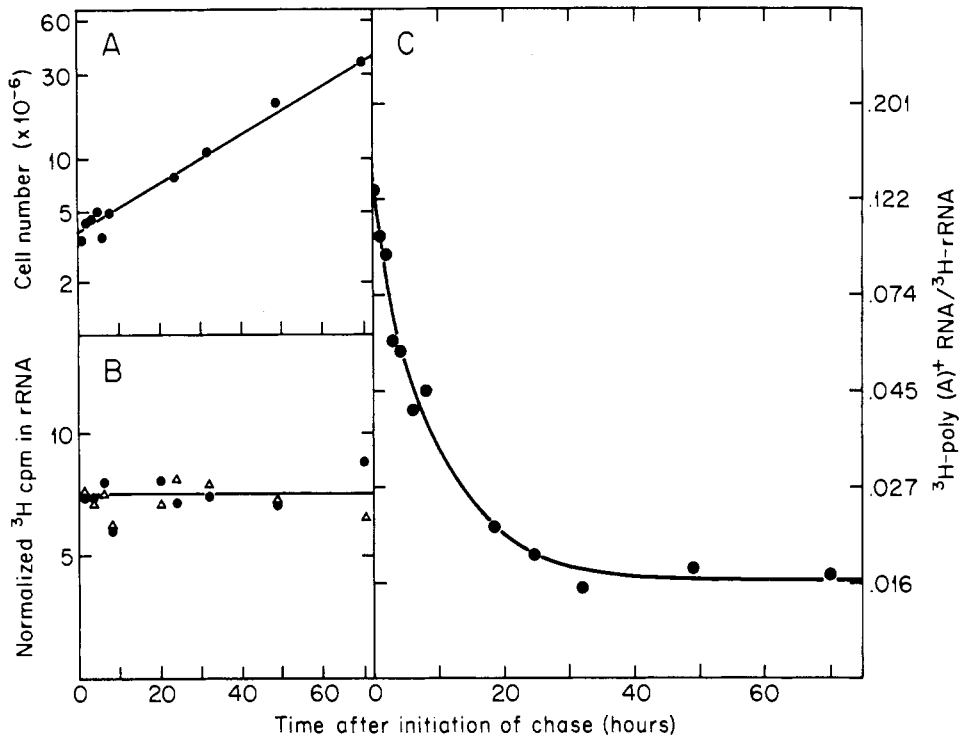

FIGURE 5: Effectiveness of 1 mM uridine and cytidine chase. Two-hundred milliliters of cells (6×10^6/mL) was labeled with [^{3}H]uridine (50 μCi/mL) for 5 h. Cells were then diluted with one-half volume of fresh medium and brought to 1 mM each of unlabeled uridine and cytidine. (A) Growth curve of cells during the 1 mM uridine and 1 mM cytidine chase. The cells were counted in a hemacytometer at various times during the chase period and corrected for volume changes due to feeding. (B) Label in rRNA during the chase. Cytoplasmic poly(A)$^-$ RNA was isolated from the culture described above at various times after the initiation of the chase and centrifuged in a 15–30% NaDodSO$_4$–sucrose gradient. (●) cpm in 19S and 26S rRNA normalized to rRNA OD_{254} and corrected for cell growth; (▲) cpm in 19S and 26S rRNA normalized to the original sample volume and corrected for cell feeding. Since the units are arbitrary the sets of corrected data were normalized to each other. (C) Decay of poly(A)$^+$ cytoplasmic RNA during the chase. Cytoplasmic poly(A)$^+$ RNA was isolated from the culture described above at various times after the initiation of the 1 mM uridine and 1 mM cytidine chase. This curve is a computer-generated least-squares fit to a linear transformation of the equation $Y = A_0e^{-K_at} + B_0e^{-K_bt} + C_0$ where A_0, B_0, and C_0 are the number of cpm at $t = 0$ in components 1, 2, and 3, respectively, K_a is the rate constant for the decay of component 1, and K_b is the rate constant for the decay of component 2. All cpm are normalized to the rRNA cpm to correct for differential yield (Singer & Penman, 1973).

Table IV: Lifetime Classes of Poly(A)$^+$ RNA

component	half-life (h) expt I[a]	expt II[b]	expt III[c]	previously published data[d]
short	1.2	1.5	1.1	1
intermediate	7.5	5.3	5.3	6–7
copia mRNA	8.5	12.5	9.5	

[a] Experiment was carried out as described in the legend to Figure 5 except that the labeling was for 18 h. [b] Experiment was carried out as described in the legend to Figure 5. [c] The computer-generated fit for this experiment is shown in Figures 5 and 6. [d] Lengyel & Penman (1977).

copia mRNA present during the chase, shown in Figure 6, was assayed by DNA-excess hybridization of labeled cytoplasmic poly(A)$^+$ RNA to filter-bound cDm1142 DNA. This curve is a computer-generated least-squares fit to a single component decay (see legend to Figure 6). Copia RNA decays as a single component with a half-life of 9.5 h. The rate of decay of copia RNA and the rates of decay of the total poly(A)$^+$ cytoplasmic RNA from several experiments are summarized in Table IV. The average cytoplasmic half-life of copia mRNA is 10 h. The stability of copia mRNA is thus 1.6 times greater than that of the intermediate-decay class of total poly(A)$^+$ cytoplasmic RNA of *Drosophila* cultured cells.

Because there are two copia RNA species in the cytoplasm, it was of interest to determine if both decay at the same rate. The size distribution of copia poly(A)$^+$ RNA was therefore determined during a cytidine–uridine chase. Poly(A)$^+$ RNA, isolated at various times after the initiation of the chase, was sedimented in sucrose gradients. Copia RNA was detected by hybridization across the gradients with filter-bound cDm1142 DNA (Figure 7). No change in the size distribution of copia RNA occurred during the chase regime; the ratio of the counts per minute in the 5-kb species to those in the 2-kb species did not change. We cannot detect any difference in the rates of decay of the 2- and 5-kb copia RNA species.

After a long period of labeling, a small amount of radioactivity appears in a species, sedimenting at 10 S, which is homologous to cDm1142 DNA (Figure 7); the amount of this species relative to the 5- and 2-kb species does not change during the chase. The 10S species is not, however, seen in RNA which was pulse labeled for 1 h (Figure 1). Furthermore, even after 16 h of labeling, it is not detected on polysomes (discussed above). These properties of the 10S copia RNA suggest that it is a degradation product of the 5- and 2-kb copia RNAs.

Discussion

Structure of Cytoplasmic Copia RNA. Copia RNA is found in the cytoplasm of *Drosophila* cultured cells in two size classes which are present in equimolar amounts. As described here, these size classes have modal *s* values of 26 S and 18 S, corresponding to the 5.2- and 2.1-kb copia RNA sequences identified by Carlson & Brutlag (1978). We have shown that after a 1-h labeling period, copia RNA has a poly(A) tract of 90 nucleotides, which is identical with the length of the

poly(A) tract of total cytoplasmic *Drosophila* poly(A)$^+$ RNA labeled for the same period of time. This length of 90 nucleotides is within the size range determined for the pulse-labeled and steady-state poly(A) tracts of the globin, silk fibroin, albumin, α-fetoprotein, and parathyroid hormone mRNAs and the total poly(A)$^+$ mRNA of *Drosophila* oocytes (Gorski et al., 1975; Nudel et al., 1976; Lizardi et al., 1975; Sala-Trepat et al., 1979; Stolarsky & Kemper, 1978; Arthur et al., 1979).

Polysomal Association and Translation of Copia RNA. When the association of copia RNA with polysomes was investigated, it was found that copia RNA cosediments with tetrasomes in a sucrose gradient. This association is not altered by sedimentation in the presence of high salt, which eliminates nonspecific binding of ribonucleoprotein particles to ribosomes (Zylber & Penman, 1970), but it is altered by EDTA treatment (Figure 3B), which is known to release mRNAs from their association with polysomes (Perry & Kelley, 1968). It is difficult to compare the release of the *Drosophila* mRNA species to that of other eucaryotic mRNAs. Although the release from polysomes by EDTA of ovalbumin and globin mRNA has been studied qualitatively, the efficiency of the release has not been satisfactorily quantitated (Palmiter, 1974; LeBleu et al., 1971). We quantitated the efficiency of the release of copia mRNA and compared this to the release of actin and histone mRNAs by hybridization of labeled cytoplasmic RNA to the appropriate recombinant DNA probes (Table III). The differential release of these mRNAs of different molecular weights by EDTA suggests that under our experimental conditions there is an inverse relationship between the efficiency of the EDTA release and the size of the mRNA.

The translation of an RNA molecule in vitro constitutes the best evidence that it functions as a messenger RNA in vivo. Using an mRNA-dependent rabbit reticulocyte lysate, we have translated hybridization-selected copia RNA. Three polypeptides of 51 000, 33 000, and 21 000 daltons were synthesized in vitro.

The fact that there are three translation products raises the question of whether the hybridization-selected copia RNA is contaminated with other mRNA sequences. This is very unlikely because of the high criterion of the hybridization procedure used to select the copia RNA for translation: the final washes of the filters prior to elution of the hybridized RNA were carried out at the T_m of a 40% G + C DNA–RNA hybrid (Ricciardi et al., 1979). This procedure is expected to wash off one-half of the hybridized copia RNA; the total amount of copia RNA obtained by the hybridization selection procedure was, in fact, only one-half of that obtained by the quantitative hybridization experiment shown in Table II. Because these stringent hybridization criteria were used to purify the copia RNA, it seems reasonable to conclude that the three polypeptides obtained in vitro are all encoded by the copia sequence. Since the complexity of the 5-kb copia sequence is sufficient to encode 150 000 daltons of polypeptide, we assume in the following discussion that the three translation products are all different polypeptides. It is possible, however, that the 33 000- and 21 000-dalton polypeptides are fragments of the 51 000-dalton polypeptide.

Because there are several size classes of copia RNA, we can ask whether all of these are translated in vivo and whether each translation product could be translated from a different size class. The majority of cytoplasmic copia RNA is present in two size classes of 5 and 2 kb (Carlson & Brutlag, 1978; Figures 1 and 7), both of which are found on polysomes (Falkenthal, 1980). In addition, a small amount of a 10S poly(A)$^+$ copia RNA species can be detected after an 18-h labeling period followed by a 5-h chase (Figure 7). The 10S species is not detected after a 1-h labeling period. This labeling behavior coupled with the absence of the 10S species from polysomes (Falkenthal, 1980) suggests that the 10S copia RNA does not function as a messenger RNA but is a degradation product. The presence of both 5- and 2-kb copia RNAs on polysomes suggests that both of these species are translated in vivo. Assignment of the translation products to the 5-kb species and/or the 2-kb species, however, will require the separation and translation of these two RNAs.

Copia mRNA in polysomes is found on foursomes (Figure 3A), the same polysome class on which histone mRNA, which encodes proteins of 12 500–25 000 daltons, is found (legend to Figure 3A). The location of copia mRNA on foursomes is thus consistent with the in vivo production of 21 000-dalton polypeptide; it is consistent with the in vivo production of the 33 000- and 51 000-dalton polypeptides if the translation of these polypeptides in vivo is initiated at a lower than average rate.

Cytoplasmic Concentration and Stability of Copia mRNA. The adenylated mRNA population of eucaryotic cells can be divided into an abundant (10 000–100 000 molecules per cell), an intermediate (100–200 molecules per cell), and a rare (1–10 molecules per cell) concentration class (Bishop et al., 1974; Levy W. & McCarthy, 1975; Axel et al., 1976; Hastie & Bishop, 1976; Hereford & Rosbash, 1977). The fact that copia RNA constitutes 2.2% of the total poly(A)$^+$ RNA of the 2-L cell line indicates that it belongs to the abundant concentration class. Assuming that there are 0.16 pg of poly(A)$^+$ mRNA per *Drosophila* 2-L cell (see Experimental Procedures) and that the 5- and 2-kb copia species are present in equimolar amounts at steady state, we calculate that there are 1900 molecules of cytoplasmic copia RNA per cultured cell. Copia mRNA molecules are thus 10- to 20-fold more concentrated in the cytoplasm than the mRNA molecules of the intermediate concentration class.

Since the steady-state concentration of a cytoplasmic RNA is equal to its rate of export to the cytoplasm divided by its rate of turnover in the cytoplasm (Greenburg, 1972), we can calculate the relative rates at which copia mRNA and mRNA species of the intermediate concentration class are exported to the cytoplasm by comparing their half-lives and steady-state concentrations. Copia mRNA turns over in the cytoplasm with a half-life of 9.5 h; this is 1.6 times longer than the average half-life of the intermediate decay class of cytoplasmic poly(A)$^+$ RNA (Lengyel & Penman, 1977; Table III). In *Drosophila* cultured cells, the intermediate decay class of mRNA consists predominantly of mRNAs which belong to the abundant and intermediate concentration classes (Lenk et al., 1978). This calculation indicates that copia mRNA is exported to the cytoplasm at a 6- to 12-fold greater rate than the mRNAs of the intermediate concentration class. The kinetics of transcription and processing of copia nuclear RNA molecules have recently been characterized in order to define the steps which control the expression of copia RNA (S. Falkenthal and J. A. Lengyel, unpublished results).

In conclusion, we have demonstrated that cytoplasmic copia RNA has the structural and metabolic characteristics of a messenger RNA and is translated *in vitro* into three polypeptides of approximately 51 000, 33 000, and 21 000 daltons. This knowledge should contribute to our further understanding of the biological role of the copia genes and of mobile DNA sequences in general.

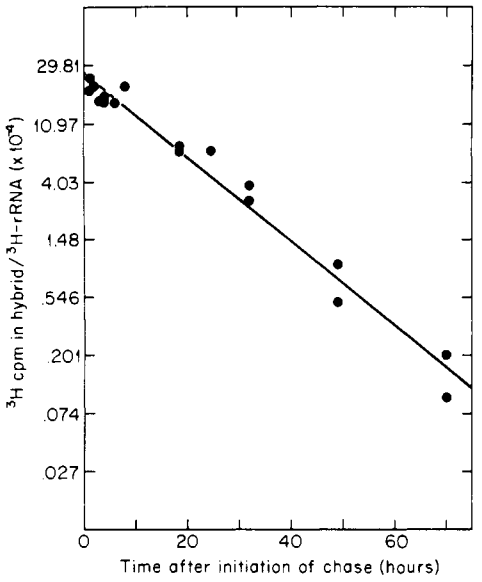

FIGURE 6: Decay of labeled poly(A)$^+$ copia RNA during a chase. After incubation with [^{3}H]uridine for 5 h, incorporation of label was terminated by the addition of unlabeled uridine and cytidine as described in the legend to Figure 5. The curve is a computer-generated least-squares fit to a linear transformation of the equation $Y = A_0e^{-K_at}$ where A_0 is the number of cpm in copia RNA at $t = 0$ and K_a is the rate constant for the decay of copia RNA. All counts were normalized to rRNA to correct for differential yield (Singer & Penman, 1973).

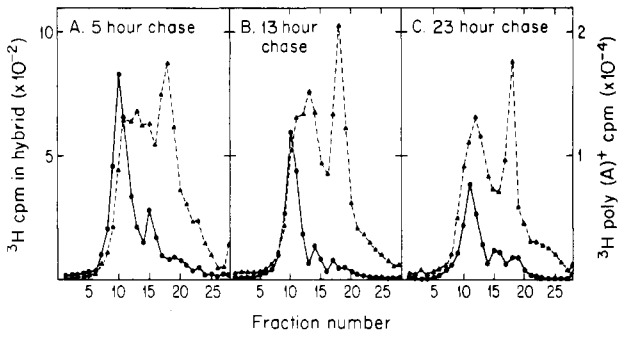

FIGURE 7: Size of copia RNA during a cytidine–uridine chase of a [^{3}H]uridine label. Two hundred milliliters of cells (2×10^6/mL) was labeled with [^{3}H]uridine (50 μCi/mL). After 18 h, incorporation of label was terminated by the addition of unlabeled uridine and cytidine as described in the legend to Figure 5. The poly(A)$^+$ cytoplasmic RNA was prepared as described under Experimental Procedures. Five percent of each sample was heat denatured at 65 °C for 5 min, cooled rapidly on ice, layered onto 15–30% LSB (10 mM Tris, pH 7.4, 1 mM EDTA, and 0.2% $NaDodSO_4$–sucrose gradients, and centrifuged at 33 000 rpm in a Beckman SW40 rotor for 18 h at 26 °C. One-tenth of each fraction was taken for total counts, and the remainder of each fraction was hybridized to filter-bound cDm1142 DNA at 65 °C for 18 h. Poly(A)$^+$ cytoplasmic RNA was isolated at the indicated time after the initiation of the 1 mM uridine–cytidine chase: (A) 5 h; (B) 13 h; (C) 23 hours. (●–●) ^{3}H cpm hybridized to filter-bound cDm1142 DNA; (▲---▲) total cytoplasmic poly(A)$^+$ ^{3}H cpm. Direction of sedimentation is from right to left.

Acknowledgments

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Regulation of Cd^{2+}/Zn^{2+}-Stimulated Metallothionein Synthesis during Induction, Deinduction, and Superinduction†

C. E. Hildebrand* and M. D. Enger

ABSTRACT: Metallothioneins (MTs) are low-molecular-weight, thiol-rich, metal-binding proteins which are synthesized in animal tissues or in cultured cells in response to Cd^{2+} or Zn^{2+} exposure. We have examined regulation of MT synthesis in a Cd^{2+}-resistant (Cd^r) Chinese hamster cell which is proficient in induction of MT synthesis following exposure to either Cd^{2+} or Zn^{2+}. The MTs synthesized by the Cd^r cell were characterized by Sephadex G-75 column chromatography, nondenaturing polyacrylamide gel electrophoresis, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The Cd^r cell MTs are cysteine rich and leucine deficient, bind Zn^{2+} and Cd^{2+} with high affinity, and are synthesized only during exposure of the cells to Cd^{2+} or to excess Zn^{2+}. Both of the major isoMTs reported to be synthesized in cells in vivo or in culture are induced coordinately in Cd^r cells during Cd^{2+} or Zn^{2+} exposure. Following addition of Cd^{2+} (2 μM) or Zn^{2+} (100 μM) to the growth medium, MT synthesis in Cd^r cells increases above the low basal level within 2 h, reaches a maximal synthesis rate (at least 30-fold > basal level) by 7 h, and thereafter declines slowly (by 24 h) to a constant rate of approximately 40–60% of the maximal rate. Induction of MT synthesis is inhibited by 5 μg/mL actinomycin D (AM), indicating that RNA synthesis is required for induction. MT synthesis also is dependent upon the continued exposure to inducing metal since withdrawal of inducer at the time of maximal MT synthesis results in a deinduction of MT synthesis with a half-time of 2–3 h. Studies of AM effects at both high (2 μg/mL) and low (0.05 μg/mL) levels on induction and deinduction kinetics revealed the phenomenon of "superinduction" of MT synthesis at the high AM level but not at the low level. Analyses of induction and deinduction kinetics, together with the results of RNA synthesis inhibitor studies, suggest that induction of MT synthesis is regulated primarily at the level of transcription of thionein mRNA. Further, examination of primary and secondary induction (i.e., secondary exposure to inducer following deinduction from a primary exposure) events indicates that translational control over MT synthesis is not a major regulatory factor in this cellular system. Finally, the existence of the AM-mediated superinduction of MT synthesis, a phenomenon originally observed in steroid-mediated induction of specific hepatic enzymes, suggests that similar regulatory mechanisms may operate in these quite different inducible systems.

Metallothioneins are low-molecular-weight, thiol-rich, metal-binding proteins (Margoshes & Vallee, 1957; Kägi & Vallee, 1960, 1961). They are ubiquitous among eukaryotes and have been reported in microorganisms [reviewed by Kojima & Kägi (1978)]. These proteins, or thioneins, are synthesized de novo by cells both in vivo (Piscator, 1964; Webb, 1972; Richards & Cousins, 1975a; Winge et al., 1975) and in culture (Lucis et al., 1970; Webb & Daniel, 1975; Hildebrand et al., 1979, and references therein) as a consequence of exposure to Zn^{2+}, Cd^{2+}, and, in some cases, Hg^{2+} or Cu^{2+} (Kojima & Kägi, 1978). Although a well-defined physiological function of metallothionein has not been established, much of the previous work on metallothionein has been performed in the context of trace-element metabolism [e.g., in homeostatic regulation of essential trace metals (viz., Zn^{2+})] (Cousins, 1979) and detoxification of nonessential heavy metals (viz., Cd^{2+} or Hg^{2+}) (Kojima & Kägi, 1978; Cherian & Goyer, 1978). Only a few studies have focused on the mechanisms involved in the regulation of induction of thionein synthesis (Richards & Cousins, 1975b; Squibb et al., 1977; Day et al., 1978; Panemangalore & Brady, 1978). Several reports suggest that the primary induction of thionein synthesis (i.e., the response occurring upon initial exposure to the inducing metal) requires transcription of thionein-specific mRNA (Richards & Cousins, 1975b; Squibb & Cousins, 1977; Squibb et al., 1977; Anderson & Weser, 1978; Hidalgo et al., 1978; Panemangalore & Brady, 1978; Enger et al., 1979b). However, some studies suggest that control of induction of thionein synthesis also is exerted at the level of translation of thionein mRNA (Squibb et al., 1977; Anderson & Weser, 1978); Panemangalore & Brady, 1978).

In the present study, we have utilized a Cd^{2+}-resistant variant of the cultured Chinese hamster cell (line CHO), which has been shown to be especially proficient in the Cd^{2+}- or Zn^{2+}-mediated induction of metallothionein (MT)[1] synthesis (Hildebrand et al., 1979), to examine the regulation of metal-mediated MT synthesis. Further, the phenomenon of ac-

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[1] Abbreviations used: MT, metallothionein; AM, actinomycin D; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; TEMED, *N,N,N',N'*-tetramethylethylenediamine; cpm, counts per minute; Cx, cycloheximide; Puro, puromycin.

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