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An analysis of the rate of metallothionein mRNA poly(A)-shortening using RNA blot hybridization

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ABSTRACT

A progressive reduction in the size of rat metallothionein-1 mRNA following induction by copper chloride or dexamethasone was demonstrated on RNA blots, and was shown to be due to shortening of the poly(A)-tail. The rate of poly(A) removal was the same in rat liver and kidney following copper chloride induction, in rat liver following dexamethasone induction, and in mouse liver following copper chloride induction. In mouse liver metallothionein-1 and 2 mRNAs were shortened at the same rate. The reduction of the poly(A) tail was more rapid in the first 5 hours (approximately 20 nucleotides/h) but much slower (approximately 3 nucleotides/h) after the poly(A)-tail had been reduced to about 60 residues. Metallothionein mRNA molecules with poly(A) tail sizes less than 30-40 nucleotides were not observed. Exonuclease digestion of the poly(A)-tail is suggested, at least in the initial rapid phase. It is hypothesized that poly(A)-tails longer than 30 are required for mRNA stability and that much longer poly(A) tails may give newly synthesized mRNA molecules a competitive advantage in protein synthesis.

INTRODUCTION

Most eukaryotic mRNA species have a sequence of adenylic acid residues added to their 3'-ends following transcription (for reviews, see refs. 1 and 2). Despite extensive investigation the reason for the existence of the poly(A) tail is still unclear. Newly synthesized mRNA molecules have poly(A) tails of about 200 nucleotides which are shortened rapidly to about 40-60 nucleotides (3). This observation led to the suggestion that the length of poly(A) may be regulating mRNA stability (3,4) and artificially deadenylated mRNA is less stable when injected into Xenopus oocytes (5,6). A tail of 30 adenylate residues was found to be sufficient to stabilize globin mRNA (7). However, newly synthesized mRNA molecules, with long poly(A) tails were shown to be equally likely to be degraded as older mRNAs with short poly(A) tails (8).

Some evidence suggests that poly(A) may have a direct role in protein synthesis. For example, Doel and Carey (9) showed that deadenylated chicken ovalbumin mRNA was translated poorly in rabbit reticulocyte lysates

compared with the fully adenylated molecule. In addition, Jacobson and Favreau (10) found that synthetic poly(A) had a specific inhibitory effect on translation of poly(A)+ mRNAs in reticulocyte lysates.

To examine hypotheses of poly(A) function, suitable methods of poly(A) size analysis preferably in specific mRNA species are required. Early procedures for analysis of poly(A) size depended upon its resistance to digestion by ribonucleases (11). Messenger RNA molecules containing different poly(A)-tail lengths have been separated using thermal elution from poly(A)-binding materials such as oligo (dT)-cellulose (12), but often this analysis has proved unsuitable for mRNA species with poly(A) below about 20 residues since these bind poorly (12). More recently the use of cloned probes bound to solid supports has facilitated analysis of poly(A)-sizes in some individual mRNAs (13). Despite the large number of studies, most conclusions about poly(A)-function have been based upon analysis of globin mRNA or of total mRNA in cultured cells. No analysis of poly(A)-shortening rates in mRNAs in different organs of an animal have been presented.

In this paper we use RNA blots to analyze the shortening of the poly(A)-tails on metallothionein mRNA in rat liver and kidney following the induction by copper chloride or dexamethasone.

MATERIALS AND METHODS

Materials

Oligo(dT) (12-18) and RNAase H were purchased from Pharmacia-PL Biochemicals. α-^{32}P-deoxycytidine triphosphate (3000 Ci/mmole) was supplied by Amersham. Dexamethasone phosphate was obtained from David Bull Laboratories, Melbourne, Victoria, Australia.

RNA Isolation

Total RNA was isolated from the livers or kidneys of adult male Sprague-Dawley rats (200g) or C57 black mice at various times following treatment with $CuCl_2$ (3mg $CuCl_2$/kg in 0.9% NaCl, intraperitoneal) or dexamethasone (4 mg/kg intraperitoneal). The RNA isolation procedure has been described previously (14).

RNA Blots

Gel electrophoresis of RNA was performed as described by Dobner et al. (15). Total RNA (5 ug per lane) was heated for 5-10 min at 65°C in a mix containing 6% w/v formaldehyde, 50% formamide, 0.02% bromophenol blue and 20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate pH 7.0. Electrophoresis was

performed using 2.2% w/v horizontal agarose gels containing 6% w/v formaldehyde, 20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate pH 7.0, for 16 hours at 30-40 mA constant current. The electrophoresis buffer was 20 mM MOPS, 1 mM EDTA, 5 mM NaAc pH 7.0. The total RNA was visualized using ethidium bromide to ensure integrity, then transferred to nitrocellulose essentially as described by Thomas (16). The filters were baked 2 hr. at 80°C in vacuo, then prehybridized 16 hours at 25°C in 50% formamide, 5 x SSCE (0.75 sodium chloride, 0.075M sodium citrate, 25 mM EDTA pH 8.0), 1 x Denhardts reagent (0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrdidone) and 250 ug/ml herring sperm DNA. The filters were then hybridized 24 hours at 25°C in the above solution which also contained 100 ug/ml of both poly(U) and poly(C) together with approx. 100 ng of mouse metallothionein-1 cDNA (17) approx. 400 bp or a mouse metallothionein-2 probe approx. 200 bp (18) nick-translated to a specific activity of $>10^8$ cpm/ug. After hybridization, filters were washed in 2 x SSC, 0.1% SDS (30 min room temp.) then in 0.5 x SSC, 0.1% SDS (60 min, 55°C). Cross-hybridization between metallothionein-1 and -2 under these conditions was <5%. Autoradiography was carried out using Kodak X-AR5 film together with Dupont intensifying screens.

RNAase H analysis of total RNA samples

Total RNA isolated from the livers of copper-injected rats (5 and 8 h post-injection) was used for the initial study. The RNAase H treatment was similar to that described by Sippel et al. (19). Total RNA (30 ug) was made 1 mM in EDTA, and heated at 100°C for 5 min. Tubes were chilled on ice, oligo(dT) (0.3 ug) added, and allowed to hybridize 10 min at 25°C. Reactions were then made 50 mM in KCl, and hybridization was continued for a further 10 min at 25°C. The final reaction mix (40 ul) contained 25 mM KCl, 28 mM $MgCl_2$, 20 mM Tris pH 8.0, 0.5 mM EDTA, 0.3 ug oligo(dT) and 30 ug total tRNA. An aliquot was removed at t = 0 min before the addition of RNAase H (0.5U, 1 U catalyses the production of 1 nmole nucleotide in 20 min at 37°C). Reactions were incubated at 37°C. Control reactions lacked oligo(dT). Aliquots of reaction were removed at 7.5 min and 15 min from experimental tubes and at 15 min from control tubes. These aliquots were extracted with phenol/$CHCl_3$ (1:1) buffered to pH 8.0 with 10 mM Tris, 1 mM EDTA. The total RNA was then precipitated, washed two times with 70% ethanol, dried and dissolved in H_2O. Sample preparation for electrophoresis was as described above. Transfer, prehybridization and hybridization conditions were also as described above.

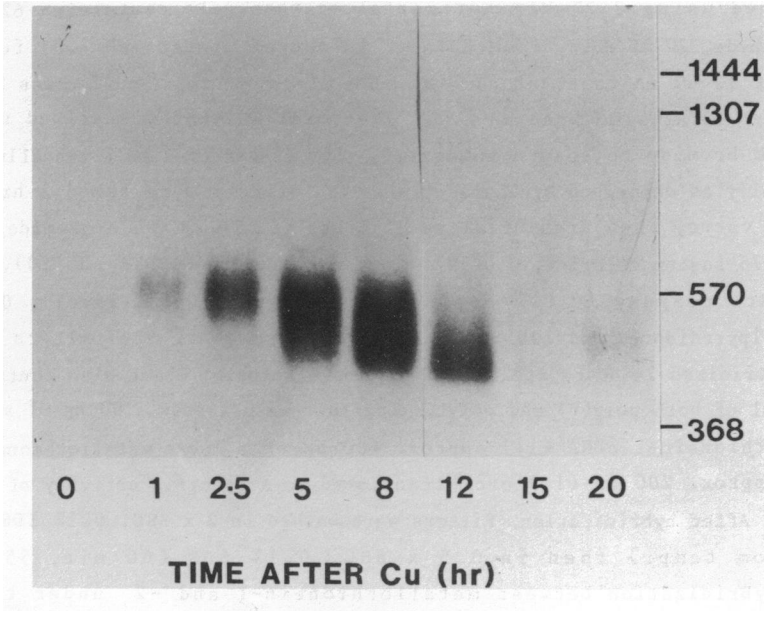

Figure 1. Size changes in metallothionein-1 mRNA in rat liver following copper chloride administration. RNA was isolated from rat liver at various times following injection (IP) of copper chloride. Total RNA (5 ug) was electrophoresed on 2.2% agarose gels containing 6% formaldehyde (15). After transfer to nitrocellulose (16) the filter was probed with a nick translated mouse metallothionein-1 cDNA (17). After washing to remove the non-hybridized probe, the metallothionein-1 mRNA was detected by autoradiography. The size markers are pBR322 digested with Taq 1.

RESULTS

The size change of metallothionein mRNA in rat liver following copper chloride injection is due to poly(A) removal

We have previously reported that metallothionein mRNA in rat liver rapidly increases (16 fold in 7 hours) following an injection of $CuCl_2$; thereafter the mRNA concentration in the liver decreases with a half-life of 3 to 4 hours (14). When total liver RNA isolated at different times following $CuCl_2$ injection was analyzed by RNA blot hybridization using a mouse metallothionein-1 cDNA probe, the RNA was seen to decrease rapidly in size from about 600 nucleotides when first detected (Fig. 1 1 hour) to about 440 nucleotides 12 to 15 hours after injection. At intermediate times (e.g. 5 or 8 hours) mRNA sizes ranged between about 600 and 470 nucleotides. At 20 hours a small amount of mRNA, larger than that present at 15 hours, was detected which probably resulted from a stress induction

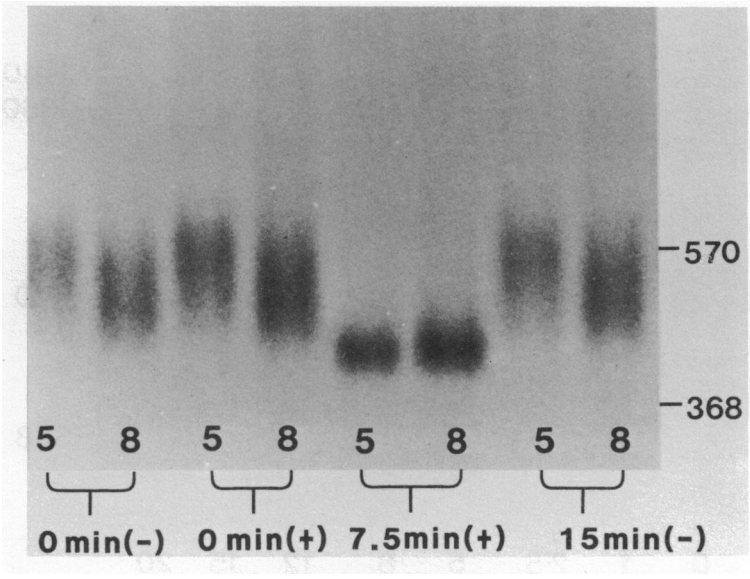

Figure 2. RNAase H treatment of metallothionein mRNA. Total RNA isolated 5 or 8 hours following copper administration was incubated for 0, 7.5 or 15 min. with ribonuclease H in the presence (+) or absence (-) of oligo(dT) as described in the text. The RNA was then analyzed by the RNA blot procedure as described for Figure 1. Only the relevant portion of the blot is shown.

rather than the $CuCl_2$ injection.

Shortening of the poly(A) tail (23) was the most likely explanation of these changes. To test this we incubated RNA in the presence of RNAase H and oligo(dT). RNAase H has the property of degrading the RNA strand of a DNA/RNA hybrid, and has been used to remove the poly(A)-tail from mRNA (12). Incubation of RNA isolated 5 or 8 hours after $CuCl_2$ injection with RNAase H and oligo(dT) for 7.5 min. reduced both RNA samples to the same size (about 400 nucleotides, Fig. 2) indicating that both the heterogeneity and size differences in the two samples was due to differing poly(A) tail lengths. Incubation for 15 min. in the absence of oligo(dT) did not cause any size alteration, showing that the size reduction was indeed due to RNAase H and not a contaminating nuclease. Significantly, the RNAase H treated sample (presumably completely deadenylated) was smaller than the smallest size (440 nucleotides) seen in 15 hours following $CuCl_2$ injection (Fig. 1).

Poly(A) removal from kidney metallothionein RNA following copper induction

The simplicity of this method of analysis suggested that we could

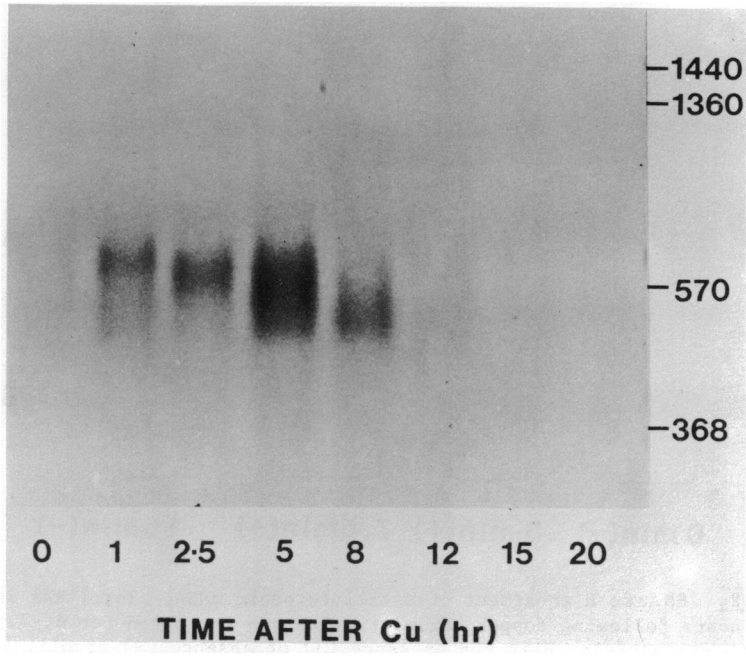

Figure 3. Size changes in metallothionein-1 mRNA in rat kidney following copper chloride administration. Total RNA was isolated from rat kidneys at various times following injection (IP) of copper chloride and analyzed by RNA blot hybridization as described in Figure 1.

study the rate of poly(A) removal and mRNA stability for a defined mRNA in different tissues and with different inducers. Figure 3 shows an RNA blot analysis of metallothionein-1 RNA from the kidneys of rats following copper chloride injection. The metallothionein-1 mRNA was found to shorten at a similar rate to that in the liver. The rapid decrease in mRNA levels between 5 and 8 hours suggested that the half-life of metallothionein-1 mRNA in the kidney may be shorter than in the liver, however, analysis of more animals failed to demonstrate a consistent difference between mRNA half-lives in the two tissues (results not shown).

Poly(A) removal from liver metallothionein RNA following dexamethasone treatment

The RNA blot shown in Fig. 4. shows that a similar shortening of poly(A) occurs with rat liver metallothionein-1 mRNA induced following dexamethasone injection. It is of interest that the 4 hour time point shows a fairly discrete band of reduced size, indicating that the poly(A) shortening is due to an exonuclease rather than a random endonuclease

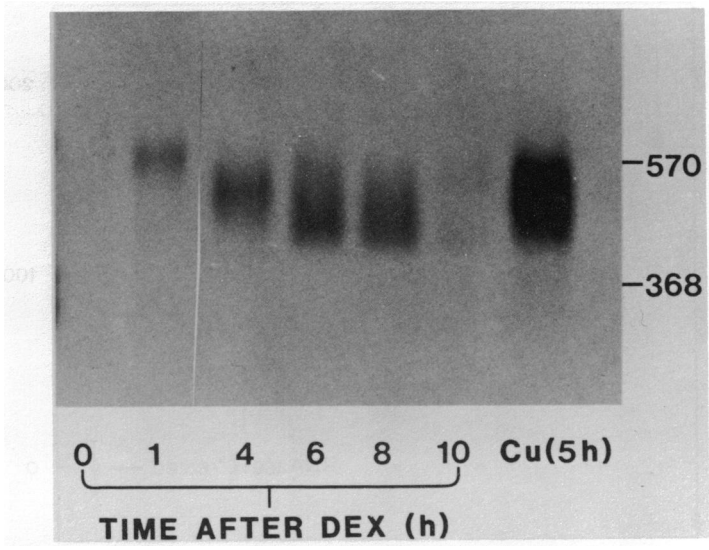

Figure 4. Size changes in hepatic metallothionein-1 mRNA following dexamethasone administration. RNA was isolated from the livers of rats at various times following treatment with dexamethasone. Metallothionein-1 mRNA was detected by RNA blot hybridization as described in Figure 1. Cu (5h) refers to a liver RNA sample 5 hours after injection of a rat with $CuCl_2$.

cleavage which would have generated a wider range of molecular sizes. The 6 and 8 hour samples show that some new transcripts (long poly(A) molecules) are reappearing, probably due to continuing release of dexamethasone from the injection site. By 10 hours the metallothionein mRNA has largely disappeared. Lane 8 shows a copper induced RNA from 5 hours after injection, illustrating that the size range was identical to the hormonally induced sample, but that the degree of induction achieved by copper was much greater than with dexamethasone at the doses used.

Comparison of the rate of shortening of poly(A) in metallothionein mRNA in liver and kidney with different inducers

To more directly analyze the rate of shortening of poly(A) under different physiological conditions, results from a number of separate RNA blots were combined for the data shown in Fig. 5. We plotted the smallest size of mRNA present at any given time because average or maximum mRNA size would be influenced by the appearance of new mRNAs over an extended period, particularly in the liver following $CuCl_2$ injection (where new transcripts appeared for up to 5 hours), or when absorption was delayed. The curves for

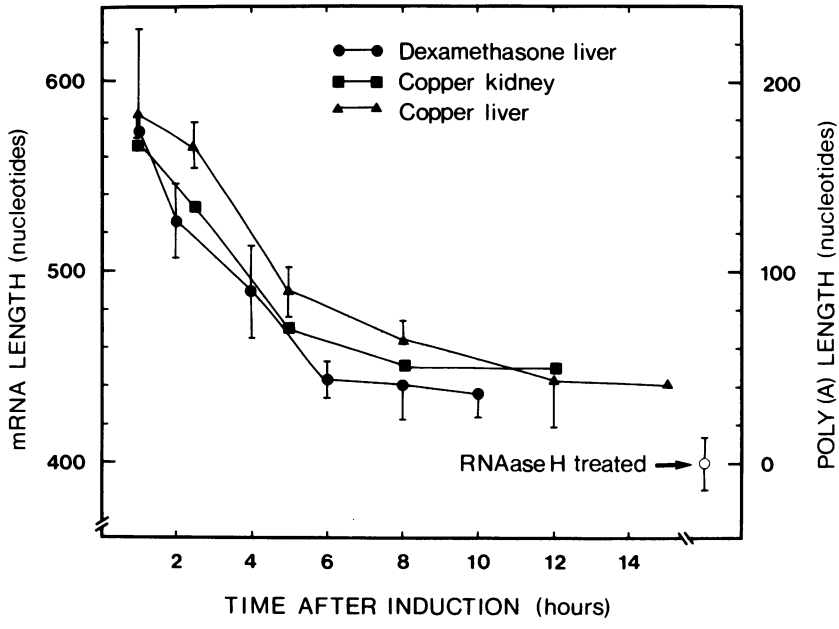

Figure 5. Comparison of the rates of poly(A) shortening in metallothionein-1 mRNA following $CuCl_2$ or dexamethasone injection. Data from 3 RNA blots were combined to produce the liver results and one blot for the kidney. The smallest mRNA detected at any one time was plotted against the time following treatment. The points are shown mean ± 2 SEM. The size of mRNA deadenylated with RNAase H is shown on the right. Liver RNA from dexamethasone treated animals ●—●; liver RNA from $CuCl_2$ treated rats ▲—▲ ; kidney RNA from $CuCl_2$ treated rats ■—■.

liver mRNA after dexamethasone and copper are an average size ± 2SE, estimated from 3 separate RNA blots. The kidney data shows results of a single blot. There is no significant difference between the rates of shortening of poly(A) in the liver and kidney or between copper and dexamethasone as inducers. Comparison of the dexamethasone and copper induced mRNA on one blot showed identity of sizes, (Fig. 4 and unpublished data). It is apparent that the poly(A) shortening was more rapid in the first 6 hours following synthesis (approx. 20 nucleotides per hour), at which point the poly(A) tail length is approximately 60 residues. Over the following 8 hours the mRNA decreased in size more slowly (approx. 3 nucleotides/hour). No RNA molecules were observed that had a poly(A) tail smaller than 30-40 nucleotides.

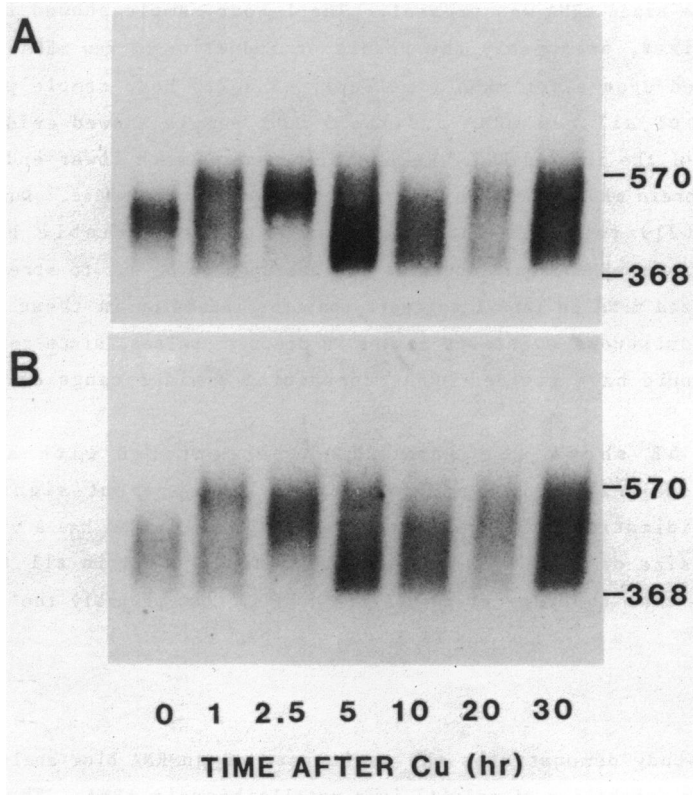

Figure 6. A comparison of the metallothionein-1 and -2 mRNA size changes in mouse liver following $CuCl_2$ administration. RNA blots of total liver RNA from mice at various times after injection were probed with either metallothionein-1 (A) or metallothionein-2 (B) cDNAs.

A comparison of metallothionein-1 and -2 mRNA sizes in mouse livers following copper chloride injection

In the mouse there are two metallothionein genes which are apparently induced co-ordinately (18). Since the molecules differ significantly in their nucleotide sequences it seemed possible that differences in poly(A) removal rate or degradation of the mRNA might be detectable. Fig. 6A shows an RNA blot analysis of mouse liver RNA following copper chloride injection using a metallothionein-1 probe. It is immediately apparent that the pattern of poly(A) shortening was not as consistent as that shown for the rat (Fig. 1). This was a consequence of the higher level of mRNA found in some untreated animals (e.g. Fig. 6 zero time). In this sample an

intermediate sized mRNA was present. The 1 hour sample showed the full range of sizes, presumably the result of induction of new mRNA (large), superimposed upon older mRNA (smaller). The 2.5 hour sample probably consisted of all new mRNA and the 5 hour sample showed evidence of shortening of the induced RNA. We generally observe much lower endogenous metallothionein mRNA levels in the rat compared with the mouse. Durnam and Palmiter (21) reported that mice have quite variable hepatic metallothionein mRNA levels and this was presumed to be due to stress. The discrete sized mRNA in lane 1 suggests that the induction in these animals is not a continuous event but occurs in discrete pulses, since continuous induction would have yielded mRNAs containing a wider range of poly(A) lengths.

Fig. 6B shows the same RNA blot reprobed with a mouse metallothionein-2 cDNA, under conditions that prevent significant cross-hybridization. Clearly the metallothionein-2 mRNA has a virtually identical size distribution to metallothionein-1 mRNA in all tracks, suggesting that the rate of poly(A)-removal is most probably the same for each.

DISCUSSION

This study demonstrates the usefulness of an RNA blot analysis for studying the metabolism of poly(A) in a metallothionein mRNA. The method is rapid and has the advantage of demonstrating poly(A) size by detection of the mRNA to which it is attached rather than by labelling of the poly(A) moiety itself. In the latter method one must adjust the measured distribution, since the contribution of radioactivity is in direct proportion to the length of the molecule and a failure to appreciate this has led to an incorrect conclusion that the synthesis inhibitor emeteine did not affect the rate of poly(A) shortening (21). The analysis is applicable to metallothionein mRNA since it is small enough for changes in poly(A) length to have an appreciable effect upon gel migration. A similar use of RNA blots and RNAase H was reported by Rosenthal et al. (22) in their study of alteration in patterns of polyadenylation following fertilization of clam eggs. Some changes in size of molecules as large as 6,000 nucleotides were detected, but detailed analysis was not possible, since the change in mobility caused by loss of the poly(A) was very small. To extend the method to larger mRNA molecules, these could be hybridized to a synthetic oligonucleotide complementary to a region about 200 nucleotides

5'- from the poly(A) addition site. Digestion with RNAase-H prior to the gel electrophoresis would split the mRNA in two, with the poly(A) on a piece long enough to allow specific probe detection but short enough to permit detailed analysis of poly(A) size changes.

Since poly(A) molecules have been shown to have an anomalous migration relative to ribosomal RNA markers in some gel systems (23), we were concerned that our mRNA size estimates may be in error. The general agreement of our poly(A) length estimates, i.e. newly synthesized molecules with poly(A)-tails of 150-200 nucleotides that shorten to about 30-40 residues, with other literature reports (1,2,21), convinced us that the denaturing gel system we used gave reasonably accurate size estimates.

We analyzed the changes in poly(A) size in metallothionein mRNA in the liver and kidney of rats and mice following induction by copper or dexamethasone, using a metallothionein-1 probe with the rat and metallothionein -1 and -2 with the mouse RNA samples. Metallothionein mRNA which first appeared following induction contained a long poly(A)-tail of about 150-220 nucleotides, similar in size to most newly synthesized mRNA molecules (3). The poly(A) underwent a rapid shortening, such that by 5 hours after induction some of the poly(A) molecules reached a length of about 60 nucleotides. The shortening after this time seemed to be much slower. The pattern of size reduction was not really consistent with the random endonuclease cleavage favoured by Sheiness et al. (21). In contrast, some samples demonstrate that the poly(A) becomes progressively shorter following an induction event (e.g. Fig. 4A, 4 hr. sample) and we conclude that the pattern of shortening is suggestive of an exonuclease removal of poly(A) from metallothionein mRNA at least during the initial rapid shortening period. The data of Sheiness et al. (21) was, however, consistent with an exonuclease action which became slower as the poly(A)-tails shortened and this is exactly the pattern we find in metallothionein mRNA (Fig. 5).

The fact that the newly synthesized molecules are longer and become progressively shorter with time allows an assessment of the recent history of induction in the animal. This was illustrated in Fig. 6, in which two size classes of metallothionein mRNA were superimposed in the mouse RNAs examined. From the sizes of the mRNA molecules, we could deduce that two different induction events were involved, one being uncontrolled, possibly due to stress and restricted in time since the mRNA deriving from this appeared as a relatively discrete band, and the other induction being due

to the copper chloride injection. Without an appreciation of the nature of the size distribution, results as shown in Fig. 6 would be difficult to interpret. We have found this analysis useful for analyzing metallothionein mRNA in neonatal rats and in tissue culture (J.F.B. Mercer and T. Stevenson, unpublished data).

We found no evidence for differential rates of poly(A)-removal in the two tissues, or with the two inducers. The rate of poly(A)-removal also appeared to be the same with both metallothionein-1 and -2 mRNAs. This suggests that poly(A) removal rates may well be constant for many messages, however, this will need to be confirmed by further analysis of a range of mRNAs. Under the conditions examined, the mRNA half-lives also seemed relatively constant, and both metallothionein-1 and metallothionein-2 mRNA species also appeared coordinately regulated and degraded (Fig. 4). It would be informative to examine the kinetics of poly(A) removal using mRNAs with different half-lives to further explore the relationship between poly(A) lengths and mRNA stability.

Others have reported that the initial phase of poly(A)-shortening is more rapid, and also a steady state population of apparently more stable mRNA species with poly(A)-tails of 40-65 nucleotides has been found (3,4). Our data and these results are consistent with a model that places a restriction on the rate of poly(A)-decay below a length of about 60 nucleotides, possibly due to a protein tightly binding to this region, perhaps one that overlaps the 3' untranslated region as has been proposed by Littauer and Soreq (1). The proteins associated with the longer sections of poly(A) may be less effective in protecting the molecule against nuclease attack. The absence of metallothionein mRNA molecules with poly(A) tails shorter than about 30 residues is in agreement with studies with other mRNAs (24,25) and it is likely that the molecules become metabolically labile as soon as these residues are removed.

Nudel et al. (26) reported that the stability of globin mRNA molecules with a poly(A)-tail length of only 34 residues was indistinguishable from those molecules with longer poly(A)-tails when injected into Xenopus oocytes. This fact, taken together with the observed equal probability of decay of new or old mRNAs (with long or short poly(A)-tails respectively) (8), suggests that above a length of 30 nucleotides the poly(A)-tail does not have a role in stabilizing mRNA. Why then should mRNA molecules be synthesized with such long poly(A) tails? One possible explanation is that the longer poly(A) tracts confer a competitive advantage in protein

synthesis, for example by increasing the rate of initiation of protein synthesis. A role for poly(A) in protein synthesis has been suggested by Jacobson and Favreau (10) who found that translation of poly(A)$^+$ mRNAs in rabbit reticulocyte lysates could be selectively inhibited by addition of free poly(A). Other studies have shown that α 2u globulin mRNA species with long poly(A)-tails more rapidly reached maximum translation rates than poly(A)-poor mRNA when injected into Xenopus oocytes (27); and Geoghegen et al. (28) found that actin mRNA molecules with short poly(A)-tails tended to be concentrated in the smaller polysomes. Both of these findings suggest the rate of initiation of translation was slower with poly(A)-deficient mRNA molecules. In contrast, an earlier study by Bard et al. (29) had concluded that species of mRNA with different poly(A)-tail lengths could reinitiate with equal frequencies in mouse L-cells after heat shock. Their data did show, however, a 14% increase in newly synthesized mRNAs in the polysomes following recovery from heat shock, but this was dismissed as a labelling artefact. It could be, however, that the initiation rate enhancement by poly(A) differs between mRNA species and analysis of bulk mRNA could obscure this effect. This argues for a more complete analysis of this question using specific probes and a simple analysis system such as we have described would be useful for this.

One can envisage that it could be advantageous for a cell to have a mechanism for enhancement of the translation of inducible mRNAs such as metallothionein. Such mRNAs are produced rapidly in response to inducers such as heavy metals and hormones, but enter a cytoplasm already full of active mRNAs most of which will have shorter poly(A)-tails. A mechanism for promoting the translation of the newly synthesized mRNAs would assist in achieving a more effective response to an inducing signal. In addition, fairly rapid removal of the poly(A)-tail would reduce this advantage, ensuring the mRNA did not out-compete the longer lived mRNAs for an extended period. Of course, other poly(A)-independent mechanisms controlling the half-life of the mRNAs are presumably involved, for example sequences in the 3'-untranslated region (30). The apparent link between protein synthesis and poly(A)-shortening (21) is not inconsistent with a role of poly(A) in protein synthesis. If the removal of the poly(A) during the rapid phase (to 60 residues) were linked to protein synthesis, then this would control the number of times that an mRNA could be translated and still maintain a competitive advantage. This is a variant of the ticketing hypothesis of Sussmann (31), but we suggest it is not mRNA stability that

is being regulated by the removal of poly(A) but its efficiency of translation.

In conclusion, our data suggests that the poly(A)-shortening of metallothionein mRNA occurs by an exonuclease activity, and is considerably slower once the poly(A)-tails have shortened below 60 nucleotides, metallothionein mRNA molecules with poly(A)-lengths of less than 30 are not found. These results and evidence in the literature lead us to suggest that poly(A) may have two roles: (i) to facilitate translation of newly synthesized mRNA molecules and (ii) a critical length of about 30 residues which is needed to maintain mRNA stability. An analysis of poly(A)-shortening and size distribution in polysomes from a number of different mRNAs could now be examined using a modified blot procedure to test these hypotheses.

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