Abundance of hepatic metallothionein mRNA is increased by protein-synthesis inhibitors

Evidence for transcriptional activation and post-transcriptional regulation

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Ongoing protein synthesis is a prerequisite in the expression of some genes. We studied the effect of various protein synthesis inhibitors on the expression of the avian metallothionein (MT) gene. Chicken embryonic hepatocytes in culture were exposed to various concentrations of cycloheximide, puromycin and pactamycin. At concentrations which decreased total protein synthesis by about 90%, each inhibitor increased MT mRNA accumulation approx. 5-fold at 9 h of incubation. Incubation with puromycin or zinc for 2 h markedly increased the rate of MT gene transcription. Estimates of the half-life of MT mRNA by using actinomycin D suggested that cycloheximide, but not puromycin, decreased the decay rate of MT mRNA. These data suggest the potential for post-transcriptional regulation of the avian MT gene. We conclude that different antibiotics increase the accumulation of hepatocyte MT mRNA by different mechanisms and that the possibility of multiple mechanisms should be considered in other studies of the role of protein synthesis in gene expression.

INTRODUCTION

Regulation of expression of the metallothionein (MT) gene has been the focus of intensive investigation. The early observation that MT induction is blocked by actinomycin D (Squibb *et al.*, 1977) has been confirmed with new technology, and it is now clear that regulation of the MT gene occurs largely, if not exclusively, by changing the rate of transcription initiation. In fact, promoter sequences for both metal and glucocorticoid induction have been identified (Hamer, 1986). At present research is focusing on the identification and isolation of *trans*-acting factors and the processes by which these factors affect transcription.

Despite a considerable knowledge of the structure of the MT promoter, the precise molecular nature of MT gene regulation is still largely unknown. An initial model (Karin & Richards, 1984) proposed that metal-free MT (thionein) was a repressor and that heavy metals stimulated transcription by removing repressor activity (derepression). This model was based largely on the finding of increased transcriptional activity and MT mRNA accumulation under conditions of decreased cellular protein synthesis (Mayo & Palmiter, 1981; Karin *et al.*, 1980). After that initial work, studies have indicated that 'This mechanism now seems unlikely since ... (various) analyses indicate positive rather than negative regulation' (Hamer, 1986). Searle (1987) reached a similar conclusion, and proposed that zinc and other heavy metals activate transcription by complexing with a hypothetical metal-regulatory protein. The metal-regulatory-protein-zinc complex presumably binds, in a sequence-specific manner, to regions of the MT promoter and thereby facilitates transcription initiation. Although the metal-regulatory protein is speculative, there is increasing evidence that metal and nuclear DNA-binding factors are involved in the activation of MT gene transcription (Seguin & Hamer, 1987; Seguin & Prevost, 1988; Westin & Schaffner, 1988; Garg *et al.*, 1989).

In the course of experiments on the transcriptional regulation of the malic enzyme gene in chick-embryo hepatocytes, we

observed that mRNA coding for MT was increased markedly under conditions of reduced protein synthesis. The simplest interpretation of these results was that a short-lived protein suppressed MT mRNA synthesis, as suggested previously by Karin & Richards (1984). However, in view of the evidence of positive regulation of the MT gene and the observations that mRNA stability can be influenced by protein-synthesis inhibitors (Brawerman, 1989), we investigated the mechanism by which protein-synthesis inhibitors affect MT mRNA accumulation. Our results suggest that the control of chicken hepatocyte MT mRNA levels may be mediated by both transcriptional and post-transcriptional processes.

METHODS AND MATERIALS

Preparation and maintenance of isolated cells

Hepatocytes were obtained from 19-day chick embryos (Back *et al.*, 1986). Freshly isolated hepatocytes were suspended in serum-free Waymouth MD705/1 medium (GIBCO Laboratories) containing penicillin (60 mg/l), streptomycin (100 mg/l) and insulin (300 µg/l). Cells were incubated at 40 °C (air/CO₂, 19:1) in untreated 100 mm-diam. Petri dishes (diSPo; Scientific Products Co.). Cell density was approx. 2 × 10⁷ cells/dish. Hepatocytes were maintained in culture for approx. 36 h before experimental treatments. Medium was replaced once, approx. 18 h after isolation. In the experimental incubation period, medium contained the above additions plus 3,3',5-L-triiodothyronine (1 µg/ml).

Isolation of total RNA and quantification of MT mRNA

Total RNA was isolated from three plates of cells for each experimental treatment (Chomczynski & Sacchi, 1987). The proportion (by vol.) of solutions as described in the above procedure was 20:2:15:3 for Solution D (4 M-guanidinium thiocyanate, 25 mM-sodium citrate, pH 7.5, 0.5% sarcosyl, 0.1 M-2-mercaptoethanol), sodium acetate, phenol and chloroform,

Abbreviation used: MT, metallothionein.

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respectively. Total RNA (10 μ g) was separated by denaturing agarose (1.2%) gel electrophoresis (Davis *et al.*, 1986) and transferred to nylon membranes (GeneScreen; New England Nuclear) by low vacuum (Vacublot; Pharmacia-LKB). MT mRNA was analysed by hybridization to 32 P-labelled synthetic oligonucleotide (30-mer) complementary to nucleotides 1–30 of chicken MT mRNA (Wei & Andrews, 1988). The probe was end-labelled (Hamilton *et al.*, 1988) and hybridized and washed by conditions described below. Membranes (10 cm $\times$ 14 cm) were prehybridized at 55 $^{\circ}$ C for 4–18 h in 12 ml of 6 $\times$ SSPE (20 $\times$ SSPE = 3.6 M-NaCl, 0.2 M-sodium phosphate, 0.02 M-EDTA, pH 7.4)/1% SDS/10 $\times$ Denhardt's (100 $\times$ Denhardt's = 10 g of polyvinylpyrrolidone, 10 g of BSA, 10 g of Ficoll 400 and water to 500 ml)/100 μ g of denatured salmon sperm DNA/ml. Prehybridization solution was removed and replaced with hybridization solution containing 6 $\times$ SSPE/1% SDS and approx. 2×10^6 c.p.m. of probe DNA/ml, and hybridized at 55 $^{\circ}$ C for 24 h. Membranes were then washed three times (10 min each) at room temperature in 6 $\times$ SSPE/1% SDS and twice (5 min each) at 55 $^{\circ}$ C in 0.3 $\times$ SSPE/1% SDS. The resulting autoradiograms were quantified by scanning densitometry (EC Apparatus), and peak areas were quantified by integration (Chromlab, Data Translation Inc.).

Measurement of transcription rates

Nuclei for transcription assays were isolated from 20 plates of hepatocytes for each treatment (Schibler *et al.*, 1983). Isolated nuclei were stored at -70° C in 100 μ l portions containing 50 mM-Hepes, pH 7.9, 75 mM-NaCl, 0.1 mM-EDTA, 5 mM-dithiothreitol, 0.125 mM-phenylmethanesulphonyl fluoride and 50% (v/v) glycerol for no more than 1 week. The elongation reaction *in vitro* was conducted essentially as described by Goldman *et al.* (1985). Each reaction consisted of 100 μ l of nuclei and 100 μ l of 2 $\times$ run-on buffer [100 mM-Hepes, pH 7.9, 200 mM-KCl, 8 mM-dithiothreitol, 60 mM-EDTA, 2 mM-ATP, 1 mM-CTP, 1 mM-GTP, 4 mM-UTP, 4 mM-MnCl₂, 70 mM-(NH₄)₂SO₄, 17.6 mM-phosphocreatine, 80 μ g of creatine kinase/ml], approx. 160 μ Ci of [α - 32 P]UTP (800 Ci/mmol; Amersham) and 30 units of RNasin (Bethesda Research Laboratories). The nuclear suspension was incubated at 26 $^{\circ}$ C for 20 min, followed by addition of 1 μ l of 1 M-MgCl₂ and 1 μ l of RNAase-free DNAase (11 μ g/ μ l; Bethesda Research Laboratories). After 10 min incubation at room temperature, the mixture was made 1 $\times$ SET (1% SDS, 5 mM-EDTA, 10 mM-Tris/HCl, pH 7.4), and proteinase K was added to a concentration of 250 μ g/ml. After incubation at 37 $^{\circ}$ C for 1 h (which occasionally was extended until the mixture clarified), the 32 P-labelled RNA was purified as described by Linial *et al.* (1985). The RNA was used directly in the hybridization procedure (Linial *et al.*, 1985) using GeneScreen (New England Nuclear) containing various plasmid DNAs. Metallothionein cDNA (approx. 0.4 kb), genomic DNA (approx. 1.5 kb) and vector DNA have been described elsewhere (Wei & Andrews, 1988; Fernando & Andrews, 1989) and were generously provided by Dr. Glen K. Andrews, University of Kansas. Glyceraldehyde-3-phosphate dehydrogenase and β -actin clones were kindly donated by R. Schwartz (Dugaiczky *et al.*, 1983) of Baylor College and D. Cleveland (Cleveland *et al.*, 1980) of Johns Hopkins University respectively. The DNA (2 μ g) probes were denatured in 0.4 M-NaOH for 10 min at room temperature, neutralized in 12 $\times$ SSC, and applied to nylon strips (GeneScreen; New England Nuclear) by using a slot-blot apparatus (Bethesda Research Laboratories). Each slot was pre- and post-washed with 6 $\times$ SSC, and membranes were dried (80 $^{\circ}$ C for 1–2 h). Autoradiograms were quantified by densitometry as described above.

Hepatocyte protein synthesis

Protein synthesis was evaluated by pulse-labelling the hepatocyte protein with [3 H]leucine. At 1 h before harvest, medium was removed and replaced with a low-leucine medium (approx. 30 μ M-leucine) containing 10 μ Ci of [4,5- 3 H]leucine (54 Ci/mmol; ICN) per ml of medium and other additions depending on experimental treatment. After 1 h, cells were scraped off plates and collected by centrifugation. After a wash with a Ca²⁺-free Krebs–Ringer modified bicarbonate buffer (4.8 mM-NaCl, 120 mM-KCl, 1.2 mM-MgSO₄, 1.2 mM-KH₂PO₄, 25 mM-NaHCO₃, pH 7.2) cells were homogenized in 0.5 ml of buffer (0.25 M-sucrose, 5 mM-Tris, 0.1 M-EDTA, 1 mM-dithiothreitol, pH 7.4). The homogenate was centrifuged (30 min at 10000 g at 4 $^{\circ}$ C) and the supernatant collected. Samples (10 μ l) were applied to glass-fibre filters (2.1 cm diam.; Whatman), dried at room temperature and washed once in ice-cold 12.5% trichloroacetic acid (30 min) and again in boiling 12.5% trichloroacetic acid (30 min). Filters were then rinsed in 90% ethanol, dried, and radioactivity was determined by liquid-scintillation counting. Total trichloroacetic acid-precipitable radioactivity was considered an estimate of protein synthesis. In preliminary experiments, the rate of [3 H]leucine incorporation was linear for 2 h. All experimental values are expressed as a percentage of the radioactivity incorporated in trichloroacetic acid-precipitable protein of control (untreated) hepatocytes.

RESULTS

Cycloheximide or puromycin for 9 h increased the concentration of MT mRNA approx. 5-fold (Fig. 1). These results probably reflect the maximum mRNA accumulation obtainable (within 9 h) with either inhibitor, since there were no differences among the various concentrations used. In other experiments, 8 μ M-cycloheximide caused comparable MT mRNA accumulation, but 2 μ M resulted in a clearly diminished response. At this latter concentration of cycloheximide, protein synthesis was inhibited by 83% (results not shown). When hepatocytes were incubated with puromycin or pactamycin, protein synthesis needed to be inhibited by almost 90% before MT mRNA accumulation was detected (Fig. 2). Thus hepatocyte MT mRNA is increased significantly by various protein-synthesis inhibitors,

Fig. 1. Effects of high concentrations of cycloheximide (CHX) and puromycin (P) on the accumulation of MT mRNA in chick-embryo hepatocytes

Hepatocytes were isolated from 19-day embryos, maintained in culture for 36 h and then incubated for 9 h with CHX or P at the indicated concentrations. In this and subsequent experiments, triiodothyronine was included in the culture medium (1 μ g/ml) during the experimental incubation period. Total RNA was isolated from three plates of hepatocytes per treatment and separated by denaturing agarose-gel electrophoresis (10 μ g/lane). Northern-blot analysis was conducted by using a 32 P-labelled synthetic MT cDNA as hybridization probe. Details of culture conditions and MT mRNA analysis are in the Methods and materials section. Hybridized membranes were washed and exposed to X-ray film at -70° C. The abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also determined. Neither CHX nor P affected the concentration of GAPDH mRNA (results not shown).

Fig. 2. Effects of various concentrations of puromycin (a) and pactamycin (b) on hepatocyte protein synthesis and MT mRNA accumulation

Chick-embryo hepatocytes were isolated as described in the Methods and materials section and the legend to Fig. 1 and incubated with several concentrations of puromycin or pactamycin. After 8 h of antibiotic treatment, the medium was replaced with one containing all experimental additions plus 10 μ Ci of [3 H]leucine/ml of medium (three plates per treatment). After 1 h, incorporation of [3 H]leucine into trichloroacetic acid-precipitable protein was determined as described in the Methods and materials section. Total RNA was analysed for MT mRNA by Northern-blot analysis (see legend to Fig. 1). In a separate analysis, the same RNA samples were analysed for glyceraldehyde-3-phosphate dehydrogenase mRNA as described in the legend to Fig. 1. Neither pactamycin nor puromycin affected the abundance of that mRNA (results not shown).

and the extent of accumulation of MT mRNA is related to the degree of inhibition of protein synthesis.

Protein-synthesis inhibitors could stimulate accumulation of MT mRNA by either increasing mRNA production or decreasing its degradation. We investigated both processes for puromycin and cycloheximide. Transcription run-on experiments were performed on three separate preparations of nuclei for each treatment. A zinc treatment was included in each experiment as a positive control to assure reliability of the transcription assay *in vitro*. The apparent transcriptional activity (assumed to reflect rate of transcription initiation) increased on average 9.5 ± 3.9 - and 8.2 ± 4.9 -fold (mean $\pm$ S.E.M., $n = 3$) for puromycin and zinc respectively (normalized to β -actin) (Fig. 3). In one experiment, cycloheximide had no effect on transcriptional activity; in two others, about 3-fold increases were observed (results not shown).

Since data for the cycloheximide treatment were equivocal, an effect of this protein-synthesis inhibitor on transcriptional activation of the MT gene could not be concluded. We therefore investigated the alternative mechanism to explain increased MT mRNA accumulation, i.e. mRNA stability. MT mRNA degradation rates were determined by assessing the change in MT

Fig. 3. Effects of Zn^{2+} and puromycin on MT gene transcription

Embryo hepatocytes were isolated as described in the Methods and materials section and the legend to Fig. 1, and then incubated for 2 h with medium without additions (C) or a medium containing either 100 μ M- $ZnCl_2$ (+Zn) or 250 μ M-puromycin (+P). Transcription was measured in isolated nuclei by run-on assay as described in the Methods and materials section. For each treatment, 2×10^7 c.p.m. of labelled nuclear RNA was hybridized with membranes containing 2 μ g each of the indicated DNAs (abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase). After 4 days of hybridization, the membranes were washed and exposed to X-ray film at $-70^\circ C$. Blots were scanned by densitometry and quantified by integration. Values obtained with genomic MT were normalized to β -actin and expressed as a percentage of control (see the Results section).

mRNA level in the presence of actinomycin D, an inhibitor of RNA synthesis. Hepatocytes were first treated with zinc (to increase MT mRNA), then subsequently treated with actinomycin D in the presence or absence of protein-synthesis inhibitors (puromycin or cycloheximide). The results of two experiments are shown in Fig. 4. Cycloheximide decreased considerably the rate of MT mRNA degradation as compared with that of hepatocytes not treated with protein-synthesis inhibitor. The concentration of MT mRNA did not change in hepatocytes treated with cycloheximide even after 16 h of actinomycin D treatment. In contrast, the presence of puromycin had little or no effect on the rate of MT mRNA degradation. These data support a role for post-transcriptional regulation in the elevation of MT mRNA levels caused by cycloheximide. To our knowledge, this is the first report of experimentally induced changes in MT mRNA stability. Owing to the variable results with cycloheximide in the run-on experiments, we cannot formally eliminate an effect of cycloheximide on transcriptional activation. It would appear, however, that a major portion of the effect of cycloheximide on MT mRNA accumulation is due to increased mRNA stability.

DISCUSSION

Protein-synthesis inhibitors increase MT mRNA in chicken hepatocytes by two different mechanisms. Puromycin significantly increased rates of transcription, but had little or no effect on mRNA stability. Cycloheximide, on the other hand, stabilized MT mRNA, but had little consistent effect on transcription. These differences could be due to relative abilities of the antibiotics to inhibit protein synthesis. On average, cycloheximide inhibited protein synthesis by no more than 92%, even at the higher concentrations. Puromycin was consistently more potent, inhibiting by 97–98% at the higher concentrations. It was these higher concentrations that were used in the transcription experiments. Others have noted that cycloheximide is less potent than other antibiotics in blocking protein synthesis (Greenberg *et al.*, 1986). Thus the contrasting effects obtained with cycloheximide

Fig. 4. MT mRNA decay in the presence of puromycin (+P) or cycloheximide (+CHX)

Embryo hepatocytes were prepared as described in the Methods and materials section and the legend to Fig. 1, and then incubated for 4 h in medium containing $20 \mu\text{M}$ - Zn^{2+} (0h- Zn^{2+}). At 20 min before the end of this period, either puromycin ($250 \mu\text{M}$) or CHX ($64 \mu\text{M}$) was added to ten plates per treatment. At 4 h, actinomycin D was added to all plates ($4 \mu\text{g}/\text{ml}$). In one treatment (-Zn), zinc-containing medium was replaced before addition of actinomycin D with medium containing no added zinc. Hepatocytes treated with Zn^{2+} (+Zn) alone continued to be incubated in medium containing $20 \mu\text{M}$ - Zn^{2+} . At various times after the addition of actinomycin D, two plates per treatment were harvested for total RNA isolation. MT mRNA was determined by Northern-blot analysis as described in the Methods and materials section. The lower panel represents the average of data from two experiments: □, +Zn; ▨, +P; ■, +CHX.

and puromycin on transcriptional activity of the MT gene may, in part, be explained by the greater ability of puromycin to inhibit protein synthesis. It is clear from data shown in Fig. 2(a) that relatively small changes in protein synthesis, i.e. that occurring between $63 \mu\text{M}$ - and $32 \mu\text{M}$ -puromycin (88% to 81% inhibition), result in marked differences in MT mRNA accumulation.

The most perplexing result of the present study was the inability of cycloheximide to increase consistently the transcription of the MT gene. Several groups have reported that cycloheximide increases accumulation hepatic MT mRNA (Karin *et al.*, 1980; Mayo & Palmiter, 1981; Imbra & Karin, 1987; McCormick & Fleet, 1989), but few have attempted to assess the mechanism. In mouse sarcoma cells treated with cycloheximide for 90 min, the rate of uridine incorporation into MT mRNA is increased approx. 2-fold (Mayo & Palmiter, 1981). These workers concluded that the MT-1 gene might be repressed by a short-lived protein. On the other hand, Friedman & Stark (1985) reported that cycloheximide ($32 \mu\text{g}/\text{ml}$ at 2 h) had 'little' effect on the rate of transcription of human MT2 gene in neuroblastoma cells. On the basis of additional data, these workers postulated that post-transcriptional as well as transcriptional events contribute to the accumulation of MT mRNA. This conclusion is supported by our data with cycloheximide, but

not puromycin. Our interpretation of the effect of puromycin is similar to that of Mayo & Palmiter (1981), i.e. that the antibiotic blocks the synthesis of a rapidly degraded repressor protein and thus stimulates transcription. We speculate that this repressor protein is normally present in large excess and that virtually complete inhibition of protein synthesis is required to obtain maximum derepression of the MT gene. The extent of inhibition of protein synthesis by cycloheximide is not sufficient, even at very high concentrations, to decrease the level of the repressor enough to obtain significant and consistent derepression; cycloheximide has its major effect on MT mRNA level by stabilization. More recently, Sadhu & Gedamu (1989) reported metal-specific differences in the rate of decline of MT mRNA in human hepatoblastoma cells (HepG2) treated with copper or zinc. They suggested that the decreased zinc-induced MT mRNA half-life is probably due to a post-transcriptional event(s).

The present study examined the effect of inhibiting protein synthesis on the expression of the MT gene in chicken hepatocytes. The inhibition of protein synthesis (by several inhibitors) resulted in the accumulation of MT mRNA. Investigation into a possible mechanism indicated that cycloheximide decreased MT mRNA degradation, whereas puromycin stimulated transcription. These contrasting effects may be related to the potency of antibiotic action. Our data highlight the importance of using more than one protein-synthesis inhibitor when studying the role of protein synthesis in gene expression.

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