Characterization of an inducible promoter system to investigate decay of stable mRNA molecules

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ABSTRACT

We have developed a system in which the decay of stable mRNAs can be studied without the use of inhibitors of transcription. The *Drosophila* hsp70 heat shock promoter linked to the bovine growth hormone (BGH) gene was used to establish stable cell lines in which the BGH gene is transcribed in a conditional manner. The BGH mRNA is synthesized only after induction at 43°C. Following a brief period of re-equilibration at 37°C during which transcription of the heat shock-driven gene ceases, the stable BGH mRNA decays with typical first-order kinetics. Hence, the decay of the mRNA can be studied without assumptions regarding radioactive labeling of precursor pools or transcriptional inhibitors. The system is applicable to any stable mRNA.

INTRODUCTION

Although regulation of gene expression via mRNA turnover has been recognized for some time, the features of mRNA that influence stability of the mRNA remain largely undetermined. The 5' cap, the 5' and 3' untranslated sequences, the initial translated sequence, the poly-A tail and secondary structure of the RNA have all been postulated or proven to be important structural elements in the control of mRNA turnover. Nonstructural features of the mRNA including the cellular environment, cell cycle stage, growth conditions, presence of hormones and extent of translation of the mRNA also influence mRNA stability in some systems. (For a review, see references 1–3).

Experiments to measure mRNA turnover have generally employed pulse-labeling or inhibition of transcription. Both methods have inherent problems, particularly when used to study decay of very long-lived messages. It is technically difficult to achieve adequate incorporation of radioactive precursors into a single message to allow one to accurately follow decay over a long period of time. Traditionally used transcriptional inhibitors such as actinomycin D are not specific for transcription and are toxic when cells are exposed for longer than a few hours;

therefore these inhibitors are not useful in studying the turnover of very stable messages. Additionally, transcriptional inhibitors could result in an apparent stabilization of mRNA by inhibiting the production of nucleases responsible for normal mRNA decay. For these reasons, most studies on determinants of RNA turnover have been carried out on short-lived mRNA molecules.

These studies have identified a conserved AU sequence in the 3' untranslated region of several lymphokine and proto-oncogene mRNAs which appears to target these messages for accelerated decay (4). It is unknown whether there exists a similarly conserved sequence which acts as a 'stabilizing element' in very long lived mRNA molecules.

We have developed a system which allows us to determine the decay of stable mRNA molecules in cells without the use of inhibitors of transcription. In this system, the gene of interest is linked to the *Drosophila* hsp70 promoter and expressed in stably transfected heterologous recipient cells. We have used the bovine growth hormone gene (BGH) as a prototype stable mRNA; in our preliminary studies using transcriptional inhibitors BGH mRNA has shown little turnover within 24 hrs.

MATERIALS AND METHODS

Cells and tissue culture

Chinese Hamster ovary (CHO B52 [APRT⁻]) cells selected for high transfection efficiency were kindly provided by Richard E. Davis and David Patterson (Eleanor Roosevelt Institute for Cancer Research, Denver, CO). Cells were grown in Dulbecco's Modified Eagle Media with 10% fetal bovine serum and nonessential amino acids.

Construction of plasmids

The *Drosophila* hsp70 promoter was provided by Dr. Susan Lindquist (University of Chicago, Chicago, IL). The 195 bp Xho I- Bam HI fragment of the 5' region (5) was linked to Exon 1 of bovine growth hormone (BGH) gene (6). Since splicing is interrupted following heat shock (7), all constructs were prepared without introns.

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Transfections

CHO cells were transfected with a 20:1 mix of hsp-BGH to pSV₂neo (8) using calcium phosphate co-precipitation essentially as described by Graham and van der Eb (9). Transfected cells were selected in 600 µg/ml (effective concentration) of the aminoglycoside G418 (Gibco Laboratories, Grand Island, NY). Individual colonies were selected and expanded for the clonal isolates; >100 colonies were combined for the pooled 'polyclonal' populations.

RNA preparation

RNA was prepared from the cytoplasm of subconfluent cells by lysis in 5 mM Tris HCl pH 7.5, 10 mM NaCl, 1% NP-40, and 5 mM vanadyl ribonucleoside complexes. The nuclei were pelleted by centrifugation at 12,000g × 10 min. The cytoplasmic component was extracted twice with phenol:chloroform (1:1), once with chloroform:isoamyl alcohol (24:1), and precipitated with ethanol. The polyadenylated (poly A+) fraction was selected using oligo-dT cellulose affinity chromatography.

Quantitation of RNA

The cells were labeled with [¹⁴C]-uridine by incubation in 0.04 µCi/ml [¹⁴C]-uridine (~500 mCi/mmol; Amersham, Inc., Arlington Hts, IL) for 36 hrs prior to heat shock. Equal amounts of radioactively labeled rRNA were used to standardize the amount of each RNA sample subjected to Northern blot analysis.

Northern blot analysis

The poly A+ fraction was separated by electrophoresis in denaturing agarose gels containing 6% formaldehyde and transferred by capillary blotting in 25 mM sodium phosphate buffer to Zetaprobe membrane (BioRad Laboratories, Richmond, CA). The membrane was baked under vacuum at 80°C for 2 hours and prehybridized in 50% formamide, 20 mM PIPES pH 6.4 in 0.8 M NaCl and 2 mM EDTA, 0.5% SDS, and 0.1 mg/ml heat-denatured, sonicated salmon sperm DNA at 42°C for 30 min. Hybridizations were carried out overnight at 42°C in the same solution containing 1–2 × 10⁶ cpm/ml linear BGH cDNA labeled with [³²P]-dCTP using the Random Primed DNA Labeling Kit (Boehringer-Mannheim, Indianapolis, IN) according to manufacturer's directions. Membranes were washed twice, for 10 minutes each, in 2 × SSC/0.1% SDS at room temperature and twice, for 30 min each, in 0.2 × SSC/0.1% SDS at 65°C. The membranes were exposed to XAR film at –80°C. The relative extent of hybridization was determined by laser densitometry. When necessary, multiple exposures were done to optimize scanning.

Elongation of nascent transcripts in isolated nuclei

Approximately 2 × 10⁷ cells were suspended in lysis buffer (15 mM HEPES, 15 mM NaCl, 14 mM mercaptoethanol, 2 mM EDTA, 0.5 mM EGTA) and disrupted with a dounce tissue grinder until >90% of cells were lysed as determined by microscopic examination. The nuclei were pelleted through a cushion of 2 M sucrose in lysis buffer by centrifugation at 200,000 g for 45 min. The nuclei were resuspended in 50% glycerol containing 20 mM Tris HCl pH 8.0, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT and 0.125 mM PMSF, counted using a hemocytometer and stored at –80°C. We found that our yield of nuclei was several fold higher when cells were lysed in a hypotonic solution without Triton X-100 or sucrose. The nuclear run-on conditions were modified from those of McKnight (10).

Approximately 1 × 10⁷ nuclei were added to transcription buffer containing 60 mM Tris HCl pH 8.0, 10% glycerol, 0.6 mM each of ATP, GTP and CTP, 2 mM MnCl₂, 2 mM MgCl₂, 0.5 mM DTT, 6 mM NaF, 10 µM creatine phosphate, 100 µg/ml creatine phosphokinase, 35 mM (NH₄)₂SO₄, and 250 uCi of [^α-³²P]-UTP. After a 20 min incubation at 29°C, 1 µg DNase was added. After a 10 min incubation at room temperature, the reaction mixture was treated with Proteinase K (50 µg) followed by phenol-chloroform extractions and ethanol precipitation. A parallel reaction contained 0.05 mg/ml alpha-amanitin to confirm that the newly synthesized RNA was the product of RNA polymerase II. Aliquots of each RNA sample containing 5 × 10⁶ cpm were hybridized with 2 µg of single stranded DNA which had been bound onto nitrocellulose filters using a 'slot blot' apparatus (Hybri-slot; BRL, Gaithersburg, MD). The DNAs fixed to the filters included both strands of BGH DNA (in separate slots) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA. GAPDH is constitutively expressed in CHO cells and served as a control for transcription conditions. Hybridizations were carried out at 65°C in 10 mM HEPES pH 7.5, 0.2% SDS, 10 mM EDTA pH 8.0, 0.3 M NaCl, and 100 µg/ml yeast tRNA in 1 × Denhardt's solution. Filters were washed 3 × in 0.1% SDS/0.2 × SSC at 65°C for 10 minutes each.

Inhibition of transcription

Cells were exposed to actinomycin D (Sigma Chemical Company, St. Louis, MO) 5 µg/ml or 5,6-dichloro-1-ribofuranosylbenzimidazole (DRB) (Calbiochem, San Diego, CA) 100 µM for periods up to 24 hr.

RESULTS

Expression of hsp70-BGH plasmid in CHO cells

The 193 bp XhoI-BamHI fragment of the *Drosophila* hsp70 5' flanking sequence was linked to the first exon of the BGH cDNA sequence as illustrated in Fig. 1. CHO cells were transfected with this plasmid and selected as described. RNA was isolated from two plates of each cell line; one plate was exposed to heat shock immediately prior to RNA isolation. The RNA was analyzed by Northern blot hybridized to BGH cDNA. In this manner, three clonal isolates and four pooled polyclonal isolates which expressed BGH mRNA after heat shock were chosen for study. Heat shock was performed by incubating cells at 43°C. In preliminary experiments, cells were exposed to periods of heat shock from 0.5 to 4 hrs. The amount of BGH mRNA increased from 0.5 to 1.5 hr of heat shock, but did not increase linearly with times of heat shock longer than 1.5 hr (data not shown). To optimize the level of transcription but minimize length of exposure to heat shock, a 1.5 hr period of heat shock was chosen for these studies. Fig. 2 shows a Northern blot of RNA from two representative clones which express different levels of BGH mRNA after heat shock. BGH mRNA is not detectable in the stable cell lines in the absence of heat shock.

Characterization of the heat shock promoter

Northern blot analysis of multiple clones showed no detectable BGH mRNA at 37°C and appearance of BGH mRNA only after incubation at 43°C (Fig. 2). To accurately determine the decay of a message over time, it is essential that there be no further transcription from the heat shock promoter following return of the cells to 37°C. To verify that under the conditions employed in these experiments, transcription from the heat shock promoter

Fig. 1. Schematic diagram of hsp-BGH plasmid. The 193 bp XhoI-BamHI fragment of the 5' flanking sequence of *Drosophila* HSP70 (which includes 2 bp beyond the transcription initiation site) was linked to the Bovine Growth Hormone cDNA sequence at the BamHI site located at the 5' end of Exon 1. The plasmid includes the native polyadenylation sequence and approximately 400 bp of BGH 3' flanking sequence.

ceases rapidly upon return to 37°C, nuclear run on assays were carried out on cells at various times after return to 37°C following heat shock. Nuclei were isolated from cells not exposed to heat shock, from cells immediately following heat shock, and from cells 15, 30, 45, 60 and 240 min after return to 37°C following heat shock. Nascent transcripts in these nuclei were elongated as described above. Fig. 3 shows the results of representative time points from this experiment. There was no detectable transcription of BGH at 37°C, and marked induction of BGH transcription occurred in nuclei isolated immediately following heat shock. Only 15 min after return to basal temperature following heat shock, the amount of BGH detected was 10% of that seen immediately following heat shock. No BGH transcripts were observed after 60 min or 240 min at 37°C. A parallel nuclear run-on reaction containing alpha-amanitin demonstrated no detectable transcripts indicating that the BGH mRNA is transcribed by RNA polymerase II.

Quantitation of RNA

The doubling time of the CHO cells used in these experiments is approximately 18 hrs. During decay experiments, aliquots of cells were removed for up to 108 hrs following heat shock. Therefore, comparisons between samples from different time points based on the presence of equal amounts of RNA would not accurately reflect the decay of heat shock-induced mRNAs because of the increase in cell number during this period. To standardize the comparison between samples, the stable rRNA fraction was labeled with [¹⁴C]-uridine as described. We demonstrated that the total amount of radioactively labeled rRNA remained constant for an aliquot of cells throughout the time of the experiment, even though the number of cells increased (results not shown). These results agree with previously published studies indicating that the rRNA is stable with little breakdown and reutilization of label (11,12,13).

Fig. 2. Northern blot analysis of poly A⁺ RNA from two stable cell lines probed with BGH. The 'Std.' is bovine pituitary RNA which demonstrates the correct size for BGH mRNA. I and II represent two separately isolated G418 resistant stable cell lines obtained after cotransfection of CHO cells with pSV₂neo and hsp-BGH. 'O' indicates the cells were not exposed to heat shock; '+' indicates the cells were exposed to 43°C for 1.5 hr (heat shock) immediately prior to RNA isolation.

Determination of the Effect of Heat Shock on mRNA Turnover

To determine the effect of the heat shock on the turnover of endogenous mRNAs in the cell, we examined the turnover of a known transcript, CHO A mRNA (14), in cells not exposed to heat shock and in cells exposed to 43°C for 1.5 hr. CHO A mRNA was found to have a half-life of 8 hrs by Darnell and coworkers using pulse labeling techniques (14). We used DRB ((100 μM) or actinomycin D (5 μg/ml) to inhibit transcription and followed the decay of the mRNA over a 24 hr period. DRB specifically interferes with RNA polymerase II transcription (15,16) while actinomycin D binds to DNA and blocks the movement of RNA polymerases.

RNA was then isolated from cells without exposure to heat shock, immediately following heat shock and at 6, 12, 24 and 48 hrs after return to 37°C following heat shock. In each of these

Fig. 3. Transcription of the chimeric BGH structural gene ceases rapidly upon return of cells to basal temperature. Hybridization of ^{32}P -labeled nascent RNA prepared in nuclei isolated from transfected cells in the absence of heat shock (O HS), immediately following heat shock (+ HS), and 15 min, 45 min, and 60 min following heat shock. The bottom panel was a parallel reaction to '+ HS' but contained alpha-amanitin in the reaction. Column 1 slots contained bound single strand BGH DNA (the complementary strand to the message); Column 2 slots contained the same sequence as the BGH RNA; Column 3 slots contained bound single strand GAPDH DNA (the complementary strand to the message).

Fig. 4. CHO A mRNA decay is unaffected by heat shock after 6 hr at basal temperature. A. Northern blot analysis of RNA isolated from cells incubated in DRB for 0 hrs (a), 6 hrs (b), 12 hrs (c) and 18 hrs (d). The cells in set I were exposed to 43°C for 1.5 hrs; DRB was added immediately following the heat shock. The cells in set II were not exposed to heat shock. DRB was added to Set III cells 6 hrs after return to basal temperature following heat shock. DRB was added to Set IV 12 hrs after return to basal temperature following heat shock. Linear CHO A DNA labeled with ^{32}P dCTP was used to probe the Northern blots. B. Northern blot analysis of RNA isolated from cells incubated in actinomycin D for 0 hrs (a), 6 hrs (b), 12 hrs (c), and 24 hrs (d). 'O HS' represents cells not exposed to heat shock; '+ HS' represents cells exposed to 43°C for 1.5 hr immediately prior to the addition of the actinomycin D. The decrease seen at 24 hrs is accounted for by the decreased number of cells remaining viable. The blot was probed with CHO A.

instances, the cells were treated with either DRB or actinomycin D. RNA was isolated at the time of addition of the inhibitor and 6, 12, 18 and 24 hours after inhibition of transcription.

Fig. 5. Decay of BGH mRNA. A. Northern blot analysis of A+ RNA prepared from one polyclonal cell line transfected with hsp-BGH in the absence of heat shock (0), immediately following heat shock (+) and at 6, 12, 24, 36, 48, 60, 72, and 84 hours following heat shock (lanes 1–8, respectively). The rRNA fraction was labeled as described in the text; equal amounts of radioactivity were loaded for each time point. B. The extent of hybridization on each Northern blot was quantitated by densitometry of autoradiograms. The mean of four separate polyclonal cell lines is shown; the standard deviations are indicated by the bars.

Cytotoxicity was observed with both inhibitors. Significant 'rounding up' of cells was noted after 18 hrs in DRB and at 24 hrs, approximately 20% of the cells detached from the plate. These effects occurred earlier and were more severe with actinomycin D than with DRB; by 24 hours, 80% of the cells were detached. In each case, the cells remaining attached excluded trypan blue. The amount of RNA loaded for each sample was quantitated as described above. Fig. 4 shows a representative selection of data from this experiment. Using DRB to inhibit transcription, the calculated half-life of CHO A mRNA in cells not exposed to heat shock is 9.4 hr and is not significantly different in cells which had been returned to basal temperatures for 6, 12, 24 or 48 hours following heat shock (calculated half-life for these is 9.5 hr, 8.6 hr, 10.1 hr and 8.3 hr, respectively). The mRNA turnover is delayed in cells treated with the transcriptional inhibitor immediately following heat shock. The half-life can not be accurately determined in these experiments, but is greater than 12 hrs. When actinomycin D was used to inhibit transcription, we were unable to demonstrate any turnover of the CHO A mRNA.

Estimation of Half-Life of BGH mRNA

Northern blot and S1 analyses were carried out on RNA isolated from three separately isolated clones and from four pooled, polyclonal cell lines stably transfected with hsp-BGH. RNA was isolated from cells not exposed to heat shock, from cells isolated immediately following heat shock, and at times up to 108 hrs after return to 37°C following heat shock. Two to four separate decay experiments were done on each cell line. A representative

northern blot is shown in Fig. 5. The average turnover time was the same in the separately isolated clones and pooled polyclonal populations. The calculated half-life of BGH mRNA was similar based on Northern blot analysis or S1 analysis done under conditions of probe excess (data not shown). Since the turnover of the CHO A mRNA in the presence of DRB indicated that processing may be altered for up to 6 hours following heat shock, the estimation of half-life was based on the decay occurring at least 6 hrs after return to basal temperature. Using our inducible promoter system, we calculate the estimated half-life of BGH mRNA is 30 hr $\pm$ 4 hr in CHO B52 cells.

DISCUSSION

We have characterized a system which will allow the measurement of decay rates of long-lived mRNAs in the absence of continuing perturbations to the cell. The *Drosophila* heat shock promoter provides no transcription of the chimeric BGH gene at 37°C and significant induction of transcription upon exposure to 43°C (as determined by Northern blot and S1 analyses and by nuclear run-on assay). Furthermore, return to 37°C is accompanied by a rapid loss of transcription, with no detectable transcription one hour after return to basal temperature. The heat shock has no apparent lasting detrimental effect on the cells. These features indicate that this is a useful system to examine the metabolism of mRNAs with low turnover rates.

Inhibition of RNA metabolism was observed immediately following heat shock; however, within 6 hours after the 1.5 hr heat shock period used in our experiments, decay of an endogenous message (CHO A) appears to be the same as that in non-heat shocked cells. Because cellular processing is disturbed immediately following heat shock, this system would be less useful for studying the metabolism of rapidly degraded mRNAs.

We believe that this system will be most useful in studying the decay of more stable mRNAs; in these settings where inhibitors must be present for several hours, the effects of these agents become more problematic. Such effects were noted when we were unable to demonstrate turn-over of CHO A mRNA in the presence of 5 μ g/ml actinomycin D, using conditions widely employed to study mRNA stability (4,17–21). Actinomycin D has been reported to interfere with the binding of mRNAs to ribosomes and inhibit translation (22). Steinberg, et al. discussed the 'multi-faceted nature' of this inhibitor with inhibition of protein synthesis and inhibition of mRNA degradation as well as blocking the synthesis of RNA (23). Our results (i.e., no observed RNA turnover with actinomycin D) indicate that significant effects on mRNA metabolism in addition to transcriptional inhibition occur and suggest caution in the use of this inhibitor in measurements of mRNA decay.

The mechanism of transcription inhibition by DRB is different from that of actinomycin D. Only minor effects on protein synthesis occur when cells are treated with DRB for several hours (24). Inhibition of mRNA synthesis is rapid and reversible (25). DRB caused less observable cytotoxicity in the CHO cells and the turnover of CHO A mRNA was similar to that reported previously using pulse labeling (14). This allowed us to compare the turnover of an endogenous, relatively short-lived message in the absence and presence of heat shock. The results of this experiment indicate that heat shock does not affect mRNA turnover after 6 hrs of recovery from heat shock.

In conclusion, this system in which chimeric genes are turned on and off with high efficiency, will allow detailed studies of

long-lived mRNA molecules. This system should be useful in characterizing the intrinsic components of mRNA structure which affect the stability and determining the effect of external signals on mRNA turnover.

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