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# A broader role for AU-rich element-mediated mRNA turnover revealed by a new transcriptional pulse strategy

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## ABSTRACT

**The widespread occurrence of AU-rich elements (AREs) in mRNAs encoding proteins with diversified functions and synthesized under a vast variety of physiological conditions suggests that AREs are involved in finely tuned and stringent control of gene expression. Thus it is important to investigate the regulation of ARE-mediated mRNA decay in a variety of mammalian cells in different physiological states. The tetracycline (Tet)-regulatory promoter system appears appropriate for these investigations. However, we found that efficient degradation of mRNAs bearing different AREs cannot be observed simply by blocking constitutive transcription from the Tet-regulated promoter with Tet, possibly due to saturation of the cellular decay machinery. In addition, deadenylation kinetics and their relationship to mRNA decay cannot be adequately measured under these conditions. To overcome these obstacles we have developed a new strategy that employs the Tet-regulated promoter system to achieve a transient burst of transcription that results in synthesis of a population of cytoplasmic mRNAs fairly homogeneous in size. Using this new system we show that ARE-destabilizing function, necessary for down-regulating mRNAs for cytokines, growth factors and transcription factors, is maintained in quiescent or growth-arrested cells as well as in saturation density-arrested NIH 3T3 cells. We also demonstrate that the ARE-mediated decay pathway is conserved between NIH 3T3 fibroblasts and K562 erythroblasts. These *in vivo* observations support a broader role for AREs in the control of cell growth and differentiation. In addition, we observed that there is a significant difference in deadenylation and decay rates for β-globin mRNA expressed in these two cell lines. Deadenylation and decay of β-globin mRNA in K562 cells is extraordinarily slow compared with NIH 3T3 cells, suggesting that the increased stability gained by β-globin mRNA in K562 cells is mainly controlled at the deadenylation step. Our strategy for studying mammalian mRNA turnover now permits a more general application to different cell lines harboring the Tet-regulated system under various physiological conditions.**

## INTRODUCTION

Regulation of mRNA turnover is now recognized as an important step for controlling the fate of cytoplasmic mRNA and consequently gene expression (1–3). In mammalian cells the abundance of a particular mRNA can fluctuate many-fold following a change in mRNA stability, without any change in transcription (2). The processes that regulate mRNA turnover can, in turn, affect how a cell grows, differentiates and responds to environmental stimuli. Therefore, it becomes important to identify the stimuli that induce changes in mRNA stability and to characterize their regulation under physiologically relevant conditions.

To elucidate the regulatory mechanisms underlying differential and selective mRNA turnover in mammalian cells we have focused on a group of so-called AU-rich elements (AREs). They are found in the 3′-untranslated regions (3′-UTRs) of many mRNAs encoding proteins with different functions synthesized under a vast variety of physiological conditions (4,5) and are now recognized as the most common RNA destabilizing element among those characterized in mammalian cells (6). The widespread occurrence of AREs in different mRNAs suggests that their function is tightly regulated. However, little is known about the regulation of ARE function. There are two major obstacles: (i) the profound mRNA decay-impeding effect of transcription inhibitors such as actinomycin D and DRB has excluded the use of these drugs as an efficient tool to study ARE destabilizing function (7,8); (ii) although the c-*fos* promoter system has been used with success to investigate the decay kinetics and key sequence features of AREs, the system has limitations that prevent it from being used as a general approach. Activation of the c-*fos* promoter requires serum or growth factor induction of quiescent cells. As a result, this system has restricted analysis of ARE function to cells undergoing the $G_0$ to $G_1$ transition (9). In addition, the use of serum induction complicates analysis of regulatory mechanisms which may affect decay of mRNA.

The recent development of the tetracycline (Tet)-regulatory promoter system has offered an opportunity to overcome these limitations (10–12). The Tet-responsive promoter ystem is based on a hybrid transactivator that specifically stimulates transcription of promoters that contain Tet operator (*tetO*) sequences (10). The hybrid transactivator protein (tTA) is composed of the DNA binding domain of the Tet repressor protein from *Escherichia coli* and the activating domain of the viral protein VP16 from herpes simplex virus. tTA is able to specifically bind to and strongly activate minimal promoters containing seven consecutive *tetO* sequences. When Tet is present, the binding of tTA to the *tetO* sequences is

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prohibited, resulting in efficient shut-off of gene transcription. While applications of the Tet-controllable system to regulation of transcription in transgenic mice (12–15) have been well documented, relatively little is known about its potential application to address the cytoplasmic aspect of post-transcriptional regulation, e.g. mRNA turnover (16,17). Possibly due to saturation of the cellular decay machinery as a result of high level constitutive expression from the Tet-regulated promoter, simply blocking constitutive transcription of the Tet-regulated promoter with Tet and then monitoring mRNA decay appears not to give an accurate measurement of mRNA half-life (see below) (16). Moreover, due to size heterogeneity of the poly(A) tails resulting from constitutive transcription, this approach did not allow an unequivocal determination of deadenylation and decay kinetics and thus the relationship between deadenylation and decay (17).

Here we report a new transcriptional pulse strategy generated via the Tet-regulated system as a general methodology to measure mRNA decay in mammalian cells. By modulating the amount of Tet and the timing of its addition to or omission from culture medium we were able to induce a short burst of mRNA synthesis from a reporter gene driven by the Tet-regulated promoter in stable lines that constitutively express tTA. Using this new system we have demonstrated that AREs can function in mouse NIH 3T3 fibroblasts under growth arrest and density arrest states. Furthermore, the ARE-mediated decay pathway is conserved in human K562 erythroleukemia cells. These *in vivo* observations indicate that the ARE-mediated decay pathway functions in regulation of mRNA expression during different phases of fibroblast growth and in an erythroid cell line. Thus AREs are more universally functional than previously realized.

## MATERIALS AND METHODS

### Plasmid construction

The construction of plasmids pBBB, pBBB+ARE$^{c\text{-}fos}$, pBBB+ARE$^{GMCSF}$ and pSVα1/GAPDH has been described previously (8,18). Plasmids pUHD15-1 and pUHC13-3 were kindly provided by Dr H.Bujard. Plasmid pTet-Splice was purchased from Gibco BRL. To construct pTet-BBB, pBBB(Stu) was first created using site-directed mutagenesis to insert a *Stu*I site at a position immediately upstream of the transcription start site of plasmid pBBB. The plasmid pBBB(Stu) was then digested with *Stu*I (fill-in) and *Kpn*I (fill-in) and subcloned between the *Eco*RI (fill-in) and *Not*I (fill-in) sites of plasmid pTet-Splice. Fragments containing the c-*fos* ARE or granulocyte/monocyte colony stimulating factor (GMCSF) ARE were synthesized by PCR as described previously (8,18), using pBBB+ARE$^{c\text{-}fos}$ or pBBB+ARE$^{GMCSF}$ as template. These ARE fragments were flanked by a *Ba*mHI site at the 5′-end and a *Bgl*II site at the 3′-end. Following *Bam*HI and *Bgl*II digestion the fragment was inserted into the unique *Bgl*II site in pTet-BBB to create pTet-BBB+ARE.

### Cell culture and Northern analysis

Mouse NIH 3T3 cells were maintained in DMEM + 10% calf serum (CS) and 500 ng/ml Tet at 37°C in an atmosphere of 8% $CO_2$ and were passed when they reached confluence. Cells were split to a density of $2 \times 10^6$/100 mm dish in DMEM with 10% CS 18–20 h before transfection by the calcium phosphate technique (19). Transfection mixtures for each plate contained 1 µg test plasmid, 2 µg internal control plasmid pSVα1/GAPDH and 17 µg carrier plasmid (pT7/T3α-18; BRL). After exposure to the plasmid precipitate for 12–16 h, cells were either serum starved in DMEM + 0.5% CS at 8% $CO_2$ for 25–28 h and then stimulated with DMEM + 20% CS or cultured for another 28 h, depending on the experiment. Isolation of total cytoplasmic RNA and Northern blot analysis were conducted as described previously (7,20).

### Establishment of stable cell lines

Stable cell transfectants of pUHD15-1 encoding tTA were obtained as follows. NIH 3T3 cells were transiently transfected as described above. Transfection mixtures for each plate contained 2 µg plasmid pUHD15-1, 0.4 µg plasmid pSV2-neo (Clontech) and 17.6 µg carrier plasmid (pT7/T3α-18; BRL). The control plate was transfected with 2 µg plasmid pUHD15-1 and 18 µg carrier plasmid only. After exposure to the plasmid precipitate for 18 h cells were rinsed and cultured in fresh medium containing 500 ng/ml Tet. At 50 h after transfection cells were diluted 80-fold and subjected to G418 (Gibco BRL) selection at a concentration of 800 µg/ml. The medium was refreshed every 3 days. The cells in the control plate died after 10 days transfection. After 12 days transfection single colonies were isolated by trypsinization within a glass cylinder. Colonies were first grown in a 12-well plate and then successively transferred to a 6-well plate, then to a 100 mm dish (Falcon Co.). When the cells reached confluence they were collected, frozen and stored in a liquid nitrogen tank. Culture, maintenance, transfection and establishment of stable cell lines of human erythroleukemic K562 cells were conducted as described previously (manuscript in preparation).

The transfectants containing the Tet-regulated transactivator (pUHD15-1) were first screened for tTA activity by transiently transfecting them with pUHC13-3 encoding luciferase. Cells were cultured in DMEM + 10% CS and 500 ng/ml Tet. At 18–20 h before transfection cells were split to two plates at a density of $2 \times 10^6$/100 mm dish, with one cultured in the presence and the other in the absence of Tet. Then, 1 µg plasmid pUHC13-3 was introduced into the cells by the calcium phosphate co-precipitation method (19). At 45 h after transfection luciferase activity was measured using a luciferase assay system (E1500; Promega). NIH 3T3 cells were lysed in 200 µl lysis buffer for 15 min at room temperature, insoluble material was pelleted by centrifugation for 2 min at 14 000 r.p.m., 20 µl supernatant was mixed with 100 µl luciferin reagent and the light produced in 10 s was measured in a luminometer (Monolight 2010; Analytical Luminescence Laboratory). Sample activity was measured within the linear range of the assay. Protein concentration of the lysates was determined as $OD_{280}$ and used to normalize luciferase activity. Cell lines with high luciferase activity in the absence of Tet and low luciferase activity in the presence of Tet were selected for further characterization.

## RESULTS

### Establishment of stable cell lines expressing the Tet-controlled transcriptional activator

In order to use the stable β-globin mRNA as a reporter message to study ARE destabilizing function we have constructed a new β-globin reporter plasmid, designated pTet-BBB. The β-globin gene to be expressed in the plasmid is under control of the Tet-regulated promoter (10). Two AREs were chosen for our initial tests: the c-*fos* ARE, a representative of the class I AUUUA-containing AREs that directs distributive digestion of

poly(A) tails; the GMCSF ARE, a typical example of the class II AUUUA-containing AREs that directs processive removal of the poly(A) tail (6,8). They were individually inserted into the unique *Bgl*II site in the 3′-UTR of the β-globin gene to generate pTet-BBB+ARE.

Initially we transiently co-transfected both plasmid pTet-BBB+ARE and plasmid pUHD15-1 (10), encoding the Tet-controlled transcriptional activator (tTA) that activates β-globin transcription from pTet-BBB+ARE by binding to the *tetO* site in the absence of Tet. However, transient co-transfection did not permit tight regulation of transcription by Tet (data not shown). We observed a high basal level of β-globin mRNA expression in the presence of Tet and inefficient repression when Tet was re-added to turn off transcription. In addition, the level of β-globin mRNA expression from *tetO* varied significantly depending on the ratio of plasmids pTet-BBB+ARE and pUHD15-1. As a result, we chose to first establish a stable cell line that produces tTA in NIH 3T3 cells. After transfection of NIH 3T3 cells with plasmid pUHD15-1 encoding tTA, individual G418-resistant clones were selected and analyzed using the transient luciferase expression assay (see Materials and Methods). pUHC13-3, which has luciferase cDNA driven by the Tet-regulated promoter, was transiently transfected into selected stable clones. Cells from individual clones were cultured in the presence or absence of 500 ng/ml Tet for 45 h prior to luciferase expression assay. Figure 1A shows that different clones of tTA-expressing NIH 3T3 cell lines achieved various levels of Tet-regulated gene expression. Clone $B_2A_2$, showing the highest luciferase activity, was selected for further analysis. It gave $>10^9$ relative luciferase units (RLU) in the absence of Tet and in the presence of Tet luciferase activity was reduced to 0.17% of its maximal level. Thus the effectiveness of Tet regulation of luciferase gene expression of this clone is >500-fold. This magnitude of expression and repression of reporter message levels is suitable for monitoring mRNA decay rates in typical experiments designed to determine rapid mRNA turnover.

Having initially characterized this stable tTA-expressing clone we then transiently transfected cells of clone $B_2A_2$ with reporter plasmid pTet-BBB and carried out RNA blot analysis of β-globin mRNA expression. As shown in Figure 1B, extremely robust expression of β-globin mRNA was achieved when transfected cells were cultured in the absence of Tet. When normalized to the control message, α/GAPDH mRNA constitutively transcribed from the SV40 enhancer-driven promoter (18), the level of expression from the Tet-regulated promoter is at least 20-fold higher than that from the c-*fos* promoter (Fig. 2). To learn the range of Tet concentration that may quantitatively regulate β-globin gene expression tTA-expressing cells were transiently transfected with pTet-BBB and cultured for 24 h in the indicated concentration of Tet prior to RNA extraction and Northern blot analysis. As shown in Figure 1B, as little as 20 ng/ml Tet is sufficient to reduce the level of cytoplasmic β-globin mRNA to an undetectable level. This feature of efficient transcriptional repression by a low concentration of Tet is particularly relevant when dealing with measurement of mRNA stability (see below).

## Development of a transcriptional pulse strategy by modulating the amount of Tet in the culture medium

Having further characterized clone $B_2A_2$ we then set out to determine the stability of β-globin mRNA in serum-induced NIH 3T3 cells. First, we performed experiments to compare

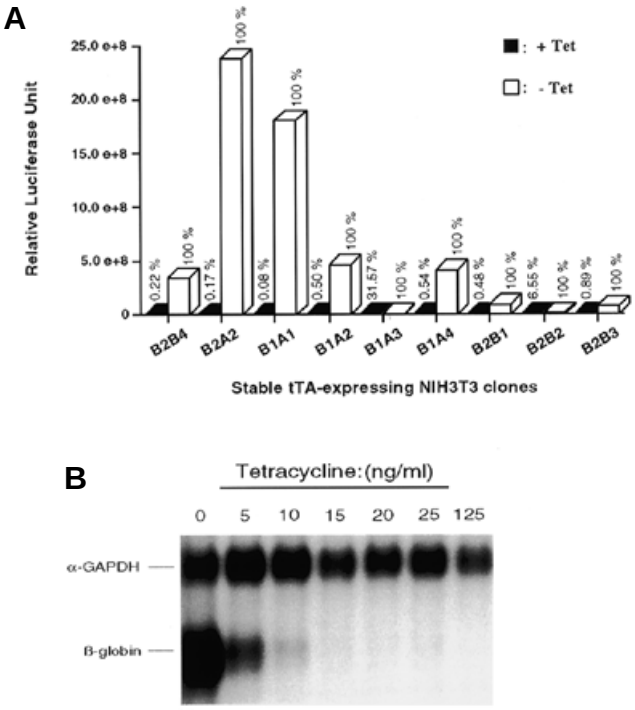


**Figure 1.** Characterization of stable transfectants expressing tTA. (**A**) Analysis of tTA-expressing NIH 3T3 cells stably transfected with pUHD15-1. Nine stable transfectant clones were analyzed for their ability to activate a luciferase reporter gene encoded by pUHC13-3 in transient transfections. Parallel transfections were performed in the presence (+Tet; black bars) or absence (–Tet; white bars) of 500 ng/ml Tet in the growth medium and luciferase activity measured as described in Materials and Methods. Luciferase activity in the absence of Tet was assigned as 100% and luciferase activity in the presence of Tet was compared with that in the absence of Tet. Percentage expression level is shown at the top of each bar. (**B**) Quantitative regulation of tTA function by Tet. Stable transfectant $B_2A_2$, showing the highest luciferase activity in the absence of Tet, was grown at a density of $2 \times 10^6$/100 mm dish in DMEM with 10% CS and the Tet concentrations indicated. After co-transfection of pTet-BBB and control plasmid pSVα1/GAPDH for 24 h total cytoplasmic RNA was isolated and Northern analysis was performed. α/GAPDH mRNA was constitutively expressed and detected by a probe to the α-globin gene. It served as an internal standard for normalizing variations derived from the transfection efficiency and sample handling.

results obtained using the Tet-regulated system with those obtained using the c-*fos* promoter system. Cells of clone $B_2A_2$ were transiently transfected with pTet-BBB and made quiescent by culturing them in medium containing 0.5% CS for 25 h. Thirty minutes after raising the serum concentration to 20% Tet was added to the culture medium to a final concentration of 500 ng/ml at the indicated times to block constitutive transcription of the β-globin gene from the Tet-regulated promoter. The decay of β-globin mRNA was then monitored as a function of time. β-Globin mRNA decayed with a half-life of >8 h, a value identical to that observed using the c-*fos* promoter system (Fig. 2A). However, when AREs were introduced into the β-globin mRNA the expected efficient degradation mediated by AREs was not observed. BBB+ARE mRNAs all decayed slowly, with half-lives similar to that of β-globin mRNA (data not shown), possibly due to saturation of the cellular decay machinery as a result of high level constitutive expression from the Tet-regulated promoter. Moreover, in contrast to the c-*fos* promoter system (Fig. 2B), deadenylation kinetics and

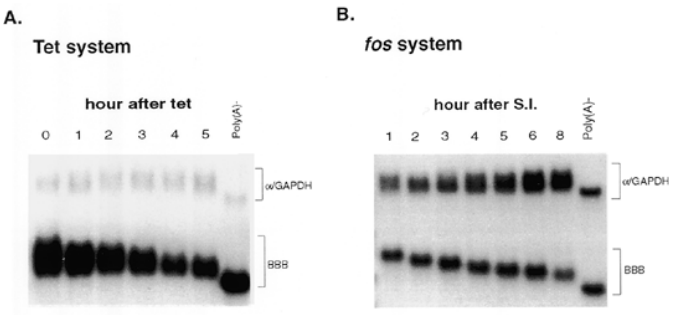


**Figure 2.** Comparison between the Tet-regulated system without transcriptional pulse and the c-*fos* promoter-regulated system for studying mRNA decay kinetics. (**A**) RNA decay study using the Tet regulatory system without pulse. Stable transfectant $B_2A_2$ was cultured in the absence of Tet and transiently co-transfected with 1 µg pTet-BBB and 2 µg pSVα1/GAPDH. After serum starvation for 25 h the quiescent cells were induced with 20% CS and 500 ng/ml Tet was added to the medium after 30 min to block constitutive transcription of the β-globin gene from the Tet-regulated promoter. Total cytoplasmic mRNA was isolated at various time intervals as indicated at the top. (**B**) RNA decay study using the c-*fos* promoter system. NIH 3T3 cells were transiently co-transfected with 3 µg test plasmid pBBB and 2 µg pSVα1/GAPDH. After serum stimulation total cytoplasmic RNA was isolated at various time intervals as indicated at the top. Poly(A)$^-$ RNA was prepared *in vitro* by treating RNA samples with oligo(dT) and RNase H from 1 h time points, when BBB mRNA still retained a full-length poly(A) tail.

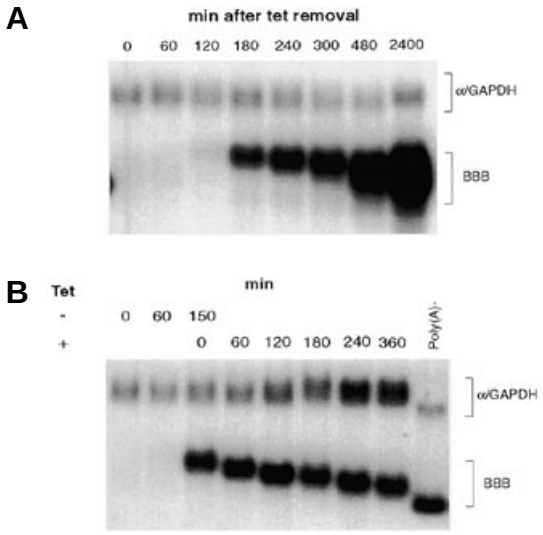


**Figure 3.** Expression kinetics of the Tet-regulated promoter. (**A**) Kinetics of transcription resumption when Tet is removed. $B_2A_2$ cells were grown in the presence of Tet (25 ng/ml). At 15 h after transient co-transfection with plasmids pTet-BBB and pSVα1/GAPDH expression of the Tet-regulated gene was induced by removing Tet. Total cytoplasmic RNA was isolated at the times indicated at the top and subjected to Northern blot analysis. (**B**) Transcriptional pulse achieved by modulating Tet in the culture medium. Cell culture, transfection and gene expression were conducted as described in (A). Transient expression from the Tet-regulated gene was induced for 150 min by transferring the cells to fresh culture medium without Tet, followed by re-addition of 500 ng/ml Tet to the medium to block transcription for the indicated time intervals to monitor mRNA decay. Time points in the upper row indicate times after Tet was removed and those in the lower row indicate times after Tet was re-added.

its relationship to mRNA decay could not be evaluated from the blot in Figure 2A, e.g. determination of whether deadenylation is the first step and precedes decay of mRNA. This is a result of size heterogeneity of the mRNA molecules, resulting from the difference of their ages in the cytoplasm.

To circumvent these problems we devised a strategy that would allow a transient burst of mRNA synthesis so that a population of mRNA homogeneous in size could be generated and decay kinetics readily followed. A transcriptional burst is also characteristic of the c-*fos* promoter system. As described in Figure 1B, clone $B_2A_2$ supports highly robust expression from *tetO*, which is at least 20-fold higher than expression from the c-*fos* promoter (compare Fig. 2A and B). Thus an expression level of at least 5% of full expression in the absence of Tet from the target gene in this clone should be sufficient to generate an acceptable signal comparable with that from the c-*fos* promoter for Northern blot analysis. In order to achieve this level of expression cells were first cultured in a low concentration of Tet (25 ng/ml), an amount sufficient to repress expression from the reporter gene (Fig. 1B), and then Tet was removed from the medium to allow transcription to resume to a level >5% of its full expression. This period was termed a transcriptional pulse. The pulse was terminated by adding 500 ng/ml Tet, which is a 20-fold higher concentration than needed for repression of transcription. This assured rapid inhibition of transcription.

As a first step we carried out experiments to evaluate the kinetics of transcription resumption when Tet was removed from the culture medium. As shown in Figure 3A, after replacing medium containing 25 ng/ml Tet with fresh medium there was a time lag of ~120 min prior to rapid appearance at the 3 h time point of a discrete band corresponding to mRNA with a 220 nt poly(A) tail (data not shown). Intensity of the signal increased linearly beyond 8 h. The results suggest that in a short time interval, i.e. between the 2 and 3 h time points after removal of Tet, significant activation of transcription was accomplished. We then proceeded to follow deadenylation and decay of β-globin mRNA as a function of time. Since the mRNA became more heterogeneous in size after a 3 h transcriptional pulse following Tet removal, 500 ng/ml Tet was re-added to the culture medium at 150 min after removal of Tet to block further transcription. The results in Figure 3B indicate a transient burst of mRNA synthesis. The β-globin mRNAs appear as a discrete band and undergo highly synchronous and slow deadenylation, which parallels the pattern seen in the c-*fos* system (compare Figs 2B and 3B). They remain very stable with little decay over the 6 h time course. Taken together, our data demonstrate the feasibility of this transcriptional pulse strategy for monitoring decay of stable mammalian mRNAs.

## ARE-mediated rapid decay in growth- and density-arrested cells

We next addressed the feasibility of this strategy for investigating rapid mRNA decay mediated by the ARE. Both c-*fos* and GMCSF AREs were inserted into the 3′-UTR of the β-globin gene to create pTet-BBB+ARE$^{c\text{-}fos}$ and BBB+ARE$^{GMCSF}$. These plasmids were then transiently transfected into cells of clone $B_2A_2$. We first examined ARE-mediated decay in serum-stimulated cells undergoing the $G_0$ to $G_1$ transition. Cells were serum starved for 25 h and then induced with serum. Ninety minutes prior to serum induction Tet was removed from the culture medium to allow transcription from the Tet-regulated promoter to resume. Sixty minutes after serum induction, which corresponds to 150 min after Tet removal, 500 ng/ml Tet was re-added for the indicated times to block further transcription. As shown in Figure 4, patterns of decay of BBB+ARE$^{c\text{-}fos}$ mRNA and BBB+ARE$^{GMCSF}$ mRNA are very

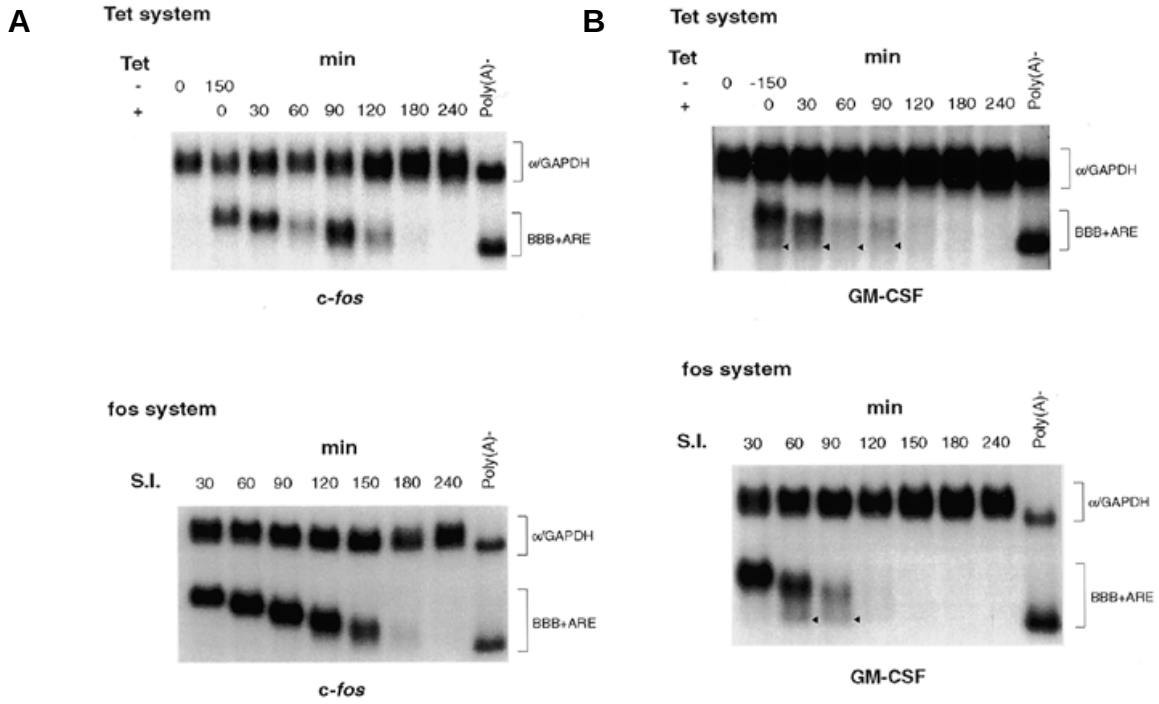


**Figure 4.** ARE-mediated mRNA decay measured using the Tet-regulated transcriptional pulse system compared with that determined using the c-*fos* promoter system. Cell culture, transfection and gene expression were conducted in the Tet system as described in the legend to Figure 3 and in the c-*fos* system as described in the legend to Figure 2B. (**A**) Comparison of c-*fos* ARE-mediated decay between the Tet pulse and c-*fos* systems. (**B**) Comparison of GMCSF ARE-mediated decay between the Tet pulse and c-*fos* systems. The bottom edges of the smears corresponding to the poly(A)$^-$ position are indicated by solid triangles.

similar to those observed using the c-*fos* promoter system. (Note that in the c-*fos* system mRNA appears 30 min after serum induction and in the Tet system the initial appearance of mRNA is designated as time 0 in Fig. 4.) BBB+ARE$^{c\text{-}fos}$ mRNAs undergo synchronous deadenylation followed by rapid decay of the RNA, consistent with distributive enzymatic removal of poly(A) tails (8,21,22). BBB+ARE$^{GMCSF}$ mRNAs display asynchronous deadenylation with smearing ending at a position corresponding to poly(A)$^-$ mRNA, consistent with processive digestion of poly(A) tails (8,21). Therefore, these results demonstrate the feasibility of this new strategy for studying deadenylation and degradation kinetics of labile mRNAs, such as ARE-bearing mRNAs.

Using this new Tet pulse strategy we then set out to test if AREs function in growth-arrested or in density-arrested cells when ARE-bearing mRNAs, e.g. cytokine, growth factor, transcription factor and proto-oncogene mRNAs, are not expressed. These experiments have not previously been possible using the c-*fos* promoter system or by simply blocking transcription with actinomycin D. After cells were transiently transfected with pTet-BBB+ARE they were maintained in 0.5% serum for 25 h to cause them to enter the $G_0$ state. Parallel cultures were kept in regular medium containing 10% CS for continuous proliferation until they reached stationary phase and exhibited contact inhibition of growth. Then, time course experiments were carried out using the transcriptional pulse strategy. As shown in Figure 5A, in the growth-arrested state both the c-*fos* and GMCSF AREs remain functionally potent. They show an increase in destabilizing function and efficiently target β-globin mRNA for rapid degradation (compare with Fig. 4). The kinetics are similar to those observed in serum-stimulated cells. While BBB+ARE$^{c\text{-}fos}$ mRNAs undergo synchronous deadenylation, BBB+ARE$^{GMCSF}$ mRNAs display asynchronous deadenylation. In the stationary phase both AREs exhibit a similar destabilizing potency to that observed in serum-stimulated cells (compare Figs 5B and 4). These results demonstrate that each of these AREs is able to direct rapid decay with unchanged or faster kinetics regardless of the physiological state of NIH 3T3 cells.

## β-Globin mRNA turnover and ARE-directed mRNA decay in K562 erythroblasts

Since NIH 3T3 fibroblasts have been our model cell system for studying key sequence features necessary for ARE destabilizing function, it is essential to learn how AREs function in non-fibroblastic cells in order to deduce general principles concerning their mechanisms. It is not known whether the ARE-directed decay pathway is conserved between different cell types. In order to answer this question we chose human K562 erythroleukemic cells for the following reasons. First, an *in vitro* decay system using cytoplasmic lysates prepared from human K562 erythroleukemic cells had been developed and used with success for many years to biochemically dissect mRNA decay (23). Unlike NIH 3T3 cells, which need to attach to a tissue culture plate for growth, K562 cells are grown in suspension. In addition, decay of c-*myc* mRNA, an ARE-bearing mRNA, has been studied in K562 cells (24,25).

As a first step stable K562 transfectants expressing tTA were selected. After extensive characterization of 20 individual clones one clone was selected for use in mRNA decay studies. This clone not only supports robust transcription from *tetO* but also exhibits rapid transcription resumption upon removal of Tet from the culture medium (data not shown).

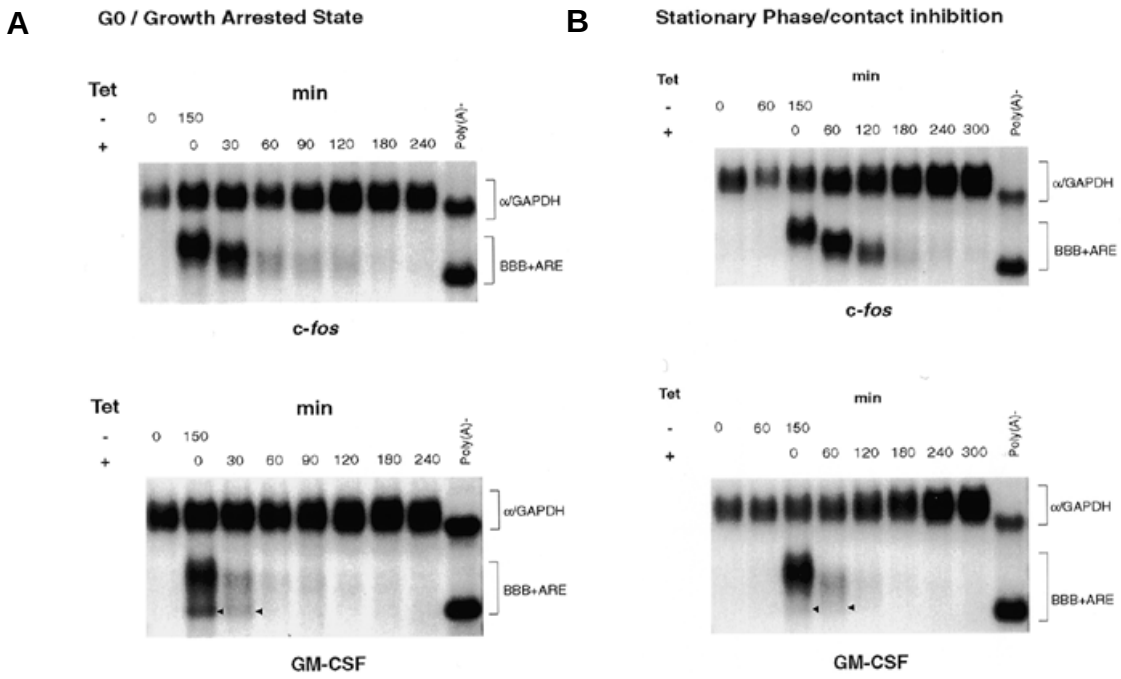


**Figure 5.** Study of ARE-mediated mRNA decay in NIH 3T3 cells under different physiological states. Cell culture, transfection and gene expression were as described in the legend to Figure 3. Total cytoplasmic RNA was isolated at the time indicated at the top. (**A**) ARE function examined in $G_0$ or quiescent cells. After serum starvation for 25 h gene expression from the Tet-regulated promoter was induced by transferring the cells to DMEM plus 0.5% CS without Tet for 150 min followed by addition of 500 ng/ml Tet for various time intervals. (**B**) ARE function examined in cells that reach confluence and exhibit density arrest of their growth. After transfection cells were grown in DMEM with 10% CS and 25 ng/ml Tet until they reached confluence. Then Tet was removed to allow expression of pTet-BBB+ARE for 150 min, followed by addition of Tet at a final concentration of 500 ng/ml.

We then transiently transfected K562 cells with pTet-BBB, pTet-BBB+ARE$^{c\text{-}fos}$ or pTet-BBB+ARE$^{GMCSF}$ by electroporation and carried out the transcriptional pulse strategy by controlling Tet in the culture medium. As shown in Figure 6, β-globin mRNA decayed with a half-life of >16 h and displayed an extraordinarily slow but synchronous deadenylation. In contrast, when AREs were introduced into β-globin mRNA both BBB+ARE$^{c\text{-}fos}$ and BBB+ARE$^{GMCSF}$ mRNA decayed significantly faster than β-globin mRNA, with half-lives of 3.1 and 1.4 h respectively. However, in comparison with NIH 3T3 cells the destabilizing function of both AREs in K562 cells is somewhat retarded. Nonetheless, the rank order of the destabilizing ability of the two AREs remained unchanged, with decay patterns parallel to those observed in NIH 3T3 cells.

## DISCUSSION

We have demonstrated the feasibility of using a transcriptional pulse strategy based on the Tet-controlled system. For the first time we are now able to address ARE function under a variety of physiological states and in various cell types. Our results reveal several novel points concerning ARE function. The ARE can act to mediate rapid decay not only during the $G_0 \rightarrow G_1$ transition but also in the growth- and density-arrested states. In each case deadenylation is the first necessary step. This observation suggests a broader role for the ARE-mediated decay pathway. First, since most ARE-containing mRNAs encode transcription factors, growth factors and cytokines involved in control of cell growth and differentiation, functioning of AREs in these non-growing states may ensure that unwanted ARE-bearing mRNAs are quickly degraded so that the growth-arrested state could be properly maintained. Second, initiation of cell growth or density arrest may require transient synthesis of certain labile mRNAs that bear AREs, e.g. growth arrest-specific (GAS) mRNAs (26–28). Thus a functional ARE-mediated decay pathway will ensure tight regulation of transient expression of these mRNAs. Therefore, the ARE-mediated decay pathway could play a critical role in initiation and maintenance of cell growth arrest and density arrest.

Our data provide a direct comparison of decay mediated by AREs between two different cell types, one growing as a monolayer and the other in suspension, and demonstrate that the pathway is conserved in non-fibroblast cells. In proliferating K562 erythroleukemic cells the c-*fos* and GMCSF AREs appear to direct decay with kinetics parallel to those observed in NIH 3T3 cells. These results support the classification of AREs and indicate that the key sequence features of AREs revealed by studies using NIH 3T3 cells and the c-*fos* promoter system should be able to serve as general principles. More importantly, these observations support a more universal destabilization effect of the ARE on mRNA degradation. Taken together, the results revealed by this new approach provide further *in vivo* evidence that AREs have a broader capacity in terms of their destabilizing function and are critical for cells to achieve tight regulation of transient mRNA expression during cell growth and differentiation.

Several important factors are particularly relevant to success of the transcriptional pulse strategy. First, the extremely high expression level achieved in tTA/Tet-controlled transcription is critical. This feature makes it possible to easily detect mRNA synthesized in a narrow time window after Tet repression is released. Our strategy produces an mRNA population nearly

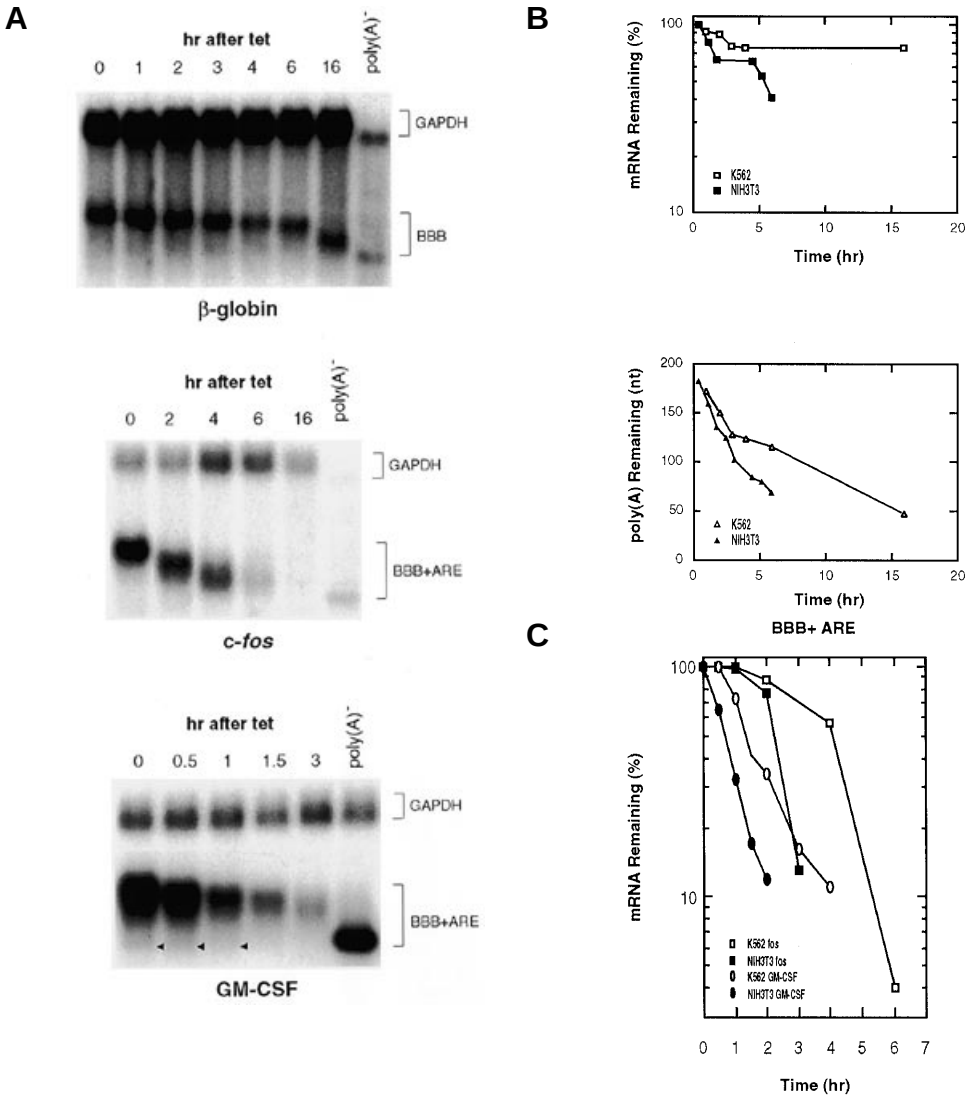


**Figure 6.** Study of mRNA decay and deadenylation kinetics in K562 erythroleukemic cells using the Tet-regulated promoter system. A stable K562 transfectant expressing tTA was cultured in RPMI1640 with 10% fetal bovine serum in the presence of Tet (30 ng/ml) and transiently transfected with plasmid pTet-BBB, pTet-BBB+ARE$^{c\text{-}fos}$ or pTet-BBB+ARE$^{GMCSF}$. At 24 h after transfection expression of the β-globin gene under control of the Tet-regulated promoter was resumed by transferring the cells to fresh medium without Tet for 150 min, followed by addition of 500 ng/ml Tet to block further transcription. (**A**) Total cytoplasmic RNA was isolated after addition of Tet at the time intervals indicated at the top and analyzed by Northern blotting. The bottom edges of the smears corresponding to the poly(A)$^-$ position are indicated by solid triangles in the RNA blot showing GMCSF ARE-mediated decay. (**B**) Semilog plot (top) and linear scale plot (bottom) showing decay and deadenylation of β-globin mRNA respectively. Poly(A) tail lengths were calculated from the difference in electrophoretic mobility between each message and cognate RNA that had been deadenylated *in vitro*. The length of poly(A) responsible for the observed differences in mobility was then estimated by comparison with the spacing of bands in a ladder of single-stranded DNA markers that had been electrophoresed in parallel. (**C**) Semilog plot showing decay of β-globin mRNA carrying the c-*fos* ARE or GMCSF ARE in NIH 3T3 or K562 cells. Note that quantitation of data was by scanning the radioactive blots with an imager (Packard).

homogeneous in size, permitting optimal determination of its deadenylation and decay kinetics (see below). Therefore, it is crucial to select a stable tTA-expressing clone that gives a maximally induced expression level of the reporter gene. Both the NIH 3T3 and the K562 clones selected for this study display >$10^9$ RLU when 2–5 × $10^6$ cells were transiently transfected with only 1 μg plasmid containing luciferase cDNA driven by the Tet-regulated promoter, pUHC13–3 (10).

Second, in order to be able to accurately determine deadenylation and decay kinetics robust yet brief transcription is necessary. Thus the resumption kinetics of transcription upon removal of Tet is a crucial factor. As shown in Figure 3, when Tet is removed from the culture medium clone $B_2A_2$ supports abrupt resumption of transcription after a 2 h lag. A significant amount of β-globin mRNA expression is detected during the 30 min time interval commencing 2 h after Tet removal. Although we were able to identify a few clones that overexpress the transfected reporter genes at equally high levels in the absence of Tet, we noticed that not all of them exhibited fast resumption of transcription after Tet removal (data not shown). This difference may be due to variations in copy number and integration site of the tTA cDNA in stable clones. Interestingly, all show an ~120 min lag before the reporter mRNA can be readily detected in the cytoplasm after removal of Tet. Therefore, care should be taken to pick a clone that is able to give at least 10% of the maximal level of expression within a 30–60 min window immediately following the 120 min lag. This will ensure that a sufficient signal representing an mRNA population homogeneous in size is generated for kinetic study. Slow resumption of transcription will require a prolonged

pulse. This in turn leads to heterogeneity in the size of mRNA molecules in the population and compromises determination of deadenylation and decay kinetics.

Third, we have selected a clone in which luciferase activity can be suppressed to 0.17% of maximal activity by the lowest possible concentration of Tet, in this case 20–30 ng/ml Tet. This facilitates rapid transcription resumption when Tet is removed from the culture medium. We have found that the lower the concentration of Tet required to repress transcription the quicker transcription is re-activated when Tet is removed. Efficient transcription shut-off is then accomplished by adding 500 ng/ml Tet to the culture medium. In HeLa cells transcription from the Tet promoter is known to turn off within 5 min upon addition of Tet, as judged by a decrease in nuclear pre-mRNA levels (M.F.Wilkinson, personal communication).

Our strategy has several advantages over other approaches used to measure mRNA stability. First, the use of Tet at a subtoxic concentration in mammalian cells allows analysis of mRNA turnover under physiologically undisturbed conditions. Second, it offers the opportunity to determine decay kinetics and the precursor–product relationship of mRNA turnover under different physiological conditions. For example, one can begin to address whether poly(A) tail removal precedes decay of β-tubulin mRNA (29) or transferrin receptor mRNA (30) under physiological conditions relevant to their regulated expression. Is there a precursor–product relationship between the full-length mRNA and the 1.8 kb mRNA suggested to be a decay intermediate in the case of insulin-like growth factor II mRNA (31,32)? Third, with an increasing collection of tTA-expressing cell lines the Tet-regulated promoter system combined with our strategy allows analysis of mRNA turnover and its regulation during cell growth and differentiation, as demonstrated in this report. For example, we found that β-globin mRNA decay in K562 human erythroleukemic cells undergoes extraordinarily slow deadenylation over a 16 h time period, which is significantly slower than deadenylation observed in NIH 3T3 cells (Fig. 6C). Our data suggest that in K562 cells deadenylation precedes decay of stable β-globin mRNA and that the unusual stability of globin mRNA is controlled largely at the deadenylation step.

The role of signal transduction in the control of ARE-directed mRNA decay has not been widely explored. Several previous studies have shown that treatment of lymphoid cells with phorbol esters, calcium ionophores or interleukins increases the steady-state level of certain cytokine and lymphokine mRNAs that contain AREs (5,33–35). Calcium ionophores like A23187 specifically stabilize IL-3 mRNA in cultured mast cells (36). A23187 also stabilizes GMCSF mRNA at least 10-fold in a thymoma cell line (37). In phorbol ester-stimulated T cells there is stabilization of lymphokine mRNA but not c-*fos* and c-*myc* mRNAs (37). Taken together these observations point to the possible differential regulation of ARE-specific mRNA degradation by calcium-dependent protein kinase C pathways. With the establishment of the Tet-controlled system in lymphoid cell lines or hematopoetic cell lines representing different lineages elucidation of the mechanisms responsible for regulation is anticipated.

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