The *c-fos* transcript is targeted for rapid decay by two distinct mRNA degradation pathways

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Rapid degradation of *c-fos* proto-oncogene mRNA is crucial for transient *c-fos* gene expression. Experiments were performed to investigate the cellular mechanisms responsible for the extremely short half-life of human *c-fos* mRNA in growth-factor-stimulated fibroblasts. These experiments demonstrate the existence of two distinct cellular pathways for rapid *c-fos* mRNA degradation. Each of these pathways recognizes a different, functionally independent instability determinant within the *c-fos* transcript. One instability determinant, which is located within the *c-fos* 3'-untranslated region, is a 75-nucleotide AU-rich segment. Insertion of this element into β -globin mRNA markedly reduces the half-life of that normally long-lived message. Nevertheless, specific deletion of the AU-rich element from *c-fos* mRNA has little effect on the transcript's cytoplasmic half-life due to the presence of the other *c-fos* instability determinant, which is located in the protein-coding segment of the *c-fos* message. Examination of mRNA decay in cells treated with transcription inhibitors indicates that one *c-fos* mRNA degradation pathway is dependent on RNA synthesis, whereas the other is not.

[Key Words: mRNA degradation; *c-fos*; AU-rich element; proto-oncogene]

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An important mechanism for regulating gene expression in all organisms is control of the cytoplasmic concentration of individual gene transcripts. mRNA levels often change dramatically during cell growth and differentiation as a result of changes in transcription, RNA processing, and mRNA decay. Although a great deal has been learned in recent years about the regulation of mRNA synthesis, much less is known about the cellular mechanisms that control mRNA degradation. Nevertheless, a number of studies have revealed that bacterial and mammalian gene expression can be modulated through changes in mRNA stability (for reviews, see Belasco and Higgins 1988; Brawerman 1987).

In mammalian cells, a class of 10–50 growth-factor-inducible genes has been identified whose transcripts appear transiently in the cytoplasm immediately after stimulation with growth factors (Greenberg and Ziff 1984; Lau and Nathans 1985; Lim et al. 1987; Almendral et al. 1988). The transient expression of these genes is a consequence not only of the brevity of their transcription but also of their transcripts' very short lifetime in the cytoplasm. The lability of this class of mRNA, which decays with a half-life of <30 min, distinguishes it from most mammalian messages, which typically are much more stable, with half-lives ranging from hours to days (Krowczynska et al. 1985). As yet, little is understood about the mechanisms by which these growth-factor-inducible transcripts are degraded.

The best characterized of the growth-factor-inducible messages is the transcript of the *c-fos* proto-oncogene.

The *c-fos* gene encodes a nuclear protein that forms a complex with the mammalian transcription factor AP-1 and is believed to regulate the transcription of a diverse set of genes important for cell growth and differentiation (Chiu et al. 1988; Rauscher et al. 1988; Sassone-Corsi et al. 1988). Expression of the *c-fos* gene is induced rapidly and transiently in a wide range of mammalian cell types by growth factors, phorbol esters, neurotransmitters, and membrane-depolarizing agents (Greenberg and Ziff 1984; Curran and Morgan 1985, 1986; Greenberg et al. 1985, 1986b). Induction of *c-fos* occurs at the transcriptional level and is independent of new protein synthesis (Cochran et al. 1984; Greenberg et al. 1986a). Minutes after its activation, transcription of *c-fos* ceases (Greenberg et al. 1985, 1986b). The newly synthesized *c-fos* mRNA is transported to the cytoplasm, where it is translated for only a brief period of time before being degraded rapidly (Kruijer et al. 1984; Muller et al. 1984). This transient accumulation of *c-fos* mRNA appears to be critical to normal cellular function because mutations that result in its deregulated expression can lead to oncogenic transformation (Miller et al. 1984; Meijlink et al. 1985; Jenuwein and Muller 1987).

There has been considerable interest in understanding the molecular basis for the rapid turnover of *c-fos* mRNA. Initial experiments showed that substitution of heterologous RNA for the 3' half of the *c-fos* transcript can have a stabilizing effect (Treisman 1985). The *c-fos* 3'-untranslated region (UTR) contains an AU-rich element (ARE, Fig. 1) that resembles an RNA segment pre-

Figure 1. The *c-fos* and β -globin transcripts. (Top) Diagrams of *c-fos* and β -globin mRNA. (Thin lines) 5'- and 3'-UTRs; (open rectangles) protein-coding regions; (solid rectangle) ARE; (A_n) 3' poly(A) tail. Arrows mark sites that correspond to DNA restriction sites used in the construction of *c-fos*/ β -globin gene fusions. (MluI) A restriction site created in the *c-fos* and β -globin genes by three point mutations near the boundary between the 5'-UTR and the protein-coding region; (BglII) a restriction site that is located immediately downstream of the rabbit β -globin translation termination codon and that was introduced into the *c-fos* gene by linker insertion at an *NaeI* site near the beginning of the *c-fos* 3'-UTR. (Bottom) Sequence of the 75-nucleotide ARE of human *c-fos* mRNA.

viously implicated in the lability of granulocyte–monocyte colony-stimulating factor (GM-CSF) mRNA (van Straaten et al. 1983; Shaw and Kamen 1986). Large deletions in the *c-fos* 3'-UTR that remove the ARE appear to retard *c-fos* mRNA degradation in actively growing fibroblasts when the *c-fos* gene is constitutively transcribed from a heterologous promoter and decay is monitored in the presence of the transcription inhibitor actinomycin D (Fort et al. 1987; Rahmsdorf et al. 1987). Nevertheless, the role of the ARE in determining the half-life of *c-fos* mRNA under normal conditions of transient gene expression in growth-factor-stimulated cells has not been established.

Here, we report experiments that reveal the existence of two distinct cellular pathways for rapid *c-fos* mRNA degradation. Each of these pathways recognizes a different *c-fos* instability determinant, either of which is sufficient to render the *c-fos* message highly labile. One of the instability determinants is the ARE in the 3'-UTR; the other is a novel element within the *c-fos* protein-coding region. Remarkably, deletion of the ARE alone has little effect on *c-fos* mRNA instability in growth-factor-stimulated fibroblasts. We hypothesize that mammalian cells have evolved multiple *c-fos* mRNA decay mechanisms to ensure that this transcript is degraded rapidly within minutes after it is synthesized.

Results

Synthesis and decay of human c-fos mRNA in NIH-3T3 cells

To identify determinants responsible for the marked lability of *c-fos* mRNA, a transient-induction assay was employed to monitor the decay of human *c-fos* mRNA in mouse fibroblasts after growth-factor stimulation. A wild-type genomic clone of the human *c-fos* gene (pF711) was introduced into mouse NIH-3T3 cells by transient transfection. After serum starvation for 25–30 hr, *c-fos* transcription was induced transiently by stimulation with fetal calf serum, and total cytoplasmic RNA

was isolated at time intervals. The relative concentrations of human *c-fos* mRNA, mouse *c-fos* mRNA, and α -globin mRNA (a cotransfected internal standard) at various times after serum stimulation were then determined by RNase-protection analysis of equal amounts of each RNA sample, using a mixture of uniformly radiolabeled RNA probes (Fig. 2, top).

Transcription of both the transfected human *c-fos* gene and the endogenous mouse *c-fos* gene was stimulated transiently by serum addition, and these two messages decayed with similar kinetics (half-lives of 15 ± 3 min and 16 ± 1 min, respectively; Fig. 2, top) following the spontaneous cessation of *c-fos* transcription that occurred within 1 hr after serum induction (Greenberg and Ziff 1984; Greenberg et al. 1985). Efficient degradation of the human message required transfection with no more than 5 μ g of pF711 per plate of 2×10^6 cells; transfection with 20 μ g of pF711 per plate increased the apparent half-life of this transcript to 90–100 min, possibly by saturating the cellular machinery involved in *c-fos* mRNA degradation. α -Globin mRNA, which was synthesized constitutively under the control of an SV40 enhancer and did not decay detectably over the time course of these experiments, served as an internal control that allowed correction for small variations in transfection efficiency and sample handling.

These findings show that the decay of human *c-fos* mRNA and endogenous mouse *c-fos* mRNA can be studied in parallel in transiently transfected NIH-3T3 cells under conditions that are physiologically relevant to normal *c-fos* gene expression and without the use of drugs to inhibit transcription.

The c-fos 3'-UTR is not required for c-fos mRNA instability

Experiments in other laboratories with *c-fos* genes transcribed from heterologous promoters have implicated an AU-rich segment of the *c-fos* 3'-UTR (Fig. 1) in causing *c-fos* mRNA instability (Fort et al. 1987; Rahmsdorf et al. 1987). To test whether the ARE is necessary for the short cytoplasmic half-life of the *c-fos* transcript after

Figure 2. Induction and decay of human *c-fos* mRNA and rabbit β -globin mRNA. NIH-3T3 cells were transiently transfected with pSV α 1 and either pF711 (*top*) or pBBB (*bottom*). Total cytoplasmic RNA was isolated at time intervals after serum stimulation and analyzed by RNase protection and gel electrophoresis. (c-fos^H) pSP6fos-derived probe fragment protected by human *c-fos* mRNA; (BBB) pT7BG-derived probe fragment protected by BBB mRNA; (M) pSP6fos-derived probe fragment protected by mouse *c-fos* mRNA; (A) pSP6 α 133-derived probe fragment protected by α -globin mRNA. Times correspond to minutes (*top*) or hours (*bottom*) after serum stimulation. Beside each autoradiogram is a semilogarithmic plot of the decay of human *c-fos* (c-fos^H) or rabbit β -globin (BBB) mRNA (solid line) and mouse *c-fos* mRNA (c-fos^M, dashed/dotted line).

growth-factor stimulation, a deletion was introduced into the human *c-fos* gene that precisely removed this 75-bp element (Table 1). The cytoplasmic half-life of the transcript of the resulting *c-fos* allele (FA1) was then determined after serum induction of transiently transfected NIH-3T3 fibroblasts. Surprisingly, the half-life of this message was found to be only 20 ± 3 min (Fig. 3, *top*), a value very similar to that measured for wild-type *c-fos* mRNA (15 ± 3 min). This finding demonstrates that the *c-fos* ARE is not required for the instability of *c-fos* mRNA in serum-stimulated fibroblasts.

To test for other structures in the long *c-fos* 3'-UTR that might contribute to *c-fos* mRNA lability, a nested

set of deletions was created within this region. These deletions removed the ARE in the *c-fos* 3'-UTR and extended unidirectionally for various distances (56–574 bp) upstream from it (FA2–FA6, Table 1). The cytoplasmic half-lives of the transcripts of the resulting *c-fos* alleles were measured as before, and all were found to decay at about the same rate as wild-type *c-fos* mRNA (Fig. 3, *bottom*; Table 1). The transcript of an additional *c-fos* deletion mutant (FA7; Table 1) that lacked a 201-nucleotide segment immediately upstream of the polyadenylation signal (AAUAAA), including the ARE, also decayed with a short half-life of 15 ± 1 min. Taken together, these findings demonstrate that no single ele-

Table 1. Half-lives of *c-fos* transcripts with deletions in the 3'-UTR

Deletion mutant	Deletion end points (kb) ^a	Half-life (min)
Wild-type	—	15 ± 3
FΔ1	1.92–2.00	20 ± 3
FΔ2	1.87–2.00	18 ± 4
FΔ3	1.67–2.00	15 ± 4
FΔ4	1.60–2.00	20 ± 2
FΔ5	1.51–2.00	21 ± 1
FΔ6	1.35–2.00	18 ± 1
FΔ7	1.87–2.07	15 ± 1

^aEnd points are measured in kilobases from the 5' terminus of human *c-fos* mRNA. In the wild-type *c-fos* message, the 5'-UTR extends from nucleotide 1 to 155, the protein-coding region from 156 to 1295, and the 3'-UTR from 1296 to 2104. The *c-fos* ARE is located between positions 1920 and 1994.

ment >30–50 nucleotides from either end of the 809 nucleotide 3'-UTR of *c-fos* mRNA, including the AU-rich element, is necessary for *c-fos* mRNA instability.

The 3'-UTR deletions described above left intact the RNA segment between the polyadenylation signal and the site of poly(A) addition. To test whether this segment contains an instability determinant, we replaced all but the first 27 nucleotides of the *c-fos* 3'-UTR with the 3'-UTR of the rabbit β -globin message. As shown below, rabbit β -globin mRNA is very stable in serum-stimulated fibroblasts. The resulting hybrid transcript, which should be properly polyadenylated at the β -globin poly(A) site, was designated FFB. This name indicates that the 5'-UTR and the coding region were derived from the human *c-fos* message, whereas the 3'-UTR was derived from rabbit β -globin mRNA. In this three-letter nomenclature for gene fusions transcribed under the control of the *c-fos* promoter, the first letter refers to the origin of the 5'-UTR (F if from human *c-fos* and B if from rabbit β -globin), the second letter indicates the source of the coding region, and the third letter refers to the source of the 3'-UTR. When the FFB gene was introduced into NIH-3T3 cells by transient transfection, cytoplasmic half-life of its transcript after serum induction was found to be only 21 ± 1 min (Fig. 4, top), about the same as that of wild-type *c-fos* mRNA. The instability of this *c-fos* mRNA variant, which lacked nearly the entire *c-fos* 3'-UTR, confirmed that the *c-fos* 3'-UTR is not necessary for *c-fos* mRNA instability.

Insertion of the c-fos ARE can destabilize an otherwise long-lived message

The lability of *c-fos* mRNA lacking the 3'-UTR could be explained in either of two ways. One possibility is that the *c-fos* 3'-UTR does not contain a signal for rapid mRNA degradation. Alternatively, there may be a determinant of mRNA instability in the 3'-UTR (e.g., the ARE) that is dispensable because of the presence of one or more additional instability determinants located up-

stream of the 3'-UTR. This additional element would ensure rapid decay of *c-fos* mRNA even when the 3'-UTR is absent. To ascertain whether the *c-fos* 3'-UTR, coding region, and 5'-UTR contain determinants of mRNA instability, we decided to substitute each of these *c-fos* mRNA segments for the corresponding segment of the stable rabbit β -globin transcript and to measure the decay rate of the resulting hybrid messages (BBF, BFB, and FBB).

As a first step, a *c-fos*/ β -globin gene fusion was constructed in which the β -globin promoter had been replaced precisely with the serum-inducible *c-fos* promoter. This gene fusion was designated BBB to indicate that it encoded a wild-type rabbit β -globin transcript whose 5' end mapped to the normal cap site. Induction and decay of β -globin mRNA transcribed from the *c-fos* promoter was then monitored in transfected NIH-3T3 cells to learn whether fusion of the *c-fos* promoter to a heterologous gene would allow transient transcription of that gene in response to serum stimulation. As in the case of the *c-fos* gene, BBB transcription was induced rapidly by addition of serum (Fig. 2, bottom). However, unlike *c-fos* mRNA, which disappears rapidly from the cytoplasm after cessation of transcription (Fig. 2, top), the cytoplasmic concentration of β -globin mRNA transcribed from the *c-fos* promoter reached a plateau within 1 hr after serum stimulation and did not decline significantly for at least 24 hr thereafter. These findings indicate that the *c-fos* promoter can direct transient synthesis of β -globin mRNA in response to serum stimulation and that the half-life of β -globin mRNA in serum-stimulated cells is very long (>24 hr).

To test whether the *c-fos* 3'-UTR contains a determinant of mRNA instability, we next analyzed the decay of a hybrid message (BBF) in which the 3'-UTR of β -globin mRNA was replaced with the corresponding segment of the *c-fos* transcript. As in the case of the BBB gene, transcription of the BBF gene was directed by the *c-fos* promoter. The cytoplasmic half-life of the chimeric BBF transcript was only 28 ± 2 min (Fig. 4, bottom), a value almost as short as that of *c-fos* mRNA itself and <2% as long as that of wild-type β -globin (BBB) mRNA. Thus, although deletion of the *c-fos* 3'-UTR does not stabilize *c-fos* mRNA, this mRNA segment is able to confer a high degree of instability upon the otherwise long-lived β -globin message.

Further analysis showed that the functional element primarily responsible for the instability of BBF mRNA was the AU-rich segment within the *c-fos* 3'-UTR. Precise deletion of the 75-nucleotide *c-fos* ARE from BBF stabilized the message considerably (BBFΔARE; Fig. 5, top). Moreover, simple insertion of the *c-fos* ARE into the 3'-UTR of an otherwise intact β -globin message (to generate BBB + ARE) was highly destabilizing; this insertion reduced the half-life of β -globin mRNA from >24 hr to only 37 ± 6 min (Fig. 5, bottom).

Taken together, these findings indicate that the *c-fos* ARE functions as an mRNA-destabilizing element. However, because deletion of the ARE or the entire 3'-UTR fails to stabilize *c-fos* mRNA, it appears that the *c-fos* message contains one or more additional deter-

Figure 3. Decay of human *c-fos* mRNA with deletions in the 3'-UTR. NIH-3T3 cells were transiently transfected with pSVα1 and either pFΔ1 (*top*) or pFΔ6 (*bottom*). Total cytoplasmic RNA was isolated at time intervals after serum stimulation and analyzed by RNase protection and gel electrophoresis. (FΔ1) pSP6fos-derived probe fragment protected by FΔ1 mRNA; (FΔ6) pSP6fos-derived probe fragment protected by FΔ6 mRNA; (M) pSP6fos-derived probe fragment protected by mouse *c-fos* mRNA; (A) pSP6α133-derived probe fragment protected by α-globin mRNA. Times correspond to minutes after serum stimulation. Beside each autoradiogram is a semi-logarithmic plot of the decay of FΔ1 or FΔ6 mRNA (solid line) and mouse *c-fos* mRNA (*c-fos*^M, dashed/dotted line)

minants of instability in the 5'-UTR or in the coding region.

The c-fos coding region contains a determinant of mRNA instability

To map the location of the additional determinant(s) of *c-fos* mRNA instability, gene fusions were constructed in which the β-globin coding region or 5'-UTR was replaced with the corresponding segment of the *c-fos* gene (BFB and FBB). The construction of these gene fusions was facilitated by the introduction of point mutations into plasmid clones of the *c-fos* and β-globin genes that

resulted in the creation of a unique *Mlu*I restriction site 5 bp downstream of the ATG translation initiation codon of each gene. These point mutations per se did not affect the half-life of the β-globin message (A.-B. Shyu, unpubl.). Transcription of each gene fusion was directed by the *c-fos* promoter and could be transiently induced by serum stimulation.

Replacement of the β-globin coding region with that of *c-fos* (BFB) had a dramatic destabilizing effect: BFB mRNA decayed with a half-life of only 17 ± 2 min (Fig. 6, *top*). Thus, like the *c-fos* 3'-UTR, the *c-fos*-coding region contains a determinant of mRNA instability that alone is sufficient to destabilize an otherwise long-lived

Figure 4. Decay of FFB and BBF mRNA. NIH-3T3 cells were transiently transfected with pSV α 1 and either pFFB (*top*) or pBBF (*bottom*). Total cytoplasmic RNA was isolated at time intervals after serum stimulation and analyzed by RNase protection and gel electrophoresis. (FFB) pSP6fos-derived probe fragment protected by FFB mRNA; (BBF) pT7BG-derived probe fragment protected by BBF mRNA; (M) pSP6fos-derived probe fragment protected by mouse *c-fos* mRNA; (A) pSP6 α 133-derived probe fragment protected by globin mRNA. Times correspond to minutes after serum stimulation. Beside each autoradiogram is a semilogarithmic plot of the decay of FFB or BBF mRNA (solid line) and mouse *c-fos* mRNA (*c-fos*^M, dashed/dotted line).

message. The instability determinant within the coding region apparently is more effective at destabilizing mRNA than even the ARE, inasmuch as the BBF transcript decays about twice as fast as BBB + ARE mRNA. In contrast to the 3'-UTR and coding region, substitution of the *c-fos* 5'-UTR for that of β -globin (FFB) hardly affected the cytoplasmic stability of the β -globin transcript, the half-life of FFB mRNA being >10 hr (Fig. 6, bottom).

Each c-fos instability determinant is the target of a distinct degradation pathway

To begin to characterize degradation mediated by each of the determinants of *c-fos* mRNA instability, we ex-

amined the effect of actinomycin D on message decay. Actinomycin D is a transcription inhibitor that acts by intercalating into nucleic acids. Interestingly, we discovered that actinomycin D differentially affects the function of the instability determinants located in the 3'-UTR and the protein-coding region. Treatment of serum-stimulated cells with actinomycin D gradually inhibited degradation of BBF mRNA. Within 0.5 hr after addition of actinomycin D, this message decayed slowly with a half-life of ~110 min, and it ceased to decay altogether within 3 hr (Fig. 7, top). In contrast, actinomycin D treatment had only a small effect on the decay of BFB mRNA, whose half-life was 28 ± 2 min in the presence of this drug and did not change with time (Fig. 7, top); nor did actinomycin D significantly affect the half-life of

Figure 5. Decay of BBF Δ ARE and BBB + ARE mRNA. NIH-3T3 cells were transiently transfected with pSV α 1 and either pBBF Δ ARE (top) or pBBB + ARE (bottom). Total cytoplasmic RNA was isolated at time intervals after serum stimulation and analyzed by RNase protection and gel electrophoresis. (BBF Δ ARE) pT7BG-derived probe fragment protected by BBF Δ ARE mRNA; (BBB + ARE) pT7BG-derived probe fragment protected by BBB + ARE mRNA; (A) pSP6 α 133-derived probe fragment protected by α -globin mRNA. Times correspond to minutes after serum stimulation. Beside the autoradiograms is a semilogarithmic plot of the decay of BBF Δ ARE mRNA (dashed/dotted line) and BBB + ARE mRNA (solid line).

the endogenous mouse *c-fos* message. These findings indicate that in serum-stimulated fibroblasts, actinomycin D inhibits decay mediated by the *c-fos* ARE but has little effect on degradation involving the instability determinant(s) present in the *c-fos* protein-coding region. This differential inhibition may be evidence that the ARE and the *c-fos* coding-region determinant are targets of two distinct cellular pathways for mRNA degradation. Alternatively, both determinants might be targeted by the same degradation pathway, and actinomycin D might inhibit ARE-mediated degradation selectively by site-specific intercalation into *c-fos* mRNA.

To distinguish between these possibilities, we examined the decay of BBF and BFB mRNA after treatment of serum-stimulated cells with 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), a transcription inhibitor that functions by interacting specifically with the RNA polymerase II transcription apparatus rather than by intercalating into DNA (Zandomeni et al. 1982; Maderious and Chen-Kiang 1984). Like actinomycin D, DRB gradually inhibited decay of the BBF transcript but had little effect on the degradation of BFB mRNA, which decayed with a uniform half-life of 25 ± 4 min in the

presence of DRB (Fig. 7, bottom). Because two transcription inhibitors with different modes of action both have the same differential effect on BBF and BFB mRNA decay, we conclude that the *c-fos* ARE and the *c-fos*-coding-region determinant direct rapid mRNA degradation by two distinct cellular pathways and that the degradation pathway that targets the ARE is transcription dependent in serum-stimulated cells.

Discussion

Our findings show for the first time that *c-fos* mRNA is targeted for rapid degradation by two distinct cellular pathways, each of which recognizes a different instability determinant within the *c-fos* transcript. One determinant of *c-fos* mRNA instability is the ARE within the *c-fos* 3'-UTR (Fig. 1). Insertion of this sequence into the 3'-UTR of β -globin mRNA reduces the half-life of that message from >24 hr down to only 37 min. Remarkably, this element is not necessary for *c-fos* mRNA instability, and its deletion has only a small effect on the half-life of the *c-fos* transcript.

We have identified a second, functionally independent

Figure 6. Decay of BFB and FBB mRNA. NIH-3T3 cells were transiently transfected with pSV α 1 and either pBFB (*top*) or pFBB (*bottom*). Total cytoplasmic RNA was isolated at time intervals after serum stimulation and analyzed by RNase protection and gel electrophoresis. (BFB) pSP6BFB-derived probe fragment protected by BFB mRNA; (FBB) pT7BG-derived probe fragment protected by FBB mRNA; (M) pSP6fos-derived probe fragment protected by mouse *c-fos* mRNA; (A) pSP6 α 133-derived probe fragment protected by α -globin mRNA. Times correspond to minutes after serum stimulation. Beside each autoradiogram is a semilogarithmic plot of the decay of BFB or FBB mRNA (solid line) and mouse *c-fos* mRNA (*c-fos*^M, dashed/dotted line).

determinant of *c-fos* mRNA instability within the protein-coding region of the message (Fig. 1). Replacement of the coding region of β -globin mRNA with the corresponding *c-fos* segment results in a dramatically reduced half-life of just 17 min. The presence of a destabilizing element in the *c-fos* protein-coding segment explains why deletion of the ARE or of the entire 3'-UTR has little effect on the decay rate of *c-fos* mRNA. Indeed, our data suggest that degradation mediated by this coding

region determinant is the primary pathway for *c-fos* mRNA decay. Recently, Kabnick and Housman (1988) have reported corroborating evidence that structural elements outside of the 3'-UTR contribute to *c-fos* mRNA instability.

Our data indicate that the ARE and the determinant(s) of instability in the *c-fos* coding region are each the target of a different cellular pathway for mRNA degradation. The *c-fos* coding region contains neither a long

Figure 7. (See facing page for legend).

AU-rich segment nor the pentanucleotide AUUUA found in all known AREs. Moreover, treatment of serum-stimulated cells with either of two transcription inhibitors (DRB or actinomycin D) gradually blocks degradation mediated by the *c-fos* ARE but has little effect on the decay pathway for which the *c-fos* protein-coding region is a target.

Previous studies have shown that the *c-fos* transcript is stabilized by treatment of cells with translation inhibitors (Greenberg et al. 1986a), which could act by blocking synthesis of a labile protein necessary for *c-fos* mRNA degradation and/or by disrupting the normal polysomal structure of the *c-fos* message. Because DRB and actinomycin D inhibit transcription by entirely different mechanisms and because DRB is not thought to bind RNA, our findings indicate that the ARE-dependent mRNA degradation pathway requires synthesis of a labile gene product whose activity is lost within 3 hr after transcription is blocked. This unstable gene product apparently is not required for the degradation pathway that targets the *c-fos* coding-region determinant. If this gene product is a protein, it might help to explain the stabilizing effect of translation inhibitors on *c-fos* mRNA; it would also imply that the message encoding this protein is itself highly labile in serum-stimulated cells, as the effect on protein synthesis of inhibiting synthesis of a stable transcript would not be apparent for many hours.

Our finding that deletion of the *c-fos* ARE hardly affects the half-life of the human *c-fos* message differs from previous studies of mouse *c-fos* mRNA (Fort et al. 1987; Rahmsdorf et al. 1987). In those experiments, the decay rate of the mouse *c-fos* transcript slowed significantly upon removal of large 3'-UTR segments containing the ARE. The difference between our results and those of others might be explained by the way the experiments were carried out. Previous studies of mouse *c-fos* mRNA decay used actinomycin D in actively growing cells to block constitutive transcription of *c-fos* from a heterologous promoter. In contrast, to ensure physiological conditions relevant to normal *c-fos* gene expression, our studies have been performed in growth-factor-stimulated cells. Moreover, we have monitored mRNA decay in the absence of transcription inhibitors to avoid the perturbation of mRNA half-life that can occur in cells treated with such inhibitors (see above; Mullner and Kuhn 1988). Finally, in creating hybrid *c-fos*/β-globin transcripts, we have been careful to minimize translational perturbations that could affect mRNA decay. For example, to avoid disrupting translation initiation signals (Kozak 1987), we have fused the *c-fos* and β-globin transcripts at sites downstream of the second codon. Furthermore, the chimeric transcripts examined

in the present study have been designed to provide ribosomal coverage for all mRNA segments that normally are translated and to prevent translation of normally untranslated RNA segments.

The method described here for studying *c-fos* mRNA decay has additional features that deserve emphasis. The observation that the lifetime of *c-fos* mRNA is unnaturally prolonged in cells transfected with an excessive amount of *c-fos* DNA is crucial. It has led us to identify transient-transfection conditions under which wild-type *c-fos* mRNA produced from the transfected gene decays at the same rapid rate as the endogenous *c-fos* message. Furthermore, because fusion of the *c-fos* promoter to other transcription units allows transient synthesis of heterologous mRNA in response to serum addition (see below), it has been feasible to examine the decay of hybrid β-globin/*c-fos* messages under conditions identical to those employed for monitoring decay of the *c-fos* transcript itself. The strategy of swapping segments between the labile *c-fos* transcript and the stable β-globin message has permitted the identification of destabilizing determinants within *c-fos* mRNA that function in growth-factor-stimulated cells. This same approach should facilitate precise mapping of the sequence elements within the ARE and coding region that are essential for rapid degradation of the *c-fos* message. It should also allow identification of the determinants that render other growth-factor-inducible mRNAs unstable.

Studying *c-fos* mRNA decay in this manner requires that transcription end within minutes after growth-factor induction. Degradation of the *c-fos* message can then be monitored in the absence of further mRNA synthesis. Our experiments indicate that, as for the endogenous *c-fos* gene, heterologous gene transcription driven by the *c-fos* promoter is turned off shortly after growth-factor stimulation. This is indicated by nuclear run-on analysis of transcription of the BBB and BFB genes (V. Rivera and M.E. Greenberg, unpubl.) and by the finding that cytoplasmic accumulation of the BBB, BFB, BBF, and BBB + ARE transcripts ceases within 60 min after serum induction. Because the only *c-fos* element shared by all of these chimeras is the *c-fos* promoter, the *c-fos* gene segment located between 14 and 711 bp upstream of the transcription initiation site must contain all of the *cis*-acting DNA sequence information necessary for stimulation and repression of transcription in response to growth factors. We conclude that in cells transfected with the BBB gene, the high level of β-globin mRNA present as late as 24 hr after growth-factor stimulation reflects the very long lifetime of β-globin mRNA and not a loss of transcriptional repression.

The existence of two pathways for rapid degradation

Figure 7. Decay of BBF and BFB mRNA in cells treated with transcription inhibitors. NIH-3T3 cells were transiently transfected with pSVα1 and either pBBF or pBFB. After serum stimulation for 30 min, actinomycin D (*top*; 10 μg/ml) or DRB (*bottom*; 30 μg/ml) was added, and total cytoplasmic RNA was isolated at time intervals and analyzed by RNase protection and gel electrophoresis. (BBF) pT7BG-derived probe fragment protected by BBF mRNA; (BFB) pSP6BFB-derived probe fragment protected by BFB mRNA; (M) pSP6fos-derived probe fragment protected by mouse *c-fos* mRNA; (A) pSP6α133-derived probe fragment protected by α-globin mRNA. Times correspond to minutes after serum stimulation. Beside each pair of autoradiograms is a semilogarithmic plot of the decay of BBF mRNA (dashed/dotted line) and BFB mRNA (solid line) in the presence of actinomycin D (*top*) or DRB (*bottom*).

of *c-fos* mRNA could serve important biological functions. For example, certain extracellular signals might activate or inhibit one decay pathway but not the other. If so, these signals could cause dramatic half-life changes for transcripts bearing a single instability determinant, without significantly affecting the half-lives of messages able to decay by more than one pathway. This would allow a greater degree of flexibility in a cell's response to its environment or state of growth. Interestingly, precise deletion of either the ARE or a sequence near the 3' end of the coding region appears to contribute to the oncogenic potential of the *c-fos* gene when it is expressed from a strong constitutive promoter (Meijlink et al. 1985). Because overexpression of the *c-fos* proto-oncogene can lead to oncogenesis, multiple degradation pathways may have evolved to ensure rapid turnover of *c-fos* mRNA in a variety of cell states.

Materials and methods

Plasmid constructions

DNA linkers were purchased from New England Biolabs. Oligonucleotides used for site-directed mutagenesis were synthesized using an Applied Biosystems 381A instrument and purified by gel electrophoresis.

The α -globin reference plasmid pSV α 1 was constructed by subcloning a 1.80-kb *Bgl*II (filled in)–*Xba*I fragment from plasmid π SVHP α 2 (Charnay et al. 1984), comprising the human α 1-globin gene driven by the SV40 early promoter and enhancer, between the *Pst*I (filled in) and *Xba*I sites of plasmid pT7/T3 α -18 (BRL).

Plasmid pF711 (Treisman 1985) contains a complete genomic copy of the human *c-fos* gene, beginning 0.71 kb upstream of the transcription initiation site and ending 1.60 kb beyond the polyadenylation site.

Plasmid pF Δ 1, a pF711 derivative, was constructed in several steps as follows. A 27-bp *Bam*HI–*Hind*III fragment was deleted from the polylinker region of pF711 to generate plasmid pF99. An *Nco*I site and a *Bam*HI site were then introduced into pF99 at the promoter–proximal end of the *c-fos* ARE by oligonucleotide-directed mutagenesis (Nakamaye and Eckstein 1986) of a 4-bp segment located 623 bp downstream of the *c-fos* stop codon (CCATGAAAAC $\rightarrow$ CCATGGATCC). Simultaneous introduction of a point mutation 704 bp downstream of the *c-fos* stop codon (CTTGAG $\rightarrow$ CTCGAG) created an *Xho*I site at the promoter–distal end of the *c-fos* ARE and generated pF100. The 76-bp *Bam*HI–*Xho*I fragment of pF100 was then excised, and the ends were filled in and religated to generate plasmid pF Δ 1.

Plasmid pF101 was created by linearizing pF100 with *Xho*I, removing the protruding 5' ends with mung bean nuclease, and inserting a *Bgl*II linker (GGAGATCTCC) by the linker-tailing method (Lathe et al. 1984).

A nested set of deletions in the 3'-UTR of the human *c-fos* gene was constructed as follows. Bidirectional deletions were created from the *Bgl*II site of pF101 by exonuclease III/exonuclease VII (BRL) digestion followed by insertion of a *Bgl*II linker (GGAGATCTCC). The complete *c-fos* gene segment downstream of the ARE was then reintroduced into the resulting plasmids by replacing the *Bgl*II–*Eco*RI fragment containing the plasmid replication origin with the intact 3.34-kb *Bgl*II–*Eco*RI fragment of pF101, to generate plasmids pF Δ 2–pF Δ 6. End points were determined by analytical restriction enzyme digestions and agarose gel electrophoresis.

To generate plasmid pF Δ 7, a 0.46-kb *Hind*III fragment

flanking the *c-fos* gene was first excised from plasmid pF4, and the ends were filled in and religated. A *Hind*III linker (CCCAAGCTTGGG) was then inserted between the *Bcl*I site and proximal *Tth*111I site of the resulting plasmid by the linker-tailing methods.

Plasmids carrying hybrid genes with segments of the rabbit β -globin gene and the human *c-fos* gene were constructed in several steps as follows.

A 2.05-kb *Bgl*II fragment containing a complete copy of the genomic rabbit β -globin transcription unit was excised from plasmid pUK4.7 by partial *Bgl*II digestion and substituted for the 1.52-kb *Bam*HI fragment of pSV α 1 to generate plasmid pSVB10. In pSVB10, the orientation of the β -globin gene was the same as that of the α -globin gene that it replaced.

Plasmid pFFB was created by replacing the 1.28-kb *Bgl*II–*Hind*III (filled in) fragment of pF Δ 6 with a 0.44-kb *Bgl*II–*Eco*RI (filled in) fragment of pSVB10 that contained the 3'-UTR of the rabbit α -globin gene.

To generate plasmid pBBB, a 2.66-kb *Nar*I (filled in)–*Bgl*II fragment of pFFB, extending from 13 bp upstream of the *c-fos* transcription initiation site to the β -globin/*c-fos* junction, was replaced with a 1.21-kb *Pvu*II–*Bgl*II fragment of pSVB10, extending from 9 bp upstream of the rabbit β -globin transcription initiation site to the end of the β -globin coding region.

Plasmid pBBB3 was generated by substituting a portion of the pUC18 polylinker region (*Kpn*I–*Hind*III) for the 0.46-kb *Kpn*I–*Hind*III fragment downstream of the polyadenylation site of pBBB.

Plasmid pBBB4 was generated by oligonucleotide-directed mutagenesis of pBBB3, into which an *Mlu*I site was introduced by changing the sequence of the third and fourth β -globin codons from CATCTG to CACGCG.

Plasmid pF106 was constructed in several steps as follows. A 0.46-kb *Hind*III fragment was deleted from pF711, and a *Bgl*II linker (GGAGATCTCC) was inserted into the *Nae*I site near the beginning of the *c-fos* 3'-UTR to generate plasmid pF104. A *Hinc*II site downstream of the *c-fos* polyadenylation site in pF104 was destroyed by insertion of a *Hind*III linker (CCCAAGCTTGGG), and an *Mlu*I site was created by oligonucleotide-directed mutagenesis of the third, fourth, and fifth *c-fos* codons (TTCTCGGGC $\rightarrow$ TACGCGTGC), to generate plasmid pF106.

To construct plasmid pBBF, a 1.91-kb *Bgl*II–*Hind*III (filled in) fragment of pF104 was substituted for the 0.44-kb *Bgl*II–*Kpn*I (filled in) fragment of pBBB. To construct plasmid pBFB, a 2.46-kb *Mlu*I–*Bgl*II fragment of pF106 was substituted for the 1.14-kb *Mlu*I–*Bgl*II fragment of pBBB4. To construct plasmid pBBB, a 263-bp *Bss*HII–*Mlu*I fragment of pF106 was substituted for the 161-bp *Bss*HII–*Mlu*I fragment of pBBB4.

Plasmid pBBF Δ ARE is a deletion derivative of pBBF and was constructed from pBBB3 by replacing the 0.45-kb *Bgl*II (filled in)–*Hind*III fragment with the 2.34-kb *Nae*I–*Hind*III fragment of pF Δ 1.

The 83-bp *Bam*HI–*Bgl*II fragment of pF101 encoding the *c-fos* ARE was subcloned into *Bgl*II-linearized pBBB to generate plasmid pBBB + ARE. The transcription orientation of the insert was the same as that in plasmid pF101.

Plasmids for generation of complementary-strand RNA probes were prepared as follows. Plasmid pT7BG was constructed by deleting a 1.17-kb *Eco*RI–*Bam*HI fragment from pSVB10. Plasmid pSP6BFB was constructed by inserting a 266-bp *Nar*I–*Hinc*II fragment of pBFB between the *Acc*I and *Hind*III (filled in) sites of pGEM3 (Promega).

Cell culture and transfection

Mouse NIH-3T3 cells were passaged in Dulbecco's modified

Eagle's medium (DMEM) with 10% calf serum. Cells were split to a density of 2×10^6 /100-mm dish 18 hr before transfection by the calcium phosphate technique (Treisman 1985). Transfection mixtures contained 2–3 μ g of the test plasmid, 1 μ g of the internal control α -globin plasmid (pSV α 1), and 17 μ g of carrier plasmid (pT7/T3 α -19; BRL). After exposure to the plasmid precipitate for 12–16 hr, cells were serum-starved in DMEM/0.5% calf serum for 26–30 hr and then stimulated with DMEM/15% fetal calf serum as described previously (Greenberg et al. 1987).

Analysis of mRNA decay

Total cytoplasmic RNA was isolated at various times after serum stimulation, as described previously (Greenberg and Ziff 1984; Greenberg et al. 1986b). Transcripts derived from transfected DNA were detected by RNase protection analysis (Treisman 1985) of equal amounts of total cytoplasmic RNA, using complementary RNA probes. Human and mouse *c-fos* mRNA and certain derivatives thereof were detected with a 588-nucleotide RNA probe derived from *Eco*RI-linearized template plasmid pSP6fos (Treisman 1985). This probe is complementary to the 5'-terminal segment of the human transcript and yields a protected fragment of 296 nucleotides or 65 nucleotides for human or mouse *c-fos* mRNA, respectively. Human α 1-globin mRNA was detected with a 270-nucleotide RNA probe that was derived from *Bam*HI-linearized plasmid pSP6 α 133 (Charnay et al. 1984) and that hybridized to a 133-nucleotide α -globin mRNA segment within the first exon. Rabbit α -globin mRNA and certain derivatives thereof were detected with a 200-nucleotide RNA probe that was derived from *Nco*I-linearized pT7BG and that hybridized to a 188-nucleotide rabbit α -globin mRNA segment within the second exon. BFB mRNA was detected with a 280-nucleotide RNA probe that was derived from *Bam*HI-linearized pSP6BFB and that hybridized to a 180-nucleotide BFB mRNA segment within the first exon.

To measure mRNA decay rates, data from autoradiograms were quantitated by densitometry on an LKB Ultrascan XL instrument. For each sample, the concentration of mRNA derived from the transfected *c-fos* gene or derivative thereof was normalized to the α -globin mRNA internal standard. The concentration of endogenous mouse *c-fos* mRNA was normalized to the concentration of total cytoplasmic RNA. mRNA half-lives were then determined by least-squares analysis of semilogarithmic plots of normalized mRNA concentration as a function of time. Half-life errors were estimated from the standard deviation of the slope of each plot. Only data from samples isolated ≥ 60 min after serum stimulation were used to ensure that mRNA synthesis directed by the *c-fos* promoter had ceased.

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The c-fos transcript is targeted for rapid decay by two distinct mRNA degradation pathways.

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