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ABSTRACT: Rapid and transient activation of both c-fos transcription and mRNA accumulation occurs when resting CCL39 hamster fibroblasts are serum-stimulated to grow. By using several combinations of serum and cycloheximide, a protein synthesis inhibitor, we showed that: i) addition of cycloheximide to resting cell elicits an increase in c-fos gene transcription located within the first 540 bases of the unit, suggesting that an "attenuation-like" mechanism, similar to that observed for c-myc, might be essential for c-fos transcriptional regulation; ii) it also prevents both transcriptional shutoff and mRNA degradation in serum-stimulated cells; iii) upon removal of cycloheximide, mRNA region leads to a stabilization of c-fos mRNA lending experimental support to a putative destabilizer element within this sequence.

INTRODUCTION

Many reports have implicated the expression of various proto-oncogenes in the regulation of normal cell cycle (1-4) and differentiation (5-8). Two of these oncogenes, c-fos and c-myc, have been shown to be expressed rapidly and transiently after resting fibroblasts are stimulated to grow (3,9-13). These genes belong to a family of induced genes, which also includes the PDGF-induced genes (14-16) and a set of genes described by Lau and Nathans (17). All these genes share two main properties: protein synthesis is not required for the accumulation of their mRNA and they are transiently expressed during the first hours of the cell cycle. Efficient induction of either cellular transformation by a transfected c-fos gene (18) or bone abnormalities during the development of transgenic mice (19), requires both the addition of a viral long terminal repeat (LTR) and alterations in the 3' end mRNA sequences. Further investigations (20,21) showed that a 5' enhancer element and sequences in the 3' part of the gene are required for serum-dependent expression and mRNA degradation, respectively.

CCL39 is a non-tumoral line of chinese hamster lung fibroblasts which can be reversibly brought to quiescence in G_0 phase by serum deprivation and subsenquently stimulated to re-enter G_1 phase in response to growth factors (22). This cell line, which represents a hamster counterpart to the murine NIH 3T3 and Balb/c 3T3 cells, provided a model for the study of early biochemical responses (23) and c-myc gene expression (24,13) after stimulation with -thrombin and insulin growth factors.

Here we investigated the expression of c-fos gene in CCL39 cells serum stimulated to grow. Our results demonstrate that inhibition of de novo protein synthesis in resting cells elicits a localized accumulation of RNA-polymerase molecules within the first 540 bases of c-fos transcription unit. In serum-stimulated cells, this same region appears to sustain a much more intense transcriptional activity than the rest of the gene. Upon inhibition of protein synthesis, transcriptional shutoff is prevented and the density of polymerases becomes evenly distributed throughout the gene. We also show that mRNA degradation requires protein synthesis and a short sequence within the 3 non-coding region. A precedent to this situation has already been established in the case of c-myc gene (25-28).

MATERIALS AND METHODS

Cell culture

CCL39 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Confluent cells were arrested in G_0/G_1 by serum deprivation in DMEM. Subsequent growth stimulation was obtained by addition of 10% FCS. Protein synthesis was inhibited (>95%) following cycloheximide (CH) treatment (10µg.ml⁻¹). RNA transcription was blocked by treating cells with 5 µg.ml⁻¹ actinomycin D. CCL39 and Ltk⁻ cells were transfected by the calcium phosphate method of Wigler *et al* (29). Individual clones (CCL39) or population (Ltk⁻) were selected in the presence of 1mg.ml⁻¹ geneticin (Gibco).

Plasmid constructs

p19/1 and p76/21 c-fos constructs were described by Ruther et al (30). p19 Δ (N-M) was obtained by deleting the 130 bp long NsiI-MstII fragment out of the c-fos 3' region of p19/1. The construct was eventually checked by DNA sequencing.

RNA extraction and analysis

Total RNA was extracted using the guanidinium isothiocyanate /LiCl procedure of Cathala *et al* (31). Poly(A)⁺ RNA was purified on oligo(dT) cellulose (Pharmacia). Total RNA(20 μ g), poly(A)⁺ RNA (corresponding to 100 μ g total RNA) were fractionated on 2M formaldehyde-containing agarose gels, transferred and bound to nylon membranes (Hybond, Amersham) as described by the supplier. Filter were hybridized using ³²P-labelled nick translated probes (32).

Nuclear run-on transcription assay

Preparation of nuclei and elongation of nascent transcripts were done as described by Greenberg and Ziff (11). Dot-spotting of DNA onto nitrocellulose as well as hybridization and washing conditions were as previously described (33). Before autoradiography, filters were RNAseA-treated in 2XSSC for 15 min. at 37°C.

RESULTS

Expression of c-fos RNA in CCL39 hamster fibroblasts

The addition of 10% FCS to G_0/G_1 arrested cells elicits a very sharp wave of accumulation of c-*fos* mRNA, identical to those obtained during growth stimulation of mouse NIH and Balb/c 3T3 cells (11,3,17,34), NGF-induced differentiation of rat PC12 cells (34,35) and TPA stimulation of human cells (36). Interestingly, the apparent size of the mRNA decreased as stimulation proceeds (Figure 1). This feature was also reported by other groups, but was not further investigated (20,36). This reduction is limited to the poly(A)⁺ fraction, as poly(A)⁻ RNAs exhibit the same size across the peak of expression. Moreover, this variation in size was abolished when these poly(A)⁺ RNAs were treated with oligo(dT) and RNAse H (data not shown). It is also of interest to note that the relative amount of poly(A)⁻ c-*fos* mRNA increases with time while the invariant GAPDH mRNA (37) remains fully polyadenylated. These results strongly suggest that the c-*fos* RNA shortening is at the expense of the poly(A) tail. A similar situation has been previously described for metallothionein (MT) mRNA (38) whose both decay and poly(A) shortening occur within 5 hours after glucocorticoid or heavy metal induction.

Effect of protein synthesis inhibition on c-fos mRNA accumulation

Alike other serum-stimulated genes, c-fos mRNA was previously shown to superaccumulate in the presence of both cycloheximide (CH) and serum (FCS) (14,3). However,



Figure 1: Accumulation of $poly(A)^+$ and $poly(A)^-$ c-fos RNA after FCS stimulation. Resting CCL39 cells were stimulated by 10% FCS. RNA was extracted at indicated times after FCS and/or CH addition. Poly(A)⁺ RNA (corresponding to 100 µg total RNA) and poly(A)⁻ RNA (20 µg) were fractionated on a 1,5% agarose gel, transferred to nylon membranes and hybridized to c-*fos* (4.5 kb BamH1 fragment from p19/1) and GAPDH DNA probes, as indicated in Materials and Methods.



Figure 2: Effect of CH and FCS on c-fos mRNA accumulation.

Poly(A)⁺ RNA corresponding to 100 μ g total RNA was analyzed as described in Material and Methods.

A: Resting cells (lane 1) were treated with both CH and FCS for 1 hour (lane 2), 2 hours (lane 5) or 3 hours (lane 7). At each time, cell samples were washed twice with DMEM-10%FCS to remove the drug and further incubated for 1 hour (lanes 3 and 6) or 2 hours (lane 4).

B: Resting cells were incubated with CH for 3 hours (lane 1), washed twice and stimulated with 10% FCS without CH for 30 min. (lane 2), 1 hour (lane 3) and 2 hours (lane 4).

this feature was never thoroughly investigated. We therefore analyzed the c-fos mRNA levels using various combinations of FCS and CH, which led to four observations: Firstly, CH addition to resting cells does not lead to any c-fos mRNA accumulation (Figure 2B), which is at variance with what was previously reported for murine cell lines (17,20). Secondly, the c-fos response of resting cells to FCS is not affected by a 3 hours (Figure 2B) or a 6 hours (not shown) CH-pretreatment indicating that no proteic factor necessary for c-fos expression has decayed during this period. Thirdly, the steady-state level of c-fos mRNA is maintained for at least 3 hours after CH and FCS addition (Figure 2A). Interestingly, CH treatment appears to slow down poly(A) shortening (Figure 1). Finally, removal of the drug one or two hours after FCS and CH addition leads to a rapid disappearance of c-fos mRNA, which indicates that the c-fos mRNA degradation is not restricted to a limited period, but remains efficient for at least 3 hours after FCS stimulation.

Effect of protein synthesis inhibition on c-fos transcription

We previously showed that stimulation of CCL39 cells by α -thrombin and insulin rapidly led to a very sharp and transient raise in c-*fos* transcription (13). This transcriptional induction was similar to that obtained in mouse and rat cell lines (11,34,35) or to that



Figure 3: Run-on c-fos transcription analysis of CCL39 cells.

A: Resting cell were stimulated by 10% FCS in the absence (-CH) or presence (+CH) of $10 \mu g.ml^{-1}$ cycloheximide (added 15 minutes before FCS). Run-on assays were performed on nuclei isolated at indicated times (in mn.) after FCS addition and ³²P-labelled in *vitro* RNA were hybridized to $5 \mu g$ of p19/1, GAPDH and pBR322 DNA immobilized on nitrocellulose (33). Autoradiographs (left) were quantitated by densitometric scanning and values were plotted (right).

B: Resting cells were incubated with (+CH) or without (-CH) cycloheximide for 3 hours and then FCS stimulated for indicated times. Subsequent ³²P-labelled RNA were hybridized to $5 \mu g$ of M13 single-stranded DNA from murine c-*fos* subclones (A:NaeI-XhoI (537bases, 121 U), B:XhoI-BgIII (479 bases, 105 U), C:XbaI-SphI (415 bases, 102 U) and D:SalI-BamHI (>749 bases, >260 U)) in the sense (+) or anti-sense (-) orientation. Mouse c-*fos* gene is schematically represented on the right (N:NaeI, X:XhoI, Bg:BgIII, Xb:XbaI, Sp:SphI, S:SalI and B:BamHI). Solid boxes indicate exons.

obtained here after FCS stimulation (Figure 3A). When CH was added 30 min. before FCS, the transcriptional raise was no more transient and persisted for at least two hours, a feature that was also reported in other cell systems (34). In order to further investigate the action of CH, we assayed for transcriptional activity along the gene by using subcloned probes (Figure 3B). These experiments show that CH addition to resting cell elicits an increase in c-*fos* gene transcription which is confined to the 540 bp long NaeI-XhoI fragment from p19/1. This feature could result either from a dramatically reduced elongation rate (less than 3 bases.mn⁻¹) or from an attenuation-like mechanism leading to abortive chains or to a pausing of RNA-polymerase. This latter possibility is supported by the fact that during a typical FCS-stimulation the internal part of the gene does not sustain as intense a transcriptional activity as the first segment (Figure 3B). Moreover, addition of both CH and FCS results in constant transcriptional activity across the entire gene. These observations show that the relief of both promoter repression and block to elongation are primary responses to FCS in



Figure 4: Effect of 3' end changes on c-fos mRNA stability.

A: Structure of the constructs used in this analysis. Hatched boxes indicate c-fos exons, the stippled box indicates the metallothionein promoter (pMT), and the solid box indicates v-fos sequences. pA: polyadenylation signal, N:NsiI, M:MstII.

B: c-fos RNA Northern blotting analysis from exponentially growing Ltk⁻ transfected cells, treated for different times by various combinations of CH and actinomycin D (actD) as indicated. 20 µg total RNA were analysed. Stars indicate the position of abnormal c-fos RNAs. Invariant signals were obtained when filters were re-hybridized to GAPDH probe (not shown).

C: CCL39 cells were transfected with p76/21. Resting cells from one individual geneticinresistant clone were FCS stimulated (+FCS). Poly(A)⁺ RNA corresponding to 100 μ g total RNA was extracted from cells stimulated for the indicated times (in min.). Estimation of endogenous and p76/21 mRNA turnover was performed by adding 5 μ g.ml⁻¹ act D after cells have been FCS stimulated for 40 minutes (40 FCS, actD). RNA was then prepared from cells isolated at various times after the addition of act D.

D: c-fos poly(A)⁺ (corresponding to 100 µg) and poly(A)⁻ (20 µg) RNA analysis from an exponentially growing CCL39 clone transfected with p19 Δ (N-M). Cells were treated with various combinations of CH and actD, as indicated. Stars indicate the position of abnormal c-fos RNAs.

hamster cells. Conversely, protein synthesis is required both for promoter repression and for restoring the attenuation.

In addition, we show that only the sense strand of the c-*fos* gene is transcribed. The stronger signal observed with probe D (Figure 3B) merely results from the higher specific radioactivity (more than twice as rich in U as probes A,B and C - see legend to Figure 3B).

A short sequence within the 3' non-coding region is essential for c-fos mRNA degradation

The rapid disappearance of c-fos mRNA following FCS stimulation (3,17) or NGF induction (35) made obvious the fact that this mRNA is highly unstable. Various reports gave

estimates ranging from 9 to 15 minutes (20,39) for c-*fos* RNA half-life in human or murine cell lines and showed its stabilization by CH. Our own experiments on hamster cells indicate that c-*fos* mRNA half-life approximates 10 minutes and increases to 2 hours upon CH addition (data not shown). In order to determine the region(s) involved in c-*fos* mRNA unstability, we transfected hamster CCL39 and murine Ltk⁻ cell lines by various constructs (Figure 4A).

First of all, we verified that the normal mouse c-*fos* gene under the control of the metallothionein promoter (pMT) yielded unstable RNAs either in CCL39 hamster cells (not shown) or Ltk⁻ mouse cells (Figure 4B). Such a construct (p19/1) gave rise to RNAs slightly less unstable than the endogenous c-*fos* RNAs $t^{1/2}$ = 20-25mn) which might result from the modifications introduced at the 5' end by the cloning procedure or from a residual effect of the protein synthesis inhibitor geneticin used for the selection of recombinant clones.

We then analyzed CCL39 cells transfected with a construct (p76/21) in which the MT promoter is enhanced by a v-fos LTR and the 3' non coding region of the c-fos has been replaced by that of v-fos. The transfected cells were found to display some transformed characteristics such as growth in soft agar and altered morphology but nevertheless could be arrested in G₀ by serum deprivation. Upon FCS stimulation, the endogenous c-fos mRNA was normally induced, while the exogenous mRNA was expressed constitutively and to a constant level during the entire stimulation period (Figure 4C). As expected, actinomycin D chase allowed to determine half-lives of approximately 3 hours and 10-15 minutes for p76/21 and endogenous c-fos mRNA respectively.

To rule out a possible stabilizing effect of v-fos 3 untranslated sequences rather than a loss of destabilizing sequences in the c-fos, we designed a new construct, p19 Δ (N-M) directly derived from p19/1 from which only a 130 bp NsiI-MstII fragment was deleted. This region was chosen because it encompasses the 67 bp region defined by Meijlink *et al* (40) and contains sequence motives similar to those found in the human GM-CSF destabilizer (41). After transfection, the whole population of geneticin-resistant Ltk⁻ mouse cells was used while an individual clone of CCL39 was selected. In both cellular contexts, c-fos RNAs are much more stable (half-life approximately 2 hours) when the 130 nt region is deleted (Figure 4B and 4D).

Further stabilization of these deleted RNAs cannot account for the superaccumulation still observed in the presence of CH but likely results from transcriptional activaction of the MT promoter.

Two additional RNA bands are detected indifferently in p19/1 and p19 Δ (N-M) transfected cells (Figure 4B and 4D). The larger (approximately 25S) behaves identically to the expected normal or deleted RNAs and might represent an aberrant unspliced transcript. The shorter (approximately 18S) is very unstable whatever the construct transfected. The structure of these atypical of these atypical RNAs is currently being investigated. However,

even though the 130 bp deletion elicits no change in the stability of the shorter RNAs, it definitely leads to a increased half-life of the larger ones. Since this work has been first submitted, Rahmsdorf *et al* (42) obtained the same results using analogous constructs on human primary fibroblasts.

DISCUSSION

-Initiation of transcription

It is well established that serum-dependent c-fos gene expression is governed by a 5'"enhancer-like" element distinct from the promoter (20). Recent reports (43-45) revealed the presence of a nuclear factor binding specifically to this enhancer. This factor is present in resting as well as in exponentially growing cells. Our results show that protein synthesis is required for preventing transcriptional initiation but not for promoting it, which would suggest that a proteic factor acting as a repressor throughout the cell cycle is transiently inactivated during growth stimulation by a mechanism independent on *de novo* protein synthesis. This repression could well be mediated by the factor that binds to the c-fos enhancer. While this work was in progress, Lau and Nathans reported on the behaviour of several growth related genes, including c-fos and c-myc (46). Although they did not investigate the effect of CH on resting cells, their experiments clearly demonstrate that these genes are transcriptionally activated by growth factors even in the absence of protein synthesis which is however required for their subsequent transcriptional shutoff. This turns out to be a general pattern of regulation that may involve the same proteic factors.

-Transcriptional elongation

We show here that transcriptional elongation is efficiently blocked withing the first 540 bp of c-fos gene. Such an attenuation-like mechanism has also been reported for murine and human c-myc genes (26-28). According to our results, this feature does not depend on ongoing protein synthesis, as it was still observed in resting cells CH-treated for 3 hours. This blockade is partially relieved when FCS is added and nearly fully so when FCS is added in conjunction with CH. One hypothesis is that a stable proteic factor which prevents RNA elongation must be rapidly degraded upon growth stimulation in order to produce full length transcripts. The transcriptional shutoff would then be mediated by newly synthesized factors acting both at the level of initiation (see above) and at the level of the elongation. The precise mechanism by which such a proteic factor could prevent RNA elongation remains unknown. As far as the c-myc gene is concerned, this attenuation mechanism is thought to result from RNA secondary structure, as it is abolished when cells are pretreated with proflavin or purified nuclei with RNAse before RNA elongation (27,28). Whether this mechanism is peculiar to a set of growth-related genes or represents a more general feature of eukaryotic gene regulation remains an interesting but open speculation.

-mRNA degradation and protein synthesis

Most growth-induced genes so far studied transiently accumulate their mRNA during the first hours of the cell cycle. The regulation of these genes has quite different requirements from those of developmentally regulated ones which are turned on for a long period of time. Regardless of whether or not the initial event is transcriptional, the rapid disappearance of the product of induced genes requires at least two post-transcriptional controls: the cessation of its production, which may result either from RNA unstability or translation repression, and its rapid degradation. Our results clearly demonstrate that de novo protein synthesis is required for c-fos RNA to be degraded. This degradation is equally efficient during the first 3 hours following growth stimulation. Several hypotheses can account for the stabilizing effect of CH: i) c-fos mRNA is no more unstable because it remains frozen in a protected state in polysomes. However, similar experiments using inhibitors of translation initiation instead of cycloheximide yielded the same results for c-fos and c-myc RNAs (34,47); ii) degradation depends on a labile proteic factor; iii) degradation can occur only when c-fos mRNA is being translated. This last hypothesis is in agreement with the very rapid resumption of c-fos RNA degradation after CH removal, an observation that has also been reported for murine Balb/c 3T3 cells and primary human fibroblasts (42).

-3' untranslated region and mRNA degradation

The first clue about the involvement of the 3' non-coding region in c-fos gene regulation was deduced from the ability of climaeric c-fos/3' non-coding v-fos constructs to transform murine cells (18). Furthermore, the regulatory element whose deletion was sufficient to render this gene highly tumorigenic could be narrowed down to a 67 bp 3' terminal c-fos sequence (48). We show here that this results from a change in mRNA turnover, as a 130 bp deletion encompassing these 67 bases leads to a stabilization of the resulting RNA. Shaw and Kamen (41) recently reported that 50 bases from human GM-CSF RNA 3' non-coding region are necessary and sufficient to destabilize a stable beta-globin mRNA. This destabilizer contains short AU-rich sequences which are also found in the 3' end of other unstable RNAs such as c-fos and c-myc (41,48). In the case of c-fos, these are localized within the 67 bases segment previously defined. Further work from our laboratory showed that addition of c-myc 3' non-coding region to a stable c-fos coding region produced an unstable RNA (Bonnieu *et al.*, submitted).

The occurence of such AU-rich sequences in most growth-related unstable RNAs suggests the presence of a unique system of degradation. However, these RNAs accumulate transiently at quite different times after serum stimulation. For instance, c-fos mRNA is already turning over at a time when c-myc RNA is only starting to accumulate. This difference in timing could result from transient production followed by degradation at a constant rate (c-fos) or modulation of the degradation itself (c-myc) (13) or a combination of

both in the case of c-myc and two PDGF-induced genes (14), KC and JE (34 and unpublished data). Another example has recently been described in the case of histone H3 whose control of mRNA degradation depends on its translation (49). Such a mechanism would explain the effect of protein synthesis inhibitors on mRNA accumulation, as well as the rapid resumption of degradation upon removal of the drug.

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