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RNA synthesis inhibition stabilises urokinase mRNA in macrophages

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Abstract Urokinase-type plasminogen activator (uPA) mRNA is induced in macrophages by the lineage specific growth factor CSF-1. Upon removal of CSF-1 from bone marrow-derived macrophages (BMM), uPA mRNA decayed with a half-life of 2 h. If RNA synthesis inhibitors actinomycin D, 5,6-dichloro-1-β-ribofuranosyl benzimidazole (DRB) or α-amanitin were added at the time as CSF-1 removal, the uPA message was stabilised. This was not a general effect on CSF-1 responsive mRNAs, as c-*myc* mRNA decayed with normal kinetics in the presence of inhibitors. The requirement for ongoing RNA synthesis for the degradation of uPA mRNA in BMM suggests that a component of the degradative pathway may be induced following removal of CSF-1.

Key words: mRNA stability; uPA; DRB; Actinomycin D; Urokinase; Macrophage

1. Introduction

Regulated mRNA decay is emerging as an important process in determining the levels of gene expression. A variety of mechanisms must be operating to result in both differential stability of a single mRNA species in different cell types, and regulated stabilities of messages within the one tissue. In the genes for c-*fos* [1,2] and c-*myc* [3] there are several determinants of rapid mRNA turnover, found both in coding and untranslated regions (UTRs). The most intensively studied instability elements are AU-rich sequences found in the 3'UTRs of genes such as GM-CSF [4], c-*fos* [1] and urokinase-type plasminogen activator (uPA) [5]. Recent analysis of the 3'UTR of uPA mRNA has shown that AU-rich sequences and at least two other regions independently confer instability to a heterologous mRNA [5]. One of these regions required ongoing RNA synthesis for its operation as an instability element.

uPA is a serine protease which cleaves plasminogen to yield plasmin, an extracellular protease of broad specificity [6]. Plasmin directly, or indirectly by activation of other proteases, catalyses the breakown of extracellular matrix allowing cell movement and changes in tissue architecture. The apparent involvement of uPA in growth and metastasis of tumours [7,8] has led to interest in the regulation of uPA expression. One study has suggested that increased metastatic potential of rat mammary adenocarcinoma lines could be a consequence of increased nuclear stability of uPA mRNA [9]. Production of uPA within tumours may also come from non-malignant stromal cells [10], amongst which macrophages are well represented [11,12].

uPA expression in macrophages may be important in migration to sites of inflammation, and is regulated by the macrophage colony stimulating factor (CSF-1), the major growth and differentiation factor for the mononuclear phagocyte lineage. In mouse bone marrow derived macrophages (BMM) uPA mRNA levels can increase up to 40-fold with CSF-1 stimulation ([13] and K. Stacey, unpublished). In this work we have studied the decline in uPA mRNA following CSF-1 removal, and shown that ongoing transcription is necessary for this decline.

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2. Materials and methods

Balb/c bone marrow derived macrophages (BMM) were prepared as described [14], and used after 7 days differentiation in culture with 10^4 U/ml recombinant human CSF-1 (Chiron Corp.).

Total RNA was extracted from cells [15] and run on MOPS/formaldehyde denaturing gels. RNA was transferred to Hybond N nylon membrane (Amersham) and hybridised with ^{32}P-labelled DNA probes. Probes used were: mouse uPA cDNA; mouse c-*myc* cDNA; 18 S rRNA, a 24 bp oligonucleotide complimentary to murine 18 S rRNA (5'-CAT GGT AGG CAC GGC GAC TAC CAT-3').

3. Results and discussion

The rate of decay of uPA mRNA after removal of CSF-1 from BMM was examined in Fig. 1. BMM were washed with PBS and re-plated in medium without CSF-1 and RNA was harvested at various times thereafter. From Fig. 1 and another 3 experiments (results not shown), uPA mRNA levels decreased after removal of CSF-1 with a half-life of approximately 2 h. Given that some new uPA mRNA may have been synthesised during this time, the real half-life of uPA mRNA in the absence of CSF-1 must be less than 2 h. In order to prevent new mRNA synthesis the cells were washed with PBS and re-plated in CSF-1-free medium with the RNA synthesis inhibitor DRB. Although it was expected that the rate of disappearance of mRNA should be greater in the presence of DRB, the inhibitor actually stabilised the message (Fig. 1), and no decay was detected in a 4 h incubation.

To determine whether the stabilisation of the uPA message was due to RNA synthesis inhibition or some other effect of DRB, two other RNA synthesis inhibitors were used. Actinomycin D (Fig. 2) and α-amanitin (result not shown) both prevented decay of the mRNA. These three inhibitors have quite different stuctures and activities. The antibiotic actinomycin D can inhibit nearly all RNA synthesis [16] by intercalation in DNA. Actinomycin D also inhibits mRNA binding to ribosomes and subsequent protein synthesis [17]. α-Amanitin is an octapeptide which inhibits RNA polymerase II and, to a lesser extent, RNA polymerase III [18]. DRB is an adenosine analogue which selectively affects RNA polymerase II-mediated transcription [19]. The fact that three RNA synthesis inhibitors all stabilised the uPA message suggests that the stabilisation is

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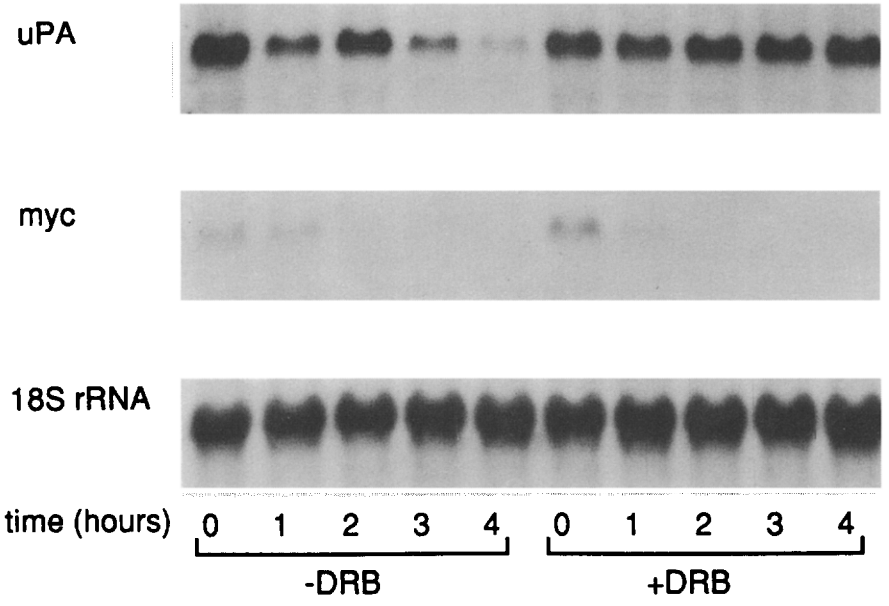

Fig. 1. Decay of uPA mRNA after removal of CSF-1, in the presence and absence of DRB. CSF-1 treated BMM were washed with PBS and re-plated in the absence of CSF-1, with or without 20 μg/ml DRB. Total RNA was collected at various times thereafter and analysed by Northern blot and hybridisation to uPA and c-*myc* cDNA probes, and an oligo specific for 18 S rRNA as a loading control.

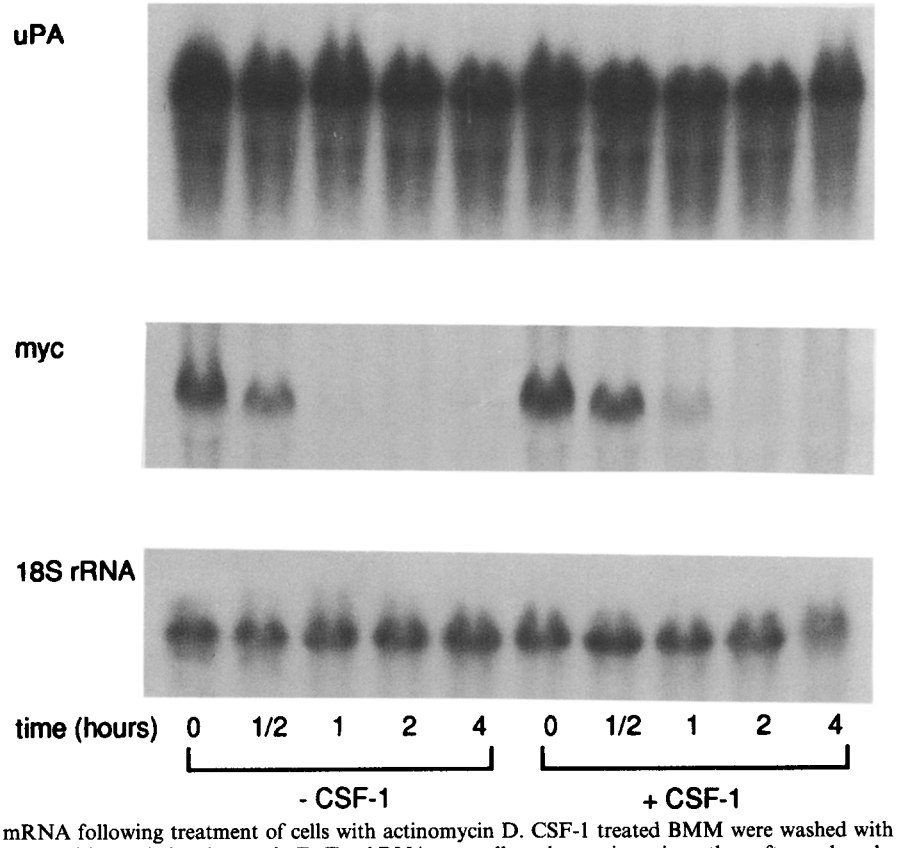

Fig. 2. Analysis of uPA mRNA following treatment of cells with actinomycin D. CSF-1 treated BMM were washed with PBS and replated in the presence or absence of CSF-1, with 5 μg/ml actinomycin D. Total RNA was collected at various times thereafter and analysed by Northern blot and hybridisation to uPA and c-*myc* cDNA probes, and an oligo specific for 1 8S rRNA as a loading control.

due to RNA synthesis inhibition itself, rather than some other property of the inhibitors. Blots were re-probed with c-*myc*, another CSF-1 inducible gene [20]. c-*myc* decayed after removal of CSF-1 with a half-life of approximately 30 min, and this was not significantly affected by DRB or actinomycin D (Figs. 1 and 2). The inhibitors are therefore not having a general effect on decay of CSF-1-inducible messages in macrophages. Clearly the rate of turnover of the message in the presence of CSF-1

could not be determined using RNA synthesis inhibitors. Determination of whether CSF-1 affects the stability of uPA mRNA would require pulse chase analysis [21].

One explanation for stabilisation of uPA mRNA by RNA synthesis inhibition is that transcription is required to produce a factor involved in uPA mRNA degradation. Such a factor could be either constitutive but unstable, or induced following CSF-1 removal. In the first possibility, uPA mRNA would always be unstable, and regulation would be via changes in the rate of transcription. After RNA synthesis inhibition, uPA mRNA would initially degrade, but gradually be stabilised as the labile degradative factor disappeared. Since uPA mRNA can be stabilised early after addition of inhibitors (Figs. 1 and 2) we favour a model where there is induction of a factor following removal of CSF-1. In either of these models, inhibitors of protein synthesis such as cycloheximide should also stabilise the uPA mRNA. Cycloheximide can prevent the decline in uPA following CSF-1 removal (result not shown), but this effect is not interpretable because cycloheximide can itself induce uPA transcription in macrophages [22].

Stabilisation of a number of other messages by RNA synthesis inhibition has been observed. Actinomycin D, α-amanitin and DRB all prevented normal decline in neurofilament mRNA levels in cultured dorsal root ganglia [23]. Actinomycin D or DRB prevented the decline in follicle stimulating hormone-β mRNA seen after inhibin or follistatin treatment of pituitary cells [24].

Significant stabilisation of uPA mRNA by RNA synthesis inhibition is not seen in all cell types. For example, a half-life of 70 min was observed in LLC-PK_1 pig kidney epithelial cells treated with DRB [21]. By expressing chimaeric β-globin uPA mRNA constructs in LLC-PK_1 cells, the uPA 3'UTR as a whole was shown to confer instability to the β-globin message in the presence of RNA synthesis inhibition [5]. When the 3' region was dissected, three regions causing instability were identified. One section apparently destabilised the β-globin message, but required ongoing RNA synthesis for the instability to be observed. Thus it may be that this region dominates in the regulation of uPA mRNA stability in macrophages, whilst its effect is masked by other instability regions in LLC-PK_1 cells. In both c-*fos*[1] and c-*myc* [3] mRNAs, instability elements were found which did not operate efficiently in the presence of RNA synthesis inhibitors. However, in the systems studied, other instability elements within the same messages were dominant, and stabilisation by DRB or actinomycin D was not detected with the full-length message.

Whilst useful information on mRNA half-life has been obtained using RNA synthesis inhibition methods, caution should be exercised in the interpretation of such experiments. In the case of macrophages, the uPA gene is inducible, so stabilisation of the message by DRB and actinomycin D was easily detected by comparison with the normal decay curve after removal of CSF-1. For cell types which have constitutive expression no such comparison can be made. Long half-lives of 30–46 h determined for uPA mRNA in tumour cell lines [25] really require confirmation with methods which do not use RNA synthesis inhibitors, such as pulse-chase analysis.

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