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Perinuclear localisation of cellular retinoic acid binding protein I mRNA

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Abstract

Retinoids are important metabolic and developmental regulators that act through nuclear receptors. The cellular retinoic acid binding protein *CRABPI* has been suggested to play a role in trafficking of retinoic acid but its exact functions and subcellular localisation remain unclear. Here we show that in CHO cells both exogenous *CRABPI* transcripts and tagged CRABPI protein have a perinuclear distribution that depends upon the 3′UTR of the mRNA. The *CRABPI* 3′UTR conferred perinuclear localisation on globin reporter transcripts. Deletion analysis indicated that the first 123nt of *CRABPI* 3′UTR are necessary for localisation of both CRABPI mRNA and protein. We propose that *CRABPI* mRNA is localised by a signal within its 3′UTR and that this partly determines the distribution of CRABPI protein.

Keywords: 3′UTR; 3′untranslated region; Messenger RNA; Trafficking; Fatty acid binding protein; Retinoic acid

Retinoic acid (RA), an active metabolite of vitamin A that is essential for normal development, exerts its effects through binding to ligand-activated transcription factors (RXR and RAR) [1]. In addition to RXR and RAR, mammalian cells contain other retinoid-binding proteins such as 15.5 kDa cellular retinoic acid binding proteins I and II (CRABPI and II) [2]. The functions, and possibly distinct roles, of CRABPI and II have not been fully defined. CRABPI has been shown to bind RA at high affinity [3] and has been reported to accelerate its metabolism [4,5]. CRABPI and II have been proposed to play a role in the metabolism and trafficking of RA within the cell, for example controlling the availability of RA for the nuclear receptors and in the shuttling of RA from cytoplasm to nucleus [6,7]. However, their subcellular distribution is a subject of debate; some immunocytochemical studies suggest that the proteins are cytoplasmic [6–9], one report proposes an association of CRABPI with mitochondria [9], whilst others indicate that they are both nuclear and cytoplasmic [10,11]. Recently, studies using a green fluorescent protein tag have suggested that CRABPII can be found in the nucleus and that this nuclear localisation of CRABPII is ligand-dependent [12].

mRNA localisation in different subcellular regions of the cytoplasm is thought to provide a mechanism for local synthesis of proteins close to where they function [13–15] and such mRNA targeting is dependent on the 3′untranslated region (3′UTR) of the mRNAs concerned [14–16]. Several mRNAs, including those encoding the nuclear transcription factors c-myc and c-fos, as well as metallothionein, which is normally cytoplasmic but which is nuclear at the G1/S transition in the cell cycle, are found associated with the cytoskeleton and localised in the perinuclear cytoplasm, and this targeting is due to signals with the 3′UTRs [17–20]. In the case of metallothionein, the 3′UTR is necessary for both mRNA localisation and subsequent nuclear localisation of the protein [20]. There is no information available regarding the subcellular localisation of *CRABPI* mRNA. The aim of this study was to use Chinese hamster ovary (CHO) cells transfected with gene constructs of tagged CRABPI with different 3′UTR sequences, immunocytochemistry, and in situ hybridisation to investigate the subcellular localisation of *CRABPI* mRNA and the

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influence of 3′UTR sequences on the distribution of both CRABPI mRNA and protein. The results indicate that the *CRABPI* mRNA contains a perinuclear localisation signal in the 3′UTR.

Materials and methods

Gene constructs. Constructs based on rabbit *β-globin* and mouse *CRABPI* sequences were made by PCR and cloned directly into pcDNA3.1/V5/his-TOPO; they are shown schematically in Fig. 1. pMLMCRABPfull and pMLMCRABPcds were obtained from a vector containing the full mouse *CRABPI* cDNA (a gift from Professor Chambon, Strasbourg) as template, using 5′-GGGAATTCCACCATGCCCA ACTTC-3′ as the forward primer and 5′-GGCAGCCAACCAGTTTA ATGAC-3′ and 5′-GCCATCGATGGTTTACTCCCGGA-3′, respectively, as reverse primers. Further constructs, namely CRABPΔ1 (bases 183–223 deleted), CRABPΔ2 (bases 123–223 deleted), and CRABPΔ3 (bases 57–223 deleted), were generated using 5′-GGTACACAAGGCA ACAAGAGC-3′, 5-′GGGTTGCCTAATATTCATGGGGG-3′, and 5′-GCATACTCCTCAGGGGAACTCG-3′ as reverse primer, respectively. A further construct was made in which the 3′UTR of CRABPI was linked to the 5′UTR and coding region of rabbit *β-globin* amplified using 5′-GAGGTACCCCATAAAAGGCAGA-3′ (forward) and 5′-GCCATC GATGGTCAGTGGTATTTGT-3′ (reverse) primers which introduced, respectively, *Kpn*I and *Cla*I sites at the 5′ and 3′ends. The *CRABPI* 3′UTR was amplified using primers 5′-GCCATCGATGGAAAGGTGGCCA-3′ (forward) and GCTCTAGAGCCAGCCAACCAGTTT-3′ (reverse) to introduce a *Cla*I and *Xba*I site at the start and end of the 3′UTR, respectively. After ligation, the chimaeric construct was introduced into the multicloning site of pcDNA3 using the *Kpn*I and *Xba*I sites. The pcKG1 construct in which the *β-globin* coding region is linked only to vector polyadenylation sequences has been described previously [19]. All constructs were verified by sequencing.

Cell culture and transfection. Chinese hamster ovary cells (CHO) and LTK$^-$ fibroblasts were grown in Ham's F-12 modified medium (ICN Biomedicals), supplemented with sodium bicarbonate (1.176 g/L), 10% foetal calf serum (FCS), penicillin (50 IU/ml), streptomycin (50 μg/ml), and amphotericin B (Fungizone, 2.5 μg/ml) at 37 °C in an atmosphere of 5% CO_2. Transfection of CHO cells was carried out using LipofectAMINE (Life Technologies). Stable transfectants were selected by culture in the presence of 1 mg/ml zeocin. After subculture, cells were left to attach and grow for two days. Comparison of both mRNA and protein distribution was carried out in cells grown in multiwell chamber slides so that the different cell lines were studied under identical conditions and the quantification of staining was directly comparable.

In situ hybridisation and immunocytochemistry. For in situ hybridisation, cells were washed 3 times with PBS prior to fixation for 10 min with 4% paraformaldehyde in PBS and processed as described previously [21]. Hybridisation was carried out overnight at 55 °C with 200 ng of digoxigenin labelled antisense riboprobes. The CRABPI probe was generated from a 120 bp *Kpn*1 fragment of CRABPI (in pcDNA3 vector) using T7 polymerase and a DIG RNA labelling kit (Roche, UK). The globin probe was a 511 bp *Xba*I–*Bam*HI fragment generated using T7 polymerase in a similar manner [18]. Bound probe was detected by incubation with alkaline phosphate-linked anti-digoxigenin (Roche,UK) and incubation with 4-nitro blue tetrazolium (Gibco, UK).

For immunocytochemistry, cells were fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature and permeabilised using 0.05% Triton X-100 in 4% PFA for 10 min [20]. Specific staining was detected using an anti-X-Press antibody (diluted 1:400 in PBS, for 45 min at room temperature) which specifically recognises the in-frame X-press tag and FITC-conjugated goat anti-mouse secondary IgG (diluted 1:50 in PBS, for 30 min at room temperature). Slides were mounted in Citifluor.

Standard microscopy was performed using an Olympus BX51 microscope and digital images of the cells were captured under the 100× oil immersionlens using an Olympus DP50 digital camera and Analysis Viewfinder Lite SIS Software. Cells were also examined under a Leica TCS NT confocal microscope, *z*-series images captured using lasersharp

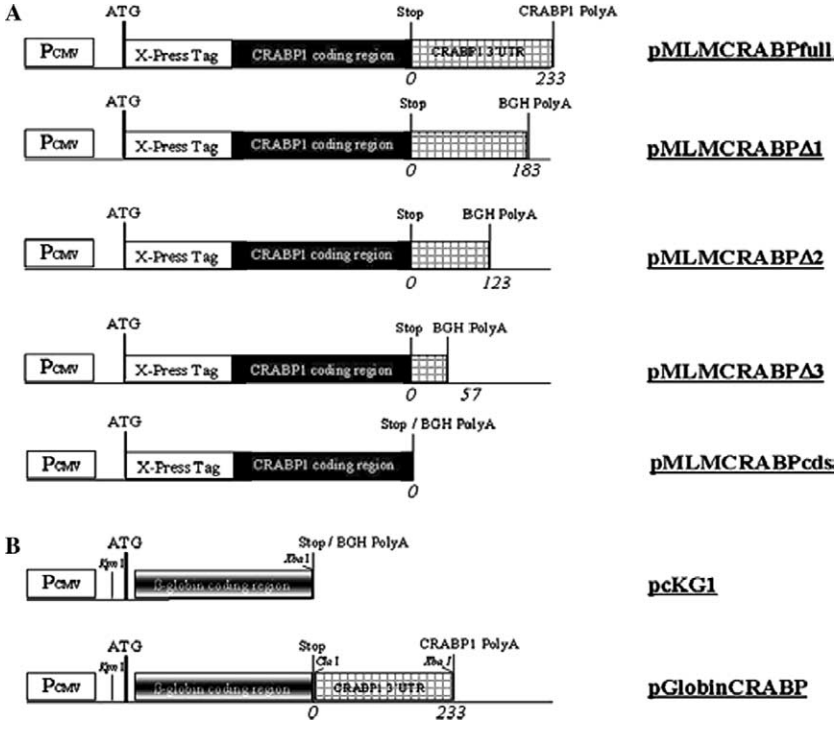

Fig. 1. Details of gene constructs. (A) The coding region of CRABPI was linked to its own 3′UTR, no 3′UTR or with increasingly large deletions so that Δ1, Δ2, and Δ3 contained, respectively, bases 0–183, 0–123, and 0–57 of the CRABPI 3′UTR. (B) The coding region of β-globin was used as a reporter gene linked either to the 3′UTR of the pcDNA3 vector to produce construct pcKG1 (19) or to CRABPI 3′UTR.