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Biochemical and Biophysical Research Communications 340 (2006) 326–331
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Perinuclear localisation of cellular retinoic acid binding protein I mRNA

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Received 30 November 2005
Available online 12 December 2005

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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doi:10.1016/j.bbrc.2005.12.006

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

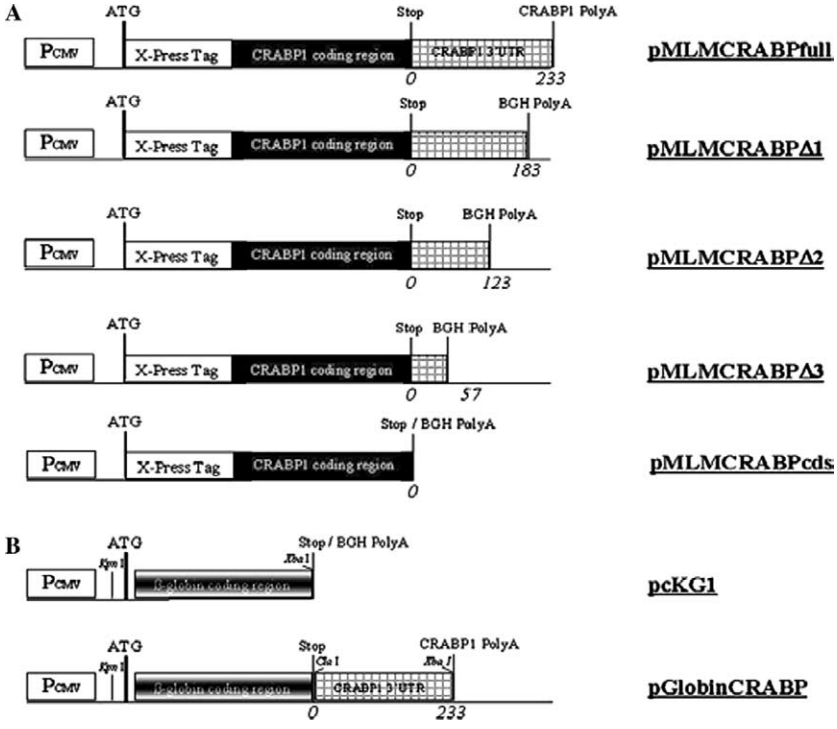

Fig. 1. Details of gene constructs. (A) The coding region of CRABPI was linked to its own 3′UTR, no 3′UTR or with increasingly large deletions so that Δ1, Δ2, and Δ3 contained, respectively, bases 0–183, 0–123, and 0–57 of the CRABPI 3′UTR. (B) The coding region of β-globin was used as a reporter gene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

software, and selected images converted into TIFF files using confocal assistant software. Photocap software was used to display the intensity of staining along an arbitrary profile across a cell. In addition, staining was quantified by examining fields of view at random and classifying cells (≈100 cells in at least 3 experiments) as exhibiting perinuclear localisation of protein or mRNA, or no localisation.

Results

CRABPI protein is localised in the perinuclear cytoplasm

Standard microscopy (results not shown) and confocal microscopy of cells expressing the full CRABPI construct showed the protein to be present in the cytoplasm and to be concentrated close to the nucleus (Fig. 2A). There was little or no staining either in the nucleus or in the cytoplasm towards the cell periphery. In contrast, cells expressing the tagged CRABPI from a construct lacking the CRABPI 3′UTR showed no perinuclear localisation of the protein (Fig. 2B) with staining being observed throughout the cytoplasm; again, there was no significant nuclear staining in these cells. Quantification of the staining, as illustrated by the profiles shown in Figs. 2C and D, confirmed the perinuclear localisation of CRABPI in the cells expressing the full CRABPI construct but no localisation in the cells expressing CRABPI without its 3′UTR.

CRABPI mRNA is localised in the perinuclear cytoplasm

The distribution of *CRABPI* mRNA was investigated in the same cell lines using in situ hybridisation. Cells expressing the full *CRABPI* showed a ring of staining around the nucleus and little or no staining in the peripheral cytoplasm, indicating that *CRABPI* transcripts were localised in the perinuclear cytoplasm (Fig. 2E). In contrast, cells

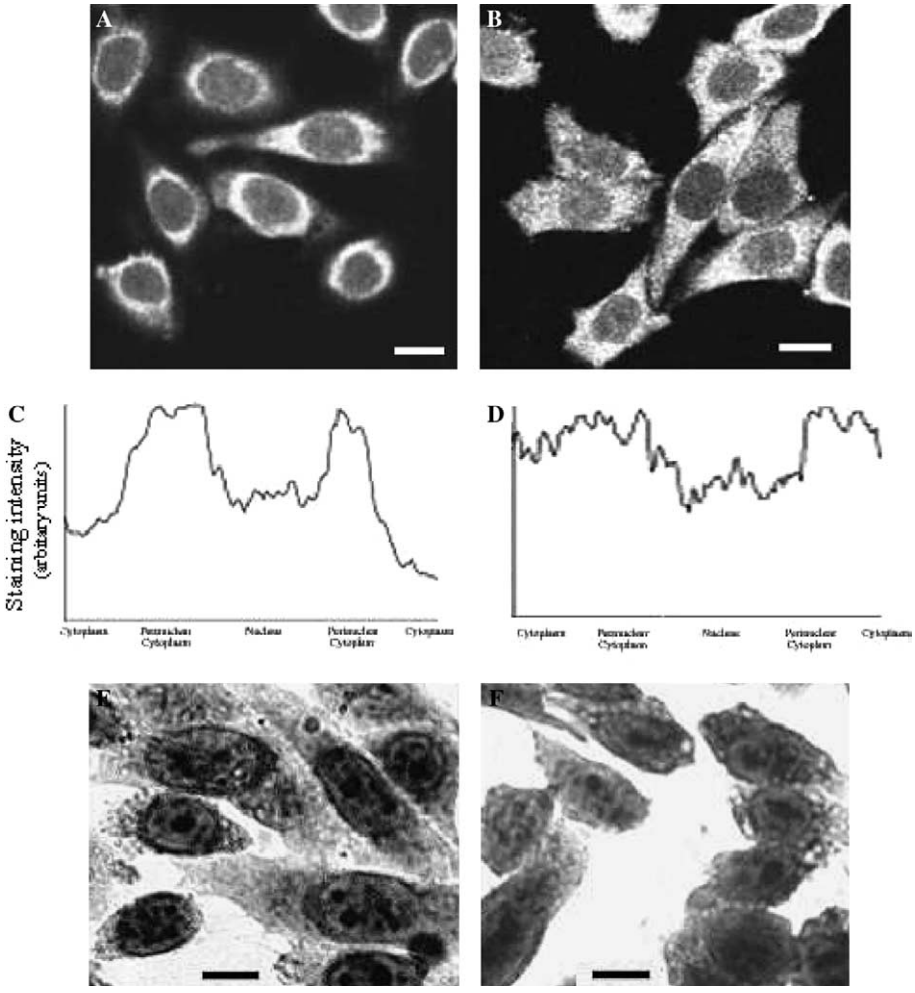

Fig. 2. Immunocytochemistry and in situ hybridisation of transfected CHO cells expressing CRABP-I gene constructs with or without its 3′UTR. (A–D) Confocal images of the pattern of immunostaining of cells after incubation with anti-X-Press antibody and FITC-conjugated goat anti-mouse secondary IgG. 0.5 μm section through CHO cells expressing either pMLMCRABP full construct (A) or pMLMCRABPcds (B) showing a perinuclear localisation of CRABPI protein with the full construct whereas the localisation is lost when the 3′UTR was removed. Profiles were obtained by drawing an arbitrary line across the cell and represent the intensity of staining along this line. Cells expressing pMLMCRABP full show peaks of staining around the nucleus (C) but pMLMCRABPcds show an even distribution throughout the cytoplasm (D). (E,F) In situ hybridisation showing distribution of specific transcripts detected using an antisense *CRABPI* riboprobe and revealed a perinuclear localisation of the *CRABPI* mRNA in cells expressing pMLMCRABP full (E) whereas no distinct localisation was visible in cells expressing the pMLMCRABPcds construct (F). Bars represent 10 μm.

expressing CRABPI without the 3′UTR showed staining throughout the cytoplasm with little or no evidence of perinuclear staining (Fig. 2F). Only background staining was observed in either transfected cells analysed with the control sense probe or in untransfected cells analysed with the antisense probe (results not shown). Overall, the data indicate that in CHO cells *CRABPI* mRNA and protein are both localised in the perinuclear cytoplasm and that this localisation is dependent on the 3′UTR, suggesting that the *CRABPI* 3′UTR contains a localisation signal.

The 3′UTR of CRABPI mRNA targets reporter sequences

To confirm the presence of such a signal, constructs were made in which the *CRABPI* 3′UTR was linked to *β-globin* reporter sequences, the constructs expressed in stably transfected cell lines, and *globin* transcript distribution assessed by in situ hybridisation. As found previously [18,20], in cells expressing the *globin* coding region with vector 3′UTR sequences, in situ hybridisation showed the *globin* transcripts to be present throughout the cytoplasm with no evidence of any localisation (Fig. 3B). In contrast, in cells expressing the *globin* coding region linked to the *CRABPI* 3′UTR the transcripts were visualised as a ring of localised staining in the perinuclear cytoplasm (Fig. 3A), indicating that this 3′UTR is sufficient to localise a reporter transcript.

Deletion analysis shows only part of the 3′UTR is required for localisation

Visual inspection of cells expressing constructs in which the *CRABPI* coding region was linked to increasingly shorter lengths of the 3′UTR (Fig. 1) showed that the CRABPI protein distribution in cells expressing the first two deletions (CRABPΔ2 and CRABPΔ3) was essentially perinuclear but that cells expressing constructs containing deletions from base 57 to 233 of the 3′UTR did not show localisation of the protein. This was confirmed by quantitative analysis (Fig. 4). In situ hybridisation showed that

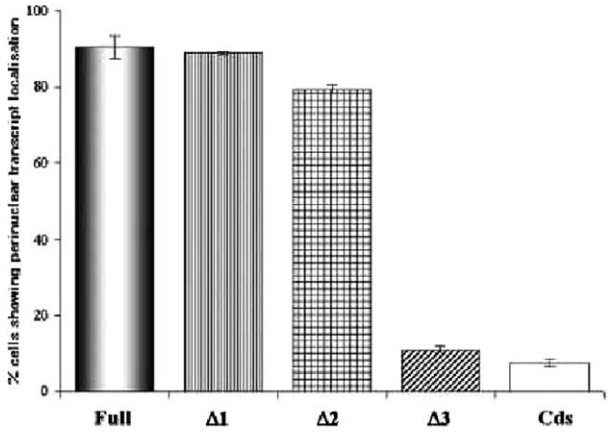

Fig. 4. Deletion analysis of the *CRABPI* 3′UTR: effects on protein distribution. CHO cells were stably transfected with constructs containing *CRABPI* coding region linked to increasingly large deletions (Δ1, Δ2, and Δ3) of the *CRABPI* 3′UTR. Cells were subjected to immunohistochemistry with anti-X-press antibody and staining was quantified by examining fields of view at random and classifying cells (≈100 cells in at least 3 experiments) as exhibiting perinuclear localisation of protein, or no localisation. There was a perinuclear localisation of CRABPI protein with both Δ3 and Δ2 deletions but not with Δ1.

these effects were paralleled by the mRNA distribution (Fig. 5): cells expressing either the full *CRABPI* 3′UTR or containing deletions from base 123 to 233 (CRABPΔ2) or 183 to 233 (CRABPΔ1) of the 3′UTR showed a perinuclear localisation of the transcripts whereas the CRABPI transcripts showing deletions from base 57 to 233 (CRABPΔ1) of the 3′UTR were not localised. Thus, the results suggest that the region between nucleotides 57 and 123 is necessary for *CRABPI* mRNA localisation, and thus protein localisation.

Discussion

The in situ hybridisation data show that *CRABPI* mRNA is localised in the perinuclear cytoplasm of transfected CHO cells. This is the first demonstration that *CRABPI* mRNA exhibits a subcellular localisation. The

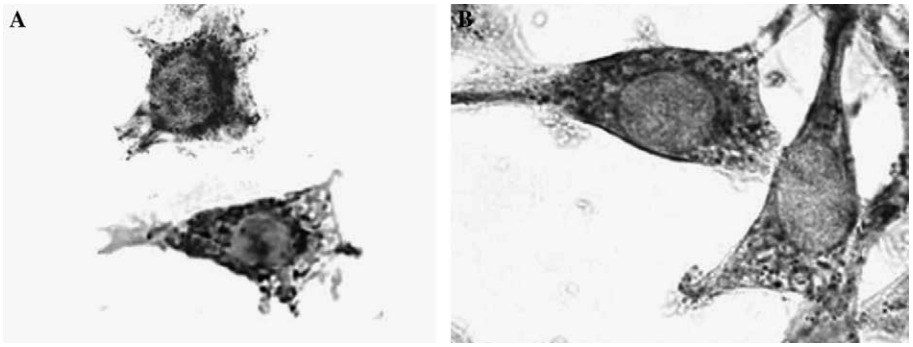

Fig. 3. In situ hybridisation showing that the *CRABPI* 3′UTR can target *β-globin* transcripts to the perinuclear cytoplasm. LTK$^-$ fibroblasts were stably transfected with either the pcKG1 construct or the pGlobinCRABP construct. Distribution of specific transcripts was detected using an antisense globin riboprobe, and this revealed a perinuclear localisation of globin transcripts in cells expressing pGlobinCRABP (A) and no distinct localisation of globin transcripts in cells expressing pcKG1 (B).

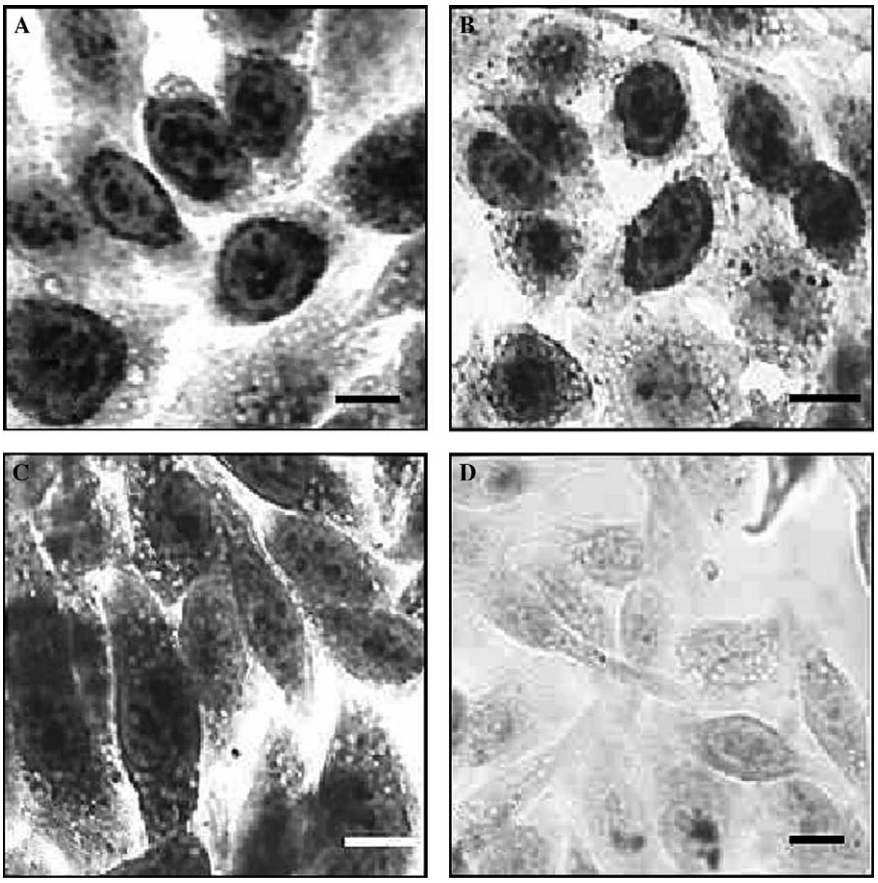

Fig. 5. Effects of 3′UTR deletions on CRABPI mRNA distribution. CHO cells were stably transfected with constructs containing CRABPI coding region linked to increasingly large deletions (Δ1, Δ2, and Δ3) of the CRABPI 3′UTR. In situ hybridisation using an antisense CRABPI riboprobe showed a perinuclear localisation of CRABPI mRNA with both Δ1 (A) and Δ2 (B) deletions whereas there was a loss of localisation with Δ3 (C) deletion. No staining was visible in the untransfected CHO cells (D). Bars represent 10 μm.

data indicate that, as found with *c-myc*, *c-fos*, and *metallothionein* mRNAs [17,18,20], this perinuclear mRNA localisation requires the 3′UTR. Furthermore, the *CRABPI* 3′UTR was sufficient to target reporter *β-globin* transcripts to the perinuclear cytoplasm. However, not all the 3′UTR was required for localisation since deletion studies showed that loss of the 110nt section from 123 to 233 had no effect on mRNA or protein localisation. Thus, the localisation signal must reside within the 123 first nt of the *CRABPI* 3′UTR. The loss of localisation after deletion of nt 57 to 233 confirms the importance of this region and indicates that some or all of the localisation signal must reside between nucleotides 57 and 123.

The present studies, using CHO cells expressing CRABPI with an N-terminal X-press tag, and immunohistochemistry with an anti-X-press antibody, showed the tagged CRABPI to be cytoplasmic in CHO cells. There was no evidence for nuclear localisation, so supporting earlier observations using antibodies that recognise the native protein [6–8]. However, CRABPI distribution was restricted to a ring around the nucleus, a location that would allow subsequent import into the nucleus in response to a suitable stimulus. Notably, *metallothionein-1* mRNA is also perinuclear and correct mRNA localisation is necessary for the protein to be perinuclear during the G1 phase of the cell cycle but nuclear as the cells enter S phase [20]. Our hypothesis is that for CRABPI, as with metallothionein-1, perinuclear mRNA localisation promotes subsequent protein import into the nucleus under certain conditions. It has been proposed that CRABPII and fatty acid binding protein exhibit ligand-activated nuclear localisation [12,22] and the observed perinuclear localisation of CRABPI is consistent with it also shuttling between cytoplasm and nucleus. Indeed, there are reports of CRABPI being present in both the cytoplasm and the nucleus [10,11].

In conclusion, the data show that *CRABPI* mRNA contains a localisation signal within the first 123 nt section of the 3′UTR. The parallel localisation of *CRABPI* mRNA and protein, and the similar lack of localisation of both after removal of sections, or all, of the 3′UTR suggest that the perinuclear localisation of *CRABPI* transcripts is a prerequisite for the observed localisation of CRABPI protein. The perinuclear localisation of the protein suggests that CRABPI functions in the cytoplasm close to the nucleus and/or in nuclear-cytoplasmic shuttling into the nucleus under appropriate conditions [10,11]. Future experiments should investigate whether CRABPI exhibits ligand-activated nuclear localisation as do CRABPII and fatty acid

binding protein [22] and if this is dependent on localisation of the mRNA by the 3′UTR signal.

Acknowledgments

M.L.-M. was supported by a post-doctoral fellowship from University of Newcastle and A.B. by BBSRC (Grant 13/C14236).

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