Perinuclear mRNA localisation by vimentin 3′-untranslated region requires a 100 nucleotide sequence and intermediate filaments

Giovanna Bermano[a,b], Rebecca K. Shepherd[c], Zendra E. Zehner[c], John E. Hesketh[a,d,*]

[a] *Intracellular Targeting Group, Rowett Research Institute, Aberdeen AB21 9SB, UK*
[b] *Department of Molecular and Cell Biology, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD, UK*
[c] *Department of Biochemistry and Molecular Biophysics, Medical College of Virginia Campus of Virginia Commonwealth University, Richmond, VA 23298-0614, USA*
[d] *Department of Biological and Nutritional Sciences, University of Newcastle, Kings Road, Newcastle upon Tyne NE1 7RU, UK*

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Abstract The role of the vimentin 3′-untranslated region (3′-UTR) in mRNA localisation was studied in cells transfected with a reporter sequence linked to subregions of the 3′-UTR. In situ hybridisation showed that nucleotides 37–137, including a previously identified protein-binding domain, were sufficient to localise transcripts to perinuclear cytoplasm. Transfection of two SW13 cell lines that do and do not express vimentin showed that perinuclear localisation due to either the vimentin or c-myc 3′-UTR requires intermediate filaments. The data suggest that both a specific protein-binding region of the vimentin 3′-UTR and intermediate filaments themselves are required to determine the site of vimentin synthesis.

Key words: mRNA; Localisation; Vimentin; Cytoskeleton; 3′-Untranslated region

1. Introduction

It is vital for correct cell function that newly synthesised proteins are delivered to the right place in the cell. One mechanism to target proteins to specific subcellular locations is to localise the mRNAs that encode them [1–4], allowing, in this way, synthesis of proteins close to their site of function. The localisation of specific mRNAs requires a mechanism to segregate such mRNAs from those that are not localised, and to sort the localised mRNAs to different locations within the cell. Most evidence indicates that the 3′-untranslated region (3′-UTR) of mRNAs is responsible for mRNA targeting [1,2,4]. In a few cases, it has been shown that proteins bind to 3′-UTR localisation elements [5,6] and there is evidence that the cytoskeleton participates in mRNA localisation [1,7], both in the transport of mRNAs within the cell and in the anchoring of them at specific sites.

Vimentin is the subunit of the intermediate filaments (IF) network expressed in cells derived from the primary mesenchyme and most cells raised in tissue culture [8,9]. Several reports suggest that IF synthesis is initiated around the nucleus and then proceeds in a vectorial fashion towards the plasma membrane [10–13]. IF assembly and turnover depend on the availability of vimentin subunits and therefore one possible mechanism to regulate the site of assembly is localised synthesis of vimentin. In embryonic muscle cells and fibroblasts, over one-half of the newly synthesised vimentin is found immediately associated with the cytoskeleton [14,15], suggesting that IF assembly occurs by a co-translation mechanism [16,17]. Furthermore, vimentin mRNA exhibits a perinuclear localisation in fibroblasts and myotubes [18,19], and in cultured chicken muscle, vimentin mRNA is localised to the costameres where its protein product is concentrated [20]. Vimentin mRNA localisation may be, therefore, a prerequisite for optimal filament formation.

The vimentin 3′-UTR targets reporter transcripts to the perinuclear cytoplasm [21] but the localisation signal has not been defined. A distinct region of the 3′-UTR has protein-binding properties [22] but the function(s) of this protein-binding region is not known. The aims of this work were to determine if the subregion of the vimentin 3′-UTR necessary for protein binding is also sufficient for mRNA localisation, and to assess whether vimentin-containing intermediate filaments are necessary for localisation of transcripts to the perinuclear cytoplasm.

2. Materials and methods

2.1. DNA constructs

The DNA constructs contained the green fluorescent protein gene as a reporter gene (pEGFP-C1, Clontech) fused to subregions of human vimentin's 3′-UTR. Constructs were prepared using unique *Mun*I and *Hpa*I sites located at positions −75 and −85, respectively, downstream of the GFP stop codon and sequences derived from a human vimentin cDNA by PCR [22]. GFP-vim11, GFP-vim37 and GFP-vim139 contain the GFP gene fused to sequences corresponding to nucleotides 11–208, 37–137 and 139–208, respectively. Each primer contained extra 2 nt, GT, to ensure maximum enzymatic digestion in addition to the 5′-*Mun*I and 3′-*Hpa*I restriction sites (in italics) plus vimentin's sequence (uppercase). For example, construct GFP-vim11 used the 5′-primer GFP1 (5′-gt*caattg*CACTCAGTGCAGCAATA-3′) which begins at base 11 and ends at base 27 downstream of vimentin's stop codon. The 3′-primer was GFP3 (5′-gt*gttaac*AAAGTATTC-TAGCACAAGA-3′) which ends at position −208. For plasmid GFP-vim37 the primers were 5′-gt*caattg*CAAGAATAAAAAA-GAAATCC-3′ and 5′-gt*gttaac*GTTAAGAACTAGAGCTTAT-3′ so as to include base pair 37–137 of vimentin's 3′-UTR. For plasmid GFP-vim139 the primers were 5′-gt*caattg*GTTCTTAACAACCGA-CAC-3′ and GFP3 so as to contain the region from position −139 to −208. In each case vimentin's 3′-UTR sequence was added after

*Corresponding author. Fax: (44)-191-222 8684.
E-mail: j.e.hesketh@ncl.ac.uk

Abbreviations: GFP, green fluorescent protein; IF, intermediate filaments; 3′-UTR, 3′-untranslated region

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the GFP stop codon and the polyadenylation site was supplied by the pEGFP-C1 vector. The content of each fusion plasmid was verified by DNA sequencing. The GFP plasmid containing vector 3′-UTR only was used as the control plasmid. The globin-myc construct has been described previously [23].

2.2. Cell culture and cell transfection

Chinese hamster ovary (CHO) cells and two clones derived from the human adrenal tumour cell line (SW13; kindly donated by Dr. R.M. Evans) which do (vim+) and do not (vim−) synthesise vimentin [24] were used to generate transfectants with the above constructs. CHO cells were grown in Ham's F-12 medium (ICN Biomedical Inc.) supplemented with 10% foetal calf serum whereas vim+ and vim− cells were grown in a 1:1 (v/v) mixture of Ham's F-12:Dulbecco's modified Eagle's medium (MEM) (Life Technologies Ltd.) containing 5% foetal calf serum. An atmosphere of 5% CO_2 was used. Transfection was carried out using LipofectAMINE™ Plus (Life Technologies Ltd.). Cells were subcultured into two-well chamber slides at a density of 2×10^4 (CHO) and 1×10^5 (vim ±) cells/well and grown until 70–80% confluent. The cells were overlaid with a mixture of 1 μg DNA, 2 μl lipofectAMINE and 5 μl Plus reagent. After 3 h, 1 ml of medium was added to each well and after 24 h the medium was replaced with a fresh one. Cells were analysed 48 h after transfection.

2.3. In situ hybridisation

Analysis of mRNA distribution was carried out following the protocol previously described [25]. Cells were hybridised with 400 ng of either digoxigenin (DIG)-labelled or FITC-labelled antisense probe in 50% formamide, 2×SSC (1×SSC: 15 mM sodium citrate, 150 mM NaCl pH 7.4). The GFP antisense probes were generated by using Sp6 polymerase from the 750 bp full GFP sequence in pcDNA3 vector, using a RNA labelling kit (Roche) with either DIG- or FITC-labelled UTP. Controls were either hybridised with labelled sense probes generated from the same plasmid using T7 polymerase or incubated with hybridisation mix containing no probe. After hybridisation, cells were washed in 5×SSC at 55°C for 30 min and subsequently in 50% formamide, 2×SSC in the same conditions. Non-specifically bound probe was removed by treatment at 37°C with 20 μg/ml RNase A in wash buffer (10 mM Tris, pH 7.5, containing 0.4 M NaCl and 5 mM EDTA). For standard microscopy, labelled transcripts were detected by incubation with alkaline phosphatase-linked anti-DIG and then with 4-nitroblue tetrazolium for 10 h as described previously [25]. The number of cells showing perinuclear staining was expressed as a percentage of the number of cells counted: for each construct, at least 100 cells were counted from each experiment.

For confocal microscopy, transcripts were detected by their hybridisation to FITC-labelled riboprobe and, after washes in 2×SSC, samples were mounted in Citifluor. Optical sections were taken and images converted into TIFF files using confocal assistant software. The staining distribution in the middle section was then quantified using Fenestra/Cyclops image analysis software (Kinetic Imaging, Liverpool, UK) to measure staining intensity in different cytoplasmic regions [25]. Squares of identical size were drawn, three over the perinuclear cytoplasm, three over the cell periphery and three outside the cell (background staining). The intensity values were corrected for background and the ratio of perinuclear:periphery staining calculated.

3. Results

3.1. A 100-nt region of vimentin 3′-UTR implicated in protein binding is responsible for mRNA localisation of a reporter gene to the perinuclear cytoplasm

CHO cells were transiently transfected with constructs containing GFP-coding region linked to different regions of the vimentin 3′-UTR. In situ hybridisation analysis of transiently transfected cells showed that, in control cells containing GFP linked to the vector 3′-UTR, there was no localisation of the transcripts in the cytoplasm: the mRNA was seen to be present throughout the cytoplasm (Fig. 1A). Thus, it appears that the vector 3′-UTR contains no localisation signal. In contrast, cells transfected with GFP-vim11 construct showed a completely different pattern in which the transcript was

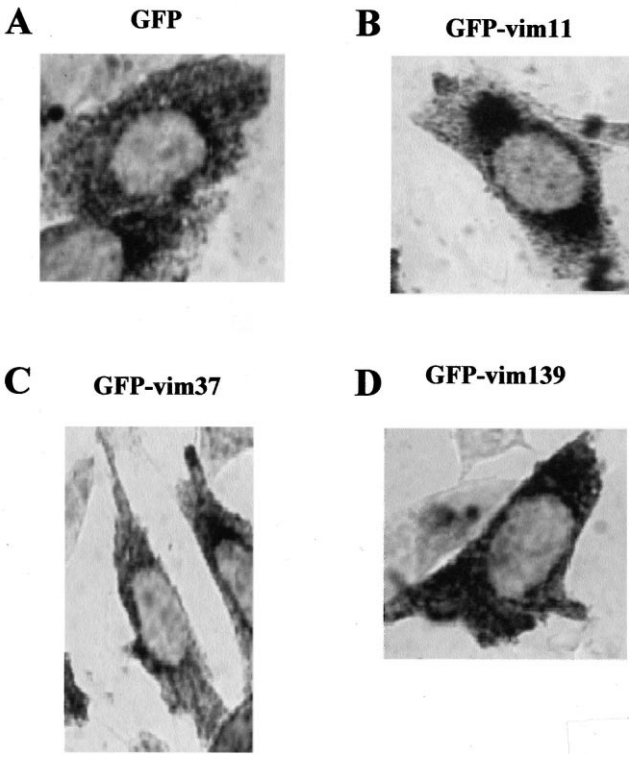

Fig. 1. In situ hybridisation showing distribution of chimaeric GFP-vimentin transcripts in transfected CHO cells. CHO cells were transfected with DNA constructs containing the GFP-coding sequence linked to the vector 3′-UTR (A) or different region of vimentin 3′-UTR. The formazan staining pattern reveals specific mRNA localisation detected by in situ hybridisation using a DIG-labelled antisense probe specific for GFP-coding sequence, alkaline phosphatase-linked anti-digoxigenin antibody and 4-nitroblue tetrazolium as substrate. Perinuclear staining was apparent in cells transfected with GFP-vim11 (B) and GFP-vim37 (C) constructs, whereas in cells transfected with GFP (A) and GFP-vim139 (D), staining was present throughout the cytoplasm.

localised predominantly in the perinuclear cytoplasm with minimal staining in the peripheral cytoplasm (Fig. 1B). Cells transfected with the GFP-vim37 construct also showed transcript localisation in the cytoplasm around the nucleus (Fig. 1C) with a pattern similar to that obtained with the GFP-vim11. In contrast cells transfected with GFP-vim139 showed no transcript localisation (Fig. 1D) with a staining pattern resembling that obtained with cells transfected with the control GFP construct. Transfected cells treated with no probe or hybridised with a sense probe and untransfected cells hybridised with the antisense probe all showed no staining (results not shown).

100 cells from each transfection and from four different experiments were analysed, in a blind study, for the distribution of the different GFP transcripts. In the case of GFP-vim11 and GFP-vim37, 57 ± S.E.M. (3)% and 59 ± 3% of the cells showed a perinuclear localisation of the GFP transcript, whereas with GFP and GFP-vim139 only 23 ± 5% and 29 ± 2% showed localisation. Overall, these data suggest that the subregion of vimentin 3′-UTR containing nucleotides 37–137 is sufficient to localise a reporter gene to the perinuclear cytoplasm.

For confocal microscopy in situ hybridisation was performed with a FITC-labelled antisense probe. Z-series images

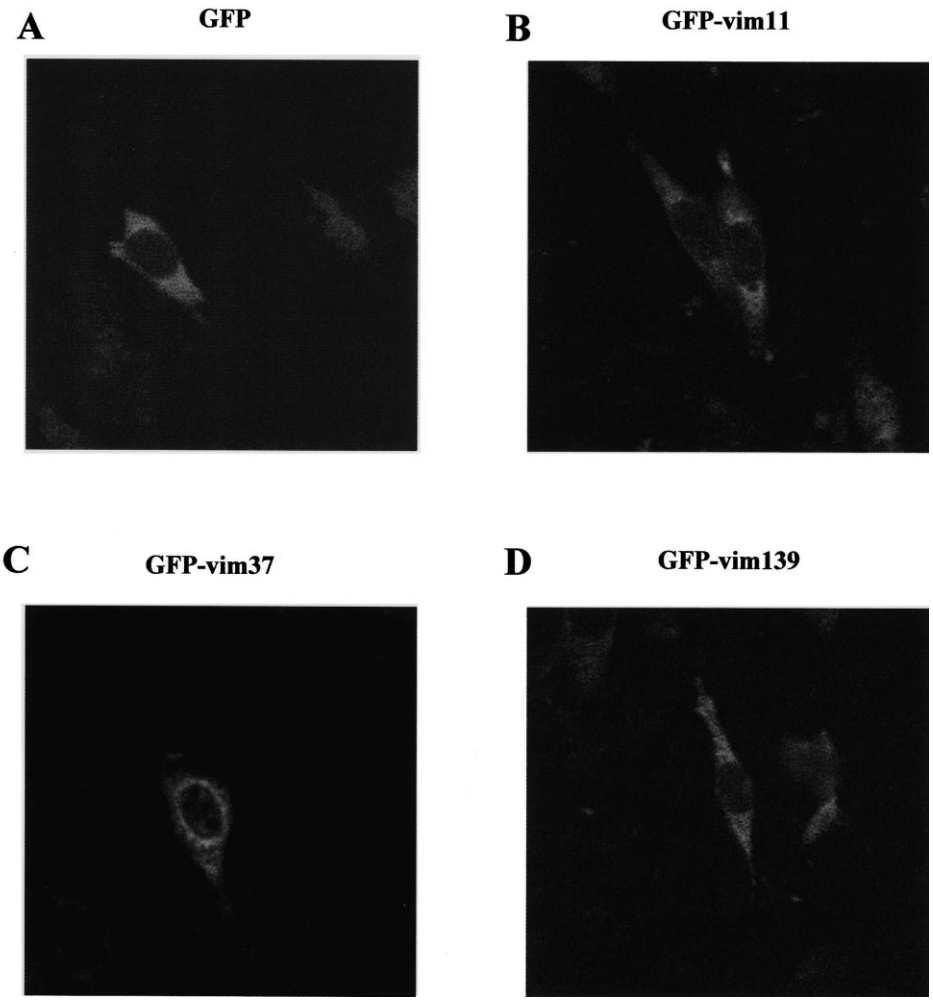

Fig. 2. Confocal microscopy of transcript distribution. CHO cells were transfected with DNA constructs containing the GFP-coding sequence linked to the vector 3′-UTR (A) and linked to different region of vimentin 3′-UTR. Specific mRNA localisation was analysed by in situ hybridisation using a FITC-labelled antisense probe specific for GFP-coding sequence. Z-series images were captured using lasersharp software and middle images are shown here. The fluorescent green signal was only concentrated around the nucleus in cells transfected with GFP-vim11 (B) and GFP-vim37 (C) constructs, whereas in cells transfected with GFP (A) and GFP-vim139 (D), staining was present throughout the cytoplasm.

were captured using lasersharp software for a minimum of 16 cells from four different experiments; typical staining patterns obtained from optical sections in the middle of the z-series are shown in Fig. 2. In cells transfected with either the control GFP construct or GFP-vim139 there was no transcript localisation with staining seen throughout the cytoplasm. In contrast, cells transfected with either GFP-vim37 or GFP-vim11 showed distinct perinuclear localisation of the transcripts. Quantification of the staining pattern demonstrated that the ratio of perinuclear:peripheral staining was greater in GFP-vim11 and GFP-vim37 (2.8 ± 0.2 and 2.9 ± 0.2, respectively) transfected cells compared to GFP and GFP-vim139 (1.3 ± 0.1 and 1.2 ± 0.1, respectively). These data obtained by confocal microscopy confirm those from conventional microscopy and eliminate the possibility that the increased staining around the nucleus is due to increased thickness of the cytoplasm in this region. In conclusion, a 100-nt region (37–137) of vimentin 3′-UTR is both necessary and sufficient for localising GFP reporter transcripts to the cytoplasm around the nucleus; this subregion also includes the minimal protein-binding domain [22].

3.2. Role of vimentin intermediate filaments in localising transcripts to the perinuclear cytoplasm

Cells that do and do not contain an IF network (vim+ and vim− clones from the SW13 cell line) were transiently transfected with the GFP and GFP-vim37 constructs. The distribution of the transcripts was analysed by in situ hybridisation using a DIG-labelled antisense probe and brightfield microscopy. Vim+ cells transfected with the GFP construct showed no localisation of the transcript (Fig. 3A), whereas when they were transfected with GFP-vim37, the transcript was localised to the perinuclear cytoplasm (Fig. 3B). This result indicates that the localisation signal in the vimentin 3′-UTR identified using CHO cells is effective in SW13 cells containing vimentin. On the contrary, when vim− cells were transfected with the same constructs in both cases no localisation of the transcripts was evident (Fig. 3C,D), suggesting that the vimentin network was necessary for the vimentin 3′-UTR to localise a reporter gene to the perinuclear cytoplasm of the cell.

In order to ascertain if the vimentin network was required only for localisation by vimentin 3′-UTR or whether localisation by other 3′-UTR signals also required intermediate

filaments, vim+ and vim− cells were transfected with a chimeric globin-myc construct. This plasmid expresses globin-myc transcripts in which globin-coding sequences are linked to the c-myc 3′-UTR and which are localised to the perinuclear cytoplasm in fibroblasts and CHO cells by a localisation signal in the c-myc 3′-UTR [23,26,27]. In situ hybridisation showed that in the transfected vim+ cells the globin transcripts were localised to the perinuclear area of the cell (Fig. 3E) whereas in vim− cells no localisation of the transcripts was evident (Fig. 3F), suggesting that the vimentin network is necessary for localising transcripts targeted by the c-myc 3′-UTR to around the nucleus.

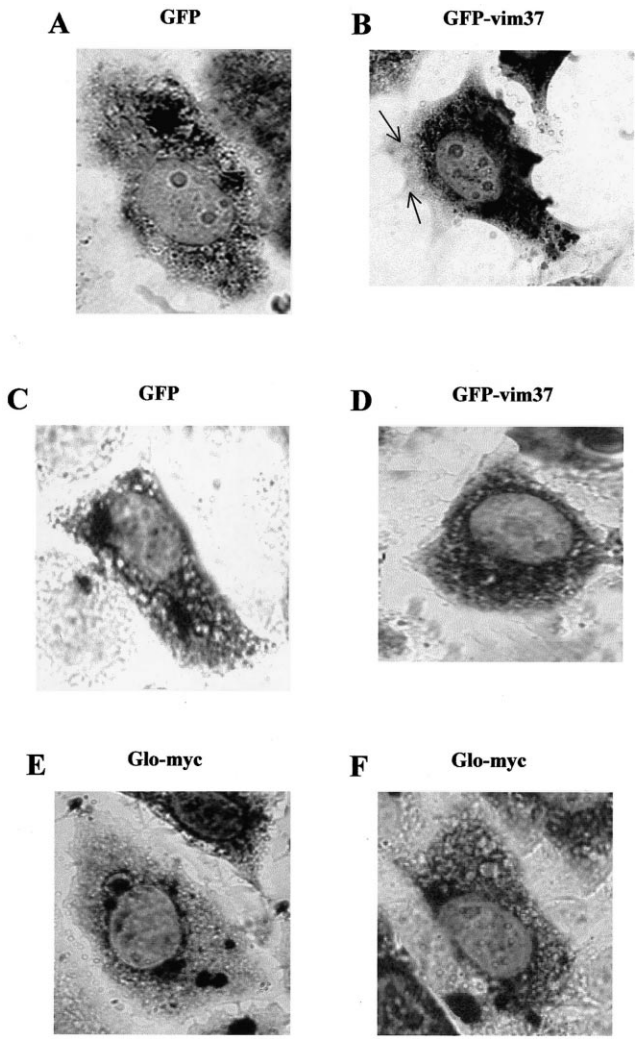

Fig. 3. In situ hybridisation showing the distribution of chimaeric GFP-vimentin and globin-myc transcripts in vim+ and vim− cells. Vim+ and vim− cells were transfected with either DNA constructs containing the GFP-coding sequence linked to the vector 3′-UTR (A,C) and to the 37–137 region of vimentin 3′-UTR (B,D) or constructs containing the globin-coding sequence linked to c-myc 3′-UTR (E,F). Specific mRNA localisation was detected by in situ hybridisation (see legend to Fig. 1). Perinuclear staining was evident only in vim+ cells transfected with either GFP-vim37 (B) or globin-myc (E) but not in cells lacking vimentin network (vim− cells) (compare B with D). Arrowheads show region of unstained cytoplasm.

4. Discussion

Earlier work has shown that vimentin mRNA is localised around the nucleus of chicken fibroblasts and myotubes [18,19] and that the vimentin 3′-UTR can target reporter transcripts to the perinuclear cytoplasm [21]. The present work extends these observations by defining the part of the 3′-UTR that contains the localisation signal and by providing evidence that an IF network is required for this localisation.

Using deletion analysis we have restricted the signal to 100 nucleotides (37–137) within the 3′-UTR (Figs. 1 and 2). It is precisely this region which has been shown previously to bind a 46-kDa protein [22] and more recently a 35-kDa protein [28], suggesting that one or more of these protein(s) are involved in localisation of vimentin mRNA to the perinuclear area of the cytoplasm. In situ hybridisation studies in SW13 cells (Fig. 3) showed that transcripts containing the vimentin 3′-UTR localisation signal are not localised in a clonal cell line (vim−) lacking vimentin. These cells contain no IF network but do contain actin and microtubule networks of grossly similar organisation to that in vim+ cells (results not shown). This suggests that it is the loss of the IF themselves rather than secondary effects on other cytoskeletal components that is the cause of the inability to localise the vimentin 3′-UTR-containing transcripts, although subtle secondary effects on the actin/tubulin networks cannot be excluded. The data thus suggest that IF play a role in the perinuclear localisation or anchoring of transcripts targeted by the 100-nt subregion of the vimentin 3′-UTR. It has been suggested that IF assembly occurs by co-translational assembly [16,17] and the present data are compatible with such a mechanism dependent on vimentin mRNA localisation.

This effect was not specific to vimentin 3′-UTR and GFP reporter, since the localisation of a different reporter transcript (globin) to the same perinuclear region by a different 3′-UTR (c-myc) was lost when the vimentin network was absent. The cytoskeletal components involved in targeting of c-myc and globin-myc transcripts have not been positively defined. These mRNAs are released into an actin-rich cytoskeletal-bound polysome (CBP) fraction [26,27,29], suggesting that microfilaments play a role in retaining these transcripts around the nucleus. However, the present data, taken together with the observations that the CBP fraction also contains significant amounts of vimentin [30], suggest that it is IF which play this role. Further work is required to dissect out the precise roles of IF and microfilaments.

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