Multiple domains control the subcellular localization and activity of ETR-3, a regulator of nuclear and cytoplasmic RNA processing events

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Accepted 3 March 2004
Journal of Cell Science 117, 3519-3529 Published by The Company of Biologists 2004
doi:10.1242/jcs.01194

Summary

Embryonic lethal abnormal vision (ELAV) type RNA binding protein 3 (ETR-3; also called NAPOR, CUGBP2, or BRUNOL3) has been implicated in the regulation of nuclear and cytoplasmic RNA processing events, including alternative splicing, RNA editing, stability and translation. Here, we report that the ETR-3 protein contains multiple regions that control its subcellular localization and are important for its activity as a splicing regulator. We cloned ETR-3 from chicken heart and fused it to the C terminus of green fluorescent protein (GFPcETR3vL). GFPcETR3vL is found predominantly in the nucleus and is an active regulator of alternative splicing in cotransfection assays with a cardiac troponin T minigene. ETR-3 contains two N-terminal RNA recognition motifs (RRMs), a 210-amino acid divergent domain, and a C-terminal RRM. We demonstrate that the C terminus contains a strong nuclear localization signal overlapping the third RRM, which can confer nuclear localization on a normally cytoplasmic pyruvate kinase chimera. Additional deletions revealed nuclear localization and export activities in the divergent domain of ETR-3, as well as regions within the first two RRMs that are important for cytoplasmic localization. The nuclear export activity of the divergent domain is sensitive to leptomycin B, indicating that export to the cytoplasm is mediated via a CRM1-dependent pathway. The C terminus and a region within the divergent domain were also shown to be important for splicing activity of ETR-3. This is the first characterization of protein domains involved in mediating the subcellular localization and splicing activity of a member of the CELF family of RNA processing regulators.

Key words: ETR-3, CELF, Subcellular localization, Alternative splicing, Protein domains

Introduction

Many RNA binding proteins serve multiple functions within the cell, affecting mRNA splicing, polyadenylation, transport, localization, stability, and/or translation. The embryonic lethal abnormal vision (ELAV) type RNA binding protein 3 (ETR-3) was first identified in a screen of genes expressed in the fetal heart (Hwang et al., 1994). It was subsequently found in a screen of genes induced during apoptosis in neuroblastoma cells, where it was called NAPOR (Choi et al., 1998), and in a screen of a mouse liver cDNA library using a CUG binding protein (CUG-BP) probe (Lu et al., 1999), after which it was also referred to as CUG-BP2. It has since been shown that ETR-3 is one member of a family of RNA binding proteins called CUG-BP and ETR-3-Like Factors [CELF (Ladd et al., 2001) or Bruno-like proteins [BRUNOL (Good et al., 2000)]. It has been suggested that ETR-3 is a shuttling protein, moving between compartments to regulate both nuclear and cytoplasmic mRNA processing (Good et al., 2000; Ladd et al., 2001). In support of this proposal, ETR-3 has been linked to regulation of both nuclear and cytoplasmic RNA processing events such as alternative splicing (Ladd et al., 2001; Charlet et al., 2002a; Suzuki et al., 2002; Zhang et al., 2002), RNA editing (Anant et al., 2001), mRNA stability (Mukhopadhyay et al., 2003) and translation (Good et al., 2000; Mukhopadhyay et al., 2003).

ETR-3 mRNA is expressed in multiple tissues, with highest expression in muscle and brain (Lu et al., 1999; Ladd et al., 2001), where it has been implicated in the regulation of cell-specific alternative splicing. ETR-3 has been shown to bind to repeat elements in rat α-actinin, upstream of a non-muscle exon and promote selection of a mutually exclusive smooth muscle-specific exon (Suzuki et al., 2002; Gromak et al., 2003). ETR-3 has been shown to activate inclusion of an alternative exon via binding to U/G-rich motifs within muscle-specific enhancer elements (MSEs) in cardiac troponin T (cTNT) pre-mRNAs (Ladd et al., 2001; Charlet et al., 2002a). In the brain, ETR-3 has been implicated in the regulation of brain region-specific alternative slicing of exons 5 and 21 of the N-methyl-D-aspartate receptor 1 (NMDA R1) in the rat (Zhang et al., 2002).

ETR-3 has also been implicated in the regulation of RNA editing. Mammalian apolipoprotein B (apoB) undergoes post-transcriptional C to U editing in small intestine to generate a truncated protein that participates in dietary lipid absorption (Young, 1990). ETR-3 binds to AU-rich sequences immediately upstream of the edited cytidine, is sufficient to block C to U RNA editing of apoB transcripts in a reconstituted system, and is necessary for repression of apoB editing in cells (Anant et al., 2001). Two models were proposed to explain repression of RNA editing by ETR-3 (Anant et al., 2001). In

one, ETR-3 binds to apoB mRNA and interacts with components of the editing complex to disrupt their function in the nucleus. In the alternative model, ETR-3 interacts with a component of the editing complex in the cytoplasm and prevents its shuttling to the nucleus.

In addition to regulating nuclear RNA processing events, ETR-3 is sometimes found in the cytoplasm and has been implicated in the regulation of mRNA stability and translation (Mukhopadhyay et al., 2003). Phylogenetic analysis indicates that the CELF family is closely related to the Hu proteins (Ladd et al., 2001), which are established regulators of mRNA stability and translation (Keene, 1999). ETR-3 binds to a Bruno response element (BRE), a cis element involved in translational control of oskar mRNA in *Drosophila* (Good et al., 2000), and may be a vertebrate homolog of Bruno, the *Drosophila* protein that mediates this process. Furthermore, ETR-3 stabilizes and inhibits translation of cycloxygenase-2 (COX-2) mRNAs via direct interaction with A/U-rich sequences in the 3′ UTR (Mukhopadhyay et al., 2003).

Here, we report that the ETR-3 protein contains multiple regions that determine its subcellular localization and contribute to its regulatory function. We used green fluorescent protein (GFP)-ETR-3 fusion constructs to conduct functional analyses for determinants of subcellular localization and splicing activity. ETR-3 contains three RNA recognition motifs (RRMs), the second and third of which are separated by a 210-amino acid divergent domain of unknown function that is unique to members of the CELF family (Fig. 1B). We found determinants for nuclear localization in RRM3 and the divergent domain, and for nuclear export/cytoplasmic localization in the first two RRMs and divergent domain. The divergent domain and RRM3 also contain regions important for splicing activity. These results suggest that ETR-3's subcellular localization is controlled by a set of elements that collectively drive a balance between a nuclear and cytoplasmic presence. This is the first characterization of localization and activation domains within a member of the CELF family of RNA processing factors.

Materials and Methods

Plasmids

RNA was extracted from embryonic day-8 and adult chicken hearts by the method of Chomcynski and Sacchi (Chomczynski and Sacchi, 1987). Full-length ETR-3 was derived by RT-PCR as previously described (Ladd et al., 2001) using primers containing the putative start and stop codons based on chicken ESTs and ETR-3 homologs in other species (primer sequences CATACTAGATCTATGAAC-GGAGCTTTGGATCA and AATTCTCTCGAGTTAGGATCAGTA-AGGTTTGCT). Two open reading frames were identified, designated variant L (GenBank accession number AY288986) and variant 4 (AY288985). Variant L corresponds to mouse and human isoforms in the databases (AF090696 and AF432906, respectively). Variant 4 matches a partial chicken ETR-3 mRNA sequence in the database (AB050498) and corresponds to published mouse, rat, human and zebrafish isoforms (AF090696, NM_017197, AF432906, and AB050496, respectively). Both open reading frames were cloned into the pcDNA3.1(+) vector (Invitrogen). The open reading frame of variant L was cloned into the pEGFP-N1 vector (Clontech) in frame with the N-terminal GFP (GFPcETR3vL). Deletions were derived from GFPcETR3vL by PCR. Gain-of-function constructs were generated by adding PCR-generated segments of ETR-3 to pyruvate kinase chimeras (GFP-PK and NLS-GFP-PK) provided by Warner Greene (University of California, San Francisco). All constructs were confirmed by mapping and complete sequencing of PCR-generated fragments. The GFPDD.4 and GFPDD.4NLS plasmids contain a point mutation in RRM3 that results in an N to D substitution at residue 461; this mutation is not believed to be significant because GFPDD.4NLS is active and RRMs in other RNA binding proteins contain this amino acid at this position. All constructs produce proteins larger than 60 kDa, the size limit for passive diffusion through the nuclear pore complex (Nigg, 1997; Görlich, 1998).

RNase protection assay

The probe used to determine the predominant splice form of ETR-3 in chicken heart was transcribed from a template amplified by PCR from the variant 4 open reading frame using the primers GATGCATCGAGCTTACCGACGGAGCCACCGTTGGAC TGA and TAATACGACTCACTATAGGGCTGCCCGCGGCACTT-TGCTG. Hybridization was performed at 60°C using 10 μg total RNA as previously described (Charlet et al., 2002b).

Cell culture, transfections, and cotransfection assays

QT35 quail fibroblast cells were cultured as previously described (Ladd et al., 2001) and transfected with 2 μg of untagged ETR-3 variant 4 or L expression plasmid using FuGene 6 (Roche Applied Science). Total protein was harvested from cells 48 hours following transfection and from embryonic day-12 chicken hearts and subjected to western blotting using an antipeptide antibody against ETR-3 as previously described (Ladd et al., 2001).

For localization experiments, COS-M6 cells were grown on coverslips in 60 mm diameter tissue culture dishes in 3 ml medium (high glucose DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotic-antimycotic). Cells were transfected at 30% confluency with 2 μg total DNA using FuGene 6 (Roche Molecular Biochemicals). QT35 cells were cultured as above but on coverslips and transfected the following day with 2 μg total DNA using FuGene 6. Primary chicken cardiomyocytes were harvested from day-14 chicken embryos. Hearts were removed and washed twice with ice cold Hanks balanced salt solution (HBSS). Hearts were minced and subjected to serial digestions at 37°C with approximately 30 Units of trypsin, 30 Units of collagenase, and 100 Units of DNase with frequent agitation. Dissociated heart cells were passed through a Percoll (Pharmacia) gradient and purified myocytes were washed three times with Ads buffer (0.68% NaCl, 0.04% KCl, 0.476% Hepes, 0.01% $MgSO_4$, 0.15% NaH_2PO_4, 0.002% Phenol Red, and 0.1% dextrose). Cardiomyocytes were plated on coverslips coated with rat tail collagen at a density of 2.5×10^6 cells/60 mm diameter plate or 6×10^5 cells/well of a 24-well plate in 3 ml or 0.5 ml growth medium (MEM supplemented with 10% heat-inactivated horse serum, 5% chick embryo extract and antibiotic-antimycotic), respectively. Cardiomyocytes were transfected with 2 μg (60 mm dish) or 0.5 μg (24-well plate) of plasmid DNA using Lipofectamine (Invitrogen). Cells were fixed 24 hours (COS-M6 cells) or 48 hours (QT35 cells and primary cardiomyocytes) following transfection in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 minutes at room temperature. Coverslips were removed and mounted in PBS on glass slides and visualized by fluorescence microscopy. In the indicated experiments, 1 μM leptomycin B (LMB; generous gift of Minoru Yoshida, RIKEN) was added to the medium for 1 hour prior to fixation. This dose was determined to inhibit CRM1-mediated NES activity by titration on cells transfected with the NES-containing GFP-PK-Vpr plasmid (provided by Warner Greene, UCSF, USA). Because LMB is dissolved in ethanol, parallel plates of cells were mock-treated with an equivalent volume of ethanol alone. No differences in morphology or viability were observed between LMB-treated and mock-treated cells. Ethanol treatment alone did not affect the localization of any of the GFP fusion constructs.

For cotransfection assays, COS-M6 and QT35 cells were cultured in 60 mm plates as above but without coverslips and transfected with 100 ng of the R35C minigene (Ladd et al., 2001) and 1.9 μg of GFPcETR3 expression plasmid DNA. Coexpression of the R35C minigene did not affect the localization of the GFP fusion proteins (data not shown). Total RNA and protein were harvested 48 hours after transfection and subjected to RT-PCR and western blotting as previously described (Ladd et al., 2001). The mean values for the extent of exon inclusion for each fusion protein were compared to the mean values obtained from the minigene alone using a two-tailed pooled *t*-test assuming a normal distribution. This test also assumes that the population variances are equal; to confirm that this assumption was valid, preliminary *F* tests were conducted where the α level was set at α=0.2.

Results

Cloning of chicken ETR-3

ETR-3 has been implicated in the regulation of alternative splicing of cTNT transcripts during chicken heart development (Ladd et al., 2001). ETR-3 cloned from embryonic chicken heart (Fig. 1A) was found to be 97% and 98% identical to the human and mouse proteins, respectively. Two variants were identified that differ by the use of an alternative 5′ splice site in an exon comparable to exon 10 in human and mouse ETR-3 that adds four amino acids (TVNS) to the divergent domain (Fig. 1A,B). Both open reading frames were transfected into QT35 quail fibroblast cells and subjected to western blotting with an anti-peptide ETR-3 antibody (Fig. 1C). Since the two variants were identified by non-quantitative RT-PCR and both proteins comigrate with endogenous ETR-3 in embryonic chicken heart, we performed RNase protection assays to determine which form was predominantly expressed during heart development. As shown in Fig. 1D, variant L is the predominant isoform expressed in both embryonic and adult chicken heart, while variant 4 is undetectable.

Identification of a C-terminal NLS

To determine the localization of ETR-3, we fused GFP at the N terminus of variant L (GFPcETR3vL) and transfected it into COS-M6 cells (Fig. 2A). This cell line was chosen for initial experiments because of its high transfection efficiency and its large, easily visualized nuclei. As shown in Fig. 2B, GFPcETR3vL is predominantly nuclear, as is variant 4 (data not shown). Western blotting with an anti-GFP antibody confirmed the proteins expressed were the expected sizes (Fig. 3B and data not shown).

The C terminus of ETR-3 is rich in arginine and lysine residues (Fig. 1A), which is

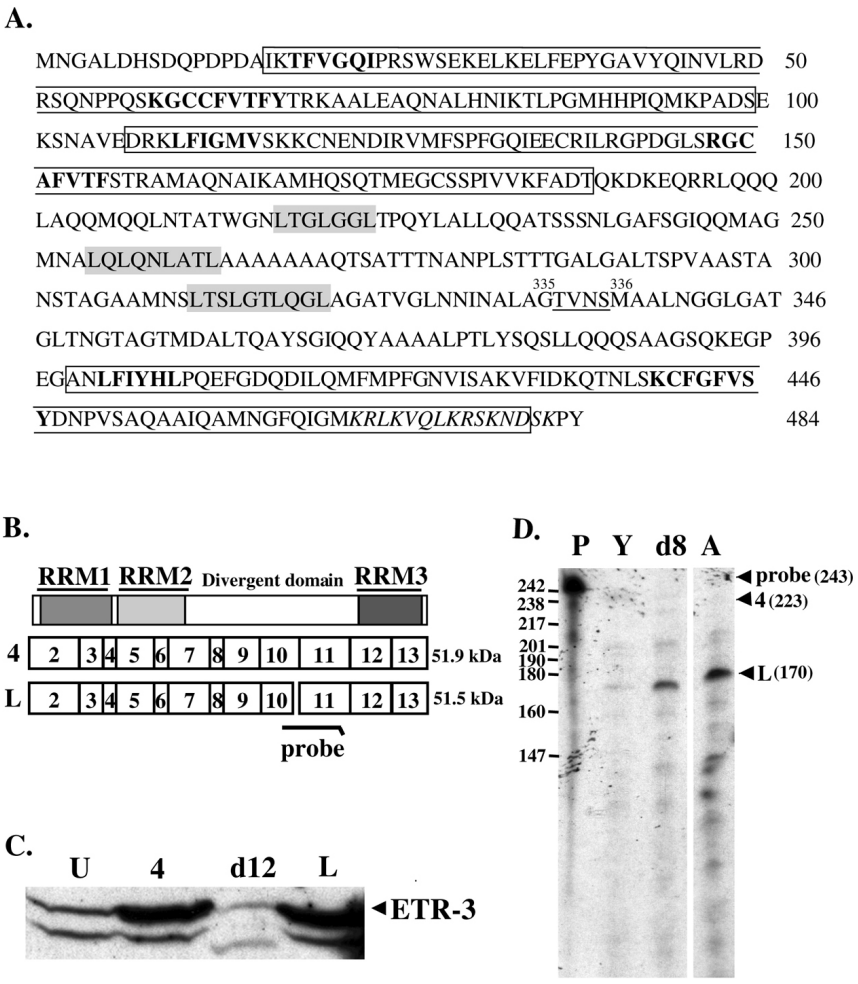

Fig. 1. Cloning of chicken ETR-3. (A) Chicken ETR-3 sequence. In human and mouse the *ETR-3* gene has at least five different first exons encoding three different N termini (Li et al., 2001) (and unpublished observations). The open reading frame of chicken ETR-3 was amplified using primers containing the termination codon and the furthest downstream start codon. RRMs are boxed, with RNP sequences in bold. Leucine-rich regions in the divergent domain are highlighted in gray, and an arginine/lysine-rich region at the C terminus is in italics. Residue numbers shown to the right are based on variant L sequence; the four amino acids unique to variant 4 (see below) fall between residues 335 and 336 of variant L and are underlined. (B) Two full-length isoforms of ETR-3 generated by alternative splicing were amplified by RT-PCR from embryonic chicken heart RNA. Variant 4 is identical to variant L except for use of an alternative 5′ splice site in an exon corresponding to human and mouse exon 10, leading to the insertion of four residues (TVNS) in the divergent domain. Exons are numbered based on comparisons to human and mouse genes. The probe used in D is shown. (C) Chicken ETR-3 variants were cloned into pcDNA3.1(+) expression vectors to express untagged proteins and transfected into quail QT35 fibroblasts. Transfected samples (4 and L) were compared to untransfected fibroblasts (U) and embryonic day 12 chicken heart (d12) by western blot with a rabbit polyclonal antibody against ETR-3. Both variants 4 and L comigrate at their predicted size (~52 kDa) with full-length ETR-3 in chicken heart. (D) RNase protection assays demonstrate that variant L is the predominant isoform expressed throughout heart development. P, undigested probe diagrammed as shown diagrammatically in B; Y, yeast RNA; d8, embryonic day 8 chicken heart RNA; A, adult chicken heart RNA. Arrowheads indicate the expected locations of the undigested probe and protected fragments for each isoform (4, variant 4 protected fragment; L, variant L protected fragment). The expected sizes for the probe and protected fragments are shown in parentheses. The two faint bands between the variant 4 and variant L protected fragment sizes are background bands, as they appear in all lanes including the yeast RNA control.

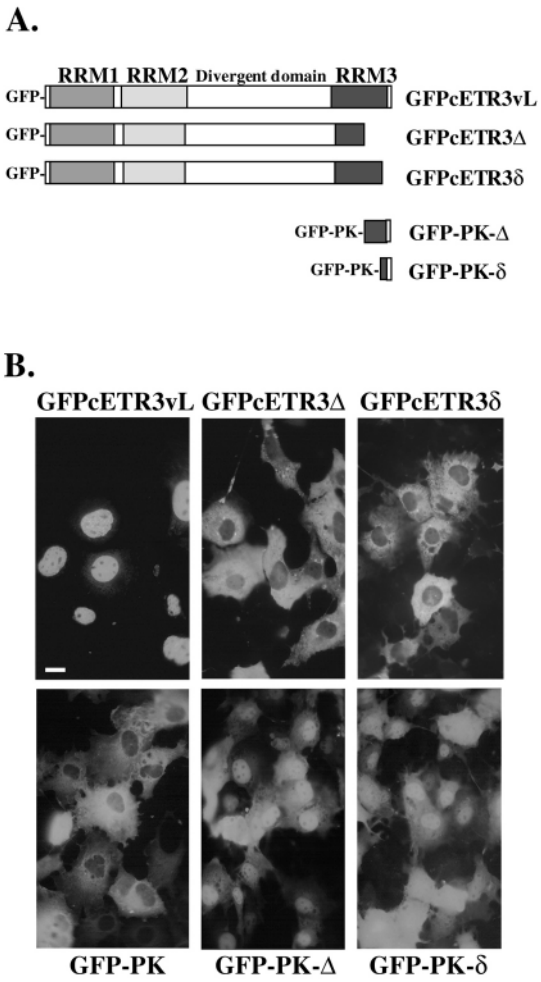

Fig. 2. Identification of a C-terminal NLS. (A) GFP was fused to the N terminus of full-length chicken ETR-3 variant L (GFPcETR3vL) and deletion constructs were subsequently made removing the last 58 (427-484) or 20 (465-484) amino acids from the C terminus, producing truncated proteins with molecular masses of 72.5 kDa or 76.6 kDa (GFPcETR3Δ and GFPcETR3δ, respectively). These C-terminal fragments were also fused to the C terminus of a GFP-PK fusion construct to produce chimeric proteins with molecular weights of 81.8 kDa (GFP-PK-Δ) or 77.7 kDa (GFP-PK-δ).
(B) GFPcETR3vL is nuclear. The C-terminal deletion constructs GFPcETR3Δ and GFPcETR3δ, in contrast, are predominantly cytoplasmic. The localization of GFP-PK is shifted to the nucleus by the addition of the C-terminal 58 or 20 amino acids of ETR-3 (GFP-PK-Δ and GFP-PK-δ, respectively). Scale bar: 10 μm.

common for NLS elements (Jans et al., 2000). To determine whether the C terminus of ETR-3 is required for its nuclear localization, deletion constructs were made that express truncated proteins missing the last 58 (GFPcETR3Δ) or 20 (GFPcETR3δ) amino acids (Fig. 2A). GFPcETR3Δ removes approximately half of RRM3, including RNP1, one of two highly conserved sequences required for the RNA binding activity of RRMs (Burd and Dreyfuss, 1994). GFPcETR3δ leaves the RNP motifs intact and removes only the arginine/lysine-rich region. Western blots confirmed both plasmids expressed protein of the expected sizes (Fig. 3B and data not shown). Both of these fusion proteins were cytoplasmic in COS-M6 cells, consistent with the presence of a C-terminal NLS (Fig. 2B).

To determine whether the C terminus is sufficient to drive nuclear localization, two gain-of-function constructs were made (Fig. 2A) that fused the last 58 (GFP-PK-Δ) or 20 (GFP-PK-δ) amino acids to the C terminus of a GFP-pyruvate kinase (GFP-PK) chimera that is known to be cytoplasmic (Sherman et al., 2001). Both the larger and smaller C-terminal segments bestowed nuclear localization on GFP-PK when expressed in COS-M6 cells, confirming the NLS activity at the C terminus of ETR-3 (compare GFP-PK-Δ and GFP-PK-δ to GFP-PK in Fig. 2B). Once again, western blots confirmed that correctly sized proteins were expressed (75.7, 81.8 and 77.7 kDa for GFP-PK, GFP-PK-Δ and GFP-PK-δ, respectively; data not shown).

Identification of divergent domain regions important for nuclear localization and export

To determine whether the divergent domain also contains regions necessary for nuclear localization, we made four serial 53-amino acid deletions in GFPcETR3vL (Fig. 3A). Western blots confirmed that all expression plasmids expressed proteins of the expected sizes (Fig. 3B). Deletions in the first three segments of the divergent domain had no effect, indicating that none of these regions is required for nuclear localization in COS-M6 cells (Fig. 3C). Deletion of the fourth divergent domain region (GFPDD.4), however, resulted in notably more cytoplasmic protein, indicating the presence of a region required for nuclear localization 66 residues upstream from the C-terminal NLS. This region is likely to promote nuclear import since an ETR-3 fusion protein lacking the C-terminal NLS accumulates in the nucleus when nuclear export is blocked despite being too large to enter the nucleus by passive diffusion (see below).

Although these results indicate that ETR-3 is predominantly nuclear and contains at least two regions that drive nuclear import, ETR-3 has been reported to be present and active in RNA processing in the cytoplasm in some cells (Mukhopadhyay et al., 2003). The divergent domain contains several leucine-rich motifs (Fig. 1A) that resemble nuclear export signals (NESs) recognized by the export protein CRM1 (Jans et al., 2000). Indeed, the predominantly cytoplasmic localization of GFPcETR3Δ or GFPDD.4 despite the presence of one region promoting nuclear import suggests the presence of at least one competing nuclear export signal. To determine whether the divergent domain contains regions required for the cytoplasmic localization of GFPcETR3Δ, we tested the effects of the four serial 53-amino acid divergent domain deletions described above on the localization of GFPcETR3Δ (Fig. 3A). Western blots confirmed that all expression plasmids expressed proteins of the expected sizes (Fig. 3B). Removal of the first two regions of the divergent domain (GFPDD.1Δ and GFPDD.2Δ) partially restored nuclear localization of protein lacking the C-terminal NLS, though the second less so than the first, consistent with the presence of nuclear export activity within these regions (Fig. 3C). Deletion of the third divergent domain region had no effect in COS-M6 cells. Deletion of the fourth region in conjunction with the C-terminal deletion (GFPDD.4Δ) gave mixed results, conveying substantial nuclear localization in half of the transfections but remaining

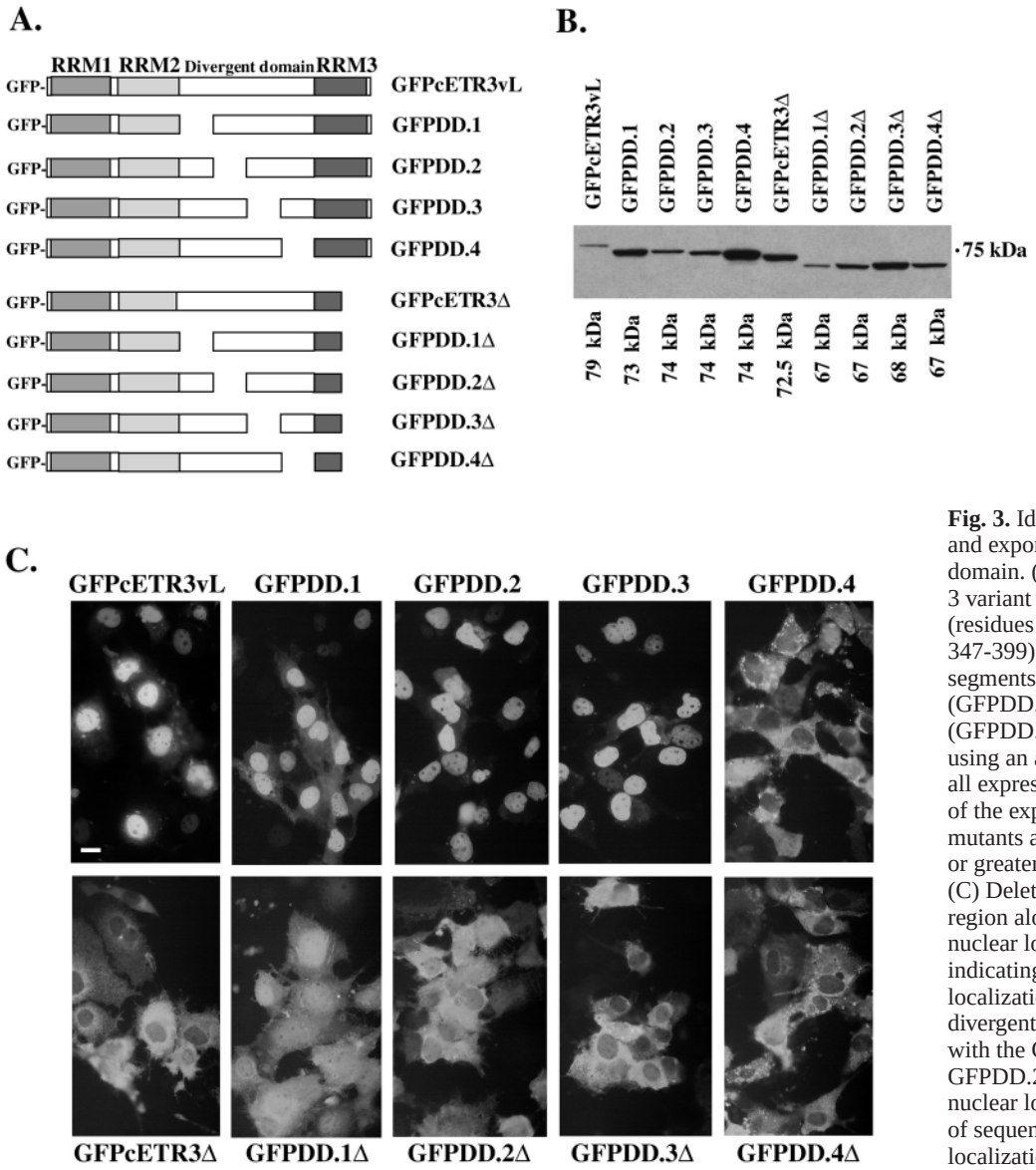

Fig. 3. Identification of nuclear localization and export elements in the divergent domain. (A) The divergent domain of ETR-3 variant L was divided into four parts (residues 188-240, 241-293, 294-346, and 347-399) and deletions of each of these four segments were made within GFPcETR3vL (GFPDD.1-4) and GFPcETR3Δ (GFPDD.1Δ-4Δ). (B) Western blot analysis using an anti-GFP antibody confirmed that all expression plasmids expressed proteins of the expected sizes, and the deletion mutants all expressed protein at comparable or greater levels than the full-length protein. (C) Deletion of the fourth divergent domain region alone (GFPDD.4) resulted in loss of nuclear localization in transfected cells, indicating this region is required for nuclear localization. Deletion of the first and second divergent domain regions in conjunction with the C terminus (GFPDD.1Δ and GFPDD.2Δ) resulted in partial restoration of nuclear localization, indicating the presence of sequences required for cytoplasmic localization. Scale bar: 10 μm.

predominantly cytoplasmic in the other half (the latter result is shown in Fig. 3C).

To determine whether a region within the divergent domain is sufficient to drive cytoplasmic localization, a gain-of-function construct was made in which the first three quadrants of the divergent domain corresponding to the regions deleted in GFPDD.1-3 (residues 188-346) were fused to the C terminus of NLS-GFP-PK, a GFP-PK chimera that has the SV40 large T antigen NLS attached to its N terminus (NLS-GFP-PK-DD.123; Fig. 4A). These constructs produced proteins of the expected sizes in COS-M6 cells (Fig. 4B). As expected, NLS-GFP-PK was restricted to the nucleus in both COS-M6 and primary embryonic chicken cardiomyocytes (Fig. 4C and data not shown). Although NLS-GFP-PK-DD.123 remained nuclear in COS-M6 cells (data not shown), in primary chicken cardiomyocytes fusion to this segment of the divergent domain caused NLS-GFP-PK to shift to the cytoplasm (Fig. 4C). These results suggest that the divergent domain does possess nuclear export activity, but it is too weak to overcome the activity of the strong SV40 NLS in some cell types.

Localization is similar in different cell types

To ensure that chicken ETR-3 is not aberrantly localized in COS-M6 cells, the full-length and deletion constructs shown in Fig. 3 were also transfected into quail QT35 fibroblasts and primary chicken cardiomyocytes. As shown in Table 1, the localization of full-length and truncated ETR-3 proteins are similar in all three cell types, with minor differences. Localization in QT35 fibroblasts is more ambiguous than in the other cell types, in that none of the proteins was found exclusively in either the nucleus or cytoplasm. Deletion of the third divergent domain region in conjunction with the C-terminal deletion (GFPDD.3Δ) resulted in partial restoration of nuclear localization in both QT35 cells and primary cardiomyocytes, but not in COS-M6 cells. Similarly, deletion of the fourth divergent domain region in the same context restored some nuclear localization in QT35 and

COS-M6 cells, but not in primary cardiomyocytes. These results suggest that the latter half of the divergent domain contains a region that is involved in nuclear export but is recognized with variable efficiencies in different cell types.

The first quadrant of the divergent domain and the C terminus of ETR-3 are required for splicing activity

ETR-3 is a potent activator of alternative exon inclusion in pre-mRNAs from cTNT minigenes (Ladd et al., 2001). To determine whether activity of ETR-3 parallels its localization, the constructs shown in Fig. 3 were cotransfected with R35C, a minigene containing a heterologous alternative exon flanked by the cTNT MSEs 1-4, which is known to be regulated by human ETR-3 (Ladd et al., 2001). Cotransfection assays were performed in both QT35 and COS-M6 cells, but not in primary cardiomyocytes as these cells express high levels of endogenous ETR-3 and already display a high basal level of exon inclusion for the R35C minigene. Full-length ETR-3 significantly promotes exon inclusion in both QT35 and COS-M6 cells (Table 1). The activity of GFPcETR3vL is similar to that of constructs expressing Xpress epitope-tagged human or chicken ETR-3 proteins (Ladd et al., 2001) (and data not shown), and GFP alone has no effect on splicing (data not shown). ETR-3 deletion mutants activate exon inclusion to varying degrees, but splicing activation did not always correlate with localization. None of the predominantly cytoplasmic forms of ETR-3 activated exon inclusion above basal levels, suggesting that their concentration in the nucleus has fallen below the threshold required for activation and/or that they lack a region required for activation. Interestingly, GFPDD.1 and GFPDD.1Δ activate poorly even though they are present in the nucleus, indicating that the first quadrant of the divergent domain contains a region important for the splicing activity of ETR-3.

GFPcETR3Δ and GFPDD.4 are predominantly cytoplasmic and are relatively inactive as regulators of cTNT alternative splicing (Fig. 2 and Table 1). To determine whether the loss of

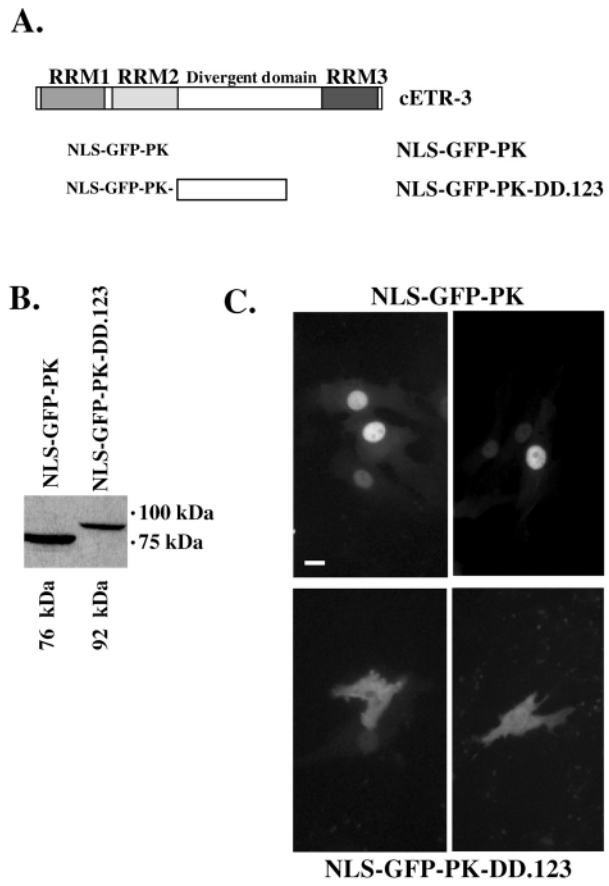

Fig. 4. A region of the divergent domain is sufficient for cytoplasmic localization in primary embryonic chicken cardiomyocytes. (A) The first three quadrants of the divergent domain (residues 188-346) of ETR-3 variant L were fused to the NLS-GFP-PK chimeric protein (NLS-GFP-PK-DD.123). (B) Western blot analysis using an anti-GFP antibody confirmed that proteins of the expected sizes were produced from both expression plasmids. (C) The NLS-GFP-PK chimeric protein is normally nuclear (top panels). Addition of the first three quadrants of the divergent domain resulted in cytoplasmic localization in transfected primary embryonic chicken cardiomyocytes (bottom panels). Scale bar: 10 μm.

Table 1. Activation of exon inclusion by GFPcETR3 constructs

	Localization			% Exon inclusion		
	CM	QT35	COS	QT35	COS	Splicing activity
cTNT alone	–	–	–	32.0±4.2	26.9±2.9	
GFPcETR3vL	N	N+c	N	55.5±0.7	67.7±7.3	+
GFPDD.1	N	N+c	N	39.5±0.7	37.5±0.7	+/–
GFPDD.2	N	N+c	N	59.5±0.7	61.0±2.8	+
GFPDD.3	N	N+c	N	61.5±2.1	63.0±0	+
GFPDD.4	N+C	N+C	n+C	41.0±1.4	35.8±1.7	+/–
GFPcETR3Δ	C	C/N+C	C	32.5±0.7	33.2±2.2	–
GFPDD.1Δ	N+C	N+C	N+C	27.0±0	26.5±0.7	–
GFPDD.2Δ	N+C	N+C	n+C	30.0±0	32.0±2.8	–
GFPDD.3Δ	N+C	N+C	C	31.5±2.1	29.0±1.4	–
GFPDD.4Δ	C	N+C	C/N+C	29.5±2.1	27.0±0	–

In addition to COS-M6 cells (COS), the constructs shown in Fig. 3 were transfected into primary chicken cardiomyocyte (CM) cultures and QT35 quail fibroblast cells and the subcellular localization was determined by microscopy. In parallel experiments, GFPcETR3 constructs were cotransfected with the R35C minigene into QT35 and COS-M6 cells. The average percentage of total mRNAs containing the alternative exon±s.e.m. for two to nine independent experiments are indicated. Where splicing activity is indicated (+ or +/–), the extent of exon inclusion differed significantly from that of the minigene alone ($P \leqslant 0.05$). Expression of the minigene did not affect protein expression or localization. RNA was harvested 48 hours after transfection and the extent of exon inclusion determined by RT-PCR analysis. N, nuclear; C, cytoplasmic. Capital letters denote substantial localization in a compartment, whereas lower case letters indicate low but detectable levels of fluorescence.

splicing activity in GFPcETR3Δ and GFPDD.4 is due to loss of nuclear localization, we fused the NLS from the SV40 large T antigen to the C terminus of GFPcETR3Δ (GFPcETR3ΔNLS) and GFPDD.4 (GFPDD.4NLS). The SV40 NLS restored nuclear localization to the truncated proteins in COS-M6 cells (Fig. 5A). Western blots confirmed that GFPcETR3ΔNLS and GFPDD.4NLS expressed proteins of the expected sizes (73.8 and 74.9 kDa, respectively) and that all four mutants expressed levels of protein at least comparable to that of the full-length protein (data not shown). Splicing activity was assessed by cotransfection with the R35C minigene. The restoration of nuclear localization did not restore the splicing activity of GFPcETR3Δ, as GFPcETR3ΔNLS activated exon inclusion only slightly above the basal level and comparable to levels induced by GFPcETR3Δ (Fig. 5B). In contrast, restoring nuclear localization to the divergent domain deletion significantly increased its splicing activity, as GFPDD.4NLS activated inclusion significantly above the level induced by GFPDD.4. These results indicate that the fourth quadrant of the divergent domain is not required for splicing activity, but the C terminus of chicken ETR-3 is important for its splicing function independent of its role in nuclear localization.

Export of ETR-3 from the nucleus is CRM1 dependent

Nuclear export of many proteins occurs via the CRM1 pathway. To determine whether export of ETR-3 from the nucleus is CRM1 dependent, we tested the sensitivity of ETR-3 localization to LMB. CRM1 is inhibited by LMB, resulting in nuclear accumulation of shuttling proteins whose export depends on CRM1 (Nishi et al., 1994; Kudo et al., 1998). Since GFPcETR3vL is already predominantly nuclear, we used the cytoplasmic GFPcETR3Δ for these experiments. Although this construct lacks the C-terminal NLS, it still retains the fourth quadrant of the divergent domain, which may be sufficient for nuclear import. GFP-PK served as our negative control as it lacks NLS and NES sequences, does not shuttle, and is cytoplasmic because it is too large to diffuse into the nucleus. A chimera possessing the first 71 amino acids of the HIV-1 Vpr protein (GFP-PK-Vpr) was used as a positive control, as this has been demonstrated to be cytoplasmic but accumulates in the nucleus in response to LMB (Sherman et al., 2001). GFP-PK, GFP-PK-Vpr or GFPcETR3Δ were transfected into COS-M6 cells, cultured for 24 hours, and treated with 1 μM LMB for 1 hour. As expected, GFP-PK remained cytoplasmic following LMB treatment, while GFP-PK-Vpr accumulated in the nucleus (Fig. 6). GFPcETR3Δ was cytoplasmic in untreated and mock-treated cells, but like GFP-PK-Vpr accumulated in the nucleus following exposure to LMB (Fig. 6 and data not shown). Nuclear accumulation of GFPcETR3Δ was consistently observed in four independent experiments and the extent of accumulation was comparable to that observed for the positive control in each case. This demonstrates that GFPcETR3Δ does possess both nuclear import and export sequences, and that its cytoplasmic localization is dependent on the CRM1 pathway for export from the nucleus.

To confirm that the CRM1 dependence of GFPcETR3Δ can be attributed to NES activity within the divergent domain, NLS-GFP-PK-DD.123, the gain-of-function construct containing the first three quadrants of the divergent domain,

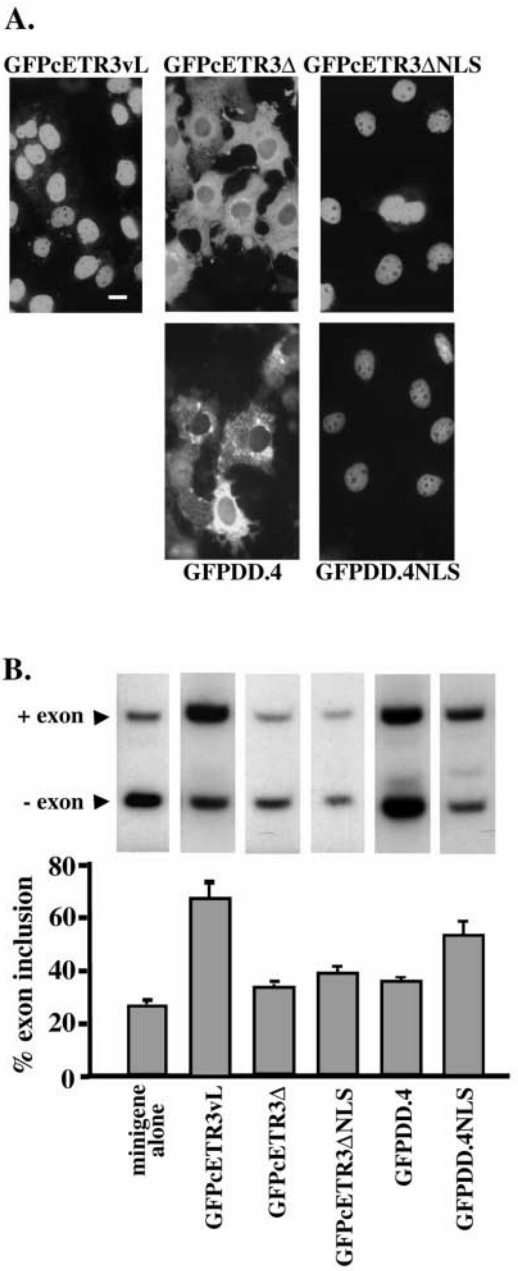

Fig. 5. Nuclear localization does not restore full splicing activity in the absence of the C terminus. (A) Incorporation of the SV40 large T antigen NLS at the C-termini of the GFPcETR3Δ and GFPDD.4 fusion proteins (GFPcETR3ΔNLS and GFPDD.4NLS, respectively) conferred nuclear localization in COS-M6 cells. Scale bar: 10 μm. (B) RT-PCR analysis was performed (upper panel) to determine the extent of exon inclusion (lower panel) in cotransfection assays with a cTNT minigene. While all of the GFP-ETR3 fusion constructs gave levels of exon inclusion that were statistically different from that of the minigene alone ($P \leqslant 0.05$), GFPcETR3Δ and GFPDD.4 activated exon inclusion only slightly above the basal level. The activity of the C-terminal deletion mutant was not enhanced by the addition of an NLS, as GFPcETR3ΔNLS did not activate levels of exon inclusion significantly higher than those observed for GFPcETR3Δ. When the divergent domain deletion was restored to the nucleus, however, GFPDD.4NLS enhanced exon inclusion significantly above the level observed for GFPDD.4 and close to the level observed for the full-length protein. The average percentages of total mRNAs containing the alternative exon plus or minus the standard error of the mean are shown for each ($n \geqslant 4$).

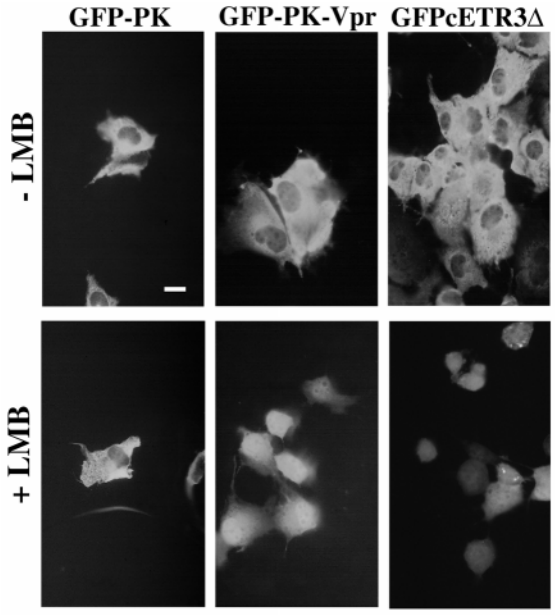

Fig. 6. Nuclear export of ETR-3 is leptomycin B (LMB)-sensitive. GFPcETR3Δ is cytoplasmic in the absence of LMB in untreated (upper right panel) or mock-treated cells (data not shown), but accumulates in the nucleus following one hour of exposure to 1 μM LMB similar to a positive control containing known NLS and LMB-sensitive NES elements (GFP-PK-Vpr). In three experiments the nuclear concentration of GFPcETR3Δ increased in the presence of LMB, but some GFPcETR3Δ was retained in the cytoplasm (this result is shown). In a fourth experiment, LMB treatment resulted in an exclusively nuclear localization of GFPcETR3Δ (data not shown). The extent of nuclear accumulation of GFPcETR3Δ in each case is comparable to GFP-PK-Vpr. A cytoplasmic fusion protein that does not enter the nucleus (GFP-PK) is not affected by LMB treatment. No differences in viability were observed in LMB-treated versus untreated or mock-treated cells. Scale bar: 10 μm.

was also tested for LMB sensitivity. GFP-PK-Vpr and NLS-GFP-PK-DD.123 are both cytoplasmic in primary cardiomyocytes but accumulate in the nucleus in response to LMB treatment, though the effective dose of LMB has a high level of toxicity in these cells (data not shown).

RRM1 and RRM2 are important for localization in the cytoplasm

To determine whether the RRMs participate in the localization of ETR-3, 89 amino acids were deleted from RRM1 or RRM2 (Fig. 7A). These RRMs have been shown to be important for binding of *Xenopus* and human ETR-3 to RNA (Good et al., 2000; Singh et al., 2004). Deletions were made in both GFPcETR3vL (to find regions important for nuclear localization) and GFPcETR3Δ (to find regions important for cytoplasmic localization). Western blots confirmed the expression of proteins of appropriate sizes from these plasmids (data not shown). As shown in Fig. 7B, when transfected into COS-M6 cells deletion of neither affects the predominantly nuclear localization in the presence of the C-terminal NLS, but both restore significant nuclear localization in its absence, suggesting the first two RRMs contain sequences important for a presence in the cytoplasm.

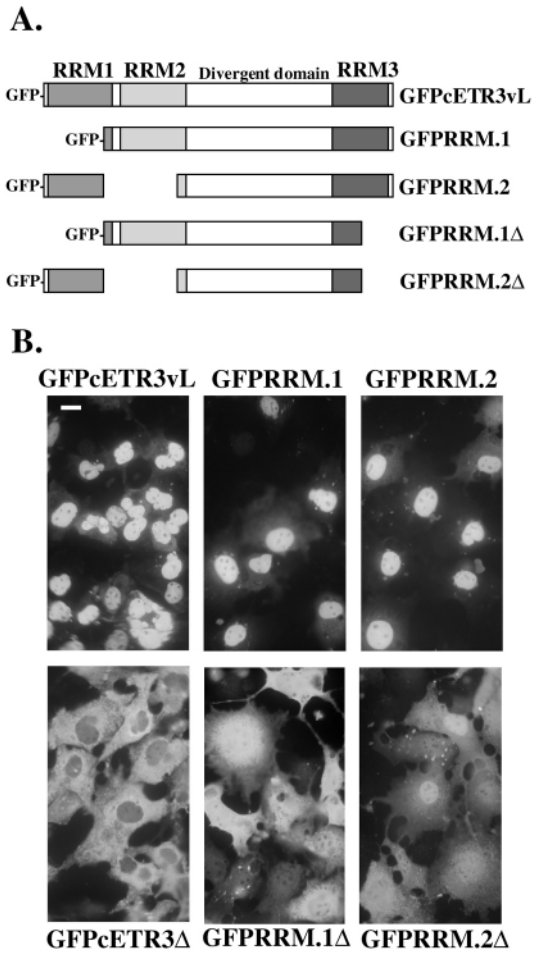

Fig. 7. Identification of regions important for cytoplasmic localization in RRM1 and RRM2. (A) Deletions within the first two RRMs of ETR-3 (residues 1-89 and 90-178, respectively) were made within GFPcETR3vL (GFPRRM.1-2) and GFPcETR3Δ (GFPRRM.1Δ-2Δ), producing truncated proteins with molecular masses of 68.9 kDa (GFPRRM.1), 69.0 kDa (GFPRRM.2), 62.4 kDa (GFPRRM.1Δ), and 62.6 kDa (GFPRRM.2Δ). (B) Deletions in RRM1 and RRM2 alone had no effect on localization, but deletions made in conjunction with the deletion of the NLS at the C terminus resulted in partial nuclear localization, indicating sequences within the first two RRMs are important for cytoplasmic localization of ETR-3. Scale bar: 10 μm.

Discussion

This study provides the first reported characterization of localization domains within a member of the CELF family. Members of this recently described family of RNA binding proteins have been implicated in the regulation of diverse nuclear and cytoplasmic RNA processing events, including alternative splicing (Philips et al., 1998; Ladd et al., 2001; Savkur et al., 2001; Charlet et al., 2002a; Suzuki et al., 2002; Zhang et al., 2002; Gromak et al., 2003), RNA editing (Anant et al., 2001), mRNA stability (Mukhopadhyay et al., 2003), deadenlyation and translation (Paillard et al., 1998; Good et al., 2000; Timchenko et al., 2001; Paillard et al., 2002; Mukhopadhyay et al., 2003; Paillard et al., 2003). All members of this family share a common domain structure (Ladd et al.,

2001), yet little is understood about these proteins outside of the highly conserved amino acids that constitute the RRMs. The discovery of functional sites within the CELF proteins may help us elucidate the mechanism(s) by which they regulate RNA processing events.

Identification of regions required for splicing activity

We identified two regions of the chicken ETR-3 protein that are required for splicing activity: the first quadrant of the divergent domain and the C terminus (Fig. 8). A protein missing either of these regions fails to activate exon inclusion even when present in the nucleus, suggesting these regions are important functional domains independent of their roles in localization. ETR-3 binds directly to its pre-mRNA targets, and probably improves recognition of the weak exon by assembling an activation complex that recruits the basal splicing machinery and/or disrupts repressive complexes that would otherwise prevent spliceosome assembly (Charlet et al., 2002a). RNA binding is mediated via the first two RRMs (Good et al., 2000), so it is unlikely that mutants lacking either the first quadrant of the divergent domain or the C terminus are inactive because of a failure to interact with pre-mRNA targets. This suggests that these domains are involved in mediating protein-protein interactions within the ETR-3 activation complex. At this time, it is not known what proteins interact with the CELF proteins to influence alternative splicing. The domains identified here may be used in the future in yeast two-hybrid or genetic screens to find CELF protein partners.

Interestingly, in contrast to the results presented here for the chicken ETR-3 protein, similar deletions of the first quadrant of the divergent domain or the C terminus of the human ETR-3 protein do not impair its splicing activity (Singh et al., 2004). This suggests that human ETR-3 has greater redundancy within its functional domains than chicken ETR-3. This is surprising given that human and chicken ETR-3 protein sequences are 97% identical. There are, however, small differences within the divergent domains of these proteins, and use of an alternative splice site results in the inclusion of six additional amino acids (VAQMLS) in the divergent domain of the human protein at the exon 10/exon 11 boundary. We are currently investigating the functional consequences of these differences.

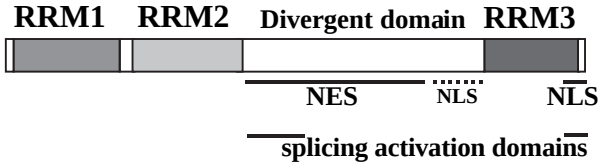

Fig. 8. Functional domains important for localization and splicing activity of chicken ETR-3. Deletion and gain-of-function analyses revealed an NLS within the last 20 amino acids at the C terminus and CRM1-dependent nuclear export activity within the divergent domain. A second region involved in nuclear localization in the last 53 amino acids of the divergent domain (dotted line) was identified by deletional analysis. Regions within the first two RRMs were also identified by deletional analysis that are important for cytoplasmic localization, though this is perhaps due to the RNA binding capacity of these domains. The first quadrant of the divergent domain and the C terminus were also found to be important for splicing activation by ETR-3 independent of its localization to the nuclear compartment.

Identification of localization elements within ETR-3

We have identified several regions of the ETR-3 protein that influence its subcellular localization (Fig. 8). The presence of both nuclear import and export elements and the ability of a truncated ETR-3 protein (GFPcETR3Δ) to accumulate in the nucleus in the presence of LMB support the proposal that ETR-3 normally shuttles between the nucleus and cytoplasm. Translocation from the nucleus to the cytoplasm may be a means of regulating its function and/or activity in a given cellular context (see below). We demonstrated that two regions of the protein contain NLS activity, the C-terminal 20 amino acids and the last 53 amino acids of the divergent domain (Fig. 8). The C-terminal NLS is rich in arginine and lysine residues, typical of conventional NLSs recognized by importin proteins (Jans et al., 2000), and is sufficient to drive nuclear localization in a heterologous context. In contrast, the fourth quadrant of the divergent domain contains very few basic amino acids and does not contain any sequences that resemble consensus NLSs. One possibility is that the divergent domain contains a novel NLS. Another possibility is that this region does not contain an NLS per se, but rather mediates the interaction between ETR-3 and another protein or complex that contains nuclear targeting signals. If so, however, the expression of this nuclear partner is not cell type-restricted, as deletion of this region was required for nuclear localization in all three cell types tested. A third possibility is that the fourth quadrant of the divergent domain does not contain NLS activity, but removal of this region prevents the NLS at the C terminus from being recognized in the mutant protein. The accumulation of GFPcETR3Δ in the nucleus when nuclear export is blocked demonstrates, however, that ETR-3 can be targeted to the nucleus without the C-terminal NLS. Since this protein is too large for passive diffusion into the nucleus, this suggests the presence of a second NLS, making this third interpretation unlikely.

The divergent domain also possesses a region that mediates nuclear export via the CRM1 pathway (Fig. 8). This region contains multiple leucine-rich motifs that resemble known CRM1-dependent NESs. Removal of 89 amino acids from either of the first two RRMs also resulted in loss of cytoplasmic ETR-3. RRMs are highly conserved domains that do not contain the leucine-rich motifs that typify CRM1-dependent NESs. These regions may contain novel NESs or domains for interacting with cytoplasmic proteins. The first two RRMs mediate binding of ETR-3 to RNA (Good et al., 2000), raising additional possibilities. For example, binding to RNA might change the conformation of ETR-3, making NES sequences in the divergent domain more accessible. Alternatively, ETR-3 might be exported to the cytoplasm while bound to exported RNA. Finally, ETR-3 could be retained in the cytoplasm by binding to RNAs that are held in cytoplasmic complexes. Interaction with these complexes would be consistent with the identification of cytoplasmic RNA processing functions for ETR-3. RNA binding activity is required for cytoplasmic accumulation of another RNA binding protein, ADAR1, which is involved in adenosine to inosine RNA editing (Strehblow et al., 2002). By constructing chimeric constructs with human

ADAR1, Shrehblow and colleagues found that the strength of RNA binding did not correlate with localization, but the type of RNA binding domain did, suggesting interaction with specific components in the cytoplasm were critical for retention of ADAR1 in that compartment. The presence of different types of nuclear import and export elements within ETR-3 may suggest that its localization is controlled by multiple, perhaps competing, mechanisms. The balance between these forces may shift depending on the cellular context, altering the ratio of ETR-3 protein distributed between the nucleus and cytoplasm.

Regulating the activity of ETR-3 via changes in localization

Because ETR-3 has both nuclear and cytoplasmic roles, changing its subcellular localization is one means by which its participation in different processes can be regulated. There is evidence supporting such a model. In the human colon cancer HT-29 cell line, ETR-3 is predominantly nuclear, but when cells are subjected to γ-irradiation ETR-3 appears at high levels in the cytoplasm as well as the nucleus (Mukhopadhyay et al., 2003). In the irradiated cells, it was also shown that ETR-3 binds to COX-2 messages and silences translation while stabilizing the RNA, promoting apoptosis (Mukhopadhyay et al., 2003). Thus the translocation of ETR-3 from the nucleus to the cytoplasm allows it to act on target RNAs in the cytoplasm, ultimately determining cell fate. So what causes ETR-3 to shift between compartments? Now that we have identified regions within the protein that are important for both nuclear and cytoplasmic localization, we can better hypothesize about the mechanisms driving translocation.

A common means of modulating nuclear to cytoplasmic ratios is to prevent recognition of targeting sequences by the import/export machinery. Masking of localization elements can occur by modification of the element itself or by interaction with heterologous molecules (Jans et al., 2000). The arginine/lysine-rich NLS at the C terminus of ETR-3 contains potential phosphorylation and glycosylation sites, which could be modified to mask the NLS. Protein-protein interactions could also affect localization. The MyoD family of transcription factors is functionally repressed by binding to the inhibitory protein I-mf, which masks an NLS and causes retention of the transcription factors in the cytoplasm (Chen et al., 1995). The divergent domain of ETR-3 has been suggested to be the site of protein-protein interactions (Good et al., 2000) and contains regions important for both nuclear localization and export. Moreover, masking molecules need not be proteins. The RNA and DNA binding regions of many nuclear proteins overlap or are in close proximity to NLSs, leading to the speculation that targeting signals within RNA- and DNA-binding proteins are at times masked by binding to their nucleic acid substrates (Jans et al., 2000). Regions within RRM1 and RRM2 are required for cytoplasmic localization of ETR-3, and the NLS at the C terminus overlaps significantly with the third RRM. It is not known whether the RNA binding activity of ETR-3 affects its localization within the cell.

The role of ETR-3 localization in the pathogenesis of disease states

ETR-3 has been implicated in the pathogenesis of a number of diseases. ETR-3 is a candidate gene for defects associated with partial monosomy 10p (Lichtner et al., 2002) and familial arrhythmogenic right ventricular dysplasia (Li et al., 2001). It has also been implicated in the misregulation of splicing in Duchenne and Becker muscular dystrophies (Sironi et al., 2003), and may play a role in the misregulation of alternative splicing in myotonic dystrophy (DM).

DM is an autosomal dominant disorder caused by expansion of an unstable CTG repeat in the 3′ UTR of the *DMPK* gene (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992) or a CCTG repeat in the first intron of the *ZNF9* gene (Liquori et al., 2001). DM is a multisystemic disorder characterized by myotonia, muscle wasting, cardiac conduction defects, insulin resistance, cataracts, testicular atrophy and cognitive dysfunction (Harper, 1998). CUG-BP, the CELF protein most closely related to ETR-3, is normally expressed in both the nucleus and cytoplasm, but aberrantly accumulates in the nucleus of cells in DM patients (Roberts et al., 1997). Three known targets of CUG-BP, cTNT, insulin receptor (IR) and muscle-specific chloride channel (ClC-1), display aberrant ratios of alternative splice products in DM muscle in a manner consistent with increased CUG-BP activity in the nucleus (Philips et al., 1998; Savkur et al., 2001; Charlet et al., 2002b). The misregulation of alternative splicing of these targets can explain insulin resistance and myotonia, two prominent symptoms seen in affected individuals (Savkur et al., 2001; Charlet et al., 2002b). Although it is not known whether ETR-3 is aberrantly localized in DM, it is normally expressed in the tissues most affected by the disease, and can regulate the alternative splicing of cTNT and IR similarly to CUG-BP (Ladd et al., 2001; Savkur et al., 2001). ETR-3 is therefore another excellent candidate for mediating altered splicing regulation in DM cells.

It is important to remember, however, that the increase of CELF protein activity in the nucleus in DM cells may only be half of the story. Loss of CUG-BP and/or ETR-3 activity in the cytoplasm also leads to misregulation of cytoplasmic target RNAs, resulting in inappropriate levels of translation or changes in mRNA stability that alter the abundance of key proteins in the cell. During skeletal muscle differentiation, the translation of p21, a cyclin-dependent kinase (cdk) inhibitor that is involved in cell cycle control, is up-regulated in normal but not DM cells (Timchenko et al., 2001). CUG-BP specifically binds to sequences in the p21 mRNA and induces translation in vitro and in vivo (Timchenko et al., 2001). The decrease in p21 in DM cells is consistent with loss of CUG-BP from the cytoplasm. The lack of p21 in turn explains elevated cdk4 levels that could contribute to differentiation defects observed in the muscle of DM patients. Interestingly, ETR-3 protein is dramatically up-regulated during skeletal muscle differentiation (Ladd et al., 2001) in both the nucleus and cytoplasm (unpublished observation). This suggests ETR-3 may also play a significant role in the cytoplasmic regulation of CELF protein targets during the differentiation process and contribute to differentiation defects in DM.

We thank Minoru Yoshida (RIKEN) for generously providing leptomycin B, Warner Greene (UCSF) for gifts of plasmids, Donnie Bundman, Nicole Nguyen and Lauren Murphy for technical assistance, and André Faustino, Thai Ho and Mary Moore for helpful discussions on the manuscript. This work was supported by grants to

T.A.C. from the NIH (HL45565). A.N.L. was supported by a postdoctoral NRSA fellowship from the National Institute of Arthritis and Musculoskeletal and Skin Diseases.

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