The EMBO Journal Vol. 22 No. 23 pp. 6356–6364, 2003

The PTB interacting protein raver1 regulates α-tropomyosin alternative splicing

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Regulated switching of the mutually exclusive exons 2 and 3 of α-tropomyosin (TM) involves repression of exon 3 in smooth muscle cells. Polypyrimidine tract-binding protein (PTB) is necessary but not sufficient for regulation of TM splicing. Raver1 was identified in two-hybrid screens by its interactions with the cytoskeletal proteins actinin and vinculin, and was also found to interact with PTB. Consistent with these interactions raver1 can be localized in either the nucleus or cytoplasm. Here we show that raver1 is able to promote the smooth muscle-specific alternative splicing of TM by enhancing PTB-mediated repression of exon 3. This activity of raver1 is dependent upon characterized PTB-binding regulatory elements and upon a region of raver1 necessary for interaction with PTB. Heterologous recruitment of raver1, or just its C-terminus, induced very high levels of exon 3 skipping, bypassing the usual need for PTB binding sites downstream of exon 3. This suggests a novel mechanism for PTB-mediated splicing repression involving recruitment of raver1 as a potent splicing co-repressor.

Keywords: alternative splicing/hnRNP/PTB/raver1/smooth muscle

Introduction

Alternative splicing is one of the major mechanisms that allows for an expressed proteome that is far more complex than predicted by the number of genes in a genome (Black, 2000; Graveley, 2001; Maniatis and Tasic, 2002; Modrek and Lee, 2002; Roberts and Smith, 2002). It allows the production of more than one protein, in some cases thousands of isoforms, from a single gene. Production of functionally distinct isoforms is usually regulated in a cell type-, developmental stage- or signal-specific manner.

Pre-mRNA splicing occurs in the multisubunit spliceosome, which assembles via recognition of the consensus splice site elements by a series of protein–RNA and RNA–RNA recognition events (Burge *et al*., 1999). Regulation of splicing of particular splice sites or exons, as occurs in alternative splicing, can be achieved by activation or inhibition (Smith and Valcárcel, 2000; Caceres and Kornblihtt, 2002). Spliceosome assembly can be assisted by additional interactions of 'SR' splicing factors with splicing enhancer sequences, which are commonly found within exons (Blencowe, 2000; Cartegni *et al*., 2002). SR proteins have a number of distinct roles in splicing and have been characterized as both constitutive and alternative splicing factors (Manley and Tacke, 1996; Graveley, 2000). A number of negative regulators have been identified, many of which are members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family (Krecic and Swanson, 1999; Smith and Valcárcel, 2000; Dreyfuss *et al*., 2002). These proteins contain RNA-binding domains as well as various accessory domains, but they do not contain the arginine-serine rich domains characteristic of the SR family. One of the better characterized splicing repressors is polypyrimidine tract-binding protein (PTB; also known as hnRNP-I; reviewed in Wagner and Garcia-Blanco, 2001). High-affinity PTB binding sites act as splicing silencers when present within or adjacent to regulated exons (Singh *et al*., 1995; Perez *et al*., 1997a; Gooding *et al*., 1998; Southby *et al*., 1999; Zhang *et al*., 1999; Chou *et al*., 2000; Wagner and Garcia-Blanco, 2002). PTB usually mediates widespread repression of target exons in multiple cell types, consistent with its widespread expression (Gil *et al*., 1991; Patton *et al*., 1991). In specific cell types, which vary according to the particular splicing event, PTB-mediated repression is somehow relieved. In some cases, this relief may be achieved by replacement of PTB by one of its tissue-restricted paralogs such as nPTB/brPTB (Markovtsov *et al*., 2000; Polydorides *et al*., 2000). In other cases, dynamic antagonism between PTB and CELF proteins may give rise to derepression (Charlet *et al*., 2002; Suzuki *et al*., 2002; Zhang *et al*., 2002; Gromak *et al*., 2003).

An exceptional example of PTB-regulated splicing is provided by the α-tropomyosin (TM) gene. PTB-mediated repression of TM exon 3 only occurs to high levels in smooth muscle (SM) cells (Wieczorek *et al*., 1988; Gooding *et al*., 1994), allowing selection of the mutually excusive TM exon 2. Exon 3 has stronger splice site elements than exon 2, particularly its 50 nt polypyrimidine tract (P3), which enforces its selection in most cells (Mullen *et al*., 1991; Zamore *et al*., 1992). Exon 2 has strong splicing enhancers, but these seem to mediate general rather than SM cell-specific activation (Dye *et al*., 1998). Repression of exon 3 requires PTB binding sites within the P3 pyrimidine tract (Figure 1A), as well as in a pyrimidine tract downstream of the exon, referred to as DY (Gooding *et al*., 1994, 1998; Perez *et al*., 1997a). In addition, two clusters of CUG/UGC motifs on either side of the exon are also required (Gooding *et al*., 1994; Gromak and Smith, 2002). Mutation of the PTB binding

sites impairs regulation of transfected constructs in SM cells, and this correlates with decreased binding of PTB to the mutated RNAs *in vitro* (Perez *et al*., 1997a; Gooding *et al*., 1998). In HeLa extracts, addition of excess PTB leads to repression of exon 3 (Lin and Patton, 1995; Singh *et al*., 1995), while addition of PTB binding RNA competitors (Gooding *et al*., 1998) or depletion of PTB (Wollerton *et al*., 2001) leads to a loss of the minor but detectable background level of exon 3 skipping. Overexpression of PTB in SM cells led to a small increase in exon 3 skipping if the long PTB4 isoform was transfected, but a decrease with the shorter PTB1 isoform (Wollerton *et al*., 2001). In contrast to all other known examples of PTB-regulated splicing, with TM exon 3, strong repression only occurs in SM cells despite the widespread expression of PTB. In this case, rather than tissue-specific relief of PTB-mediated repression, cell-specific regulation presumably involves additional factors that enhance the repressive effect of PTB.

The protein raver1 was recently identified in two-hybrid screens by its ability to interact with the cytoskeletal proteins α-actinin and vinculin. It was also found to interact with, and co-localize in the nucleus with, PTB (Hüttelmaier *et al*., 2001). Raver1 contains three RRM type RNA binding domains, a long proline rich C-terminal extension, and both nuclear localization and nuclear export signals. Consistent with its two-hybrid interactions with both nuclear and cytoskeletal proteins, raver1 is a shuttling protein that can be located predominantly in either the nucleus or the cytoplasm. Although its function in either compartment is not clear, obvious possibilities include the modulation of activities that are mediated by PTB, including alternative splicing (Hüttelmaier *et al*., 2001). TM alternative splicing is an interesting candidate, since PTB is known to be necessary but not sufficient for regulation.

Here, we show that raver1 can switch selection of the TM mutually exclusive exons towards use of the SM-specific exon 2. This effect is achieved by inhibition of exon 3 and it is mediated primarily via the PTB-binding *cis*-acting regulatory elements, but not by the UGC motif regulatory elements. Direct recruitment of just the C-terminus of raver1 can bypass the requirement for an essential PTB binding element. Our data suggest a novel mechanism for PTB-mediated splicing repression involving recruitment of a potent co-repressor.

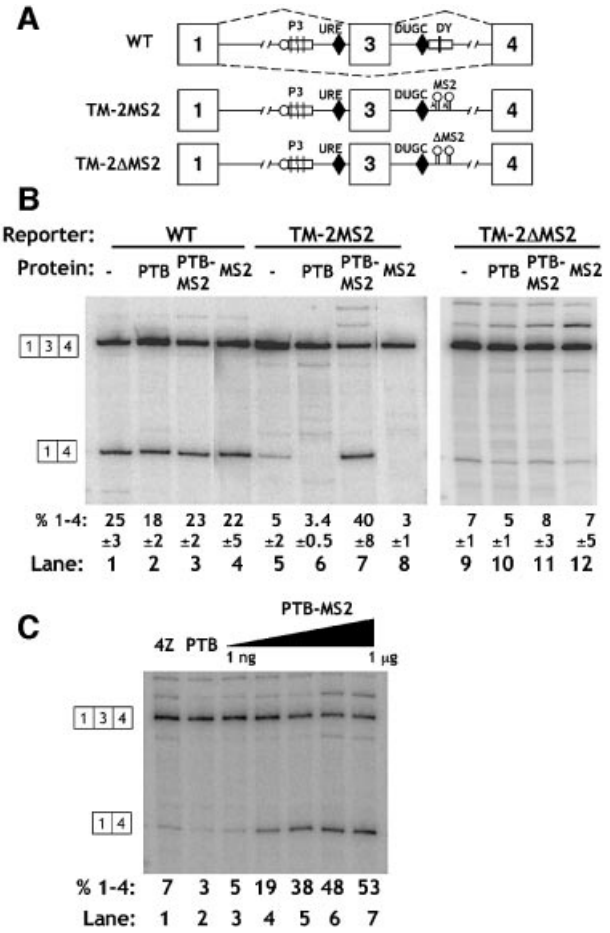

Fig. 1. Regulation of TM splicing by artificial recruitment of PTB. (**A**) The wild-type TM construct (pT2) contains exons 1, 3 and 4 with the four defined regulatory elements flanking exon 3. Deletions of exon 2 and flanking sequences and in the intron between exons 3 and 4 are denoted by the diagonal lines. P3 and DY are pyrimidine tracts (rectangles) containing optimal PTB-binding UCUU motifs (vertical lines). URE and DUGC contain clusters of UGC motifs (diamonds). Construct TM–2MS2 has the DY tract replaced by two MS2 binding sites, while TM–2ΔMS2 has two mutant MS2 sites, lacking the essential bulged A in the stem–loop. (**B**) The three reporters were co-transfected into PAC-1 SM cells with expression constructs for PTB, PTB–MS2, MS2 or pGEM4Z (4Z) negative control. Spliced RNA was analyzed by RT–PCR. PTB–MS2 was able to restore exon skipping to TM–2MS2 (compare lanes 1 with 5 and 5 with 7). (**C**) TM–2MS2 reporter was co-transfected with 1 μg pGEM4Z (lane 1), 1 μg PTB expression plasmid (lane 2), or 1, 10, 100, 500 or 1000 ng of PTB–MS2 plasmid (lanes 3–7). PTB–MS2 caused a dose-dependent increase in TM exon 3 skipping.

Results

Artificial recruitment of PTB restores regulation of splicing

While various lines of evidence point to the importance of PTB as a regulator of TM splicing (see Introduction), experiments in SM cells have been restricted to correlating the effects of mutations that impair PTB binding *in vitro* with impairment of splicing regulation in PAC-1 SM cells (Perez *et al*., 1997a; Gooding *et al*., 1998), or over-expression of PTB (Wollerton *et al*., 2001). To obtain more direct evidence for the role of PTB in SM cells, we adopted a heterologous recruitment approach using the bacteriophage MS2 coat protein (Graveley and Maniatis, 1998; Del Gatto Konczak *et al*., 1999; Dauksaite and Akusjarvi, 2002). We replaced the PTB-binding DY element with two copies of the binding site for MS2 coat protein (construct TM–2MS2; Figure 1A). A control construct (TM–2ΔMS2) contained two MS2 sites with a deletion of a bulged adenosine that is essential for high-affinity binding of coat protein. In transfected PAC-1 cells, the constructs with the MS2 sites showed 4- to 5-fold lower levels of exon skipping than the wild-type construct (Figure 1B, compare lanes 1, 5 and 9), consistent with the replacement of the essential DY regulatory element (Gooding *et al*., 1998). Co-transfection of a PTB–MS2 fusion protein completely restored exon skipping to the TM–2MS2 construct so that levels of exon skipping actually exceeded those with the wild-type construct

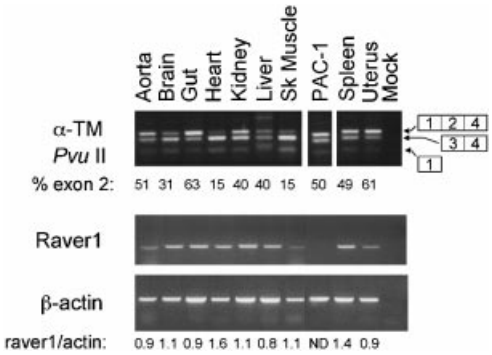

Fig. 2. Raver1 is expressed in tissues that regulate TM splicing. TM splicing was analyzed by RT–PCR followed by digestion with *Pvu*II, which digests the 134 product at the exon 1:3 junction, but leaves the 124 product intact. The percentage of exon 2 containing product is indicated below each lane. Raver1 and β-actin expression were analyzed by semi-quantitative RT–PCR using RNA from various rat tissues and from the rat PAC-1 smooth muscle cell line. The ratio of raver1/β-actin RT–PCR product levels are shown underneath the β-actin panel, with the exception of PAC-1 cells (ND, not determined) where the raver1 was undetectable with the number of PCR cycles used. Raver1 was widely expressed in tissues including gut, aorta and uterus, where the major TM splicing pattern is exon 2 inclusion.

(Figure 1B, lane 7), but had negligible effect upon the wild type or mutant MS2 constructs (lanes 3 and 11). The experiment shown used PTB1–MS2, but experiments with PTB4–MS2 produced identical results (F.Robinson and C.W.J.Smith, unpublished observations) despite the fact that PTB4 is a more potent repressor when overexpressed as a non-fusion protein (Wollerton *et al*., 2001). Co-expression of either component part of the fusion protein also had no major effect upon splicing of any of the constructs (PTB or MS2 alone; Figure 1B, lanes 2, 4, 6, 8, 10, 12), except that PTB transfection caused a small decrease in exon skipping by the wild-type construct, as reported previously (lane 2) (Wollerton *et al*., 2001). Titration of PTB–MS2 plasmid with the TM–MS2 reporter showed a gradual dose-dependent increase in exon skipping (Figure 1C, lanes 3–7). Transfection of 1 μg of PTB–MS2 plasmid produced >10-fold the amount of exon skipping observed with an equivalent PTB co-transfection (Figure 1C, lanes 2 and 7). These data indicate that artificial recruitment of PTB downstream of exon 3 can complement the defect caused by deletion of the DY regulatory element. This provides strong support for the involvement of PTB in regulation of TM splicing in PAC-1 cells.

Raver1 regulates TM splicing

The preceding data confirm the importance of PTB in mediating repression of TM exon 3 in PAC-1 cells. However, PTB is not sufficient for repression of TM exon 3 (see Introduction). Raver1 was recently characterized as a PTB-interacting protein, but currently no function has been ascribed to it (Hüttelmaier *et al*., 2001). We therefore decided to test whether raver1 might be able to influence TM alternative splicing. We first investigated the expression of raver1 in a range of rat tissues where TM splicing is regulated (Figure 2). Raver1 and β-actin expression were detected by RT–PCR, while TM splicing was analyzed by RT–PCR followed by digestion with *Pvu*II, which cuts the 1-3-4 spliced product, but not the 1-2-4 SM-specific isoform. Raver1 was expressed in all the SM tissues where TM splicing is regulated, although, as reported previously, it is also widely expressed in other tissues (Hüttelmaier *et al*., 2001). The levels of raver1 relative to β-actin in cultured PAC-1 SM cells were far less than in any of the tissue samples, and were undetectable at PCR cycle numbers necessary to prevent saturation of the PCR from tissue samples. However, higher cycle numbers of PCR and western blot (see below) showed that raver1 is expressed in PAC-1 cells.

Having established that raver1 is expressed in tissues where TM splicing is regulated, we next tested the effects of raver1 overexpression in PAC-1 and HeLa cells. Both cell types express similar levels of endogenous raver1 and PTB, as detected by western blots (data not shown). Raver1 was co-transfected with the construct TS23D (Figure 3A), which contains TM exons 1–4 and is appropriately regulated in transfected PAC-1 cells (Figure 3A, lanes 3) (Gooding *et al*., 1994). Since the two alternatively spliced products give rise to identically sized 291 bp RT–PCR products, they were digested with either *Pvu*II, which cuts specifically at the splice junction of exon 1 with exon 3, or with *Xho*I, which cuts at the 5′ end of exon 2. Compared with negative control co-transfections (pGEM4Z or pCMV-βGal; Figure 3A, lanes 1–4 and 9–12) raver1 co-transfection led to an increase in inclusion of TM exon 2 in both HeLa and PAC-1 cells as indicated by the increase in the amount of either the *Pvu*II-resistant 291 bp band, or the 141 bp *Xho*I product (compare lane 6 with lane 2 or 10, and lane 7 with lane 3 or 11). In HeLa cells, the quantity of exon 2 containing product was still relatively minor, but in PAC-1 cells it became the major product (Figure 3A, lanes 6 and 7). Therefore, overexpressed raver1 can switch selection of the TM mutually exclusive exons towards the regulated pattern.

Previous analyses have shown that TM splicing in SM cells is regulated via inhibition of exon 3 (Gooding *et al*., 1994, 1998; Perez *et al*., 1997a; Gromak and Smith, 2002). We therefore tested whether the raver1-mediated regulation of TM splicing also occurred independently of exon 2 by co-transfecting raver1 with the construct pT2, in which exon 2 has been deleted (Figure 3B). This construct typically gives rise to 20–30% skipping of TM exon 3 in PAC-1 cells. Titration of a co-transfected raver1 expression plasmid had a dose-dependent effect, increasing exon 3 skipping by up to 3-fold (Figure 3B). Therefore, raver1 exerts a regulatory effect upon TM splicing by inhibiting exon 3.

Raver1 represses TM exon 3 via PTB binding elements

Four negative regulatory elements are essential for skipping of TM exon 3. These include PTB binding sites within the P3 polypyrimidine tract and the downstream DY pyrimidine tract, and two clusters of UGC motifs designated URE and DUGC (Figure 4, top). We tested whether the raver1 effect upon splicing could be linked to any of these elements by co-transfecting raver1 with a series of mutant constructs in which the elements had been mutated, either individually or in pairwise combinations. The mutations of the UGC elements were short deletions

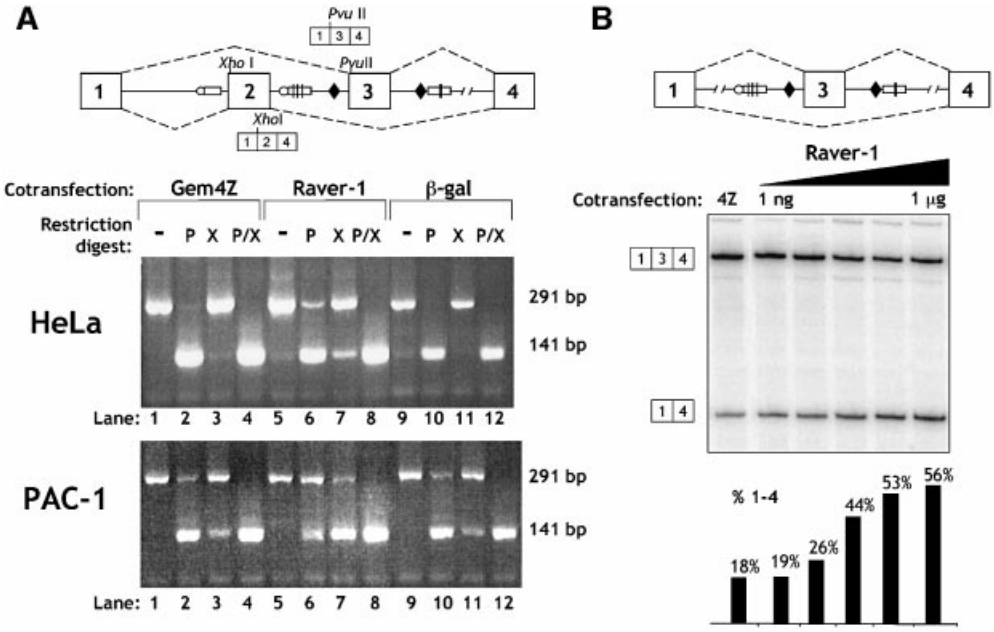

Fig. 3. Raver regulates TM splicing. (**A**) The TM splicing reporter TS23D, containing TM exons 1–4, was transfected into HeLa and PAC-1 SM cells along with pGEM4Z, or expression plasmids for raver1 or β-gal. Splicing was analyzed by RT–PCR followed by digestion with *Pvu*II (exon 3 specific, lanes 2, 6 and 10), *Xho*I (exon 2 specific, lanes 3, 7 and 11) or both enzymes (lanes 4, 8 and 12). Raver1 promoted splicing of exon 2, as indicated by the increase in the 141 bp *Xho*I product in lanes 7. (**B**) Increasing amounts of raver1 expression plasmid (1, 10, 100, 500, 1000 ng) were co-transfected into PAC-1 cells with the reporter plasmid pT2, which lacks exon 2 but retains all essential regulatory elements. RNA was analyzed by RT–PCR and PhosphorImager. Raver1 caused a dose-dependent increase in TM exon 3 skipping.

(15 and 23 nt for URE and DUGC, respectively). The mutations of the P3 element (P3Δ123) involved point mutations at each of the three PTB consensus sites, while mutations of the DY pyrimidine tract involved complete deletion (ΔDY) or deletion of 12 nt including the two overlapping optimal PTB sites (DYΔPC).

As expected, compared with the wild-type construct all of the mutants had decreased amounts of exon skipping in PAC-1 cells (Figure 4, compare '–raver' lanes). However, they exhibited distinct responses to raver1 co-transfection. The constructs with deletions of the UGC elements (ΔURE, ΔDUGC and ΔURE/ΔDUGC) still exhibited a robust response to raver1. For instance, while the wild-type construct increased exon skipping by 35% in response to raver1, the ΔUREΔDUGC double mutant had a 26% response. In contrast, the PTB binding site mutants (P3Δ123, ΔDY, DYΔPC) had a significantly reduced response (6, 10 and 2%). The double PTB site mutants (P3Δ123ΔDY and P3Δ123DYΔPC) showed no response to raver1, similar to the mutant in all four regulatory sites. These data clearly indicate that the response of raver1 is highly dependent upon the previously characterized PTB binding *cis*-acting regulatory elements, but independent of the UGC motif elements.

Raver1 itself has three RRM type RNA binding domains. One interpretation of the dependence of the raver1 effect upon PTB binding sites is that raver1 competes for binding to the same sites as PTB, but that it is a stronger repressor. If this were the case, over-expression of PTB would be expected to antagonize the effect of raver1. In order to test this possibility, a limiting amount of raver1 expression plasmid (100 ng) was co-transfected with the TM reporter. As previously, this led to a significant increase in exon skipping (Figure 5, lanes 1 and 2). Titration of a co-transfected PTB1 expression plasmid (Figure 5, lanes 3–7), had no major effect. There was a slight increase in exon skipping with intermediate

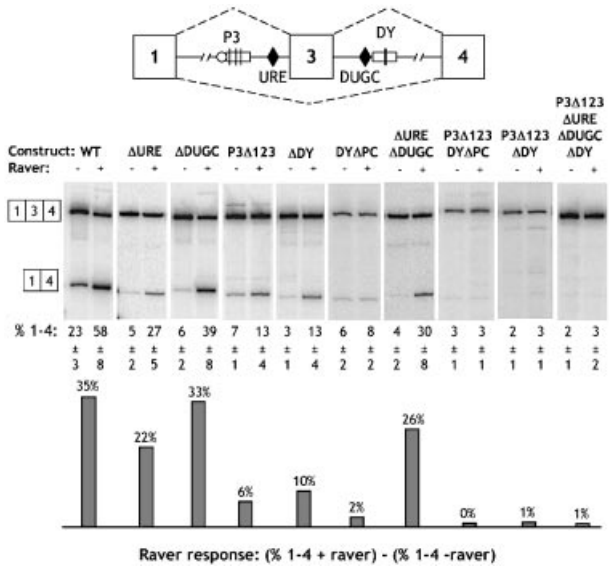

Fig. 4. Raver regulation is mediated by essential PTB binding elements. TM constructs containing mutations in one or more of the four essential regulatory elements (P3, URE, DUGC, DY) were transfected in the presence or absence of a raver1 expression plasmid into PAC-1 cells. RNA was analyzed by RT–PCR and quantified by PhosphorImager. The percentage of exon skipping is mean ± SD for at least three independent experiments. The 'raver response' was quantified as the arithmetic difference in the percentage of exon 3 skipping in the presence and absence of raver1, and is illustrated in the histogram below. Raver response was critically dependent upon the PTB binding elements, but not upon the UGC motif elements.

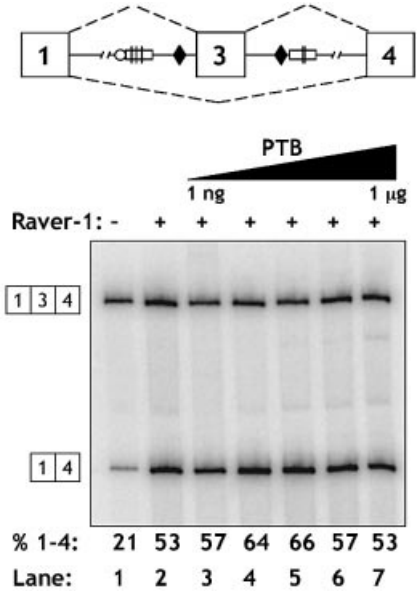

Fig. 5. Raver1 is not antagonized by PTB. The wild-type pT2 TM reporter was co-transfected in the presence or absence of 100 ng raver1 expression plasmid. A titration of PTB1 expression plasmid (1, 10, 100, 500, 1000 ng) was carried out in the presence of the raver plasmid. Splicing was analyzed by RT–PCR and quantified by PhosphorImager.

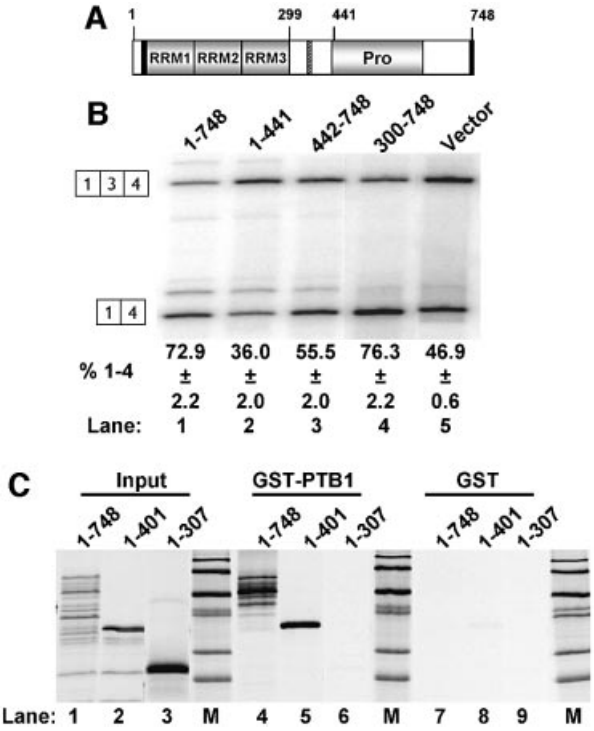

Fig. 6. Raver1 activity depends upon a PTB-interacting segment. (**A**) Schematic structure of raver1, with amino acid numbering. The N-terminal part contains the three RRMs and interacts with PTB. The C-terminal region contains a proline-rich region. The two nuclear localization signals are shown as black boxes and the nuclear export signal as a dashed box. (**B**) The wild-type TM reporter was co-transfected into PAC-1 cells along with constructs expressing the indicated segments of raver1 (lanes 1–4) or empty vector (lane 5). Note that the degree of regulated exon skipping of the reporter construct in this series of experiments (lane 5) was approximately twice that observed in the other experiments shown. Numbers below each lane represent mean ± SD of three experiments. (**C**) [^{35}S]methionine labeled *in vitro* translated raver1 proteins corresponding to amino acids 1–748, 1–401 or 1–307 were incubated with GST–PTB1 (lanes 4–6) or GST (lanes 7–9), and then pulled down with glutathione–agarose beads. The 'input' lanes contain 20% of the equivalent reactions shown in the pull-down lanes. Lanes 'M' contain radiolabeled protein markers (97, 67, 58, 56, 43 and 36 kDa).

levels of PTB expression plasmid (Figure 5, lanes 4 and 5), but with a 10-fold excess of the PTB plasmid the level of exon skipping was the same as with raver1 alone (compare lanes 2 and 7). This therefore suggests that raver1 and PTB do not compete for binding to the same sites. The more likely explanation for the dependence of raver1 upon the PTB binding elements is that it interacts directly with PTB (Hüttelmaier *et al*., 2001) and that PTB binding to these elements helps to recruit raver1.

Raver C-terminal domain is a potent splicing repressor

The N-terminal half of raver1 contains the three RRM domains and is able to interact with PTB and vinculin in a two-hybrid assay, while the C-terminal half interacts with actinin (Figure 6A; Hüttelmaier *et al*., 2001). As a first step to analyzing the requirements for different domains of raver1 for splicing regulatory activity, we tested the deletion constructs raver1 1–441 and 442–748, which lack the C- and N-terminus, respectively. Both constructs contained additional nuclear localization signals to ensure that the deletions did not cause relocalization to the cytoplasm. Full-length raver1 again caused a significant increase in exon skipping (Figure 6B, compare lane 1 with 5), although in this series of experiments the high levels (~46%) of regulated exon skipping limited the extent of the raver1 effect. Neither of the deletion constructs 1–441 or 442–748 had a similar effect to full-length raver1. Construct 1–441 caused a reproducible decrease in exon skipping (Figure 6, lane 2) while the C-terminal 442–748 led to a small increase in exon skipping, which was one-third of that achieved by full-length raver1 (lane 3). Strikingly, a mutant entirely lacking the N-terminal RRM domains (300–748; Figure 6, lane 4) was as active as full-length raver1 in inducing exon skipping. Thus the RRMs of raver1 are dispensable for alternative splicing activity in this assay. Previous two-hybrid assays had shown that raver1 interacts with PTB within the N-terminal 441 amino acids. In order to further analyze the regions of raver1 that mediate the interaction with PTB and their relationship to raver1 activity, we performed *in vitro* pull-down assays using GST–PTB1 and [^{35}S]methioine *in vitro* translated proteins corresponding to full-length raver1 (1–748) or amino acids 1–401 and 1–307. While full-length raver1 and 1–441 were able to interact with PTB (Figure 6, lanes 4 and 5), 1–307 was not. Thus, a region encompassing raver1 amino acids 307–401 is necessary for the interaction with PTB. Significantly, deletion of this region between constructs 300–748 and 442–748 caused a substantial loss in the alternative splicing activity of raver1 (Figure 6B). Taken together, the data shown in Figure 6B and C suggest that the effect of raver1 upon TM alternative splicing depends upon its ability to interact with PTB.

The dependence of raver1-mediated effects upon binding sites for PTB in TM RNA (Figure 4) and upon domains that mediate its interaction with PTB (Figure 6) suggest

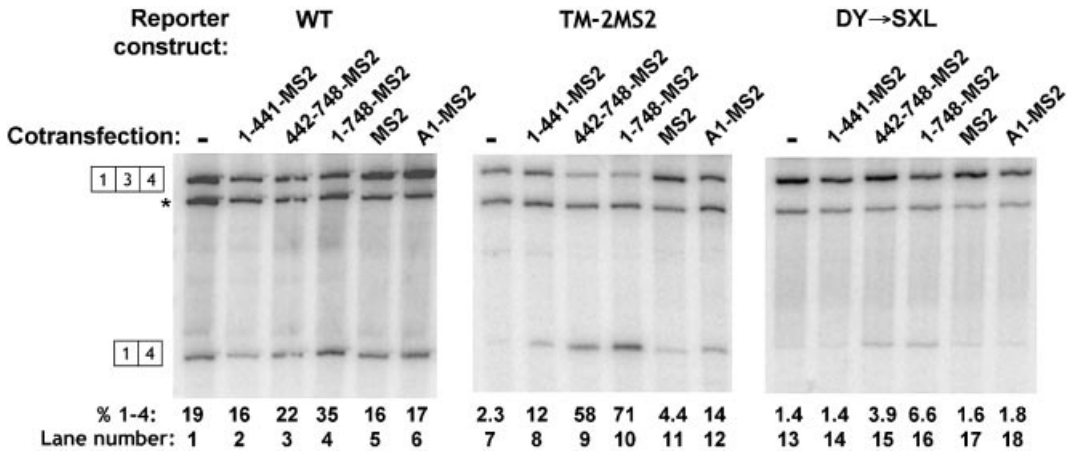

Fig. 7. Direct recruitment of raver1 C-terminal domain bypasses PTB binding site requirement. Tropomyosin alternative splicing reporters (500 ng) were co-transfected into PAC-1 cells with expression constructs (60 ng) for various MS2 fusion proteins. Lanes 1–6, wild-type TM reporter (WT); lanes 7–12, TM–2MS2 reporter with DY element replaced by two MS2 binding sites; lanes 13–18, DY→SXL reporter with DY element replaced by SXL binding site (Gooding *et al*., 1998). Co-transfection was with pGEM4Z negative control (lanes 1, 7 and 13), raver1 1–441–MS2 (lanes 2, 8 and 14), raver1 442–748–MS2 (lanes 3, 9 and 15), full-length raver1 1–748–MS2 (lanes 4, 10 and 16), MS2 alone (lanes 5, 11 and 17) or hnRNPA1-MS2 (A1-MS2; lanes 6, 12 and 18). The band marked with an asterisk is a PCR artifact that does not appear consistently between experiments (compare with Figures 1–5). Numbers below each lane represent the percentage exon skipping in the experiment shown. The results are qualitatively reproducible, but due to variations in the experimental procedures between repeats, SDs are not given.

that PTB might recruit raver1 to the RNA. In this case it might be possible to bypass the requirement for PTB by direct recruitment of raver1. We therefore tested the ability of raver1–MS2 fusion protein to induce exon skipping using the TM–2MS2 construct. Consistent with the recruitment model, co-transfection of raver1–MS2 with TM–2MS2 caused a dramatic increase in exon 3 skipping from 2 to 71% (Figure 6, lanes 7 and 10). In contrast, raver1–MS2 had more modest effects upon the wild-type TM construct (Figure 6, lanes 1 and 4), or a negative control in which the DY PTB binding site had been replaced by a SXL-binding pyrimidine tract (DY→SXL; Figure 6, lanes 13 and 16; Gooding *et al*., 1998). This result shows that artificial recruitment of raver1 to the site downstream of TM exon 3 can bypass the normal requirement for PTB binding at this site. We tested two further MS2 fusion proteins containing either the N-terminus (1–441–MS2) or C-terminus (442–748–MS2) of raver1 fused to MS2. Strikingly, fusion of the C-terminal domain of raver1 to MS2 was sufficient to confer potent exon skipping activity to the fusion protein, increasing exon skipping of the TM–2MS2 construct from 2 to 58% (Figure 6, lane 9), while having negligible effects upon the wild-type or DY→SXL controls (lanes 3 and 15). In contrast, 1–441–MS2 had little effect, even though it is the N-terminal domain that interacts with PTB (Hüttelmaier *et al*., 2001) (Figure 6C). Control experiments showed that 1–441–MS2 protein was abundantly expressed (data not shown). These data suggest that the C-terminus of raver1 has potent splicing repression activity and that the specificity of repression is conferred by the interactions between the N-terminus of raver1 with PTB and between PTB and its target regulatory sites.

Discussion

The experiments reported here provide the first evidence for a functional role of raver1, showing that it acts as a splicing repressor in the regulation of TM exon 3. Raver1 has the strongest effect upon TM alternative splicing of any potential regulator that we have tested to date. Consistent with its two-hybrid interactions and co-localization with PTB (Hüttelmaier *et al*., 2001), raver1's activity in the TM system appears to be intimately linked with that of PTB. Activity depends upon domains that interact with PTB, and upon the presence of intact PTB binding sites in the substrate pre-mRNA. Various lines of evidence suggest that the reliance upon PTB binding sites is not due to direct raver1 binding to these sites in place of PTB. Raver1 activity was not antagonized by overexpression of PTB (Figure 5), and FRET experiments show that co-transfected raver1 and PTB are present in a complex in the nuclei of transfected cells (S.Hüttelmaier and R.Singer, unpublished observations). Deletion of the three RRMs of raver1 did not affect its activity (Figure 6B, construct 300–748). However, further N-terminal deletion to 442–748, which removes residues shown to be critical for PTB interaction, significantly impaired activity (Figure 6B and C). In contrast, amino acids 442–748 were sufficient for full activity when recruited via the MS2 interaction (Figure 7). The data from the MS2 recruitment system provides strong evidence that transfected raver1 acts directly on TM splicing. The simplest model to accommodate all the data is that PTB binds to its high affinity sites, raver1 is recruited via protein–protein interactions with PTB, and that the recruited C-terminal domain of raver1 then acts as a potent repressor of the adjacent splice sites. The apparent dominant-negative activity of the raver1 1–441 mutant (Figure 6B) also lends support to this model. The RRMs of raver1 might also make additional RNA–protein contacts, which need not necessarily be sequence specific. However, the unimpaired activity of deletion mutant 300–748, which lacks the RRMs, shows that direct RNA binding by raver1's RRM domains probably plays at most a marginal role in regulation of TM alternative splicing (Figure 6B).

Raver1 therefore appears to satisfy the definition of a co-repressor, by conferring repression when recruited by a protein that binds to defined silencer elements. Future experiments to test this co-repressor model will require recombinant raver1 protein for *in vitro* RNA binding and splicing assays, and investigation of the effects of raver1 after depletion of PTB either *in vitro* (Southby *et al*., 1999) or *in vivo* (Wagner and Garcia-Blanco, 2002).

Conventional models of splicing repression by PTB involve either simple binding competition with U2AF^{65} at the polypyrimidine tract (Singh *et al*., 1995; Perez *et al*., 1997a), or co-operative binding of PTB to a series of sites (Chou *et al*., 2000), which could involve packaging of an extended region of RNA into a repressed state (reviewed in Wagner and Garcia-Blanco, 2001). Our data suggest that in the TM system, the requirement for PTB at the downstream site can be completely bypassed by direct recruitment of the raver1 C-terminal domain. This suggests that in the wild-type setting, one role of PTB might be to recruit the splicing repressor activity of raver1 to this site. This novel model for PTB activity might be peculiar to systems like TM, where the PTB-mediated repression only occurs strongly in a limited subset of cell types. In the majority of PTB-regulated systems, PTB is sufficient to confer repression without the apparent need of a co-repressor like raver1. Indeed, we have found that raver1 overexpression has only a modest effect upon repression of splicing of PTB exon 11, which is itself repressed by PTB (M.C.Wollerton and C.W.J.Smith, manuscript in preparation). We are currently using the MS2 system to define more precisely the splicing repressor domain within raver1. In splicing repressors such as SXL and hnRNP-A1, glycine-rich domains distinct from the RRMs are necessary for co-operative binding to RNA and for repression of splicing (Wang and Bell, 1994; Del Gatto Konczak *et al*., 1999). In the case of SXL, the glycine-rich domain also contacts splicing factor SPF45 and thereby inhibits step 2 of splicing (Lallena *et al*., 2002). It will be of interest to determine which of these two models for splicing repression—general repressive packaging or inhibitory interactions with splicing factors—is used during splicing repression by raver1.

The MS2 recruitment experiments also provide the most direct evidence to date for the role of PTB in the regulation of TM splicing *in vivo* (Figure 1). Restoration of splicing repression using PTB–MS2 fusion proteins has also been demonstrated in the FGFR2 system, where the role of PTB was also emphatically demonstrated by RNAi knockdown (Wagner and Garcia-Blanco, 2002). We have carried out RNAi knockdown of PTB in PAC-1 cells, but in general the cells become de-differentiated during the experiment, so that effects upon TM splicing are difficult to analyze. MS2 coat protein recruitment provides a very useful model system in which a structure–function analysis of PTB can be carried out. Deletion analyses of PTB have previously been carried out (Kaminski *et al*., 1995; Perez *et al*., 1997b; Oh *et al*., 1998; Conte *et al*., 2000; Liu *et al*., 2002), and in particular RRMs 3 and 4 appear to be responsible for high-affinity sequence-specific RNA binding. The artificial recruitment system should allow us to identify effector domains within PTB that are separable from the RNA binding function. It is an interesting possibility that such an effector domain could correspond to the region of PTB that is sufficient for interaction with raver1. A limitation of the MS2 system is that we can only analyze the role of PTB at the downstream DY site. The upstream PTB binding sites are embedded in the exon 3 polypyrimidine tract, where their replacement by MS2 sites would interfere with both steps of splicing when exon 3 is selected.

Despite the identification of raver1 as an important component of the regulatory machinery that represses TM exon 3 it is not the crucial cell-specific factor that switches TM splicing. Like PTB, raver1 is widely expressed and is not restricted to SM cells (Hüttelmaier *et al*., 2001) (Figure 2). Overexpression of raver1 in HeLa cells (Figure 3) demonstrates that abundant raver1 and PTB together are not sufficient for a complete switch in TM splicing. The fact that raver1 overexpression in PAC-1 cells has a strong effect on TM splicing suggests first that raver1 levels in PAC-1 cells are limiting, and second that PAC-1 cells contain factors other than PTB and raver1 that promote skipping of TM exon 3. One possibly interesting *trans*-acting factor is a PTB paralog, smPTB, which is abundantly expressed in a number of rodent SM tissues (Gooding *et al*., 2003). However, smPTB is not detectably expressed in PAC-1 cells, and is also not restricted to SM-containing rodent tissues. Moreover, unlike raver1 it does not have a strong effect upon TM splicing in co-transfection assays. More likely candidate regulators are the elusive factors that interact with the CUG/UGC regulatory elements flanking exon 3 (Gromak and Smith, 2002). These elements are essential for regulation of TM splicing, but they are not binding sites for PTB and raver1 acts independently of them (Figure 3).

While our experiments address a nuclear function of raver1 in regulation of alternative splicing, the nature of its possible cytoplasmic role is as yet unknown. Raver1 co-localizes with vinculin and α-actinin containing microfilament attachment structures, and an attractive possibility for its cytoplasmic role is in the localization of vinculin and α-actinin mRNAs to the sites where the proteins are required (Hüttelmaier *et al*., 2001). In addition to its direct role in the cytoplasm, alterations in the nuclear concentration of raver1 due to altered nuclear/cytoplasmic distribution could have an important effect in altering various splicing patterns, as has been shown for export of hnRNP-A1 in response to the p38 stress pathway (van der Houven van Oordt *et al*., 2000). Raver1 redistributes from nucleus to cytoplasm during differentiation of myogenic cells. It will be interesting to determine whether this redistribution plays a role in switching any of the large number of alternative splicing events that are altered during this differentiation program.

Materials and methods

Constructs

The raver1 expression construct contained the open reading frame of mouse raver1 cloned as an *Eco*RI–*Xho*I fragment with an N-terminal FLAG tag into pcDNA3 (Invitrogen) (Hüttelmaier *et al*., 2001). pCIMS2-NLS-FLAG is a vector for *in vivo* expression of MS2 coat protein driven by pCMV promoter (Del Gatto Konczak *et al*., 1999). hnRNPA1-MS2 contains hnRNPA1 cloned into a unique *Stu*I site downstream of FLAG tag in pCIMS2-NLS-FLAG (Del Gatto Konczak *et al*., 1999). To assist subsequent directional cloning a pair of oligonucleotides containing *Mlu*I, *Bss*HII and *Avr*II sites were cloned into the *Stu*I site. The *Mlu*I and *Avr*II

sites were used to insert the indicated amino acids from raver1. For expression of raver1 fragments without the MS2 fusion (Figure 6), the MS2 segment was removed by *Bam*HI digestion leaving raver1 fragments with a C-terminally added nulear localization signal. PTB expression constructs contained human PTB1 in the pCMV vector (Wollerton *et al*., 2001). The PTB–MS2 construct was created by subcloning the MS2 coding sequence as a *Bam*HI fragment downstream of PTB1 in pCMV. The wild-type TM splicing reporter pT2 was as described previously (Gromak and Smith, 2002) and contains TM exons 1, 3 and 4 in the context of flanking regulatory intron sequences. Expression of wild type is driven by the SV40 early promoter and enhancer. TS23D is a splicing reporter which contains TM exons 1–4, including mutually exclusive exons 2 and 3 as described previously (Gooding *et al*., 1994). TM–2MS2 reporter was cloned by replacing DY sequence in wild type with oligonucleotides (5′-GCACGCGTACACGATCACGGTACGCTGAA-TTAGATCTCTGCGGATAGCATGAGGATCACCCATGCTCCCG-GGG-3′) comprising two copies of the binding site for MS2 coat protein. TM–2ΔMS2 reporter contained a deletion of the bulged adenosine (underlined above) within MS2 sequence essential for high-affinity binding of coat protein. DY→SXL has the DY pyrimidine tract replaced by a SXL binding pyrimidine tract (Gooding *et al*., 1994). The ΔURE and ΔDUGC constructs have 15 and 27 nt deletions of the respective regulatory elements (Gromak and Smith, 2002). ΔURE/ΔDUGC construct contains both deletions. ΔDY was created by deletion of the complete DY sequence between *Fsp*I and *Eco*RI sites of wild type. ΔDRE construct was generated by deletion of the DRE sequence between *Bst*XI and *Eco*RI sites of wild-type reporter. ΔPC contains the deletion of 12 nt including two overlapping PTB binding sites within the DY sequence (Gooding *et al*., 1998). P3Δ123 contains three mutations within the PTB binding sites of P3: CUCUU→UUUUU, UCUU→UUUU and UCUU→CCUU. P3Δ123/ΔDY and P3Δ123/ΔPC reporters combine the corresponding individual mutations. P3Δ123/ΔU/ΔDRE contains mutations of all regulatory elements. All constructs were verified by DNA sequencing.

Cell culture, transfections and RNA analysis

Cell culture transfections and detection of expressed RNA was carried out as described previously (Gromak and Smith, 2002; Gromak *et al*., 2003). Radiolabeled splicing products were visualized on a Molecular Dynamics Storm 840 PhosphorImager. The relative ratios of the spliced products were determined using Molecular Dynamics ImageQuant software (version 1.11). The percentage of exon 3 skipping was expressed as [1-4/(1-3-4 + 1-4)] × 100%. All experiments were repeated at least three times independently to ascertain the reproducibility of results. For the detection of pTS23D RNA the PCR was carried out with SV5′2 (5′-GGAGGCCTAGGCTTTTGCAAAAAG) and TM4 (5′-CAGAGATGC-TACGTCAGCTTCAGC) primers. PCR consisted of 30 cycles: 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, followed by a final 2 min extension at 72°C. PCR products were phenol extracted, ethanol precipitated and resuspended in 35 µl of H_2O. The mixture was aliquoted in separate tubes (7 µl each) for undigested control, *Xho*I, *Pvu*II and *Xho*I–*Pvu*II digests in 10 µl reactions. The resulting digests were electrophoresed on 2.5% agarose gels.

Rat tissue RNAs were harvested using Tri-Reagent (Sigma). Reverse transcriptions were carried out on 2.5 µg total RNA using a mixture of oligo(dT) and random hexamer primers and AMV reverse transcriptase. PCR detection of raver1 used primers 5′Rav (5′-GTACATGACCTCCT-GAGCGACTAC) and 3′Rav (5′-GAGTCTTTCTTCATGTACTCTG-CAA-3′). Thirty cycles of PCR were carried out at 94°C for 30 s, 57°C for 30 s and 72°C for 30 s. For β-actin, PCR primers were: F, 5′-ATGGTGGGTATGGGTCAGAAGGACTC; and R, 5′-TAGGAGC-CAGGGCAGTAATC. Twenty-five cycles of PCR were carried out at 94°C for 30 s, 57°C for 30 s and 72°C for 30 s. For tropomyosin, PCR primers were: TM1, 5′-CGAGCAGAGCAGGCGGAG and TM4, 5′-CAGAGATGCTACGTCAGCTTCAGC. Thirty-five cycles of PCR were carried out at 94°C for 30 s, 62°C for 30 s and 72°C for 30 s. All PCRs used a hot start procedure and had a final extension at 72°C for 2 min. For raver1 and β-actin, cycle numbers were restricted to maintain amplification in the exponential phase. TM PCR samples were digested with *Pvu*II, which digests the 204 bp 1-3-4 product to 150 and 54 bp products, but leaves the 204 bp 1-2-4 product intact.

GST pull-down assays

Full-length and truncated [^{35}S]methionone labeled raver1 proteins were synthesized in 20 µl *in vitro* translation reactions. Five percent of the total reaction was retained to load directly on the gel and the remainder was further divided into two aliquots, which were diluted into 500 µl of binding buffer D (20 mM HEPES, pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 0.3% Tween 20). These were incubated with either 1 µg GST–PTB1 or 0.3 µg GST protein, which had been pre-bound to glutathione–Sepharose 4B beads. The sample was rotated at 4°C for 60 min. Beads were washed three times in binding buffer, resuspended in 30 µl SDS loading buffer, and 15 µl were loaded onto a 20% Laemmli gel.

Acknowledgements

Many thanks to Brigitte Jockusch for providing raver1 plasmids and monoclonal antibodies, and for helpful discussions and comments on the manuscript, Richard Breathnach for the MS2 vectors, and Sushma Nagaraja-Grellscheid for tissue RNA samples. This work was funded by a grant from the Wellcome Trust (059879) (to C.W.J.S.). A.R. is supported by a Wellcome Trust studentship.

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Received May 7, 2003; revised September 19, 2003; accepted October 13, 2003