



# Tethered function assays using 3' untranslated regions

Jeffery Collier<sup>\*,1</sup> and Marvin Wickens

*Department of Biochemistry, University of Wisconsin—Madison, Madison, WI 53706, USA*

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## Abstract

Proteins that regulate mRNA metabolism are often bipartite: an RNA binding activity confers substrate specificity, and a “functional” domain elicits the biological outcome. In some cases, these two activities reside on different polypeptides that form a complex on the mRNA. Regardless, experimental separation of RNA binding from function facilitates analysis of both properties, liberating each from the constraints of the other. “Tethered function” assays bring a protein to a reporter RNA through a designed RNA–protein interaction. The function of the tethered protein—whether that be stability, translation, localization, or transport, or otherwise—is then assessed. We refer to this approach as a “tethered function” assay, since it can be examined. The approach does not require knowledge of the natural RNA binding sites, or of the composition of the naturally occurring protein complexes. It can be useful in dissecting how proteins that act on RNAs work, and in identifying active components of multiprotein complexes. RNA-binding proteins previously have been linked to foreign RNA-binding specificities, for a wide variety of purposes. We emphasize here the particular value of tethering to the 3' untranslated region of eukaryotic mRNAs, and the opportunities it presents for the analysis of how those mRNAs are regulated. We discuss experimental design, as well as published and potential applications. © 2002 Elsevier Science (USA). All rights reserved.

## 1. Introduction and rationale

Proteins involved in controlling specific mRNAs often must bind RNA and elicit a “function.” Functions include alterations in RNA stability, processing, translational activity, and cellular localization, as well as covalent modifications. In some cases, RNA binding and biological function are inseparable: for example, the binding of RNA substrates to certain ribonucleases is inseparable from nucleolytic cleavage. In other instances, and arguably in the majority of instances involving mRNAs, binding activity and function are distinct. In these cases, the separation of the two activities from one another provides a powerful inroad into understanding how these proteins work, just as does the analogous approach to dissecting transcription factors.

To separate function from binding, the protein of interest can be tethered to an mRNA through its 3'

untranslated region (3'UTR), using known RNA–protein interaction (Fig. 1) [1,2]. By manipulating the tethered polypeptide, one can analyze the protein's biological activity stripped of the need to bind to RNA. This logically simple technique is termed a “tethered function” assay because a polypeptide to be tested is tethered with an aim of assessing any biological function—turnover, transport, translation, or localization, for example. Indeed, these four functions in particular have all been assessed using this strategy.

Of course, mRNA-binding proteins have previously been linked to foreign RNA binding specificities, with a variety of successful applications. Here, we specifically concentrate on applications in which the 3'UTR of a eukaryotic mRNA is the location of the tether. 3'UTRs have special biological interest and experimental advantages in such assays, particularly for analyzing proteins that control mRNA activity and biogenesis. A few of these advantages are listed below.

- *A common site of control elements:* Sites that regulate many steps in an mRNA's life often reside naturally in the 3'UTR. Thus tethering to that region places regulators where they might naturally function.

\* Corresponding author. Fax: 520-621-3709.

*E-mail address:* jmcoller@email.arizona.edu (J. Collier).

<sup>1</sup> Present address: Department of Molecular and Cellular Biology, Howard Hughes Medical Institute, University of Arizona, Tucson, AZ 85721, USA.

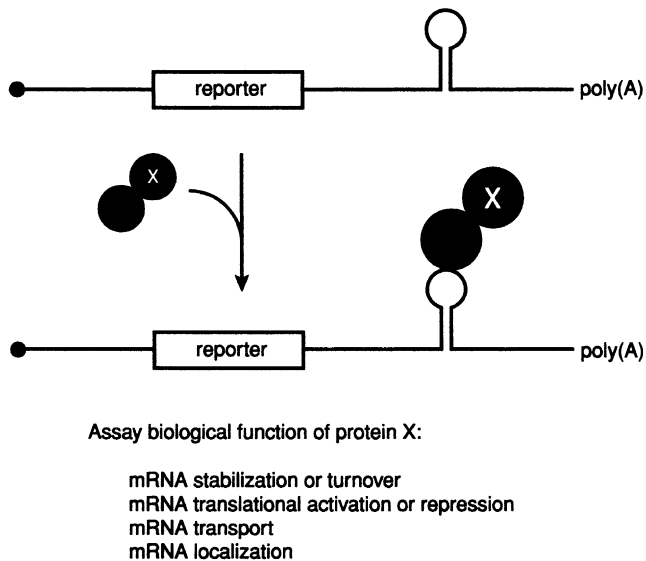


Fig. 1. Tethered function assays using the 3'UTR. A protein, X, is brought to a reporter mRNA through an artificial RNA–protein interaction. The function of the tethered protein in any aspect of the mRNA's metabolism or function can then be assayed.

- *Flexibility*: The exact location of several 3'UTR regulators within the 3'UTR is not critical for their function, implying that precise spatial positioning is not critical.
- *Noninterference with translation*: 3'UTRs have fewer constraints for insertion of tethering sites than do 5'UTRs or open reading frames (ORFs). Perturbances in the 5'UTR can affect initiation frequency, and RNA–protein complexes placed in the ORF may be displaced by the elongating ribosome. On the other hand, the intercistronic region of bicistronic mRNAs is relatively unconstrained and also has been used for tethered function experiments [3,4].

Certain regulatory proteins must reside in precisely the “right” position to function. For example, specific RNA–protein interactions prevent translation initiation when placed appropriately within 5'UTR of the mRNA (e.g., [5]); similarly, splicing regulators sometimes must be positioned correctly relative to splice sites. In such instances, tethering to the 3'UTR will not work.

## 2. Experimental strategy

### 2.1. *A priori* considerations

#### 2.1.1. Multiprotein complexes

mRNA regulatory events often occur through multiprotein complexes formed through protein–protein and protein–RNA interactions. The protein that contacts the mRNA may not always contain the activity responsible for the effects of the complex on mRNA

metabolism; conversely, the “active” protein may not directly contact the RNA. Tethered function assays permit an active molecule to be identified and analyzed (e.g., [6]). In certain instances, however, multiple proteins may be required (e.g., [7]).

#### 2.1.2. The role of RNA binding in function

The interaction of RNA with a protein can change the conformation of the RNA, the protein, or both [8]; as a result, function may require the RNA–protein complex, as opposed to the protein alone. The RNA ligand can influence the biological activity of the bound protein [9], much as in cases of DNA–mediated allostery with transcription factors [10]. In addition, the context of the natural binding site may be important because other factors are bound in the neighborhood, and are essential for function. These considerations have two implications. First, negative results in a tethered function assay are meaningless, even if the RNA and protein do interact on the reporter. Second, the outcome observed in a tethered function assay may differ when the protein is associated with its natural RNA binding site. The same is true of transcription factors, and has been useful in elucidating their activities [10].

#### 2.1.3. Analyzing function without knowing the target

In many cases, putative RNA-binding proteins have been identified but their RNA target is unknown. An asset of the tethered function approach is that analysis of function does not require knowledge of the natural RNA target.

#### 2.1.4. Analyzing the function of essential genes

In some cases, the RNA-binding protein under investigation may be essential for cell viability; as a result, traditional genetic techniques may be complicated by pleiotropy. Tethered function assays allow the protein of interest to be modulated on one mRNA reporter in a wild-type background, thus eliminating global cellular effects.

## 2.2. Designing the assay

To test the effects of protein X on mRNA activity or biogenesis, a fusion between X and a tethering protein, such as MS2 coat protein, is created by recombinant DNA methods and expressed *in vivo*. A reporter mRNA carrying an ORF to be monitored (such as *lacZ* or luciferase) and binding sites for the tether within the 3'UTR is expressed in the same cells. The effects of the fusion protein on the mRNA are then assayed by conventional means (Fig. 1). While the assay is relatively straightforward, several technical issues merit discussion.

#### 2.2.1. The fusion protein

Criteria for selecting a tethering protein include its affinity, specificity, and subcellular location. Perhaps the

most common tether used to date has been bacteriophage MS2 coat protein [1,2,6,11–18]. However, the iron regulatory protein (IRP), bacteriophage N-protein [3,4], and U1A protein have also been used successfully [19,20].

MS2 coat protein has several desirable features for such assays. First, it is relatively small (14 kDa). Second, it binds with high affinity and selectivity to a 21-nucleotide RNA stem–loop [21]. Third, the biochemistry of MS2 binding to its target sequence has been analyzed extensively. Mutants in the binding site are available that increase or decrease affinity. In particular, the substitution of a single U within the stem–loop with a C increases affinity at least fivefold over wild type [21]. Fourth, MS2 coat protein interacts as a dimer with a single RNA stem–loop, thereby increasing the concentration of the tethered protein on the reporter. Lastly, MS2 binds cooperatively to adjacent stem–loops, further increasing occupancy of sites [22,23]. Since MS2 coat protein binds as a dimer to a single site and interacts with adjacent sites cooperatively, a large (and not trivial to determine) number of protein molecules may be bound to the targeted 3'UTR.

The relative positions of the tethering moiety and the polypeptide to be tested are a second issue. In the case of MS2/PAB fusions, fusion proteins in which MS2 was present at the N terminus gave a slightly higher stimulation of translation than those at the C terminus (N. Gray and M.W., unpublished). It is unclear whether this is generally the case. Thus it may be desirable to test both orientations.

Another issue concerns any trans-effects that expression of the fusion protein may exert *in vivo*. Commonly, the fusion protein is expressed in a wild-type background, creating a situation in which the protein of interest is overexpressed in a “mutant” form. In the case of MS2/Pab1p fusions in yeast, the chimeric protein slightly affected the ability of yeast to grow under nutrient-poor conditions (unpublished).

### 2.2.2. The reporter mRNA

The design of the reporter mRNA is important, and is dictated by the particular function being assayed. A wide variety of open reading frames have been used to follow activity. In yeast, the effects of tethered polypeptides on mRNA stability have been assessed using the *MFA2*, *PGK1*, *YAP1*, and *LacZ* genes as reporters [1,14]. Translational activity and mRNA localization have been determined using the *HIS3*, *CUP1*, *GFP*, and *LacZ* genes (unpublished observations, [2,11]). In *Xenopus*, luciferase has been used, while in mammalian cell culture,  $\beta$ -globin and CAT mRNAs have been tested [3,4,6,16].

For certain experiments, the intrinsic behavior of the reporter (in the absence of the tethered protein) is an important aspect of the experimental design. For ex-

ample, if one wants to assess whether a tethered protein can elicit mRNA degradation, then the reporter needs to be relatively stable in the absence of the tethered protein; conversely, if one wants to assess whether the tethered protein stabilizes the mRNA, the reporter needs to be relatively unstable in the absence of the protein. It is also important to determine whether the desired characteristics of the reporter are unaffected by the tether *per se* (e.g., MS2 coat protein alone). Often, reporters with suitable properties can be designed by manipulating their 5' and 3'UTRs.

The number and location of binding sites are additional considerations. In several instances, two sites have been sufficient to see an effect [1,2,14,18]. However, as many as 8 and 16 sites have been used [6,11,16,19]. Increasing the number of binding sites can increase the sensitivity of the assay [16], though a systematic comparison of reporters with different numbers of sites has not yet been reported. The position of the sites within the 3'UTR is a second variable. Because wild-type 3'UTRs contain regulatory information, it is important that the tethering sites not interfere with normal expression. Thus, the behavior of the reporter mRNA with and without the sites should be compared in the absence of the chimeric protein.

## 3. Case studies

In the following sections, we discuss a few specific examples with general principles in mind. Table 1 includes examples from the literature in which tethered function assays have been performed, organized by the function analyzed. In the following section, we discuss a few examples of the utility of the approach. This is not intended to be a complete account of possible or published uses.

### 3.1. Standard controls and assays

Whatever the biological problem being studied, a subset of control experiments are similar. One set of controls, drawn from the initial work describing tethered function assays in 3'UTRs [1], is depicted in Fig. 2. In these experiments, tethered poly(A)-binding protein (Pab1p) was shown to stabilize a reporter mRNA in yeast, increasing its half-life in pulse–chase experiments to approximately 20 min (Fig. 2A). The specificity of this effect was shown by tethering other RNA-binding proteins, none of which stabilize the reporter. Similarly, removal of the tethering sites in the 3'UTR also abrogates the effect. Additional standard controls include a demonstration that the tethered protein is functional (e.g., complements a mutant) and that it does not interfere with the activity of the wild-type protein in the same cells.

Table 1  
Uses and adaptations of tethered function assays

Key issue	Protein	Organism	Tether	Reporter	Effects	Ref.
Analysis of essential gene	Pab1p	Yeast	MS2	MFA2, PGK1	Tethered Pab1p stabilizes mRNAs, functions independent of poly (A)	[1]
Separation of multiple functions	PAB1, Pab1p	<i>Xenopus</i> Yeast	MS2	Luciferase CUP1	Distinct regions of tethered PAB1 stimulate translation and stabilize mRNA in vivo	[1,2]
	Xp54	<i>Xenopus</i>	MS2	Luciferase	Tethered Xp54 represses or stimulates translation of poly(A) minus reporters	[18]
	Pub1p	Yeast	MS2	YAP1	Tethered Pub1p prevents NMD of mRNA with uORF	[14]
Dissection of complex	hUPF1, hUPF2, hUPF3a, hUPF3b	Mammalian cells	MS2	$\beta$ -globin	Tethered UPFs transform a normal mRNA into a message subject to NMD	[16]
	RNP S1, Y14, DEK, REF2, SRm160	Mammalian cells	MS2	$\beta$ -globin	Tethered RNP S1 stimulates NMD on a normal mRNA	[6]
	eIF-4E	Mammalian cells	N	CAT/Luc	Tethered eIF-4E stimulates translation when tethered to 5'UTR	[3]
	eIF-4G	Mammalian cells	IRP	CAT/Luc	Tethered eIF-4G stimulates translation	[4]
Identifying localization functions	She2p, She3p	Yeast	MS2	LacZ	Tethered She2p is sufficient to stimulate the localization of ASH1 mRNA	[13]
	REV, TAP1, NXF3	Mammalian cells	MS2	CAT	Tethering nuclear export factors enhance transport	[17]
Analysis of modifying enzymes	PAP1	<i>Xenopus</i>	MS2	Luciferase	Tethered PAP1 polyadenylates mRNAs in the cytoplasm and stimulates their translation	[15]
Following localized mRNAs	GFP	Yeast, mammalian cells	MS2	Various	Tethered GFP allows for the visualization of cytoplasmic mRNA localization in live yeast cells and mammalian neurons	[11–13,20,26,27]
	GFP	Yeast	U1A	RPL25, PGK1, SSA4	Tethered proteins monitor nucleocytoplasmic transport	[19] <sup>a</sup>

<sup>a</sup> See also article by Brodsky and Silver [32].

The details of how the reporter mRNA and tethered protein are produced and analyzed are not described in detail here, since they vary with the cells used and the problem studied. Suffice it to say that a broad range of cell types, reporters, and vectors have been used successfully (Table 1). Tethered proteins have been produced from transgenes in yeast and in mammalian cells, and from injected mRNAs in *Xenopus* oocytes (see Table 1 for references and details).

### 3.2. Analyzing essential genes

Tethered function assays allow the presence of a particular RNA-binding protein to be modulated on a target mRNA without consequences to cell viability. For example, in *Saccharomyces cerevisiae*, poly(A)-binding protein (Pab1p) is an essential gene involved in many different aspects of mRNA metabolism. Genetic

studies of *PAB1* show that this protein is required for translation, coupled deadenylation and decay, and regulation of poly(A) length. Detailed analysis of these functions in vivo is complicated by the multiple roles of Pab1p and the fact that Pab1p is essential for viability. A tethered function assay bypassed these complications, and allowed genetic analysis of Pab1p on a single mRNA in a wild-type background [1].

In yeast, the effects of Pab1p mutations on mRNA stability and translation could be analyzed even when the mutant was insufficient to support viability [1,2]. Pab1p tethered to the 3'UTR of an unstable reporter in yeast increased reporter half-life (Fig. 2A); similarly, Pab1p tethered to the 3'UTR of a stable mRNA increased mRNA translation, modestly in yeast and more dramatically in frog oocytes [1,2]. The effects of four mutant forms of Pab1p ( $\Delta 1$  through  $\Delta 4$ ) were analyzed in yeast carrying wild-type (non-MS2-containing)

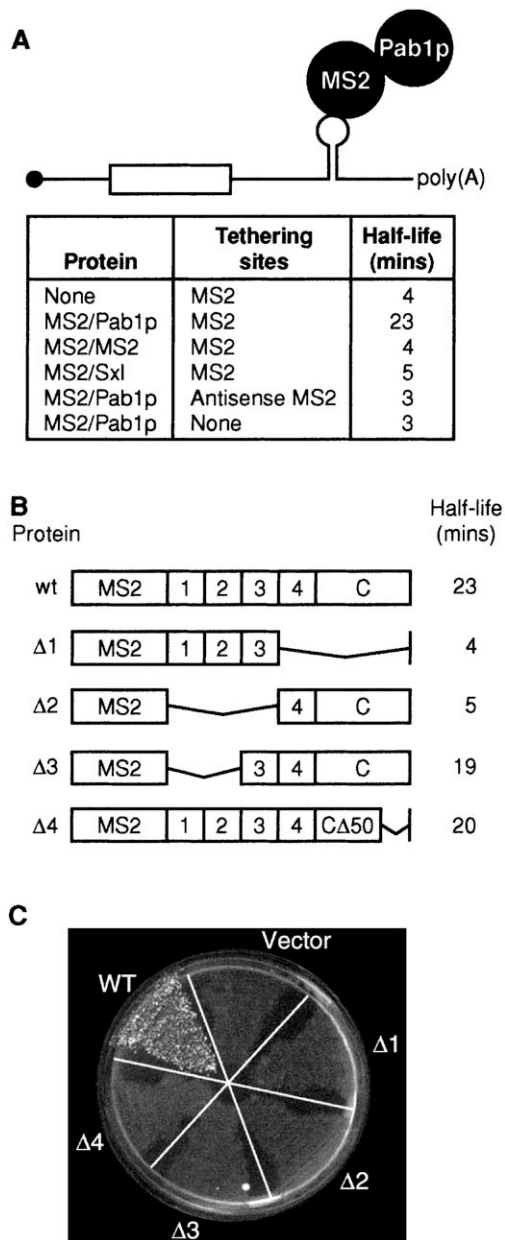


Fig. 2. Standard controls, mutational analysis, and analyzing essential functions. (A) Standard controls: Pab1p tethered by MS2 coat protein to the 3'UTR of an otherwise unstable mRNA increases its half-life from 4 to 20 min [1]. As in other tethered function experiments, necessary controls include other fusion proteins and mRNA without tethering sites. Ideally, the proteins used as controls are of approximately the same size and type as the one being tested; in this case, Sxl possesses three RRM motifs, and Pab1p, four. (B) Mutational analysis: A series of deletions in the Pab1p part of an MS2/Pab1p fusion protein were analyzed ( $\Delta 1$ – $\Delta 4$ ). Each protein was produced in a strain containing wild-type (non-MS2-containing) Pab1p. Pab1p consists of four consecutive RRM domains (labeled 1–4) and a more divergent C terminus. mRNA half-life was determined by a pulse-chase assay. See Ref. [1] for details. (C) Viability: The same *Pab1* deletions analyzed in (B) were tested for their ability to provide Pab1p's essential function, using a plasmid shuffle assay [1]. Only the "wt" MS2–Pab1p construct supports viability in the absence of the *PAB1* gene. Nonetheless, the function of each tethered protein can be analyzed (B).

Pab1p: two mutants ( $\Delta 3$  and  $\Delta 4$ ) stabilize the reporter mRNA while the other two do not (Fig. 2B). None support viability in circumstances in which they are the sole source of Pab1p in the cell (Fig. 2C). These findings illustrate that the functions of essential proteins can be analyzed genetically using the assay.

### 3.3. Dissecting complexes in the absence of genetics

Tethered function assays are particularly useful when genetics is not available. For example, multiprotein complexes control many mRNA regulatory events. Some proteins in the complex provide RNA binding; others provide protein–protein bridges; some possess the ultimate "activity" of the complex. When genetics is unavailable, dissecting the different contributions of individual proteins can be difficult. Tethered function assays can identify the active ingredient.

Analysis of nonsense-mediated decay (NMD) in mammalian cells is exemplary. Mammalian mRNAs are targeted for rapid turnover when they contain a stop codon that is greater than 50 nucleotides upstream of the last exon–exon boundary. A group of at least five proteins, and perhaps more, form a complex 20–25 nucleotides upstream of the exon–exon (E/E) junction of mammalian mRNAs after they are spliced (Fig. 3A) [24]. This complex was proposed to trigger NMD if located after a premature nonsense codon. To identify proteins in the complex that have that activity, Lykke-Andersen et al. (2001) tethered each of the constituent proteins to the 3'UTR of a normal mRNA, thus placing them downstream of the last splice site and the normal termination codon (Fig. 3B). Tethering one protein, RNP S1, and, to a lesser extent, another, Y14, caused NMD; tethering the other three proteins had little effect (proteins "a" to "c" in Fig. 3B). Thus the tethered function approach identified RNP S1 as an active principle in the E/E complexes, and suggested that its deposition was critical for NMD. In turn, this provided an entre to the dissection of its biological activity, as opposed to its assembly onto the RNA or into the complex.

This approach to dissecting multicomponent complexes can be complicated in three ways. First, tethering of any protein in a complex can, in principle, bring in all the other members of the complex. If this is the case, then the region required for the tethered activity will precisely coincide with that required for interaction with the other protein. Second, activity may require multiple proteins, not simply the one being tethered. Thus it may be necessary to provide other proteins, particularly when the tethered protein comes from another species or cell type. Third, the lack of activity observed with certain tethered proteins is difficult to interpret unambiguously.

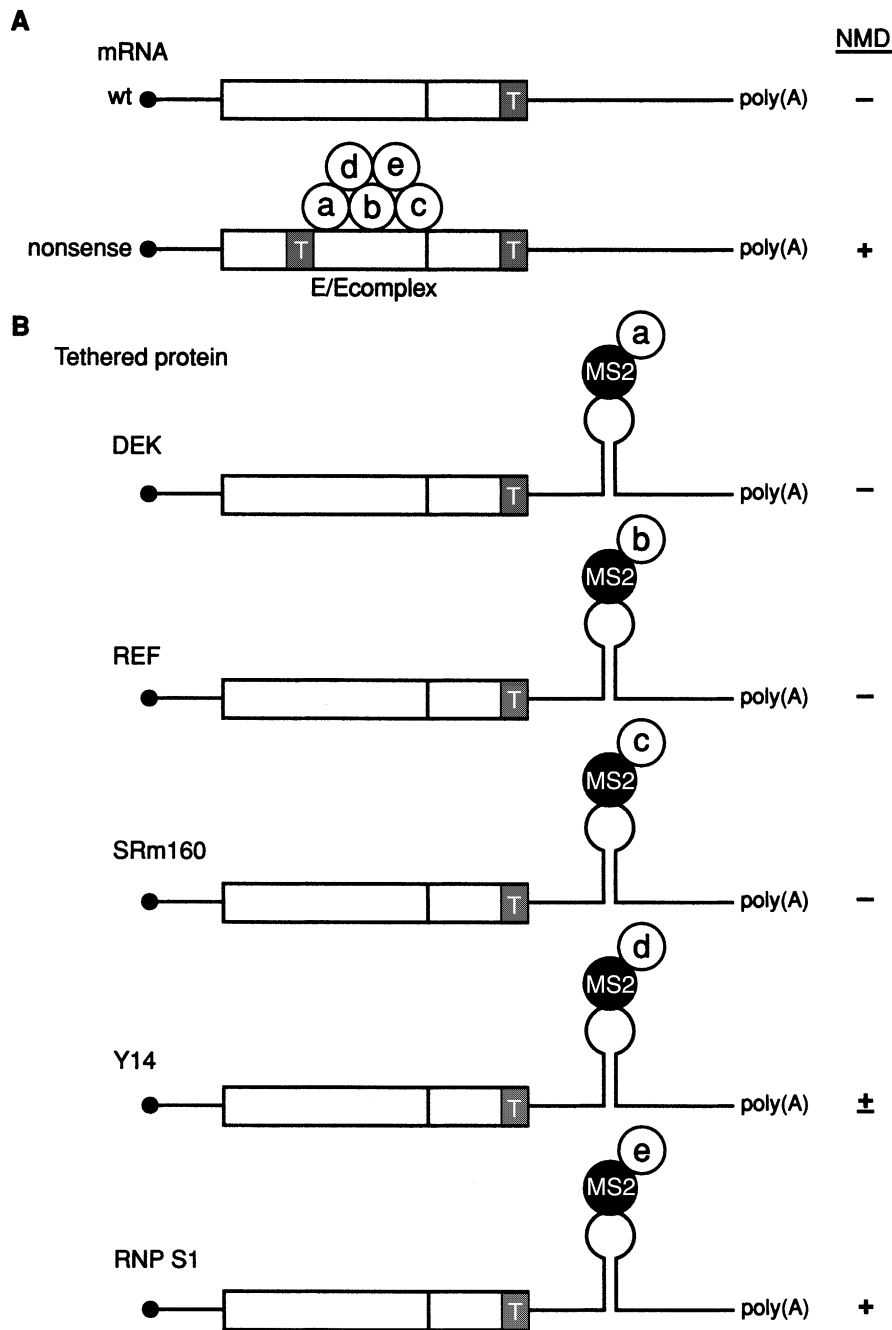


Fig. 3. Dissecting a multiprotein complex in the absence of genetics. (A) The E/E complex and NMD: A wild-type mRNA is shown that contains an exon/exon junction (vertical line) and a single termination codon (black “T”) at the end of its ORF. This mRNA is stable. An identical mRNA containing a premature termination codon (black “T” within the ORF) is subject to NMD [24]. At least five proteins (“a” through “e”) form a complex on the mRNA upstream of the E/E junction, and were proposed to trigger NMD. In this model, NMD is triggered because the complex is present downstream of the splice junction. The drawing of the complex is not meant to imply that any particular proteins contact the mRNA directly. See text and Le Hir et al. [24] for details. (B) Identifying the component of the E/E complex that possesses NMD activity [6]: Each of the five known components of the E/E complex was tethered individually to the 3’UTR of a reporter. In this design, the tethered proteins are downstream of the termination codon, and so are predicted to elicit NMD. Protein “e” (RNP S1) and, to a lesser extent, protein “d” (Y14) elicit NMD; the other three proteins (DEK, REF, and SRm16) do not [6].

### 3.4. Separation of multiple functions in the same protein

Tethered function assays can be used to dissect one function from another in a multifunctional protein. For

example, Pab1p both stimulates translation and stabilizes mRNAs. By tethering various deletion mutants of Pab1p in yeast, distinct alleles were created that affect one function of Pab1p but not the other [1,2].

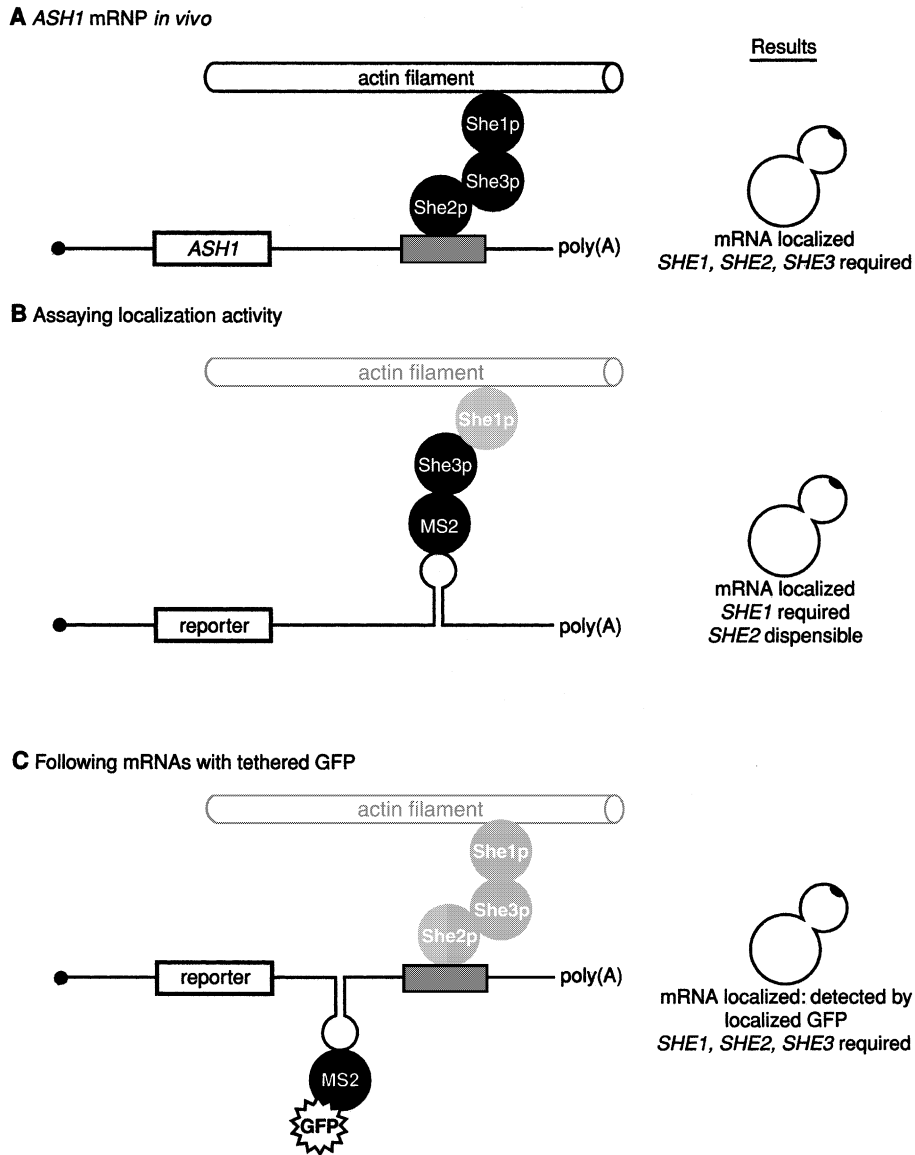


Fig. 4. mRNA localization and tethered assays. (A) Model of *ASH1* mRNA localization in yeast, based in part on tethered function assays: She2p binds to an element in the 3'UTR of *ASH1* mRNA (black box). It also binds to She3p protein, which interacts with a myosin (She1p/Myo4p), which in turn binds to the actin filament to localize the mRNA to the bud tip. Localization can be detected by nucleic acid hybridization, as illustrated. (B) Tethered She3p causes localization of the mRNA: This process is dependent on factors “downstream” of She3p (such as She1p and the actin filament) (gray in figure), but not on She2p. (C) Tethered GFP can be used to monitor localization in living cells: GFP is tethered to the 3'UTR or elsewhere in the mRNA, as a means of “tagging” the mRNA. Localization of the GFP fluorescence, and hence the mRNA, requires all of the components required in the unperturbed localization process.

Mutagenesis of tethered proteins can also be useful in identifying unique gain-of-function alleles. For example, Minshall et al. have recently shown that the *Xenopus* homolog of the RCK1/p54 helicase family of proteins, Xp54, is sufficient for repression of mRNA when tethered to the 3' end of a reporter [18]. Interestingly, mutants within the putative DEAD box motif of this protein transform this helicase from a translational repressor into a translational stimulator. These results may indicate that Xp54 may serve two roles in mRNA metabolism that are dependent on modulation of its helicase activity.

### 3.5. Identifying mRNA localization functions

Proteins that cause an mRNA to move to a particular location within a cell can be assayed using the tethered function approach. In yeast, *ASH1* mRNA is distributed into, daughter cells during budding, regulating yeast mating type switching (Fig. 4A) (reviewed in [25]). Signals in the 3'UTR of *ASH1* mRNA are sufficient to localize the mRNA. Yeast She2p and She3p are present in a complex on the *ASH1* 3'UTR, and they interact with a myosin, She1p/Myo4p, which in turns interacts with cytoskeletal actin (Fig. 4A). Tethering She3p to the

3'UTR of a reporter mRNA was sufficient to localize that mRNA to the bud tip (Fig. 4B) [13]. As expected, this process required *SHE1*; moreover, localization by tethered She3p obviated the need for *SHE2*, implying that She2p is the natural tether, and is dispensable if She3p is brought to the 3'UTR by other means.

Adaptations of the tethered function assay have been developed to tag an mRNA for further analysis, rather than study the effects of a particular protein. Although these are not strictly tethered function assays (as the protein is merely a passive tag), we mention them here because they are so closely related technically and historically. Several groups have tethered green fluorescent protein (GFP) to study mRNA localization in living cells (e.g., [11–13,19,20,26,27,32]). To follow the movement of a mRNA in living cells, MS2 sites are introduced into a reporter mRNA in cells expressing an MS2/GFP fusion (Fig. 3C). Such experiments have been performed with reporters bearing the *ASH1* 3'UTR, and demonstrated localization to the bud tip. In a more reductionist form of the same experiment [27], MS2 sites have simply been inserted into a 3'UTR on its own (absent an ORF) using an RNA polymerase III vector for the purposes of the three-hybrid system [28]. U1A protein and its binding sites also have been used to the same end [19,20].

Tethered GFP assays can be used to identify the factors involved in localization. For example, certain mutants (e.g., *she1* $\Delta$  through *she5* $\Delta$ ) perturb *ASH1* localization, as monitored by the tethered GFP [11,27]. Furthermore, the same assay can detect RNA movement to any subcellular location that can be identified cytologically. For example, the 3'UTR of yeast *ATM1* mRNA, encoding a mitochondrial ABC transporter, moves to mitochondria as monitored by tethered GFP [29]. CaMKII $\alpha$  mRNA, to which GFP is tethered, moves through neurons to synapses [26].

### 3.6. Analyzing mRNA modifying enzymes

Tethered function assays can be used to address the effects of various proteins on mRNA processing or maturation. Nuclear polyadenylation of eukaryotic mRNAs is accomplished via recognition sequences embedded within the 3' end of the mRNA. These sequences recruit a complex of proteins that bring the enzyme poly(A) polymerase (PAP1) to the RNA. Elongation of poly(A) tails by PAP1 in the cytoplasm appears to underlie translational activation of certain mRNAs in oocytes and early embryos, and is triggered at a specific time during development (reviewed in [30,31]). Tethering PAP1 directly to the 3' end of a reporter mRNA in oocytes caused poly(A) elongation in the cytoplasm, stimulated translation, and did so in a way that circumvented the normal signals that control polyadenylation [15]. While the active site of PAP1 was necessary,

the region that appears to bind to other components of the polyadenylation complex was not [15]. Thus a tethered mRNA modification enzyme can elicit enzymatic activity while linked to a reporter mRNA, enabling in vivo analysis of the activity and how the activity is regulated.

## 4. Prospects

In summary, tethered function assays provide a simple means to address the role of a given RNA-binding protein on the metabolism of a reporter. The approach provides a unique platform for the study of suspect regulators of unknown target specificity and function. Of particular interest are simple phenotypic screens that allow the rapid identification of tethered proteins on the metabolism of a given reporter.

As the genome sequences of more species become available, methods to analyze the function of mRNA regulatory proteins are needed. Familial resemblance of sequences or structures is insufficient for this purpose. Tethered function assays may provide a rapid screen to sort proteins into functional families, based on their biological activities. Combined with physical and genetic linkages to other proteins, this should provide new perspectives on the logic of mRNA control.

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