CHAPTER FOURTEEN

Tethered Function Assays: An Adaptable Approach to Study RNA Regulatory Proteins

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Contents

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Abstract

Proteins and protein complexes that regulate mRNA metabolism must possess two activities. They bind the mRNA, and then elicit some function, that is, regulate mRNA splicing, transport, localization, translation, or stability. These two activities can often reside in different proteins in a complex, or in different regions of a single polypeptide. Much can be learned about the function of the protein or complex once it is stripped of the constraints imposed by RNA binding. With this in mind, we developed a "tethered function" assay, in which the mRNA regulatory protein is brought to the 3′ UTR of an mRNA reporter through a heterologous RNA–protein interaction. In this manner, the functional activity of the protein can be studied independent of its intrinsic ability to recognize and bind to RNA. This simple assay has proven useful in dissecting numerous proteins involved in posttranscriptional regulation. We discuss the basic assay, consider technical issues, and present case studies that exemplify the strengths and limitations of the approach.

1. Introduction and Rationale

In studying proteins that regulate mRNA metabolism, it often is useful to experimentally separate function from mRNA binding. In many instances, the natural mRNA target for a given protein is unknown; any assay of function must therefore be performed independent of the natural RNA–protein interaction. In addition, because posttranscriptional regulatory steps often are coupled, genetic analysis of functions *in vivo* can be complicated by indirect effects. Lastly, mutations in many critical RNA-binding proteins have pleiotropic effects on the cell and make it impossible to deduce which functions are direct. To circumvent these problems, we have developed a useful technique that allows the function of a protein to be analyzed, unconstrained by that protein's natural ability to interact with its mRNA target. We commonly refer to the technique as a "tethered function assay." The approach is adaptable and overcomes multiple complications in the study of mRNA-binding proteins.

In tethered function assays, the polypeptide of interest is tethered to a reporter mRNA through a heterologous RNA–protein interaction

(Fig. 14.1). Usually, the tethering site lies in the 3′ untranslated region (UTR) of the mRNA; this region is relatively unconstrained evolutionarily, and the natural site of action of many mRNA regulators. Tethered function assays have been used to show the role of proteins in control of mRNA transport, translation, localization, and stability (Coller and Wickens, 2002). Different reporters need to be used to assay each of these processes.

The tethered function assay takes advantage of the observation that many nucleic acid-binding proteins are modular. For example, many DNA transcription factors are bipartite, with separate DNA-binding and transcriptional activation domains (Hope and Struhl, 1986; Keegan *et al.*, 1986). Often the activities of these two domains are autonomous and separable; in other instances, they reside in distinct members of a multi-polypeptide complex. RNA-binding proteins display similar modularity. The rationale of the tethered function approach is to examine solely the "functional" activity of an RNA-binding protein tethered artificially to an mRNA, circumventing the constraints imposed by natural RNA binding.

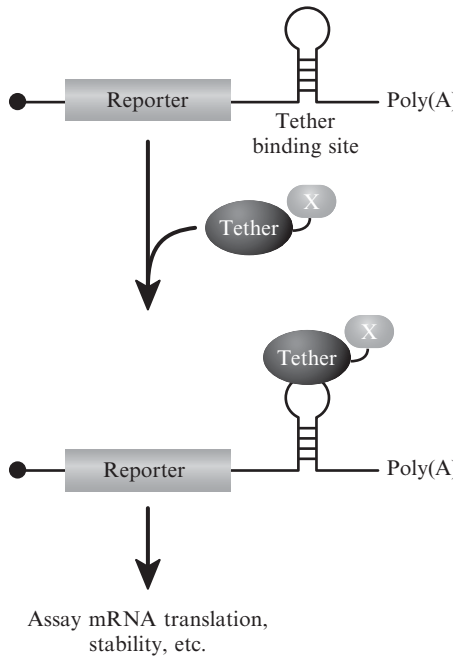


Figure 14.1 Tethered function assays using the 3′ UTR. A protein (X) is brought to a reporter mRNA through an artificial RNA–protein interaction (tether). In this example, the tethered binding site has been shown in the 3′ UTR of the reporter, but other locations have been used. The function of the tethered protein in any aspect of the mRNA's metabolism or function can then be assayed by conventional methodology.

In some cases, RNA binding and function may not be readily separable. For example, in nucleases and helicases, the nucleic acid-binding site is also the active site of the protein. Moreover, the interaction of a protein with its natural RNA-binding site can regulate the protein's activity; in these instances, it may be impossible to assay the function of the tethered protein in the absence of its cognate site.

2. The Basic Design of the Tethered Function Assay

The design of the tethered function assay is relatively straightforward. To determine the effects of a protein X on mRNA metabolism, a chimeric protein is expressed *in vivo* in which protein X is continuous with a tethering polypeptide (see Fig. 14.1). The tethering protein is an RNA-binding protein that recognizes an RNA tag sequence with high specificity and affinity. The effect of the fusion protein on mRNA metabolism is determined by coexpressing the chimera with an mRNA reporter (such as lacZ or luciferase) into which a tag RNA sequence has been embedded. The fusion protein's effects on mRNA metabolism are assayed by conventional means [i.e., Western blot, Northern blot, reverse transcriptase polymerase chain reaction (RT-PCR), etc.]. While the assay is relatively straightforward, several issues discussed in the following sections should be considered at the outset in designing a tethering experiment.

The assay, though powerful, is artificial. Only positive results are meaningful: lack of effects cannot be interpreted. Some RNA-binding proteins may require other proteins or their cognate RNA-binding sites to function, or be inactive as chimeras, or require appropriate positioning on the mRNA.

2.1. Position of the tethering site

A first consideration when designing a tethered function assay is the position in the mRNA of the tag sequence (i.e., the tethering site). While different laboratories have used tethered function assays and placed tag sequences within all regions of the mRNA, the most useful and common site is the 3′ UTR (Coller and Wickens, 2002). The tethering of proteins to the 3′ UTR has particular biological and experimental advantages. Importantly, many sites that regulate diverse steps in an mRNA's life, including its transport, cytoplasmic localization, stability, and translational activity, often reside in the 3′ UTR. Thus, tethering to that region places regulators where they might well function. In addition, it is known that the exact location of several 3′ UTR regulators is not critical for their function, implying that

precise spatial positioning is not critical. Lastly, the 3′ UTR has fewer constraints than either the 5′ UTR (which can affect translational initiation frequency) or the open reading frame. The intercistronic region of bicistronic mRNAs also is relatively unconstrained and has been used for tethered function experiments using the same rationale (De Gregorio *et al.*, 1999, 2001; Furuyama and Bruzik, 2002; Shen and Green, 2006; Spellman *et al.*, 2005; Wang *et al.*, 2006).

3. The Tether

In choosing which protein to use as the tether, it is necessary to consider affinity and specificity for the RNA tag, subcellular localization, and impact of the tether on the activity of the test protein. The most common tether is the bacteriophage MS2 coat protein (Beach *et al.*, 1999; Bertrand *et al.*, 1998; Coller *et al.*, 1998; Collier *et al.*, 2005; Dickson *et al.*, 2001; Dugre-Brisson *et al.*, 2005; Gray *et al.*, 2000; Kim *et al.*, 2005; Long *et al.*, 2000; Lykke-Andersen *et al.*, 2000, 2001; Minshall and Standart, 2004; Minshall *et al.*, 2001; Ruiz-Echevarria and Peltz, 2000). However, the iron response element binding protein (IRP), a derivative of bacteriophage λ N-protein (De Gregorio *et al.*, 1999, 2001), and the spliceosomal U1A protein have been used successfully (Brodsky and Silver 2000; Finoux and Seraphin, 2006). In the following sections we will discuss each of these specific tethers and their merits and drawbacks.

3.1. The MS2 bacteriophage coat protein as a tether

The MS2 coat protein has been a popular choice for several reasons. First, this protein is relatively small (14 kDa), thus minimizing potential disruptions to the test protein. Second, the biochemistry of the MS2 coat's binding to its target sequence has been well established. Specifically, the MS2 coat is known to bind with high specificity and selectivity to a 21-nucleotide RNA stem–loop ($K_d = 1$ nM; Carey and Uhlenbeck, 1983). In addition, mutations in the binding site are available that increase or decrease affinity. In particular, the substitution of a single U within the stem–loop to a C increases affinity 50-fold over wild type (Lowary and Uhlenbeck, 1987). Moreover, use of MS2 allows a high dosage of tethered proteins to be present on the mRNA: the MS2 coat interacts with its target sequence as a dimer; thus for every stem–loop present in the mRNA reporter, two tethered proteins are present. Lastly, MS2 binds cooperatively to two stem–loops, further increasing the occupancy of sites (Witherell *et al.*, 1990). In some applications, the more protein that is bound, the better; each of these factors contribute to a strong signal in the functional assay.

On the other hand, the MS2 coat protein is not the simplest option when it is necessary to carefully control the number of tethered protein molecules bound. Since the 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 mRNA.

3.2. N-peptide as a tether

The bacteriophage λ N protein is often used in the tethered function assay (Baron-Benhamou *et al.*, 2004). N-protein regulates bacterial transcriptional antitermination by binding to a 19-nucleotide RNA hairpin within early phage operons called boxB (Scharpf *et al.*, 2000). Importantly, the N-peptide/boxB interaction occurs with high affinity ($K_d = 1.3$ n*M*). The particular advantage of the N-peptide in tethering assays is the result of its extremely small size; only 22 amino acids are required for the high affinity interaction with boxB RNA. Because of this, many laboratories have opted to use the N-peptide rather than MS2 coat protein, reasoning that it minimizes potential interference with the fusion protein's function (Baron-Benhamou *et al.*, 2004). Another desirable feature of N-peptide is that unlike the MS2 coat, the protein binds 1:1 to its RNA target.

3.3. U1A protein and IRP as tethers

Both the U1A protein and IRP have been used successfully as tethers (De Gregorio *et al.*, 1999; Finoux and Seraphin, 2006). U1A is a U1 small nuclear ribonucleoprotein (snRNP)-specific protein that binds with high specificity and affinity to a 30-nt RNA hairpin ($K_d = 5$ n*M*; van Gelder *et al.*, 1993). IRP also binds to a 30-nt RNA hairpin that normally resides within the UTRs of target mRNAs with high affinities ($K_d = 90$ p*M*; Barton *et al.*, 1990). Like N-peptide, the concentration of both U1A and IRP on the reporter mRNA is theoretically 1:1 (protein:RNA tag). Unlike N-peptide, however, both of these proteins are relatively large: 38 kDa for U1A and 97 kDa for IRP. As a result, they have not commonly been used in tethered function assays.

In general, the MS2 coat provides the highest concentration of tethered proteins to be bound to the reporter per binding site. This may allow phenotypes to be observed without greatly increasing the overall length of the mRNA reporter, an undesired situation in some applications. N-peptide, on the other hand, allows the delivery of a single tethered protein per binding site. The cost of this control of stoichiometry can be a need to introduce multiple tandem binding sites (more than four) in order to observe a robust phenotype (see below); the trade-off is an increase in reporter length. Nonetheless, the relative merits of MS2 coat protein, N-peptide, U1A, or IRP are situation specific. All have been successfully used to measure effects on mRNA

translation, turnover, and transport. Direct comparisons between different tethers have not been made.

3.4. N-terminal or C-terminal fusions

The relative positions of the tethering protein and the protein of interest can be important. For example, in our own experience, tethering the MS2 coat protein to the N-terminus of the poly(A)-binding protein (PAB) resulted in much more activity than if the tether was located at the C-terminus (data not shown). This will have to be determined on a case-by-case basis; both orientations should be tested.

3.5. Trans-effects

A third important issue to consider is that the fusion protein may have *trans*-acting effects. Often, the tethered function assay is performed in a wild-type background with the endogenous copy of the test protein present. The presence of the tethering moiety may create a dominant negative allele that blocks the function of the normal protein *in vivo*, seriously complicating analysis. As a result, controls to ensure that any observed effects occur only in *cis* with respect to the mRNA reporter are important (see below).

4. The Reporter mRNA

The tethered function assay can be adapted to measure the effect of a tethered protein on many steps in mRNA metabolism and function. The adaptability comes mainly from the choice of reporter mRNA and the final assay performed. We will discuss only some of the reporters and assays that have been put into practice.

The choice of reporter mRNA obviously is dictated by the effect to be assayed. For example, translational activity can be measured in yeast using the LacZ, *HIS3*, and *CUP1* mRNAs, while in metazoans, luciferase, CAT, and epitope tags are most common (De Gregorio *et al.*, 1999, 2001; Gray *et al.*, 2000; Pillai *et al.*, 2004). In determining the effects of a tethered protein on mRNA stability, *MFA2, PGK1*, and *YAP1* have been used as reporter mRNAs in yeast, and β-globin and luciferase have been used in mammalian systems (Amrani *et al.*, 2006; Chou *et al.*, 2006; Coller *et al.*, 1998; Finoux and Seraphin, 2006; Kim *et al.*, 2005; Lykke-Andersen *et al.*, 2001, 2001; Ruiz-Echevarria and Peltz, 2000).

The intrinsic behavior of the reporter mRNA is an important consideration. To determine whether a tethered protein stabilizes an mRNA, the mRNA must be unstable in the absence of the protein; conversely, to determine whether a tethered protein destabilizes the mRNA, the

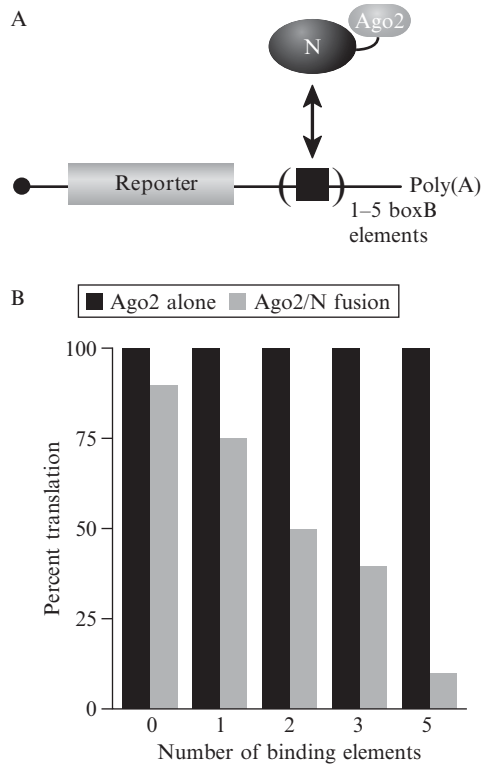


Figure 14.2 The number of tethered binding sites can influence phenotypic read-out. (A) Shown is the effect of increasing the number of tethered binding sites on translational repression mediated by tethered Ago2 (Pillai *et al.* 2004). Specifically, 0, 1, 2, 3, or 5 boxB elements were introduced into the 3′ UTR of a reporter gene expressing *Renilla* luciferase (RL). (B) The reporters were transfected into HeLa cells expressing either Ago2 (black bars) or an N-peptide Ago2 fusion (gray bars) and translation measured by enzymatic assay. As shown, increasing the number of tethered binding sites dramatically influences the repression observed.

mRNA reporter must be stable without the protein. The same reasoning applies to effects on other aspects of mRNA metabolism such as translation and subcellular localization.

4.1. The number and location of tethered binding sites

The number and location of tether binding sites are important variables. First, it should be decided where the tethered sites should be positioned, i.e., the 5′ UTR, 3′ UTR, or coding region. This depends on the suspected role of the protein in mRNA metabolism. For example, a protein thought to regulate polyadenylation might logically be placed in the 3′ UTR. It is important that the placement of the tethered binding sites not interfere on

its own with the mRNA. For example, in testing the role of tethered PAB on mRNA stability, sites were placed in a region of the *MFA2* 3′ UTR that was known not to affect the mRNA's half-life (Coller *et al.*, 1998; Muhlrad and Parker, 1992). Placement elsewhere would have dramatically altered the normal turnover rate of this message. It is helpful, therefore, to select as a reporter an mRNA whose *cis*-acting sequences are well characterized. Obviously these issues make it important that the behavior of the reporter mRNA with and without tethering sites be compared in the absence of the chimeric protein (see below, and Fig. 14.2).

A second issue in designing a reporter concerns the number of tethered binding sites. In many cases using the MS2 bacteriophage coat as the tether, two stem–loops have been sufficient to observe an effect (Coller *et al.*, 1998; Gray *et al.*, 2000; Minshall *et al.*, 2001; Ruiz-Echevarria and Peltz, 2000). However, many more sites have been used, ranging from 6 to 24 (Bertrand *et al.*, 1998; Fusco *et al.*, 2003; Lykke-Andersen *et al.*, 2000, 2001; Pillai *et al.*, 2004). The effect of the number of binding sites has been evaluated systematically in two reports (Lykke-Andersen *et al.*, 2000; Pillai *et al.*, 2004). Increasing the number of binding sites can increase the signal and enhance the assay's sensitivity. In Fig. 14.2, the extent of translational repression achieved by a tethered protein is proportional to the number of binding sites (Pillai *et al.*, 2004).

5. A Priori Considerations About the Logic of the Assay

5.1. Multiprotein complexes

mRNA regulatory events often occur through multiprotein complexes formed via protein–protein and protein–RNA interactions. In such cases, RNA binding may occur via one critical protein, which tethers the activity of another protein to the mRNA. Thus, the "active" protein may not directly contact the RNA. One strength of the tethered approach is its ability to assay the "activity" independent of RNA binding.

5.2. The role of RNA binding in function

The interaction between RNA and protein in some cases is essential for activity. RNA–protein interactions can change the conformation of the RNA, the protein, or both; not surprisingly, some complexes are biologically active, while the free RNAs or proteins are not (Williamson, 2000). Certain RNA ligands likely can influence activation or repression activity, much as in DNA-induced allosteric effects on transcription factors (Lefstin and Yamamoto, 1998; Scully *et al.*, 2000). In addition, the context of the

natural binding site may be important for the protein's activity because essential factors are bound in the neighborhood.

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 seen—for example, mRNA stabilization by a particular tethered protein—may differ when the protein is associated with its natural RNA-binding site. The same issues apply to DNA-binding transcription factor complexes, which have been powerfully dissected via comparable tethering approaches.

5.3. Analyzing function without knowing the target

In many cases, putative RNA-binding proteins have been identified, but their respective RNA target is unknown. One asset of the tethering approach is that a protein's activity can be determined without knowing the natural RNA target.

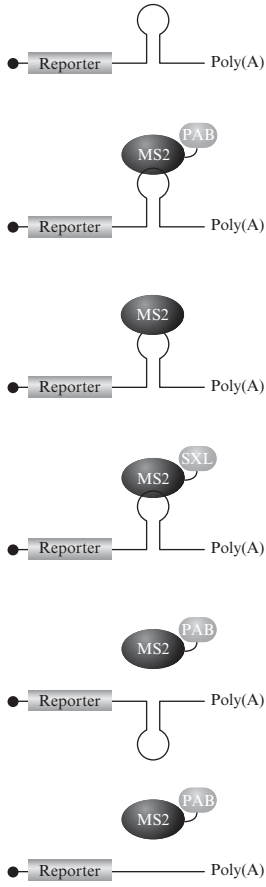
5.4. Analyzing the function of essential genes

In some cases, the RNA-binding protein under investigation is essential for cell viability; as a result, traditional genetic techniques are complicated by pleiotropic effects. The tethered function assay allows the function of the protein to be examined on just one mRNA species in an otherwise wild-type cell.

6. Important Controls

Several controls are critical in tethered function assays, and should always be performed (Fig. 14.3). It is necessary to ensure that (1) the tethered binding site does not affect the mRNA on its own, (2) the tethering protein alone (e.g., MS2 coat protein) does not have an impact, and (3) any observed effects should occur only in *cis* (that is, when the protein is bound to the mRNA). To control for possible *trans*-acting effects, the chimeric protein should be expressed alongside a reporter that lacks binding sites. This set of controls can ensure that an observed effect is specific to the protein of interest, and occurs only when it is associated with the mRNA in *cis* (see Fig. 14.3).

This concludes the general discussion of the design of a basic tethered function assay. In the following section we discuss a few specific examples with the aforementioned general principles considered. These case studies are not meant to be comprehensive of the literature but rather provide a sample of the uses of the tethered function assay to address certain biological issues. An overview is provided in Table 14.1.



Protein	Tethering site	Half-life (min)
None	MS2	4
MS2-PAB	MS2	**23**
MS2	MS2	4
MS2-SXL	MS2	5
MS2-PAB	Antisense MS2	3
MS2-PAB	None	3

Figure 14.3 Important controls to consider when performing a tethered function assay. Shown is a representation of experiments we performed to demonstrate the effects of PAB on mRNA stability (Coller *et al.*, 1998). First, the effect of the tether was evaluated by determining half-lives of the reporter in cells expressing just the MS2 coat protein alone or MS2 fused to Sxl-lethal, a distinct RNA-binding protein of similar size to PAB (MS2-SXL). Second, we determined that the observed increase in mRNA stability was a consequence of tethering PAB in *cis*, by measuring reporter half-life when the mRNA cannot bind MS2-PAB; either the tethering sites were not present or the sites were in the antisense orientation. This latter experiment also controlled for the contribution of the tethering sites to the stability of the reporter. From these controls it was possible to conclude that the observed reporter stabilization was specific to PAB and occurred only when it was tethered.

Table 14.1 Uses and adaptations of tethered function assays

Key issue	Protein	Organism	Tether	Reporter	Effects	Reference
Analysis of essential genes	Pab1p	Yeast	MS2	MFA2, PGK1	Tethered Pab1p stabilizes mRNA, functions independent of poly(A)	Coller *et al.*,1998
Separation of multiple functions	PAB1, Pab1p	*Xenopus*, yeast	MS2	Luciferase, CUP1	Distinct regions of tethered PAB1 stimulate translation and stabilize mRNA *in vivo*	Gray *et al.*, 2000
	Xp54	*Xenopus*	MS2	Luciferase	Tethered Xp54 represses or stimulates translation of poly (A) minus reporters	Minshall *et al.*, 2001
	SF2/ASF	*Xenopus*, HeLa cells	MS2	Luciferase	Tethering SR proteins demonstrates they have a novel role in translation	Sanford *et al.*, 2004
Dissection of complex	Ago2, Ago4	HeLa cells	N-peptide	Luciferase	Tethered Ago proteins repress translation, suggests that miRNA functions to guide Ago proteins to message	Pillai *et al.*, 2004
	hUPF1, hUPF2, hUPF3, hUPF3b	Mammalian cells	MS2	β-Globin	Tethered UPFs transform a normal message into a message subject to NMD	Lykke-Andersen *et al.*, 2000
	RNP, S1, Y14, DEK, REF2, SRm160	Mammalian cells	MS2	β-Globin	Tethered RNP S1 stimulates NMD on a normal mRNA	Lykke-Andersen *et al.*, 2001

Identifying localization functions	She2p, She3p	Yeast	MS2	LacZ	Tethered She2p is sufficient to stimulate the localization of ASHI mRNA	Long *et al.*, 2000
Analysis of modifying enzymes	PAP1	*Xenopus*	MS2	Luciferase	Tethered PAP1 polyadenylates mRNAs in the cytoplasm and stimulates their translation	Dickson *et al.*, 2001
	GLD-2	*Xenopus*	MS2	Luciferase	Tethering of GLD-2 homologs demonstrates these proteins are poly(A) polymerases	Kwak *et al.*, 2004
Following localized mRNAs	GFP	Yeast, mammalian cells	MS2	Various	Tethered GFP allows for the visualization of cytoplasmic mRNA localization in live cells	Reviewed in Singer *et al.*, 2005
Tethering of proteins to different areas of the reporter can have different effects	Staufen	HeLa cells	MS2	Luciferase (3′ UTR MS2 sites)	Tethering of Staufen to 3′ UTR of reporter in HeLa cells results in stimulation of NMD	Kim *et al.*, 2005
	Staufen	HEK293T cells	MS2	Luciferase (5′ UTR MS2 sites)	Tethering of Staufen to 5′ UTR of reporter in HEK293T cells results in stimulation of translation	Dugre-Brisson *et al.*, 2005

7. Examples of the Tethered Function Assay in the Literature

7.1. Analyzing essential genes

Tethered function assays allow the presence of essential RNA-binding proteins to be modulated on a target mRNA without affecting cell viability. For example, in *Saccharomyces cerevisiae,* PAB is an essential gene involved in many different aspects of mRNA metabolism. Studies of *PAB1* function using conditional alleles or genetic suppressors have shown that this protein is required for efficient mRNA translation, coupled deadenylation and decay, and polyadenylation. Detailed analysis of these functions *in vivo* is complicated by the breadth of PAB's roles and the fact that it is essential. Tethered function assays were used to circumvent these pleiotropic effects. Using this approach, PAB was shown to stabilize an mRNA to which it was tethered (Coller *et al.*, 1998). The activities of mutant forms of PAB (as tethered proteins) have been determined, and the active regions identified, even though yeast carrying the equivalent mutants would not be viable (Coller *et al.*, 1998; Gray *et al.*, 2000).

Tethered function assays have also facilitated analysis of essential translation initiation factors. For example, eukaryotic initiation factor (eIF)4G, a critical member of the cap-binding complex, is thought to recruit the 40S ribosome to the mRNA by simultaneously binding both cap-binding factors (eIF4E) and a 40S ribosome-associated complex (eIF3). A wealth of biochemical data has illuminated the contribution of eIF4G to translation *in vitro*. De Gregorio *et al.* (1999) used a tethered function approach to reveal mechanisms of eIF4G action *in vivo*. They first determined that eIF4G tethered to the intergenic region of a bicistronic reporter mRNA was sufficient to drive mRNA translation independent of the cap. This enabled identification of a conserved core domain of eIF4G that is required for translational stimulation (De Gregorio *et al.*, 1999). Similar studies with translational initiation factor eIF4E demonstrated that it stimulates translation independent of its ability to bind the cap (De Gregorio *et al.*, 2001). This latter study pioneered the use of N-peptide as a tethering device (Baron-Benhamou *et al.*, 2004).

7.2. Separation of multiple functions that reside within the same protein

Many posttranscriptional events are coupled. For example, splicing and 3′ polyadenylation influence one another and these events influence transport, degradation, and translation of the mRNA. In several cases, proteins involved in an upstream event can also have a dramatic role in a downstream

event. This complicates the use of conventional mutational analysis in pinpointing the protein's direct effects. In such cases, tethered function assays can help determine which of many affected steps are due directly to the activity of the protein.

In one example of this approach, SR proteins were shown to directly affect both splicing and translation (Sanford *et al.*, 2004). SR proteins are a large family of nuclear phosphoproteins required for constitutive and alternative splicing. A subset of SR proteins is known to shuttle between the nucleus and cytoplasm, suggesting that these proteins play important cytoplasmic roles in mRNA metabolism. Since many alterations in SR proteins *in vivo* impact splicing, it was difficult to determine whether any observed effects on translation were a direct effect of the SR defect or an indirect consequence of the splicing defect. To overcome this limitation, Sanford *et al.* (2004) used a tethered function assay in which they injected reporter mRNA bearing the MS2-RNA binding element with an MS2-SF2/ASF (an SR protein) protein fusion into the cytoplasm of *Xenopus* oocytes. The data demonstrated that tethered SF2/ASF stimulated translation by approximately 6-fold over the appropriate controls. This was also shown to be a general property of SF2/ASF by demonstrating that similar phenotypes were observed in HeLa cell-free translation extracts.

These findings resulted in the conclusion that SR proteins can promote mRNA translation after they are deposited on the mRNA via splicing. From the standpoint of this review, the important point is that the tethered function assay allowed the elucidation of a role for SR proteins in mRNA translation by removing the complication of the upstream event, i.e., splicing.

7.3. Dissecting complexes

Tethered function assays can be particularly useful when genetics is complex or unsuited to the problem. Many regulatory events are controlled by multiprotein complexes. Discrete components of the complex provide RNA binding and recognition, which in turn recruit the functional activity to the site of regulation.

7.3.1. Protein complexes: NMD

Analysis of non-sense-mediated decay (NMD) 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 process termed NMD. A group of proteins binds to the exon–exon (E/E) junction of mammalian mRNA subject to NMD (Le Hir *et al.*, 2000a,b; Singh and Lykke-Andersen, 2003). Although this complex is primarily found on NMD substrates, it was unclear if their presence was a cause or effect of the transcript being targeted for NMD. Lykke-Andersen *et al.* (2001)

used a tethered function approach to test whether the placement of any of these proteins on a normal mRNA would elicit an NMD response. While the E/E complex consists of at least five proteins, only tethered RNP S1 elicited NMD. In this case, the tethered function approach revealed a role of a specific protein in eliciting the function of a multiprotein complex (E/E complex), and showed it was a cause, rather than an effect, of the NMD process.

7.3.2. RNA–protein complexes: miRNAs

The tethered function assay has helped identify key components in the RNA protein complex associated with miRNA-mediated gene silencing. Ten years ago, a small, noncoding RNA of approximately 21 nucleotides, lin-4, was shown to bind the 3′ UTR of lin-14 mRNA in the nematode *Caenorhabditis elegans*, and to silence its translation (Pasquinelli *et al.*, 2005). Since that initial discovery, miRNAs have emerged as ubiquitous regulators of mRNA translation and stability.

Numerous factors are required for miRNA maturation and for the assembly of the miRNA into a ribonucleoprotein (RNP) complex that represses translation of the target mRNA. The RNA interference silencing complex (RISC) has been shown to be necessary for cessation of mRNA translation by an miRNA (Filipowicz, 2005; Sontheimer, 2005). Tethered function assays made it possible to dissect the repression function of RISC from the miRNA: specific components of RISC, namely Ago1–2, are sufficient to translationally repress reporter mRNAs to which they are artificially bound (Behm-Ansmant *et al.*, 2006; Pillai *et al.*, 2004; Rehwinkel *et al.*, 2005).

7.4. Mutagenesis of tethered proteins can also be useful in identifying unique gain-of-function alleles

Because the effects of a tethered protein are examined on a single reporter mRNA, the effects of many manipulations of the protein sequence can be examined readily and conclusively. This can reveal novel molecular properties in the protein.

This general approach has been applied to the Dhh1p/RCK1/p54 family of RNA helicases (Minshall and Standart, 2004; Minshall *et al.*, 2001). The *Xenopus* homolog, Xp54, is sufficient to repress the translation of an mRNA to whose 3′ UTR it is tethered. 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 conformation or helicase activity.

7.5. Tethering of proteins to different areas of the reporter can have different effects

It should be noted that the tethered function assay measures the effect of an mRNP complex in its nonnative context and thus may induce emergent properties of the protein. Moreover, the protein of interest may have distinct functions when positioned differently on the mRNA reporter. Indeed, it has been documented that similar proteins when tethered to different areas of an mRNA can have distinct outcomes.

For example, the conserved mRNA-binding protein Staufen is important during early embryonic development in *Drosophila* and has been identified as an important regulator of mammalian mRNA processes. Tethering of mammalian Staufen to the 5′ UTR of reporter mRNAs stimulates translation without impacting mRNA stability in HEK293T cells and rabbit reticulocyte lysates (Dugre-Brisson *et al.*, 2005). Interestingly, tethering mammalian Staufen to the 3′ UTR in HeLa cells does not stimulate translation, but instead destabilizes the mRNA (Kim *et al.*, 2005). These two reports are from distinct cells types, and so require further analysis. However, it may be that Staufen possesses different activities, dependent on its location in the mRNA. This property would echo that of IRP; bound to the 5′ UTR of ferritin mRNA, it inhibits translation; bound to the 3′ UTR of transferrin mRNA, it inhibits mRNA decay (Hentze *et al.*, 2004). It may turn out to be important to compare the effects of proteins tethered to different locales to reveal region-specific differences.

7.6. Identifying mRNA localization functions and visualizing tagged mRNAs *in vivo*

Proteins that cause an mRNA to move to a particular location within a cell can be assayed using the tethered function approach. For example, yeast She2p and She3p are present in a complex on the *ASH1* 3′ UTR. Tethering either She2p or She3p to the 3′ UTR of a reporter gene was sufficient to stimulate that mRNA's localization to the bud tip (Long *et al.*, 2000). These findings directly demonstrate a localization function, and should enable its genetic dissection away from formation of the complex or binding to RNA.

Several adaptations of the tethered function assay have been developed to tag an mRNA for further analysis, rather than study a particular protein's effects. Although these are not strictly tethered function assays (as the protein is merely a tag), we mention them here because they are so closely related technically. They now are widely used, and have been reviewed in their own right (Beach *et al.*, 1999; Singer *et al.*, 2005); we discuss only a single, early pioneering example.

Bertrand *et al.* (1998) used the tethered function approach to facilitate the study of *ASH1* mRNA localization in living yeast cells. *ASH1* mRNA is distributed into daughter cells during budding, regulating asymmetric switching of yeast mating type. To determine how various mutants affect *ASH1* mRNA localization, MS2 sites were inserted into the 3′ UTR of a LacZ reporter containing the *ASH1* 3′ UTR. The localization of this RNA was then monitored in living cells by tethering an MS2/green fluorescent protein (GFP) fusion to the MS2 sites (Fig. 14.4). Tethered GFP allows for simple detection of the RNA and provides a unique perspective of *ASH1* mRNA localization in real time (Bertrand *et al.*, 1998). This assay has also been successfully used to identify the factors involved in the process. For example, certain mutants (*she2* and *she3*) perturb localization monitored by tethered GFP (Bertrand *et al.*, 1998).

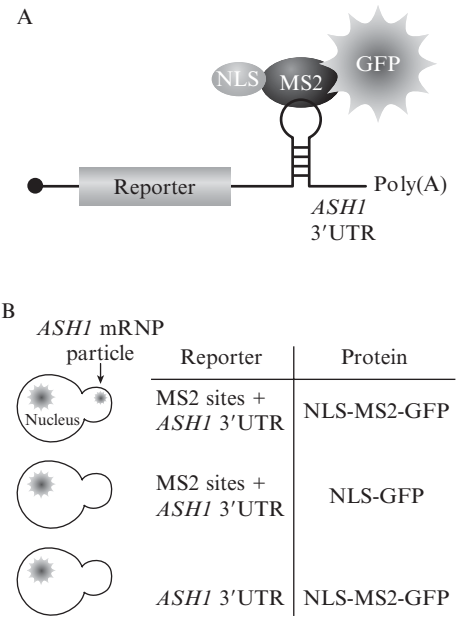


Figure 14.4 mRNA localization and tethered assays. (A) Tethered GFP can be used to monitor mRNA localization in living cells: GFP is tethered to the 3′ UTR or elsewhere in the mRNA, as a means of "tagging" the mRNA. Localization of the GFP fluorescence, and hence the mRNA, can then be monitored by microscopy. (B) Often the MS2–GFP fusion is tagged with a nuclear localization signal (NLS) as a means to reduce cytoplasmic noise. In this example, Bertrand *et al.* (1998) monitored the localization of the *ASH1* mRNA in yeast to the bud tip. Importantly, this ASH1 mRNP particle was observed only when the tethering sites were present in the reporter, and GFP was fused to the MS2 coat.

7.7. Tethered function can be used to detect both stimulatory and inhibitory events

As mentioned, the tethered function assay is highly adaptable. Tethered function assays have been used to monitor stimulatory and inhibitory effects of mRNA metabolism factors. For instance, in *Xenopus* it was demonstrated that tethered DAZL stimulates translation (Collier *et al.*, 2005), while using the same reporters others have shown that tethered Xp54 inhibits mRNA translation in *Xenopus* (Minshall and Standart, 2004; Minshall *et al.*, 2001). Similar results have been seen for assaying effects on mRNA stability. Certain classes of AU-rich binding proteins will stabilize mRNA when tethered, while others destabilize the mRNA (Barreau *et al.*, 2006; Chou *et al.*, 2006). Thus, tethered function assays provide flexibility in allowing a range of phenotypes to be observed.

7.8. Analyzing mRNA modifying enzymes

Tethered function assays have been used to identify enzymes involved in mRNA processing. Sequences near the 3′ end of an mRNA recruit a complex of proteins that promotes 3′ end cleavage and polyadenylation. By tethering the relevant poly(A) polymerase directly to the 3′ end of the reporter, that enzyme was shown to be sufficient for the elongation of poly (A) tails in oocytes and to stimulate translation as a result (Dickson *et al.*, 2001). Sites for interaction with other components of the complex are dispensable (Dickson *et al.*, 2001). The same general approach has been used to identify other divergent poly(A) adding enzymes, termed the GLD-2 family, from *C. elegans,* flies, frogs, mice, and humans (Kwak *et al.*, 2004; J. E. Kwak *et al.*, unpublished observations; Wang *et al.*, 2002).

A strength of the tethered approach is that many candidate open reading frames (ORFs) can be tested rapidly. A limitation is that false negatives arise. For example, two *Saccharomyces cerevisiae* proteins, Trf4p and Trf5p, that are known to be poly(A) polymerases, differ dramatically as tethered proteins. Trf5p is active, and Trf4p is not (J. E. Kwak *et al.*, unpublished observations). This may reflect a difference in their substrate specificity, requirements for RNA or protein partners, or be an artifactual consequence of an inactive conformation in one chimeric protein.

Tethering assays can reveal unanticipated biochemical activities. In the same group of tethering experiments that identified the GLD-2 family, certain relatives of these PAPs turn out not to add poly(A) at all, but to add poly(U) instead (J. E. Kwak *et al.*, unpublished observations). Investigations into the biological role of these newly discovered poly(U) polymerases are currently underway. The key point here is that tethered assays enabled facile biochemical identification of the RNA modifications they catalyze.

8. Prospects

Tethered function assays provide a simple means to address the role of specific RNA-binding proteins on mRNA metabolism and function. Their use is certainly not limited to the few examples mentioned here and in Table 14.1. The tethered function approach provides a unique platform for the study of suspect regulators of mRNA metabolism that have unknown target specificity and/or functional activity. 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 function beyond familial sequence resemblance are needed. Tethered function assays may provide a rapid screen to sort proteins into functional families.

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