Stoichiometry of a regulatory splicing complex revealed by single-molecule analyses

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Splicing is regulated by complex interactions of numerous RNA-binding proteins. The molecular mechanisms involved remain elusive, in large part because of ignorance regarding the numbers of proteins in regulatory complexes. Polypyrimidine tract-binding protein (PTB), which regulates tissue-specific splicing, represses exon 3 of α-tropomyosin through distant pyrimidine-rich tracts in the flanking introns. Current models for repression involve either PTB-mediated looping or the propagation of complexes between tracts. To test these models, we used single-molecule approaches to count the number of bound PTB molecules both by counting the number of bleaching steps of GFP molecules linked to PTB within complexes and by analysing their total emissions. Both approaches showed that five or six PTB molecules assemble. Given the domain structures, this suggests that the molecules occupy primarily multiple overlapping potential sites in the polypyrimidine tracts, excluding propagation models.

As an alternative to direct looping, we propose that repression involves a multistep process in which PTB binding forms small local loops, creating a platform for recruitment of other proteins that bring these loops into close proximity.

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Introduction

The scale of alternative splicing is breathtaking. It has been estimated on the basis of high-throughput sequencing of mRNA that there are about 100,000 significant alternative splicing events in humans, derived from about 90% of the multiexon genes; this corresponds to an average of seven events per multiexon gene (Pan et al., 2008; Wang et al., 2008). The actual number of alternative isoforms of mRNA depends on the combinations produced by each gene, but the number could exceed a million. Despite such overwhelming numbers, it seems that alternative splicing is not the result of errors (Pan et al., 2008) and that the error rate of splicing is so low that any errors may be in large part the result of misincorporation during transcription (Fox-Walsh and Hertel, 2009).

Many, possibly most, alternative splicing events are represented more abundantly in particular tissues (Castle et al., 2008; Wang et al., 2008). The introns flanking tissue-specific exons are enriched in a number of sequence motifs, the most abundant of which contain UCUCU (Castle et al., 2008; Wang et al., 2008). This is recognized as a site, or partial site, for binding of polypyrimidine tract-binding protein (PTB) (Chan and Black, 1995, 1997). PTB was characterized as a PTB repressor of a muscle-specific exon (Mulligan et al., 1992). Subsequently, it was shown to regulate the expression of a number of mRNA isoforms, particularly some specific to neural and muscle tissue (Grabowski and Black, 2001; Black and Grabowski, 2003; Boutz et al., 2007; Spellman et al., 2007; Venables et al., 2008). As the level of inclusion of exons with nearby intronic UCUCU motifs is inversely correlated among tissues with the abundance of PTB (Castle et al., 2008), repression by PTB seems to be a widespread and important contribution to tissue-specific gene expression. Indeed, genome-wide analysis of PTB–RNA association suggests that PTB is associated with transcripts from over 40% of annotated human genes (Xue et al., 2009).

PTB has four RNA-binding domains of the RRM type, with flexible linkers after domains 1 and 2 (Petoukhov et al., 2006). There are three isoforms of PTB, which differ in the length of the linker between RRMs 2 and 3. PTB4 has the longest linker and is the most effective repressor of Tpm1 exon 3 (Wollerton et al., 2001). The individual RRMs bind RNA with low affinity and weak specificity for short pyrimidine tracts (Oberstrass et al., 2005). Structural analyses of the individual RRMs and of RRMs 3 and 4 together suggest that the first three RRMs could bind a consecutive sequence of at least 15 nucleotides but that the fourth RRM would require linking sequences before binding to a short pyrimidine tract (Oberstrass et al., 2005; Lamichhane et al., 2010). In contrast, the occluded binding site size on poly(U) for the intact protein was estimated to be 5 nts (Perez et al., 1997). The protein was found by selection experiments to recognize a pyrimidine-rich consensus of 26 nts (Singh et al., 1995), although experiments with natural substrates identified shorter high-affinity motifs of UCUCUCU (Chan and Black, 1997) or UCUU (Perez et al., 1997). In the absence of other proteins, PTB binds to RNA with canonical
motifs to form small complexes with nanomolar affinity, and then larger complexes non-cooperatively (Singh et al., 1995; Amir-Ahmady et al., 2005; Clerte and Hall, 2006). The numbers of PTB molecules in the larger complexes were hard to predict but seemed to correlate more with the overall length of the pyrimidine tract than with specific motifs. An analysis of genome-wide binding sites suggested that the level of pyrimidines remains elevated over tens of nucleotides around each site (Xue et al., 2009).

The mechanisms by which PTB association represses splicing are unclear. In several cases, pyrimidine-rich tracts are located on both sides of the regulated exon or splice site (Wagner and García-Blanco, 2001; Amir-Ahmady et al., 2005). In the case of the neural exon of the Src gene, which is repressed in most tissues by the binding of PTB, two separate pyrimidine tracts on either side of the exon cooperate to form an ATP-resistant complex containing unknown numbers of proteins (Chou et al., 2000). The effect of this is to prevent the interaction of U1 snRNPs, bound to the 5′ exon, with components at the downstream 3′ splice site (Sharma et al., 2005, 2008), but the nature of the impediment is unknown. Pyrimidine tracts are found on both sides of the alternative exon 3 of α-tropomyosin (TPM1), but, compared with the Src gene, they are longer and the pattern of regulation is different. Exons 2 and 3 of TPM1 are mutually exclusive (Figure 1). Exon 3 is used in most tissues because it contains strong splicing signals (Mullen et al., 1991), and the switch to exon 2 in smooth muscle cells is primarily the result of repression of exon 3 through the pyrimidine-rich tracts in the flanking introns (Gooding et al., 1994; Perez et al., 1997). It is likely that the longer pyrimidine tracts (P3 and DY) are bound by PTB in all cells (Singh et al., 1995; Perez et al., 1997; Gooding et al., 1998), but that the strong splicing signals override this except in smooth muscle cells. When the branch site is weakened by mutations, exon 3 is strongly repressed in HeLa cells (Gooding et al., 2006) by the P3 and DY PTB-binding elements (CG and CWJS, unpublished data).

Although it is clear that PTB has a preference for binding to pyrimidine tracts, the connection between PTB binding and inhibition remains elusive. Two speculative models have dominated thinking, both of which have been invoked also to explain the activities of another repressor, hnRNP A1. One suggestion is that repressor molecules bound to sequences flanking the regulated sites associate in a complex, forcing the exon or site into a loop (Blanchette and Chabot, 1999; Chou et al., 2000; Eperon et al., 2000; Wagner and García-Blanco, 2001; Nasim et al., 2002; Oberstrass et al., 2005; Spellman and Smith, 2006). However, direct evidence for this is lacking. Moreover, looping is not easily reconciled with evidence that some other exons are regulated by single-pyrimidine tracts (Shen et al., 2004; Izquierdo et al., 2005), and an alternative model is that extended complexes propagate along the RNA from the initial high-affinity site(s) (Eperon et al., 2000; Wagner and García-Blanco, 2001; Zhu et al., 2001; Spellman and Smith, 2006). Given that estimates for the length of sequence bound or recognized by pure PTB vary so widely and that there is a strong concentration-dependent ability to form large complexes, it is impossible to infer the nature of the repressor complexes from the properties of the pure protein.

A critical factor in developing mechanistic models for any regulated splicing reactions will be the ability to determine the numbers of proteins bound to RNA in complexes formed under splicing conditions. However, there are no methods available for doing this, whether with purified complexes or crude extracts, because of the complexity of the reactions: pre-splicing complexes assemble in the presence of numerous competing RNA-binding proteins, many of which have low specificity and are likely to bind dynamically and sometimes cooperatively. Such complexity may make it difficult to establish general principles for regulation, because different sites may involve different configurations of bound proteins. Despite considerable progress in identifying regulatory proteins and their target sites, we are in the unhappy situation that we do not know the molecular mechanisms operating at even one site.

To address this critical limitation, we have developed single-molecule methods for analysing the stoichiometry of protein–RNA complexes formed in nuclear extracts. We show here that five or six molecules of PTB assemble around exon 3 of rat Tpm1 pre-mRNA, and we propose a model for the organization of the complex. This is the first report describing the measurement of the stoichiometry of proteins in complexes in nuclear extracts, and the method will be of widespread use in investigations of many aspects of gene expression.
Results

It has been shown previously that PTB binds to the pyrimidine-rich tracts flanking exon 3 and that these tracts are essential for repression (Gooding et al., 1994, 1998; Perez et al., 1997). To follow the binding of PTB to RNA among all the other proteins in nuclear extracts, GFP-labelled PTB (isoform 4) was expressed in HEK 293T cells and nuclear extracts were prepared. RNA transcripts corresponding to various portions of exon 3 and its flanking intron sequences (Figure 1) were transcribed from genomic fragments cloned in plasmid pGEM4Z (Gooding et al., 1998; Gromak et al., 2003a) cut with EcoRI or AccI (TM1 Trunc) and annealed to an oligoribonucleotide analogue complementary to the first nine nucleotides of the transcript that had been conjugated with a fluorescent label (Cy5) and biotin. Constructs TM2 and TM4 contain mutations in the three UCUU motifs in element P3 (Gromak et al., 2003a), and constructs TM3 and TM4 contained deletions of two short U-rich tracts in element DY (Gooding et al., 1998). The transcripts were incubated in the extracts under splicing conditions, and the complexes formed were captured from diluted reaction mixtures on streptavidin-treated silica slides. The signals from the GFP and Cy5 fluorophores were detected by total internal reflection fluorescence (TIRF) microscopy (Conibear and Bagshaw, 2000), separated with a beam splitter and acquired on two halves of an emCCD chip (Figure 2). Representative time courses of GFP fluorescence from single molecules of RNA are shown in Figure 3. The survival times of the fluorescence from single molecules were no shorter than those from GFP molecules deposited directly on the surface, indicating that fluorescence was terminated by bleaching rather than by dissociation from the complex.

Quantification of PTB molecules in each transcript complex by single-step photobleaching

Novel strategies were needed to quantify the numbers of GFP-PTB molecules associated with each transcript molecule. Unlike analyses done with membrane proteins (Leake et al., 2006; Ulbrich and Isacoff, 2007) or small organic fluorophores (Shu et al., 2007), counting the number of steps in which GFP molecules became progressively bleached was impracticable because the variations in amplitude between individual GFP molecules (see below) effectively masked the individual steps in many cases (compare Figures 3B and C). Selecting only those spots that produced clear steps would leave open the possibility of accidental bias. However, it was readily possible to distinguish complexes with only one GFP-PTB, which behaved like the individual GFP molecules seen in the absence of RNA (Figure 3A), from those containing more than one, which could be identified by the duration of the signal and the larger intensities (Figure 3C). This allowed us to count the numbers of complexes with one GFP-PTB and those with more than one. The total number of PTB molecules in the complexes could be calculated by assuming that (1) the total number of PTB molecules bound was constant and (2) the proportions of PTB and GFP-PTB followed a binomial distribution. The probability \( p \) that a molecule

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**Figure 2** Representative image showing co-localization of RNA (red) and PTB protein (green). Nuclear extract containing GFP-PTB was incubated with TM1 RNA previously annealed to Cy5-labelled biotinylated oligonucleotide. The mixture was diluted and injected into a chamber on a prepared silica slide. Fluorescence from molecules attached to the surface was detected by TIRF. Images obtained in the Cy5 and GFP channels have been superimposed. White circles show co-localized Cy5 and GFP signals.

**Figure 3** Time recordings of GFP-PTB molecules. (A) Trace from GFP-PTB in nuclear extract in the absence of added RNA, showing the numbers of photons collected in frames of 300 ms versus time of observation. After about 23 s, the molecule bleached in a single step. (B) Trace from GFP-PTB co-localized with TM1 RNA. Five possible discrete steps are marked by lines. (C) As in (B), but in this case, discrete steps cannot be assigned.
of PTB bound to RNA was GFP-PTB, rather than endogenous unlabelled PTB, which was determined by cross-linking of protein in the extract to radioactive TM1 trunc RNA (Figure 4) and found to be about 0.45.

The results from the analysis of about 1600 molecules (Table Ia) show that the fraction of RNA molecules associated with GFP-PTB bleaching in a single step rose from 18%, with the longest wild-type transcript, to 68% with the shortest. Table Ib shows the fraction of molecules expected to show single-step bleaching with $p = 0.45$. The observed value of 18% for the wild-type transcript TM1 falls between the values expected for 5 or 6 molecules of PTB. Similarly, the complexes formed on TM2 and TM3 contain 4–5 molecules, those on TM4 contain 3–4 and those on TM1 trunc contain 2. The observation that removing the three high-affinity UCUU motifs from the P3 element (TM2) reduced the number of bound PTB molecules by only one was surprising and suggested that the common practice of predicting PTB-binding sites by high-affinity motifs is misleading.

Table II shows the fraction of labelled RNA molecules co-localized with GFP-PTB (Table II), which reflects the affinity of the interactions. The fractions were progressively reduced by mutations in either polypyrimidine tract (TM2 and TM3), both tracts (TM4) and by truncation of the RNA immediately downstream of P3 (TM1 trunc). These results provide an independent confirmation of the significance of the single-step photobleaching results in Table Ia.

Quantification of PTB molecules in each transcript complex by measurement of integrated intensities

Our second strategy involved counting the total number of detected photons (TNPs) from GFP in each complex before bleaching. For single GFP-PTB molecules, in the absence of RNA, the probability density function for the TNP fits a normal probability density function (Figure SB). On this basis, a probability density function was calculated assuming that the TNP would be Gaussian for each GFP-PTB within a complex and that the number of complexes with $i$ bound GFP-PTB molecules ($i = 1,2,3, \ldots b$) would follow a binomial distribution, as above. The observed TNP for all molecules in each experiment was converted to a cumulative distribution and fitted by the corresponding cumulative distribution calculated from the function described above (Figure 5). The fitting procedure was done with various values of $p$, the probability that a PTB molecule bound to the RNA was GFP labelled. The best global fit was obtained with $p$ close to 0.45 (Figure 5), suggesting that TM1 binds 5–6 molecules, TM2 and TM3 bind 4–5, TM4 binds 3–4 and TM1 trunc binds 1–2. These results are in good agreement with the results from the first method (Tables I and II), although the first strategy is more sensitive to the numbers of molecules in smaller complexes, and the second strategy is more sensitive to the numbers of molecules in the larger complexes.

Together, our results show that five or six molecules of PTB are bound to the wild-type $z$-tropomyosin RNA. Even though these results were derived from non-muscle extracts, the complexes are likely to represent the repressed state of exon 3. Mutations that weaken the exon 3 branch site lead to high levels of exon 3 repression in HeLa cells (Gooding et al, 2006), which remains dependent on the P3 and DY PTB-binding elements (CG and CWJS, manuscript in preparation). As our transcripts do not contain the branch site, there would be no interference with formation of a repressed complex.

**Table Ia** Observed proportions of Cy5 (RNA) molecules in which associated GFP-PTB is bleached in a single step

| Substrate       | TM1 | TM2 | TM3 | TM4 | TM1 trunc |
|-----------------|-----|-----|-----|-----|-----------|
| % Single step   | 18  | 28  | 29  | 40  | 68        |
| No. measured    | 472 | 288 | 319 | 342 | 159       |

The fraction of GFP spots that co-localized with Cy5 spots and exhibited single step bleaching is shown for each substrate tested (% single step). The values in parentheses show the highest value for the fraction when ambiguous assignments are included (see Materials and methods). The total number of measurements with each RNA substrate is shown.

**Table Ib** Proportions of molecules showing single step bleaching predicted with various values of $n$

| Total number of PTB/RNA $(b)$ | 6  | 5  | 4  | 3  | 2  | 1  |
|-------------------------------|----|----|----|----|----|----|
| $p = 0.45$                    | 14 | 22 | 33 | 49 | 71 | 100|

Theoretical values for the fraction of spots exhibiting a single bleaching behaviour were calculated using a binomial distribution for the complexes formed by $b$ bound PTB molecules with the fraction of PTB-GFP molecules equal to 0.45. The fraction of GFP spots exhibiting single bleaching in extract in the absence of any RNA substrate was >90%.
Table II  Efficiency of formation of the complexes for various substrates

|                | TM1 | TM2 | TM3 | TM4 | TM1 trunc | Oligonucleotide |
|----------------|-----|-----|-----|-----|----------|-----------------|
| No. Cy5 spots  | 334 | 657 | 866 | 1344| 824      | 1724            |
| % Associated with GFP-PTB | 66  | 47  | 52  | 32  | 23       | <5              |
| No. measured   | 334 | 657 | 866 | 1344| 824      | 1724            |

The fraction of Cy5 spots that co-localized with GFP spots relative to the total number Cy5 spots detected within imaging area is shown for each substrate tested.

Figure 5  Cumulative distributions of total photon counts. (A) Observed cumulative distributions of the total photons collected for each RNA in nuclear extract. Numbers were normalized to the total number of spots analysed for each transcript, shown in Table Ia. The abscissa is in counts/pixel, that is, total counts over the 8 x 8 area per pixel. (B) The cumulative distribution for GFP-PTB in extract in the absence of added RNA, with a curve fitted assuming that each spot contained only one fluorescent protein molecule. Inset, plot of residuals. Abscissa as in (A). (C–G) Cumulative distributions for GFP-PTB bound to transcripts, with different fits fitted assuming that the total number of PTB molecules bound was as shown in the individual legends.
Fitting PTB to the polypyrimidine tracts
With the knowledge that only five or six molecules of PTB are bound to TM1, and that the first three RRM domains of each PTB molecule contact in total only about 15 nucleotides (Oberstrass et al, 2005), with additional binding by RRMs 4 elsewhere, our first conclusion is that we can exclude models in which PTB molecules occupy the RNA for 500 nucleotides continuously between the upstream and downstream sites. Instead, PTB must act directly through the discrete upstream and downstream regions that it recognizes directly.

As the assignment of high-affinity sites does not predict the number of PTB molecules bound, nor their sites of binding, we have combined the data in Table III with the sequence specificities derived for each domain from the structures (Oberstrass et al, 2005) to model PTB in the repressed complex, assuming that in nuclear extract competition with other proteins would tend to enforce binding only with optimal sites. We allowed the inter-domain separation to range from 1 to 6 nucleotides (based on an average inter-domain distance of 4.5 nm (Petoukhov et al, 2006)). RRMs 4 has a low-binding specificity (YCN) and, because of the back-to-back packing of RRMs 3 and 4, it binds preferentially 15 or more nucleotides away from RRMs 1–3 (Oberstrass et al, 2005; Lamichhane et al, 2010). As there would be a YC sequence at a distance >15 nts from any possible site for RRMs 1–3, RRMs 4 binding does not provide a constraint and was not taken into account. Analysis of the entire sequence of TM1 showed that there were numerous overlapping candidate sites, almost entirely located within the known P3 and DY-binding elements (Figure 6A). The maximum number of proteins that could be accommodated simultaneously in P3 was three, although there are a number of possible arrangements (Figure 6B). To test whether up to three proteins could bind in practice, gel shift assays were done with TM1 trunc RNA and various proportions of recombinant N-terminally truncated PTB1 and full-length PTB4 proteins at a saturating total PTB concentration of 4 μM, in the absence of nuclear extract. The results confirm that this region could accommodate three PTB molecules (Figure 6C).

The correspondence between the binding we observed in nuclear extract and the structure-based predictions was underscored by the mutant TM2, in which mutations had altered all three of the UCUU sequence motifs previously believed to be the high-affinity binding sites in P3 (Perez et al, 1997). These mutations reduce repression of exon 3 in smooth muscle (Gromak et al, 2003b) and HeLa cells (CG and CWJS, manuscript in preparation). Our results in Tables I and III showed a reduction by one in the number of PTB molecules bound in nuclear extract, and the number of molecules predicted as described above to bind to P3 was also reduced by one. In contrast, gel shift assays showed that the mutations did not affect the binding of pure PTB to TM1 trunc (Figure 6D). This discrepancy in the gel shift assays is explained by the use of the very high concentrations of pure PTB and the high protein:RNA ratio used to measure stoichiometry by gel shifts, under which conditions and in the absence of competing or accessory nuclear proteins the protein may bind to suboptimal sites.

If three molecules of PTB are bound to element P3 in TM1, then only two or three could be bound to DY. The mutations in TM3 cause loss of only one PTB from DY. Predictions based on the domain structures, as above, suggest that up to two molecules could bind DY simultaneously at optimal-binding sites (Figure 6E), and that only one molecule would be able to bind this region in TM3. Gel shift assays of DY with recombinant PTB appeared to show two bands with the N-terminally truncated PTB1-N (Figure 6F), but up to three extra bands appeared as PTB4 was added. Moreover, the background was more prominent, suggesting that the complexes were less discrete or less stable than those on P3. One interpretation of this is that each molecule of DY RNA was bound by either two or three molecules of PTB. This might be explained by a suboptimal arrangement accommodating three molecules that is favoured when high concentrations of pure protein are used, as noted above for the occupancy of mutant P3 sequences. If this occurred to some extent in nuclear extracts, then the presence of complexes containing either two or three molecules of PTB in DY would account for the observation that all transcripts except TM1 trunc (which lacks DY) appear to be bound by either n or n+1 molecules of PTB.

We conclude that the use of single-molecule methods has enabled us to measure the numbers of proteins in a splicing repressor complex assembled in nuclear extracts on wild-type and mutant sequences. These measurements have allowed us to apply data from domain structures to identify probable sites of binding of the proteins and to test the ability of the model to accommodate the effects of the mutations.

Discussion
We have described here the first use of single-molecule methods to measure the number of molecules of a protein in a complex assembled in nuclear extract. This is essential information for any mechanistic models, but it has been entirely lacking in all earlier research into the regulation of pre-mRNA splicing and other steps in gene expression. The difficulty arises partly from the nature of the proteins involved and from the complexity of the reactions. Bioinformatic and selection approaches to identify sequence motifs that affect the selection or use of splice sites have produced so many motifs (Coulter et al, 1997; Liu et al, 1998; Schaal and Maniatis, 1999; Fairbrother et al, 2002; Wang et al, 2004, 2005, 2008; Zhang and Chasin, 2004; Han et al, 2005; Zhang et al, 2005, 2008; Castle et al, 2008) that about three quarters of all the nucleotides in an exon (Zhang et al, 2009) or a quarter of all possible hexamer motifs (Wang and Burge, 2008) are likely to affect splicing. This is borne out by experiments (Pagani et al, 2003; Singh et al, 2004). It is presumed that most of these sequences are recognized by proteins, but predicting which proteins bind where is not straightforward. Numerous proteins bind pre-mRNA (Gabut et al, 2008), but there are very few cases in which their target sequences are well defined. Even in cases where the properties have been studied extensively, the binding sites
Figure 6 Ways of accommodating five PTB molecules on TM1 pre-mRNA. (A) Possible binding sites for PTB monomers (domains 1–3), based on the sequence specificities inferred from the structures (Oberstrass et al., 2005). Each vertical line represents the first nucleotide in the potential binding site. The ordinate shows the number of potential binding sites that begin at the same nucleotide; such sites arise because of the range of values allowed for the distance between the motifs recognized by each domain. The abscissa shows the position in the regulatory region, with the P3 and DY elements and exon 3 delineated. (B) Possible arrangements for the binding of three molecules of PTB to the P3 element. Each horizontal line shows one possible arrangement. Coloured bars show the region bound by the three separate molecules in each arrangement. Identical arrangements arise on different horizontal lines when RRM2 is predicted to bind at different positions within a monomer but RRM domains 1 and 3 are at the same positions. The region marked ends at the last specific nucleotide recognized by domain 3 and does not include the two additional nucleotides bound by the domain (Oberstrass et al., 2005). (C) Gel shift analysis of PTB binding capacity of TM1 trunc RNA. The left-hand lane contained RNA only. The other lanes contained, from the left, RNA incubated with His-tagged PTB4 at 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.5 and 0 mM, and sufficient His-tagged PTB1-N (containing a deletion of the N-terminal 54 amino acids) to maintain a total PTB concentration of 4 mM. Electrophoresis was done on a 4% polyacrylamide gel. Labels to the right of the image show our interpretation of the compositions of the bands. (D) Gel shift analysis of PTB binding capacity of mutant TM1 trunc RNA. The substrate is derived from TM2 and contains the mutations in the P3 element that change the three putative high-affinity UCUU motifs in P3 to UUUU, UUUU and CCUU and strongly reduce repression. The protein concentrations and conditions of electrophoresis are as in Figure 6C. (E) Possible arrangements for the binding of two PTB molecules to the DY element, labelled as in (B), in which both molecules are located at optimal sites. (F) Gel shift analysis of PTB binding capacity of RNA from the DY element. The transcript contains nucleotides 449–562, that is, all of the DY element shown in Figure 6E. The protein concentrations and conditions of electrophoresis are as in Figure 6C. Possible assignments are shown.
attributed to an important protein such as SF2/ASF depend on whether they are assayed with pure protein (Tacke and Manley, 1995), functional selection (Liu et al., 1998; Smith et al., 2006) or cross-linking in cells (Sanford et al., 2008) and in all cases they are diverse. It is likely, therefore, that the composition of pre-mRNP is the result of competition among proteins with overlapping binding preferences, each with a range of affinities for different sequences (Abdul-Manan and Williams, 1996) and sometimes able to bind cooperatively. Prediction is therefore difficult, experimental validation in bulk assays requires purification with all the artefacts that entails, and purified proteins will have opportunities that would be denied to them in the nucleus. However, the problem of pre-mRNP composition can be addressed by single-molecule methods.

We expected to be able to count the number of fluorescent proteins attached to the pre-mRNA by following bleaching steps (Figure 3). However, this proved impracticable and subjective for a high proportion of molecules due to the differences in amplitudes of emission from individual molecules. The presence of a significant fraction of unlabelled PTB in the extract was an advantage, because it allowed a large fraction of the complexes to form with only one GFP-PTB molecule, which can be identified readily. We assumed that the proportions of complexes containing various numbers of labelled and unlabelled PTB followed a binomial distribution. This approach was supported by the discovery that the proportions of complexes containing a single GFP-PTB increased as expected with increasing numbers of mutations in the substrates that compromised PTB binding (Table I). Moreover, there was an inverse relationship between this proportion and the proportion of RNA molecules associated with GFP-PTB, as predicted. In addition, we measured the TNPs from co-localized GFP-PTB/RNA spots until they bleached, which produced a modified Gaussian distribution (see Materials and methods). This produced results that were almost entirely consistent with the first method (Table III).

These approaches allowed the number of bound proteins to be determined, within errors due to uncertainties intrinsic to the splicing complex and TIRF microscopy. For instance, we modelled the distributions of complexes assuming that each molecule of each transcript type contained the same number of PTB molecules. This is partly supported by gel shift assays of TM1 in nuclear extract, which produce a single, well-defined band (Supplementary Figure S1), and by single-molecule studies with recombinant PTB that show that TM1 has 5–6 binding sites for PTB (Y Kafasla and DC, data not shown). Another source of uncertainty arose from the large variations of emission amplitudes. These may be the result of restricted movement of tethered complexes randomly oriented on the imaging surface, strong polarization of the evanescent field and the collection efficiency function of the objective. The close proximity of bound PTB molecules to each other (distances <10 nm) does not exclude homofRET occurring between GFP molecules. The latter can modulate (in a non-linear manner) the overall intensities of GFP molecules and their life times (Luczhowksi et al., 2008). The fraction of complexes with single GFP-PTB molecules might be reduced by the slight tendency of eGFP to dimerize, although our results in the absence of RNA suggest that this affects <10% of the complexes. Nevertheless, we believe that these uncertainties are relatively small and our simple models give good approximations of the experimental data.

Knowing that TM1 contains five or six molecules of PTB, it became feasible to predict the binding sites on the substrate. Potential sites were mapped by combining the binding preferences of individual domains (Oberstrass et al., 2005) with estimates for the lengths of flexible linkers between them (Petoukhov et al., 2006). Interestingly, this approach revealed a multiplicity of arrangements that satisfied the conditions, but it was possible at most to fit only three optimally bound molecules on P3 and two on DY. The multiplicity of possible sites for the protein in P3 and DY and the multiplicity of arrangements possible for three domains within each site could contribute to a much higher apparent affinity for the first PTB molecule to bind. The lack of experimental evidence for binding to other candidate sequences in TM1 (at ~340 and 410 nucleotides in Figure 6A) might be the result of weaker apparent binding, because the level of multiplicity is much reduced, as well as competition with other proteins. The first PTB molecule bound to P3 or DY might interfere with subsequent binding by additional molecules. However, PTB comprises flexibly linked domains that each recognize only two specific nucleotides (Oberstrass et al., 2005), and we suggest that this arrangement might permit relatively facile shuffling on pyrimidine tracts, thereby reducing the interference with binding of second and third molecules.

Our results provide important insights into the nature of the repressor complex. Figure 7A represents models in which repressor complexes use cooperative interactions to propagate along the RNA away from high-affinity sites (Wagner and Garcia-Blanco, 2001; Spellman and Smith, 2006). PTB
does not itself show strong protein–protein interactions, but Raver1 contributes to repression of exon 3 (Gromak et al., 2003a) and could fulfill this function. It has four potential PTB-binding motifs that interact with the α-helical face of RRM2 (Rideau et al., 2006), and it was possible that it could facilitate the formation of extended complexes. However, the length of RNA bound by a single molecule of PTB is quite insufficient for five molecules to span over 500 nucleotides, and we consider that this model can be rejected for PTB.

As both P3 and DY are essential for repression through binding of PTB, models have been suggested in which there are looping interactions between proteins bound to the two regions. Loops might repress splicing, as seen in the effects of secondary structures around exons in vitro (Solnick, 1985). RRM domains 3 and 4 of PTB associate back-to-back and bind the RNA in opposite orientations, with a preference for at least 15 nucleotides between them (Oberstrass et al., 2005; Lamichhane et al., 2010). A natural suggestion is therefore that the 450 nts between P3 and DY are placed in such a loop (Oberstrass et al., 2005; Spellman and Smith, 2006). It is difficult to predict the likelihood of collision between PTB attached to one strand with a second RNA sequence, because the binding of proteins to the sequences within the potential exon-containing loop will affect the flexibility of the RNA. However, we note that other interactions that might involve looping are restricted to a range of <300 nts (Lavigne et al., 1993; Graveley et al., 1998), and it would be expected that shorter loops would be more likely to form.

As the only known requirements for loop formation by RRM4 are that the distance from RRM3 is 15 nts or more and the domain binds YC (Oberstrass et al., 2005; Lamichhane et al., 2010), it is not possible to predict with any confidence where RRM4 might bind. RRM4 is known to be essential for the repression of splicing but to make little or no contribution to the affinity of PTB (Liu et al., 2002). If the probability of binding by RRM4 is increased by proximity (beyond 15 nts) to RRM3 and by an abundance of cytosines, then it is possible that it binds heterogeneously to any of a number of C-rich tracts downstream of P3 and upstream of DY, forming small loops as shown in Figure 7C. Raver1 may interact with PTB proteins on each side of the exon or it may bridge the now-smaller distance across the exon. The small loops would contain the UGC-rich URE and DUGC elements. Muscleblind-like protein (MBNL) is known to bind these sequences and we have evidence that it contributes to repression of exon 3 (CG and CWJS, manuscript in preparation). MBNL binds structured regions (Warf and Berglund, 2007; Yuan et al., 2007), and it may be that one function of PTB is to facilitate re-structuring of the RNA. MBNL can self-associate and even form large rings (Yuan et al., 2007), and it would be a good candidate for stabilizing a loop that sequesters the 3′ and 5′ splice sites of the exon (Figure 7D). What we suggest, therefore, is that assembly proceeds in at least two stages, and that PTB association provides a platform for subsequent recruitment of Raver1 and/or MBNL or other regulatory proteins. It is now an important and practical objective to determine the numbers of molecules of Raver1 and MBNL in these complexes. We believe that the single-molecule approaches described here will prove to be of widespread use in revealing the stoichiometry of many other complexes involved in gene expression.
localized with GFP signals. In the presence of oligonucleotide alone, the fraction of co-localized Cy5 spots was <5%. Time series intensities were analysed for co-localized spots using an area of 8 × 8 pixels, as the average apparent width (FWHM of a Gaussian fit) of the spots was 2.6–2.8 pixels. Spots that deviated significantly from a Gaussian fit were not counted (<5%).

Analysis of bleaching steps

More than 90% of the signals from individual GFP-PTB molecules in the absence of exogenous RNA showed a single-bleaching step. The duration of emission varied significantly in the range 2–30 s with the mean of about 10 s. Emissions from GFP-PTB associated with Cy5-labelled RNA showed a noticeably longer duration, with a dramatic increase in the number of apparent bleaching steps but a reduction in their resolution. Emissions from a fraction of these GFP-PTB molecules exhibited single-bleaching behaviour similar to that observed in the absence of RNA. Assuming that the number of bound PTB molecules, b, is constant but specific for each RNA tested, and taking into account that complexes formed with unlabelled PTB only are not detectable, the fraction of complexes with only one bound GFP-PTB molecule, \( P_b^{\text{exp}} \), can be calculated using a binomial distribution,

\[
P_b^{\text{exp}} = \frac{\sum_{i=1}^{b} \binom{i}{b} p^i (1-p)^{b-i}}{\sum_{i=0}^{b} \binom{i}{b} p^i (1-p)^{b-i}},
\]

where \( p \) is the probability that bound PTB is GFP labelled (\( p \) is close to 0.5). Calculating the fraction of the complexes exhibiting a single-bleaching behaviour allows \( b \) to be estimated for each type of complex.

There are possibly several uncertainties in \( b \). One of them is partial GFP bleaching at the beginning of the experiment (<10%). From the above formula it follows that \( \Delta P_b^{\text{exp}} = \Delta P_b^{\text{exp}} \Delta \text{GFP/Ep} \), and therefore \( \Delta P_b^{\text{exp}} \) is in the range 0.04–0.06 when \( P \) is close to 0.5 and \( b = 2–6 \). The latter can be translated into an error of \( b \) using \( \Delta P_b^{\text{exp}} = \Delta P_b^{\text{exp}} \Delta \text{GFP/Ep} \). This means \( b \) can be underestimated due to partial GFP bleaching by ~0.6 for \( b = 5 \), ~0.45 for \( b = 4 \), ~0.3 for \( b = 3 \) and ~0.15 for \( b = 2 \). Another source of errors is the large variation of intensities and/or duration of the emission for individual GFP molecules (see below), obscuring the detection of complexes exhibiting a single-step bleaching. These uncertainties (given in Table Ia) lead to similar errors in the estimation of \( b \) as above but with the opposite sign.

Analysis of the TNPs

The majority of the signals (>90%) from individual GFP-PTB molecules in the absence of exogenous RNA showed a single-bleaching step behaviour characteristic to single molecules, albeit with a wide range of amplitudes from 50 to 250 photons per time bin with large variance. Emissions from GFP-PTB associated with Cy5-labelled RNA showed a much larger amplitude and variance indicative of several GFP-PTB molecule within each complex. To calculate the TNPs, the intensities for each time trace were used for background calculation. In the absence of RNA, the distribution of TNP has a Gaussian-like shape spanning the region from ~200 to 10 000 photons, with a maximum around ~1200 photons (Supplementary Figure S2), whereas in the presence of TM1 substrate RNA, the distribution is much broader with an apparent maximum around ~3500 photons. To gain insight into this distribution, we applied the Kolmogorov–Smirnov (KS) statistic, which is superior for small sample sizes (Lilliefors, 1967). We note that the small fraction of the molecules (~10%) that did not exhibit single-bleach behaviour was excluded from the analysis. Briefly, we took the range of intensities from 0 to 10 000 photons, divided it into with 2000 steps and binned the data accordingly. Normalization by \( N \), the number of measured GFP spots, yielded a discrete density function, \( g(i) \), \( i = 1 \), \( m = 2000 \) in our case. Such a distribution density is related to a Gaussian p.d.f. of the variable \( I \), with \( \mu \) and variance, \( \sigma^2 \):

\[
g(I, \mu, \sigma) = \frac{1}{\sigma \sqrt{2\pi}} \exp \left[ -\frac{(I - \mu)^2}{2\sigma^2} \right]
\]

The cumulative distribution of the experimental data, \( E(i) = \sum_{i=1}^{n} e(i) \), was fit by the cumulative distribution of the function \( g(I, \mu, \sigma) \):

\[
\Pr \{ I_{\text{app}}>Z \} = \frac{1}{2} \left[ \text{erf} \left( \frac{Z - \mu}{\sigma \sqrt{2}} \right) + \text{erf} \left( \frac{\mu}{\sigma \sqrt{2}} \right) \right]
\]

The best fit, varying \( \mu \) and \( \sigma \), was obtained by minimizing the KS statistic:

\[
D = \sqrt{\max \{ \Pr \{i\} - E(i) \}}
\]

The D value was found (using Mathematica 5 and Global Optimization module) to be ~0.70, which is below the 0.20 significance level (\( D_{0.20} = 0.736 \); Lilliefors, 1967), implying that the experimental distribution of TNP can be adhered to a normal probability density function. The distribution of TNP did not depend on the intensity of the 488 nm laser in the range from 20 to 50 mW. We note that our aim was to define a model describing the distribution of TNP rather than absolute values for the mean and variance.

Cumulative distributions were built similarly for the data from each experiment with TM RNA. Using the above assumptions, that is (1) the number of bound PTB molecules, \( b \), is constant (\( b > 1 \)) but specific for each RNA, (2) the fraction of complexes with 0, 1, 2… \( b \) PTB-GFP molecules follows a binomial law, (3) the complexes lacking a fraction of TNP are not detectable and (4) PTB and GFP-PTB exhibit similar affinity for the specific sites, the corrected fraction for the complexes with \( i = 1, 2, b \) PTB-GFP molecules is equal to

\[
P_b^{\text{exp}} = \frac{\sum_{i=1}^{b} \binom{i}{b} p^i (1-p)^{b-i}}{\sum_{i=0}^{b} \binom{i}{b} p^i (1-p)^{b-i}} = \frac{\binom{i}{b} p^i (1-p)^{b-i}}{1 - (1 - p)^b}
\]

Assuming that each GFP-PTB molecule in a complex would behave likewise, the p.d.f. for the complexes with \( i \) bound PTB-GFP molecules will be:

\[
\text{GFP}(i, \mu, \sigma, b) = \frac{1}{\sigma \sqrt{2\pi}} \exp \left[ -\frac{(i - \mu)^2}{2\sigma^2} \right] \left( \frac{b}{i} \right) p^i (1-p)^{i-b}
\]

The cumulative distribution of \( \text{GFP}(i, \mu, \sigma, b) \) would be:

\[
\Pr \{ I_{\text{app},b}>Z \} = \frac{1}{2} \sum_{i=1}^{b} \left[ \text{erf} \left( \frac{Z - \mu}{\sigma \sqrt{2i}} \right) + \text{erf} \left( \frac{\mu}{\sigma \sqrt{2i}} \right) \right] \left( \frac{b}{i} \right) p^i (1-p)^{i-b}
\]

Individual fits for each RNA substrate were optimized first by varying \( \mu \), \( \sigma \) and \( b \) with \( p \) around 0.45 to estimate \( b \). It became clear that \( b \) corresponds to 5–6 for TM1, 4–5 for TM2 and TM3, 3–4 for TM4 and ~2 for truncated TM1 RNA. The combination 5, 4, 3, and 2 for TM1, TM2, TM3, TM4 and TM1 trun RNA, respectively, is fitted better by slightly higher values of \( p \) (e.g. 0.47, as shown in Figures 5C–G), whereas the combination 6, 5, 5, 4 and 2 is fitted better by slightly lower values, such as 0.42. As every experimental cumulative distribution exhibits small deviations from the expected distribution, seen as a deficiency at the smaller numbers of TNP and overpopulation for the middle values, the expected distributions with the values of \( b \) that fit most closely for each substrate with the same values for \( \mu \), \( \sigma \) and \( p \) are shown in Figure 5C–G. We note that the assumption about homogeneity of the complexes, that \( b \) is constant for each substrate, is not very stringent. Simple calculations show that heterogeneity of the complexes, that is the presence of complexes containing ~1 or ~2 bound PTB molecules of up to 20–30%, will effectively lead to the cumulative distributions within the indicated boundaries. The TNP distribution can also be analysed by a novel
method using an exponential approximation for the data beyond the maximum (DC, unpublished results). This gives similar results.

**Prediction of sites and arrangements of binding**

The binding preferences for RRM domains 1–4 of PTB were inferred from NMR structures to be YCUN1, YCUN2, YCUN3, and YCU, respectively (Oberstrass et al., 2005). On the basis of an average inter-domain distance of 4.5 nm for domains 1, 2 and 3 (Piotukhov et al., 2006), we constrained their separation to be 1–6 nucleotides. RRM4 was omitted because there is no clear distance constraint (Oberstrass et al., 2005; Lamichhane et al., 2010). Thus, an optimal-binding site was taken to be YCUN1g, YCUN2g, YCUN3g, and YCU, where N is any nucleotide. The total number of configurations is 144, which is the product of the variable lengths of the spacers and the two pyrimidines (Y). An array of 22 bases was defined in C+ and all allowed configurations were generated, which were then compared with the RNA sequence at every position. Each nucleotide position matching the optimal site was given a value of 1, and the sum of these taken as a score, the maximum score possible being 8. Alignments were ranked according to their score. The figures show positions that scored the maximum. For determining whether two or three PTB molecules would fit on a sequence, an initial array was scored and those showing a perfect match held while a second array was scored and so on. In this case, gaps of at least three bases between adjacent arrays were required. Configurations scoring the maximum possible (24) were plotted.

**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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