A bichromatic fluorescent reporter for cell-based screens of alternative splicing

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ABSTRACT

Alternative splicing is the primary source of proteome complexity in metazoans and its regulation shapes the proteome in response to shifting physiological requirements. We developed a bichromatic splicing reporter that uses a peculiar feature of some fluorescent protein coding regions to express two different fluorescent proteins from a single alternative splicing event. The mutually exclusive expression of different fluorescent proteins from a single reporter provides a uniquely sensitive approach for high-throughput screening and analysis of cell-specific splicing events in mixed cell cultures and tissues of transgenic animals. This reporter is applicable to the majority of alternative splicing patterns and can be used to quantify alternative splicing within single cells and to select cells that express specific splicing patterns. The ability to perform quantitative single-cell analysis of alternative splicing and high-throughput screens will enhance progress toward understanding splicing regulatory networks and identifying compounds that reverse pathogenic splicing defects.

INTRODUCTION

Alternative splicing exponentially increases the number of proteins expressed from a surprisingly limited number of genes. Beyond simply generating proteome diversity, the regulation of alternative splicing is responsible for a recently recognized level of complexity of post-transcriptional regulation of gene expression. Recent biocomputational and microarray analyses have demonstrated the extent to which regulation of alternative splicing modulates proteome diversity in response to dynamic cellular requirements and indicate the presence of regulatory networks interconnected with the intricate control mechanisms of gene expression [reviewed in (1)]. There is also growing awareness of the significant role that the disruption of splicing plays in human disease (2,3).

Identification of splicing regulators has been accomplished primarily through biochemical and genetic approaches (4). The combination of this gene-by-gene approach with global approaches would greatly enhance progress toward deciphering signaling events and regulatory networks that control splicing. Recently, several reports have demonstrated the utility of a fluorescent protein read out for alternative splicing in cell culture and transgenic mice (5–12). These splicing reporters have been used in screens for RNA binding proteins or upstream signaling pathways that modulate specific splicing events or for identifying cells within a mixed population that express a splicing pattern that differs from the majority of the cells in the population. This approach typically utilizes alternative splicing to control on–off expression of GFP such that inclusion or skipping of a variable region puts GFP in frame. The downside to using a monochromatic readout for alternative splicing is that the splicing pattern producing GFP cannot be quantified relative to the other splicing pattern(s) expressed from the reporter. Detection of GFP does not determine whether the GFP mRNA represents a majority or a small minority of the mRNAs from the reporter. In addition, monochromatic reporters require all-or-none splicing decisions for on or off expression of GFP because it is difficult to compare fluorescence intensity of different cells without an internal control. As the majority of alternative splicing events are not regulated as all or none, splicing events must be manipulated to fit into an all-or-none output. One successful approach that has been utilized in cell cultures and transgenic animals is to co-express GFP and mRFP1 from two separate reporters (10,12).

We developed a novel approach to quantify the ratio of two alternative splicing pathways expressed from a single reporter in which enhanced green fluorescent protein (EGFP) is expressed from one splicing pathway and dsRED is expressed from the other. The approach uses an unusual feature of some fluorescent proteins that contain an alternate reading frame that lacks stop codons. The reporter described here contains dsRED upstream of EGFP such that dsRED and EGFP are expressed from the two different reading frames; one for dsRED, and the other for the dsRED alternate reading frame fused with EGFP. Inclusion or skipping of a variable region of the appropriate size located upstream of dsRED allows toggling between the dsRED and EGFP reading

frames. The reporter can be used with all alternative splicing patterns that generate internal variability within an mRNA (cassette and mutually exclusive exons, alternative 3′ and 5′ splice sites and retained introns). The resulting mutually exclusive expression of dsRED and EGFP proteins provides high sensitivity and a quantitative measure of the ratio of splicing patterns. We demonstrate that this reporter can be used to quantify the ratio of alternative splicing events within individual cells, for flow cytometry and is applicable to Fluorescence Activated Cell Sorting (FACS) analysis, and can be used to identify cells expressing different splicing patterns within a mixed cell culture. The reporter is engineered such that genomic segments containing variably spliced regions of interest can be easily 'cut and paste' into convenient restriction sites. This alternative splicing reporter will be useful for high-throughput screening to identify direct regulators and the signaling pathways that modulate a specific splicing event or agents that reverse aberrant splicing events that are the cause of disease.

METHODS AND MATERIALS

Plasmid construction

The FRE5 expression plasmid was constructed by PCR amplifying EGFP from pEGFP-N1 (Clontech) using primers that removed the EGFP translation initiation codon. The EGFP open reading frame (ORF) was place into pcDNA3.1-HisC (Invitrogen) in which the Xpress tag had been replaced by the FLAG epitope tag and a downstream nuclear localization signal (NLS). The primers used to PCR amplify EGFP were designed such that EGFP was flanked by restriction enzymes that are unique to the final RG6 plasmid. The dsRED ORF was PCR-amplified from pCX-dsRed (obtained from Dr A. Nagy) without its initiation codon. The dsRED primers also flanked the ORF with restriction enzymes that are unique to the final RG6 plasmid (Figure 3A). The dsRED sequence was placed upstream of EGFP such that the alternate ORF of dsRED was in frame with the downstream EGFP. The reading frame was also designed such that the initiating ATG for FLAG–NLS was in frame with EGFP in FRE5 and that addition of 2 nt (filled in ClaI site generating FRE5Cf, Figure 1B) shifted the reading frame to dsRED.

The RG6 minigene was derived from FRE5 by inserting PCR-generated segments containing introns 4 and 5 of chicken cardiac troponin T (13). The chicken cTNT genomic fragment was amplified in two segments to introduce the natural introns 4 and 5, an artificial alternative exon in place of exon 5 and the 3′ splice site of intron 5 + 35 nt of exon 6 which contains purine rich motifs resembling an exonic splicing enhancer (14). The artificial exon is flanked by restriction sites that are unique to the plasmid. Several exons of different size and sequence composition were tested to generate a construct that produced 1:1 exon skipping:inclusion in COSM6 cells. The complete sequence of the RG6 plasmid is posted at http://www.bcm.edu/pathology/labs/cooper/reagreq.htm.

RG6ME (minus exon) and RG6PE (plus exon) constructs contain cDNAs of the RG6 mRNAs that lack and include, respectively, the alterative exon. The XbaI/AgeI fragments
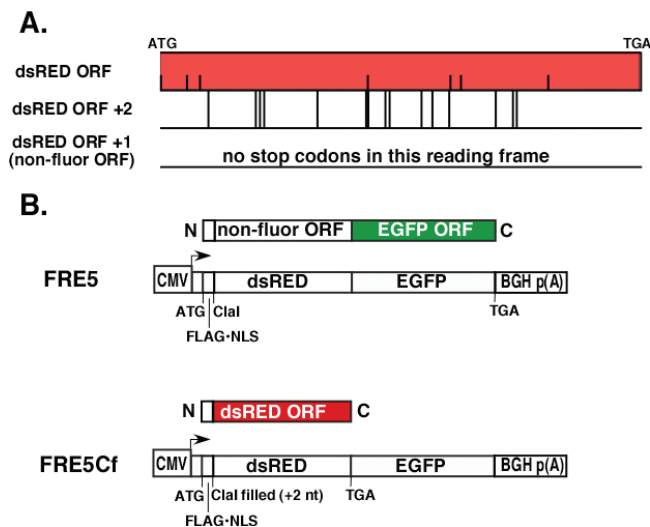

Figure 1. Strategy for bichromatic expression of dsRED and EGFP from alternative splicing pathways. (**A**) The three reading frames of dsRED showing the dsRED ORF (red) and illustrating that the +1 reading frame lacks translation stop codons. Lines that extend across the whole reading frame indicate translation stop codons and lines that extend to the bottom third of the reading frame indicate ATG codons. (**B**) Expression constructs to determine whether the alternate ORF of dsRED is efficiently expressed in mammalian cells. FRE5 and FRE5Cf differ by insertion or deletion of 2 nt (ClaI filled in) between the NLS and dsRED coding regions. The predicted proteins are shown above the gene diagram. Transcription initiation is indicated by the arrows.

from the amplified PCR products from the spliced RG6 mRNAs that include or exclude the alternative exon were cloned into the XbaI/AgeI sites of the RG6 plasmid (see Figure 1A). Therefore, RG6ME and RG6PE express mRNAs identical to those from the spliced mRNAs from RG6 that lack and include the alternative exon, respectively.

Transfection, RNA extraction and RT–PCR

Cell cultures for COSM6, C2C12 and primary chicken breast muscle cultures were prepared, maintained and transiently transfected as described previously (15,16) except that 1 μg of plasmid DNA was used. RT–PCR was performed as described previously (15) using RGf (CAAAGTGGAGGAC-CCAGTACC) and RGr (GCGCATGAACTCCTTGATGAC).

Fluorescence microscopy

COSM6 cells were plated at a density of 1.5×10^5 cells per well on No. 1 coverslips (Fisher brand) for transfection. Forty-eight hours post-transfection, coverslips were washed once with chilled 1× PBS (phosphate-buffered saline). Cells were fixed in 4% paraformaldehyde for 30 min on ice, followed by three washes with 1× PBS at RT. To minimize autofluorescence, cells were quenched with 0.1 M ammonium chloride in 1× PBS for 10 min at room temperature, followed by two washes with 1× PBS. Cells were then permeabilized with 0.5% Triton X-100 for 5 min at room temperature, followed by three washes with 1× PBS, and a final rinse with ddH$_2$O. Fixed coverslips were mounted on VWR micro slides (Superfrost Plus) $25 \times 75 \times 1$ mm with Vectasheild hard set

mounting media containing DAPI (Vector). Slides were allowed to dry in the absence of light for 15 min at RT.

Images were collected using FITC channel 488 nm and Texas Red channel 594 nm. Healthy cells with intact nuclei as visualized by DAPI staining and cells which expressed both red and green were selected. Images were collected with the exposure time kept constant between the red and green channels for each individual cell. The collected images were exported as TIFF files and opened with Image J software. Nuclei were identified by DAPI staining and a region of interest (ROI) was drawn by hand to incorporate the entire nucleus. Image J software was used to compute the average red and green intensity for the ROI. The percent green expression was calculated by dividing the green intensity by the sum of the red and green intensity multiplied by 100.

Flow cytometry analysis

A total of 1.5×10^5 cells were plated per six well plate, transiently transfected with RG6 alone or with Xpress-ETR-3 or FLAG-MBNL3 (transfection efficiency ∼45%). Forty-eight hours following transient transfection, cells were dissociated in 0.25% trypsin with vigorous pipetting to completely dissociate cells, centrifuged a 800× g for 5 min with slow stop and resuspended into 0.5% FBS/1× PBS. Cells were spun down again and resuspended in 1% paraformaldehyde in PBS for flow cytometry. Flow cytometry was performed on FACScan (Becton Dickinson), and was analyzed using FlowJo software (Tree Star).

RESULTS

Our goal was to develop a splicing reporter in which each of two alternative splicing pathways would express different fluorescent proteins. We noticed a peculiar feature of dsRED in that the +1 reading frame contains no stop codons (Figure 1A). This feature allowed us to design an expression construct with EGFP placed downstream from dsRED in mutually exclusive reading frames: one for dsRED and the other for a fusion protein between the dsRED alternate reading frame and EGFP (Figure 1B). Insertion or removal of a variably spliced region located upstream of dsRED and EGFP can be used to toggle between the dsRED and EGFP reading frames based on the size of the variable region.

To test whether this was a viable strategy, and in particular to determine whether the individual reading frames will express the expected proteins, we generated two expression plasmids for either the dsRED or EGFP reading frames (FRE5Cf and FRE5, respectively, Figure 1B). Of particular concern, it was unclear whether the dsRED alternate reading frame would be expressed in mammalian cells. In the mRNAs from both plasmids (which represent the two reading frames from the planned bichromatic reporter), translation initiates from the same start codon at position 90 of the mRNA and both proteins contain an N-terminal FLAG epitope tag for detection by western blotting and a NLS to concentrate the fluorescent proteins in the nucleus for increased sensitivity. The predicted protein expressed from FRE5 contains the dsRED alternate reading frame (231 amino acids) fused to EGFP for a total of 498 amino acids (MW = 55.7 kDa). FRE5Cf was derived from FRE5 by

filling in a unique ClaI restriction site between the FLAG–NLS and the dsRED ORF to add 2 nt and switch the reading frame to dsRED (MW = 29.4 kDa). FRE5 and FRE5Cf plasmids were transiently transfected in COSM6 cells. Forty-eight hours after transfection, cell cultures were used to prepare whole cell protein extracts for western blotting or mounted for fluorescence microscopy. Western blot analysis using anti-FLAG antibodies demonstrated that FLAG-tagged proteins of the expected size were expressed from FRE5 and FRE5Cf (Figure 2A). Trace amounts of additional proteins were detected upon overexposure of the blot, suggesting expression of minor amounts of longer (FRE5Cf) and truncated (FRE5) proteins containing the FLAG epitope. The minor FRE5 protein was slightly larger than GFP. Antibodies to EGFP detected only the expected protein from the FRE5 plasmid (Figure 2A) indicating that the smaller protein was not due to internal initiation upstream of EGFP and will not produce interfering GFP fluorescence.

Fluorescence microscopy demonstrated that cells transfected with FRE5 and FRE5Cf expressed green and red fluorescence, respectively, which was localized to nuclei (Figure 2B). Equal exposures of cells transfected with either plasmid demonstrated that red fluorescence was not detected in cells transfected with FRE5 and green fluorescence was only minimally detected in cells transfected with FRE5Cf. We conclude that both the dsRED and EGFP fluorescent proteins are efficiently expressed and expression of the two reading frames is mutually exclusive. Specifically, the alternate reading frame of dsRED efficiently expresses an EGFP fusion protein of the expected size. We also demonstrated that FLAG-NLS-tagged protein from the dsRED alternate reading frame lacking EGFP that was readily detectable by western blotting was not detectable by fluorescence microscopy using filters to detect DAPI, dsRED or EGFP (data not shown). Therefore, the protein from the alternate reading frame of dsRED does not interfere with detection of dsRED or EGFP by fluorescence microscopy.

Quantification of alternative splicing pathways using a bichromatic reporter

Having demonstrated that the different fluorescent proteins from mutually exclusive reading frames can be expressed, we next generated a construct in which alternative splicing would toggle between the two reading frames. We chose to use the well characterized chicken cardiac troponin T (cTNT) regulated alternative splicing event. Two genomic fragments containing the introns and exons flanking the cTNT alternative exon (exon 5) were placed downstream of the FLAG–NLS coding segment and upstream of the dsRED/EGFP cassette to generate the RG6 reporter plasmid (Figure 3A). Splicing of cTNT exon 5 responds strongly to co-expressed CUG-BP and ETR-3 Like factors (CELF) and muscle-blind like 3 (MBNL) proteins which promote exon inclusion and skipping, respectively (16,17). The natural cTNT alternative exon is 30 nt and would maintain the reading frame. Therefore, the natural exon was replaced by an artificial exon of 28 nt that shifted the reading frame from dsRED (exon skipping) to EGFP (exon inclusion) (Figure 3A). Previous results have demonstrated that the specific exon sequence is not required for cTNT exon 5 regulation (14). The alternative
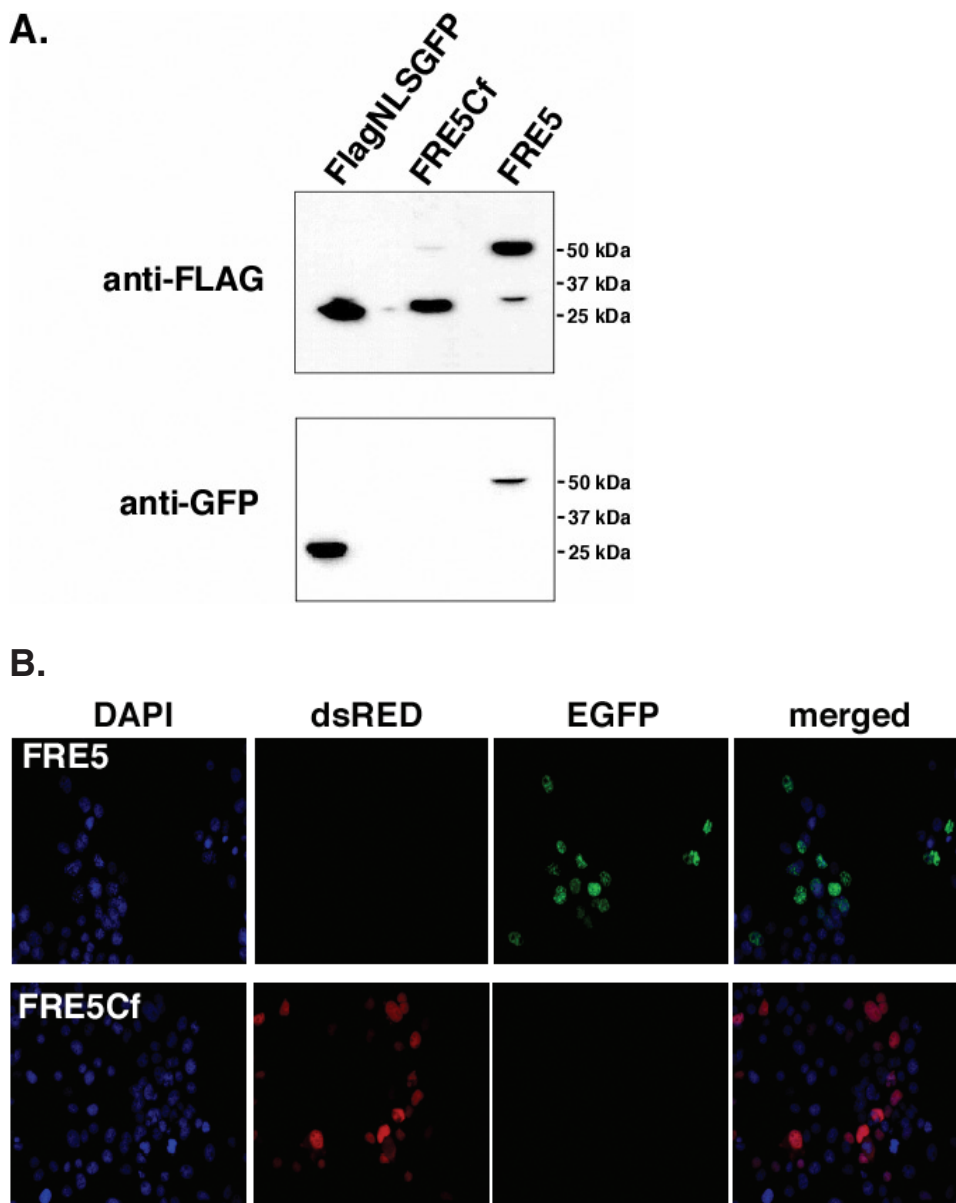
Figure 2. Expression of dsRED and EGFP from FRE5 and FRE5Cf mRNAs with alternate open reading frames. (**A**) Western blot analysis demonstrated efficient expression of FRE5 (EGFP) and FRE5Cf (dsRED) FLAG-tagged proteins. FlagNLSGFP is a FLAG-tagged version of GFP used for a positive control. (**B**) Nuclear expression of dsRED from FRE5Cf and EGFP from FRE5. Fluorescence microscopy demonstrates that the FLAG–NLS-alternate dsRED ORF–EGFP fusion protein is expressed in nuclei with enhanced nucleolar localization while FLAG–NLS–dsRED shows more diffuse nuclear distribution. Nucleolar localization of the EGFP fusion protein is variable between cell lines (data not shown).

exon is flanked by unique restriction sites to allow easy manipulation of the region to be spliced. The length and composition of the alternative exon was designed to balance splicing such that equivalent levels of exon inclusion and skipping was expressed in the COSM6 cells used for this analysis.

RG6 was transfected alone or with ETR-3 or MBNL3 expression plasmids. The effects on exon inclusion were examined using three assays: RT–PCR using primers located in the flanking exons (positions indicated in Figure 3A), western blot analysis for FLAG-tagged dsRED and EGFP proteins, and fluorescence microscopy. RT–PCR analysis demonstrated that the RG6 minigene expressed ~1:1 exon inclusion to skipping and, as expected, ETR-3 promoted

exon inclusion and MBNL3 promoted exon skipping (from 52 to 85% and 19% inclusion, respectively, Figure 3B). Shifts in the ratios of the two reading frames were also detectable by western blotting of FLAG-tagged dsRED and EGFP (Figure 3B).

An important potential feature of this bichromatic reporter is the ability to quantify the ratio of alternative splicing in individual cells using quantitative fluorescence microscopy. The majority of cells transfected with the RG6 minigene alone contained both nuclear dsRED and EGFP fluorescence (Figure 3C). Coexpression of either ETR-3 or MBNL3 significantly altered dsRED and EGFP expression consistent with promotion of exon inclusion by ETR-3 and exon skipping

by MBNL3 (Figure 3C). Nuclear fluorescence intensity for individual nuclei was quantified using ImageJ software and the results are presented as percent green fluorescence intensity (resulting from alternative exon inclusion) in Table 1. This analysis indicated that the different splicing patterns induced by ETR-3 and MBNL3 are detected by quantitative fluorescence of individual nuclei. We conclude that the ratio of red and green fluorescence expressed from the RG6 minigene can be used to quantify the ratios of splicing patterns within individual cells. The loss of one fluorescent protein and gain of the other due to mutually exclusive use of alternate reading frames provides a highly sensitive assay.

To determine whether this reporter was applicable to cell sorting, transiently transfected COSM6 cells were trypsinized and used for flow cytometry. Consistent with results from fluorescence microscopy above, cells transfected with the RG6 minigene contained a mixture of cells expressing red and green fluorescence, however there was a large population exhibiting predominantly green fluorescence which did not respond to ETR-3 or MBNL3 expression (RG6 alone, Figure 4). This population could represent cells that are expressing both dsRED and EGFP but dsRED is below the level of detection of FACscan. The population expressing relatively high levels of both red and green fluorescence
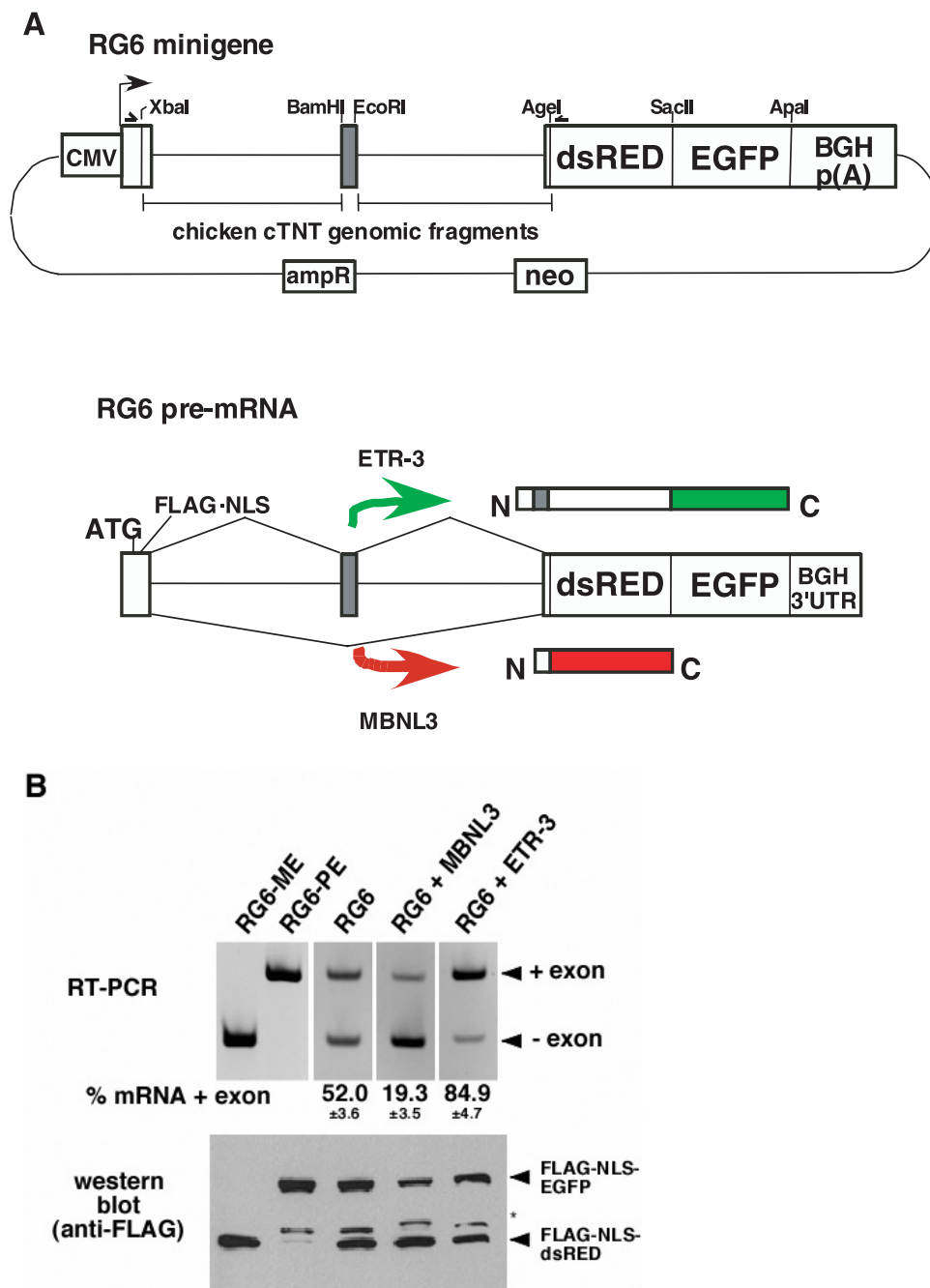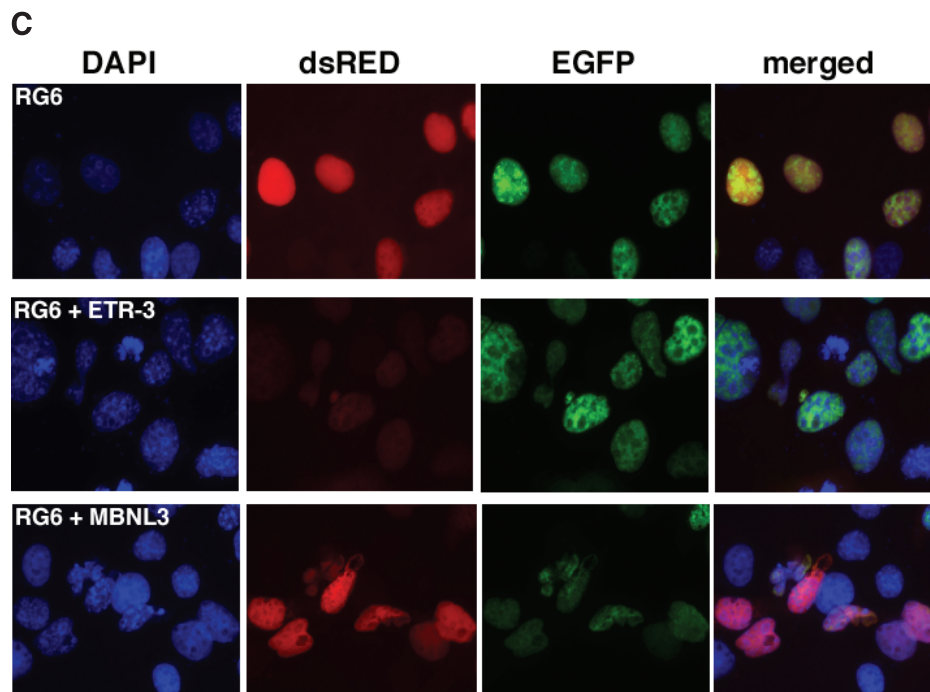
Figure 3. A bichromatic alternative splicing reporter for cTNT. (**A**) Diagram of RG6 minigene. Alternative splicing of a 28 nt cassette exon shifts the reading frame between dsRED and EGFP. Restriction sites shown are unique to the plasmid. (**B**) RG6 was transiently transfected into COSM6 either alone or co-expressed with Xpress-ETR-3 (promotes exon inclusion) or FLAG–MBNL3 (promotes exon skipping). RT–PCR analysis demonstrates exon repression by MBNL3 and activation by ETR-3. Percent exon inclusion was calculated by intensity of the top band divided by the sum of the intensities of the top and bottom bands × 100. The results are from four independent transfections. Western blot analysis using anti-FLAG antibodies demonstrates expression of FLAG-tagged EGFP and dsRED proteins. RG6PE (plus exon) and RG6ME (minus exon) are expression constructs for the spliced mRNAs containing and lacking the alternative exon, respectively. The additional band in the RG6+MBNL3 lane (indicated by asterisk) is FLAG–MBNL3. (**C**) Fluorescence microscopy of the RG6 reporter plasmid. Cells within cultures expressing RG6 alone contain nuclear staining for both dsRED and EGFP consistent with ~1:1 exon inclusion:skipping detected by RT–PCR in panel B. RG6+ETR-3 clearly exhibited a shift towards nearly exclusive expression of EGFP and RG6+MBNL3 exhibited a strong shift toward dsRED. Quantification of EGFP versus dsRED fluorescence is presented in Table 1.

was divided in half into boxes A and B to approximate the 1:1 ratio of dsRED and EGFP mRNAs detected by RT–PCR. Cultures expressing MBNL3 exhibited a significant shift in cell population expressing higher red fluorescence and lower green fluorescence while cultures expressing ETR-3 shifted toward higher green and lower red fluorescence (Figure 4). By setting the RG6 plasmid results at 1:1 and then calculating the percentage of cells in box A (green = exon inclusion) the flow cytometry analysis indicated that ETR-3 induced 82.6% of the cells into box A and MBNL3 reduced the fraction of cells in box A to 8.3%. These results are consistent with the results obtained from RT–PCR (88.9 and 17.3%, respectively, shown in Figure 3B). We conclude that the bichromatic reporter provides a sensitive assay for sorting cell populations based on predominant alternative splicing patterns.

Analysis of cell-specific splicing patterns in mixed cell populations

To determine whether the RG6 minigene could be used to detect splicing changes driven by endogenous regulatory programs, we tested its expression in differentiated and undifferentiated C2C12 mouse skeletal muscle cultures. C2C12 myoblasts proliferate as mononucleated cells in high serum medium and differentiate into multinucleated myotubes after 3–4 days in low serum medium. Analysis of chicken

Table 1. Percent green fluorescence in individual nuclei within cultures transfected with RG6 alone or with MBNL3 or ETR-3

RG6	41.7 ± 3.8 ($n = 16$)
RG6 + MBNL3	15.1 ± 2.7 ($n = 16$)
RG6 + ETR-3	65.4 ± 3.7 ($n = 22$)

cTNT minigenes previously indicated that both exon 5 inclusion and skipping was detected in undifferentiated C2C12 myoblast cultures while exon inclusion was strongly favored in differentiated myotubes (16). In undifferentiated C2C12 cultures transfected with the RG6 plasmid, the majority of transfected cells expressed both red and green nuclear fluorescence indicating that both splicing patterns are expressed in each cell (RG6, undiff; Figure 5A). In contrast, chains of nuclei located within differentiated myotubes contained only nuclear EGFP, indicating that the splicing switch to the exon inclusion pathway is readily detectable based on the bichromatic read-out (Figure 5B). Differentiated C2C12 cultures also contain a subpopulation of cells that have lost the ability to undergo myogenic differentiation. In addition to the exclusively green nuclei consistently observed in myotubes, we detected mononucleated cells that expressed both red and green fluorescence consistent with a failure to differentiate and expression of both splicing patterns (lower panel, open arrows, Figure 5C). This result indicates that
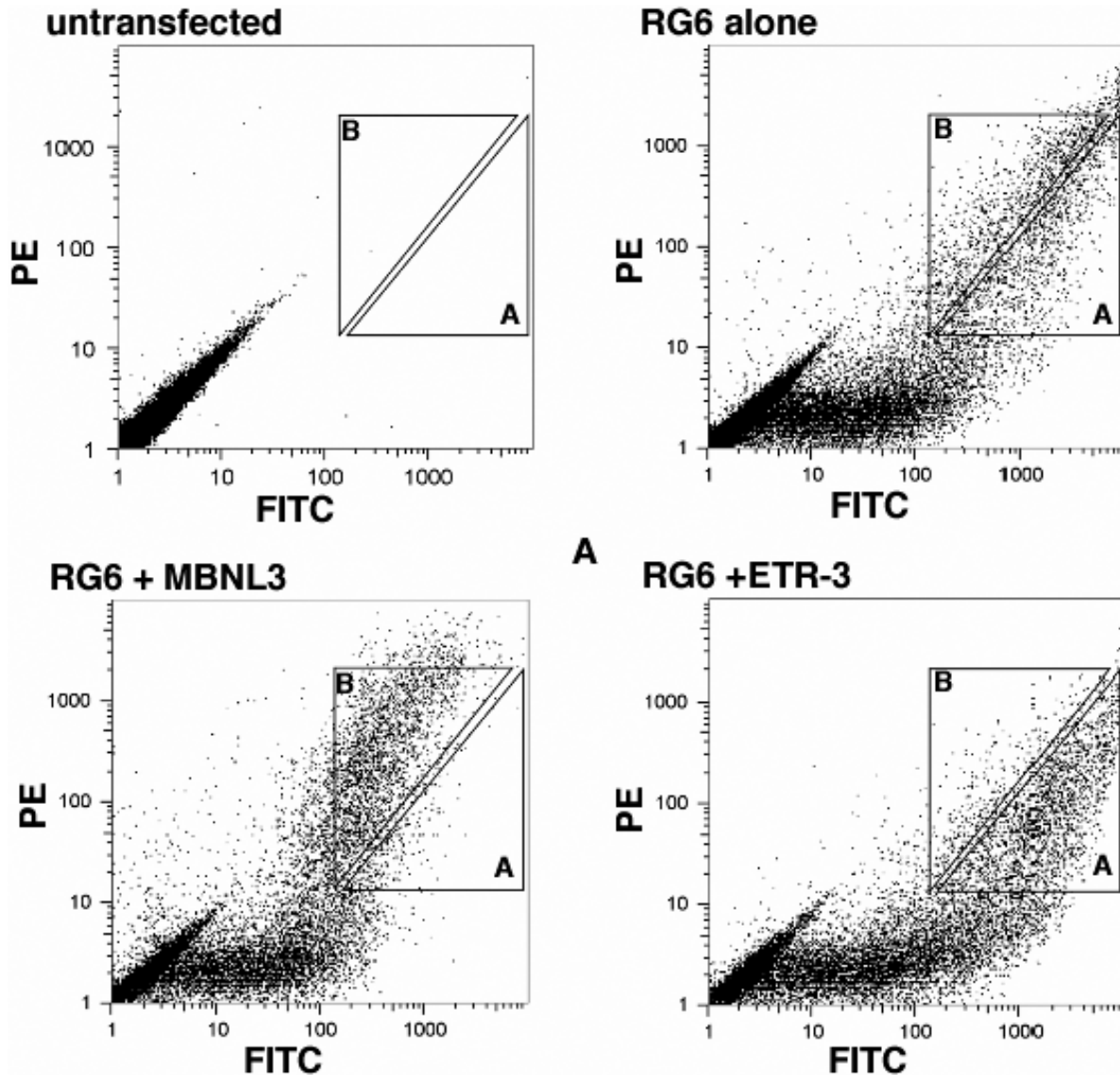
Figure 4. Analysis of RG6 expressing cells by flow cytometry. Cells determined to be expressing both red and green were gated in the RG6 alone sample so that 50% of cells are located in box A (green) and the other 50% in box B (red). The percent of cells favoring box A was calculated for cells co-transfected with MBNL3 or ETR-3. This percentage represents the relative shift of cells expressing more green and less red indicative of an alteration in splicing regulation. The y axis (PE) is intensity of red fluorescence and the x axis (FITC) is the intensity of green fluorescence.

the red:green fluorescence output can be used to readily identify cells within a mixed cell population that express different splicing patterns.

To further address the resolution with which the RG6 mini-gene can be used to detect individual differences between cells in mixed cell populations, we transfected RG6 into chicken primary breast muscle cultures and allowed these cultures to differentiate. Differentiated primary cultures contain a mixture of differentiated myotubes and fibroblasts. Avian fibroblasts have previously been shown to express predominantly exon skipping and a low level of exon inclusion (14). Fluorescence microscopy demonstrated that all chains of nuclei found in differentiated myotubes expressed only EGFP and no detectable dsRED (Figure 6A). In contrast, non-muscle cells were detectable by nuclear fluorescence from both dsRED and EGFP (open arrows, Figure 6B).

These results demonstrate the utility of this reporter to identify individual cells expressing divergent splicing patterns within a mixed cell population.

DISCUSSION

Using a peculiar property of dsRED (also found in EGFP), we have developed a bichromatic readout for alternative splicing in which a single reporter expresses dsRED from one alternative splicing pathway and EGFP from the other. Inclusion or skipping of variable regions introduced into the mRNA by alternative splicing upstream of the dsRED/EGFP cassette allows toggling between the dsRED or EGFP reading frame. The system is amendable to the majority of alternative splicing patterns and requires only that the variable
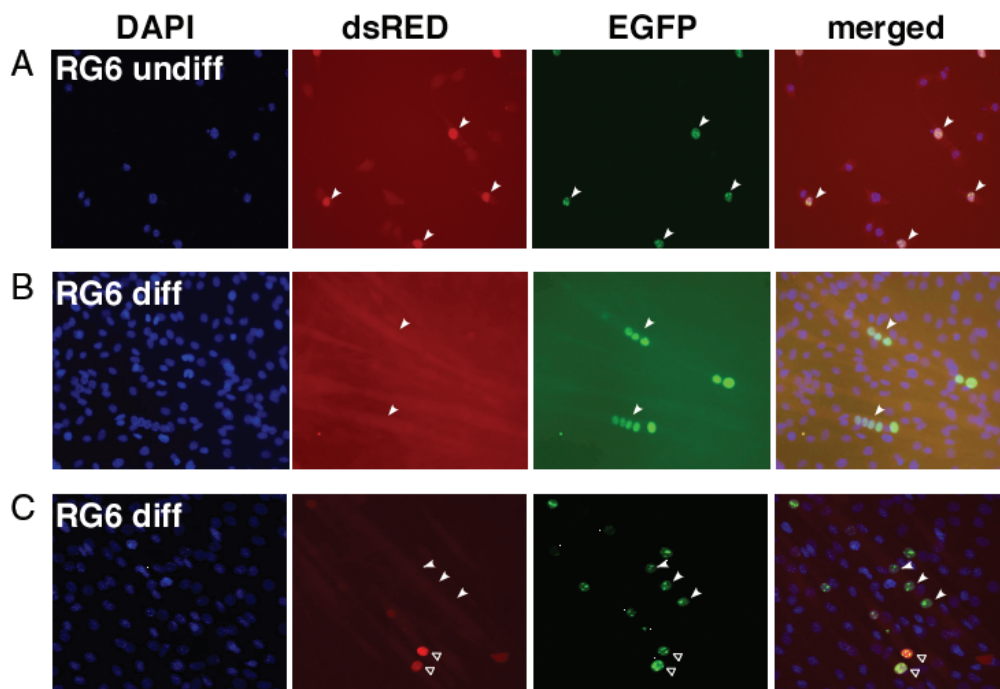
Figure 5. Expression of the RG6 bichromatic reporter in undifferentiated (**A**) or differentiated (**B** and **C**) C2C12 mouse skeletal muscle cultures. The cTNT alternative exon shifts from exon skipping and inclusion to exon inclusion during differentiation of C2C12 myoblasts (16). The vast majority of cells in undifferentiated cultures expressing the RG6 minigene contain red and green nuclear fluorescence (panel A). Differentiated cultures contain chains of nuclei in differentiated myotubes which express exclusively EGFP (exon inclusion) (B and C, solid arrows). Undifferentiated mononucleated cells in the same culture express nuclear red and green fluorescence indicative of both splicing patterns (panel C, open arrows).
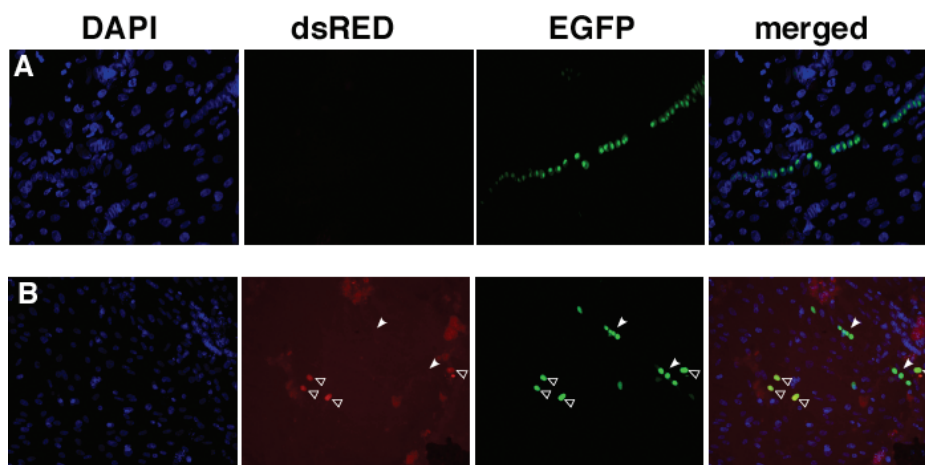

Figure 6. Expression of the RG6 bichromatic reporter in primary skeletal muscle cultures (both **A** and **B**). Chicken breast muscle cultures were transiently transfected with RG6 then examined for fluorescence. (A) nuclear chains found within differentiated myotubes express EGFP with no detectable dsRED indicative of the expected exon inclusion splicing pattern. (B) Exclusive expression of EGFP in myotubes (solid arrows) and both red and green fluorescence in fibroblasts (open arrows).

region not be a multiple of three and that it lacks a translation stop codon.

Expression of different fluorescent proteins from each of the two alternative splicing patterns expressed from a single reporter has several advantages compared to a monochromatic read-out or the use of two separate constructs to perform a bifluorescent read-out. First, expression of dsRED and EGFP is mutually exclusive so that a splicing transition results in both a gain of one fluorescent signal and the loss of

the other enhancing the change in signal. Second, having a read-out for both of two splicing pathways allows quantification of the complete output of a gene. This is particularly useful for quantitative assessment of alternative splicing within single cells. Third, using a single bichromatic reporter removes the variability associated with coexpression of two separate reporters that express different fluorescent proteins.

This reporter is directly applicable for high-throughput analyses of libraries of cDNAs, shRNAs and small molecules

that alter splicing pattern of a specific alternative splicing event. In addition, a large fraction of human diseases are caused by mutations that disrupt normal splicing and high-throughput screens can be used to identify compounds that restore correct splicing patterns. For example, reversing an exon skipping event of the SMN2 gene could reverse, in a large fraction of cases, the protein deficit in spinal muscular atrophy (SMA) (18). While compounds that affect this splicing event have been identified (19), a high-throughput screen would enhance the likelihood of identifying compounds with enhanced specificity for the aberrant splicing event and reduce potential side effects from a general disruption of splicing.

This reporter will also be useful to identify the range of alternative splicing patterns expressed by individual cells within cultured cells and tissues of transgenic animals. Single cell RT–PCR has directly demonstrated that different cells within a tissue express different splicing patterns (20,21). A GFP reporter was used in transgenic mice to demonstrate appropriate cell-restricted expression of the smooth muscle specific exon of alpha tropomyosin (6). Recently, an RFP splicing reporter for FGFR2 used in AT3 prostate tumors transplanted into syngenic animals revealed an unexpected minor population of cells expressing a splicing pattern indicative of a mesenchyme to epithelial transition (12). The ability to use a bichromatic reporter to quantify the ratios of alternative splicing patterns in individual cells will enhance our understanding of the diversity of splicing patterns in individual cells with in tissues and mixed cell culture.

Both dsRED and EGFP proteins contain the identical N-terminal FLAG–NLS. Nuclear localization concentrates the fluorescent signal for enhanced sensitivity and also rules out spurious expression of fluorescent proteins from internal translation initiation. Earlier versions of these constructs were found to express low levels of cytoplasmic dsRED and EGFP protein that were not detectable on western blots by FLAG antibodies but were detectable by antibodies to dsRED or GFP (data not shown). Cytoplasmic localization and the absence of the FLAG epitope indicated an inappropriate internal translation initiation at the dsRED and EGFP translation initiation codon. The current reporter lacks the initiating methionines of both dsRED and EGFP and does not express detectable levels of internally initiated proteins.

The variable region upstream of the dsRED–EGFP cassette must be of the appropriate size to change reading frames between dsRED and EGFP. For variable regions that are a multiple of three, this can be accomplished by altering the size of the variable region by one nucleotide. Variable regions that contain stop codons in either of the reading frames required for expression of dsRED or EGFP would need to be converted to an ORF. Such changes simply require single nucleotide substitutions or insertion/deletion.

It is of interest that two fluorescent proteins from very different sources, EGFP from the *Aequorea* genus of jelly fish and dsRED from the *Discosoma* genus of coral, both have an alternate reading frame that is devoid of both translation stop codons and start codons. In addition to providing the opportunity to generate a bichromatic readout for alternative splicing, it is likely that this unusual property has biological implications with regard to how fluorescent proteins or adjacent or overlapping genes are expressed and regulated.

REFERENCES

1. Blencowe,B.J. (2006) Alternative splicing: new insights from global analyses. *Cell*, **126**, 37–47.
2. Faustino,N.A. and Cooper,T.A. (2003) Pre-mRNA splicing and human disease. *Genes Dev.*, **17**, 419–437.
3. Cartegni,L., Chew,S.L. and Krainer,A.R. (2002) Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nature Rev. Genet.*, **3**, 285–298.
4. Black,D.L. (2003) Mechanisms of alternative pre-messenger RNA splicing. *Annu. Rev. Biochem.*, **27**, 27–48.
5. Sazani,P., Gemignani,F., Kang,S.H., Maier,M.A., Manoharan,M., Persmark,M., Bortner,D. and Kole,R. (2002) Systemically delivered antisense oligomers upregulate gene expression in mouse tissues. *Nat. Biotechnol.*, **20**, 1228–1233.
6. Ellis,P.D., Smith,C.W. and Kemp,P. (2004) Regulated tissue-specific alternative splicing of enhanced green fluorescent protein transgenes conferred by alpha-tropomyosin regulatory elements in transgenic mice. *J. Biol. Chem.*, **279**, 36660–36669.
7. Wang,Z., Rolish,M.E., Yeo,G., Tung,V., Mawson,M. and Burge,C.B. (2004) Systematic identification and analysis of exonic splicing silencers. *Cell*, **119**, 831–845.
8. Wu,J.Y., Kar,A., Kuo,D., Yu,B. and Havlioglu,N. (2006) SRp54 (SFRS11), a regulator for tau exon 10 alternative splicing identified by an expression cloning strategy. *Mol. Cell. Biol.*, **26**, 6739–6747.
9. Levinson,N., Hinman,R., Patil,A., Stephenson,C.R., Werner,S., Woo,G.H., Xiao,J., Wipf,P. and Lynch,K.W. (2006) Use of transcriptional synergy to augment sensitivity of a splicing reporter assay. *RNA*, **12**, 925–930.
10. Newman,E.A., Muh,S.J., Hovhannisyan,R.H., Warzecha,C.C., Jones,R.B., McKeehan,W.L. and Carstens,R.P. (2006) Identification of RNA-binding proteins that regulate FGFR2 splicing through the use of sensitive and specific dual color fluorescence minigene assays. *RNA*, **12**, 1129–1141.
11. Wagner,E.J., Baines,A., Albrecht,T., Brazas,R.M. and Garcia-Blanco,M.A. (2004) Imaging alternative splicing in living cells. *Methods Mol. Biol.*, **257**, 29–46.
12. Oltean,S., Sorg,B.S., Albrecht,T., Bonano,V.I., Brazas,R.M., Dewhirst,M.W. and Garcia-Blanco,M.A. (2006) Alternative inclusion of fibroblast growth factor receptor 2 exon IIIc in Dunning prostate tumors reveals unexpected epithelial mesenchymal plasticity. *Proc. Natl Acad. Sci. USA*, **103**, 14116–14121.
13. Cooper,T.A. and Ordahl,C.P. (1985) A single cardiac troponin T gene generates embryonic and adult isoforms via developmentally regulated alternate splicing. *J. Biol. Chem.*, **260**, 11140–11148.
14. Xu,R., Teng,J. and Cooper,T.A. (1993) The cardiac troponin T alternative exon contains a novel purine-rich positive splicing element. *Mol. Cell. Biol.*, **13**, 3660–3674.
15. Singh,G. and Cooper,T.A. (2006) Minigene reporter for identification and analysis of cis elements and trans factors affecting pre-mRNA splicing. *Biotechniques*, **41**, 177–181.
16. Ladd,A.N., Charlet-B., N. and Cooper,T.A. (2001) The CELF family of RNA binding proteins is implicated in cell-specific and developmentally regulated alternative splicing. *Mol. Cell. Biol.*, **21**, 1285–1296.
17. Ho,T.H., Charlet-B., N., Poulos,M.G., Singh,G., Swanson,M.S. and Cooper,T.A. (2004) Muscleblind proteins regulate alternative splicing. *EMBO J.*, **23**, 3103–3112.

18. Sumner,C.J. (2006) Therapeutics development for spinal muscular atrophy. *NeuroRx*, **3**, 235–245.
19. Jarecki,J., Chen,X., Bernardino,A., Coovert,D.D., Whitney,M., Burghes,A., Stack,J. and Pollok,B.A. (2005) Diverse small-molecule modulators of SMN expression found by high-throughput compound screening: early leads towards a therapeutic for spinal muscular atrophy. *Hum. Mol. Genet.*, **14**, 2003–2018.
20. Wang,Z.H. and Grabowski,P.J. (1996) Cell- and stage-specific splicing events resolved in specialized neurons of the rat cerebellum. *RNA*, **2**, 1241–1253.
21. Graf,E.M., Bock,M., Heubach,J.F., Zahanich,I., Boxberger,S., Richter,W., Schultz,J.H. and Ravens,U. (2005) Tissue distribution of a human Ca v 1.2 alpha1 subunit splice variant with a 75 bp insertion. *Cell Calcium*, **38**, 11–21.