Design of a GFP reporter for splicing analysis in mammalian cells

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Research note

Keywords: pre-mRNA splicing, GFP, reporter system

Posted Date: June 3rd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-20486/v2>

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Abstract

Objective: The great majority of eukaryotic genes are formed by exons and introns. Pre-RNA transcripts are extensively processed in the nucleus, with the addition of a cap group at the 5' end, intron removal and exon ligation (splicing) followed by addition of a poly-A tail at 3' end. Splicing is performed by specialized macromolecular machinery named spliceosome, composed of five small ribonucleoprotein particles (snRNPs) and several proteins. The activity of this complex is highly accurate due to coordinated activity of its components. Altered splicing has already been related to the development of several diseases as amyotrophic lateral sclerosis and different types of cancer. Detailed understanding of splicing regulation in eukaryotic cells can be achieved using splicing reporter systems.

Results: We designed a new splicing reporter plasmid suitable for analysis in mammalian cells. Our reporter is based on splicing of the GFP sequence. The greatest advantages of this system are the ease of visualization of the splicing outcome, by using a fluorescence microscope to confirm GFP expression from the reporter. Also, quantification of splicing efficiency using real-time PCR is possible. The use of this system allows rapid and easy detection of the splicing results in cultured cells.

Introduction

Pre-mRNA splicing is an essential processing step in eukaryotes. Eukaryotic genes are formed of exons, which remain in the mature RNA sequence, and introns, which are removed during splicing. Splicing depends on the assembly of a complex machinery, the spliceosome, composed of 5 U-rich small nuclear RNAs (snRNAs) and associated proteins, forming the 5 U-snRNPs (small nuclear ribonucleoproteins: U1, U2, U4, U5 and U6). Besides these 5 U-snRNPs, the spliceosome is composed of more than 100 proteins, most of which are conserved from budding yeast to humans (1). During pre-RNA processing, the splice sites, which are conserved sequences within the intron and in the exon-intron borders, are recognized by spliceosome components and trigger the sequential assembly of this complex. U1 snRNP binds to the 5' splice site, located at the border of 5' exon and intron. Association of U2 snRNP to a branchpoint-binding protein promotes its association to the branchpoint site in the intron, leading to rearrangements and recruitment of the tri-snRNP particle (U5-U4/U6). After that, several RNA-protein rearrangements release U1 and U4 snRNPs and promote the association between U2 and U6 snRNAs, creating a catalytic core center (2). Two sequential trans-esterification reactions remove the intron and ligate the exons in a mature RNA. Besides the canonical splicing described above, most genes go through alternative splicing processes. In this case, different splice sites along exons or introns are recognized and result in an alternative transcript isoform (3, 4). The most common mechanism of alternative splicing is exon skipping, which results in the exclusion of an exon in the mature mRNA. Another mechanism is intron retention, in which mature mRNA maintains intronic sequences (5). In all cases, these transcripts will generate different proteins.

A major challenge in exploring splicing efficiency and catalysis is the difficulty to analyze it in a living cell. First, because different transcripts might have different splicing rates, especially due to differences in

splice signal sequences (6). Second, splicing can be affected by the cellular environment, which includes the presence of splicing inhibitors and regulatory proteins (7). To analyze splicing efficiency, real-time RT-PCR can be the method of choice (8). It is simpler than methods that require autoradiography and cheaper than high-throughput methods, as for example RNA-seq, which spends high workflow for each experimental group tested (9). At the same time, the use of reporter plasmids based on fluorescent proteins, such as GFP, allows a simple and quick screening by fluorescence microscopy. To allow a precise characterization of splicing efficiency in cultured cells, we developed a reporter system based on GFP coding sequence. We also analyzed the effect of an intronic non-coding RNA (snR38A) on splicing efficiency. Our reporter transcript allows for visualization and quantification of splicing reactions in cultured cells by fluorescence microscopy and real-time RT-PCR.

Methods

Construction of GFP reporter: The GFP coding sequence was amplified by PCR using pEGFP vector (Invitrogen) as DNA template in 2 separate fragments, named “exon 1” and “exon 2”. “Exon 1” was amplified using primers PC 20 (5' ACGCTGGATCCATGGTGAGCAAGGGCGAGG 3') and PC 21 (5' GGAGTGAATTCACCTCGGCGCGGGTCTTG 3'), flanked by BamHI and EcoRI restriction sites. “Exon 2” was amplified using primers PC 28 (5' GAAACTGCGGCCGCCACAGGTGAAGTTCGAGGGCGACACCC 3') and PC 29 (5' GCCGCTTCTAGATTACTTGTACAGCTCGTCCATGCC 3'), flanked by NotI and XbaI restriction sites. The intron sequence was amplified from AdML precursor, using plasmid pHMS388 (10). Primers used contained EcoRI and NotI restriction sites (PC26: 5' GAAACTGAATTCACCTCCCTCTCAAAGCGGGC 3', and PC 27: 5' GAAACTGCGGCCGCAAAAAAGGGACAGGGTCAG 3').

“Exon 1”, “exon 2” and the intron amplification products were separately cloned into pGEM-T easy vector (Promega), according to manufacturer's instructions, and sequenced to confirm their integrity. After confirmation of sequence integrity, the fragments were sub-cloned into pcDNA 3.1(+) vector (Invitrogen), using BamHI and XbaI enzymes, resulting in the splicing reporter pGFP-spl. The pcDNA 3.1(+) vector contains ampicillin resistance gene and pUC19 origin of replication, suitable for maintenance in *E. coli*. It also contains CMV promoter, for high-level expression in mammalian cells, and geneticin resistance marker, allowing for transient selection in cell culture.

To insert snR38A sequence in the pGFP-spl intron, this sequence was firstly amplified from HeLa genomic DNA using primers PC 1: 5' ATAGCCTCGAGCACAAGCCTATGATGG 3' and PC 2: 5' ATGATAAGCTTAAGCCTCAGAATAGA 3', flanked by XhoI and HindIII restriction sites. The PCR product was then cloned in pGEM-T easy vector, according to manufacturer's instructions. snR38A fragment was then sub-cloned into the pGEM-intron, and this intron carrying snR38A sequence was interchanged with the intron in pGFP-spl, to finally construct pGFP-spl-snR38A plasmid.

Cell culture and transfection: HEK-293T cells were cultivated in 100 mm plates in 10 mL of DMEM / F12 with 10% FBS; 3.2 g / L sodium bicarbonate (NaHCO₃); in a humidified, controlled atmosphere incubator

(5% CO₂) at 37 °C. Transfections were performed when cells reached 70-80% confluence. 1.5 µg of each plasmid (pGFP-spl, pGFP-spl-snR38A and pEGFP) were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen), according to manufacturer's instructions. pEGFP was used as a positive control. Transfected cells were selected using up to 1.6 mg/mL of geneticin (G418, Amresco). After selection, cells were collected and subjected to cell extract preparation.

Cell analysis by fluorescence microscopy: Transfected HEK293-T cells were analyzed by fluorescence microscopy to check for GFP expression. 48 h after transfection cells were visualized and photographed in a fluorescence microscope (Axio Vert. A1, Zeiss), at a 50X magnification. Untransfected cells were used as negative controls. The photos were analyzed with the ImageJ software (1.46r, 32-bit version; Windows), to quantify the fluorescence intensity. For each cell group, 3 photos of different areas of the plates were analyzed, selecting the total area of each photo.

RNA analysis and real-time RT-PCR: Cells were collected and washed in PBS buffer (3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl, 135 mM NaCl, pH 7.4). Cell extracts were prepared using buffer A (KCl 10 mM, MgCl₂ 1.5 mM, Tris-HCl 20 mM [pH 7.5], DTT 0.5 mM) followed by homogenization using Douncer. Total RNA was extracted from cellular extracts using Trizol reagent (Invitrogen), according to manufacturer's instructions. After Trizol extraction, samples were precipitated using 3 M sodium acetate pH 5.2 and 100% ethanol. After resuspension, samples were used for cDNA synthesis using the Superscript kit (Invitrogen) and random primers. 100 ng of these cDNAs were analyzed by real-time RT-PCR, using SYBR Green® reagent (Thermo) to verify splicing efficiency. Two pairs of primers were used: for the exon junction and exon 2 ("mature RNA") (primers PC 157: 5' GCGCCGAGGTGAAGTTC 3' and PC 156: 5' GATGCCCTTCAGCTCGATGC 3'; expected amplicon length: 59 bp) and a pair annealing at both exons of the precursor ("total RNA") (primers PC 155: 5' GACGACGGCAACTACAAGAC 3' and PC 156: 5' GATGCCCTTCAGCTCGATGC 3'; expected amplicon length: pGFP-spl: 204 bp; pGFP-spl-sn38A: 234 bp; pEGFP: 81 bp). The "mature RNA" amplicon was detected only when splicing reaction was successful, since primer PC 157 depends on the juxtaposition of exons for annealing. Importantly, splicing efficiency was measured as a ratio between total RNA and mature mRNA amplifications. Also, b-actin primers were used to normalize reactions (primers PC 168: 5' ACCTTCTACAATGAGCTGCG 3' and PC 169: 5' CCTGGATAGCAACGTACATGG 3').

Results And Discussion

AdML intron and GFP coding sequence were amplified with specific primers. GFP coding sequence was amplified in two separate exons (Fig 1). The three fragments were first cloned into pGEM-T easy (Promega). After confirming sequence integrity by DNA sequencing, fragments were separated and ligated into pcDNA 3.1(+) vector. Correct fragment order was confirmed by restriction enzyme analysis, according to the plasmid map generated after GFP and intron cloning (Fig S1).

We then transfected the splicing reporters pGFP-spl and pGFP-spl-snR38A, and the positive control pEGFP into HEK-293T cells, as described in the Methods section, and proceeded to splicing analysis using

fluorescence microscopy (Fig 2). Fluorescence analysis showed untransfected cells did not show green fluorescence, as expected, and pEGFP showed fluorescence due to EGFP expression. Transfection of our reporter plasmids, pGFP-spl, and pGFP-spl-snR38A, resulted in fluorescence after 48h. Quantification of fluorescence intensity showed pGFP-spl and the positive control pEGFP are similar, indicating our reporter was successfully spliced and resulted in mature GFP protein (Fig 2B). Despite the slightly higher fluorescence intensity shown by pGFP-spl-snR38A transfected cells, no statistically significant alteration was observed in comparison to pEGFP.

Formation of mature RNA was also confirmed by RT-PCR and by real-time RT-PCR (qPCR) (Fig 3). In order to assess splicing efficiency, we amplified mature RNA and total RNA, with two different pairs of primers (as described on Methods). Importantly, “total RNA” includes both pre-mRNA and mRNA molecules, once primers are located on exon 1 and exon 2. RT-PCR revealed mature RNA was present in pGFP-spl and on the positive control, as expected. The same was observed for pGFP-spl-snR38A (Fig 3A). Splicing efficiency was calculated after qPCR, by a ratio of cycle threshold numbers (Ct's) observed after total RNA and mRNA amplifications. In the plot shown on Fig 3, higher bars indicate higher splicing efficiency. As expected, positive control shows a ratio of 1, once it does not have an intron and only mature GFP is present. pGFP-spl showed a ratio of 0.67 of splicing efficiency, revealing mature RNA was successfully generated. Splicing of a precursor containing snR38A in the intron shows a slight reduction in splicing efficiency (rate around 0.64) but still retains splicing activity. At the same time, these samples still retain unspliced pre-RNA, which is expected considering pre-RNA splicing reactions dynamics (11) (Fig 3B). Importantly, these results suggest our reporter is functional and performs splicing efficiently. The inclusion of a non-coding RNA in the intron showed a mild reduction on splicing activity (in comparison to pEGFP-spl).

Previous works measured canonical and alternative splicing using fluorescence-based plasmids. A system based on an interrupted fluorescent protein gene to analyze alternative splicing was also constructed (12). In this system, the sequence of mCherry, which is translated in a red fluorescent protein, is interrupted by an alternative exon. Depending on the alternative splicing pattern, the sequence that interrupts mCherry gene can be removed or skipped. The removal of this interrupting sequence allows for mCherry gene reconstitution and RFP expression. In another work alternative splicing efficiency of vascular endothelial growing factor A (VEGF-A) was analyzed (13). This gene has two isoforms, one of which excludes part of exon 8. With the use of a plasmid reporter based on dsRED sequence, they were able to detect alternative splicing of this exon. Zheng (9) developed a fluorescent-based plasmid to detect alternative splicing activators or repressors. In this system, the alternative splicing of a given sequence can be studied based on the GFP or RFP expression. The inclusion or not of sequences during the splicing process determines which fluorescent protein will be expressed, allowing for a rapid detection of alternative isoforms. Fluorescence-based plasmids have been used to study a diversity of splicing transcripts, with different applications. In order to analyze the effects of different transcripts, our reporter system is a single-intron mini-gene system that provides a new tool to analyze splicing in cultured cells, allowing for a characterization of effects that stimulate or inhibit splicing in a straightforward manner. Different from approaches based on multiple introns, our splicing reporter system is simpler and more

direct, requiring only a fluorescent microscope and allowing for a rapid mensuration of GFP splicing outcome in cell cultures. At this stage of the analysis, procedures such as collecting cells and preparing them for flow cytometry are not necessary. In addition, our system allows the inclusion of regulatory sequences in the intron, as for example miRNAs and other types of non-coding RNAs, to analyze its effect on splicing efficiency. Some other possible applications would be the study of different elements that affect splicing dynamics, such as splice site mutations and the use of spliceosome targeting drugs.

Limitations

Our work describes a new approach to visualize and quantify splicing reactions using cultured cells. Despite the ease of visualization using the fluorescence microscope, we consider extremely important that analyses are performed along with a negative control, to overcome possible background fluorescence intensities of different cells. In order to confirm different amounts of unspliced and spliced products, RT-PCR should be performed with previously validated primers.

List Of Abbreviations

snRNP - small ribonucleoprotein particles

GFP – green fluorescent protein

PCR – polymerase chain reaction

pre-mRNA – precursor messenger RNA

cDNA – complementary DNA

snRNA - small nuclear RNAs

RT-PCR – reverse transcriptase – polymerase chain reaction

RNA-seq – RNA sequencing

snR38A – small nuclear intronic non-coding RNA 38

AdML – adenovirus major league

DMEM / F12 – Dulbecco's modified Eagle's medium/ factor F12

FBS – fetal bovine serum

NaHCO₃ - sodium bicarbonate

G418 - geneticin

PBS – phosphate buffered saline

KH_2PO_4 – monopotassium phosphate

KCl – potassium chloride

NaCl – sodium chloride

MgCl_2 – magnesium chloride

Tris-HCl – acid Tris buffer

DTT - dithiothreitol

mRNA – messenger RNA

Ct – cycle threshold

RFP – red fluorescent protein

VEGF-A - vascular endothelial growing factor A

miRNA - microRNA

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: The dataset used on this study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

Funding: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) grant 2017/06994-9 and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) grant 474672/2013-1.

Author's contributions: ATM and HN conceived and performed experiments; wrote the manuscript. PPC conceived the study and wrote the final manuscript. All authors read and approved the final manuscript.

Acknowledgements: We are grateful to Gisela T. Ramos for excellent technical support.

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Figures

Fig 1

Figure 1

Amplification of GFP exons and AdML intron. (A) Schematic representation of EGFP amplification (upper part) and intron amplification (lower part), arrows show primers used. (B) Electrophoresis of PCR amplification products. Expected product lengths are shown in the bottom. Upper band represents an excess of plasmid DNA template. MW, molecular weight ladder 1 kb (Thermo).

Fig 2

Figure 2

Fluorescence analysis of GFP expression after 48 hours of transfection on HEK293T cells. (A) Cells were transfected with pEGFP plasmid (Invitrogen) as a positive control. Untransfected cells were used as a negative control. Green fluorescence is shown on the left and bright field is on the right. Magnification 50X. (B) Fluorescence intensity quantification from 3 fields, standard deviation is shown. pEGFP, pGFP-

spl and pGFP-spl-snR38A intensities were compared. Y axis show arbitrary units after quantification on Image J software.

Figure 3

Figure 3

RT-PCR and real-time RT-PCR (qPCR) analysis. Cells were transfected with pGFP-spl, pGFP-spl-snR38A, and pEGFP (Invitrogen) as a positive control. Reactions were performed using primers to detect (1) total RNA and (2) mRNA. (A) Electrophoresis of RT-PCR amplification products using pGFP-spl [expected

lengths (1): 204 bp; (2): 59 bp], pGFP-spl-snR38A [expected lengths (1): 234 bp; (2): 59 bp], and positive control [expected lengths (1): 81bp; (2): 59bp]. Red arrowheads on the left points to reference size bands for comparison. MW. Molecular weight ladder 1 kb (Thermo). (B) qPCR using samples of pGFP-spl, pGFP-spl-snR38A, and positive control, as indicated. The plot shows relative splicing efficiency of each sample by generating a ratio with the number of Ct's observed for total RNA and mature RNA (y-axis), after normalization with β -actin. Higher bars indicate higher splicing efficiency.

Supplementary Files

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