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Protocol

The in vivo minigene approach to analyze tissue-specific splicing

Oliver Stoss [a], Peter Stoilov [a], Annette M. Hartmann [a], Oliver Nayler [b], Stefan Stamm [a,*]

[a] *Max-Planck Institute of Neurobiology, Am Klopferspitz 18a, D-82152 Martinsried, Germany*
[b] *Max-Planck Institute of Biochemistry, Am Klopferspitz 18a, D-82152 Martinsried, Germany*

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Abstract

The exact mechanisms leading to alternative splice site selection are still poorly understood. However, recently cotransfection studies in eukaryotic cells were successfully used to decipher contributions of RNA elements (*cis*-factors), their interacting protein components (*trans*-factors) or the cell type to alternative pre-mRNA splicing. Splice factors often work in a concentration dependent manner, resulting in a gradual change of alternative splicing patterns of a minigene when the amount of a *trans*-acting protein is increased by cotransfections. Here, we give a detailed description of this technique that allows analysis of large gene fragments (up to 10–12 kb) under in vivo condition. Furthermore, we provide a summary of 44 genes currently investigated to demonstrate the general feasibility of this technique.

Themes: Cellular and molecular biology

Topics: Gene structure and function: general

Keywords: Alternative splicing; Minigene; RT-PCR; Transfection

1. Type of research

- A general method to create minigenes suitable for in vivo splicing experiments.
- (Co)-Transfection assay to determine the (alternative) splicing pattern of a given minigene.
- RT-PCR conditions to analyze specific minigenes.

2. Time required

- Generation of the minigene: 1 month.
- Cotransfection and RT-PCR analysis: 3 days.

3. Materials

3.1. Construction of minigenes

- Subcloned genomic DNA fragment (in bacterial, or yeast artificial chromosome or in lambda phage).
- PCR primers.
- Long-range PCR reagents: e.g., SAWADY Long PCR System (Peqlab Biotechnologie, Erlangen, Germany).
- 10 × Long-range PCR buffer: 500 mM Tris–HCl pH 9.1, 150 mM $(NH_4)_2SO_4$, 20% DMSO, 1% Tween-20
- 25 mM $MgCl_2$ solution
- 10 mM dNTP mix
- pCR XL TOPO cloning kit (Invitrogen, Carlsbad, USA)
- pcDNA1.1 (Invitrogen) or any other suitable eukaryotic expression vector

3.2. Transfection of cells

- Six-well tissue culture plate (35 mm) (Falcon, Becton Dickinson Labware, NJ, USA), HEK293 cells (ATCC, Manassas, VA, USA), Dulbecco's modified Eagle medium with glutamax, supplemented with 10% fetal calf serum (GIBCO BRL Life Technologies, Eggenstein, Germany).
- Vortex mixer.
- 1 M $CaCl_2$ solution (Dissolve 5.4 g $CaCl_2 \cdot 6H_2O$ in 20 ml H_2O, sterilize by filtration, store at −20°C).

* Corresponding author. Fax: +49-89-8578-3749; E-mail: stamm@pop1.biochem.mpg.de; www.neuro.mpg.de/stamm.htm

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- 2 × HBS (Dissolve 1.6 g NaCl (280 mM), 0.074 g KCl (10 mM), 0.027 g $Na_2HPO_4 \cdot 2H_2O$ (1.5 mM), 0.2 g dextrose (12 mM) and 1 g HEPES (50 mM) in 90 ml H_2O. Adjust pH to 7.05 with NaOH, then bring up to a total volume of 100 ml with H_2O. Sterilize by filtration, store at −20°C).
- 5% CO_2 incubator.
- 3% CO_2 incubator.

3.3. RT-PCR

- 10 mM dNTPs (Pharmacia-Biotech Europe, Freiburg, Germany).
- Taq polymerase, 5 U/μl (Applied Biosystems-Perkin Elmer, Weiterstadt, Germany).
- Thermocycler (Biometra, Göttingen, Germany).
- 5 × RT buffer: 250 mM Tris–HCl (pH 8.3), 200 mM KCl, 20 mM $MgCl_2$.
- RNase inhibitor 50 U/μl (Boehringer, Mannheim, Germany).
- H^- reverse transcriptase, 200 U/μl (GIBCO BRL Life Technologies).
- 100 mM DTT (1.4 dithiothreitol).
- 10 × PCR buffer, 100 mM Tris–HCl, pH 8.3; 500 mM KCl, 15 mM $MgCl_2$; 0.01% (w/w) gelatin (Applied Biosystems-Perkin Elmer).
- Primer sequences for amplification: TAATACGACTCACTATAGGG (X16-T7), CCTGGTCGACACTCTAGATTTCCTTTCATTTGACC (X16-r) GTTTTCTCCTCCGAGCCGCTCCGA (E1A-f).
- CTCAGGCTCAGGTTCAGACACAGG (E1A-r).
- Agarose (GIBCO BRL Life Technologies).
- 1 × TBE (10.8 g Tris base (89 mM), 2.1 g boric acid (89 mM), 4 g 0.5 M EDTA (2 mM), in 1 l).

4. Detailed procedure

An overview of the complete procedure is shown in Fig. 1.

4.1. Construction of the minigenes

- A minigene is best constructed from genomic subclones in lambda phages or artificial chromosome systems. The genomic clones containing the alternatively spliced exon(s) together with the flanking constitutive exons are verified by Southern Blot hybridization using standard procedures [58]. If no suitable genomic clones are available, genomic DNA prepared by standard procedures [58] can be used as a template for PCR amplification. However, the PCR amplification from genomic DNA is often more difficult. Restriction site mapping is performed directly with the PCR product or with the genomic clones to identify absent restriction sites.
- Restriction sites absent from the PCR fragment or the genomic clones can be used to clone the minigene by introducing them into the PCR primers. They should be placed in the most 5′ part of the primer (Fig. 1A). The part of the primers complementary to the genomic clone should have an annealing temperature between 62 and 65°C to ensure specificity of the reaction. The calculation of annealing temperatures can be performed under http://mbcf.dfci.harvard.edu/docs/oligocalc.html.
- Long-range PCR amplification is performed according to the protocol supplied by the manufacturer (SAWADY Long PCR System, Peqlab Biotechnologie). For target sizes less than 30 kb, the following reaction setup can be used: 36.5 μl H_2O, 5 μl 10 × long-range PCR buffer, 2.5 μl 10 mM dNTPs, 4.5 μl 25 mM $MgCl_2$, 1 μl template DNA (10 pg/μl), 0.5 μl of a mixture of Taq and a high fidelity thermostable polymerase with proofreading activity. Assemble the reactions on ice and perform the amplification using the following thermocycler settings: Initial denaturation for 2 min at 93°C; 10 cycles with 10 s denaturation at 93°C, extension at 68°C (allow 30 to 60 s extension per 1 kb); 15 to 20 cycles with 10 s denaturation at 93°C, 30 s annealing at 65°C, extension at 68°C. Increase the extension time (30 to 60 s per 1 kb) for 20 s every cycle to compensate for enzyme inactivation; final extension for 7 min at 68°C. Analyze 5 to 10 μl from the PCR reaction on a 0.8% agarose gel.
- The gel purification and cloning of the PCR product into the pCR-XL-TOPO vector (Invitrogen) is performed according to the manufacturer's protocol with the following modification: mix the cloning reaction by adding 0.5 μl pCR-XL-TOPO vector to 2 μl of the gel purified PCR product. After incubation for 5 min at room temperature, use the entire reaction for bacterial transformation.
- Finally, the minigene is recloned from the pCR-XL-TOPO vector into an eukaryotic expression vector, e.g., [49] using the unique restriction sites introduced by the PCR primers. We found that SV40 promotors [63] or CMV promotors [53] work well for minigene analysis in many cell lines.

4.2. Transfection of cells

- Transient transfection of adherent HEK293 cells is performed using the calcium phosphate method [11] on 35-mm plates (six-well tissue culture plate). The day before transfection 3.0×10^5 cells/plate are seeded in 3 ml DMEM/10% FCS. This leads to approximately 40%–60% confluency on the day of transfection. After splitting, the cells are incubated at 37°C in 5% CO_2 for 17–24 h.
- Splicing assays are based on the titration of increasing amounts of plasmid DNA expressing a splicing factor to a constant concentration of minigene DNA. To avoid

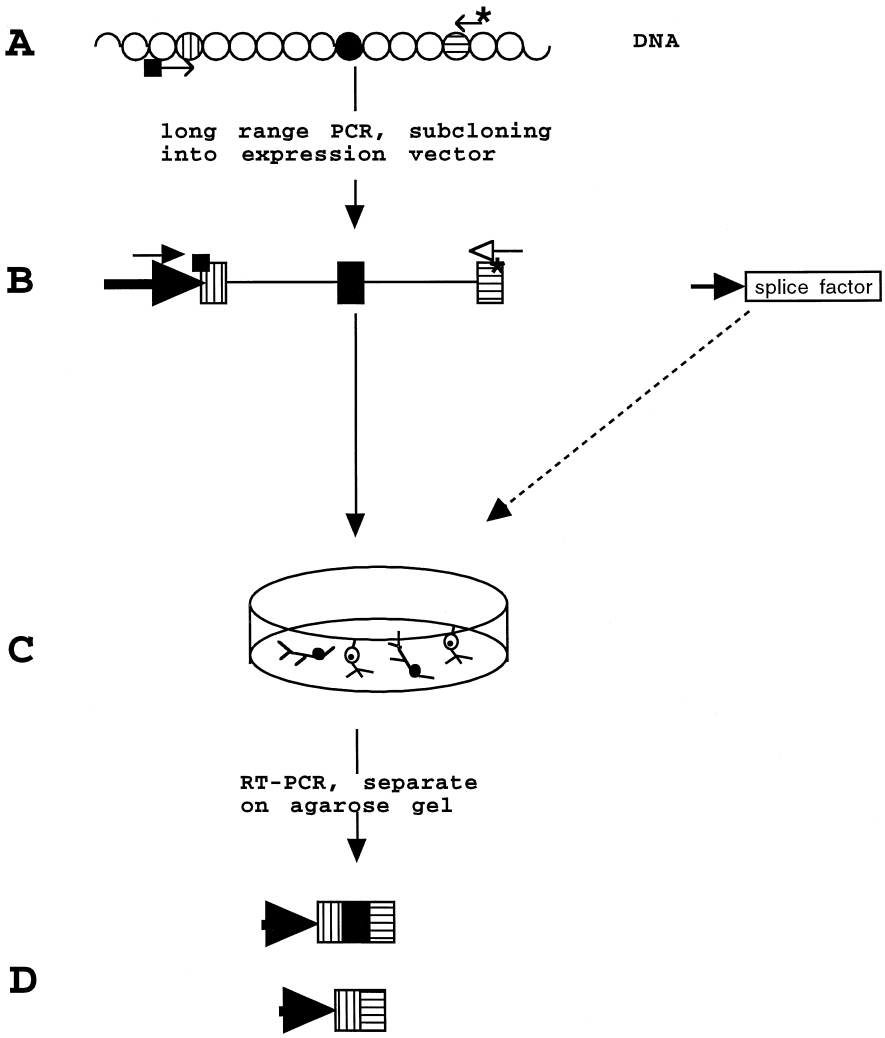


Fig. 1. Overview of in vivo splicing analysis with the minigene approach. (A) Using long-range PCR, the alternatively spliced exon (black circle) and its flanking constitutive exons (striped circles), as well as intergenic regions (open circles) are amplified from a genomic DNA clone. The restriction sites introduced by the PCR primers are indicated with a star and a box. (B) After subcloning into a suitable TOPO vector, the minigene is recloned into an eukaryotic expression vector using the unique restriction sites introduced by PCR (star and box). The eukaryotic promoter is indicated by a thick arrow. Exons are shown as boxes, introns as lines. After transfection, the resulting RNA is analyzed by RT-PCR using an antisense primer against the downstream flanking exon (open arrow) and a sense primer against a vector-derived sequence (closed arrow). (C) The minigene can be cotransfected with putative splicing factors to test putitative *trans*-acting factors or it can be transfected into different cell types to analyze them for their splicing ability. (D) The resulting PCR products can be discriminated by size or hybridization pattern, due to the presence or absence of the alternatively spliced exon.

''squelching'' effects, the 'empty' parental expression plasmid containing the promotor is added to ensure a constant amount of transfected DNA (Fig. 2A, top).

- The standard assay employs five reactions, each containing 2 μg of minigene DNA and an increasing amount of plasmid DNA expressing a splicing factor. 0, 0.5, 1, 1.5 and 2 μg of splicing factor DNA is a good start point for this titration. The appropriate amount of empty vector (2, 0.5, 1, 1.5 and 0 μg) is added to ensure that equal amounts of DNA are transfected. The DNA solutions are brought to a total volume of 75 μl with water and 25 μl 1 M $CaCl_2$ are added. While mixing the DNA/$CaCl_2$ solution with a vortex, 100 μl of 2× HBS is added dropwise.
- The mixture is incubated for 10–20 min at room temperature to allow the calcium phosphate-DNA precipitate to form.
- The precipitates are resuspended by pipetting and the complete mixture is added dropwise to the cultured cells.
- The dishes are incubated at 37°C in 3% CO_2 overnight.
- After the incubation, a fine precipitate is visible on the cells. The transfection efficiency can be estimated by fluorescence microscopy if an EGFP-tagged construct is used and should be at least 50% with HEK293 cells. If the splicing factor itself is not EGFP tagged, the use of pEGFP-C2 (Clontech, Heidelberg, Germany) as an 'empty' vector can help to monitor the transfection.

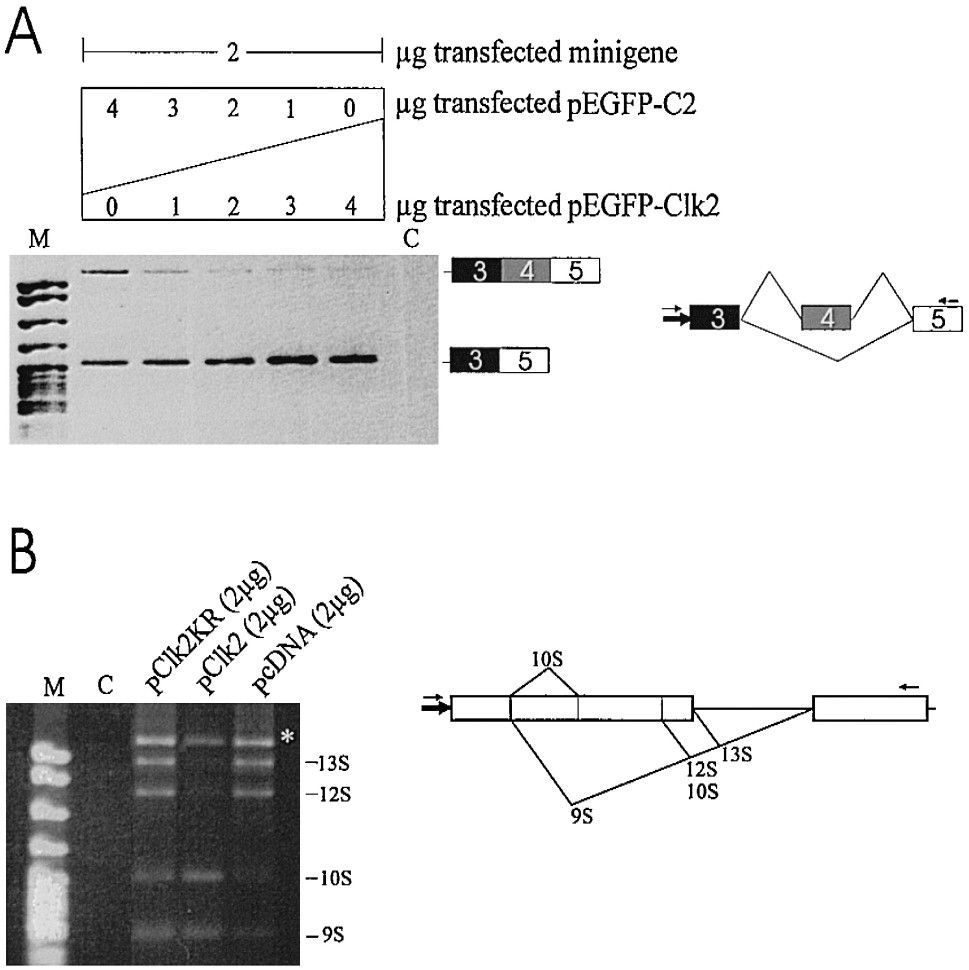


Fig. 2. Example of a minigene analysis. (A) Change of the splicing pattern of the SRp20 minigene [32] by titrating the SR-protein kinase CLK2. Top — Transfection scheme: The amount of transfected DNA is indicated in µg. The concentration of the splicing factor CLK2 is increased by adding 0, 1, 2, 3, 4 and 5 µg of its expression plasmid pEGFP-Clk2. The total amount of transfected DNA was kept constant by adding empty vector pEGFP-C2. A total of 2 µg of the minigene was added in each reaction. Bottom — Agarose gel of the PCR products generated by RT-PCR. The structure of the reaction products is shown on the right. Overexpression of Clk2 repressed inclusion of exon 4. C: PCR control using RNA without reverse transcription. Right — Schematic representation of the SRp20 minigene structure. Exon 4 is alternatively spliced. Small arrows indicate the position of the primers used for PCR amplification, the large arrow represent the CMV promoter. (B) Change of the splicing pattern of the E1A minigene [53] by overexpressing the SR-protein kinase CLK2 and its catalyticly inactive form CLK2-KR. The structure of the E1A minigene and the splicing patterns that create the 13, 12, 10 and 9S splice variants is shown on the right. The location of the gene specific and vector specific primers is indicated with arrows. pClk2, but not pClk2KR and expression vector alone (pcDNA) represses usage of the 12 and 13 S RNA, which is most likely achieved by phosphorylation of splicing components. pClk2KR slightly increases the formation of the 10S and 9S band, which could be a result of splicing component sequestration [52,53]. C: PCR control using RNA without reverse transcription. The star indicates an unspliced band.

4.3. RT-PCR analysis

- RNA is isolated 17–24 h after transfection using an RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA is eluted in 40 µl RNAse free H_2O.
- Best results are achieved when reverse transcription and following PCR are performed immediately after the RNA purification, thus avoiding freezing of the RNA or reverse transcription reaction.
- For reverse transcription, 2 µl of isolated RNA are mixed with 5 pmol antisense minigene specific primer in 0.5 µl H_2O, 2 µl 5× RT buffer, 1 µl 100 mM DTT, 1 µl 10 mM dNTP, 3 µl H_20, 0.25 µl RNase inhibitor and 0.25 µl H^- reverse transcriptase. In one sample, the RNA is substituted with water as a control. After a brief centrifugation, the tubes are incubated for 45 min in a 42°C water bath.
- During this incubation period, the PCR mixture is prepared. It consists of 50 pmol of sense and antisense primer each, 100 µl 10× PCR buffer, 20 µl 10 mM dNTPs in a total of 1000 µl water. The optimal $MgCl_2$ concentration for amplification has to be determined empirically in trial experiments and is usually in a range of 1.5–3.0 mM final.
- For six reactions, 1 µl Taq polymerase is added to 300 µl PCR mixture. 2 µl of the RT reaction are added to 50 µl of this mix and PCR is performed.

A: minigenes containing one cassette exon

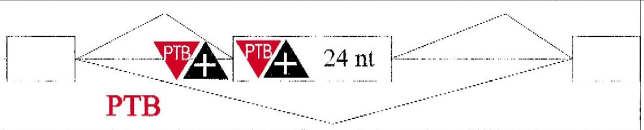
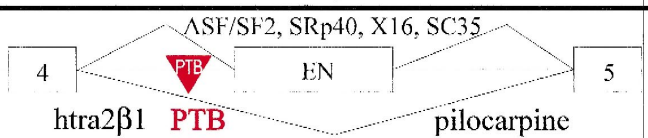
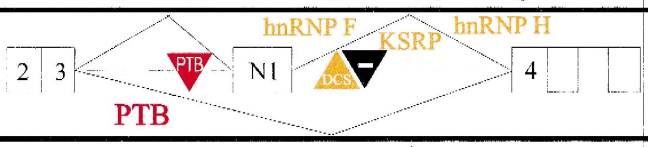
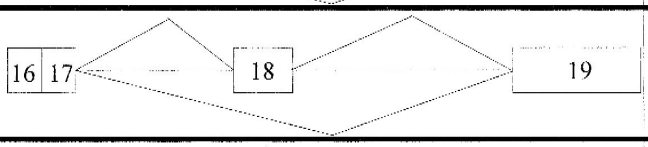
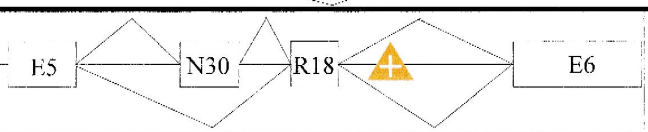
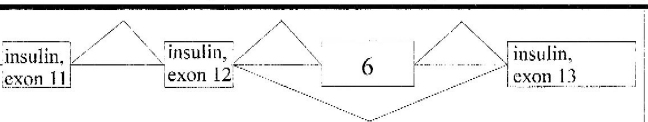
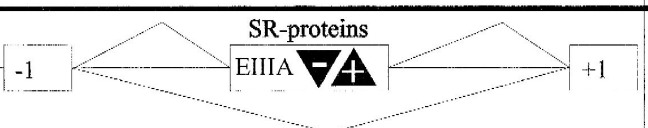
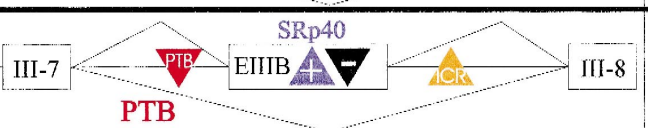
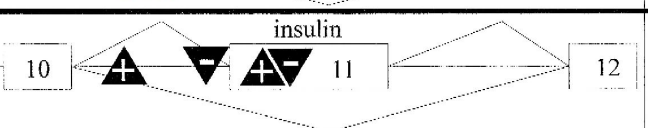
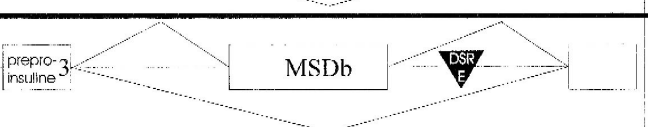
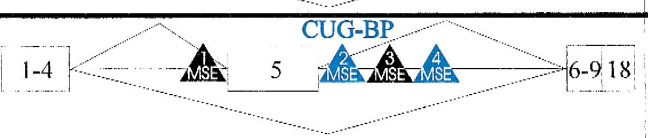
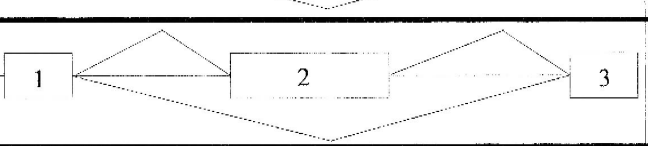
No	name	species	tissue specificity	minigene	reference
1	GABA$_A$γ2, 24 nt exon	rat	DE neuronal / non-neuronal		[2,74, 75]
2	clathrin light chain B, exon EN	rat	DE neuronal / non-neuronal		[62,63, 74]
3	src, exon N1	mouse	neuronal / non-neuronal		[9,10, 48,74]
4	NCAM, exon 18	mouse	N2a neuro-blastoma		[33,67]
5	MHC-B, exon N30	human	neuronal		[34]
6	tau, exon 6	human	skeletal muscle, brain		[72]
7	fibronectin, EIIIA	mouse	DE liver		[8,51]
8	fibronectin, EIIIB	rat	DE liver		[13,41]
9	insulin receptor, exon 11	human	liver, muscle, kidney		[37]
10	NCAM, exon MSDb	mouse	DE muscle myogenesis		[33]
11	cTNT, exon 5	chicken	DE embryonic striated muscle / adult striated muscle		[14,15]
12	AMP deaminase 1, exon 2	rat	DE adult muscle / fetal muscle		[47]

Fig. 3.

A: minigenes containing one cassette exon

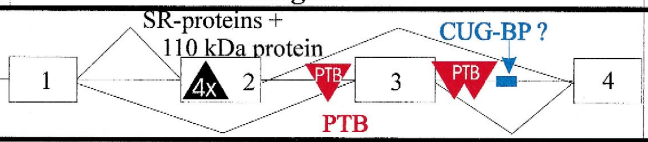
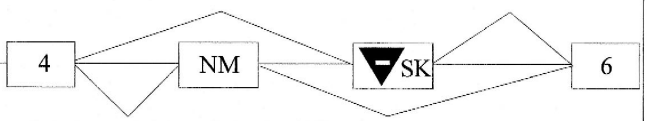
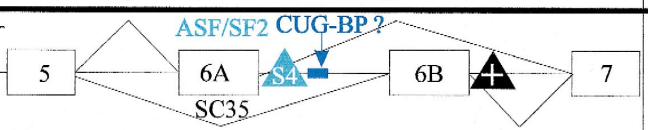
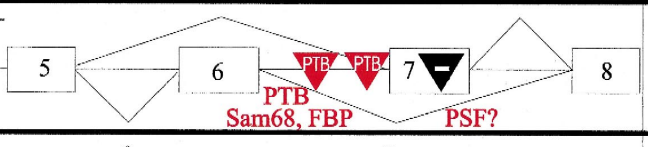
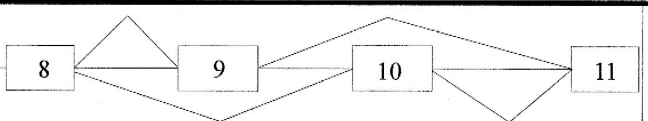
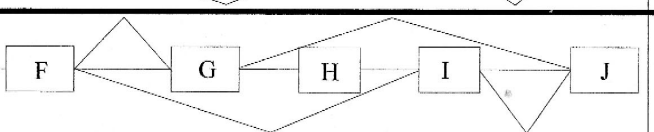
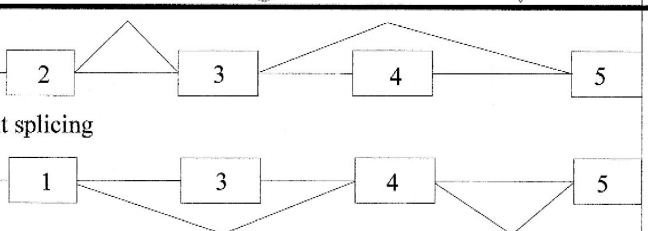
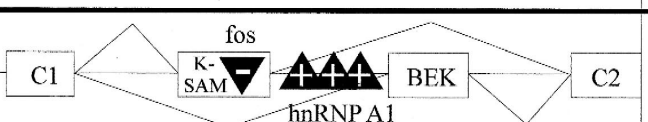
No	name	species	tissue specificity	minigene	reference
13	CD44, exon 5	mouse	HaCaT keratinocytes HT-3 cervix carcinoma	phorbolester v5	[36]
14	FGFR-1 , exon α	human	NT-2, JEG-3 T98G, SNB 19 glioblastoma cells	α γ hmT1-3	[17]
15	myosin heavy chain, exon 18	Drosophila	DE Drosphila larvae	17 18 19	[28]
16	FcγRIIA, exon Tm	human	neutrophils HeLa, Dami cells	EC2 Tm C1	[35]
17	interleukin-3α, exon 8	mouse	A/J mice	7 8 9	[30]
18	DHFR, exon 2A, reporter gene	hamster	inactive DHFR active DHFR	1 2A 2 3-6	[12]
19	HIV-1, exon 6D	HIV-1		cTNT exon 4 6D cTNT exon 6	[73]
20	SRp20, exon 4	human		3 SRp20 4 5 SRp30c ASF/SF2 Nop30	[32]
21	PPT, exon 4	rat	γ-PPT	3 4 5	[40]

B: minigenes containing multiple cassette exons

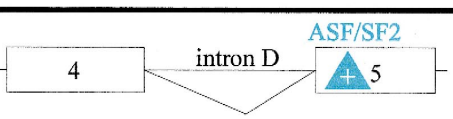
22	fast skeletal TnT, exons 4-7	rat		31 combinations of inclusion 1-3 9	[6]
23	CD45, exons 4-6	human, mouse	B-cells T-cells	3 4 5 6 7 HsSWAP ASF/SF2	[56,57, 77]

Fig. 3.

C: minigenes containing mutually exclusive exons

No	name	species	tissue specificity	minigene	reference
24	α-tropo-myosin, exons 2,3	rat	smooth muscle		[22,50, 54,61]
25	α_s -tropo-myosin, exons NM, SK	human	non-muscle skeletal muscle		[26]
26	β-tropo-myosin, exons 6A,B	chicken	smooth muscle+ non-muscle skeletal muscle		[4,23, 60]
27	β-tropo-myosin, exons 6,7	rat	smooth muscle+ non-muscle skeletal muscle		[27]
28	pyruvate kinase M, exons 9,10	human	skeletal muscle, heart and brain		[68]
29	albumin, exons G, H	rat			[66]
30	MLC, exons 1/3	rat	promoter upstream of exon 2 promoter dependent splicing promoter upstream of exon 1		[24]
31	FGFR, K-SAM	human	epithelial cells		[19]

D: minigenes containing a retained intron

32	bGH, intron D	bovine	anterior pituitary somatotrophs		[20]

E: minigene containing incremental combinatorial exons

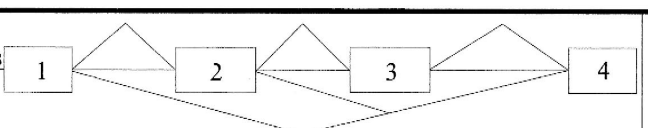
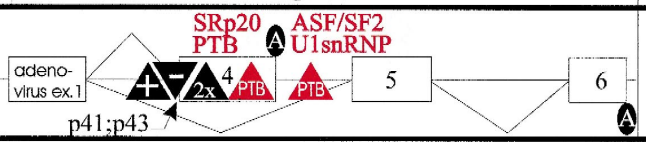
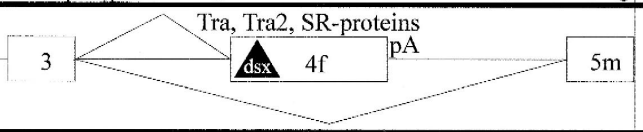
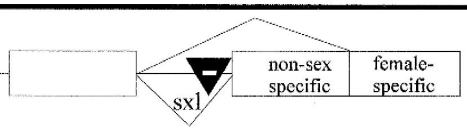
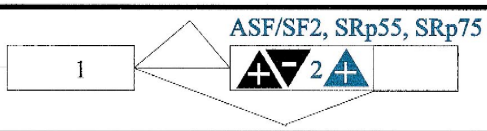
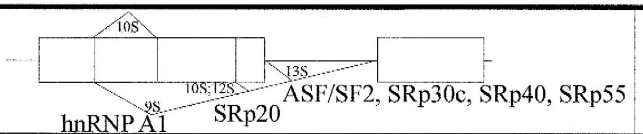
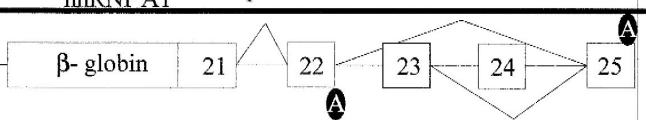
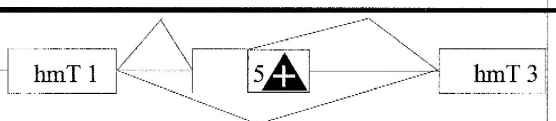
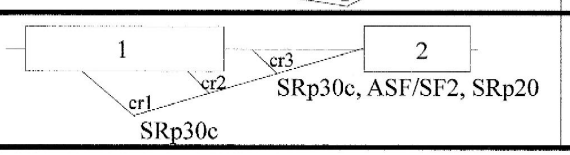
33	tau, exons 2,3	human	DE adult neurons, COS-, SKN-cells fetal neurons		[1]

Fig. 3.

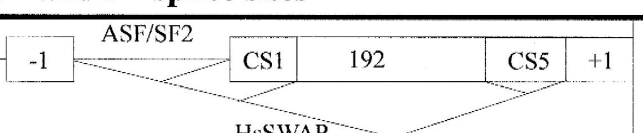
F: minigenes containing alternative 3' splice sites

No	name	species	tissue specificity	minigene	reference
34	CT/CGRP	human	HeLa, calcitonin (thyroid) CGRP (neurons)		[18,42, 55]
35	dsx RO	Drosophila	female male		[31]
36	M-tra	Drosophila	female male		[60,69]
37	BPV-1	BPV-1	DE		[76]

G: minigenes containing alternative 5' splice sites

38	E1A	Adeno-virus			[7]
39	SERCA2, exon 2a	hamster	muscle brain		[3,70]
40	Caldesmon	human	DE smooth muscle non-muscle		[29]
41	SWAP	human			[59]
42	SV40 t-antigen	SV40	HEK 293 HeLa		[25,71]
43	β-globin, β-thalassemic allele	human			[7,38]

H: minigene containing alternative 3'- and 5'- splice sites

44	fibronectin, exon IIICS	human	DE liver, adult: skipping of CS1 and CS5		[43,59]

- The PCR program must be optimized for each minigene in trial experiments as we found that often identical programs show variations of amplification products when different thermocycler models are used [64]. Using the biometra trio thermoblock 050–000, we apply the following program for the X16 minigene: Initial denaturation for 2 min at 94°C; 30 cycles: 30 s denaturation at 94°C, annealing at 55°C for 1 min, extension at 72°C for 1 min, after 30 cycles a final extension at 72°C for 20 min and cooling to 4°C. For the E1A minigene, we use the following touchdown [21] program: Initial denaturation for 2 min at 94°C; 20 cycles: 30 s denaturation at 94°C, annealing at 65°C for 1 min, a 0.5°C decrease of the annealing temperature in each cycle, extension at 72°C for 2 min, after 20 cycles 10 more cycles with 30 s denaturation at 94°C, annealing at 55°C for 1 min, extension at 72°C for 2 min, final extension at 72°C for 20 min and cooling to 4°C. Both these programs can be used as starting points when optimizing a new reaction.
- The PCR reaction products are analyzed on a 0.3- to 0.4-cm-thick 2% agarose TBE gel.

5. Results

The results of two typical splicing assays are shown in Fig. 2 using the CDC2 like kinase CLK2 as an example of a *trans*-factor that acts on the SRp20 [32] and E1A [7] minigenes. The CLK2 protein has been shown to phosphorylate splicing factors [52]. Fig. 2A shows a titration experiment using the SRp20 minigene system [32]. Here, an increase of pEGFP-CLK2 concentration leads to skipping of exon 4. Fig. 2B shows the comparison of three factors at a constant concentration in the E1A minigene system [53]. Here, CLK2 overexpression inhibits formation of the 13 and 12 S splice variant. In contrast, the catalytic mutant CLK2 KR that lacks kinase activity [52] and empty pcDNA vector have no effect on the 13 and 12 S variants.

6. Discussion

This technique, summarized in Fig. 1, has been applied for the analysis of several genes listed in Fig. 3. In comparison to a biochemical analysis, the major advantages of analyzing splicing patterns with minigenes in vivo are: that the length of the analyzed minigene is not limiting, that a large number of cell types can be analyzed and that the analysis is based on the in vivo situation. In addition, indirect effects, such as phosphorylation or cellular differentiation, e.g., Refs. [4,14,15,23,26,47,60] can be addressed. Several parameters can be changed to analyze factors that affect alternative exon recognition. Firstly, the cell type used for transfection can be changed, e.g., tropomyosin minigenes have been transfected in muscle and nonmuscle cells [4,22,23,26,27,60] and clathrin light chain B minigenes were transfected into primary neuronal cultures, as well as nonneuronal cells [63]. In both cases, the splicing pattern of the minigenes reflected the exon usage observed for the endogenous genes in the appropriate cell system and allowed the analysis of regulatory factors.

Secondly, parts of the minigene can be changed by site-directed mutagenesis. Often, alternative exons are surrounded by weak splice sites and their improvement leads then to a constitutive exon usage [5,65]. Another parameter that is often analyzed by mutagenesis of minigenes are splicing enhancers or silencers.

Finally, minigenes can be cotransfected with putative alternative splicing factors to identify possible *trans*-acting factors. This can be used to verify in vitro data collected in biochemical systems [7], to analyze genes that do not show splicing activity in vitro [63], or to analyze systems such as differentiated neurons where biochemical systems are difficult to apply.

6.1. Troubleshooting

6.1.1. Transfection efficiency

The most crucial parameter for the success of an in vivo splicing experiment is the transfection efficiency, especially when cotransfections with putative *trans*-acting factors are performed. We therefore usually employ EGFP-tagged cDNA in cotransfection experiments that allow an easy monitoring of the transfection efficiency that can reach 90% with HEK293 cells. Reasons for lower efficiencies are usually dense seeding of cells, a high passage number of cells or a deviation of the pH of the transfection

Fig. 3. Summary of minigenes that have been used to analyze alternative splicing patterns in vivo. The structures of the various genes are schematically indicated, however, the drawings are not to scale. The various genes are sorted according to their splicing mechanism (A–H). Stimulatory effects of *trans*-acting factors binding to exonic splicing enhancers (ESE) or intronic enhancers (ISE) are indicated by an upward triangle marked with a ''+''. An inhibitory effect by *trans*-acting factors binding to exonic (ESS) or intronic (ISS) splicing silencers is indicated with a downward triangle marked with a ''–''. S: splicing is regulated by a secondary structure, DE: splicing is developmentally regulated. A: alternative polyadenylation site, dsx: double sex repeat, dcs: downstream control sequence, icr: intronic control region, MSE: muscle specific splicing enhancer, PTB: polypyrimidine tract binding protein binding site. The tip of the triangle points towards the resulting splicing pattern. *cis*-Elements containing identical sequence elements are marked by the same color. Identified tissue specific *trans*-acting factors are shown on top or bottom of the minigenes, depending on the stimulatory or inhibitory effect, respectively. When a direct correlation between binding of a splicing factor to a *cis*-element and a change of splicing patterns has been demonstrated, *cis*-elements and *trans*-acting factors are shown in the same color. An updated collection is available at www.neuro.mpg.de/stamm.htm.

solution caused by not transfecting in a 3% CO_2 atmosphere.

6.1.2. Reproducibility

In vivo splicing assays are generally well reproducible when several parameters are kept constant. For transfection, cells should be always plated at the same density. It is also important to keep the time between seeding and transfection, as well as the actual transfection time constant.

6.1.3. Autoregulation

Several splicing factors seem to autoregulate their expression levels, e.g., Ref. [32]. This can result in a substitution of the endogenous protein by the transfected cDNA, which means that the concentration of this splicing factor will not be dramatically changed. The autoregulation needs some time to occur and if observed, the time between transfection and cell harvesting can be shortened. Therefore, it is best to perform the analysis in transient transfection systems.

6.1.4. Contamination

As with all PCR-based methods, DNA contaminations are a major problem. It is therefore advisable to make aliquoted stocks of all solutions and if possible to separate the PCR setup form the DNA analysis.

6.1.5. Heterodimers

Often, the simultaneous generation of two PCR products that differ only in the presence or absence of short exonic sequences results in the formation of a heteroduplex that consists of two DNA strands differing by this exonic sequence [78]. The heteroduplex usually migrates as a third PCR product (e.g., Ref. [62]). In our hands, heteroduplex formation increases when the reaction products are stored for longer time and if too many cycles in the PCR amplification are used. These parameters should therefore be minimized.

6.2. Alternative methods

Most *cis*- and *trans*-acting elements governing alternative splicing were identified using biochemical methods that employ a cell-free nuclear extract and radioactively labeled in vitro synthesized pre-mRNA [39]. Although this approach allows the analysis of direct protein RNA interactions, it has several limitations. So far, nuclear extracts functional in pre-mRNA splicing have only been made from transformed cell lines, almost exclusively fibroblasts, which complicates analysis of, e.g., alternative splicing in adult neurons. Furthermore, synthesis and analysis of in vitro transcribed pre-mRNA is limited to small RNA molecules (< 600 nt), which is smaller than the size of introns flanking most alternatively spliced exons. Finally, there is increasing evidence that pre-mRNA splicing, transcription and polyadenylation are coupled processes [16,44,53,54]. The interdependence of these processes can only be addressed by studying intact cells.

So far, most alternative exons studied by the in vivo minigene approach were alternatively spliced cassette exons that can be analyzed easily by RT-PCR due to the different size of their PCR products. Another common alternative splicing mechanism are mutually exclusive exons (see Fig. 3C). Most often, the mutually exclusive exons are similar in size, making their PCR products indistinguishable by their length. In these cases, the products can be identified with exon-specific restriction sites or by Southern blotting employing exon specific probes. Sometimes, alternative mRNAs are produced by the usage of different polyadenylation sites. Here, the downstream sequences are different, prohibiting the RT-PCR analysis used for cassette exons. It is possible, however, to use $(T)_nG$ primers in RT-PCR to analyze these splicing events.

Most minigenes are analyzed by RT-PCR as described here. However, different methods, such as RNAse protection analysis [67], or a functional assay, where a selection maker depends on the splicing pattern [12] have been employed as well.

7. Quick procedure

- Construct or obtain a minigene containing the desired alternative exon flanked by constitutive exons.
- Transfect this minigene alone, or together with varying amounts of cDNA expressing splicing factors into cells.
- Analyze the resulting RNA by RT-PCR.

8. Essential literature references

General PCR methods: Refs. [45,46].
Example of minigene analysis: Refs. [7,63]

9. References cited in Fig. 3

[1–3,6,8–10,13,17–20,24,25,28–31,33–38,40–43,48,50,51,55–57,59,61,66,68–77]

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