A novel strategy for the simultaneous detection of alternatively spliced transcripts: A study of MEF2C exon covariation

BECKMAN COULTER

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INTRODUCTION

Myocyte Enhancer Factor 2 (MEF2) proteins are transcription factors that exist in all metazoans and play pivotal roles in development and differentiation of tissues. There are 4 vertebrate Mef2 genes, Mef 2A, B, C and D, and these have different temporal and spatial expression patterns. Mef2A, C and D encode protein variants by virtue of alternative splicing of primary transcripts, and these genes have similar structures and alternative splicing patterns that are conserved across evolution. The alternative splicing involves mutually exclusive exons (alpha1 and alpha2), a cassette exon (beta), and alternative splice acceptors that flank a short region (gamma). The corresponding short polypeptide domains encoded by these alternative segments are nested within the Mef2 carboxy-termini and are structurally conserved across isotypes. These domains confer specific functions, important for binding co-activators, transactivation and transrepression. In an effort to elucidate the roles of Mef2 alternative splicing variants, we have developed an RT-PCR long fragment assay in which all eight Mef2C mRNA isoforms are simultaneously monitored in cell and tissue samples. Capillary electrophoresis with laser induced fluorescence (CE-LIF) was performed using the GenomeLab™ GeXP to separate and detect these fragments. CE-LIF successfully resolved all eight fragments, the longest of which was 1049 nucleotides and only six nucleotides longer than the next largest fragment. This assay was used to confirm and extend prior observations of regulated Mef2 alternative splicing among tissues, during development and during muscle differentiation. The technique is well suited for rapid qualitative evaluation of splicing variant expression, and could be effectively used for candidates with established splicing variants or for the validation of findings observed with sequencing or fragment analysis. Importantly, this strategy uniquely allows for the evaluation of co-variations in multiple alternative splicing events for primary transcripts of a given gene.

§ The PCR process is covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffman La Roche, Ltd.

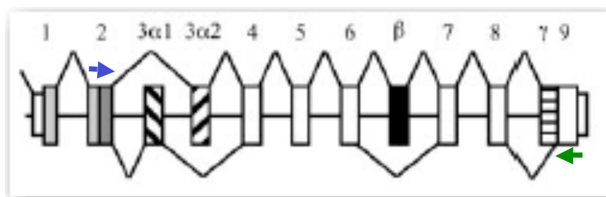
Figure 1. Mef2c Alternative Splicing: Primer design for amplification of all eight transcript variants

Primer Set: Mef2c +alpha1+beta+gamma — **full-length product = 1049nt**

Forward: D4-AGCCGGACAAACTCAGACAT — nt 232-251

Reverse: CAGCTGCTCAAGCTGTCAAC — nt 1280-1261

Note: This primer set amplifies mouse and human Mef2c.



alpha 1 or 2 — beta + or - — gamma + or -

variant	fragment size (nt)	variant	fragment size (nt)
α1βγ	1049	α2βγ	1043
α1β	1025	α2β	1019
α1γ	953	α2γ	947
α1	929	α2	923

METHODS

cDNA and RNA Samples. Purified and quantitated cDNA and RNA samples were obtained from the Burnham Institute. RNA was isolated from C2C12 cell lines or mouse tissues by Trizol* extraction with DNase treatment. The C2C12 cells were untreated (blasts), treated for 1 day with either 2% Horse Serum (D1_HS) or 10% Fetal Bovine Serum (D1_FBS), or allow to differentiate for 6 days into myotubes (D6_tubes).

Primer Design. Primers for the full-length variant of Mef2c (a1,b+,g+) were designed such that all eight transcript variants would be amplified (Fig. 1). The forward primer was labeled with WellRED D4 dye for detection with the GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter).

RT-PCR. A one step (one-tube) RT-PCR reaction (Promega Access* RT-PCR System) was performed with 200 ng of RNA or 1 ug of cDNA per reaction according to the manufacturer's instructions.

Separation by Capillary Electrophoresis (CE). PCR product separation, detection and analysis was performed with the GenomeLab GeXP Genetic Analysis System. PCR products were diluted in a mixture of Sample Loading Solution (Beckman Coulter) and MapMarker* WellRED D1-1000 (Bioventures) size standard and then separated by capillary electrophoresis at 3kV for 180 minutes. Custom analysis parameters (Dye Mobil. Calib. = PAver1, Slope Threshold = 1, Include Peaks = 1%, Size Standard 50-1000, Quartic model) were used in the Fragment Analysis module of GenomeLab Genetic Analysis System software.

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RESULTS

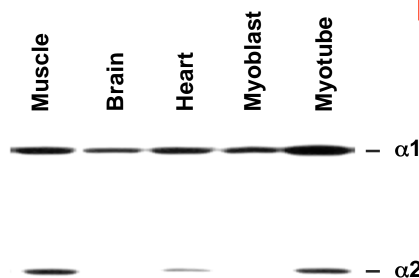

Figure 2. RNase-Protection Assay detects the expression of Mef2c alpha domain in mouse tissues and C2C12 cells. Alpha 1 is detected in all the tissues and both cell stages. Alpha 2 is only detected in muscle, heart and myotubes.

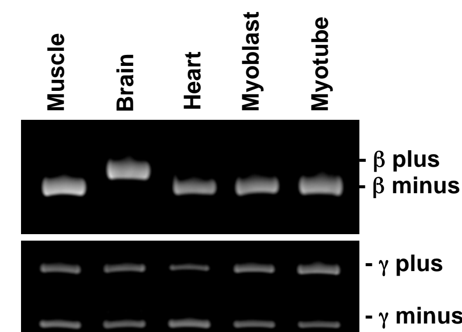

Figure 3. RT-PCR Assay detects the expression of single alternative exon site from either Mef2c beta or gamma domains in mouse tissues and C2C12 cells . Beta domain is detected exclusively in brain tissue. Both gamma-plus and gamma-minus variants are found in the samples.

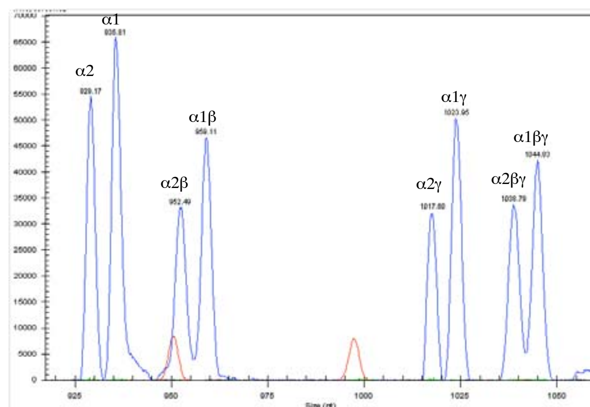

Figure 4. Electropherograms of the 8 human *Mef2c* variants amplified from cDNAs. GeXP is able to resolve and distinguish all eight *Mef2c* fragments.

Mef2c Variant	Expected Size (nt)	GeXP Apparent Size (nt)
α1βγ	1049	1045
α2βγ	1043	1039
α1γ	1025	1024
α2γ	1019	1018
α1β	953	959
α2β	947	953
α1	929	936
α2	923	929

Table 1. Expected and Apparent fragment sizes for the eight *Mef2c* variants.

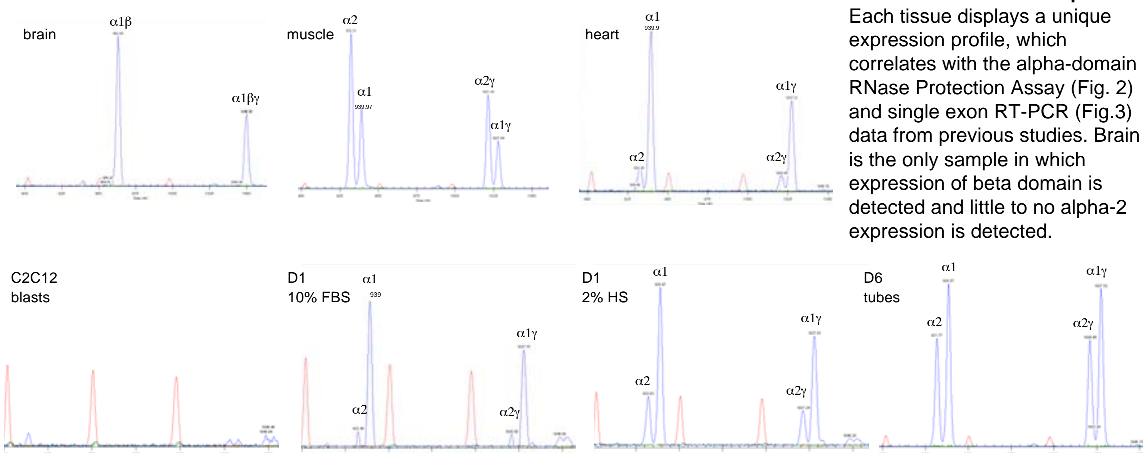

Figure 5. Electropherograms of RT-PCR products amplified with D4-*Mef2c* primer from various mouse tissue RNA samples. Each tissue displays a unique expression profile, which correlates with the alpha-domain RNase Protection Assay (Fig. 2) and single exon RT-PCR (Fig.3) data from previous studies. Brain is the only sample in which expression of beta domain is detected and little to no alpha-2 expression is detected.

Figure 6. Electropherograms of C2C12 cells. Cell were left untreated as myoblasts (blasts), or treated with either 10% fetal bovine serum (FBS) or 2% horse serum (HS) for 1 day (D1), or differentiated for six days (D6) into myotubes (tubes). Very little to no expression of *Mef2c* was detected in undifferentiated myoblasts, whereas increasing amounts of the α1, α2, α1γ and α2γ transcript variants were detected in the cells treated with 10% FBS or 2% HS with maximal expression of these four variants detected in the differentiated myotubes. This correlates with previous RNase-protection and single-exon RT-PCR data for all but the myoblasts, in which some alpha-1 domain expression was detected. Further optimization of the GeXP-Long Fragment RT-PCR is necessary.

CONCLUSION

Traditional RT-PCR and RNase Protection assays are limited to the detection of the expression of a single alternative splicing event. The GenomeLab GeXP Genetic Analysis System is capable of resolving and distinguishing fragments as long as1050 nucleotides, with sizes differing as little as six nucleotides, allowing for simultaneous amplification of transcript variants that contain multiple, alternatively spliced exons. This strategy uniquely allows for the evaluation of co-variation in multiple alternative splicing events for primary transcripts of a given gene.

The GenomeLab GeXP is for Laboratory Use Only; not for use in diagnostic procedures.