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THE IMPACT OF PROMOTERS AND CELL-LINE TYPES ON ALTERNATIVE SPLICING OF RECOMBINANT CONSTRUCTS

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SUMMARY

Alternative intron splicing is a process where introns are excised from pre-mRNA following different patterns. The consequence of it is a presence of several different forms of mature mRNAs differing in length and in content of the whole exons or their parts. Studies in the past decade lead to the view that a complex and extensively coupled network has evolved to coordinate the activities of gene expression pathway, which includes transcription, several pre-mRNA processing steps and the export of mature mRNA to the cytoplasm. Coupling of transcription to splicing was revealed in studies showing that transcription of pre-mRNA by different promoters can generate different alternatively spliced mRNAs. The main goal of this study was to determine the impact of different milk gene promoters on alternative splicing under in vitro conditions. Besides, we also investigated how different cell types influence the splice site selection. With this experiment, we confirmed our predictions that promoter structure and cell-type have an impact on alternative splicing. Differences were observed in quantity of transcripts, as well as in splice-site selection.

Key-words: alternative splicing, milk-protein gene promoters, cell lines

INTRODUCTION

Alternative intron splicing is a process where introns are excised following different patterns. The consequence of it is a presence of several different forms of mature mRNAs differing in length and in content of the whole exons or their parts, but representing transcripts of the same gene (Collins and Guthrie, 2000).

Studies in the past decade lead to the view that a complex and extensively coupled network has evolved to coordinate the activities of gene expression pathway, including transcription, several pre-mRNA processing steps and the export of mature mRNA to the cytoplasm (Bentley, 1999). Both the carboxy-terminal domain (CTD) of the RNA polymerase II and transription elongation factors play central roles in coupling transcription to pre-mRNA processing (McCracken et al., 1997).

Coupling of transcription to splicing was also found out in studies showing that transcription of pre-mRNA by different promoters can generate different alternatively spliced mRNAs (Cramer et al., 1997; Cramer et al., 1999). The impact of *cis*-elements and *trans*-acting factors on alternative intron splicing could be determined by *in vitro* studies using plasmid constructs, containing parts of genes with one or more weak exons. By inserting different promoters in such constructs it was shown that, in addition to the control exerted by splicing factors in splice site selection, alternative splicing is sensitive to the kind of promoter, possibly reflecting the particular arrangement of binding sites for basal and regulatory transcription factors (Caputi et al., 1994; Cramer et al., 1997).

The impact of *cis*-elements and *trans*-acting factors on alternative intron splicing could be determined by *in vitro* studies using plasmid constructs, containing parts of genes with one or more weak exons. The pSVEDA-Tot vector includes first three exons of human α-globin gene with its functional promoter. The EDA human fibronectin exon has been inserted in the *Bst*EII site of the third α globin exon. EDA is the second exon of human fibronectin gene and is alternatively included in mature fibronectin mRNA because of atypical donor site in intron 1 (Caputi et al.,1994).

Promoters of the milk protein genes are not only important for the regulation of milk gene expression, but also for the transgenesis in the mammary gland.

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It was shown that milk is currently the best available bioreactor. Extensive studies have shown that it can be the source of a variety of recombinant proteins, for example human IGF1, human lactoferrin, human fibrinogen, human parathyroid hormone, etc. To express foreign genes into the mammary gland, milk protein gene promoters must be used. These promoters showed different potency. Those of κ- and α_{S2}-casein gene are particularly weak. All the other promoters are being used with variable success (Houdebine, 2000). When expressing a human growth hormone gene under the transcriptional control of WAP2 gene promoter in different animal models, different splicing products were observed. Apart from already-known physiological mRNA splice products, previously undescribed processed hGH transcripts were found. It was suggested that the species specific hGH mRNA patterns may be caused by species and tissue-specific differences in *trans*-acting splice factors (Aigner et al., 1999).
With the cell-transfection experiment on different cell-lines with different recombinant constructs integrated into the pSVEDA-Tot vector (kindly provided by dr. Muro Andres, ICGEB research center, Triest) we studied the impact of the milk-protein gene promoters and of the cell-type on alternative splicing under *in vitro* conditions.

MATERIAL AND METHODS

The impact of different lactoprotein gene promoters was studied in *in vitro* system by using heterologous plasmid construct, containing different lactoprotein gene promoters inserted into plasmid vector pSVEDATot. Two different constructs were prepared, containing mouse WAP promoter and porcine α_{s1}-casein promoter. Constructs were transfected to 4 different cell types (BME-UV, Caco-2, HC11, COS7) to determine the impact of the cell type on splicing. Original pSVEDATot was used as control.
Number of 488 bp of porcine α_{s-1} casein gene promoter and 1028 bp long fragment of the murine WAP gene 5'UTR was PCR-amplified from genomic DNA. Oligonucleotide primers with designed restriction site for *Sca*I and *Pau*I restriction endonucleases were used for amplification: PASScaI.F: 5'GGG AGT ACT GAT GAT AAA TCT TCT C-3', PASPauI.R: 5'AAA GCG CGC CGG AAA GAA GCA A-3'; WAPSca.F: 5'-TTT AGT ACT CGA GGT TAC AGC CGA GAG G-3', WAPPau.R: 5'-TTT GCG CGC ACA GAC TCA CAG ACC CTA CG-3' (restriction sites are underlined).
Products were isolated with *JETquick Spin Column Technique* kit (Genomed) from 1 % agarose gel and further used for cloning into p-GEM T Easy vector (Promega). Plasmid DNA was isolated with *JETSTAR Plasmid Midiprep* kit (Genomed) and restricted with appropriate restriction enzymes, *Sca*I and *Pau*I. Promoter fragments were subcloned into pSVEDA Tot plasmid vector, previously cut with *Sca*I and *Pau*I restriction enzymes.
Transfections of BME-UV, HC11, Caco-2 and COS7 cell lines were performed with FuGENE 6 transfection reagent. 6-well plates were used for transfection. For transfection of one well, 2 µg of plasmid DNA, 3 µl of FuGENE 6 transfection reagent, and Opti-MEM serum-free medium to final volume 100 µl were used.
Forty eight h after transfection, medium was removed and cells were washed with PBS buffer. Total RNA was isolated with *TRIzol* reagent (Invitrogen). cDNA, complementary to mRNA, was synthesised with reverse transcription reaction. Specific pSVcDNA (5'-GGT ATT TGG AGG TCA GCA-3') was used for reverse transcription with a sequence complementary to pSVEDA-Tot sequence in 3' direction from the exon FN+1. Obtained one-strand cDNA was used as a template in the PCR with oligonucleotide primers PSV5'J and PSV3'J (described in Caputi et al., 1994). Products were run on Agilant capilary electrophoresis.

RESULTS AND DISCUSSION

With this experiment, we wanted to demonstrate the impact of different promoters, and different cell-types on alternative splice site selection. In all epithelilal cell lines (BME, HC11, Caco2), we observed two fragments by RT-PCR method employing primers PSV5'J and PSV3'J. One fragment is 231 bp long and another 501 bp long. Those fragments resulted from amplification of cDNA, transcribed from two different forms of mRNA with alternatively spliced exon EDA. Both fragments were already described in previous experiments with plasmid pSVEDA-Tot. As expected, we were also able to

demonstrate the impact of different promoters on splicing. Differences between promoters were observed regarding relative amount of both fragments. Relative quantity of the short, 231 bp fragment, was always highest when original pSVEDA-Tot vector with human α-globin promoter was used. Relative amount of the longer form (501 bp) was highest in case of the porcine α_{s1}-casein gene promoter- pSVEDA-Tot recombinant construct.

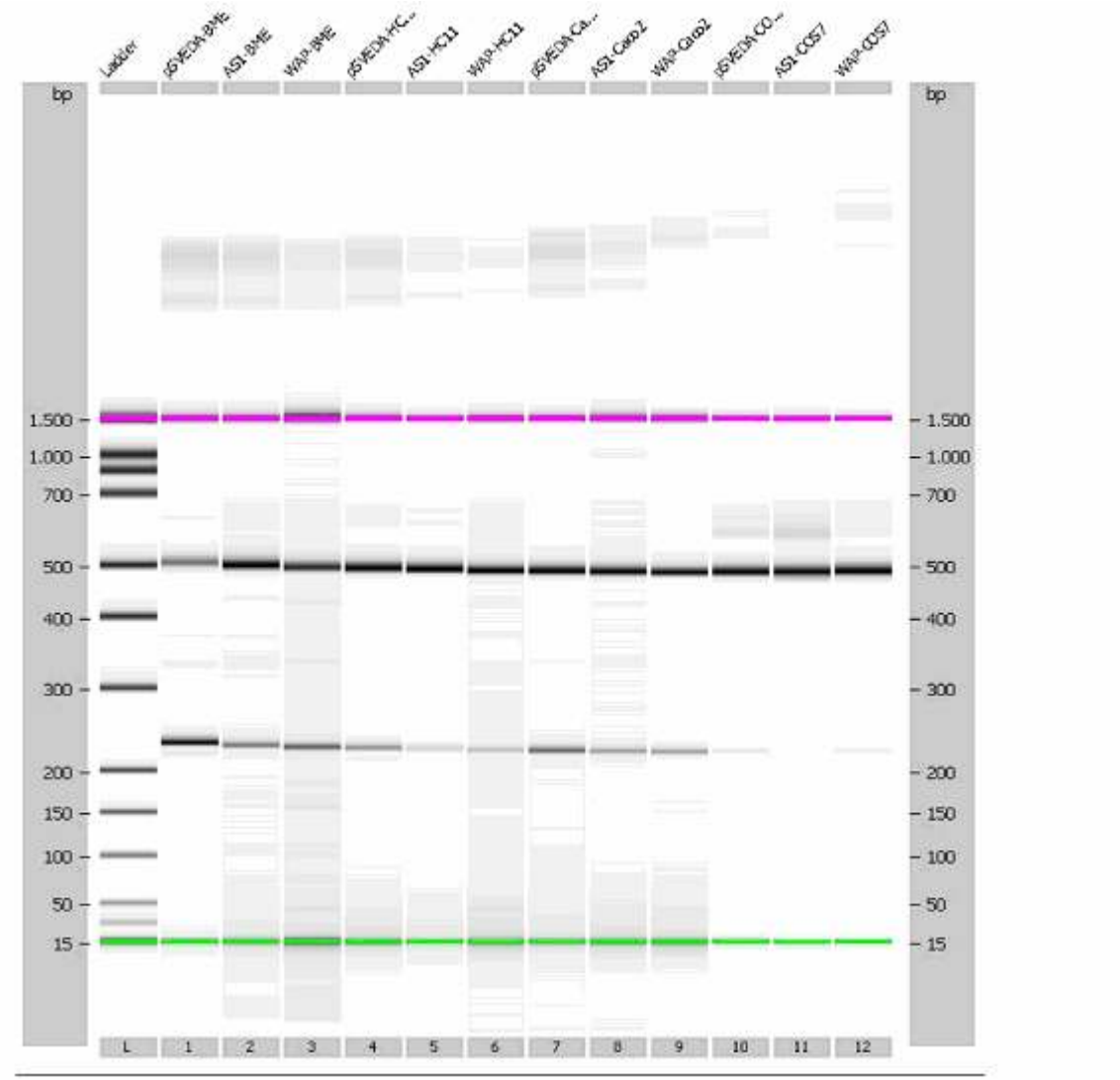

Figure 1. Capillary electrophoresis gel image

Capillary electrophoresis gel image shows RT-PCR products of recombinant constructs transfection experiment in different cell lines: rows 1-3: BME-UV1 cell line; rows 4-6 : HC11 cell line; rows 7-9: Caco2 cell line; rows 10-12: COS7 cell line. The type of the promoter in recombinant constructs is indicated above each row: pSVEDA- original pSVEDA Tot plasmid containing human fibronectin promoter; AS1- recombinant construct containing porcine CSN1S1 promoter; WAP- recombinant construct containing mouse WAP promoter.

In the case of fibroblast-like COS7 cell line, majority of transcripts were 501 bp long (long form with included exon EDA). Traces od short form without exon EDA were observed when using original human α-globin promoter and mouse WAP promoter. Beside these two forms, another form around 560 bp was observed in all three recombinant constructs. The fragment of approximately the same length was observed in the experiment with horse CSN2 gene promoter-pSVEDA-Tot recombinant construct, transfected in bovine BME-UV cells (Lenasi, 2006). With sequencing, she found that this fragment resulted from atypical splice site selection. This fragment does not contain intron FN-1 and alternative exon EDA, but it contains part of intron FN+1 and the whole exon FN+1.This form is probably result of atypical splice-site selection.

Results, showing RT-PCR products of recombinant constructs transfection experiments, were run on Agilant capillary electrophoresis, and are presented in Figure 1.
With this experiment, we approved our predictions, that promoter structure and cell-type have an impact on alternative splicing. Differences were observed in quantity of transcripts, as well as in splice-site selection. Our observations go in line with experiment in transgenic animals, expressing human growth hormone under the control of mouse WAP promoter (Aigner et al., 1999). Compared to the transcription products in transgenic mice harboring the same gene construct, expression analysis in transgenic pigs and rabbits found out different mRNA splice patterns with regard to the proportion of the processed transcripts. Even differences between both mammary-gland cell lines (BME-UV and HC11) did not include different splice forms. Only the quantity of both alternative transcripts was different. The short form without exon EDA was stronger present in BME-UV cells compared to HC11 cells.

CONCLUSION

Our results support the predictions, that alternative splicing is species- and tissue- specific. Also, we approved that promoters influence splicing. Impact of promoters on alternative splicing could be explained through hypothesis of coupling between transcription and splicing processes, and cooperation between RNApolII and different factors involved in splicing, which is in line with the fact that RNA splicing should be considered as co-transcriptional rather than post-transcriptional event.
The obtained results could be also important for further researches on transgenesis in the mammary gland because of the potential impact of the milk gene promoter on splicing pattern of a transgene.

REFERENCES

1. Aigner, B., Pambalk, K., Reichart, U., Besenfelder, U., Bosze, Z., Renner, M., Gunzburg, W.H., Wolf, E., Muller, M., Brem, G. (1999): Species-specific alternative splicing of transgenic RNA in the mammary glands of pigs, rabbits, and mice. Biochem Biophys Res Commun. Apr 21;257(3):843-50.
2. Bentley, D. (1999): Coupling RNA polymerase II transcription with pre-mRNA processing. Current Opinion in Cell Biology, 11(3):347-351.
3. Caputi, M., Casari, G., Guenzi, S., Tagliabue, R., Sidoli, A., Melo, C.A., Baralle, F.E. (1994): A novel bipartite splicing enhancer modulates the differential processing of the human fibronectin EDA exon. Nucleic Acids Research 22(6):1018-1022.
4. Collins, C.A., Guthrie, C. (2000): The question remains: is the spliceosome a ribozyme? Nature Structural Biology 7(10):850-854.
5. Cramer, P., Pesce, C.G., Baralle, F.E., Kornblihtt, A.R. (1997): Functional association between promoter structure and transcript alternative splicing. Proceedings of the National Academy of Sciences of the United States of America 94(21):11456-11460.
6. Cramer, P., Caceres, J.F., Cazalla, D., Kadener, S., Muro, A.F., Baralle, F.E., Kornblihtt, A.R. (1999): Coupling of transcription with alternative splicing: RNA pol II promoters modulate SF2/ASF and 9G8 effects on an exonic splicing enhancer. Molecular Cell, 4(2):251-258.
7. Houdebine, L.M. (2000): Transgenic animal bioreactor. Transgenic Research 9: 305–320.
8. Lenasi, T., Peterlin, B.M., Dovc, P. (2006): Distal regulation of alternative splicing by splicing enhancer in equine beta-casein intron 1. RNA 12 (3): 498-507
9. McCracken, S., Fong, N., Rosonina, E., Yankulov, K., Brothers, G., Siderovski, D., Hessel, A., Foster, S., Shuman, S., Bentley, D.L. (1997): 5'-Capping enzymes are targeted to pre-mRNA by binding to the phosphorylated carboxy-terminal domain of RNA polymerase II. Genes & Development 11(24):3306-3318.

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