Transgenic Research **13:** 75–79, 2004.

Short communication

On the use of post-transcriptional processing elements in transgenes

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Received 27 August 2003; accepted 2 October 2003

Key words: biotechnology, gene silencing, RNA processing, transgene, vector

Abstract

RNA processing events modulate final productivity of a given transgene. We have evaluated a series of RNA elements for their ability to enhance α1-antitrypsin production in mammary cells. Our results indicate the need for a case-by-case assessment of each construct design and the occurrence of gene silencing events *in vivo*.

Introduction

The ability to introduce genes into the germline of animals has allowed novel biotechnological applications to be developed. One of the most promising has been the generation of animal bioreactors (Houdebine, 2000). The primary focus has been to produce novel proteins in milk, enabling repeated harvest of the protein. The basic transgene design is simple. The promoter of a gene normally active in the mammary gland during lactation, usually a milk protein gene, is linked to a cDNA or genomic fragment encoding the gene of interest. In this way, expression of genes normally active in the liver are directed to the mammary gland and hence into the milk. Attempts to improve transgene expression have largely focussed on enhancing transcription frequency within a population of founder animals (Clark et al., 1992), increasing transcription level within a line (James et al., 2000; Pantano et al., 2003) or through the use of large genomic fragments that are considered to contain the necessary regulatory elements (Giraldo & Montoliu, 2001).

The production of mRNA that can be translated by the ribosome into protein is the combinatorial result of transcription and numerous RNA processing events (Green, 1991; Harris & Hope, 2000). Some of the sequences regulating RNA maturation have been shown to be transferable, in that they can be incorporated into a heterologous gene construct and still function to regulate RNA processing (Huang & Yen, 1995; Liu & Mertz, 1995; Vassilakos et al., 1995; Donello et al., 1996). In the belief that enhancing RNA processing would increase productivity (Yull et al., 1995; Houdebine, 2000), we have now attempted to enhance transgene expression in the mammary gland through the use of RNA processing elements.

Materials and methods

Construct generation

The 566 bp PRE sequence was isolated from pGEM7.PRE (Huang & Yen, 1995) as a *Sph*I–*Hin*dIII fragment, blunted and inserted in both orientations into *Ban*I restricted ptgpoly (Lathe et al., 1987), enabling subsequent excision with *Eco*RV and *Sma*I and insertion in either orientation into *Eco*RV restricted pBJ41 (Sola et al., 1998), to generate pBJ41.PRE5′ and pBJ41.PRE3′. The *Hin*dIII–*Acc*I 1345 bp α1-antitrypsin cDNA fragment (Whitelaw et al., 1991), after blunting, was inserted into the *Eco*RV site of pBJ41.PRE5′ and pBJ41.PRE3′ to generate pPRE-5′

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(PRE upstream of cDNA) and pPRE-3′ (PRE downstream of cDNA). The 98 bp BP sequence was PCR amplified from p334 (Vassilakos et al., 1995) with *Sma*I and *Eco*RV restriction site sequences introduced into the forward (TCCCCCGGGAACTGCTAA and GGGTGAGATATCAACAACTGC) and reverse (TTTCCGATATCCATTAGAGCC and GATCCCGGG GAGCCAAGCAT) primers. Digestion with *Sma*I and *Eco*RV released the PPE sequence for insertion into the *Eco*RV site of pBJ41 with insertion of α1-antitrypsin cDNA as above to generate pBP-5′ and pBP-3′. The 119 bp PPE sequence was PCR amplified from pTGTK1 (Wallace et al., 1991) with *Sma*I and *Eco*RV restriction site sequences introduced into the forward (TCCCCCGGGGAGGCG GCGGTG and GGGTGAGATATCGGCCGGGGA) and reverse (TCCGATATCGGGATGGCGGTC and TCCCCCGGGATGGCGGTCGAA) primers. Digestion with *Sma*I and *Eco*RV released the PPE sequence for insertion into the *Eco*RV site of pBJ41 with insertion of α1-antitrypsin cDNA as above to generate pPPE-5′ and pPPE-3′.

Construct testing

Cell transfection and Northern blot analysis was as described previously (Donofrio et al., 1998) using an α1-antitrypsin cDNA probe generated from pUC8α1AT73 by *Acc*I–*Hin*dIII restriction and gel purification of the resulting 1345 bp fragment containing the α1-antitrypsin cDNA, normalised to β-casein signal to correct for loading as described before (Farini & Whitelaw, 1995). Protein levels were assessed by SDS-PAGE and Western blotting as described before (Archibald et al., 1990) and by ELISA (Wright et al., 1991). Transgenic mice were generated as described previously (Clark et al., 1992) with the PRE-3′ isolated as a *Sal*I–*Xba*I fragment. Transgenic mice were identified by PCR using internal PRE primers (TGCGTGGAACCTTTCT GGCT and TGCGCAGACCAATTTATGCCTA) and subsequently by Southern blot (data not shown).

Results and discussion

Three RNA elements were selected on their reported ability to retain function after incorporation into heterologous transcript. The 98 nucleotide (BP) sequence from the bovine bovine pre-prolactin 3′UTR is proposed to prolong translation (Vassilakos et al., 1995). The 119 nucleotide sequence called post-transcriptional enhancer (PPE) from within the coding region of Herpes Simplex virus thymidine kinase gene enhances RNA transport (Liu & Mertz, 1995). The 566 nucleotide sequence called post-transcriptional regulatory element (PRE) from within the coding region of Hepatitis B virus S gene enhances nuclear export of intronless transcripts (Huang & Yen, 1995; Donello et al., 1996). The first sequence is derived from an intron-containing mammalian gene while the other two derive from viruses. The latter are predicted to function on intronless genes. Each sequence was inserted either just upstream (termed-5′) or just downstream (termed-3′) of the α1-antitrypsin cDNA in AAT-D (Whitelaw et al., 1991). A fourth sequence, the constitutive transport element (CTE) of Mason Pfizer monkey virus genome (Saavedra et al., 1997) was also tested and gave comparable results to that of the BP (data not shown).

Transgenes incorporating RNA processing elements

All constructs were tested by stable transfection of mouse HC11 cells which we have previously used to evaluate splicing of β-lactoglobulin gene constructs (Donofrio et al., 1996). In none of the hybrid constructs was an increase in steady-state mRNA observed. In one construct, PPE-3′, mRNA levels were comparable to that of the parental construct, AAT-D; in all other hybrid constructs tested mRNA levels were <50% that of AAT-D. A trend was observed in that -5′ constructs gave lower mRNA levels compared to -3′ constructs. We measured protein levels by Western blot and ELISA for α1-antitrypsin. The -5′ constructs, all giving <40% α1-antitrypsin protein levels compared to AAT-D, were again consistently worse than their -3′ counterparts (70–160% of AAT-D). Notably, PRE-3′ gave the highest levels at 160% that of AAT-D. This was the only example were protein levels were increased above that of the parental construct. This appreciable increase in protein production promotes the use of this element in production systems.

In a separate experiment, we inserted these RNA elements upstream of the α1-antitrypsin genomic sequences in AAT-B (Archibald et al., 1990). Transfection of these constructs into HC11 cells resulted in very low levels of mRNA and no detectable α1-antitrypsin protein.

We have summarised the productivity of each AAT-D based construct in Figure 1. The presence of

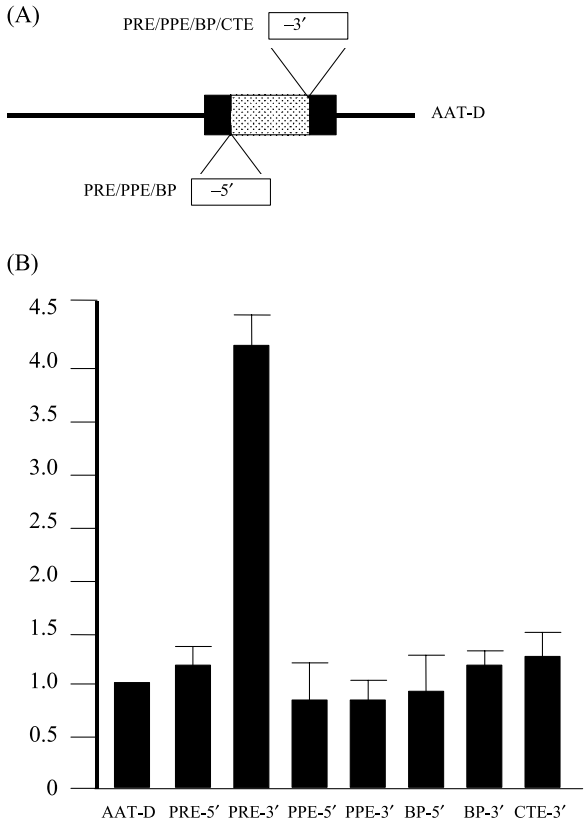

Figure 1. Protein/RNA ratio for transfected HC11 cells. (A) Schematic of constructs: black line, 5′- and 3′ β-lactoglobulin flanking sequences; black boxes, 5′- and 3′ β-lactoglobulin untranslated regions; hatched box, human α1-antitrypsin cDNA; open box, RNA element (inserted either 5′ or 3′ to cDNA). No intronic sequences are present in these constructs. (B) Histogram of protein/RNA ration for stably transfected HC11 pools. Note that the parental transgene AAT-D is nominally given the value of 1.0 and all test transgenes are relative to AAT-D. RNA measured by phosphorimager quantitation of northern blot normalised to β-casein signal. Protein levels measured by ELISA corrected for protein concentration by Pierce BCA assay. The graph shows the mean ratio determined from three separate transfection experiments for each plasmid, with RNA levels determined twice and protein levels measured in triplicate for each sample. Standard deviation values are represented.

the RNA element sequence reduced expression at both the RNA and at the protein level resulting in similar protein:RNA ratios. The exception was PRE-3′. Based on this we generated transgenic mice with an AAT-DPRE-3′ transgene. We utilised transgene co-injection rescue since this strategy is considered to facilitate transcription of cDNA-based transgenes (Clark et al., 1992). Basically the cDNA containing transgene is co-injected with a genomic transgene, in this case the genomic β-lactoglobulin transgene BLG.SX which has been pre-determined to be efficiently expressed (Whitelaw et al., 1992). Milk was harvested at mid-lactation (11th day of lactation) female mice and α1-antitrypsin protein assayed by ELISA and Western blot. We tested seven founder G0 females and G1 females for four lines established from male founder animals in comparison to milk obtained from BAD/135 mice that have been reported to carry an active AAT-D transgene (Clark et al.,1992). No α1-antitrypsin protein was detected by ELISA nor Western blot. In addition, Northern blot of these mice did not detect any α1-antitrypsin mRNA in total RNA prepared from lactating mammary tissue. The presence of the PRE-3′ transgene in these mice was confirmed by Southern blot and PCR analysis; founder mice may have been mosaic with regard to the presence of the transgene and this could have contributed to the lack of transgene expression in these samples.

Bearing in mind that this construct gave an appreciable increase in productivity *in vitro*, this result highlights the discrepancies between *in vitro* and *in vivo* expression properties of a vector (Petitclerc et al., 1995). This contrasts with the previous demonstration of similar RNA splicing events when compared *in vitro* and *in vivo* (Donofrio et al., 1996). One intriguing possibility is that insertion of a 566 bp viral sequence into a mammalian derived transgene is sufficient to stimulate some form of gene silencing mechanisms. This has been recognised before for reporter genes such as *LacZ* (Cohen-Tannoudji et al., 2000; Montoliu et al., 2000; Chevalier-Mariette et al., 2003) and other intronless sequences (Clark et al., 1997).

Transgene silencing

To generate PRE-3′ transgenic mice we utilised the transgene rescue strategy since the genomic β-lactoglobulin transgene BLG.SX facilitates expression of co-injected cDNA-based transgenes (Clark et al., 1992). The resulting mice, therefore, carry both the test cDNA-based transgene and the genomic β-lactoglobulin transgene. Although BLG.SX is anticipated to be expressed in all lines generated (Whitelaw et al., 1992), in some cases the cDNA-based transgene can silence BLG.SX expression (Clark et al., 1997). Somewhat surprisingly, when PRE-3′ and BLG.SX are co-injected, even though we do not see any expression of PRE-3′ transgene, we do see silencing of the BLG.SX transgene in four of the seven founders and two of the four lines analysed (Figure 2). Previous reports encountered a similar silencing frequency: 9 out of 11, 9 out of 11 and 6 out of 9 (Clark et al.,

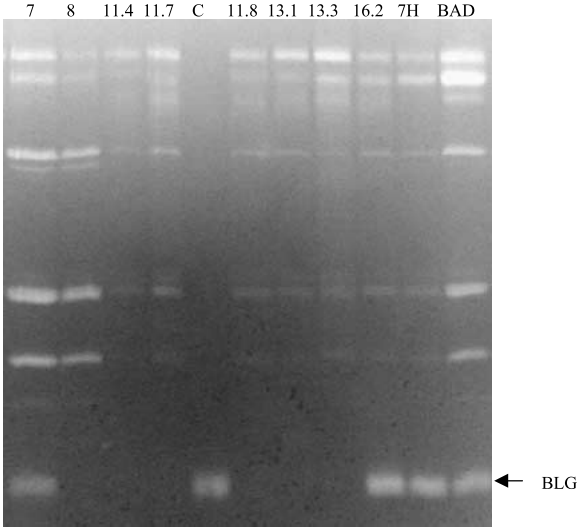

Figure 2. SDS-PAGE of transgenic milk. Representative SDS-PAGE of de-fatted mid-lactation milk samples stained with Coomassie-Blue. Transgenic founders are represented with a number, for example, 7; G1 animals represented by two numbers, for example, 11.4; C, purified β-lactoglobulin standard; 7H, β-lactoglobulin transgenic mouse (Opsahl et al., 2002); BAD, BAD/135 transgenic mouse (Clark et al., 1992). Note the variable presence of transgenic β-lactoglobulin protein (BLG).

1997). Southern blot confirmed that both transgenes are present in all lines analysed in this study.

We have not analysed this silencing event further but consider it likely that it is similar to that proposed previously (Clark et al., 1997). If so and because no expression of the test transgene was detected, this may indicate that it is the transgene array that initiates the silencing event. Regardless of the silencing mechanism, we suggest that some caution is applied when incorporating this type of sequence into vectors designed for expression in animals.

Conclusion

We have analysed three RNA-processing elements in an attempt to enhance transgene expression. Initial evaluation *in vitro* clearly demonstrated that most construct designs reduced productivity. The exception was a construct that had the PRE sequence inserted downstream of the α1-antitrypsin cDNA were a substantial increase in the protein:RNA ratio was measured *in vitro*. Unfortunately, transgenic mice carrying this transgene failed to expression any α1-antitrypsin in their milk. We conclude, therefore, that although the incorporation of this type of element into *in vivo* expression vectors is growing in popularity (Loeb et al., 1995; Schambach et al., 2000; Barry et al., 2001; Tiscornia et al., 2003), the amassed evidence support our conclusion that each element and construct requires to be assessed on a case-by-case basis.

Acknowledgements

We are grateful to John Webster, Cationa Quirk, Katrina Gordon and Margaret Opsahl for help and advice; Tom Johnson (PPL Therapeutics for assistance with the ELISA). We thank Ben Yen for pGEM7.PRE, David Andrews for p334, Eliza Izaurralde for the CTE sequence and Helen Wallace for pTGTK1. This work was funded by the BBSRC and from the British Council (British Hungarian Exchange Programme GB-10/98).

References

Archibald AL, McClenaghan M, Hornsey V, Simons JP and Clark AJ (1990) High-level expression of biologically active human α1-antitrypsin in the milk of transgenic mice. *Proc Natl Acad Sci USA* **87**: 5178–5182.

Chevalier-Mariette C, Henry I, Montford L, Capgras S, Forlani S, Muschler J et al. (2003) CpG content affects gene silencing in mice: evidence from novel transgenes. *Genome Biol* **4**: R53.

Clark AJ, Cowper A, Wallace R, Wright G and Simons JP (1992) Rescuing transgene expression by co-integration. *BioTechnology* **10**: 1450–1454.

Clark AJ, Harold G and Yull FE (1997) Mammalian cDNA and prokaryotic reporter sequences silence adjacent transgenes in transgenic mice. *Nucl Acids Res* **25**: 1009–10014.

Cohen-Tannoudji M, Babinet C and Morello D (2000) *LacZ* and ubiquitously expressed genes: should divorce be pronounced? *Transgenic Res* **9**: 233–235.

Donello JE, Beeche AA, Smith GJ, Lucero GR and Hope TJ (1996) The hepatitis B virus posttranscriptional regulatory element is composed of two subelements. *J Virol* **70**: 4345–4351.

Donofrio G, Bignetti E, Clark AJ and Whitelaw CBA (1998) Comparable processing of β-lactoglobulin pre-mRNA in cell culture and transgenic mouse models. *Mol Gen Genet* **252**: 465–469.

Farini E and Whitelaw CBA (1995) Ectopic expression of β-lactoglobulin transgenes. *Mol Gen Genet* **246**: 734–738.

Giraldo P and Montoliu L (2001) Size matters: use of YACs, BACs and PACs in transgenic animals. *Transgenic Res* **10**: 83–103.

Green MR (1991) Biochemical mechanisms of constitutive and regulated pre-mRNA splicing. *Ann Rev Cell Biol* **7**: 559–599.

Harris ME and Hope TJ (2000) RNA export: insights from viral models. *Essays Biochem* **36**: 115–127.

Houdebine LM (2000) Transgenic animal bioreactors. *Transgenic Res* **9**: 305–320.

Huang ZM and Yen TS (1995) Role of the hepatitis B virus posttranscriptional regulatory element in export of intronless transcripts. *Mol Cell Biol* **15**: 3864–3869.

James RM, Neil C, Webster J, Roos S, Clark AJ and Whitelaw CBA (2000) Multiple copies of β-lactoglobulin promoter do not function as LCR. **272**: 284–289.

Lathe R, Vilotte JL and Clark AJ (1987) Plasmid and bacteriophage vectors for excision of intact inserts. *Gene* **67**: 193–201.

Liu X and Mertz JE (1995) HnRNP L binds a cis-acting RNA sequence element that enable intron-independent gene expression. *Genes Dev* **9**: 1766–1780.

Loeb JE, Cordier WS, Harris ME, Weitzman MD and Hope HJ (1995) Enhanced expression of transgenes from adeno-associated virus vectors with the woodchuck hepatitis virus posttranscriptional regulatory element: implications for gene therapy. *Hum Gene Ther* **10**: 2295–2305.

Montoliu L, Chavez S and Vidal M (2000) Variation associated with *LacZ* in transgenic animals: a warning note. *Transgenic Res* **9**: 237–239.

Opsahl ML, McClenaghan M, Springbett A, Reid S, Lathe R, Colman A et al. (2002) Multiple effects of genetic background on variegated transgene expression in mice. *Genetics* **160**: 1107–1112.

Pantano T, Rival-Gervier S, Prince S, Bourhis CM, Maeder C, Viglietta C et al. (2003) *In vitro* and *in vivo* effects of a multimerized alpha s1-casein enhancer on whey acidic protein gene promoter activity. *Mol Reprod Dev* **65**: 262–268.

Petitclerc D, Attal J, Theron MC, Bearzotti M, Bolifraud P, Kann G et al. (1995) The effect of various introns and transcription terminators on the efficiency of expression vectors in various cultured cell lines and in the mammary gland of transgenic mice. *J Biotechnol* **40**: 169–178.

Saavedra C, Felber B and Izaurralde E (1997) The simian retrovirus-1 constitutive transport element, unlike the HIV-1 RRE, uses factors required for cellular mRNA export. *Curr Biol* **7**: 619–628.

Schambach A, Wodrich H, Hildinger M, Bohne J, Krausslich HG and Baum C (2000) Context dependence of different modules for posttranscriptional enhancement of gene expression from retroviral vectors. *Mol Ther* **2**: 435–445.

Sola I, Castilla J, Pintado B, Sanchez-Morgado JM, Whitelaw CBA, Clark AJ et al. (1998) Transgenic mice secreting coronavirus neutralising antibodies in milk. *J Virol* **72**: 3762–3772.

Tiscornia G, Singer O, Ikawa M and Verma IM (2003) A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc Natl Acad Sci USA* **100**: 1844–1848.

Vassilakos A, Hughes MJ and Andrews DW (1995) The 3′ untranslated region of bovine preprolactin contains a transferable non-poly(A) mRNA sequence that prolongs translation. *FEBS Lett* **359**: 206–210.

Wallace H, Ledent C, Vassart G, Bishop JO and al-Shawi R (1991) Specific ablation of thyroid follicle cells in adult transgenic mice. *Endocrinology* **129**: 3217–3225.

Whitelaw CBA, Archibald AL, Harris S, McClenaghan M, Simons JP and Clark AJ (1991) Targeting expression to the mammary gland: intronic sequences can enhance the efficiency of gene expression in transgenic animals. *Transgenic Res* **1**: 3–13.

Whitelaw CBA, Harris S, McClenaghan M, Simons JP and Clark AJ (1992) Position-independent expression of the ovine beta-lactoglobulin gene in transgenic mice. *Biochem J* **286**: 31–39.

Wright G, Carver A, Cottom D, Reeves D, Scott A, Simons P et al. (1991) High level expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. *BioTechnology* **9**: 830–834.

Yull F, Harold G, Wallace R, Cowper A, Percy J, Cottingham I et al. (1995) Fixing human factor IX (fIX): correction of a cryptic RNA splice enables the production of biologically active fIX in the mammary gland of transgenic mice. *Proc Natl Acad Sci USA* **92**: 10899–10903.