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Unexpected high testis-specific transcriptional activity of the cyclin T1 promoter in transgenic mice

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Abstract The ubiquitously expressed cyclin T1 gene encodes for a protein involved in human immunodeficiency virus type 1 (HIV-1) transcription activation. The goat gene was recently shown to share an expression pattern similar to that of its endogenous counterpart when incorporated into mice using a BAC insert. To assess if its promoter could target ubiquitous expression of the bovine *Prnp* in transgenic mice, two constructs carrying either 1 or 30 kb of cyclin T1 5′-flanking sequences were built and microinjected. Both constructs resulted in the unexpected high male germ cell-specific expression of the prion protein. These data re-question the suspected location of the cyclin T1 gene regulatory elements.
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Key words: Cyclin T1 promoter; Transgenic mouse; Testis; Transcription

1. Introduction

The cyclin T1 (CycT1) protein was identified as being a cofactor for the human immunodeficiency virus type 1 (HIV-1) Tat protein, in association with the cyclin-dependent kinase-9 (CDK-9) [1,2]. This complex is involved in the RNA polymerase II transcription elongation. Indeed, binding of Tat to CycT1 induces cooperative binding of the positive transcription elongation factor complex, P-TEFb, onto nascent HIV-1 transcription response element (TAR) RNA [3]. The interaction of the Tat protein strongly increases the affinity and specificity of the Tat protein for TAR, making a stable RNA stem-loop structure at the 5′-end of nascent viral transcripts [1,2,4]. Inhibition of the P-TEFb complex can involve the binding of the 7SK small nuclear RNA [5,6] while CycT1 itself was shown to be implicated in the ubiquitination and degradation of CDK-9 [7].

The human, murine and goat CycT1 genes are known to have a ubiquitous expression [8–12]. Functional analysis of the human promoter in cell cultures suggested that its activity resides in the 545 nucleotides upstream to the translation initiation site [8,12] while expression in transgenic mice of the goat gene, alongside 30 kb and about 90 kb of 5′- and 3′-flanking sequences (BAC 41), respectively, was shown to be ubiquitous [13].

To further assess the potentiality of this promoter in vivo, a short (1 kb) and a long (30 kb) promoter of the goat CycT1 gene was used to express a bovine *Prnp* cDNA in *Prnp* knock-out mice. Because of the ubiquitous reported transcriptional activity of this gene, creation of mice expressing the bovine prion protein in most if not all of their cells was expected. Such animals could in return be useful models to study bovine spongiform encephalopathy (BSE), as it is now well documented that PrP is a major determinant of the species barrier in transmissible spongiform encephalopathy (TSE) transmission [14–18].

An unexpected high testis expression of the bovine *Prnp* was observed in transgenic mice carrying either transgenes. Occurrence of the prion protein was only observed in the germ cells. These results suggest that some *cis*-regulatory elements involved in the transcriptional regulation of the CycT1 gene are located within the transcription unit of this gene and/or its 3′-flanking region.

2. Materials and methods

2.1. Construction of the transgenes

Two overlapping fragments were polymerase chain reaction (PCR) amplified: one containing the entire bovine six octarepeats *Prnp* open reading frame (ORF) (using oligonucleotides 5′-GACGCCGAGA-AGTGCTTGCAGCCGCCGCCGCTGCCTTCTGGTTGAAGCAC-TATGGTGAAAAGCCACATAGG-3′ and 5′-GCTTGGTCGAC-CTGATACTTCCCG-3′ and the corresponding cloned cDNA as template) and the second corresponding to about 1 kb of the goat CycT1 promoter region and the 5′-untranslated region (UTR) of the cDNA (using oligonucleotides 5′-TGCATTGTCGACGGAAATGG-CAACCC-3′ and 5′-ATGTGGCTTTTCACCATAGTGCTTCAAC-CAGAAGGC-3′ and the cloned CycT1 gene as template). These two fragments were joined together in a third PCR made with the purified amplified products and the oligonucleotides 5′-GCTTGGT-CGACCTGATACTTCCCG-3′ and 5′-TGCATTGTCGACGGAA-ATGGCAACCC-3′. The amplified DNA, named CRG11, was digested with *Sal*I, cloned into the corresponding site of pUC19 and sequenced. The CRG11 insert was purified out of the plasmid following a *Sac*I restriction digest.

The 3′-end (exons V–VII) of the sheep β-lactoglobulin gene and its 3′-flanking region were released from the pBJ39 recombinant plasmid (quoted in [23], a kind gift from Dr C.B.A. Whitelaw, Roslin Institute, Scotland) as an *Eco*RV/*Xba*I fragment and cloned into pUC19 *Sma*I/*Xba*I. This plasmid was then digested by *Sac*I and the CRG11 insert cloned into it. Its orientation was assessed by an *Eco*RI digestion, leading to the CRβLg2 plasmid. Prior to microinjection, the A construct was released by a *Sal*I digestion of this plasmid and gel purified.

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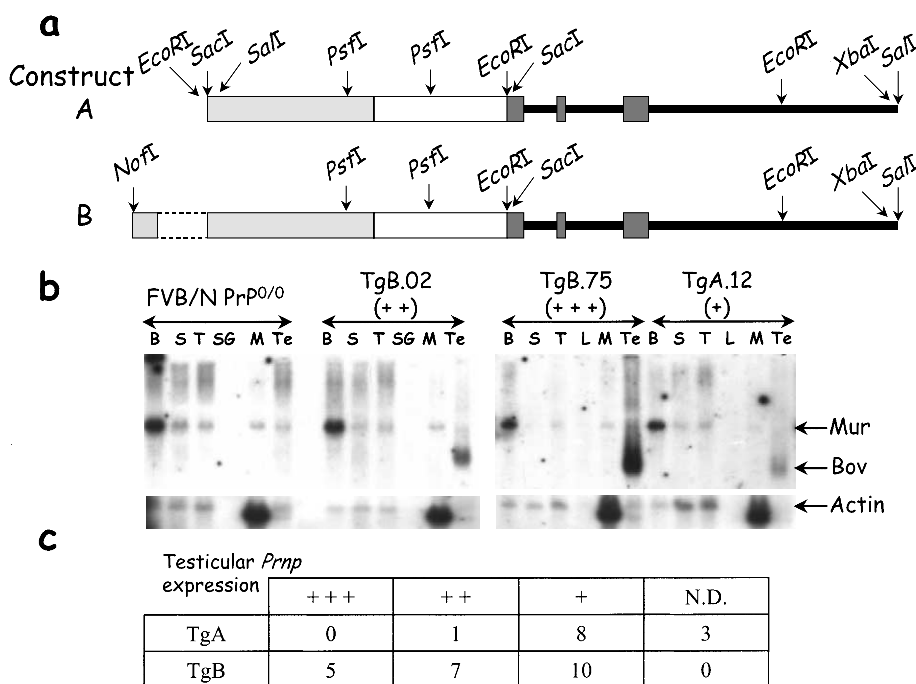

Testicular *Prnp* expression	+ + +	+ +	+	N.D.
TgA	0	1	8	3
TgB	5	7	10	0

Fig. 1. Schematic representation of construct A and B and expression analysis. a: Schematic representation of A and B constructs. Light grey box: goat CycT1 5′-flanking region and 5′-UTR. These regions cover about 1 kb in A and 30 kb in B. White box: bovine *Prnp* ORF. Dark grey boxes: sheep β-lactoglobulin exons V–VII and 3′-flanking region (1.7 kb). Known locations of restriction sites are indicated. This figure is not at scale. b: Northern analysis performed using 20 μg of total RNA and hybridised with an ovine *Prnp* cDNA probe. Mur, murine *Prnp* signal. This expected signal results from the strategy used to knockout the *Prnp* locus [25]. Bov, bovine *Prnp* signal; B. brain; S, spleen; T, thymus; SG, salivary gland; L, liver; M, skeletal muscle; Te, testis. Origins of the sample are indicated on the top. Crosses refer to the Tg classification shown below. The blot was stripped and rehybridised with a mouse actin cDNA probe to assess the equal loading of the RNA samples derived from the same tissue. c: Classification of TgA and TgB lines according to the transgene testicular expression level. N.D., not detected.

The *Sal*I-A insert was subcloned into the pSV1RecA plasmid and inserted by homologous recombination into the BAC 41 [13], following previously described procedures [24]. Recombinant BAC clones were analysed by Southern blotting analyses of their DNA and the B construct gel purified out of them following a *Not*I/*Sal*I double digest prior to its microinjection.

2.2. Generation of transgenic mice

Transgenic founders were obtained by pronuclear microinjection of the constructs into FVB/N $Prnp^{0/0}$ (knockout) mouse eggs (a kind gift from Dr S. Prusiner, University of California, San Francisco, CA, USA), using standard procedures. Transgenic mice were identified by PCR using oligonucleotides 5′-GCTCACCTAGACGTGG-CACTG-3′, located within sheep β-lactoglobulin gene exon VI (Fig. 1) and 5′-CTTTCCTCATTTTTCTCATAGTAG-3′, located within the bovine ORF cDNA (Fig. 1), with 40 cycles: 30 s at 94°C, 30 s at 60°C, 30 s at 72°C. Southern blot analysis on *Pst*I-digested genomic DNA of G1 mice was performed to assess the number of copies of the transgene integrated in the different lines using a bovine *Prnp* cDNA as probe.

2.3. Transgene expression analysis

Northern and Western blot analyses were performed as previously described [19] using anti-PrP antibodies 18 (a kind gift from Dr S. Hawke, Imperial College, London, UK). Immunohistochemical analysis of cellular PrP distribution in testis was performed using anti-PrP antibodies 12F10 [20], as described [21]. For reverse transcription (RT)-PCR experiments, the RT step was performed on 5 μg of DNase I-treated total RNA using oligonucleotide 5′-GAAGGAGAAGCT-GCCCAGAGC-3′, located within exon VII of the sheep β-lactoglobulin gene (Fig. 1), while the PCR step was as described for the identification of the transgenic mice (data not shown). Since one of the oligonucleotides is located within the bovine ORF cDNA while the second is complementary to part of the sheep β-lactoglobulin gene exon VI (Fig. 1), the size of the amplified fragment will discriminate between DNA contamination and RT mRNA products.

3. Results and discussion

3.1. Testis prevalence transcriptional activity of a short goat CycT1 promoter in transgenic mice

The bovine *Prnp* ORF was inserted 5′ of an ovine β-lactoglobulin polyadenylation signal and 3′ of a 1 kb goat CycT1 promoter (construct A in Fig. 1a). In vitro and in silico analyses suggested that the regulatory elements controlling the CycT1 transcriptional activity are located within the proximal CycT1 5′-flanking region [8,10,12]. Thus the promoter used in this transgene was supposed to contain this critical region. The construct was injected into eggs derived from mouse *Prnp* knockout and 15 transgenic founders (named TgA) were identified out of the 86 analysed pups. One female founder was found to be sterile. This percentage of sterility is in the range of what was previously obtained in our hands with various other transgenes and no correlation could be observed with the expression levels of the constructs in this mouse (data not shown). Two lines did not transmit the transgene to their progeny, reflecting their potential mosaic status [22].

Transgene expression was first assessed by Northern analyses performed on total RNA samples from various tissues (brain, spleen, liver, skeletal muscle, ovary, kidney, salivary gland, heart, intestine, lung, epididymis and testis) of 5 weeks old G1 transgenic mice. A testicular transgene expression was observed in the nine lines while no detectable hybridisation signal could be revealed in any of the other samples tested, despite the loading of 20 μg of total RNA (Fig. 1b and data

not shown). The number of expressing lines suggest that this pattern of expression is due to the transgene transcriptional activity and not to an integration site effect. No signal was visible in all analysed tissues from three other lines (data not shown). It probably results from the integration of the transgene in silent chromatin domains in these three lines.

The CycT1 gene is known to be ubiquitously expressed in human, mouse and goat [8–11] while the goat gene was shown to behave similarly when introduced into mice [13]. We next assessed whether the observed testicular expression of the transgene reflected a tissue-restricted transcription of this gene or its specific overexpression in this tissue. To this aim, RT-PCR experiments were performed on the RNA samples already analysed by Northern analysis. Weak ubiquitous expression of the transgene was detected in one transgenic line that has a moderate level of *Prnp* mRNA in its testis, while it was not observed in the remaining eight lines that have lower PrP expression levels in their testis.

This relatively low level of expression of the transgene in tissues other than the testis could result from the location of enhancer elements further upstream from the promoter sequence used in the A construct. To test this hypothesis, a new construct with increased CycT1 5′-flanking region was tested.

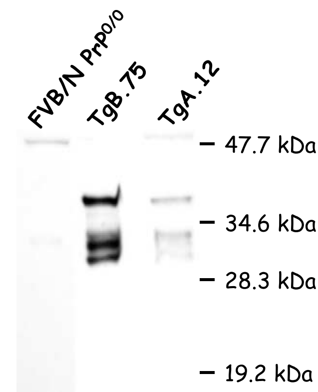

Fig. 2. Western blotting analysis of PrP expression. 50 μg of total testis protein extract were loaded per sample. Coomassie blue staining of the gel was also performed to confirm equal loading between samples (data not shown). Origins of the sample are indicated on the top line. Distances of migration of molecular mass markers are indicated on the right margin. Western blot analysis was performed as previously described [22] using anti-PrP antibodies 18 (a kind gift from Dr S. Hawke, Imperial College, London, UK).

Fig. 3. Immunohistochemical analysis of PrP distribution in testis. Tissue sections were counterstained with hematoxylin. Specific PrP germ cell labelling is observed in TgA and TgB mice. The labelling observed in Leydig cells corresponds to background as observed in A. Immunohistochemical analysis of cellular PrP distribution in testis was performed using anti-PrP antibodies 12F10 [20], as described [21]. A: FVB/N *Prnp* knockout mouse. B, C: TgA.12 mouse. D–F: TgB.75 mouse. Magnitudes: D: 200×. A, B, E: 400×. C, F: 1000×.

3.2. Increasing the length of the 5′-flanking region does not influence its expression pattern

The B construct is identical to the A transgene but encompasses 30 kb of the 5′ CycT1 flanking region (Fig. 1a). 24 transgenic founders (named TgB) were identified out of the 112 analysed pups. Two male founders were found to be sterile and two did not transmit the transgene to their progeny.

A testicular transgene expression was consistently observed by Northern analysis in the 22 lines analysed while no detectable hybridisation signal could be revealed in any of the other tissues tested (Fig. 1 and data not shown). Comparative analysis of the hybridisation signals observed in the testis revealed various levels of expression between transgenic lines, with all highest expressing lines belonging to the TgB group (see Fig. 1c). No correlation was detected between the number of copies of the B transgene that were integrated and the bovine *Prnp* testicular expression levels, suggesting an influence of the site of integration on the transcription rate despite 100% of expressing lines (data not shown).

As in TgA lines, RT-PCR experiments were performed on the RNA samples already analysed by Northern analysis. Weak ubiquitous expression of gene was detected in TgB expressing high to moderate levels of *Prnp* in their testis, labelled +++ and ++ in Fig. 1c, and no expression was observed but in the testis in transgenic mice labelled +.

Overall, this pattern of expression is identical to that observed in TgA mice and is in sharp contrast with the one observed in transgenic mice bearing the original BAC 41 [13]. It is unlikely that this profile results from sequences located within the *Prnp* cDNA and/or the 3′-region of the sheep β-lactoglobulin since (i) non-testicular tissue-specific expression of *Prnp* has been repeatedly achieved in mice and (ii) the β-lactoglobulin is a mammary-specific gene. It rather strongly suggests that regulatory elements are present within the transcription unit of the CycT1 gene or its 3′-flanking region that enhance its expression in non-testis tissues, or inversely repress its testicular expression. The similarity of the expression pattern observed in TgA and TgB mice argues for the occurrence of some critical regulatory elements within the proximal CycT1 5′-flanking region, as already suggested by sequence comparisons and transfection experiments [8,10,12]. However, enhancers and/or protective sequences against position effect might reside in regions more 5′-distal to the start of the transcription unit, explaining the overall higher level of expression observed in TgB mice.

3.3. The testicular bovine PrP protein expression is restricted to the germ cells

Western blot analysis performed on total testicular protein extracts revealed the occurrence of three PrP bands in both TgA- and TgB-expressing lines, corresponding to different glycosylation patterns (Fig. 2 and data not shown). A more precise biochemical characterisation of this protein will be reported elsewhere. To further assess cellular distribution of the transgene expression, immunohistochemical analysis was performed. The bovine prion protein was detected on germ cells, including spermatozoa, but not on Sertoli or Leydig cells (Fig. 3).

Overall TgA and TgB mice demonstrate an unexpected CycT1 promoter transcriptional activity in vivo with a marked germ cell prevalence directed by a relatively short 5′-flanking region. These data also suggest that regulatory elements controlling the transcriptional activity of this promoter are located outside the 5′-flanking region.

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