Position-independent and copy-number-related expression of a goat bacterial artificial chromosome α -lactalbumin gene in transgenic mice

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A bacterial artificial chromosome goat insert comprising the α -lactalbumin-encoding transcription unit with approximately 150 and 10 kb of 5'- and 3'-flanking sequences, respectively, was micro-injected into mouse eggs. In six out of seven transgenic lines, the level of mammary tissue- and stage-specific expression was position-independent and copy-number-dependent. The exogenous α -lactalbumin yield, about 0.8 mg/ml of milk per copy, compared favourably with the α -lactalbumin content of mouse and goat milks, about 0.8 and > 1 mg/ml, respectively. This

suggests that the insert contains most if not all of the *cis*-acting elements involved in the full and specific expression of the goat α -lactalbumin gene and opens up opportunities to use this vector to target expression of foreign genes in the lactating mammary gland of transgenic animals. The transgene was silent in the seventh line for an unknown reason.

Key words: insulator, locus-control region, mammary gland, milk.

INTRODUCTION

Micro-injection into eggs of hybrid constructs driven by regulatory elements from milk-protein-encoding genes has been a successful method for producing pharmaceutical or nutraceutical proteins in the mammary glands of transgenic animals [1,2]. However, one major drawback is the unpredictable behaviour of most transgenes, in particular the variable expression level related to different integration sites. This so-called 'position effect' [3,4] means that several independent transgenic lines have to be created in order to obtain animals expressing a given transgene in the appropriate manner, which increases the cost of such experiments.

The position-independent expression of a number of genes in transgenic animals has been related to the constitutive occurrence of dominant *cis*-regulatory elements such as insulators and locus-control regions [5,6]. Unfortunately, such dominant regulatory elements are yet to be identified in milk-protein gene loci and the use of heterologous insulators has led to miscellaneous results. An alternative is the use of long genomic fragments, based on yeast artificial chromosome (YAC) or bacterial artificial chromosome (BAC) inserts, which are more likely to contain the whole set of regulatory elements required for the appropriate expression of the transcription unit [7]. Recently, position-independent expression of a 210 kb human α -lactalbumin (α lac) YAC was reported in transgenic rats [8]. However, the four available lines contained only a single copy of the transgene, preventing analysis of copy-number effect. In the present study, we report position-independent and copy-number related expression of a 160 kb goat α lac BAC insert in mice.

EXPERIMENTAL

Isolation of the goat α lac BAC clone

Construction and characterization of the goat BAC library was previously reported [9]. The library was screened by PCR using

primers that amplified the sequence between nt 441 and 786 of the goat α lac gene [10], as described previously [9].

BAC DNA analyses

Mini-preparations of two of the four available α lac clones were used for restriction-mapping analysis, end-sequencing of the insert and fluorescent *in situ* hybridization (FISH) to evaluate possible chimaerism, as described previously [9].

Isolation of the BAC insert for micro-injection

BAC DNA prepared with the Hybaid DNA preparation kit was digested to completion with *NotI*, size-fractionated by field-inverted gel electrophoresis (FIGE) using a 1% low-melting-point agarose gel and the BAC insert isolated as described [11]. The concentration of the purified DNA was estimated on a 1% agarose minigel using λ DNA of known concentration as standard and its integrity was verified by FIGE. DNA was micro-injected into the pronuclei of C57Bl6 X CBA fertilized oocytes. Transgenic mice were identified by Southern blotting. Purified DNA tail was digested to completion with *PstI* and size-fractionated in 1% agarose gel. The blot was probed with a bovine full-length α lac cDNA probe, as previously described [12]. Under the experimental conditions used, this probe does not cross-hybridize with murine DNA ([12] and results not shown). In order to estimate the copy number of integrated transgene in each line, Southern blots were hybridized with a mixture of bovine and murine full-length α lac cDNA probes. Signal quantification was performed by scanning autoradiographs using a Pharmacia LKB image-master DTS scanning system, according to the manufacturer's guidelines. The murine α lac signal was used as an internal standard to normalize the DNA loading between samples. The ratio of the signals derived from the murine and bovine probes was determined to estimate the copy number of the transgene.

Abbreviations used: α lac, α -lactalbumin; BAC, bacterial artificial chromosome; FIGE, field-inverted gel electrophoresis; FISH, fluorescent *in situ* hybridization; YAC, yeast artificial chromosome.

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Expression analysis of the goat α lac transgene

Northern-blot analyses were performed as described previously [12], using 20 μ g of total RNA per sample size-fractionated in formaldehyde/agarose gels. Western blots were performed on 14% SDS/PAGE gels transferred on to a nitrocellulose membrane, using a rabbit anti-bovine α lac antiserum as described previously [12]. Experimental conditions were such that the endogenous murine α lac was not detected. Furthermore, this protein was previously shown to have a different apparent molecular mass by SDS/PAGE to its goat counterpart when expressed in transgenic mice [13]. The goat α lac content of the milk was estimated using purified bovine α lac samples of known concentrations and by scanning the blots.

RESULTS AND DISCUSSION

BAC characterization and generation of transgenic mice

Four positive clones were found by PCR screening with α lac-specific primers of a goat BAC library. The primers were designed to amplify 345 bp of the proximal 5'-flanking region of the gene in order to minimize the potential amplification of α lac-related sequences that are present in ruminants [10,14,15]. DNA from two α lac clones was prepared for further characterization. Insert sizes were estimated to be 160 and 170 kb, respectively, by FIGE. FISH analysis showed that both mapped to chromosome 5q21 (results not shown), as expected [16], and that neither were

Figure 1 Southern-blot analysis of mouse tail DNA

Southern-blotting experiments were performed using *Pst*I-digested purified mouse tail DNA as indicated. The membrane was hybridized with a mixture of bovine and murine full-length α lac cDNA probes. The nature and size of the detected and previously characterized hybridizing fragments are indicated on the right [13,23].

Figure 2 Northern-blot analysis of goat α lac expression

Northern blots were performed using 20 μ g of total RNA from 7-day-old lactating mice as indicated and hybridized with a bovine α lac cDNA probe. Positions of the 28 S and α lac RNAs are indicated on the right. Non-Tg, non-transgenic.

chimaeric. Partial restriction mapping and Southern analyses revealed that both contained the α lac transcription unit but that only the 170 kb insert contained α lac-related sequences, located 3' of the gene (results not shown). Further characterization of the BAC clone with the 160 kb insert revealed that it contained the α lac-encoding gene-transcription unit (2.2 kb) with around 150 and 10 kb of 5'- and 3'-flanking sequences, respectively. This

Table 1 Quantification of the Southern and Western analyses

Values are means $\pm$ S.E.M. of independent experiments performed on two animals from each line.

Line	Southern-blot analyses		Western-blot analyses
	Goat α lac/murine α lac hybridization signal ratio	Estimated transgene copy number	Estimated goat α lac milk content (mg/ml)
04	0.8 $\pm$ 0.02	2	1.45 $\pm$ 0.05
07	1.2 $\pm$ 0.1	3	3.07 $\pm$ 0.5
15	0.77 $\pm$ 0.07	2	1.4 $\pm$ 0.2
17	0.4 $\pm$ 0.01	1	Not detected
23	0.45 $\pm$ 0.05	1	0.72 $\pm$ 0.1
31	0.4 $\pm$ 0.01	1	0.8 $\pm$ 0.1
35	1.3 $\pm$ 0.05	3	2.8 $\pm$ 0.1

Figure 3 Developmental regulation of the goat α lac transgene in the mammary gland

Northern blotting was performed using 20 μ g of total mammary RNA from lines 07 and 35. Origins of the samples are indicated at the top. The membrane was hybridized successively with a bovine α lac cDNA probe, stripped, rehybridized with a murine α lac cDNA probe, stripped and finally hybridized with a 28 S RNA probe, as indicated on the right. Following each probe stripping, the membrane was exposed to ensure complete removal of the probe (results not shown). Note, on the 7th day of lactation, potential murine α lac pre-mRNAs were detected.

insert was purified out of the BAC vector and micro-injected into mouse eggs to produce transgenics.

Seven transgenic animals were identified by Southern analysis of 38 pups. All founders transmitted the transgene to their progeny and animals from the first generation were studied for transgene expression. The numbers of integrated copies were estimated by Southern analysis of *Pst*I-digested genomic DNA of two mice per line probed with both bovine and murine α lac cDNAs, using the latter as an internal standard (Figure 1 and Table 1). Assuming the occurrence of one copy in lines 17, 23 and 31, lines 04 and 15 carried two copies and lines 07 and 35 carried three copies.

Expression analysis of goat α lac transgene

The presence of abundant goat α lac mRNA was shown by Northern-blot analysis of total RNA from lactating mammary glands in all lines except line 17, for which no α lac mRNA was detected (Figure 2). The transgene was specifically expressed in the mammary gland: no ectopic expression was detected in liver, kidney, thymus, salivary gland or brain, or in the testes of males of the seven lines. Developmental regulation of the transgene analysed in lines 07 and 35 was similar to that of the endogenous murine α lac gene (Figure 3). No obvious feature was responsible for the lack of detectable expression in line 17. Sequencing of the edges of the BAC insert allowed the design of specific sets of

Figure 4 Western-blot analysis of milk

Origins and amounts of α lac and milk samples are indicated at the top. Under the conditions used, the polyclonal rabbit anti-bovine α lac antibody did not detect murine α lac, which has a different apparent molecular mass by SDS/PAGE compared with its goat counterpart [13].

Figure 5 Copy-number related expression of goat α lac

For each line, goat α lac content was estimated by scanning several Western blots and plotted against the estimated number of integrated transgene copies for the six expressing lines, assuming one integrated copy in lines 23 and 31. The broken line indicates the line of best fit.

primers to analyse the presence of these ends in the genome of the transgenic mice, revealing a deletion of the 5' end of the transgene in mice from line 31 (results not shown). Southern-blot analyses of *Pst*I- or *Eco*RI-digested genomic DNA showed no rearrangement of the goat α lac transcription unit or the proximal flanking sequences in line 17 (Figure 1 and results not shown). We cannot exclude mutations within this transgene fragment, or its integration into an unfavourable chromosomal region, resulting in the silencing of expression in line 17.

Confirming the RNA analysis, Western-blot analysis of milk from at least two 7-day-old lactating mice per line showed the occurrence of goat α lac in all but line 17 (Figure 4, Table 1 and results not shown). The α lac content was copy-number dependent, with a linear relationship ($R^2 = 0.9$; Figure 5). The estimated goat α lac yield per transgene copy, in the region of 0.8 mg/ml, compared favourably with α lac content in mouse and goat milk, about 0.8 and > 1 mg/ml, respectively. According to previous experiments with bovine and goat α lac transgenes

[12,13], the 0.4 kb sequence upstream from the bovine transcription unit can confer mammary-tissue specificity. Nevertheless, the level of expression of a goat transgene with as much as 8.5 and 9.5 kb of 5'- and 3'-flanking regions, respectively, differed widely between transgenic lines. The present BAC insert appeared to contain most, if not all, essential regulatory sequences needed for the full expression of the goat α lac gene. Furthermore, elements that enable copy-dependent expression appear to reside distal to the transcription unit, most probably in the 5'-flanking region.

Potential use of the BAC insert as a targeting vector

So far, only a few milk-protein genes have been expressed in a position-independent fashion in transgenic mice: sheep β -lactoglobulin [17], rat WAP (whey acidic protein) [18] and human α lac YAC [8]. In the first two examples, position-independent expression was restricted to transgenes with a given size. Transcription units with longer flanking regions were not as efficiently expressed, perhaps indicating that an optimal spatial organization of the transgene rather than the occurrence of dominant *cis*-regulatory elements is important [19,20]. The 210 kb human α lac YAC seems to contain such elements, as the level of mammary-specific expression was similar in the four available lines carrying one copy of the transgene [8]. Our present study with the 160 kb goat α lac BAC transgene, which behaved similarly to its endogenous counterpart during mammary-gland development and functioning, has confirmed the position-independent expression of large α lac fragments and has shown a linear relationship between the number of integrated copies and the level of expression.

The mammary gland has been the target of biotechnological applications of gene-transfer technology [1,2], although still limited by the variability of transgene-expression profiles [3,4]. Functional analysis of truncated α lac inserts should allow the identification of the presumed dominant *cis*-regulatory elements that might be incorporated into existing mammary expression cassettes to improve their efficiency. Alternatively, a native or shortened α lac BAC insert might itself be used as a vector. Efficient homologous recombination in yeast has already been used to introduce mutations into genes cloned in YAC vectors and further expressed in transgenic mice [21]. More recently, a procedure has been described to perform homologous recombination of BAC inserts in *Escherichia coli* [22]. Thus, the goat

α lac BAC could be used as an expression vector to produce foreign proteins in the mammary glands of transgenic animals by exchanging *in vitro* the transcription units encoding α lac and any protein of interest.

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