Nucleic Acids Research, 1997, Vol. 25, No. 5 **1009–1014**

Mammalian cDNA and prokaryotic reporter sequences silence adjacent transgenes in transgenic mice

A. J. Clark*, G. Harold and F. E. Yull

Roslin Institute, Roslin, Midlothian EH25 9PS, UK

Received November 19, 1996; Revised and Accepted January 12, 1997

ABSTRACT

The ovine β-lactoglobulin gene is expressed efficiently and at high levels in the mammary gland of transgenic mice. In contrast, when this gene is linked to a second gene construct comprising a mammalian cDNA or a *CAT* reporter sequence it fails to be expressed in the majority of transgenic lines generated. We suggest that mammalian cDNAs and prokaryotic reporter sequences can serve as active foci for gene silencing in the mammalian genome.

INTRODUCTION

The use of cDNA constructs in transgenic mice often results in poor expression, even when the same constructs are expressed efficiently in cell culture (1,2). Similar problems are encountered with prokaryotic reporter sequences such as β-galactosidase (*lacZ*) or chloramphenicol acetyltransferase (*CAT*) (3) even if these sequences are linked to regulatory elements such as the globin locus control region (LCR), which can mediate position-independent expression of other genes (4). We have constantly encountered these problems in our attempts to target the expression of foreign genes to the mammary gland of transgenic animals (5,6). In these studies we have used regulatory elements derived from the ovine β-lactoglobulin (*BLG*) gene. The unmodified gene is expressed very efficiently in the mammary gland of transgenic mice (7) and only 400 bp of the proximal promoter are required to mediate position-independent expression (8). In contrast, hybrid gene constructs comprising the *BLG* promoter linked to human α-1-antitrypsin (*α1AT*) or factor IX (*fIX*) cDNAs (5) or *CAT* reporter sequences (6) are expressed very inefficiently and these transgenes are silent in the majority of the lines generated.

To overcome the problem of poor expression of cDNA constructs we developed a strategy involving co-integration with the unmodified *BLG* gene and showed that this approach can be used to rescue the expression of poorly expressed gene constructs. For example, a *BLG–fIX* construct, FIXD, was completely silent in transgenic mice but was expressed in most of the transgenic lines generated when it was co-integrated with *BLG* (9). Transgenic mice expressing relatively high levels of human fIX in the milk have been made using this strategy (10). Notwithstanding the success of the approach, the level of expression of the rescued gene was usually less than the expression of the co-integrated *BLG* gene and the degree of rescue was highly variable when different transgenic lines were compared. Other workers have also noted this when using this approach to enhance transgene expression (11).

Co-injection generally results in the co-integration of the two transgenes, but their mutual arrangement is highly variable when different lines are compared (9). We reasoned that this variability in the structure of the transgene arrays contributes to the unpredictability of the rescue effect. To address this problem we constructed plasmids in which *BLG–fIX* or *BLG–CAT* sequences were linked to the *BLG* gene prior to injection so that their relative arrangement in the transgene arrays would be more consistent. The major effect seen was not so much improvement of expression of the hybrid genes but, surprisingly, complete silencing of the adjacent *BLG* gene in the majority of the transgenic lines generated.

MATERIALS AND METHODS

Constructs

The genomic *BLG* gene (BLGΔDp), *BLG–CAT* construct (ΔDpCAT) and *BLG–fIX* construct (FIXD) have been described previously (5,6,8). For CAFI the BLGΔDp gene was linked at the *Xba*I site in the 3′ flanking region to the 5′-end of the 400 bp *BLG* promoter contained in a modified FIXDΔ3′ construct lacking the 3′ *Bam*HI–*Xba*I flanking region. The *fIX* cDNA sequences have a small deletion in the 3′-UTR that removes a cryptic acceptor splice site, preventing aberrant splicing(10). In BRAT, the 5′-end of the 400 bp *BLG* promoter in ΔDpCAT was linked to the 3′ *Xba*I site and in TARB the two constructs were joined at their respective 3′ *Xba*I sites. All three constructs were generated in pBluescript SK2 (Stratagene) and were excised from the vector sequences by *Sfi*I digestion. All constructs are depicted in Figure 1.

Generation and characterization of transgenic mouse lines

Injection fragments were purified by agarose gel electrophoresis and injected into pronuclear eggs from C57/Bl6 × CBA mice as previously described (7). Tail biopsies were analysed by PCR and Southern blotting using *BLG* primers and probes and transgenic lines were established by breeding from positive founder mice. Southern blotting of *Eco*RI and *Hin*dIII digested tail and liver DNA was used to confirm transgenic status and to ensure the

*To whom correspondence should be addressed. Tel: +44 131 527 4200; Fax: +44 131 440 0434; Email: johnclark@bbsrc.ac.uk

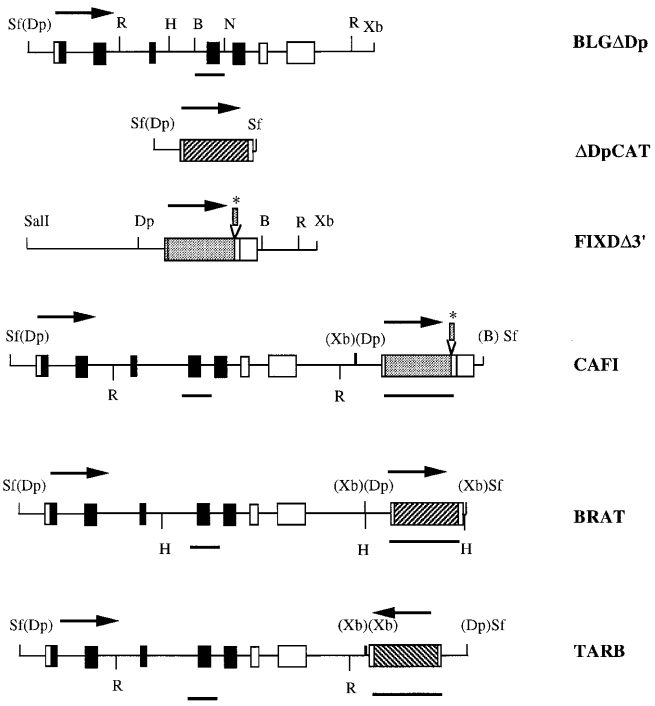

Figure 1. DNA constructs. CAFI comprises the *BLG* gene linked in a tail-to-head fashion to a modified FIXDΔ3′. BRAT comprises *BLG* linked to ΔDpCAT in a tail-to-head fashion, whereas for TARB the two genes are linked tail-to-tail. These three constructs each contain two copies of the 0.4 kb *BLG* promoter. Line, 5′, 3′ and intronic *BLG* sequences; open box, untranslated *BLG* exonic sequences; closed box, translated *BLG* exons; hatched box, *CAT* reporter sequences; stippled box, *fIX* cDNA sequences (*, small deletion in the 3′-UTR of *fIX* removes a cryptic splicing site). The arrows show the direction of transcription of the various genes. Relevant restriction sites: Sf, *Sfi*I; Dp, *Dpn*I; Xb, *Xba*I; Sa, *Sal*I; B, *Bam*HI; N, *Nru*I. *Eco*RI (R) and *Hin*dIII (H) sites were used to map the transgenic loci with the probes indicated.

integrity of the transgenic loci using a *BLG*-specific *Bam*HI–*Nru*I probe spanning exon IV, *fIX* cDNA sequences isolated from the plasmid p5′G 3′cVI (a gift from G.Brownlee) and *CAT* reporter sequences (Fig. 1). Transgene copy numbers were estimated by reference to dilutions of the constructs run in parallel on the same gel.

Analysis of expression

Expression analysis was normally carried out in generation 1 (G_1) or generation 2 (G_2) mice, although in some cases expression was assessed in the founder (G_0) animals. Positive females were selected, mated and killed at day 12 of lactation. Mammary tissue and milk was taken. fIX protein was measured in milk samples by ELISA as previously described (10). CAT assays were carried out on protein extracts prepared by homogenizing and then centrifuging small amounts of frozen mammary tissue. The protein concentration in each sample was measured using the BioRad protein assay kit. CAT activity was assayed in a final volume of 200 µl using 10 µg protein extract in 0.25 M Tris, pH 8.8, with [^{14}C]chloramphenicol. The reaction was started with 5µl 50 mM acetyl-CoA, incubated at 37°C for 3 h and stopped with an equal volume of ethyl acetate. Samples were freeze dried, resuspended in 30 µl ethyl acetate, spotted on to TLC plates and run in chloroform: methanol 95:5. BLG expression was determined by Northern blotting analysis on 1% agarose–MOPS gels. After electrophoresis the gels were blotted onto Hybond N and probed with BLG and GAPDH or a mouse casein probe to control for RNA loading. Hybridization signals were imaged with a PhosphorImager and the levels of BLG mRNA in the expressing lines estimated relative to a standard BLG-containing RNA sample included on the blots using ImageQuant software.

RESULTS

Human *fIX* sequences silence an adjacent *BLG* transgene

We have reported previously that the *BLG–fIX* transgene FIXD, comprising human *fIX* cDNA sequences linked to the *BLG* promoter, was not expressed in transgenic mice (5). The expression of this construct and its derivative FIXDΔ3′ (Fig. 1) were, however, rescued by co-injection with the unmodified *BLG* gene (9,10). Nevertheless, the levels of expression of these *BLG–fIX* transgenes were still unpredictable and, for example, the fIX levels in the milk of different transgenic lines carrying FIXDΔ3′ varied from <100 ng/ml to ~60 µg/ml and expression levels bore no relationship to copy number (10). Co-injection of two genes generates highly variable transgene arrays when different lines are compared and we hypothesized that this contributed to these unpredictable levels of expression. Therefore, we elected to link the *BLG* and *BLG–fIX* constructs prior to injection so as to generate a more consistent arrangement of the two constructs. CAFI comprises the BLGΔDp transgene linked to a modified FIXDΔ3′ transgene (Fig. 1). Eleven transgenic lines were generated which carried this transgene. Restriction blotting analysis with *BLG*- and *fIX*-specific probes indicated that the transgene was not rearranged significantly in any of these lines and they all yielded the expected *Eco*RI fragments. The mice were analysed in the G_0 and G_1 generations for fIX and BLG expression in the mammary gland. Only two of the 11 CAFI lines showed detectable fIX expression either in the milk (Fig. 2) or as mRNA transcripts (not shown). The expression in these lines was low, with the highest line exhibiting only 0.38 µg/ml fIX in the milk. This level of fIX was nearly two orders of magnitude lower than was obtained when FIXDΔ3′ was co-injected with the *BLG* gene and fIX concentrations in the milk >100 µg/ml were achieved (10).

BLG expression was analysed by Northern blotting experiments using total mammary gland RNA. Nine of the 11 transgenic lines failed to express the *BLG* transgene and the two BLG-expressing lines exhibited only low levels of BLG mRNA (Fig. 2). The highest BLG expressor corresponded to the line which expressed the highest level of fIX. Expression of fIX and or BLG did not appear to relate to copy number, the highest expressing line carrying ~10 copies of the transgene with non-expressing lines with both higher and lower copies being observed (Fig. 2).

The failure of BLG to be expressed in the majority of the transgenic lines was rather surprising, since the BLGΔDp transgene itself was expressed very efficiently in transgenic mice (Fig. 2). All 10 lines carrying this construct introduced as a single transgene expressed high levels of BLG mRNA, varying from 0.2 to 1.5 times the steady-state levels estimated for lactating sheep mammary gland RNA. These levels showed a rough correlation with transgene copy number; the highest expressors carrying 20–25 copies of *BLG* and the lowest carrying 2–3 copies (Fig. 2; 8,12). The frequency of BLG expression in CAFI versus BLGΔDp transgenic mice was compared using a χ^2 test and was significantly lower ($P < 0.05$). We conclude that the otherwise

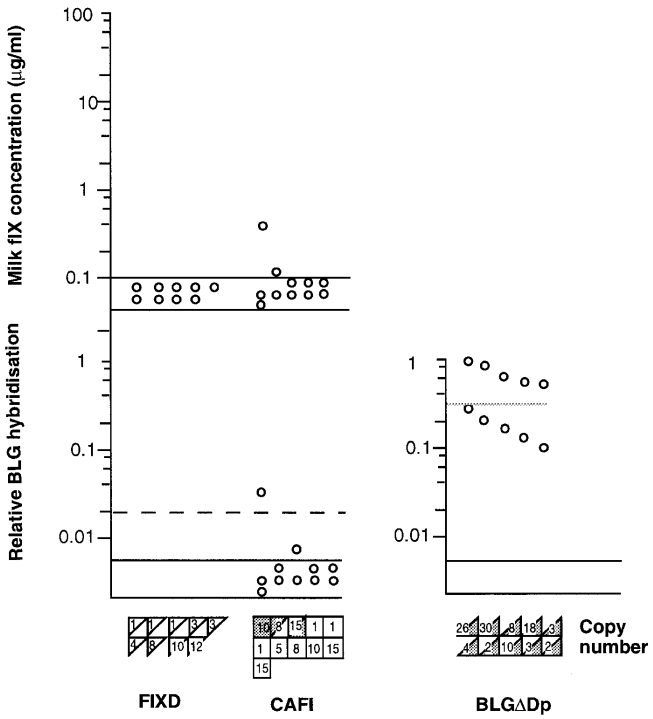

Figure 2. fIX and BLG expression in CAFI mice. Each circle represents the average fIX level or BLG mRNA in mid-lactation transgenic mice. fIX levels were estimated by ELISA and BLG mammary mRNA levels were estimated relative to the hybridization signal obtained from a standard sample of RNA from mouse line BLGΔDp21, the highest expressing BLGΔDp transgenic line (8) included on the blots. The transgene copy numbers estimated for each line are tabulated on the corresponding matrices below the ordinate. For each line a shaded upper quadrant corresponds to detectable fIX expression in the line and the shaded lower quadrant to detectable BLG expression; unshaded quadrants correspond to non-expressing lines. The sensitivity of the fIX ELISA was 0.1 μg/ml and for estimating BLG mRNA levels was 0.02 of the control RNA signal on the Northern blot. For BLG, circles grouped below the upper (dashed) ordinate represent transgenic lines in which a very low unquantifiable BLG signal was seen in one or more individuals of the line. For fIX and BLG, circles grouped below the ordinate represent lines in which no detectable expresssion was observed. For comparison the transgene expression levels previously reported for the transgenic lines carrying FIXD (5) and BLGΔDp (8) are shown. For BLGΔDp the dotted line shows the relative steady-state level of BLG mRNA per *BLG* gene copy estimated for lactating sheep mammary gland RNA (5).

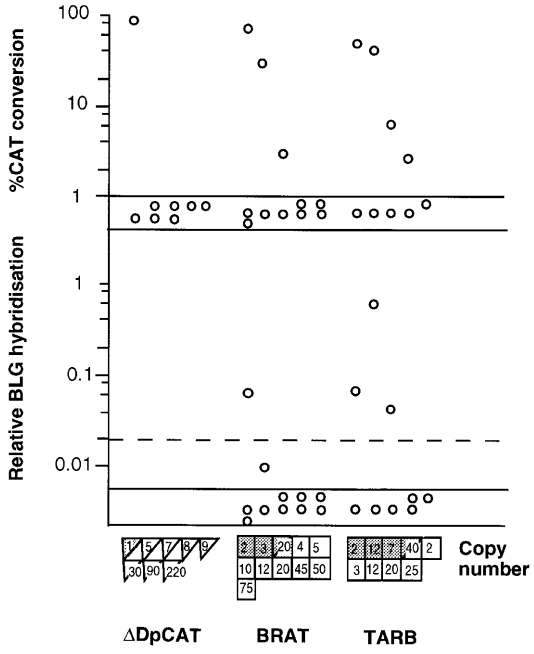

Figure 3. CAT and BLG expression in BRAT and TARB mice. Expression analysis was carried out on mid-lactation transgenic mice. Each circle represents the average CAT conversion level or BLG mRNA level in a transgenic line. Copy numbers are tabulated on the corresponding matrices below the ordinate, which are shaded to distinguish expressing and non-expressing lines. CAT activity was determined as described in Materials and Methods using 10 μg protein extract in each assay; the sensitivity was estimated as 1% CAT conversion (corresponding to the average background). BLG mRNA levels were estimated relative to mouse line BLGΔDp21.

efficiently expressed *BLG* transgene was silenced by linking it to a second transgene containing *fIX* cDNA sequences.

CAT reporter sequences also silence the *BLG* gene

The 0.4 kb *BLG* promoter is sufficient to target expression of a heterologous reporter gene to the mammary gland in transgenic mice. The construct ΔDpCAT (Fig. 1) containing the 0.4 kb *BLG* promoter linked to *CAT*, however, was expressed with low efficiency and only one of eight lines expressed this transgene (Fig. 3; 6). In an attempt to rescue expression of this construct it was linked to BLGΔDp in a head-to-tail fashion to generate a double gene construct named BRAT (Fig. 1). Eleven lines of mice were generated with this construct, characterized by Southern blotting after *Hin*dIII digestion and investigated for CAT and BLG expression. Linking the ΔDpCAT construct to BLGΔDp improved its expression marginally, with three out of 11 lines showing measurable levels of CAT activity (Fig. 3). Northern blotting experiments showed that nine of the 11 BRAT lines analysed failed to express the linked *BLG* gene and, as for the CAFI construct described above, the predominant effect was silencing of the *BLG* gene.

At the outset of these experiments we also wished to determine whether the relative arrangement of the *BLG* and *BLG–CAT* transgenes would influence the efficiency of rescue. To address this question the TARB construct was generated which comprises BLGΔDp linked to *BLG–CAT* in a tail-to-tail fashion, the opposite orientation to BRAT. Nine lines were generated with this construct and characterized by Southern blotting. Four were shown to exhibit CAT expression in the mammary gland (Fig. 3), suggesting a modest improvement in the frequency of CAT expression compared with ΔDpCAT. Analysis of BLG expression showed that six of the nine lines failed to express BLG, in agreement with the results obtained with the BRAT construct, demonstrating that silencing of the BLGΔDp transgene by the adjacent *CAT* construct is independent of the relative orientation of the two transgenes.

No strong effects of transgene copy number were observed on BLG or CAT expression with either construct, although three of the four highest expressing CAT lines were quite low copy number and carried less than five transgene copies (Fig. 3). Interestingly, the only line expressing ΔDpCAT was single copy (Fig. 3; 6). For both BRAT and TARB lines analysis by χ^2 showed that the frequency of BLG expression was significantly lower than for BLGΔDp lines ($P < 0.05$). For both lines there was a strong correlation between BLG and CAT expression. Taken together, all five lines in which BLG was detected also expressed CAT, although in the two lines with the lowest average CAT expression we failed to detect BLG mRNA. This suggests that the

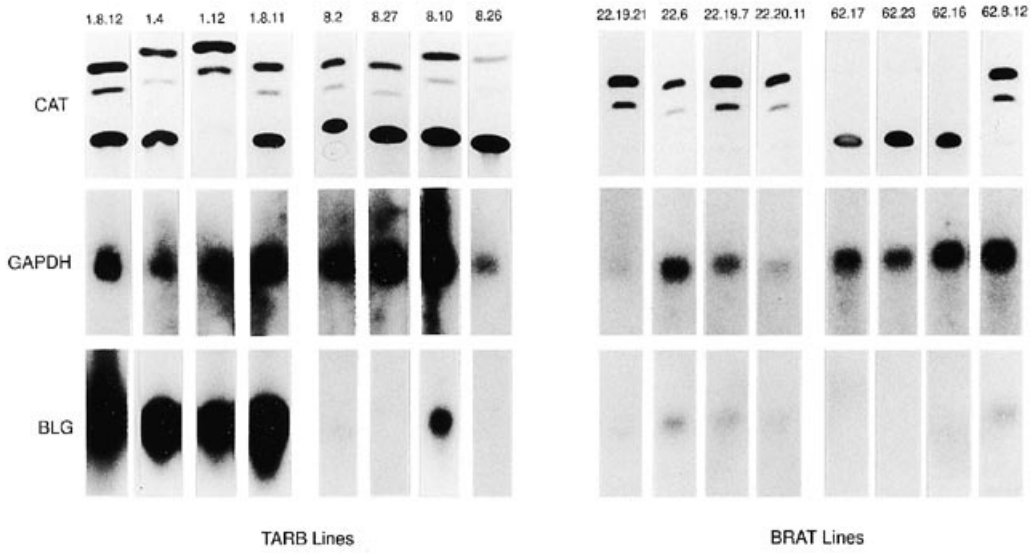

Figure 4. Variable transgene expression within BRAT and TARB transgenic lines. CAT assays and Northern blotting analyses for BLG and GAPDH are shown for mammary gland samples from mouse lines TARB 1, TARB 8, BRAT 22 and BRAT 62. The numbers at the top refer to the individual mice in the lines.

activities of the two genes are interdependent and that the silencing effects observed tend to act on a transgenic locus as a whole.

Variable silencing within transgenic lines

For most of the transgenic lines in this study expression was analysed in two G_1 females and the line was scored negative if both mice failed to express either transgene. For two of the BRAT and two of the TARB lines additional expressing females within the line were also analysed for expression.

In some of these lines we observed differing levels of CAT and/or BLG expression on comparison of individuals within the line. In some cases this variability was extreme. For example, in line BRAT 62 CAT was only seen in one of the four individuals analysed (Fig. 4) and, likewise, BLG was only detectable in this individual. In contrast, mice from line BRAT 22 showed a more stable pattern of CAT expression, with all the individuals exhibiting high levels of CAT expression and low levels of BLG mRNA. All the mice analysed in line TARB 1 showed a significant level of CAT activity and BLG mRNA. In contrast, individuals from line TARB 8 all showed some CAT activity but only one exhibited detectable BLG expression (Fig. 4). Southern blotting analyses indicated that the differences in expression within the variably expressing lines were not due to segregation of two or more transgenic loci or instability of the DNA sequences at the locus (data not shown).

DISCUSSION

Silencing of endogenous genes has been reported for a variety organisms, including mice, *Drosophila* and yeast (13–15). The silenced state is stable and clonally heritable and is thought to be due to the packaging of the affected DNA into stable heterochromatic structures (16). There is increasing evidence that transgenes are subject to similar effects and, for example, in *Drosophila* it has been proposed that transgene arrays act as foci for the formation of heterochromatin leading to variegated patterns of expression (17).

The silencing of BLG transgenes observed in this present study contrasts with our previous reports (9,10). Thus, when *BLG* was co-injected with FIXD or FIXDΔ3′ (Fig. 1) the main effect was activation of expression of the *fIX* construct; silencing of *BLG* was not observed in any of the lines (Table 1). There are two possibilities for these differences. Firstly, co-integration was achieved by co-injection in our previous experiments, rather than by linking the two constructs together prior to injection. The ratio of *BLG* to the co-injected second *BLG–fIX* transgene was 3:1 and this was broadly reflected in the average composition of the arrays. These were multicopy and complex comprising *BLG* in tandem, FIXD in tandem as well as the two transgenes adjacent to one another (9). We suggest that *BLG* transgenes in a region of the array containing few copies of FIXD escape silencing and are expressed. Presumably, these actively expressed *BLG* transgenes suppress the silencing of one or more of the FIXD transgenes and this accounts for the rescue effect. There appears to be a balance between gene activation and gene silencing and in the transgene arrays resulting from co-injection of *BLG* and FIXD the former prevails. The efficient silencing of *BLG* observed in the present experiments is presumably due to the fact that the *BLG* gene is invariably adjacent to the *fIX* cDNA or *CAT* sequences because they are physically linked. Although this changes the spacing between ΔDpBLG transgenes in the arrays, this is almost certainly not the cause of silencing, because transgenes comprising ΔDpBLG plus various lengths of 5′ or 3′ *BLG* flanking sequences are expressed at high levels in all transgenic lines (8). In the present experiments the *BLG* gene was not silenced in seven of the 31 lines analysed and this was generally coincident with expression of the second transgene. We do not know whether this reflects some positive influences at the site of integration or the structure of the arrays in these lines. We have not attempted to elucidate the precise structure of the arrays, since previous experience has taught us that when there are multiple copies at a locus it can be very difficult, if not impossible, to deduce the map (9).

The failure of *fIX* cDNA sequences to silence *BLG* in co-injection experiments (9) could also reflect the fact that greater lengths of 5′ and 3′ flanking *BLG* sequence were incorporated in

the co-injected constructs than were included in the CAFI, BRAT or TARB constructs. Therefore, the *CAT* and *fIX* cDNA sequences will be positioned closer to the promoter of the *BLG* gene in these tandem arrays than was the case for the co-injected transgenes. It is, therefore, interesting to note that we have observed silencing of *BLG* when *BLG* and a *BLG–α1AT* construct (AATD) comprising 4.2 kb of 5′ and 2.2 kb of 3′ *BLG* flanking sequences were co-injected. In these experiments *BLG* and AATD (analogous to FIXD, but comprising *α1AT* cDNA sequences) were co-injected in a 1:1 ratio. Expression of the AATD construct was rescued (7/9 lines expressed, Table 1) but the two lines failing to express α1AT did not express BLG either (9). Thus, even though the frequency of gene silencing observed was lower than in the present experiments, it suggests that these silencing effects can be transmitted over longer stretches of 5′ and 3′ *BLG* sequence and that they may occur in arrays generated by co-injection.

Analysis of additional animals in some of the expressing BRAT and TARB lines showed variable penetrance of *BLG* silencing. It is possible, therefore, that some of the negative lines in this study would eventually yield an expressing mouse if more individuals had been analysed. The key point, however, is the comparison with expression of the BLGΔDp transgene alone, which was expressed at high levels in every mouse in all 10 of the lines analysed (8). Nevertheless, unmodified *BLG* transgenes do appear to be subject to a less extreme form of gene silencing. This manifests itself as variable levels of BLG expression within some but not all lines, corresponding to a heterocellular pattern of BLG expression in the mammary gland (18). However, even the lowest expressors exhibited high levels of BLG compared with most of the expressing lines described in this present study. The variable silencing seen with some of the BRAT and TARB lines may be a more extreme version of that observed for *BLG* transgenes alone and the complete silencing observed for the majority of lines the end point.

Repressive effects are thought to be mediated by chromatin at the transgene insertion site and different insertion sites vary in their ability to repress expression. The same transgene may be expressed at one integration site but not at another, giving rise to so-called ‘position effects’. Predictable expression of reporter sequences such as *CAT* or cDNA constructs in transgenic mice has always been elusive (3) and these types of construct seem particularly susceptible to these effects. In the light of our data this may be something of an understatement, since the cDNA and *CAT* reporter constructs appeared to silence the expression of an adjacent *BLG* transgene in a high proportion of the transgenic lines generated. Perhaps the problem encountered with expressing prokaryotic reporters or cDNAs is not that they lack the appropriate regulatory elements or that they are prone to repressive position effects but, rather, that they themselves serve as active foci for gene silencing. Indeed, these effects are reminiscent of those described some years ago for plasmid and λ vector sequences, which were shown to silence linked transgenes when retained in the constructs (19). Similarly, a review article (1) referred to observations that v-*src* sequences severely inhibited the expression of an *MT–hGH* transgene in the liver of transgenic mice when the two sequences were juxtaposed. Our present study with *BLG* transgenes illustrates how potent this type of silencing can be, although the degree of the effect will depend on a number of factors, including the transgene sequences involved, the structure and size of the array and the site of integration.

One of the best characterized systems of gene silencing is the repression that occurs at the telomere in yeast. The telomere exerts its suppressive effect through the collaboration of telomere-specific factors and histones; terminal DNA sequences are recognized by binding of RAP1 and this initiates polymerization of SIR proteins which interact with the N-termini of histones H3 and H4, repressing the adjacent domain. Alternative models for gene silencing in other systems postulate loop domains wherein factors bound to DNA at specific sites interact to isolate domains topologically and inactivate the genes therein (20). Possibly, mammalian cDNAs and prokaryotic reporter and vector sequences bind ubiquitous factors that catalyse polymerization or inactive loop formation and these effects spread to adjacent transgenes and silence them. Alternatively, they could also serve as foci for methylation or histone H1 deposition. An obvious feature of both types of sequence is that they lack introns and the corollary to this is that introns serve to ameliorate these silencing effects. In direct comparisons of intron-containing genes with their intronless counterparts in transgenic mice, introns dramatically enhance the efficiency of expression (2,5,21). Recently introns have been shown to stimulate nucleosome alignment in the rat growth hormone gene, showing that their presence can directly influence chromatin structure (22). We suggest that the chromatin state formed in the absence of introns may be highly repressive, spread to adjacent genes and probably resembles heterochromatin.

Table 1. Rescue and silencing of co-integrated transgenes

Transgenes		Frequency of expressors (%)		Comments	References
BLG	2nd transgene	BLG	2nd transgene		
BLG	–	3/3 (100%)	–	4 kb promoter	5
BLGΔDp	–	10/10 (100%)	–	0.4 kb promoter	5
	AATD	–	1/7 (14%)	4 kb promoter	8
	FIXD	–	0/9 (0%)	4 kb promoter	8
	ΔDpCAT	–	1/8 (12.5%)	0.4 kb promoter	6
BLG	AATD	7/9 (78%)	7/9 (78%)	co-injected	9
BLG	FIXD	12/12 (100%)	10/12 (83%)	co-injected	9
BLG	FIXDΔ3′	9/9 (100%)	8/9 (89%)	co-injected	10
BLGΔDp	ΔDpFIXDΔΔ3	2/11 (15%)	2/11(15%)	lig. head-to-tail (CAFI)	this paper
BLGΔDp	ΔDpCAT	2/11 (15%)	3/11(22%)	lig. head-to-tail (BRAT)	this paper
BLGΔDp	ΔDpCAT	3/9 (33%)	4/9 (44%)	lig. tail-to-tail (TARB)	this paper

The frequencies of expression of BLG and BLG-derived transgenes after co-integration by co-injection or ligation prior to injection are compared.

ACKNOWLEDGEMENTS

We thank Roberta Wallace and Francis Thomson for the production and maintenance of transgenic mice, Claire Neil for technical assistance, Ian Cottingham and Judith Percy for carrying out the fIX ELISA assays, Anthea Springbett for statistical advice and Bruce Whitelaw for critical discussions. This work was supported by the BBSRC and PPL Therapeutics Ltd.

REFERENCES

1 Palmiter,R.D. and Brinster,R.L (1986) *Annu. Rev. Genet.*, **20**, 465–499.
2 Brinster,R.L, Allen,J.M., Behringer,R.R., Gelinas,R.E. and Palmiter,R.D. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 836–840.
3 Pardy,K. (1994) *Mol. Biotechnol.*, **2**, 23–27.
4 Guy,L.G., Kothary,R., DeRepentigny,Y., Delvoye,N. and Wall,L. (1996) *EMBO J.*, **15**, 3713–3721.
5 Whitelaw,C.B.A., Archibald,A.L., Harris,S., McClenaghan,M., Simons,J.P. and Clark,A.J. (1991) *Transgenic Res.*, **1**, 3–13.
6 Webster,J.,Wallace,R.M., Clark,A.J. and Whitelaw,C.B.A. (1995) *Cell Mol. Biol. Res.*, **41**, 11–15.
7 Simons,J.P., McClenaghan,M. and Clark,A.J. (1987) *Nature*, **328**, 530–532.
8 Whitelaw,C.B.A., Harris,S., McClenaghan,M., Simons,J.P. and Clark,A.J. (1992) *Biochem. J.*, **286**, 31–39.
9 Clark,A.J., Cowper,A., Wallace,R., Wright,G. and Simons,J.P. (1992) *BioTechnology*, **10**, 1450–1454.
10 Yull,F., Harold,G., Cowper,A., Percy,J., Cottingham,I. and Clark,A.J. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 10899–10903.
11 McKnight,R.A., Wall,R.J. and Hennighausen,L (1995) *Transgenic Res.*, **4**, 39–43.
12 Burdon,T., Maitland,A., Demmer,J., Clark,A.J., Wallace,R. and Watson,C.J. (1994) *Mol. Endocrinol.*, **8**, 1528–1536.
13 Cattenach,B.M. (1974) *Genet. Res. Camb.*, **23**, 291–306.
14 Tartof,K.D. and Bremmer,M (1990). *Development*, **110** (suppl.), 35–35.
15 Allshire,R.C., Javerzat,J.P., Redhead,N.J. and Cranston,G. (1994) *Cell*, **76**, 157–169.
16 Karpen,G.H. (1994) *Curr. Opin. Genet. Dev.*, **4**, 281–291.
17 Dorer,D.R. and Henikoff,S. (1994) *Cell*, **77**, 993–1002.
18 Dobie,K.W., Lee,M., Fantes,J.A., Graham,E., Clark,A.J., Springbett,A., Lathe,R. and McClenaghan,M. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 6659–6664.
19 Townes,T.M., Lingal,J.B., Chen,H.Y., Brinster,R.L. and Palmiter,R.D. (1985) *EMBO J.*, **4**, 1715–1723.
20 Felsenfeld,G. (1996) *Cell*, **86**, 13–19.
21 Palmiter,R.D. Sandgren,E.P., Avarbock,M., Allen,D.D. and Brinster.R.L. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 478–482.
22 Liu,K., Sandgren,P., Palmiter,R.D. and Stein,A. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 7724–7728.