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Multiple Effects of Genetic Background on Variegated Transgene Expression in Mice

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ABSTRACT

BLG/7 transgenic mice express an ovine β-lactoglobulin transgene during lactation. Unusually, transgene expression levels in milk differ between siblings. This variable expression is due to variegated transgene expression in the mammary gland and is reminiscent of position-effect variegation. The BLG/7 line was created and maintained on a mixed CBA × C57BL/6 background. We have investigated the effect on transgene expression of backcrossing for 13 generations into these backgrounds. Variable transgene expression was observed in all populations examined, confirming that it is an inherent property of the transgene array at its site of integration. There were also strain-specific effects on transgene expression that appear to be independent of the inherent variegation. The transgene, compared to endogenous milk protein genes, is specifically susceptible to inbreeding depression. Outcrossing restored transgene expression levels to that of the parental population; thus suppression was not inherited. Finally, no generation-dependent decrease in mean expression levels was observed in the parental population. Thus, although the BLG/7 transgene is expressed in a variegated manner, there was no generation-associated accumulated silencing of transgene expression.

POSITION-effect variegation (PEV) was first characterized in *Drosophila melanogaster* 70 years ago (Muller 1930). This involved a mutation-induced translocation of the *white* eye color gene from its normal euchromatic site to an integration point close to centromeric heterochromatin. The juxtaposition led to variegated expression of the gene, the physical manifestation being the mottled eye phenotype. In the intervening years PEV has been extensively studied in Drosophila, where the combination of numerous characterized strains and short generation time has allowed complex genetical studies. This work has shown that many modifiers of PEV exist (Henikoff 1990; Singh 1994), with the relative abundance of a given modifier or group of modifiers influencing the ratio of silenced to expressing cells for a gene displaying PEV.

Variegated expression patterns have recently been observed in transgenic studies in mammals (McGowan *et al.* 1989; Dobie *et al.* 1996; Festenstein *et al.* 1996; Robertson *et al.* 1996; Giraldo *et al.* 1999; Sutherland *et al.* 2000); PEV may occur in mice (Cattanach 1974). Although in some cases this variegated transgene silencing appears to reflect a PEV-like phenomenon (Dobie *et al.* 1997), in others it seems to be at odds with the classical characteristics of PEV. For example, in some reports the extent of variegation remains constant between siblings within a line (Robertson *et al.* 1996). To be precise, in these mice the amount of transgene product or number of cells expressing the transgene remains constant between individuals of the same line. In contrast, we identified a line of transgenic mice (BLG/7) where there were clear within-line differences in the extent of variegation (Dobie *et al.* 1996). This case is different from the previous because the amount of product or number of cells expressing the transgene varies between individuals (siblings) of the same line. In this line of mice, the β-lactoglobulin transgene is integrated close to the centromere of chromosome 15 as a tandem array of ~25 copies. The variegated expression of the transgene in this line therefore exhibits some of the characteristics of PEV (Dobie *et al.* 1997).

Different genetic backgrounds can have widely differing effects on transgene expression (McGowan *et al.* 1989; Elliot *et al.* 1995; Schweizer *et al.* 1998), reflecting the presence of strain-specific modifiers of expression. The experimental overexpression of a single heterochromatin protein can modify the extent of transgene variegation in mice (Festenstein *et al.* 1999; McMorrow *et al.* 2000). BLG/7 was originally created on a mixed CBA × C57BL/6 background, and it can thus be argued that strain-specific modifiers could affect the observed variegation. However, initial analysis of 3rd generation backcrosses provided no evidence for a

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major strain-specific modifier of variegation affecting the transgene array at this locus (Dobie *et al.* 1996). There was, nonetheless, a small trend toward a lower mean expression level for both populations, suggesting that minor modifiers may exist. In view of this observation, we continued the backcrossing regime for 13 generations. This allowed us to investigate the existence of minor modifiers. In addition, the activity of the transgene with respect to inbreeding and generation effects could be assessed.

MATERIALS AND METHODS

Mice: Transgenic mouse line BLG/7 carries ~25 copies of a 16-kb *SalI-SalI* ovine genomic fragment encompassing the milk protein gene β-lactoglobulin (Simons *et al.* 1987; Whitelaw *et al.* 1992; Dobie *et al.* 1996) as a tandem array inserted close to pericentric heterochromatin of chromosome 15 (Dobie *et al.* 1996). The BLG/7 line was generated by microinjection of the transgene into F_1 CBA × C57BL/6 eggs with subsequent line maintenance through mating transgenic males with F_1 CBA × C57BL/6 females (termed the parental population). This mating regime has been maintained in parallel with backcross matings generating isogenic lines (termed the backcross populations). Generation of isogenic lines was by mating male transgenic mice with either CBA or C57BL/6 female mice. Outcross mice were generated by mating isogenic C57BL/6 BLG/7 males to female CBA mice, using resulting F_1 females for our analysis. Due to breeding difficulty, the CBA backcross population was not maintained beyond the 13th generation.

Milk collection and processing: Milks were collected at day 11 of lactation and processed as described (McClenaghan *et al.* 1995). Individual 1/250 diluted samples were analyzed on protein gels, 50 μl of the diluted samples were run for the backcross milks, and 40 μl of the diluted samples were run for the parental milks.

Total milk protein content was determined by micro-Kjeldahl analysis of pooled milk samples from backcross and parental populations.

Protein gels: SDS/PAGE analysis of milks was performed using 17.5% discontinuous gels (37.5:1 acrylamide:bisacrylamide, Anachem), 1-mm-thick, 20-cm plates with a standard 4% stacking gel (Bio-Rad Protean II rig used), and stained overnight with Coomassie Blue G-250. Purified sheep β-lactoglobulin standards, whose protein content had been determined using the micro-Kjeldahl technique (Aschaffenburg and Drewry 1957), were run on each gel. Quantification of β-lactoglobulin in transgenic samples (average of three repeats) was by pixel intensity (Multi Analyst Program version 1.1, Bio-Rad, Hercules, CA). Values were normalized for loading using the β-casein of the nontransgenic control.

Statistical analysis: Satterthwaite's approximation was used to calculate the degrees of freedom (d.f.) for a *t*-test between sample means in cases where the sample variances differed significantly. This gives a more conservative test than the standard *t*-test. Coefficients of variation (100 × SD/mean) were also used to compare variation between samples. These give a measure of variability as a percentage of the mean value, which is appropriate for samples from populations whose variation tends to increase with the mean.

FISH: Fluorescent *in situ* hybridizations (FISH) were performed on 5-μm sections from paraformaldehyde-fixed, wax-embedded mammary tissue.

Prehybridization: Sections were deparaffinized using two 20-min and one 5-min washes in xylene and then rehydrated following standard procedures. This was followed by 2 min 1× PBS, 10 min 4% paraformaldehyde (PFA) in PBS (pH 7.2), two 2 min 1× PBS, 7.5 min Proteinase K digestion (50 mM Tris, 5 mM EDTA, 5 μg/ml Proteinase K), 1 min 1× PBS, 2 min 4% PFA in PBS (pH 7.2), 10 sec H_2O, 30 sec 0.1 M TEA (or TCA), two 5 min 0.1 M TEA (or TCA) with 312.5 μl/100 ml acetic anhydride, 2 min each 1× PBS and 0.85% NaCl, and then sections were dehydrated using standard procedures and air dried.

Hybridization: Sections overlaid with buffer containing probes were heated for 6 min at 90°, iced for 1 min, and hybridized O/N at 37°. Sections were washed 10 + 30 min at 37° in 2× SSC and then 10 + 30 min RT in 0.1× SSC and mounted using Vectashield mounting medium with antifading agent. Probes were antisense 30′ oligos based on cDNA sequences labeled with either Cy3 (BLG) or fluorescein (β-casein). BLG probes were for positions 1621–1650, 1681–1710, 2591–2620, 3851–3880, and 4581–4610 using GenBank sequence X12817. β-Casein probes were for positions 9291–9320, 9411–9440, 9471–9500, 9531–9560, and 9621–9650 using GenBank sequence X13484. Synthesis and labeling were performed by MWG-BIOTECH AG (Ebersberg, Germany). The oligos were combined to a final concentration of 40 ng/ml in the hybridization buffer (40% formamide, 2× SSC, 1× Denhardt's, 10% dextran sulfate, 50 mM phosphate buffer, 50 mM dithiothreitol, 0.250 mg/ml tRNA, and 0.5 mg/ml denatured salmon sperm DNA).

Visualization: Images were captured using a Nikon Microphot-SA microscope fitted with a cooled CCD camera (Digital Pixels, Brighton, UK) and analyzed with IPLab software (Scanalytics, Fairfax, VA).

RESULTS

In the transgenic line BLG/7, variable expression of the ovine β-lactoglobulin (the transgene) protein in milk reflects variegated transgene expression in mammary epithelial cells (Dobie *et al.* 1996). This locus did not display age-related progressive silencing as the expression level for an individual mouse is stable between lactations (Dobie *et al.* 1996). To determine the effect of backcrossing, the transgenic locus was bred on two different backgrounds for 13 generations. These mice were congenic, with ~99.4% of chromosomal loci being homozygous (excluding those loci closely linked to the transgene integration locus). Milk samples were collected from 18 BLG/7 CBA mice and 21 BLG/7 C57BL/6 mice. Nineteen milk samples were taken from a parental population of BLG/7 transgenic mice with a mixed CBA × C57BL/6 genetic background, also bred for 13 generations.

Variegation occurs irrespective of genetic background: We determined β-lactoglobulin protein levels on polyacrylamide gels by comparison to known β-lactoglobulin standards for the backcross and parental populations. Values were corrected for loading differences by comparison to mouse β-casein levels, which we have previously shown to be expressed uniformly in BLG/7 mice (Dobie *et al.* 1996). Similar results were also apparent when serum albumin was used as the loading control protein (data not shown). Clearly, the BLG/7 transgene displays variable expression in both backcross populations (Figure 1). *In situ* hybridization for β-lactoglobulin expression in mammary tissue of backcross mice shows

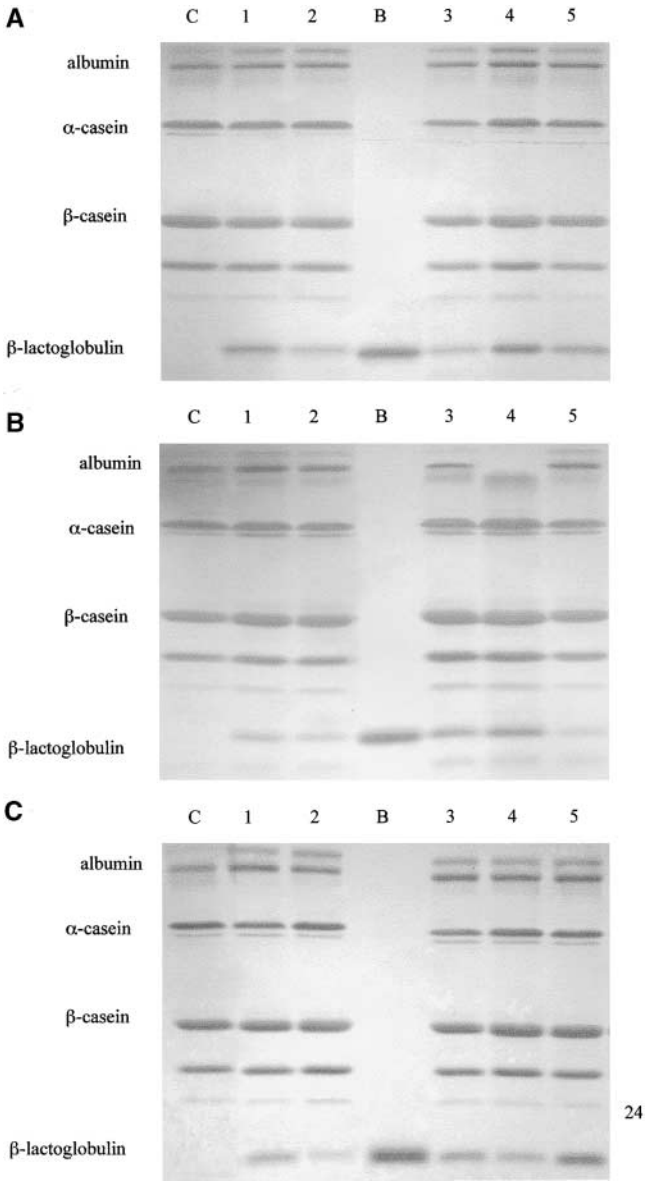

FIGURE 1.—BLG/7 locus displays variable expression. SDS/PAGE protein gels showing milk samples taken from 13th generation backcross CBA (A), C57BL/6 (B), and parental mice (C). Lane C, nontransgenic control milk; lane B, standard β-lactoglobulin sample: 2 μg for A and B, 3 μg for C. All samples were a 1/250 dilution of milk samples taken at day 11 of lactation from individual mice with 50 μl (A and B) and 40 μl (C) loaded per sample. Identities of milk proteins are indicated.

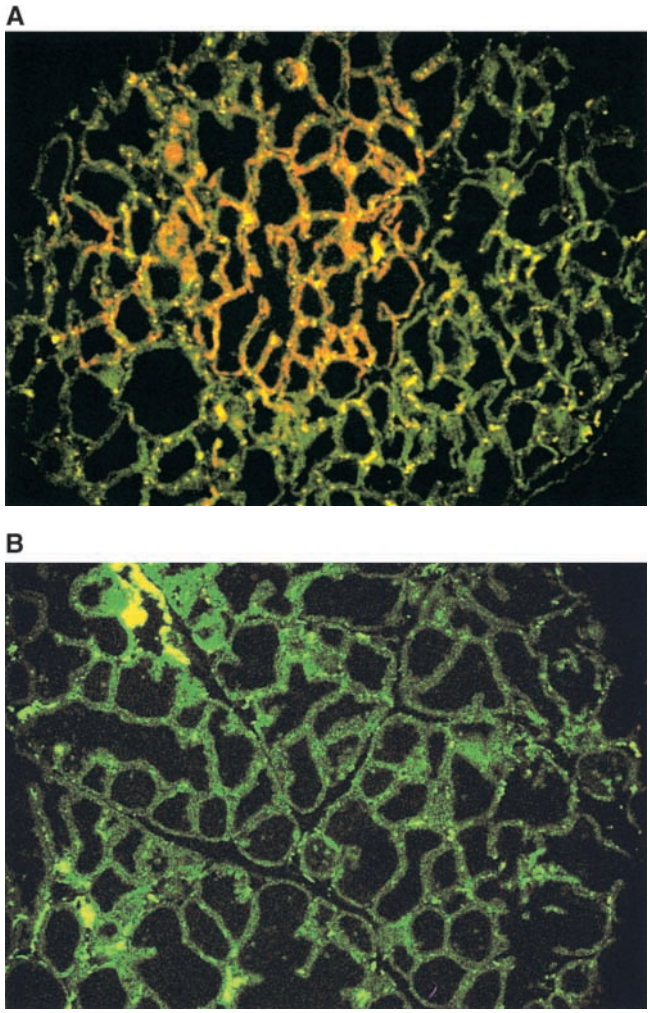

FIGURE 2.—BLG/7 locus displays variegated expression pattern. (A) FISH of backcross wax-embedded mammary tissue probed simultaneously for BLG (Cy3) and β-casein (fluorescein) expression. (B) Nontransgenic control tissue probed for BLG (Cy3) and β-casein (fluorescein). Note that residual wax fluoresces bright yellowish-white.

that the variable expression is due to variegation (Figure 2). The extent of variegation reflects β-lactoglobulin levels in milk for the parental population (M. L. OPSAHL, A. SPRINGBETT, R. LATHE, A. COLMAN, M. MCCLENAGHAN and C. WHITELAW, unpublished results) and for those backcross mice analyzed (data not shown). Expression level for individual mice from each backcross population and the parental population were determined (Figure 3) and the mean, standard deviation, and coefficient of variation for each population were calculated (Table 1).

Expression of transgenic protein levels was compared. Several effects were observed. The variance of β-lactoglobulin protein levels in the C57BL/6 population was significantly greater than for the CBA population ($P < 0.05$). The mean for the C57BL/6 population was also significantly higher than for the CBA population ($P < 0.01$). Thus, although BLG/7 still variegates after extensive backcrossing into CBA or C57BL/6 genetic backgrounds, there are significant differences in expression levels between these populations. For both backcross populations, the mean and variance differed significantly from the parental population (both $P < 0.01$), with values considerably lowered in both cases. A mild trend toward a reduction of mean expression and variegation in the CBA third generation backcross (DOBIE *et al.* 1996) is significantly enhanced in the 13th generation population, and similar trends that were not apparent in the 3rd generation C57BL/6 background have now become evident. Therefore, modifiers that down-

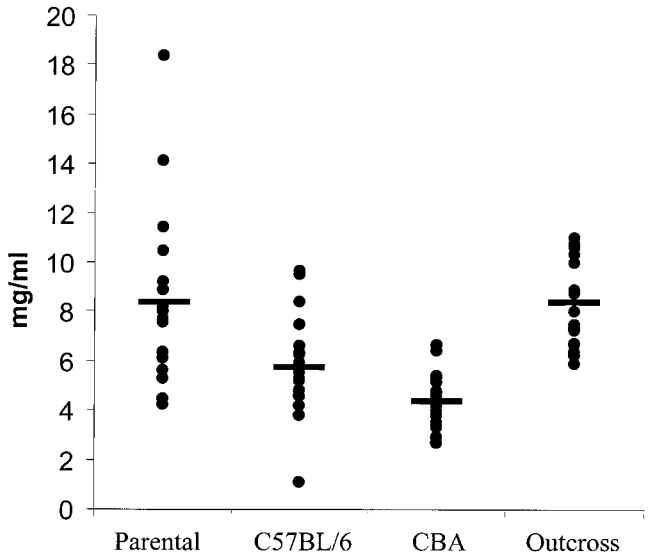

FIGURE 3.—Comparison of BLG/7 expression on different genetic backgrounds. Yield (milligrams per milliliter) of β-lactoglobulin from individual mice in the mixed parental, backcross C57BL/6, backcross CBA, and outcross populations. Each dot represents an average of three estimates for each individual mouse. Mean levels for each population are shown as a horizontal bar.

regulate without silencing expression of the variegating transgene exist. In addition, the maintenance of transgene expression levels in the parental population indicates that there is no progressive silencing of the transgene through repeated rounds of germline transmission.

The BLG/7 transgene is selectively sensitive to inbreeding depression effects: Inbreeding depression led to a slight reduction in total milk protein levels for both backcross populations (Table 2), with total protein content down by 15%. Unexpectedly, transgene expression was found to be depressed to a greater extent (down 29% in the C57BL/6 population and 47% in the CBA population). Thus, factors contributing to inbreeding depression may selectively suppress the transgene.

Outcrossing rescues β-lactoglobulin expression to parental levels: To determine if the inbreeding suppression of transgene expression could be maintained in a mixed genetic background we outcrossed one of the backcross populations (C57BL/6 BLG7 males to CBA, generating first-generation outcross females). Variegation was still very much evident in these mice. The mean β-lactoglobulin expression level reverted to that of the parental BLG/7 population, *i.e.*, 8.2 mg/ml. This differed significantly from the backcross C57BL/6 BLG/7 population (Table 3; Figure 3). This further supports our conclusion that there is no progressive silencing of the transgene through repeated rounds of germline transmission when in a mixed genetic background.

The coefficient of variation was reduced for the outcross *vs.* parental mice (Table 3), although expression was always within the range seen for the parental population (Figure 3). Differences in genetic composition could account for this. The outcross mice are very nearly 50% CBA and 50% C57BL/6, while the parental mice are maintained on a mixed CBA × C57BL/6 background through $F_2 \times F_1$ matings. Thus, individuals in the parental population can be homozygous (for CBA or C57BL/6) for a variable number of loci.

In conclusion, the reduced transgene expression seen in the backcross populations is not due to heritable change at the transgenic locus. Rather, this indicates that the inbreeding and background effects reflect recessive allele(s) with the manifestation of the allele(s) not being inherited.

TABLE 1

Analysis of expression in 13th generation backcross and parental populations

Genotype	Mean (mg/ml)	*n*	SD	Coefficient of variation (%)
CBA BLG/7	4.3	18	1.17	27.3
C57BL/6 BLG/7	5.8	21	1.92	33.1
Parental BLG/7	8.2	19	3.46	42

DISCUSSION

We have investigated the influence of genetic background on expression of a known variegating transgene. Variable transgene expression levels were seen on both backgrounds studied, even after 13 generations of backcrossing. The effects of inbreeding depression were more severe for our transgene than endogenous milk genes. Outcrossing resulted in complete reversion to parental expression levels.

Modifiers of variegation: The BLG/7 locus displays variable expression in both 13th generation CBA and C57BL/6 backcross populations. Therefore, the variegation observed is an inherent property of the BLG/7 transgene array at its site of integration—a position effect. This epigenetic property is inherited through generations. Variegating gene expression has been reported in many different organisms (CATTANACH 1974; HENIKOFF 1990; MARTIN and WHITELAW 1996; DOBIE *et al.* 1997). Apart from those that are the result of mutations, most examples derive from translocation or transgene insertion into unfavorable positions in the genome. These positions are often close to heterochromatic chromatin, and the cause of the subsequent mosaic expression pattern is believed to be a variable spread of heterochromatic proteins into the inserted gene array (PARO and HOGNESS 1991). Modifiers of PEV have been identified, many being components of chromatin (SINGH 1994), and there is evidence that the mammalian homologs can modify expression of a variegating gene (STAN-

TABLE 2

Micro-Kjeldahl analysis of total milk protein content

Genotype	β-Lactoglobulin[a]	Total protein[a]	Protein minus β-lactoglobulin[a]	% of endogenous proteins[b]	% reduction in β-lactoglobulin[b]
CBA BLG/7	4	85	81	85	47
C57BL/6 BLG/7	6	87	81	85	29
Parental BLG/7	8	103	95	100	0

[a] Protein in milligrams per milliliter.
[b] Relative to parental BLG/7 values.

kunas *et al.* 1998; Aagaard *et al.* 1999; Festenstein *et al.* 1999; McMorrow *et al.* 2000). Presumably some aspect of the BLG/7 transgene locus renders it a target for this type of nuclear factor.

Evidence for strain-specific major modifier locus effects in mice has been found (Allen *et al.* 1990; Schweizer *et al.* 1998; Mayeux-Portas *et al.* 2000). These reported strain differences are generally evident by the second or third generation backcross, with clear segregation patterns emerging that are indicative of a single locus. In addition, overexpression of a single protein can induce a major modification of expression in a variegating transgene (Festenstein *et al.* 1999; McMorrow *et al.* 2000). For the BLG/7 locus, we concluded previously from the third generation backcross that no single major modifier is present (Dobie *et al.* 1996). Nevertheless, in the 13th generation backcrosses we see both a lowering in the mean β-lactoglobulin level and an accompanying reduction in the variability of transgene expression. This suggests that rather than a single major modifier affecting the BLG/7 transgene, multiple loci exist, each having a subtle but cumulative effect on expression. There are also differences between the two backcross populations, implying that the modifiers differ in either composition or concentration in the CBA and C57BL/6 backgrounds.

Inbreeding depression selectively downregulates transgene expression: As a consequence of inbreeding depression the total milk protein content of both backcrosses was slightly reduced compared to the parental milks. We additionally observed a marked selective reduction of β-lactoglobulin levels with respect to that of endogenous milk proteins. We believe this is the first reported case of selective "discrimination" of a transgene in an inbred environment. Upregulation of β-lactoglobulin upon outcrossing rules out the presence of sequence mutations or heritable epigenetic modifications at the β-lactoglobulin locus.

TABLE 3

Analysis of expression in parental and outcross populations

Genotype	Mean (mg/ml)	*n*	SD	Coefficient of variation (%)
Parental BLG/7	8.2	19	3.46	42
Outcross BLG/7	8.2	16	1.80	22

We propose that, in contrast to the endogenous mouse milk proteins, the BLG/7 transgene locus is specifically susceptible to inbreeding depression. It is not clear what aspect of the BLG/7 locus confers this increased sensitivity. The transgene may lack regulatory regions present at the endogenous milk protein gene loci that provide a compensatory mechanism. Alternatively, the multicopy nature of the transgene array may be recognized in some way (Dorer and Henikoff 1994; Matzke and Matzke 1995; Hsieh and Fire 2000). Whatever the cause, it presumably reflects a recruiting disadvantage for the β-lactoglobulin transgene sequences with regard to the endogenous genes. In support of this being a general property of transgenes, the overexpression of the suppressive heterochromatic protein M31 (the mammalian homolog of the Drosophila heterochromatic protein 1) in transgenic mice selectively silenced a transgene without causing any phenotypic changes (Festenstein *et al.* 1999). Subsequently, and again without reported phenotypic changes, dosage-dependent effects of enhancers and suppressors of variegation were observed in a transgene (McMorrow *et al.* 2000). As heterochromatic factors are involved in the organization of higher-order chromatin, it is tempting to speculate that the BLG/7 transgene locus is compromised with regard to its spatial organization within the nucleus. Furthermore, because variable expression is inherited while inbreeding suppression is not, some differences in the mechanisms leading to these two forms of silencing must exist.

It has been proposed that the extreme consequence of inbreeding depression could be that a gene is transcriptionally silenced (Matzke *et al.* 1993). We did not observe such total silencing of expression for the BLG/7 transgene, only downregulation of expression in both genetic backgrounds. Should this phenomenon prove to be common, then current backcrossing strategies designed to alleviate unstable transgene expression may exacerbate the problem instead of curing it.

Genome policing: In plants it has been proposed that

a genome defense system operates to inactivate foreign or invasive sequences such as transgenes and transposable elements (Matzke and Matzke 1998). One aspect of this policing of the plant genome is that the extent of silencing increases through generations (Assaad *et al.* 1993; Guo *et al.* 1998) with similar effects reported in insects (Jensen *et al.* 1999) and fish (Amsterdam *et al.* 1995). We are unaware of any reported generation-associated silencing of transgenes in mammals. In our study, in contrast to the selective silencing of the β-lactoglobulin transgene during backcrossing, we saw no evidence for a generation-associated progressive silencing of our variegating transgene when present in a mixed genetic background. Indeed, expression of this transgene has remained essentially constant since its generation in 1987 (Simons *et al.* 1987). Therefore, if a genome-policing mechanism exists in mammals to selectively silence foreign sequences, then some transgenes, *e.g.*, BLG/7, can evade detection.

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