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Enhancing the Efficiency of Transgene Expression

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SUMMARY

Two strategies for enhancing gene expression in transgenic animals are described with particular reference to targeting expression to the mammary gland. Gene constructs in which the protein-encoding DNA sequences are contained within a genomic segment (comprising most or all of the natural introns of the corresponding gene) are shown to be expressed more efficiently than their intronless counterparts. Secondly, co-integrating an otherwise poorly expressed transgene in the vicinity of an actively expressed transgene can dramatically improve its efficiency of expression.

1. INTRODUCTION

The ability to manipulate the germline of animals offers a number of biotechnological opportunities. Recent interest has focused on the use of transgenic animals for the production of recombinant proteins, in particular human biomedical proteins. The approach generally taken has been to target expression to the mammary gland and produce the desired protein in milk. Livestock such as sheep or cows, or even pigs, synthesize and secrete large amounts of protein in their milk during lactation. Using gene transfer techniques this synthetic capacity can be harnessed to produce other valuable proteins, thus offering a relatively cheap method of production compared to conventional fermentation-scale mammalian cell culture (Clark *et al.* 1987; Whitelaw & Clark 1989).

Milk protein genes from a variety of species have been isolated and shown to function appropriately in the mammary gland of transgenic mice (Simons *et al.* 1987; Vilotte *et al.* 1989; Bayna & Rosen 1990). Regulatory sequences from these and other milk protein genes have been used to target expression of human proteins in transgenic mice (Gordon *et al.* 1987; Yu *et al.* 1989; Archibald *et al.* 1990; Meade *et al.* 1990; DiTullio *et al.* 1992), rabbits (Buhler *et al.* 1990) sheep (Clark *et al.* 1989; Wright *et al.* 1991) and goats (Ebert *et al.* 1991). Proteins produced by this route are biologically active demonstrating that the mammary gland is capable of carrying out the appropriate post-translational modifications. In many cases, however, the efficiency of expression of these hybrid transgenes was shown to be low, both in terms of the frequency and levels of expression. The inefficient and unpredictable mode of expression of transgenes is a major constraint to the adoption of this technology. This is particularly the case when transgenic livestock are required for large-scale production, given that the efficiency of gene transfer in these species is low and the procedures involved both expensive and time-consuming (Pursel *et al.* 1989; Clark *et al.* 1991). It is clearly important to understand more fully the processes that control the expression of transgenes and, if possible, manipulate them to improve their efficiency.

To target expression to the mammary gland we have used sequences derived from the sheep milk protein gene β-lactoglobulin (BLG, Ali & Clark 1988). The unmodified gene, itself, is expressed efficiently and specifically in transgenic mice (Simons *et al.* 1987; Harris *et al.* 1991). We have shown that essential regulatory elements are located within the proximal 406 b.p. of 5′ flanking sequences (Whitelaw *et al.* 1992) and that this region contains the binding sites for a putative mammary gland-specific transcription factor (Watson *et al.* 1991). BLG transgenes comprising this region, the transcription unit and 3′ flanking sequences are expressed in a position-independent manner: they are invariably expressed, and the levels of expression are related to the copy number of integrated transgenes. In this paper we describe two approaches for improving the expression of hybrid genes containing regulatory element from the BLG gene. Firstly, we demonstrate a requirement for introns for efficient expression and, secondly, we show that chromosomal position effects at the site of integration can be manipulated to enhance expression.

2. INTRON RESCUE

The DNA sequences encoding a particular protein of interest are often only available as cDNA sequences. This may reflect the original cloning experiments or the fact that many candidate proteins are encoded by very large genes (for example, the gene encoding human factor VIII is about 180 kb in length; Gitschier *et al.* 1984) and not conveniently manipulated by conventional recombinant DNA techniques. As such, cDNA segments lack introns and although no specific

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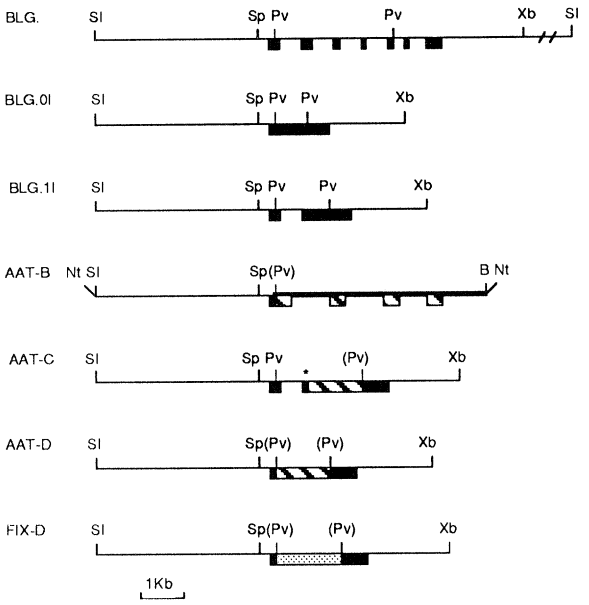

Figure 1. Structure of BLG and BLG hybrid genes introduced into transgenic mice. Thin line, BLG flanking sequences and introns; thick line α_1AT flanking sequences and introns; filled boxes, BLG exons and/or cDNA; hatched boxes, α_1AT exons and/or cDNA; lightly shaded box, fIX cDNA; * inserted termination codon. Relevant restriction sites; B, *Bam*HI; Nt, *Not*I; Sl, *Sal*I; Sp, *Sph*I; Pv, *Pvu*II; Xb, *Xba*I. BLG sites lost during ligation are shown in brackets. Data from Whitelaw *et al.* (1991).

role in controlling gene expression has been ascribed to them, their absence is a common feature of many the poorly performing transgenes that we and other workers have described.

Figure 1 shows a number of different BLG-derived constructs designed to evaluate whether introns are required for efficient expression. BLG, BLG-0I and BLG-1I are derived solely from the BLG gene and comprise the unmodified gene, an intronless derivative and an intron-1 containing derivative, respectively. AAT-B, AAT-C and AAT-D are hybrid genes comprising various configurations of human alpha-1-antitrypsin (α_1AT) encoding sequences with BLG sequences. AAT-B comprises ~4.0 kb of 5′ flanking sequences of the BLG gene fused to a genomic minigene encoding human α_1AT. In this construct three of the four 'natural' introns present in the human α_1AT gene are retained. AAT-D comprises an intronless BLG/α_1AT hybrid gene in which the 5′ and 3′ segments of BLG have been linked to a cDNA segment encoding human α_1AT. AAT-C comprises the same α_1AT cDNA segment but, by analogy with BLG-1I, the first intron of BLG has been included upstream. Finally, FIX-D comprises a cDNA segment encoding human factor IX (fIX) fused to the 5′ and 3′ flanking sequences of BLG; it contains no introns and is analogous to BLG-0I and AAT-D.

These constructs were introduced into transgenic mice by microinjection into the pronuclei of fertilized eggs from superovulated (C57BL/6 × CBA) F1 female mice (Simons *et al.* 1987). Expression was analysed in G_0 or G_1 (when the G_0 was male) female transgenic mice. Mid-lactation females were milked and milk samples were analysed by SDS-PAGE, Western blotting and radioimmunoassay as described previously (Simons *et al.* 1987; Archibald *et al.* 1990). After milking, the mice were killed and total RNA was prepared from the mammary gland as well as a small

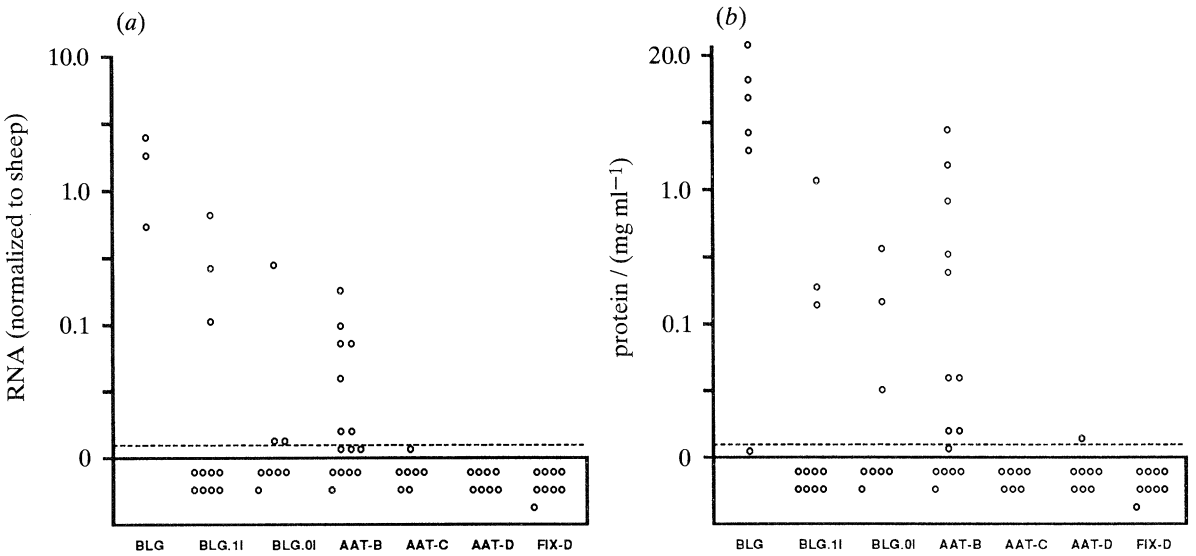

Figure 2. Comparison of transgene expression. Steady state BLG and α_1AT mRNA levels in the mammary gland (*a*) and protein levels in mouse milk (*b*) are compared for the various constructs shown in figure 1. Each circle corresponds to the levels determined for a G_0 or G_1 female transgenic mouse; values below the dotted line were detectable but could not be accurately determined. Note that RNA levels were not determined for all BLG mice. Statistical comparison of expression frequencies (Fisher's Exact Test) determined that BLG-0I and BLG-1I were significantly different from BLG ($p < 0.01$ and $p < 0.05$, respectively); AAT-D and FIX-D were significantly different from AAT-B ($p < 0.05$ and $p < 0.01$, respectively) while AATC was nearly significantly different from AAT-B ($p < 0.06$); no significant difference was detected between BLG and AAT-B. For full details see Whitelaw *et al.* (1991).

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selection of other tissues. RNA levels in the mammary gland were quantified by probing dot blots loaded with serial dilutions using sheep mammary and human liver RNAs as controls.

Figure 2 summarizes the data obtained from these experiments. A clear difference emerges when constructs comprising most or all of their native introns (BLG and AAT-B) are compared with constructs that lack introns (BLG-0I, AAT-D, FIX-D) or contain just one BLG intron (BLG-1I, AAT-C). Thus, all mice (or lines) that carried the unmodified BLG gene expressed it, and in five of the six cases high levels of BLG (greater than 1 mg ml^{-1}) were obtained in the milk. The efficient expression of native BLG transgenes has been confirmed in an additional set of experiments involving a further 24 transgenic mice (or lines) carrying comprising various amounts of 5′ flanking DNA, but otherwise identical to the construct shown in figure 1. In every case significant expression was observed and, furthermore, the level of expression showed a significant correlation with the copy number of integrated transgenes (Whitelaw *et al.* 1992). By contrast only three of the eight mice (or lines) carrying the intronless gene, BLG-0I, expressed the transgene. In those mice that did express the average level of expression was approximately one twentieth the level obtained with the unmodified BLG transgene. A similar result was obtained with BLG 1I, which contains only the first BLG intron. Only three of the 11 mice (or lines) expressed this construct and the average level of expression in these animals was, again, lower than was observed with BLG.

A similar difference in the efficiency of expression was observed when the genomic α_1AT construct AAT-B was compared to the the two α_1AT cDNA constructs AAT-D and AAT-C: ten of the 15 AAT-B mice (or lines) expressed this construct in the mammary gland. The highest expressing line produced milk concentrations of human α_1AT of between 7 and 8 mg ml^{-1} and, generally, there was a good correlation between the steady-state mRNA levels and the level of protein detected in milk (Whitelaw *et al.* 1991). The level of expression of this transgene, however, was more variable than observed with BLG and no relationship was observed between expression levels and copy number. In contrast to AAT-B, only one of the eight AAT-D transgenics expressed detectable α_1AT protein and only one of the eight AAT-C animals expressed detectable RNA (figure 2). No expression was observed in the nine mice (or lines) which carried the fIX cDNA construct FIX-D.

Transcript sizes and tissue specificity were determined in expressing animals by Northern blotting experiments. BLG and BLG-minigenes gave rise to the appropriate sized transcripts and expression was restricted to the mammary gland, showing that BLG introns do not contain sequences required to limit tissue specificity. Northern blotting of RNA samples from AAT-B mice also showed transcripts of the expected size in the mammary gland of the ten expressing mice (or lines). By contrast to BLG constructs, four of these mice (or lines) were also shown to express AAT-B transcripts in the salivary gland. Additionally, four of the five animals that did not express AAT-B in the mammary gland showed detectable expression in the salivary gland. We do not understand at present why the AAT-B transgene is expressed at such a high frequency in the salivary gland. It is possible that downstream BLG sequences contain a salivary gland-specific negative regulatory element or that regulatory elements within the α_1AT genomic sequences direct expression to this tissue.

Other workers have also investigated the requirement for intronic sequences in transgenic mice (Brinster *et al.* 1988; Palmiter *et al.* 1991; Choi *et al.* 1991). In these studies intron-containing and intronless gene constructs were targeted to various tissues in transgenic mice. Introns were shown to improve expression in a variety of tissues showing that this may be a general requirement for the efficient expression of transgenes.

We do not fully understand the mechanism(s) whereby introns enhance transgene expression. The failure of cDNA-based hybrid genes to be expressed at high frequency in the mammary gland does not appear to due to a sinple requirement for splicing because AAT-C (which contains the first intron of BLG) was expressed as poorly as its intronless counterpart. Secondly, BLG-1I (which contains the first BLG intron) was expressed no more efficiently than its intronless counterpart, BLG 0I.

The performance of BLG hybrid genes and BLG minigenes in transgenic mice is reminiscent of the behaviour of transgenes carrying weak promoters (see, for example, Bonnerot *et al.* (1990); Allen *et al.* (1988)) which are particularly sensitive to chromosomal position effects. All the constructs described in this study comprise identical 5′ flanking regions and we have postulated that it is the interaction between the 5′ promoter sequences and *cis* elements downstream that determines how effectively chromosomal position effects are overcome and, hence, how efficiently a given construct will function (Whitelaw *et al.* 1991). Intron containing constructs such as BLG and AAT-B may function relatively efficiently because they contain such *cis* acting elements rather than any obligatory requirement for splicing. One possibility is that these putative *cis*-acting sequences enable a transgene to adopt the appropriate chromatin configuration and allow access to the soluble transcription factors which then drive expression from the 5′ promoter segment. The precise nature of these sequences is not known: they may be discretly organized or distributed throughout the transcription unit.

From a practical point of view a general requirement for introns has a number of implications for transgene design. Limitations are imposed on the construction of transgenes particularly those in which the structural region encompasses large regions of chromosomal DNA (e.g. factor VIII; Gitschier *et al.* 1984). Genomic minigenes such as AAT-B do function quite efficiently and, therefore, it should be possible to produce efficiently expressing constructs that comprise a mixture of cDNA and genomic sequences. Nevertheless, given our lack of understanding as to how introns enhance gene expression the choice of which to include in any given gene construct will not be

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Table 1. *Production of β-lactoglobulin-alpha$_1$-antitrypsin (BAD) and β-lactoglobulin-human factor IX (BIX) transgenic mice*

(Tail DNA samples from G_0 mice were screened initially by the polymerable chain reaction (PCR) using primers which detect all three transgenes. PCR-positive samples were further analysed by Southern blotting analysis, using BLG-specific, AATD-specific and FIXD-specific probes (BLG, β-lactoglobulin gene; AATD, α-1-antitrypsin-D hybrid gene; FIXD, human factor IX-D hybrid gene). Data from Clark *et al.* (1992).)

		double transgenics		single transgenics		
	total G_0 mice	BLG + AATD	BLG + FIXD	BLG	AATD	FIXD
BAD	173	11		1	8	
BIX	135		20	10		0

straightforward and may require the *ad hoc* evaluation of a number of different alternatives.

3. TRANSGENE RESCUE

Poorly expressed transgenes (such as those lacking the appropriate introns) appear to be highly influenced by chromosomal position effects which are presumed to act at the site of integration. We reasoned, therefore, that engineering the site of integration rather than the construct *per se* might provide a strategy to rescue poorly expressed transgenes. For targeting expression to the mammary gland the vicinity of an actively expressed milk protein could constitute a position which would rescue the expression of, otherwise, poorly expressed constructs. Gene targeting by homologous recombination in embryonic stem cells (Capecchi 1989) would, in principle, enable the insertion of constructs adjacent to endogenous milk protein genes, for example, within the cluster of casein genes. Gene targeting into the germline is, however, technically difficult and, for the present, limited to mice since no proven ES cells have yet been isolated from other mammalian species. We have favoured an

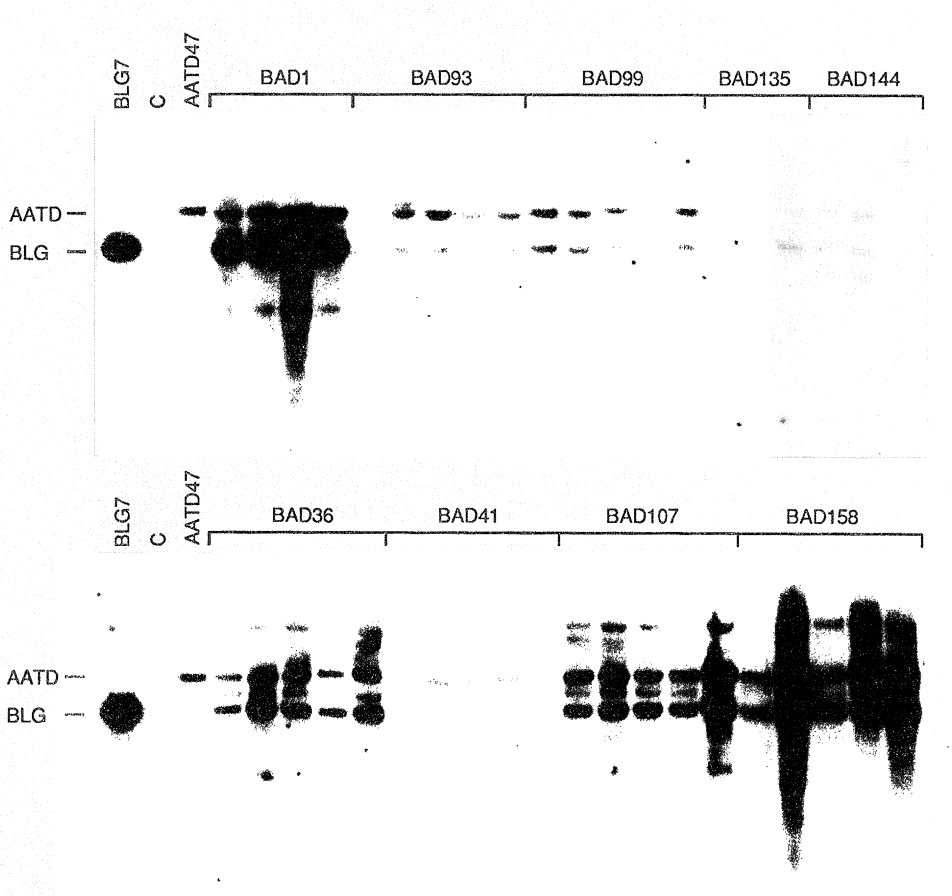

Figure 3. Co-segregation of BLG and AAT-D in BAD lines. Tail DNA samples from a number of individuals from each BAD line (BAD 1–BAD 158) and controls were cleaved with *Eco*RI and analysed by electrophoresis and Southern blotting. The filters were probed with 5′BLG sequences which hybridize to both AAT-D and BLG transgenes. BLG7, DNA from a single BLG transgenic mouse; C, control non-transgenic DNA; AATD47, DNA from a single AAT-D transgenic line. The AAT-D-specific and the BLG-specific fragments are indicated. Data from Clark *et al.* (1992).

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alternative approach that involves the co-injection of different DNA constructs into pro-nuclei, since this can result in the co-integration of the DNAs at a single site (Storb *et al.* 1986; Behringer *et al.* 1989). The BLG gene is efficiently expressed in transgenic mice and, moreover, seems to contain all the sequences necessary for overcoming chromosomal position effects (Whitelaw *et al.* 1992). Therefore, to test the rescue hypothesis, transgenic mice were produced by co-injection of the BLG gene with either the AAT-D or FIX-D constructs which are expressed very inefficiently when integrated alone (figure 2).

In the first experiment the BLG and AAT-D constructs (figure 1) were co-injected into mouse eggs in a 1:1 molar ratio. The constructs were not ligated prior to microinjection. The co-integration frequency was high and more than 50% of the G_0 transgenic mice obtained had integrated both BLG and AAT-D transgenes (table 1). From the 11 G_0 BLG/AAT-D (BAD) mice, nine transgenic lines were established. Southern blotting experiments carried out on a number of G_1 progeny showed the same pattern of AAT-D and BLG-specific fragments within each line (figure 3). Thus in each line the BLG and AAT-D transgenes have co-segregated, suggesting a single site of integration. One exception was line 93 in which an additional fragment was observed in some of the G_1 mice, most probably resulting from a second site of integration. More detailed Southern blotting analyses of DNA prepared from the BAD mice identified junction fragments between BLG and AAT-D confirming co-integration of the two transgenes. Transgene copy number varied widely between lines although within a line, BLG and AAT-D copy numbers were similar, reflecting the molar ratio of the DNAs injected (figure 3).

Milk was collected from transgenic mid-lactation G_0 or G_1 females and assayed by ELISA. Human α_1AT was detected in the milk of mice from seven of the nine lines. The concentrations of the foreign protein ranged from ~ 1 μg ml^{-1} to over 600 μg ml^{-1} (figure 4). Total RNA was prepared from the mammary gland and a small selection of other tissues and probed for AAT-D transcripts (figure 5). The expected transcripts were detected in mammary gland RNA in six of the nine BAD lines and their steady-state levels correlated approximately with the concentration of human α_1AT in milk (we were unable to detect AAT-D transcripts in the lowest expressing BAD line). No expression of AAT-D was detected in other tissues. The efficiency of expression of AAT-D when cointegrated with BLG contrasts markedly with expression of the singly integrated AAT-D construct. In the latter case low level expression was detected in only one of eight mice (or lines), whereas in the BAD mice AAT-D expression was detected in seven of the nine lines, two of which expressed more than 100 μg ml^{-1} of the foreign protein.

The second rescue experiment was carried out using the FIX-D construct (figure 1). In this experiment the BLG fragment was injected in a threefold molar excess over the FIX-D fragment. Again the efficiency of co-integration was high and 20 of the 30 G_0 animals

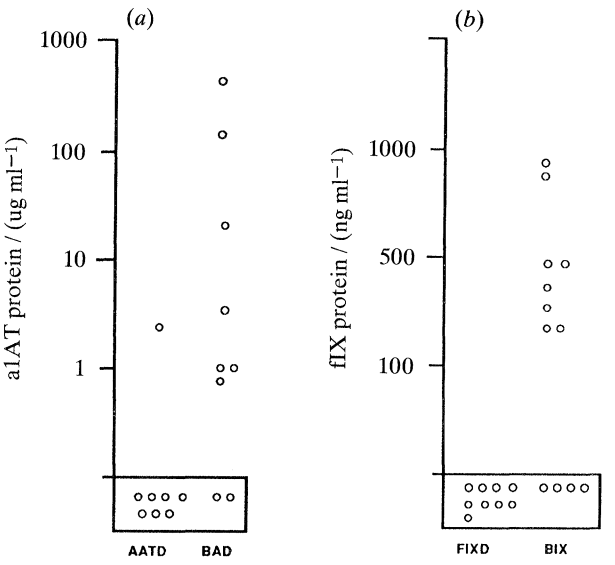

Figure 4. Expression of co-integrated transgenes. α_1AT (*a*) and fIX (*b*) protein levels (measured by ELISA) in BAD and BIX mice were compared to the corresponding single transgenics carrying AAT-D and FIX-D. The circles correspond to the levels determined for independently-derived G_0 or G_1 females. For full details see Clark *et al.* (1992).

obtained carried both FIX-D and BLG transgenes (table 1). From 13 of the BLG/FIX-D (BIX) transgenic mice germline transmission was obtained from 11. As for the BAD mice the two genes co-segregated and the majority were co-integrated at one site only. By contrast to the BAD lines, the BLG copy numbers in the BIX lines were higher than those of FIX-D, indicating that the composition of the integration sites reflects the ratio of the input DNAs microinjected (not shown).

Milk and RNA samples were collected from mid-lactation females. Factor IX was detected by ELISA in milk from eight of 12 BIX transgenic mice, although the concentrations were low ranging from 0.2–1 μg ml^{-1} (figure 4). FIX-D transcripts were detected specifically in the mammary gland (figure 5) in samples from ten of the 12 mice (or lines) analysed. Steady-state levels varied considerably between mice (or lines) but those in which fIX was detected in the milk (eight of 12) exhibited relatively abundant transcripts; these were considerably higher (up to 60-fold) than the level of human fIX mRNA in human liver, the normal site of expression of factor IX. The FIX-D transcripts were, however, approximately 400 nt shorter than predicted. Additional Northern blotting experiments (not shown) demonstrated that this was due to an internal deletion of the transcripts, presumably due to aberrant splicing. Thus the low concentration of fIX detected in milk may result from the translation of a small proportion of non-deleted FIX-D transcripts, the innefficient translation of the truncated mRNA or the innefficient secretion of an aberrant fIX. Notwithstanding, the problems of mRNA processing, co-integration of FIX-D with BLG dramatically enhanced its efficiency of expression: in none of nine single transgenics was expression detected

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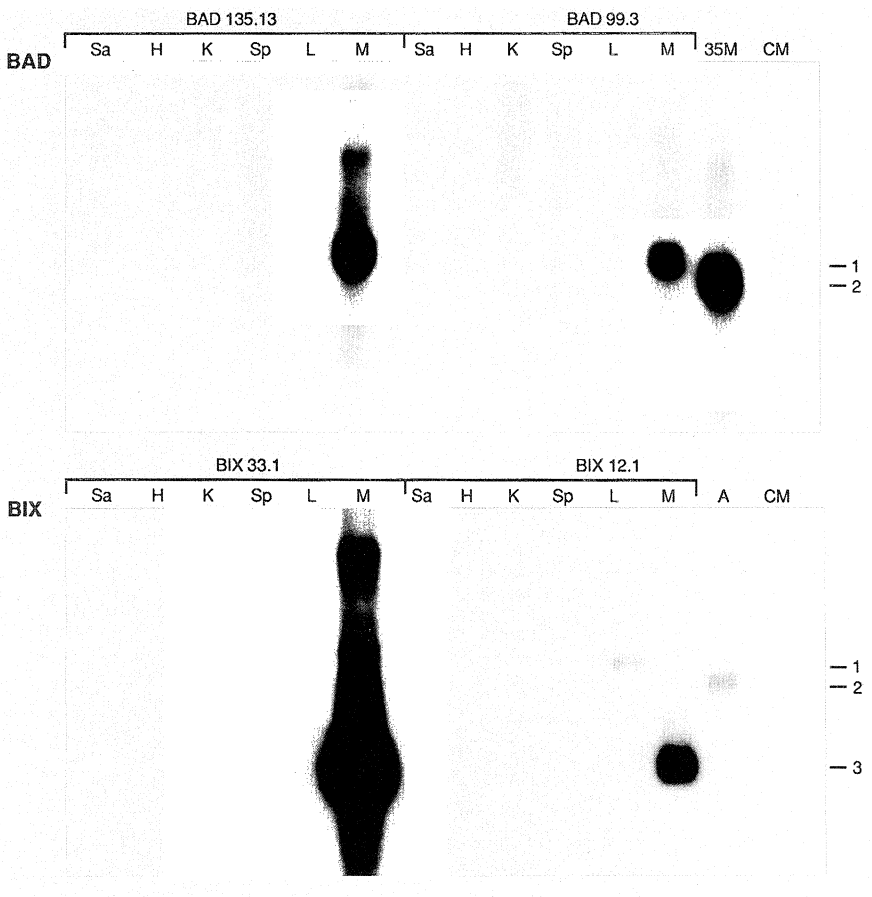

Figure 5. Tissue-specific expression in BAD and BIX mice. Total RNA samples (10 μg) from controls, BAD, and BIX mice were analysed by Northern blotting. Top panel, BAD RNA samples: CM, control mouse mammary RNA; 35M, mammary gland RNA from mouse AAT-B35 (Archibald *et al.* 1990); M, mammary gland; L, liver ; Sp, spleen; K, kidney; H, heart; Sa, salivary gland. The ~1600 nt AAT-D (1) and ~1400 nt AAT-B (2) transcripts are indicated at the right-hand side of the panel. Bottom panel, BIX RNA samples: A, mammary gland RNA from FIXA51 a transgenic mouse expressing FIXA, a fIX construct we have previously shown to be expressed in transgenic sheep (Clark *et al.* 1989); other tracks as for the top panel. The ~2600 nt endogenous mouse fIX transcript (1), ~2400 nt FIXA transcript (2) and ~1450 nt FIX-D (3) transcripts are indicated at the right-hand side of the panel. For further details see Clark *et al.* (1992).

(figure 1), whereas the majority (ten of 12) BIX mice (or lines) expressed the construct.

The data from both BAD and BIX experiments indicates that engineering the site of integration for a particular construct can be used as a strategy to enhance expression. At present we do not understand the mechanism whereby co-integration with BLG rescues the expression of an otherwise poorly expressed construct. Possible mechanisms include the abrogation of negative position effects, whereby sequences within the BLG gene insulate the hybrid genes from the chromosomal sequences flanking the integration site (Kellum & Schedl 1991). Alternatively, it is possible that the adjacent BLG gene provide positive effects on expression. Such positive effects could operate via interactions of the BLG gene and the rescued genes through enhancer or LCR-like activity (Grosveld *et al.* 1987). Alternatively, the open chromatin structure surrounding the expressed BLG gene may spread to encompass the flanking sequences, including the rescued genes.

4. DISCUSSION

We have described two studies on improving the efficiency of gene expression in transgenic animals. The first shows that the inclusion of introns in gene constructs can improve their performance. In our hands constructs containing most or all of their natural introns were expressed more efficiently than their intronless counterparts. Inclusion of one BLG intron did not improve the efficiency of expression indicating that a requirement for splicing was not the reason for the poor performance of these constructs. Other workers (Brinster *et al.* 1988; Palmiter *et al.* 1991; Choi *et al.* 1991) have demonstrated similar results with a variety of constructs and have also suggested that insertion of a heterologous intron between the promoter and coding region can enhance expression. From a practical point of view, all these studies support the use of genomic constructs wherever possible for transgenic experiments, with the next best strategy being the use of a minigene in which as much

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as possible of the genomic sequence is retained but large intronic regions are removed. This latter strategy is exemplified in this current work by construct AAT-B. Not only does this minigene construct function efficiently in transgenic mice it also performs very well in transgenic sheep. Each of the four G_0 females expressed human α_1AT in milk; all expressed at levels above 1 mg ml^{-1} with the highest expressor producing a sustained level of $\sim$35 mg ml^{-1} (Wright *et al.* 1991).

If the appropriate genomic or genomic minigene constructs are not readily available then the second strategy presented here, transgene rescue, may provide a solution for enhancing transgene expression. Co-injection provides a simple method for introducing two gene constructs into the germline but suffers from the problem that complex and variable transgene arrays are generated at the different sites of integration. The variable structure of these arrays may be partly or wholly resposible for the wide variation of expression levels between different lines and suggests that there is an optimal configuration of the two transgenes. Building double-contructs prior to injection could provide more control over the structure of the arrays by ensuring that the optimal configuration is present. Finally, although the present data are limited to targeting gene expression to the mammary gland it seems likely that transgene rescue will work in other tissues, given that a suitable 'rescuing' gene is available.

It is tempting to speculate that intron rescue and transgene rescue involve a common mechanism(s) at the chromosomal level, for example, by the generation of the appropriate chromatin configurations. Whatever the mechanisms involved it is clear that the design of efficiently expressing gene constructs for gene transfer entails more than simply linking a 'strong' promoter to the appropriate coding sequences. As well as providing some practical solutions to transgene design the results presented in these two studies suggest that other, as yet poorly understood, processes are critical for efficient gene expression.

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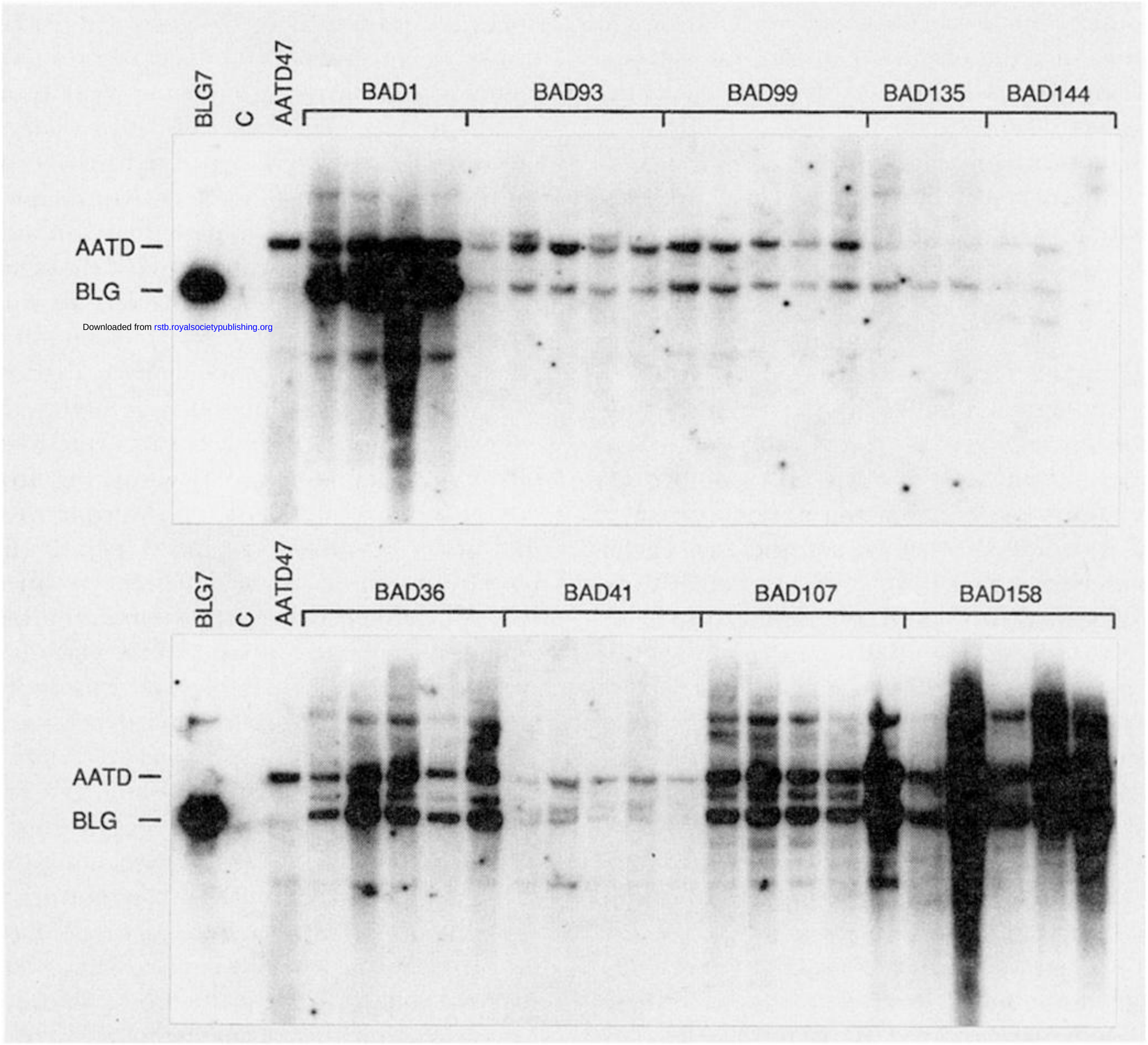
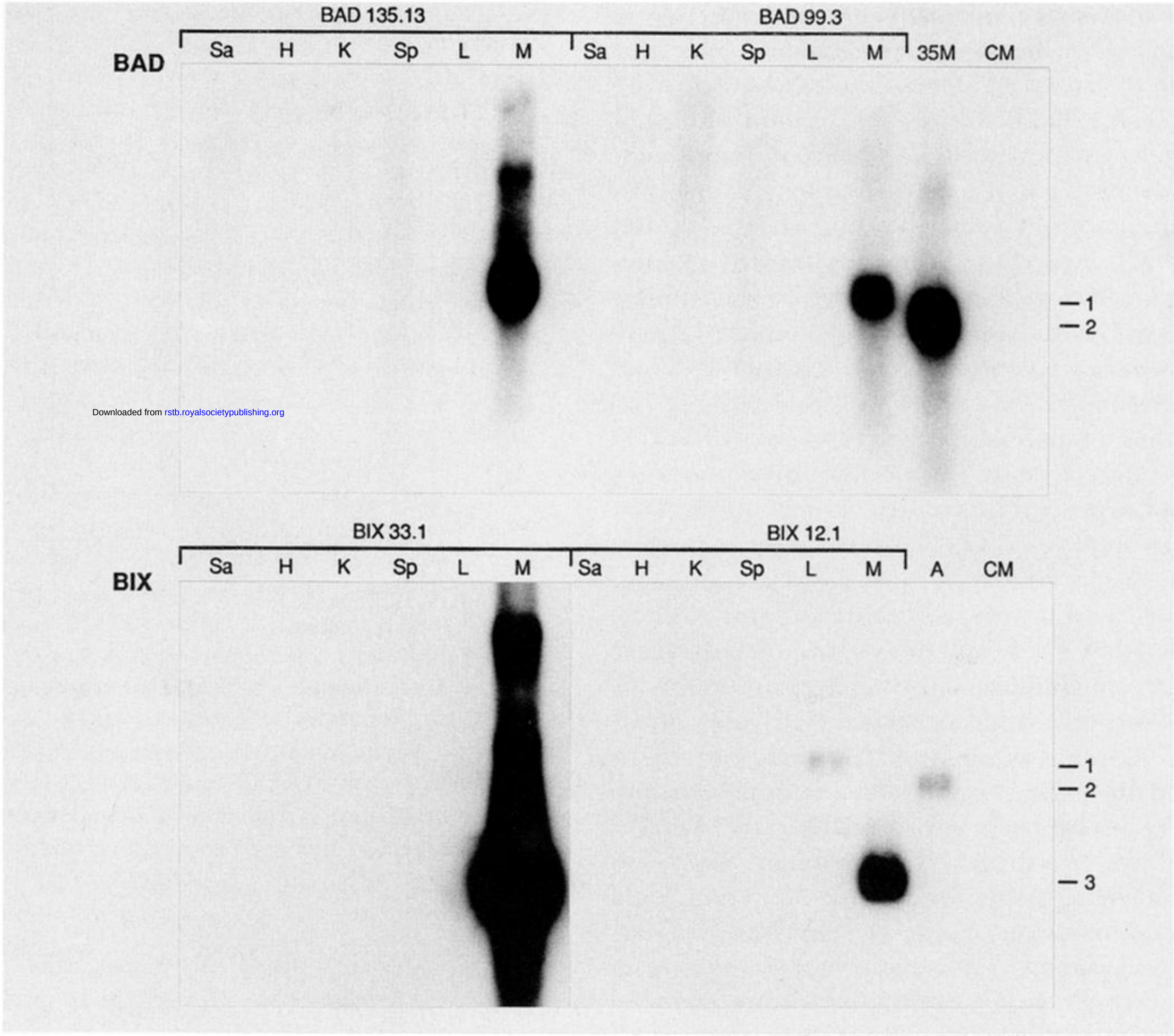


Figure 3. Co-segregation of BLG and AAT-D in BAD lines. Tail DNA samples from a number of individuals from each BAD line (BAD 1–BAD 158) and controls were cleaved with *Eco*RI and analysed by electrophoresis and Southern blotting. The filters were probed with 5′BLG sequences which hybridize to both AAT-D and BLG transgenes. BLG7, DNA from a single BLG transgenic mouse; C, control non-transgenic DNA; AATD47, DNA from a single AAT-D transgenic line. The AAT-D-specific and the BLG-specific fragments are indicated. Data from Clark *et al.* (1992).



igure 5. Tissue-specific expression in BAD and BIX mice. Total RNA samples (10 μg) from controls, BAD, and IX mice were analysed by Northern blotting. Top panel, BAD RNA samples: CM, control mouse mammary NA; 35M, mammary gland RNA from mouse AAT-B35 (Archibald *et al.* 1990); M, mammary gland; L, liver ; Sp, leen; K, kidney; H, heart; Sa, salivary gland. The ~1600 nt AAT-D (1) and ~1400 nt AAT-B (2) transcripts are dicated at the right-hand side of the panel. Bottom panel, BIX RNA samples: A, mammary gland RNA from IXA51 a transgenic mouse expressing FIXA, a fIX construct we have previously shown to be expressed in ansgenic sheep (Clark *et al.* 1989); other tracks as for the top panel. The ~2600 nt endogenous mouse fIX anscript (1), ~2400 nt FIXA transcript (2) and ~1450 nt FIX-D (3) transcripts are indicated at the right-hand de of the panel. For further details see Clark *et al.* (1992).