Dissection of the locus control function located on the chicken lysozyme gene domain in transgenic mice

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ABSTRACT

The entire chicken lysozyme gene locus including all known cis-regulatory sequences and the 5' and 3' matrix attachment sites defining the borders of the DNase I sensitive chromatin domain, is expressed at a high level and independent of its chromosomal position in macrophages of transgenic mice. It was concluded that the lysozyme gene locus carries a locus control function. We analysed several cis-regulatory deletion mutants to investigate their influence on tissue specificity and level of expression. Position independent expression of the gene is lost whenever one of the upstream tissue specific enhancer regions is deleted, although tissue specific expression is usually retained. Deletion of the domain border fragments has no influence on copy number dependency of expression. However, without these regions an increased incidence of ectopic expression is observed. This suggests that the domain border fragments may help to suppress transgene expression in inappropriate tissues. We conclude, that position independent expression of the lysozyme gene is not controlled by a single specific region of the locus but is the result of the concerted action of several tissue specific upstream regulatory DNA elements with the promoter.

INTRODUCTION

Transcriptional activation of a gene locus leads to distinct changes in the structure of the surrounding chromatin. Active genes or gene clusters are located within regions of general DNase I sensitivity, which are not restricted to the coding region, but extend into 5' and 3' flanking chromatin (1–3). Active cis-regulatory elements usually located within the domain of general DNase I sensitivity adopt a distinct non-nucleosomal conformation, which renders them hypersensitive with respect to DNase I digestion [For review: (4)]. It is not known whether chromatin decondensation is autonomous and has to preceed the

transcriptional activation of a gene, or whether the general DNase I sensitivity of active gene loci is the consequence of a topological constraint on the chromatin as a result of transcription itself (5). The first model would imply a hierarchy of cis-regulatory DNA elements some of which would function as master switches responsible for the initial, differentiation dependent commitment of a gene locus towards gene expression. Evidence for these type of regulatory elements comes from experiments describing the features of DNA elements having a dominant control function in the activation of gene expression, the locus control regions (LCRs). LCRs were first described in the human β-globin gene cluster (6,7) and have been detected in a variety of developmentally regulated genes (8–15). LCRs have transcriptional enhancer activity, are necessary for high level, tissue specific expression and direct transcription independent of the random chromosomal position of integrated transgenes (6,16). They are often associated with developmentally stable DNase I hypersensitive chromatin sites (DHSs), which are present before specific mRNA can be detected (7,17,18). In the human β-globin system the formation of DNase I sensitive chromatin is dependent on the presence of at least parts of the LCR (19). However, chromatin analysis of deletion mutants of the chicken β-globin locus in transgenic mice indicate the dependence of the establishment of generally DNase I sensitive chromatin on the presence of both the enhancer/LCR and the promoter (20).

The chicken lysozyme gene, one of the major egg white protein genes is expressed in the oviduct. The same gene is also expressed in the myeloid lineage of the hematopoietic system. Expression is gradually switched on during macrophage differentiation, reaching its highest level in the mature, activated macrophage (21,22). Lysozyme gene transcription is regulated by a complex set of cis-regulatory DNA elements consisting of several tissue specific enhancers, a silencer and promoter elements (21–25). The activity of these cis acting elements is marked by tissue specific DHSs (26–28). The active gene locus including all cis acting elements is located within a domain of general DNase I sensitivity, which spans 24 kb (2). The DNase I sensitive domain

is flanked by DNA sequences which have a high affinity for the nuclear matrix *in vitro* (29). The domain border fragment containing the 5' matrix attachment region was shown to be able to stimulate transcription in stable transfections and to buffer linked reporter genes from chromosomal position effects (30,31). When the complete chicken lysozyme locus is introduced into the germline of mice it is expressed as a macrophage specific, independent regulatory unit regardless of its random position in the host genome (32). We therefore conclude, that this DNA construct contains the locus control function of this gene.

Two regions within the lysozyme locus outside the immediate promoter area carry well characterized macrophage specific enhancers and could serve as candidates for a LCR. One enhancer is located 2.7 kb, the second one 6.1 kb upstream of the transcription start (23,33,34). The activity of both enhancers is marked by the presence of a DHS in the chromatin of macrophage cells. The DHS at the −6.1 kb enhancer is present in all active or potentially active cell types (oviduct, all retrovirally transformed macrophage precursor cell lines and primary macrophages) regardless of the level of lysozyme mRNA (22,27). In contrast, the DHS at the −2.7 kb enhancer appears only in cells representing late stages of macrophage differentiation where the transcriptional activity of the lysozyme gene is high. It is not present in the oviduct (24,27). These results suggested a differential temporal regulation of the activity of the two enhancers during development (22). Transfection assays have shown both enhancers to be tissue specifically active in myeloid cells (21,23,33,34). However, from these experiments it could not be decided whether the activation of the lysozyme locus during development can be mediated by the independent action of both enhancers or whether the activiation of the −2.7 kb enhancer and the subsequent activation of lysozyme gene expression is dependent on the previous function of the −6.1 kb enhancer. It is also not known whether position independent expression can be assigned to one or several *cis* acting elements.

In this report, we describe the role of these two regulatory regions and the domain border fragments with respect to the activation of the chicken lysozyme gene domain in macrophages of transgenic mice. We show that both enhancer regions do not act in a hierarchical order since they can individually activate the lysozyme gene in a tissue specific manner. In the presence of the complete set of regulatory sequences deletion of the domain border fragments has no consequences on macrophage specificity and copy number dependency of transgene expression. However, absence of the domain border fragments in constructs carrying incomplete regulatory units results in a higher incidence of ectopic expression. Copy number dependency of expression can not be assigned to a single regulatory element. Only the presence of the complete set of *cis*-regulatory sequences ensures position independency of transgene expression at random chromosomal positions.

MATERIALS AND METHODS

Construction of deletion mutants of the chicken lysozyme domain

All constructs and their subsequent injected fragments were derived from plasmid polIIIilys (32). The *XbaI−SmaI* XS fragment was purified from a polyIIIilys plasmid in which the second *XbaI* site located in the second intron was inactivated (pIIIilys-X2). The Xba' (X') site shown in Figure 1A is not cleaved in procaryotic DNA due to an overlapping dam-methylation sequence. The KS fragment was purified from polyIIIilys digested with *KpnI* and *SmaI*. The dXK construct was prepared by ligating the 3 kb B-1X1 fragment (30) carrying the 5' matrix attachment sites into polyIIIi K1X4 (32) cut with Asp718. The dSS construct was prepared as follows: The 1.6 kb fragment reaching from *SacI* site [S4; (35) at −900 to the *BamHI* site at +700 (B3)] was ligated into plasmid polyIIIi (pIIIiS4B3) followed by the ligation of the 5.0 kb *BamHI* fragment reaching from *BamHI* site 3 to *BamHI* site 4 which carries the rest of the coding region and the poly A addition point into the *BamHI* site of pIIIiS4B3. This construct was named pIIIilysS4B4. pIIIilysS4B4 was then digested with *EcoRV* which cuts in the polylinker of the vector immediately upstream of the S4 site and with *SpeI* which cuts at +2600 in the second intron. The 3.5 kb *EcoRV−SpeI* fragment was ligated into polyIIIilys digested with *SpeI* which cleaves at −3000 and at +2600. The injected dSS and dXK fragments were liberated from the vector by digestion with *XhoI*. The injected XSdSS fragment was generated from the dSS construct by digestion with *XbaI* and *SmaI*.

Generation and characterization of transgenic mice

The fragments carrying different parts of the chicken lysozyme gene domain were purified from vector sequences as described (36) and injected into fertilized oocytes from CBAxC57 bl mice (37). Transgenic mice were identified by PCR analysis of tail DNA using chicken lysozyme specific primers and Southern blotting. Copy numbers of integrated transgenes were determined by phosphorimager analysis of genomic Southern blots from *EcoRI* digested genomic DNA hybridized simultaneously with a lysozyme specific probe recognizing a 2.7 kb *EcoRI* fragment located in the 3' half of the gene (35) and a Thy1 probe (6) recognizing the endogenous mouse gene to control loading differences. Single copy mice in the KS-series were identified by cleavage of tail DNA with *BamH1*, which cuts only once in the integrated fragment. *EcoRI* digested plasmid polyIIIilys or chicken genomic DNA were used as a standard for the calculation of actual copy numbers. In order to eliminate founder mice chimeric for the injected gene, DNA from liver, spleen, brain and kidney was tested for the copy number of the lysozyme transgene by Southern blotting. Most other mouse lines were bred before expression analysis to generate F1 generations if not otherwise indicated. Mouse lines 0a and 0b were generated from one founder mouse carrying two different nonlinked transgene clusters which could be bred into two lines carrying one integration site respectively, as demonstrated by Southern blot analysis of end fragments. Homozygous mouse line 4 (32) carries 100 copies of the complete wild type (wt) lysozyme gene domain.

RNA preparation and S1 protection analysis

RNA from various tissues of at least six week old mice were prepared with LiCl/urea (38). RNA from cultured chicken cells and from primary mouse macrophages was prepared by the acid guanidinium thiocyanate−phenol−chloroform extraction method (39). A 530 bp *BstNI* fragment spanning the promoter region served as a chicken lysozyme specific S1 hybridization probe (21), a 320 bp *BamHI−AvaI* fragment from pHFβA-1 (40) was used as a β-actin specific probe. S1 analysis for chicken lysozyme and β-actin mRNA was performed as described (32). To construct a mouse lysozyme hybridization probe a 320 bp *EcoRI−PstI*

fragment of plasmid pmLc1 carrying the mouse lysozyme M cDNA (41), containing exons 1 and 2 was cloned into Bluescribe (M13+) and named pmLc2. A 315 bp *Bst*NI fragment isolated from pmLc2 containing vector sequences provided a mouse lysozyme specific internal hybridization probe. Hybridization with this probe was performed at 50°C overnight and digestion with 100 units S1 nuclease was performed for 1.5 h at 37°C. S1 resistant fragments were run on a 7.5% polyacrylamide/urea gel. S1 hybridization signals were quantified by phosphorimager analysis. To determine the expression levels per gene copy S1 hybridization signals for β-actin and chicken lysozyme were quantified. The chicken lysozyme expression value was then calibrated with respect to the β-actin specific signal. To prove that the calculated expression level per gene copy in XS mice was correct, an S1 hybridization assay was performed in which RNA samples from transgenic mouse macrophages were diluted with macrophage RNA from nontransgenic mice to contain the same amount of specific RNA as determined in the previous assay and the same amount of total RNA. The first S1 hybridization assay containing the same amount of transgenic mouse macrophage RNA was then repeated.

Cell cultures

HD11 cells (42) were grown in standard Iscove's medium supplemented with 8% fetal calf serum and 2% chicken serum. Mouse L-cell fibroblasts used to prepare conditioned medium were grown in Iscove's medium, 10% fetal calf serum. Primary mouse macrophages were prepared by peritoneal flush 4 days after injection of 3% Thioglycolate medium (Gibco) in PBS into the peritoneal cavity of transgenic mice. Cells were cultured overnight in Iscove's medium plus 10% L-cell conditioned medium (32).

RESULTS

The generation of transgenic mouse lines carrying deletion mutants within the chicken lysozyme gene domain

We generated a series of transgenic mice carrying constructs in which one or several DNA elements of the complete lysozyme gene domain were deleted (Figure 1A). Two constructs carry internal deletions. In one of them a 3.4 kb fragment containing the early −6.1 enhancer was removed (dXK). In addition this fragment included DNA sequences with no apparent *cis*-regulatory function adopting a constitutive DHS at −7.9 kb in the chromatin of all cell types investigated to date. In the other construct a 2 kb fragment carrying three *cis*-regulatory elements including the −2.7 kb enhancer (dSS) was deleted. The latter deletion in addition removes the silencer element at −2.4 kb (43) and the steroid responsive element necessary for expression of the gene in the oviduct (44). The DHSs marking the activity of the silencer is present in macrophage progenitor cells, non expressing cells and the oviduct (27). Its disappearance parallels the appearance of the −2.7 kb DHS during macrophage maturation (22). In mature primary macrophages only the −2.7 kb DHS is present and the −2.4 kb DHS is absent (27,28). In another series of constructs carrying either the complete set of *cis* acting elements or internal deletions as described above the 5′ and 3′ domain border fragments were removed (XS, KS, XSdSS). The 3′ domain border fragment carries a DHS, which is only present in oviduct and non-expressing cells, but absent in macrophages (27).

For each construct between 4 and 12 different mouse lines were generated (Figure 1B). Copy number and integrity of the integrated transgenes was investigated by Southern blotting. No evidence for rearrangements within the integrated gene clusters was found (data not shown). The variation in copy numbers of integrated transgenes in the different mouse lines appeared to be random and independent of the type of insert (Table 1). Most founder mice were bred for the analysis of the F1 generation to avoid quantitation mistakes due to mosaicism. Founder mice used in this analysis were examined carefully to be non-mosaic (see Methods).

Each enhancer region can direct chicken lysozyme transgene expression into macrophages

The wild type construct (wt, Figure 1A) is exclusively expressed in macrophages, spleen and in the brain. Brain expression is specific and is found in the same cell type in several independently investigated transgenic mouse lines (45). Expression in spleen is due to the presence of macrophages in this organ (32,45). Within the limits of the assay, expression could not be found in any other cell type (Figure 1B). Without the upstream region harboring the early −6.1 kb enhancer (dXK) macrophage specific lysozyme expression is observed in three of five mouse lines (Figure 2A, mice 0, 2, 6 with 2, 13 and 13 gene copies, respectively). One mouse line did not express the gene in any tissue (mouse 9 with 8 gene copies) and one mouse line expressed the gene at approximately the same level in four different tissues including macrophages (Figure 2A, mouse 25 with 43 gene copies). In all mouse lines brain expression was below the level of detection. Without the intermediate region carrying the −2.7 kb enhancer/ −2.4 kb silencer region (dSS) transgene expression

Figure 1. (A) Map of the chicken lysozyme locus (upper part) and of the injected DNA fragments (lower part). The intron−exon structure and the coding region are indicated by black vertical bars, the major transcription start is indicated by an arrow. The positions of DNase I hypersensitive chromatin sites (DHS) are indicated by arrows above the map. The domain border fragments harboring matrix attachments regions are indicated by the line with the hook. The position of restriction sites are indicated above and below the map. X: *Xba*I, (X): *Xba*I site which has been removed in some of the injected fragments, X′: *Xba*I site which is not cleaved in procaryotic DNA, K: *Kpn*I, Sp: *Spe*I, Sa: *Sac*I, S: *Sma*I, Xh: *Xho*I. wt : mouse lines described in (32). (B) Summary of the results presented in this paper. WT expression pattern refers to expression observed in macrophages only or macrophages and brain as observed with the wild type construct, nonspecific expression refers to expression in other tissues. In some cases the wild type pattern and additional nonspecific expression is observed. Expression in lung is regarded as nonspecific. Mouse lines not listed in the table do not express the transgene in any tissue. var: variable levels per gene copy in different transgenic lines.

Table 1. Expression of deletion mutants within the lysozyme gene domain in transgenic mice

construct	mouse line	lysozyme mRNA levels in macrophages (arbitrary units) [a]	β-actin mRNA levels in macrophages (arbitrary units) [a]	lysozyme mRNA level in macophages (normalized values) [b]	copy number [c]	normalized lysozyme mRNA level per transgene copy (+/- 30%)
WT	4[d]	41825	11112	41825	100	418
dXK	25	2757	69729	439	43	10
	2	67066	31641	23552	13	1811
	6[e]	25052	42474	6554	13	504
	9[e]	0	63979	0	8	0
	0	14066	45041	3470	2	1735
dSS	4	1442	55984	286	35	8
	18	614	20416	334	34	10
	34	43215	30231	15884	24	661
	7	17255	23268	8233	13	633
	8	5546	25543	2412	11	219
	15	1424	56684	279	10	3
	12	307	19944	171	8	21
XS	0b	198600	23820	92646	74	1251
	13	46951	12660	42488	15	2832
	15[e]	45852	17892	28476	10	2847
	0a	49596	16488	33424	9	3713
	N	44316	25752	19122	7	2731
KS	C11[e]	0	13561	0	63	0
	A5[e]	29606	19133	17194	54	318
	C13[e]	7608	23972	3526	38	92
	B2[e]	0	8850	0	15	0
	B5[e]	0	33872	0	8	0
	C1[e]	0	32852	0	4	0
	C3[e]	1241	14580	945	3	315
	B0[e]	7608	11970	7062	2	3531
	C6[e]	0	26678	0	2	0
	A13[e]	9837	14103	7750	1	7750
	B1[e]	923	20661	496	1	496
	A0[e]	0	24003	0	1	0
XSdSS	26	809	15282	588	138	4
	5	8875	24998	3945	71	55
	0	3088	28278	1213	46	26
	28	59328	22325	29529	16	1845

[a]Expression levels were quantified by phosphorimager analysis.

[b]Lysozyme expression levels were normalized with respect to WT β-actin expression levels. The expression levels per gene copy in XS mice were determined by repeated S1 analysis as described in Methods.

[c]Copy numbers were determined by quantitive Southern blotting.

[d]Bonifer et al., 1990.

[e]Founder mice were analyzed.

could be detected in macrophages of all mouse lines. All mice except mouse 12 showed a significant level of brain expression (see also Figure 5). Expression in macrophages of four of seven mouse lines was higher than in brain (Figure 2B, mice 34, 7, 8, 18 with 35, 13, 11 and 34 gene copies, respectively), in two mouse lines it was lower (Figure 2B, mice 15 and 4 with 10 and 35 gene copies; see also Figure 5A and Table 1) and one mouse expressed the transgene at a very low level (Figure 2B, mouse 12 with 8 gene copies; see also Table 1). No lysozyme mRNA was found in any other tissue. Taken together, we conclude that deletion of either enhancer region from the chicken lysozyme domain does not disable transgene expression in macrophages.

The presence of the complete set of *cis*-regulatory sequences results in high level and macrophage specific expression

Lysozyme DNA constructs carrying two copies of the 5' matrix attachment region flanking the −6.1 kb enhancer, the lysozyme promoter and the CAT reporter gene have in previous experiments been stably transfected into chicken promacrophage cells. They were expressed in a position independent manner with an exponential relationship between copy number and expression level. This result led to the hypothesis that the border elements buffer the chromatin mediated position effect usually observed after stable transfection (30). We were interested to examine the role of the domain border fragments in the context of the whole lysozyme gene domain in the more stringent transgenic mouse system.

The first construct we examined (XS) lacked the domain border fragments, but still carried all *cis*-regulatory sequences. All five different mouse lines carrying this construct expressed the transgene at a high level in macrophages (Figure 3). The expression pattern was very similar to that observed in mice carrying the wild type construct (32). In addition, lysozyme expression levels followed transgene copy numbers (Table 1, see below). Two mouse lines showed a low level of chicken lysozyme expression in thymus and lung (Figure 3, mice 0a and 0b with
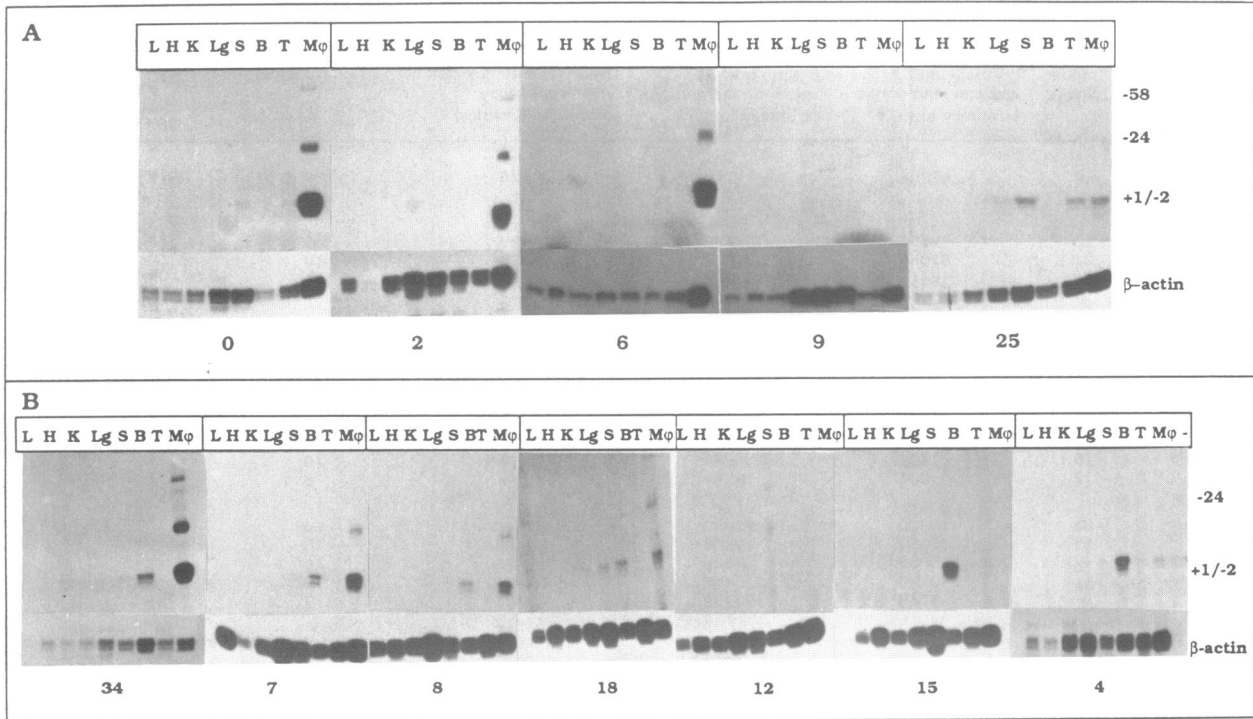
Figure 2. (A) Expression of the dXK construct in transgenic mouse tissues. RNA was prepared from liver (L), heart (H), kidney (K), lung (Lg), spleen (S), brain (B), thymus (T) and macrophages (Mφ). 7 μg of RNA were subjected to S1 hybridization analysis with a chicken lysozyme specific probe (upper panel) or a β-actin probe (lower panel). S1 nuclease resistant fragments were analyzed on a 7.5% polyacrylamide/urea gel. +1/−2, −24 and −58 bp refer to the three main transcriptional start sites on the lysozyme promoter (47). (B) Expression of the dSS construct in transgenic mouse tissues. 7 μg of RNA prepared from various mouse tissues were examined by S1 hybridization analysis as described in the legend of Figure 2. (−): no RNA. For copy numbers and nomenclature see Table 1.
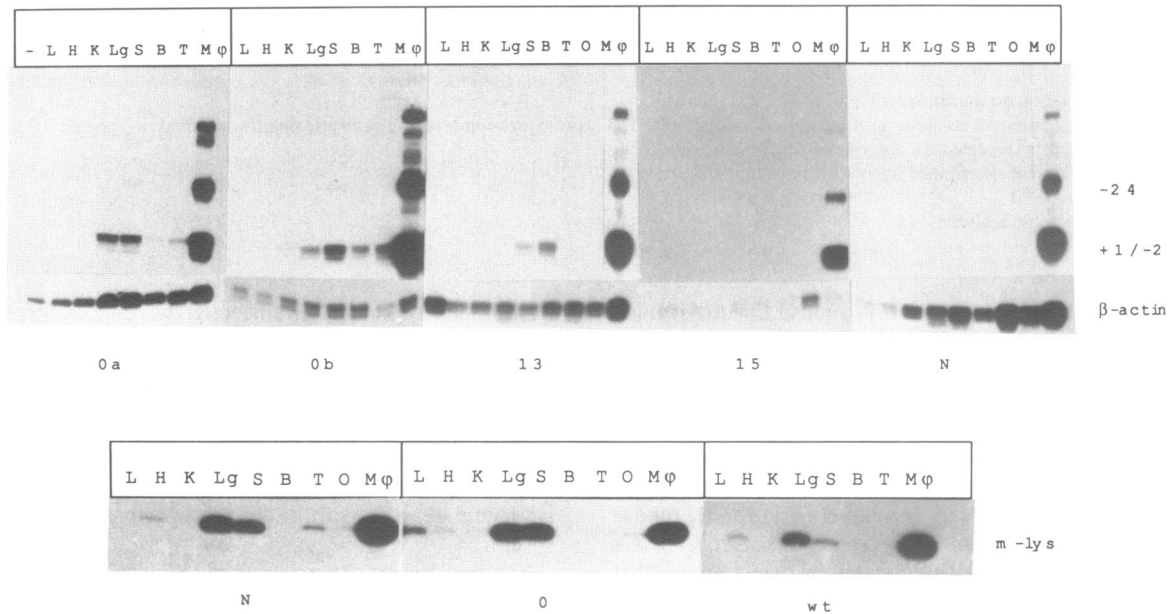

Figure 3. Expression of the XS construct in transgenic mouse tissues. 7 μg RNA prepared from different mouse tissues as described in the legend of Figure 2 were analyzed in the S1 protection assay with probes specific for chicken lysozyme and β-actin (upper panels), as well as for mouse lysozyme (m-lys; lower panel). O = oviduct. wt refers to a mouse line carrying 100 copies of the wild type construct. 0 refers to an offspring of the original founder still carrying both transgene integration loci before breeding into lines 0a and 0b. (B) Expression of the KS construct in transgenic mouse tissues. 7 μg RNA prepared from various mouse tissues were subjected to an S1 analysis as described in the legend of Figure 2. (+): RNA prepared from macrophages of mice carrying the wild type construct. For copy numbers and nomenclature see Table 1.
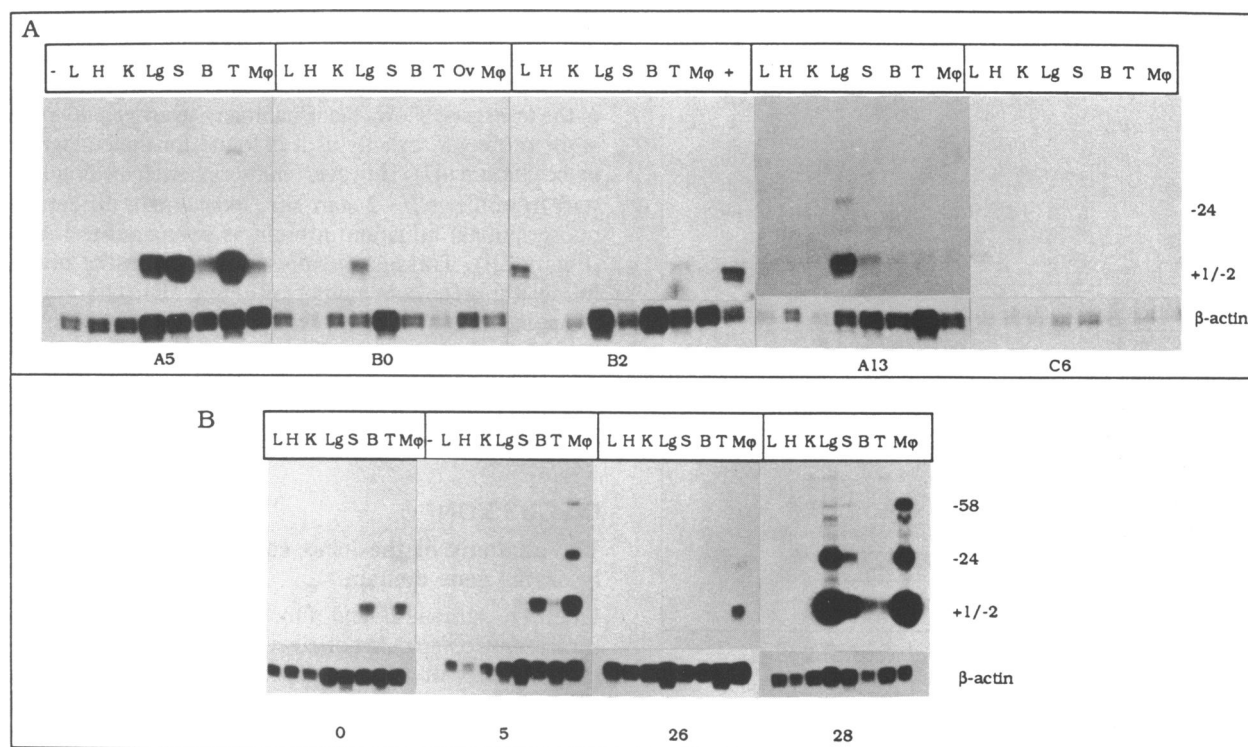
Figure 4. Expression of the XSdSS construct in transgenic mouse tissues. 7 μg RNA prepared from various mouse tissues were subjected to an S1 analysis as described in the legend of Figure 2. For copy numbers and nomenclature see Table 1.

9 and 74 gene copies, respectively). For comparison we measured endogenous mouse lysozyme expression in the different tissues of three different transgenic mouse lines (Figure 3, lower panel). Each of them showed a significant amount of endogenous mouse lysozyme mRNA in lung and spleen, as it has been seen before (41,46). However, we were unable to detect lysozyme mRNA in lungs of mice carrying the wild type construct, therefore we regard expression of the transgene in the lung as nonspecific. No differences in transgene expression could be found between individual members of each line (data not shown). It is obvious that deletion of the domain border fragments has no effect on the expression of the transgene in macrophages.

Incomplete regulatory units without domain border fragments show an increased incidence of nonspecific expression

The next constructs we investigated lacked the domain border fragments and carried only one of the enhancer regions. The first construct (KS) contained only the −2.7 kb enhancer region. The analysis of transgene mRNA levels in various tissues of 12 founder mice showed, that half of the mice did not express the transgene at all. Most of the remaining mice showed low transgene expression levels in macrophages (Figure 4A). Furthermore, we found lysozyme mRNA to be expressed ectopically. One mouse (Figure 4A, mouse A5 with 54 gene copies) expressed the gene at different levels in thymus, spleen and lung but at a low level in macrophages. In four other mice lysozyme mRNA was found in the liver (Figure 4A, mice B2 and C6 with 15 and 2 gene copies, respectively) or in the lung (Figure 4A, mice A13 and B0 with 1 and 2 gene copies, respectively). One mouse (C13 with 38 gene copies) expressed

the gene at a very low level in all tissues analyzed (data not shown). Transgenic mice carrying a construct with only the −6.1 kb region (XSdSS) showed a similar expression pattern as mice carrying the dSS construct (Figure 4B). Expression in the brain could be observed in all mice including mouse 26 (a specific signal was visible after longer exposure of the film, data not shown). One mouse, however, expressed a very high level of lysozyme mRNA in the lung (Figure 4B, mouse 28). In lung cells the three lysozyme specific transcriptional start sites at the promoter are not used with the same relative frequency as in macrophage cells. Only the +1/−2 and the −24 start sites are used, whereas macrophages use an additional start at −58 (47). With both constructs a significantly increased incidence of nonspecific expression is observed when compared to mice carrying the same *cis*-regulatory elements plus the domain border fragments. Only 1 out of 11 expressing dXK and dSS mice expressed the lysozyme transgene ectopically, whereas this is the case for 7 out of 10 expressing KS and XSdSS mice (Figure 1B).

Position independency of expression is a result of the cooperation of all *cis*-regulatory elements within the chicken lysozyme gene domain

One of the key definitions of locus control regions is their ability to direct copy number dependent expression of transgenes irrespective of their random position of integration into host chromatin. We examined the precise transgene expression level in macrophages (Figure 5A) and determined the expression levels per gene copy (Table 1). All chicken lysozyme expression values were calibrated with respect to β-actin mRNA levels in order to compare absolute expression levels (Table 1). The average
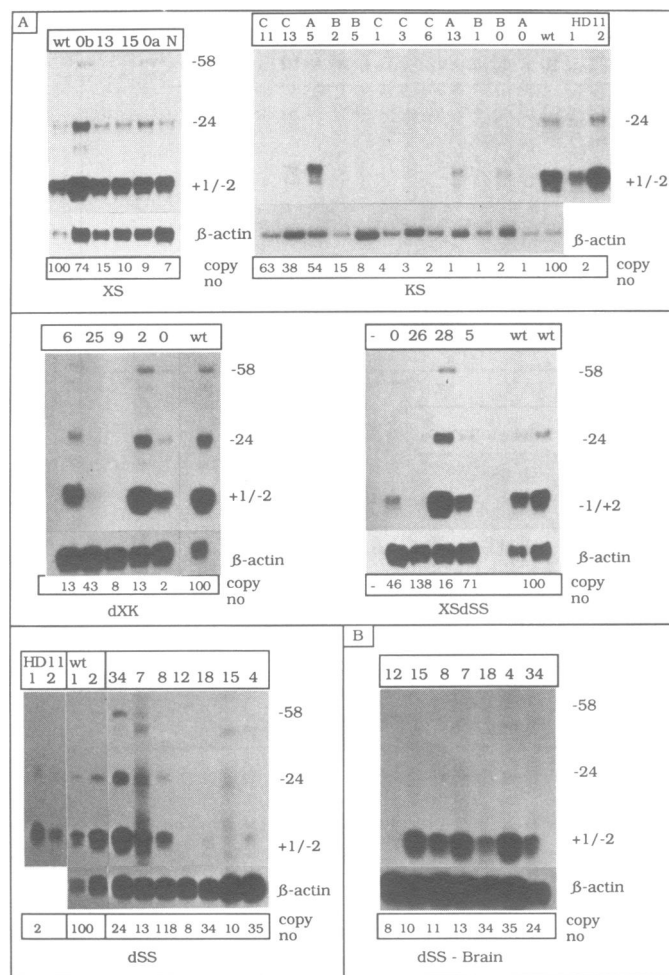
Figure 5. (A) Comparison of the chicken lysozyme expression level in macrophages of different mouse lines carrying the various constructs. For nomenclature see Table 1. Macrophage RNA was subjected to S1 protection analysis as described. KS, dXK: 7 µg RNA, dSS, XSdSS: 3.5 µg RNA, XS: 1.5 µg RNA, respectively. RNA prepared from macrophages carrying the wt construct served as standard. wt control in XS, dSS and XSdSS: 0.5 µg, 1.75 µg and 3.5 µg RNA, respectively. 15 µg (1) and 30 µg (2) HD 11 RNA served as an additional standard (Panel KS and dSS). The gels were exposed to a Kodak XAR film with an intensifier screen at −70°C. Exposure times was adjusted with respect to the wt standard. (B) Comparison of the chicken lysozyme expression level in brains of mice carrying the dSS construct. For nomenclature see Table 1. 7 µg RNA was subjected to S1 hybridization analysis as described.

expression level per gene copy for each of the constructs lacking one enhancer region (KS, XSdSS, dXK, dSS) is very low. All 28 mouse lines carrying these constructs show highly variable levels of transgene expression in macrophages indicating the influence of strong chromosomal position effects. The only exception, in addition to the previously shown wt construct (32), is construct XS which also carries all *cis*-regulatory DNA elements. Expression levels in macrophages of XS mice are consistently higher in macrophages than in any other tissue (Figure 3) and follow transgene copy number. The expression level per gene copy is similar for all five individual lines.

Brain expression in mice carrying the wild type construct was copy number dependent and thus independent of the genomic position (32). To investigate the role of the different *cis*-regulatory

regions in the brain, we took advantage of the mouse lines carrying the dSS construct which express the chicken lysozyme transgene in macrophages and brain. In macrophages transcription of the transgene shows the usual microheterogeneity of start sites at the promoter, exactly as it is found for the endogenous gene in the chicken (47). However, transcription in the brain did mostly start from the +1/−2 start site, indicating a different mode of transcriptional initiation in brain as compared to macrophages (Figure 2B). Transgene expression levels in the brain did not follow transgene copy numbers (Figure 5B). The comparison of brain and macrophage expression levels in the same transgenic animal showed that high expression in macrophages does not parallel high expression in the brain (Figure 4B), despite the fact that in the two different tissues the same construct is integrated at the same chromosomal position.

DISCUSSION

The anatomy of the locus control function located on the lysozyme gene domain

Extensive structural and functional studies have revealed a surprisingly complex set of *cis*-regulatory DNA elements involved in the control of the tissue specific activation of the lysozyme gene locus in oviduct and in macrophages [for review: (24,25)]. In the analysis described here we focused on two regions containing the −6.1 kb enhancer and the −2.7 kb enhancer which are the main myeloid specific stimulatory elements located within the lysozyme gene domain. The anatomy of both enhancers was extensively characterized. The −6.1 kb enhancer core is composed of seven different modules harboring binding sites for the NF1, AP1, C/EBP transcription factor families and two unknown proteins (23). The −2.7 kb enhancer has a less complex structure, it consists of binding sites for proteins of the *ets*-, AP1-, and C/EBP-transcription factor families (48,49). The experiments described in this paper show the contribution of each regulatory region to correct transgene regulation:

In ten of twelve mouse lines macrophage specific expression of the transgene can be achieved with constructs flanked by the domain border fragments and containing only one of the two enhancer regions. However, the two enhancer regions have different capabilities, which is most apparent in constructs lacking the domain border fragments. In that case the −6.1 kb enhancer region can still direct macrophage specific expression. The −2.7 kb region is unable to do so, which may be the result of its less complex modular structure or its different temporal regulation. The finding that neither enhancer region is capable of directing position independent expression on its own differs from results obtained with subregions of the β-globin LCR. Such elements can direct position independent gene expression, albeit at lower level (50) and only when the gene constructs are present in multiple copies (51), suggesting that β-globin LCR subregions in contrast to the lysozyme elements are able to interact with themselves to provide LCR activity.

Lysozyme constructs containing the domain border fragments but only one enhancer region were not expressed in a copy number dependent way. Hence, the domain border fragments are not sufficient to insulate a gene lacking one essential *cis*-regulatory element from position effects in lysozyme expressing cells. However, mice carrying the same deletions but lacking the domain border fragments show a significantly higher incidence of ectopic transgene expression (7 of 10 expressing KS and

XSdSS mice) than those with border fragments present (1 of 11 expressing dXK and dSS mice). This result indicate that the domain border fragments help to suppress ectopic transgene activation by random neighbouring *cis* acting elements. It would be premature to make any suggestions about the molecular nature of this insulating function on the basis of the present results. It is also difficult to compare the buffering effect of the two domain border fragments with the position effect buffering activity of the 5' domain border fragment observed in stably transfected retrovirally transformed promacrophage cells in culture (30). The transfected constructs were artificial combinations of lysozyme regulatory sequences and the CAT reporter gene. In transgenic mice we analyzed constructs of a more complex nature which, with the exception of the deletions, have the correct spatial arrangement of regulatory sequences. Secondly, retrovirally transformed monocytes reflect only one differentiation status of the myeloid lineage and DNA transfected into cultured cells will most likely integrate into active chromatin regions. In the transgenic mouse the DNA is integrated at an early stage where transcription is absent (52) and without selection for chromatin regions active in myeloid cells. During development integrated DNA constructs have to be able to reorganize chromatin structure, to activate transcription in the right cell type and to suppress activation in all other cells. Constructs which are expressed correctly in transfected cultured cells might therefore not fulfil all demands of the more stringent transgenic animal system.

The concerted action model for the correct function of transgene promoters

The DHS pattern of the early −6.1 kb enhancer and the late −2.7 kb enhancer in various chicken cells suggested a stepwise activation of the gene locus during macrophage differentiation (22). It was therefore speculated, that the −6.1 kb enhancer and not the −2.7 kb enhancer could act as a dominant control element (25) and by that being a prerequisite for the activation of the later acting *cis*-regulatory elements. The experiments described in this paper show, however, that if flanked by domain border fragments each individual enhancer region is by itself capable of activating lysozyme transcription in macrophage cells. The activity of one enhancer is not dependent on the previous function of the other, instead they cooperate.

We conclude that only the concerted action of all regulatory elements of a gene locus leads to a correct developmental regulation of a transgene. It is, therefore , no surprise that the transfer of transgenes on yeast artificial chromosomes (YACs) into the mouse germ line results in a correct regulation of transgenes (53,54). Due to their large size YACs will usually include the complete regulatory domains of gene loci even when their structural and functional extensions are not yet determined. Most likely the regulatory protein complex at the promoter requires a saturating amount of interacting transcription factors bound to other *cis*-regulatory elements provided by a complete gene locus, otherwise deregulating position effects become apparent. Support for this interpretation comes from experiments examining the chromatin structure of the different lysozyme transgenes in mouse macrophages. In macrophage nuclei of all mouse lines carrying position independently expressed transgenes all DHSs indicating the activity of *cis*-regulatory elements are formed in the chromatin of all transgene copies. This is not the case in mouse lines carrying transgenes with position dependent

expression (55). Currently we examine the interaction of each individual *cis*-regulatory element with the promoter region in order to define their exact role in the tissue and stage specific transcriptional activation of the lysozyme locus in transgenic mice.

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