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Contrasting Effects of the SATB1 Core Nuclear Matrix Attachment Region and Flanking Sequences of the Keratin 18 Gene in Transgenic Mice

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The 2.3 kb and 3.5 kb of DNA that flank the human keratin 18 (K18) gene and synthetic nuclear matrix attachment regions (MAR) composed of the binding sites for the SATB1 nuclear protein were fused to a reporter gene that utilizes the mouse metallothionein promoter and the human growth hormone gene (MThGH). Transgenic mice were generated from both constructions and the control MThGH gene to test K18 and SATB1 MAR sequences for the ability to insulate the reporter gene from integration site-specific position effects. The MThGH control gene was variably expressed in brain, heart, intestine, kidney, liver, and testes, confirming previous studies. In contrast, the MThGH gene insulated by the K18 flanking sequences was expressed in the same tissues of four independent transgenic animals at levels correlated with the copy number except for intestine. The average level of expression on a per gene basis of the K18 insulated gene was from 9- to 49-fold higher than the control. The MThGH gene linked to the SATB1 MAR sequences was completely repressed in the brains and kidneys of all six transgenic mice. However, expression was nearly as efficient in testes as the K18-insulated gene. Both the SATB1 MAR and the K18 flanking sequences confer position-independent transcriptional status on the reporter gene in some or many tissues. However, the effects are stimulatory for the K18 elements and generally suppressive for the SATB1 MAR elements.

INTRODUCTION

How are genetic regulatory domains defined within the chromatin environment of the mammalian nucleus (Felsenfeld, 1992)? Several types of DNA elements have been implicated in the definition of independent chromosomal regulatory domains. Locus control regions (LCR), originally discovered associated with the β-globin locus, appear to act as strong, positive transcriptional regulatory elements that ensure tissue-specific expression independent of the particular site of integration (Grosveld *et al.*, 1987; Ryan *et al.*, 1989; VanAssendlft *et al.*, 1989; Felsenfeld, 1992; Palmiter *et al.*, 1993). LCRs are composed of multiple elements each of which may contribute to appropriate transcriptional regulation, position-independent expression, and/or the initiation of DNA replication (Aladjem *et al.*, 1995; Fiering *et al.*, 1995; Jones *et al.*, 1995). DNA sequences that have high affinity to the nuclear matrix, called nuclear matrix attachment regions (MAR; also called SARs for scaffold attachment regions), may define the attachment sites of chromosomal DNA to nuclear structures and to separate chromatin into topologically and functionally independent loop domains (Cockerill and Garrard, 1986; Gasser and Laemmli, 1986; Gross and Garrard, 1988; Laemmli *et al.*, 1992). The co-localization of transcriptional regulatory elements and topoisomerase II sites to MARs and their capability of activating linked genes in stable transformants (Cockerill and Garrard, 1986; Gasser and Laemmli, 1986; Bode *et al.*, 1992; Poljak *et al.*, 1994) have further reinforced the potential importance of MAR elements in physically defining chromosomal regulatory domains. MAR sequences, which are generally AT rich, contain a core element that readily

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relieves negative superhelical strain by base unpairing or unwinding (Kohwi-Shigematsu and Kohwi, 1990). This core MAR is important for high affinity binding to the nuclear matrix and for stimulating transcription of MAR-linked genes in stable transformants (Bode *et al.*, 1992). SATB1, a protein expressed primarily in thymocytes, specifically binds to core elements within a MAR (Dickinson *et al.*, 1992). However, the role of MARs in defining the boundaries of chromatin domains remains controversial, in part because the effects of MARs may depend upon the particular combination of MARs and other regulatory elements and also because of the relative large size of MAR binding fragments that have been tested. All MARs do not function as chromatin domain boundaries (Poljak *et al.*, 1994). However, MARs have been reported to have positive effects upon expression in both transgenic mice (Phi-van *et al.*, 1990; McKnight *et al.*, 1992; Brooks *et al.*, 1994; Thompson *et al.*, 1994) and in stably transfected cells (Stief *et al.*, 1989; Bode *et al.*, 1992; Poljak *et al.*, 1994; Yu *et al.*, 1994). The importance of MARs in tissue-specific expression was illustrated for MARs associated with an immunoglobulin heavy chain (IgH) locus. These are essential for transcription of a rearranged Ig gene in transgenic B lymphocytes (Forrester *et al.*, 1994).

Another type of DNA activity implicated in defining regulatory domains of genes is a "boundary" or "insulator" type activity. An example of this activity are the scs and scs' sequence elements that flank the *Drosophila melanogaster* hsp70 gene in the 87A7 heat shock locus. These elements can confer upon a transposable reporter gene the characteristic of integration site-independent expression (Kellum and Schedl, 1991). In addition, they function to block the effect of distally positioned enhancer elements (Kellum and Schedl, 1992). A protein BEAF-32, which binds to the scs' elements and may participate in boundary activity, has been identified and cloned (Zhao *et al.*, 1995). Similarly, a fragment of the chicken β-globin locus upstream of the LCR, containing the DNase hypersensitive site four was shown to substitute for the *Drosophilia* scs type sequence in conferring position-independent expression and is active in enhancer-blocking assays (Chung *et al.*, 1993). This element is distinct from the strong enhancer activity associated with the β-globin LCR region.

In transgenic mice, random integration of exogenous genes commonly results in variable expression due to the interaction of positive or negative regulatory elements located near the site of integration with the newly integrated gene (Palmiter and Brinster, 1986). The predominant arrangement of integration is a head to tail tandem duplication of the exogenous DNA fragments. The silencing effect of a particular site of integration has been confirmed by rescuing a silenced transgene and showing that it was capable of expression in a different site of integration (Al-Shawi *et al.*, 1990). An insulator or LCR-like activity has been implicated in the behavior of the keratin 18 (K18) gene in transgenic mice. Unlike most fragments used to generate transgenic mice, the 10-kb K18 gene was expressed in every transgenic animal and at levels directly proportional to the number of gene copies integrated (Abe and Oshima, 1990; Neznanov *et al.*, 1993; Thorey *et al.*, 1993). Deletion analysis of the requirements for this behavior in transgenic mice revealed that optimal activity required sequences from both the 5′ and 3′ flanking regions of the gene (Thorey *et al.*, 1993). These flanking sequences did not contribute to the transcriptional activity of the gene in transient transfection analysis. Furthermore, transgenic mice generated from the Herpes simplex virus thymidine kinase gene placed between the K18 flanking sequences expressed the gene in an integration site-independent and copy number-dependent fashion (Neznanov *et al.*, 1993). However, the extremely restricted tissue-specific expression of this transgene prevented conclusions concerning the utility of these putative insulator elements in tissues other than brain and testes or their possible influence on tissue-specific expression.

In the present study, we compared the flanking sequences of the K18 gene and SATB1 binding MAR sequence for their potential insulating activity and their effect on gene expression of a reporter gene in transgenic mice. We employed the mouse metallothionein promoter fused to the human growth hormone gene (MThGH), which is well characterized and widely expressed in various tissues. Although expression of this gene results in accelerated growth and significant pathology in certain organs, the large number of previously characterized, independent, transgenic mice provides a strong basis for evaluating changes in the sensitivity of this particular reporter gene to its site of integration (Palmiter *et al.*, 1983). The position dependency and the level of expression of this reporter gene was determined in transgenic mice for the same gene flanked by either the K18 putative insulator elements or the SATB1 core MAR. Transgenes containing the K18 candidate insulator elements were expressed at elevated levels proportional to the number of integrated genes. This suggests that each copy of the transgene could function independently of both the site of integration and the other neighboring copies of the tandem array of transgenes. In contrast, the MThGH gene flanking by SATB1 binding sites was completely repressed in the brain and kidney, but highly stimulated in the testes of male transgenic animals. These results demonstrate that SATB1 MAR elements when combined with the regulatory elements of the metallothionein promoter and hGH gene, function as active suppressors in multiple tissues but are activating in testis. Both the K18 flanking elements

and the SATB1-binding MAR sequence appear to confer position independence on the transcriptional status of the reporter gene in some or many tissues. However, the effects on transcription are stimulatory for the K18 elements and generally suppressive for SATB1-binding MAR sequence.

MATERIALS AND METHODS

hGH Vectors

The MThGH111 plasmid (Palmiter *et al.*, 1983) was kindly provided by R. Palmiter (University of Washington, Seattle, WA). The *Bst*EII/*Nde*I fragment of the plasmid was used for generating additional MThGH transgenic mice. The nn-MThGH gene was made by cloning the *Bst*EII/*Nde*I fragment with *Xba*I linkers into the unique *Xba*I site of the NNTG vector that contains the 5′ and 3′ distal flanking regions of the K18 gene (Kulesh and Oshima, 1989; Neznanov *et al.*, 1993). *Hin*dIII and *Not*I restriction enzymes were used to remove the construction from the plasmid for generating transgenic mice. For the mar-MThGH construction, the MThGH fragment with *Xba*I linkers was cloned into the BSKS M13$^+$vector. A heptamer of the 25-bp SATB1 binding sites [wild-type $(25)_7$] (Bode *et al.*, 1992; Dickinson *et al.*, 1992), was inserted into a *Not*I site upstream of the MThGH gene and between a *Sma*I and *Hin*dIII site downstream of the gene. The orientation of wild-type $(25)_7$ relative to the MThGH gene is the same as the wild-type $(25)_7$-linked luciferase gene, Lu $(25)_7$ previously reported (Bode *et al.*, 1992). For transgenic mice, the MThGH gene with the MAR flanking sequences was excised by digestion with *Sac*II and *Sal*I.

Transgenic Mice

Transgenic mice were prepared by the standard procedures as previously described (Abe and Oshima, 1990; Neznanov *et al.*, 1993; Thorey *et al.*, 1993) by the Transgenic Mouse Facility at The Burnham Institute. Strain FVB/N mouse eggs were injected and transferred to CD-1 foster mothers. Founder nn-MThGH and MThGH animals identified by dot blot hybridization of tails DNAs were killed for analysis between 8 and 12 wk of age because they failed to breed, due to both direct and indirect effects of excessive hGH production (Bartke *et al.*, 1994). The mar-MThGH-8 and mar-MThGH-24 founders were fertile and were mated once to generate progeny for analysis of thymus. The two founders were sacrificed for RNA analysis.

Nucleic Acid Analysis

Transgene copy number was determined by dot blot or Southern blot hybridization with the hGH probe, followed by quantitation in an Ambis radioactivity image analyzer or by scanning exposed films with a laser densitometer (Ultroscan XL; LKB, Alameda, CA). DNA concentration was determined fluorometrically (Labarca and Paigen, 1980). To minimize variation due to DNA loading or retention on the filter, each DNA spot or band was normalized by hybridizing the stripped filter with mouse L32 ribosomal protein gene or mouse MT-1 cDNAs hybridization probes. The estimation of the copy number of the transgene was performed as previously described (Neznanov *et al.*, 1993). RNA was purified from dissected mouse tissues with the use of guanidine isothiocyanate and ultra centrifugation in cesium chloride (Chirgwin *et al.*, 1979). The levels of hGH RNA were determined by Northern blot analysis and RNase protection assay using a T3 RNA polymerase transcript of the 795-bp *Bgl*II/*Eco*RI fragment of the hGH gene cloned into the BSKSM13$^+$ vector. The protected hGH signal was normalized to the signal obtained for the endogenous mouse metallothionein MT-1 RNA, which was measured simultaneously in the same RNA sample in RNAse protection assays and sequentially with Northern blots. The MT-1 probe was derived from the SP6 RNA polymerase transcription of the *Eco*RI-digested pSP-MTI vector containing the MT-1 cDNA (Durnam *et al.*, 1980). The probe was 405 nts, which protects a 321-nt fragment of the MT-1 RNA. Synthetic MT-1 RNA was synthesized from the same MT-1 cDNA fragment after cloning into pGEM4 and transcription of the *Hin*dIII-digested plasmid by SP polymerase. The poly A and poly T tails added to the cDNA during cloning (Durnam *et al.*, 1980) are likely responsible for the trimming of the 405-nt protected region to approximately 350 nts. Estimates of the absolute amount of hGH RNA were obtained by comparing the signal of hGH to those of synthetic standard RNAs of thymidine kinase and a thymidine kinase probe of the same specific radioactivity as the hGH probe as described previously (Neznanov *et al.*, 1993).

Northern blot filters were analyzed by hybridization with a 274-nt single stranded DNA hybridization probe, representative of the hGH gene from the *Bgl*II site in the last exon to 116 bp downstream of the polyadenylation signal. This fragment was subcloned into BSKSM13$^+$. The probe was made by polymerase chain reaction with one oligonucleotide primer as previously described (Neznanov and Oshima, 1993). After appropriate exposures the filters were stripped of probe by heating to 100°C and hybridized with a probe for MT-1 or the ribosomal protein L32. The amount of RNA was estimated by quantitation in an Ambis radioactivity image analyzer. The estimated abundance of the hGH RNA was then normalized to the signal generated by the endogenous mouse MT-1 RNA to correct for variation in loading and induction of the MT-1 promoter. Duplicate samples analyzed by RNase protection provided references for conversion of the relative values of the Northern blots. The amount of the hGH protein in the serum of transgenic mice was measured with the immunoassay system for the quantitative determination of the human growth hormone levels (Allegro).

RESULTS

To test the potential utility of K18 flanking sequences and the SATB1 core MAR to protect genes from position effects of integration, transgenic mice were generated from the three constructions shown in Figure 1. The nn-MThGH construct contains the 5′ distal 2.3 kb and the 3′ distal 3.6 kb of the K18 genomic fragment. The mar-MThGH construct contains seven copies of the 25-bp core sequence (wild-type $(25)_7$) derived from the MAR, located 3′ of the IgH gene enhancer (Cockerill and Garrard, 1986). This 25-bp sequence contains the core unwinding element of the MAR because mutation of this element abolishes the unwinding capability of the entire MAR segment and decreases binding to the nuclear matrix (Bode *et al.*, 1992; Dickinson *et al.*, 1992). Multimerization of the 25-bp sequence was necessary for high affinity binding to the nuclear matrix in vitro. Wild-type $(25)_7$ binds tightly to the nuclear matrix, and is, therefore, operationally defined as an MAR (Bode *et al.*, 1992). Wild-type $(25)_7$ was positioned both upstream and downstream of the MThGH gene in a unidirectional manner capable of augmenting transcription of a linked gene when integrated in chromosomal DNA in cultured cells.

Figure 2 shows Southern blot analysis of representative transgenic mice of all three constructions. In the three cases of the control MThGH and nn-MThGH mice shown, digestion with the enzyme *Bgl*II, which cuts each gene only once, resulted in primarily unit

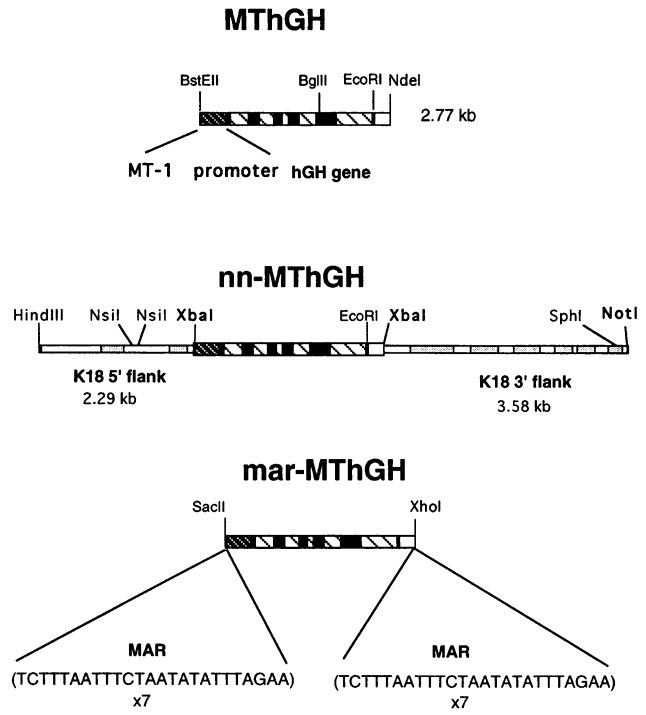

Figure 1. MThGH constructions tested in transgenic mice. The MThGH gene represents the *Bst*EII/*Nde*I fragment of the MThGH111 plasmid. The exons of the hGH gene are shown as filled boxes. The introns are shown as large hatching. The MT-1 promoter is indicated by the small hatching. The nn-MThGH consists of the MThGH gene flanked by sequences from the K18 gene (Neznanov and Oshima, 1993). The shaded regions within the K18 flanking sequences represent Alu repetitive elements. The mar-MThGH construction is flanked by the seven reiterated copies of the SATB1 binding element.

length fragments of the expected size (Figure 2A, lanes 1–3 and 4–6). Additional fragments likely represent rearranged copies or the integration site-specific fragments representing the first and last fragments of the array. Analysis of additional lines not shown was consistent with the same head to tail pattern of integration commonly found in transgenic mice except for the mouse nn-MThGH-23, which was found to have a single copy of the gene integrated. The expected unit length fragment was detected in all mar-MThGH mice (Figure 2A, lanes 7–9; Figure 2B, lanes 1–6). Each line contained multiple copies of the original fragment in tandem head to tail arrays. Quantitative dot blot analysis was performed to estimate the copy number of each line (Table 1).

MThGH Gene Expression Is Integration Site Dependent

Previous studies by other investigators (Palmiter *et al.*, 1983) had demonstrated that the MThGH gene was expressed widely in transgenic mouse tissues and generally reflected the tissue-specific expression of the endogenous MT-1 promoter. However, expression levels were highly variable and not correlated with copy number. This indicated that the MThGH gene was sensitive to its particular site of integration and that tandem arrays of the gene could not be expressed efficiently. Three additional lines of mice were generated from the MThGH gene to confirm the behavior of the gene in our hands. RNA from multiple organs of each animal was analyzed by Northern blot analysis first for hGH and a second time for endogenous mouse metallothionein MT-1 RNA for standardization of the MT-1 promoter activity. Variable expression was evident from the three control MThGH strains (Figure 3). All three expressed MThGH in brain. However, only two lines accumulated significant amount of hGH RNA in heart, kidney, and liver and only one line expressed detectable levels hGH RNA in intestine and spleen. Although the levels of expression were easily detectable in many organs, expression was not correlated with copy number (Table 1). Overall the patterns of expression were similar to those previously reported for the MThGH transgene (Palmiter *et al.*, 1983).

K18 Flanking Sequences Confer upon MThGH Position-independent Expression

The MThGH gene was placed between the 2.3 kb and 3.6 kb of the 5′ and 3′ flanking sequences of the K18 gene to determine if the K18 sequences could insulate the MThGH gene from position effects of integration and determine if such sequences influenced the tissue-specific expression of the gene (Figure 1, nn-MThGH). Four transgenic mice carrying the nn-MThGH construct, containing both flanking regions, were identified and analyzed for expression of the hGH gene. None of these lines were able to reproduce, as previously noted by other investigators (Palmiter *et al.*, 1983; Wanke *et al.*, 1992), so only founder animals were analyzed. Figure 4 shows that high levels of hGH RNA were detected in multiple organs, even though the number of gene copies was modest (Table 1 and Figure 1). Every transgenic animal expressed hGH RNA in all tissues analyzed except spleen, in which endogenous MT RNA was not detectable. RNase protection experiments were used to analyze the levels of hGH and MT-1 RNAs simultaneously (Figure 5). These and additional unpublished results confirm a higher signal for the transgenic hGH RNAs relative to the endogenous MT-1 RNA. Analysis of multiple samples by both the RNase protection method and the Northern blot method provided a basis for direct comparison of the samples from multiple blots (Table 1). The variation in RNA levels/gene of individual nn-MThGH transgenic mice was relatively uniform for brain, heart, kidney, liver, and testes indicative of

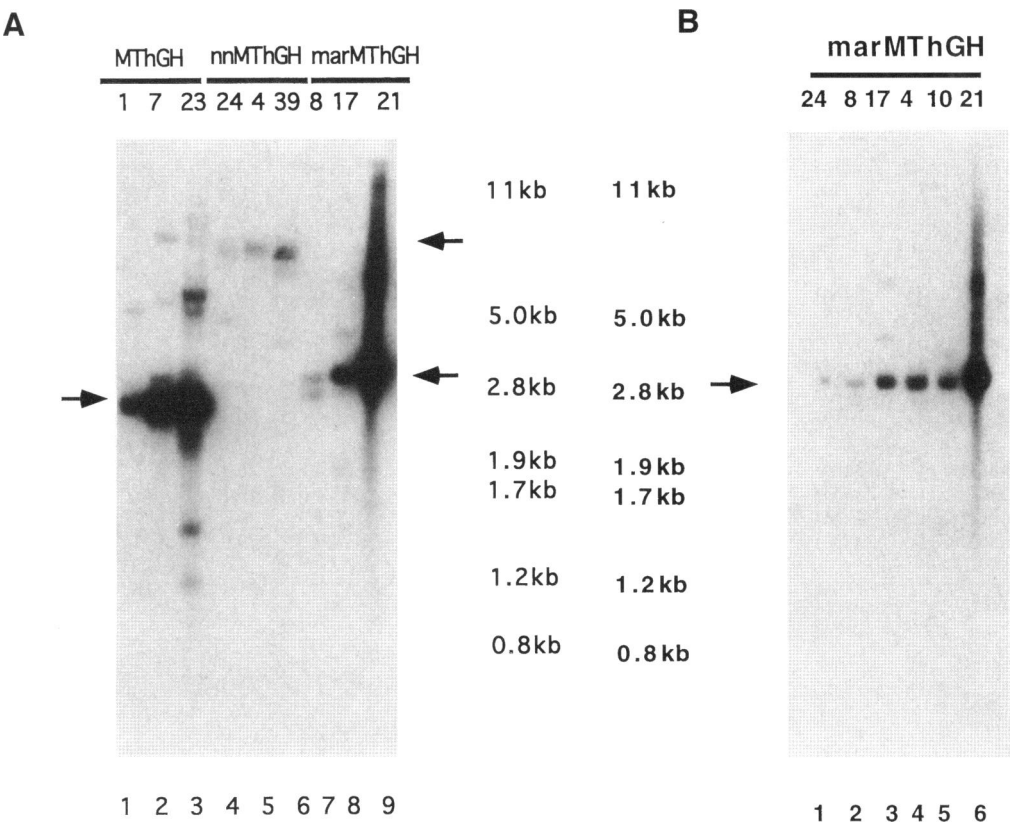

Figure 2. Southern blot analysis of DNA from transgenic mice. (A) Ten micrograms of mouse genomic DNA was digested with *Bgl*II which cuts all three constructs only once. The *Bgl*II/*Eco*RI fragment of the 3′ end of the hGH gene (Figure 1) was used as a hybridization probe. Autoradiographic exposures of x-ray film in the presence of intensifying screens is shown. The position of the unit length gene fragments, corresponding to each construction, are indicated by the arrows. The mouse number is indicated beneath the construct name. The positions of size markers are shown at the far right. (B) Analysis of additional mar-MThGH mice. The experiment was performed as indicated for panel A. Mouse numbers of the marMThGH mice are indicated at the top of each lane. Note that the preponderance of unit length copies of mouse 21 (lane 6) and mouse 8 (lane 2) is seen better in this exposure than in panel A, lane 9.

copy number-dependent expression. However, variation in intestinal tissues was high. Overall, the profile of nn-MThGH expression was similar to the control vector but highly elevated and much more efficient on a per gene basis. The hGH RNA signal correlates roughly with the transgene copy numbers of 1, 3, 3, and 6 for mice 23, 24, 4, and 39, respectively.

All four nn-MThGH mice grew more rapidly than nontransgenic animals and contained detectable hGH in the serum. Levels ranged from 0.5 μg/ml to as high as 4800 μg/ml for mouse nn-MThGH-39. The hGH concentrations of all but one mouse (nn-MThGH-23, copy number = 1) were in excess of the highest level (64 μg/ml) previously reported for a large series of MThGH mice (Palmiter *et al.*, 1983). Mouse nn-MThGH-23 was also the only mouse to have normal kidney size and appearance at the time of sacrifice, unlike the common kidney pathology associated with excessive hGH production (Wanke *et al.*, 1992). The elevated levels of hGH RNA per gene and the consistent expression in each transgenic animal indicates that position-independent, copy number-dependent expression can be conferred upon the MThGH gene by the flanking sequences of the K18 gene in tissues other than intestine. K18 flanking sequences enhanced expression in heart even though this organ expresses little or no endogenous or transgenic K18. Furthermore, the efficient expression from a single copy of the transgene in nn-MThGH mouse 23 demonstrates that the K18 flanking sequences are active on a single integrated copy of a transgene.

mar-MThGH Mice

In contrast to the results obtained for MThGH mice and nn-MThGH mice, no detectable hGH RNA was found in brain and kidney or in the intestines of five of six mar-MThGH animals. Furthermore, although expression was detectable in four of six animals in both heart and liver, the levels of expression was generally less than either the control or nnMThGH animals. On the other hand, all three males expressed high levels in the testes (Figure 6). This overall pattern of expression was dramatically different from both the control MThGH and nn-MThGH animals. One animal (mar-MThGH-8) expressed only in testes (Figure 6, lane 33). Another female (mar-MThGH-24) failed to express detectable hGH RNA in any organ tested (Figure 6, lanes 2, 8, 20, and 26). These two founder mice that had no detectable expression in any tissue except testes

Table 1. MThGH RNA expression of transgenic mice[a]

Vector	Line (sex)	Copy no.	Brain			Heart			Intestine		
			RNA	RNA/gene	Avg	RNA	RNA/gene	Avg	RNA	RNA/gene	Avg
MThGH	1 (F)	18	4.8	0.27		63.3	3.5		5.4	0.30	
MThGH	7 (M)	180	22.6	0.12		80.7	0.4		4.0	0.02	
MThGH	23 (F)	270	7.0	0.03		12.9	0.1		0.0	0.00	
					0.1 ± 0.1			1.3 ± 1.8			0.1 ± 0.1
marMThGH	8 (M)	3	0			0.0	0.0		0.0	0.0	
marMTHGH	24 (F)	3	0			0.0	0.0		0.0	0.0	
marMThGH	4 (F)	8	0			3.2	0.4		0.0	0.0	
marMThGH	10 (M)	8	0			3.5	0.4		0.0	0.0	
marMThGH	17 (M)	8	0			7.1	0.9		0.0	0.0	
marMThGH	21 (F)	80	0			6.4	0.1		7.3	0.1	
					0			0.4 ± 0.3			<0.1
nnMThGH	23 (M)	1	6.3	6.3		28.4	28.4		10.7	10.7	
nnMThGH	24 (M)	3	9.6	3.2		57.4	19.1		6.0	2.0	
nnMThGH	4 (M)	3	14.6	4.9		29.1	9.7		0.7	0.2	
nnMThGH	39 (M)	6	52.1	8.7		174.0	29.0		12.4	2.1	
					5.8 ± 1.7			21.6 ± 7.1			3.8 ± 3.5

			Kidney			Liver			Testes		
			RNA	RNA/gene	Avg	RNA	RNA/gene	Avg	RNA	RNA/gene	Avg
MThGH	1 (F)	18	32.0	1.8		55.2	3.07				
MThGH	7 (M)	180	43.7	0.2		66.7	0.37		59.7	0.3	
MThGH	23 (F)	270	0.0	0.0		3.9	0.01				
					0.7 ± 0.7			1.1 ± 1.3			
marMThGH	8 (M)	3	0			0.0	0.0		48.8	16.3	
marMTHGH	24 (F)	3	0			0.0	0.0				
marMThGH	4 (F)	8	0			0.3	0.04				
marMThGH	10 (M)	8	0			4.8	0.60		48.0	6.0	
marMThGH	17 (M)	8	0			2.3	0.29		36.5	4.6	
marMThGH	21 (F)	80	0			30.7	0.38				
					0			0.3 ± 0.2			8.9 ± 4.5
nnMThGH	23 (M)	1	7.2	7.2		9.2	9.20		14.2	14.2	
nnMThGH	24 (M)	3	25.7	8.6		17.5	5.83		57.3	19.1	
nnMThGH	4 (M)	3	24.1	8.0		19.4	6.47		65.6	21.9	
nnMThGH	39 (M)	6	115.9	19.3		102.6	17.10		61.3	10.2	
					10.8 ± 4.5			9.6 ± 3.7			16.3 ± 4.1

[a] MThGH RNA was measured by Northern blot analysis and was normalized to the amount of MT-1 RNA in the same probe. RNA values are presented as picograms of MThGH RNA per 10 μg of total RNA. Average values represent means and SD. nnMThGH vector is flanked by K18 sequences. marMThGH is flanked by synthetic matrix attachment sites. MThGH is the basic vector.

were also the only two mice that reproduced. The ability to reproduce appears to be a consequence of the suppression of hGH expression in these two mice because all other founders failed to productively mate. Others have shown that females expressing the MThGH gene are usually sterile and males exhibit reduced fertility (Bartke *et al.*, 1994) due to both direct and indirect effects of the hormone. Thus, the inclusion of wild-type $(25)_7$ actively suppressed the expression of the reporter gene in various tissues and, at least in brain and kidney, this suppression was independent of position of integration. This integration-independent suppression by wild-type $(25)_7$ may also apply to intestine, although we have no obvious explanation for the one exception (nn-MThGH-21). No hGH RNA was detected in the thymuses of the progeny of mar-MThGH-8 or mar-MThGH-24. However, no endogenous MT-1 RNA was detected either. It is likely that the MT promoter of the MThGH gene is not active in thymus, where SATB1 protein expression is highly expressed (Dickinson *et al.*, 1992).

In testis, expression was dramatically enhanced, most likely in a position-independent manner, as judged by the high level of expression obtained from all three male mice (Table 1). These results are sum-

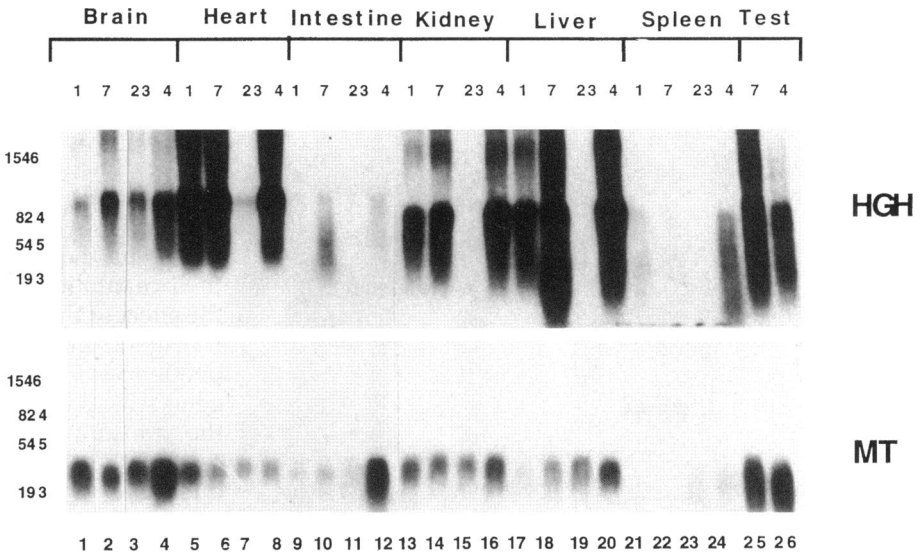

Figure 3. Northern blot analysis of hGH RNA from control MThGH and one nn-MThGH transgenic mouse. Ten micrograms of total RNAs from the indicated organ were separated by electrophoresis and transferred to a filter and hybridized with an hGH probe. After exposure to film and quantitation in an Ambis radioactivity image analyzer the probe was removed and the blot hybridized again with a probe for mouse metallothionein (MT). The animal number is indicated above each lane. Mice 1, 7, and 23 carry approximately 18, 180, and 270 copies of the MThGH gene per cell. Animal 4 (lanes 4, 8, 12, 16, 20, 24, and 26) carries three copies of the nn-MThGH construct. The position of molecular weight markers is shown in nucleotides on the left side.

marized and compared with the other two types of transgenic mice in Figure 7. This expression profile is unique to mar-MThGH mice and was unexpected in light of the previous finding that the same MAR sequences exhibited strong enhancing activity of a linked reporter gene in stable transformants of tissue culture cells (Bode *et al.*, 1992).

DISCUSSION

The expression of the MThGH gene, like most genes used in transgenic animals (Palmiter and Brinster, 1986; Pursel *et al.*, 1989), is quite sensitive to the particular site of integration. The pattern and levels of expression found in the control MThGH transgenic

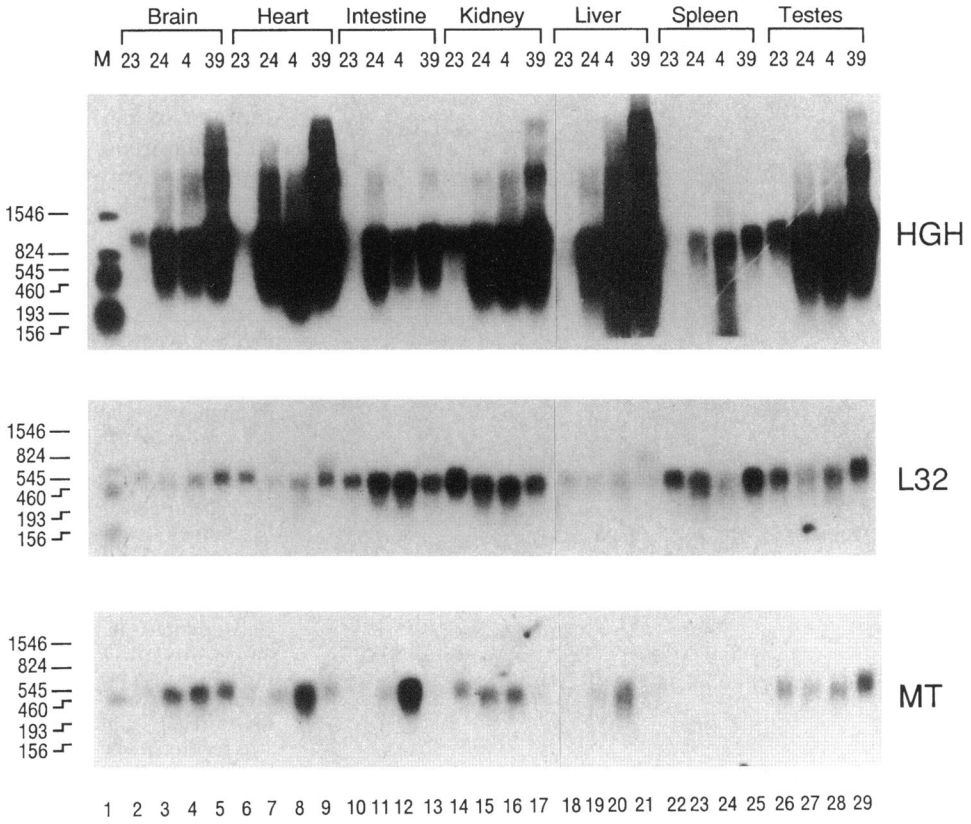

Figure 4. Northern blot analysis of RNA from nn-MThGH transgenic mice. RNAs from the indicated nn-MThGH mice (above each lane) were analyzed as described for Figures 3 and 4. However, after hybridization for MT-1, the filter was probed a third time for the L32 ribosomal protein to show the presence of mRNA in spleen samples that were very low in MT-1.

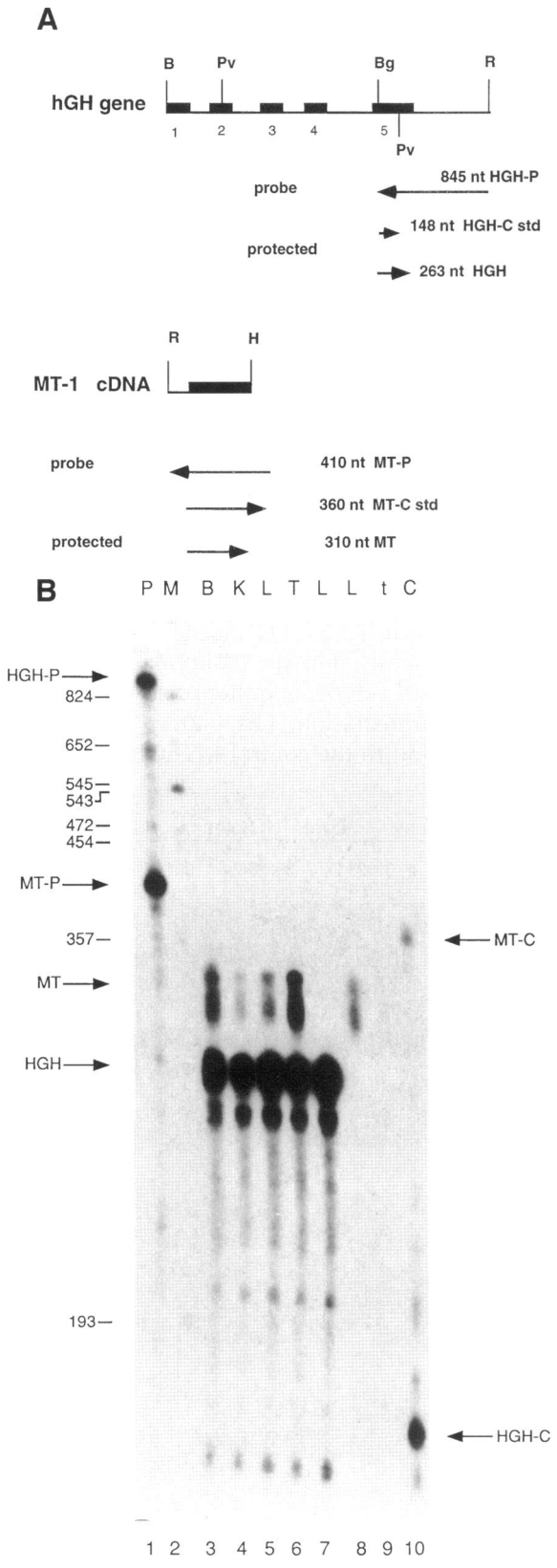

Figure 5.

mice we generated are completely consistent with published results for the much larger series of original animals (Palmiter *et al.*, 1983, 1993). One distinguishing characteristic of the expression pattern of these control mice is the variable expression in particular tissues. Furthermore, the levels of expression were independent of the number of genes integrated. We examined two different types of DNA sequences, a MAR and the flanking sequences of the K18 gene, for their capabilities of protecting this MThGH gene from the effect of the neighboring sequences in chromatin of transgenic mice. In most tissues, integration-site independence of the transcriptional status of the MThGH gene was achieved by either of these sequences, but the transcription was generally suppressed for the case of the MAR and enhanced for K18 flanking sequences.

The SATB1 Core MAR Suppresses Expression

The addition of the MAR sequence to both sides of the MThGH gene resulted in a complete suppression of transcription in brain and kidney in all six mice. The suppression in these tissues is, therefore, integration-site independent. Although expression of hGH RNA was detected in heart and liver for some mar-ThGH mice, the levels were substantially lower than those detected in MthGH and nn-MThGH mice. Two of the six mar-MThGH mice had undetectable levels of RNA in these tissues. In contrast to the suppressive effects in most organs, testes of all three male transgenic animals expressed hGH RNA at a high level, which may be correlated with copy number. Even in the case of mouse mar-MThGH-8 that did not express the MThGH gene in any other tissues, the gene was uniquely expressed at a high level in testis, indicating that the MAR exerts a positive effect on its linked gene expression in the testes chromatin. The negative regula-

Figure 5 (cont). RNA protection analysis of the hGH and MT-1 RNAs from transgenic mouse nn-MThGH-39. (A) Map of the promoterless hGH gene is shown. Exons of hGH are shown as filled rectangles. The antisense RNA for hGH probe is 845 nt. The hGH RNA protects the 263 nt of the last exon. A synthetic standard (Std) RNA of 148 nt was generated from the *Bgl*II–*Pvu*II fragment of the last exon as a positive control. Restriction enzyme sites: B, *Bam*HI; Bg, *Bgl*II; and Pv, *Pvu*II. (B) Ten micrograms of total RNA from brain (B), kidney (K), 5 μg from liver (L), and 10 μg from testes (T) of mouse nn-MThGH-39 or 10 μg of tRNA (t) was hybridized with both hGH (HGH-P) and MT-1 (MT-P) probes (lanes 3–6 and 9), digested with RNases A and T1, and analyzed by the acrylamide gel electrophoresis in 8 M urea and autoradiography. The size markers are shown at the left in nucleotides. The arrows mark the position of undigested probes (HGH-P and MT-P), the major protected fragments (HGH and MT), and the position of the synthetic control RNAs (HGH-C and MT-C). Lane 1, hybridization probes; lane 2, size markers; lane 7, liver RNA with only the hGH probe; lane 8, liver RNA with only the MT probe; lane 9, tRNA control; and lane 10 represents the signals with both control synthetic hGH and MT RNAs.

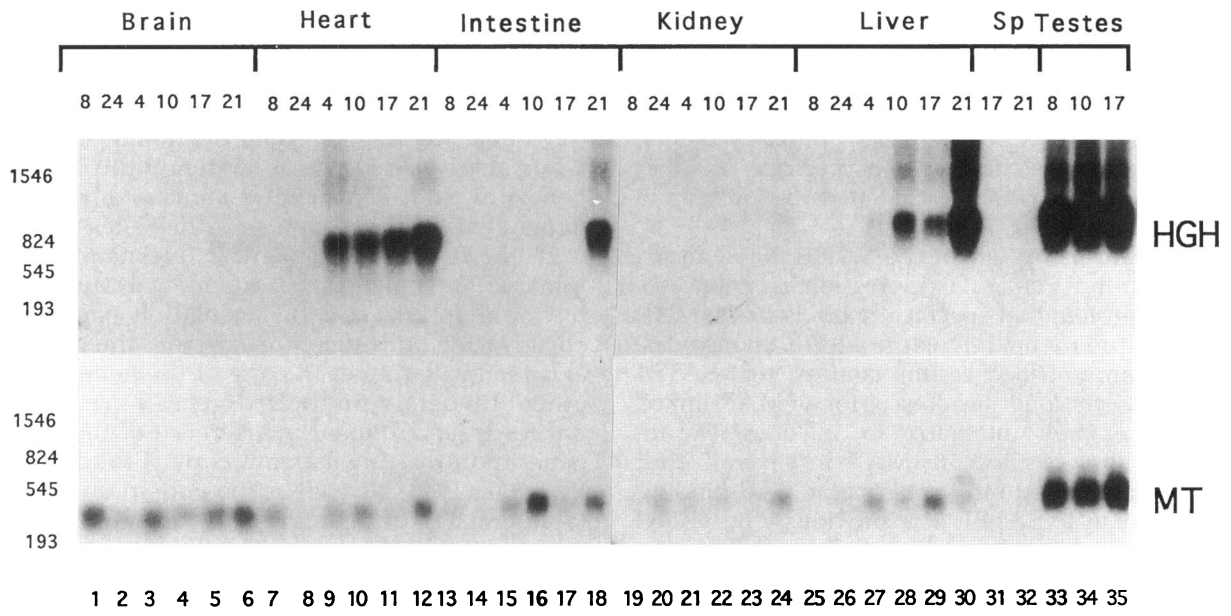

Figure 6. Northern blot analysis of the RNA from the various organs of mar-MThGH transgenic mice. Mice carrying the mar-MThGH construct (Table 1) are indicated above each lane. RNAs for hGH and MT-1 were detected as described for Figure 3. The position of the molecular weight markers is shown in nucleotides at the left.

tory effect of the SATB1 core MAR sequence in certain tissues contrasts with the positive regulatory effects observed with the same MAR sequence on a different reporter gene (Bode *et al.*, 1992) or with other MARs in stable transfection analysis of cultured cells (Stief *et al.*, 1989; Phi-van *et al.*, 1990; Laemmli *et al.*, 1992; Kalos and Fournier, 1994; Poljak *et al.*, 1994; Yu *et al.*, 1994).

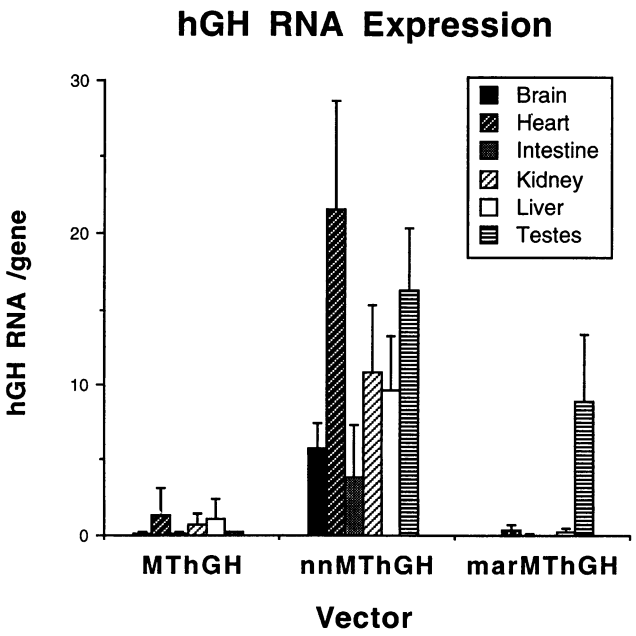

Figure 7. Summary of expression of hGH RNA. Relative values for hGH RNA/gene for individual animals (Table 1) were averaged. Error bars indicate the SD.

Our demonstration that the SATB1 core MAR suppresses MThGH gene expression contrasts with other tests of MARs in transgenic mice. MAR sequences were found to be essential for appropriate expression of Ig μ heavy chain (Forrester *et al.*, 1994). In addition, a MAR derived from the human IFN-β gene stimulated expression of a reporter gene composed of the HSP 70.1 promoter and the luciferase gene in a copy number-dependent fashion during preimplantation mouse development but not in differentiated tissues (Thompson *et al.*, 1994). Furthermore, the lysozyme MAR was reported to confer position-independent hormonal regulation but not copy number-dependent expression on the WAP gene in transgenic mice (McKnight *et al.*, 1992). However, removal of the MAR elements from the lysozyme gene still results in position-independent and copy number-dependent expression in transgenic mice (Huber *et al.*, 1994). Other positive effects of MAR elements in transgenic mice have also been observed (Brooks *et al.*, 1994). It appears that the specific effect of MAR elements may depend upon the specific combination of MAR and regulatory elements used.

In contrast to testing large MAR segments that might contain additional regulatory elements, we examined a short and well-defined core unwinding element of the MAR 3′ of the IgH enhancer. Although

this element, when concatemerized, confers high affinity to the nuclear matrix comparable to naturally occurring MAR segments, the effect is due to the small subregion specifically recognized by SATB1. An active suppression of reporter gene transcription by this element may be alleviated by the presence of other positive-acting cis elements that may be present in larger MAR segments.

The mechanism by which the SATB1 MAR mediates position-independent suppression is unknown. SATB1, the protein that specifically binds to the MAR sequence, is predominantly expressed in thymocytes. Additional unpublished results confirm that SATB1 acts as a transcriptional suppressor for an MAR-linked reporter gene stably integrated in cell lines. The repressive activity of the core MAR in several adult tissues might be due to the presence of MAR-binding activities similar to SATB1. Our previously published and unpublished results identify other proteins that can specifically bind core MAR. However, these proteins appear to be found only in tissues of early development and in cancer cells. For example, nucleolin, which can act as a transcriptional suppressor (Yang *et al.*, 1994) and has high MAR-binding activity in human erythroleukemia cells, rapidly disappears upon differentiation induced by hemin (Dickinson and Kohwi-Shigematsu, 1995, unpublished results). Furthermore, we have been unable to identify such specific core MAR binding activity in several negatively affected tissues. The repressive activity of the SATB1 core MAR in transgenic mice may be due to a nonpermissive chromatin state established during development. However, in testes, chromatin repression may be relieved by a positive core MAR binding activity that acts directly as a transcriptional activator.

Sequences Flanking the K18 Gene Insulate the MThGH Gene from Position Effects and Confer Copy Number Dependence

In contrast to mar-MThGH mice, each of the four nn-MThGH mice expressed detectable hGH RNA in every tissue examined except for the spleen of a single copy transgenic mouse. The consistent expression in all tissues of all four mice reinforces the view that the K18 flanking sequences facilitate appropriate expression by insulating the MThGH gene from position effects. The present example complements the previous demonstration that the HSV thymidine kinase gene flanked by the K18 sequences is capable of position-independent, copy number-dependent expression in brain and testes (Neznanov *et al.*, 1993). The K18 sequences may be useful for uniform expression in heart, kidney, and liver. The positive effects for heart suggest that the K18 flanking sequences may be effective even in tissues that do not normally express K18 if appropriate regulatory elements are included in the transgene. The consistent expression per gene of the nn-MThGH construct suggests that all or most copies of the nn-MThGH gene were active in all but one tissue analyzed. This conclusion has been confirmed for transgenic K18 genes by the demonstration that all or nearly all copies of tandemly duplicated K18 genes in transgenic mice contained expression-dependent, DNase-hypersensitive sites associated with the first intron enhancer (Neznanov and Oshima, 1993). If all genes of a tandem array are active, it would follow that the insulation effect may be effective for both the chromosomal integration site and for insulating each copy of the gene from each other. Tandemly duplicated genes have the potential for transcriptional interference of the upstream gene upon the downstream copy (Proudfoot, 1986; Wu *et al.*, 1990), as well as the potentially detrimental interaction of the regulatory element of one gene with a neighboring copy. The flanking sequences of the K18 gene do not contain MARs nor transcriptional enhancers detectable by normal transient transfection assays. Previous deletion analysis of the K18 gene in transgenic mice indicated that the characteristics of position-independent expression and copy number-dependent expression have different sequence requirements, suggesting multiple different activities may be important for the function of these sequences (Thorey *et al.*, 1993). The characteristics of the flanking sequences of the K18 gene fit the general definition of an LCR as conferring high-level, position-independent expression. However, whether the K18 sequences act primarily as LCRs (Grosveld *et al.*, 1987; Forrester *et al.*, 1989; Ryan *et al.*, 1989; VanAssendlft *et al.*, 1989; Fiering *et al.*, 1995), as insulators like the chicken β-globin region (Chung *et al.*, 1993), or both remains to be determined. However, the positive effects found for the K18 flanking elements in tissues that do not normally express K18 and the lack of DNase-hypersensitive sites or transcriptional enhancer activity of the 3′ flanking element may point to differences from other LCRs. It will be of interest to determine if the K18 flanking regions have position-dependent enhancer blocking activity characteristic of the chicken β-globin insulator (Chung *et al.*, 1993) and the *Drosophila* scs chromatin boundary elements (Kellum and Schedl, 1992).

The K18 flanking regions may be useful for the efficient, controlled expression of other genes in transgenic mice. Their activity appears similar to the 10-kb and 7-kb DNA regions flanking the mouse metallothionein I and II genes (Palmiter *et al.*, 1993). Copy number-dependent expression in transgenic mice would provide the opportunity of extending the utility of the system to evaluate quantitative as well as qualitative questions of the biological effects of transgene expression.

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