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Alu Sequence Involvement in Transcriptional Insulation of the Keratin 18 Gene in Transgenic Mice

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The human keratin 18 (K18) gene is expressed in a variety of adult simple epithelial tissues, including liver, intestine, lung, and kidney, but is not normally found in skin, muscle, heart, spleen, or most of the brain. Transgenic animals derived from the cloned K18 gene express the transgene in appropriate tissues at levels directly proportional to the copy number and independently of the sites of integration. We have investigated in transgenic mice the dependence of K18 gene expression on the distal 5′ and 3′ flanking sequences and upon the RNA polymerase III promoter of an *Alu* repetitive DNA transcription unit immediately upstream of the K18 promoter. Integration site-independent expression of tandemly duplicated K18 transgenes requires the presence of either an 825-bp fragment of the 5′ flanking sequence or the 3.5-kb 3′ flanking sequence. Mutation of the RNA polymerase III promoter of the *Alu* element within the 825-bp fragment abolishes copy number-dependent expression in kidney but does not abolish integration site-independent expression when assayed in the absence of the 3′ flanking sequence of the K18 gene. The characteristics of integration site-independent expression and copy number-dependent expression are separable. In addition, the formation of the chromatin state of the K18 gene, which likely restricts the tissue-specific expression of this gene, is not dependent upon the distal flanking sequences of the 10-kb K18 gene but rather may depend on internal regulatory regions of the gene.

The mouse form of keratin 18 (mK18 or EndoB; K18 in humans) is expressed in the trophectoderm of the blastocyst embryo and is subsequently restricted to a variety of simple epithelia where, by copolymerization with the complementary keratin 8 (mK8), it forms intermediate filaments. In adults, K18 is found primarily in simple or single-layered epithelial tissues, including liver, intestine, lung, pancreas, and kidney, but is not expressed in skin, most of the brain, or most mesodermal derivatives such as skeletal muscle, cardiac muscle, or blood cells (1, 22). The tissue-specific expression of the K18 gene appears to be due, at least in part, to its chromatin state, which may limit accessibility of necessary transcription factors (24, 25, 28). Previous analyses of transgenic mice revealed that a 10-kb fragment of the K18 gene contained sufficient genetic information to ensure both adult tissue specificity (1, 23) and appropriate developmental expression (38). In contrast to the results of many other transgenic experiments, K18 was expressed in every transgenic mouse line, independently of the different sites of integration. Furthermore, the level of K18 RNA was directly proportional to the number of transgenes and comparable, on a per-gene basis, to the level of the endogenous mK18 gene. This combination of integration site-independent and copy number-dependent expression implies a mechanism of insulating the K18 gene from the *cis*-acting effects of regulatory elements flanking random sites of integration and of preventing inappropriate interaction between regulatory elements of each copy of the tandemly duplicated transgene normally found in transgenic mice. The distal 3′ flanking sequence of the K18 gene is important for efficient K18 transgenic expression in liver. However, expression in kidney and lung was unaffected by deletion of the 3′ flanking sequence (23). Furthermore, a herpes simplex virus thymidine kinase gene inserted between the flanking sequences of the K18 gene was found to be transcriptionally insulated in transgenic mice (23). Thus the distal sequences flanking the K18 gene and not the internal regulatory elements (24, 26) may confer transcriptional insulation.

Position effects of transgene expression have been attributed to *cis*-acting regulatory elements flanking the sites of integration (2, 3, 29). Integration site-independent expression has been reported for some genes (1, 4, 5, 7, 13–15). Integration site-independent, copy number-dependent expression of the β-globin gene requires a locus control region (LCR) located about 20 kb upstream of the first gene in the β-globin cluster (15). The globin LCR contains a powerful, tissue-specific enhancer which confers high-level, tissue-specific expression on linked genes but does not necessarily ensure copy number-dependent expression (12, 33). A similar dissection of transcriptional enhancer activity from sequences conferring integration site-independent expression has been reported for the lysozyme gene (21, 36). In this case, the element which confers integration site-independent expression in transgenic mice is associated with nuclear matrix attachment activity (36). A 585-bp fragment of the 5′ end of a human class I histocompatibility antigen gene with LCR activity contains multiple binding sites for transcription factors and may act similarly to the enhancer portion of the globin LCR (7). In contrast to these examples, a 630-bp fragment of the first intron of the adenosine deaminase (ADA) gene has been implicated in the position-independent and copy number-dependent expression of an ADA-chloramphenicol acetyltransferase (CAT) reporter gene construction. This fragment is predominantly *Alu*-type repetitive DNA (4). Several observations have prompted us to consider the involvement of *Alu* sequences in the transcriptional insulation of the K18 gene in transgenic mice. First, two DNase-hypersensitive sites which correlate with expression of the K18 gene map to an *Alu* element immediately up-

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stream of the K18 promoter (24). Second, transcription of this *Alu* sequence is directly correlated with K18 expression (24). Finally, the placement of this *Alu* element (B1 repeat in the mouse) is conserved in the mouse and human K18 genes (16, 18).

In this study, we investigated the sequence requirements for the transcriptional insulation of the K18 gene in transgenic mice, with special emphasis on the necessity of the transcriptional competence of the K18 *Alu* element. The characteristics of integration site-independent expression and copy number-dependent expression are separated by the effects of a mutation of the *Alu* promoter. Active transcription of the *Alu* sequence may relieve transcriptional interference of tandemly arranged transgenes, resulting in copy number-dependent expression. However, other elements associated with an 825-bp genomic fragment containing the *Alu* sequence appears to ensure expression in every transgenic mouse.

MATERIALS AND METHODS

K18 vectors. The RNA polymerase III (Pol3) promoter of the *Alu* sequence proximal to the K18 promoter was mutated by deleting the B-box (K18-dB) or both A- and B-box (K18-dAB) elements of the split intragenic promoter (30). The deletions were constructed by polymerase chain reaction synthesis of two fragments, extending from the margins of the target sequence upstream beyond the restriction site for *Nsi*I (nucleotide [nt] 1456) or downstream beyond the unique *Xho*I site (nt 2281). The primer pairs used (K18 sequences are indicated in uppercase) were GGT GTG CAG AAG TCA GG at nt 1440 and ggc aga tct CAT CCT AGC CAA CAT GG at nt 2096; ggc aga tct CTG ACC TCG TGA TACC GC at nt 2124 and ATG GAC ACG GAC AGC AG at nt 2300 for the B-box mutation; and ggc aga tct CGG TCA AGA CTC CCA AA at nt 2191 and ATG GAC ACG GAC AGC AG at nt 2300 for the A- and B-box deletion. The primers flanking the deletion site created an additional *Bgl*II site. The polymerase chain reaction fragments were digested with *Bgl*II, ligated together, cut with *Xho*I and *Nsi*I, gel purified, and cloned into the K18 gene between the *Xho*I and *Nsi*I sites.

Transgenic mice. Transgenic mice were prepared by standard procedures as previously described (1) by the Transgenic Mouse Facility at the La Jolla Cancer Research Foundation. Strain FVB/N mouse eggs were injected and transferred to CD-1 foster mothers. Founder animals identified by dot blot hybridization of tail DNAs were sacrificed without further breeding. Mosaic animals identified by immunofluorescent staining of intestine and liver sections with a K18-specific monoclonal antibody were excluded from further analysis. All K18-XX, K18-NX, and K18-NBX mice were analyzed.

Transfection and RNA analysis. HR9 parietal endodermal cells were transfected by the calcium phosphate precipitate method (35) with 20 μg of DNA per 9-cm-diameter dish of cells. All plasmids were cotransfected with 2 μg of plasmid pMC1neopA (37) to normalize for transfection efficiency. RNA was purified by acidic phenol extraction of cells lysed in 0.5% sodium dodecyl sulfate (SDS)–20 mM EDTA (35). Total RNA was treated with RNase-free DNase I at 37°C for 60 min in the presence of RNase inhibitor (Stratagene, La Jolla, Calif.). K18 and Neo^r RNAs were quantitated by RNase protection analysis using [^{32}P]UTP-labeled probes. The RNA probe for K18 RNA was a T7 RNA polymerase transcript of a 431-bp fragment of the K18 gene (*Xho*I at nt 2284 to *Xho*II at nt 2715) overlapping the RNA start site (18). For the Neo^r probe, a 245-bp *Eco*RI-to-*Nar*I fragment of pMC1neopA was subcloned first into Bluescript KS and then into the pGEM-1 vectors via a fragment generated by *Eco*RI and *Xho*I, resulting in plasmid NeoSP6rp. For detection of the Neo^r RNA, plasmid NeoSP6rp was digested with *Eco*RI and transcribed with SP6 RNA polymerase. Both probes together were added to RNA from transfected cells for hybridization at 43°C. Protected probe was revealed by digestion with RNases A and T_1 followed by acrylamide gel electrophoresis in 8 M urea and autoradiography.

Nucleic acid analysis of transgenics. Transgene copy number was determined by dot blot hybridization of 2 μg of tail DNA with the K18 cDNA (27) followed by quantitation in an Ambis radioactivity image analyzer, using K18 transgenic animals with known copy numbers and multiple concentrations of plasmid DNA as standards. DNA concentrations were determined fluorimetrically (20). The DNA load of each dot was normalized by rehybridizing the stripped filter with a random-primed total mouse DNA probe. The average signal of all dots was considered 2 μg of DNA. Southern blotting was performed with 5 μg of tail DNA from each mouse to determine the arrangement of the integrated transgenes and to confirm the dot blot quantitation. In all cases reported, the predominant integrated organization was a head-to-tail tandem array of duplicated unit-length gene fragments (data not shown).

RNA was purified from mouse organs with the use of guanidine isothiocyanate and ultracentrifugation in $CsCl_2$ (9, 40). K18 RNA was quantitated by Northern (RNA) blot analysis, including K18 synthetic mRNA standards and samples from organs of K18TG mice as controls. Northern blot filters were analyzed by hybridization with the random-primed K18 cDNA probe under conditions sufficiently stringent to exclude cross-hybridization with the mouse homolog, mK18 (final washes in 0.1× SSPE–0.1% SDS at 65°C). After appropriate exposures, the filters were stripped of probe by boiling and rehybridized with a probe for the L32 ribosomal protein RNA (6, 13). Signals obtained by densitometer tracing of autoradiographs for K18 were normalized to those of L32. The mean L32 value of all samples of the same organ was considered 10 μg. RNA levels were determined by interpolation of the standard curve and are presented as picograms of K18 RNA per 10 μg of total RNA.

In vitro transcription of *Alu* promoter mutations. Fragments of the K18 gene containing the *Alu* element proximal to the K18 transcriptional initiation site and either of two mutations of the *Alu* Pol3 promoter were subcloned into Bluescript KS plasmids. The plasmids were transcribed in vitro in the presence of [^{32}P]UTP with the use of partially purified *Xenopus laevis* Pol3 transcription factors as previously described (32). *Alu* transcripts were detected by an RNase protection assay by hybridization with 100 ng of a synthetic, nonradioactive RNA probe and subsequent digestion with RNase T_1 and acrylamide gel analysis (24).

RESULTS

An 825-bp fragment of the K18 gene needed for efficient, copy number-dependent expression. We have previously shown that deletion of 1.46 kb of 5′ flanking sequence of the 10-kb K18 gene did not alter expression in liver, intestine, or kidney in transgenic mice. However, deletion of 3.5 kb of 3′ flanking sequence abrogated efficient expression in liver (23). We have now examined the effects of further 5′ deletions with and without the previous 3′ deletion. The fragments

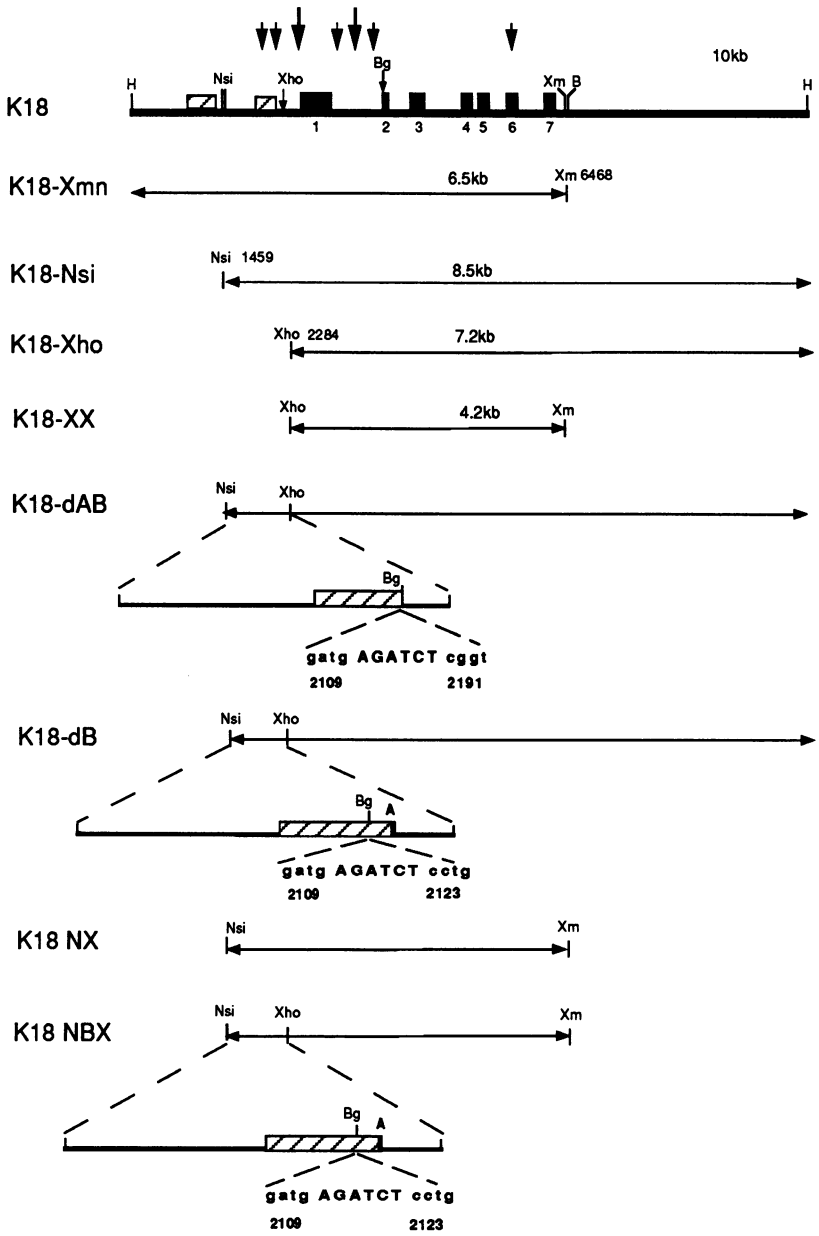

FIG. 1. K18 gene constructions tested in transgenic mice. The K18 gene is represented at the top, with exons designated by black boxes and two *Alu* repetitive elements indicated by the hatched boxes. Arrows above the map indicate the positions of DNase-hypersensitive sites (24). Restriction enzyme sites: H, *Hin*dIII; Nsi, *Nsi*I; Xho, *Xho*I; Bg, *Bgl*II; Xmn, *Xmn*I; B, *Bam*HI. Fragment K18-dAB contains a deletion of the A-box and B-box elements of the *Alu* transcription unit proximal to the K18 gene. The sequence of the region is shown below the expanded portion of the fragment. K18 sequences are indicated in lowercase; the *Bgl*II site is indicated in uppercase. Numbers refer to K18 base pairs, using the continuous-numbering convention. Transcription initiates at approximately nt 2533. The K18-dB fragment contains a substitution of a *Bgl*II site for the essential B-box element of the *Alu* promoter. The K18-NBX fragment contains the same B-box substitution within the context of the smaller fragment define by *Nsi*I and *Xmn*I.

introduced into transgenic mice are indicated in Fig. 1. All of the constructions alter sequences outside of the transcribed region of the K18 gene and thus are expected to generate the same K18 mRNA. Northern blot analysis confirmed the expected size of the K18 mRNA for all vectors, and RNase protection analysis confirmed the correct 5′ start sites on RNAs of selected animals (data not shown).

All of the mice carrying the 2.3-kb 5′ deletion of the transgene (Fig. 1, K18-Xho) expressed K18 RNA with the same tissue specificity as found for K18 mice (Fig. 2 and Table 1). Expression was detected in liver, intestine, kidney, lung, and brain but not in spleen, skeletal muscle, or heart (some data not shown). However, in comparison with previously characterized mice carrying either the whole gene (K18) or a smaller 1.46-kb deletion of the 5′ end (K18-Nsi), the K18-Xho mice contained greatly decreased levels of K18 RNA in liver and brain but near normal levels in intestine and kidney (Fig. 3A). These results may indicate that regu-

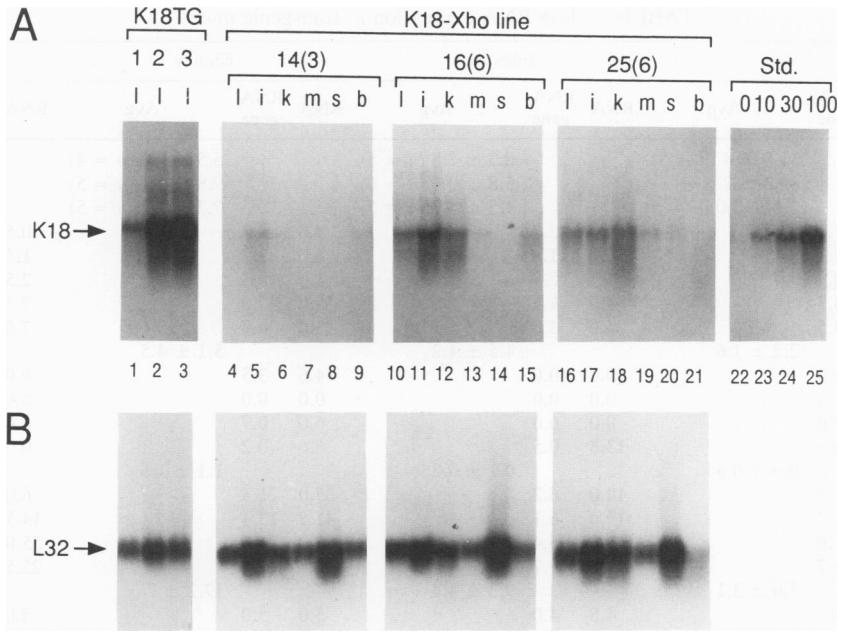

FIG. 2. Northern blot analysis of RNAs from K18-Xho mice. (A and B) Intensifier screen-enhanced autoradiographic signal obtained after sequential analysis of a single filter with the K18 (A) and L32 ribosomal protein (B) radioactive gene probes. Positions of the expected signals are indicated at the left. The transgenic lines are indicated at the top. K18TG represents the previously characterized K18 transgenic mice. The specific line designations with the integral copy number of the K18-Xho lines in parentheses are indicated above the organ designations. Std., standard synthetic K18 mRNAs. The numbers indicate picograms of RNA loaded in 5 μg of carrier tRNA. Organ designations: l, liver; i, intestine; k, kidney; m, muscle; s, spleen; b, brain. Five micrograms of total RNA was loaded in each lane.

latory signals that modulate expression in liver and brain are located between the *Nsi*I and *Xho*I sites within the 5′ flanking sequence of the K18 gene. However, these results contrast with those of experiments utilizing transient transfection of cell cultures that revealed little difference between constructions differing in this region of the K18 5′ flanking sequence (26).

The unusual characteristics of the K18 gene which result in its expression in every transgenic animal and the linear dependence of K18 RNA levels on the number of integrated genes (1, 24) can be efficiently represented by the average variation of the level of K18 RNA divided by the copy number of the transgene of all founder lines for a particular tissue. The variation in expression efficiency (RNA per gene copy) was much higher in K18-Xho transgenic mice than in previously characterized K18 and K18-Nsi mice (Fig. 3B and Table 1). This variation is illustrated in a comparison of the standard deviation of the average of all lines of each construct as shown in Fig. 3B. The standard deviations of the averages of all K18 and K18-Nsi transgenic mice are less than 50% of the mean. In contrast, the values for intestinal RNAs and kidney RNAs of the K18-Xho mice are much in excess of the 50% level. Individual values contributing to this variation ranged from 0.3 to 12 pg of K18 RNA per gene for kidney (Table 1). Variations in liver and brain were less, but the significance of the brain values may be tempered by difficulty of accurately quantitating low levels of RNA. Thus, deletion of the 5′ end of the K18 gene to the *Xho*I site, 250 bp upstream of the start of transcription, results in decreased expression in liver and brain and increased variability of expression in kidney and intestine.

Expression of K18 in transgenic mice is dependent upon inclusion of either the distal 5′ or 3′ flanking sequence. Transgenic mice containing deletions of both the 5′ and 3′ ends of the K18 flanking sequences (K18-XX) expressed little or no K18 RNA in any tissue (Fig. 4). Low levels of RNA were detected in the livers of only two of the four transgenic lines. RNA was detectable in the intestine of only one of the lines. These results are in contrast with those for all other K18 transgenic constructions, which resulted in K18 RNA in the livers, intestines, and kidneys of every transgenic animal. For example, deletion of the 5′ flanking sequence to the *Xho*I site results in loss of copy number-dependent expression, but every transgenic mouse line still expressed the transgenes (Table 1). Similarly, all transgenic animals carrying the 3′ deletions (K18-Xmn) expressed detectable levels of RNA in permissive tissues, albeit at low levels in the livers. We conclude that expression is dependent upon inclusion of either the 5′ or 3′ flanking fragment.

As neither the 5′ nor 3′ flanking sequence is essential for transient expression of K18-driven reporter genes (26), the silence of the K18-XX transgenes may be due to position effects of the sites of integration. Because transgenes are normally found as tandemly duplicated, head-to-tail arrays of the injected fragments, the inclusion of either flanking element would result in the placement of the element both upstream and downstream of most gene copies in the array. Inclusion of either the 5′ or 3′ flanking fragment is apparently sufficient to ensure expression. However, the loss of the linear dependence of RNA expression on gene copy number suggests additional, tissue-dependent regulatory elements present in both flanking elements.

Mutations of the *Alu* RNA Pol3 promoter. The most prominent sequence element located between the *Nsi*I and *Xho*I sites of the 5′ end of the K18 gene is an *Alu*-type repetitive sequence. Two DNase-hypersensitive sites in K18 transgenic liver nuclei mapped to this region. Transcription of the *Alu* sequence was correlated with expression of the K18

TABLE 1. K18 RNA expression of transgenic mice[a]

Vector	Line	Copy no.	Liver			Intestine			Kidney			Brain		
			RNA	RNA/gene	Avg	RNA	RNA/gene	Avg	RNA	RNA/gene	Avg	RNA	RNA/gene	Avg
K18					11.9 ± 4 (n = 5)			8.3 ± 2.8 (n = 5)			6.5 ± 2.3 (n = 4)			4.9 ± 3.9 (n = 4)
Nsi					12.4 ± 2.6 (n = 6)			5.8 ± 0.8 (n = 6)			9.8 ± 2.1 (n = 5)			5.0 (n = 2)
Xmn					2.6 ± 2.0 (n = 6)			4.5 ± 5.7 (n = 5)			7.7 ± 4.2 (n = 5)			3.9 ± 1.4 (n = 6)
Xho	29	1.5	1.5	1.0		4.0	2.7		4.0	2.7		0.5	0.3	
Xho	27	2.2	2.5	1.1		26.0	11.8		8.0	3.7		1.5	0.7	
Xho	14	2.7	2.5	0.9		7.5	2.8		1.0	0.3		2.5	0.9	
Xho	16	6.2	27.0	4.3		90.0	1.5		75.0	12.0		7.5	1.2	
Xho	25	6.4	22.0	3.5		24.0	3.8		43.0	6.7		7.0	1.1	
					2.2 ± 1.6			4.5 ± 4.2			5.1 ± 4.5			0.8 ± 0.4
XX	4	1.3	0.0	0.0		0.0	0.0		4.6	3.5		0.0	0.0	
XX	6	5.0	8.0	1.6		0.0	0.0		0.0	0.0		0.0	0.0	
XX	1	8.4	0.0	0.0		0.0	0.0		6.0	0.7		0.0	0.0	
XX	11	26.3	9.0	0.3		13.8	0.5		6.6	0.2		0.0	0.0	
					0.5 ± 0.8			0.1 ± 0.2			1.1 ± 1.6			0
dB	28	1.6	10.0	6.2		10.0	6.2		35.0	21.5		6.0	3.2	
dB	54	3.1	14.5	4.7		19.0	6.1		41.5	13.4		14.5	4.7	
dB	33	4.4	51.0	11.6		68.0	15.4		108.0	24.5		55.0	12.5	
dB	37	6.6	37.5	5.7		64.0	9.7		62.5	9.4		25.5	3.8	
					7.0 ± 3.1			9.3 ± 4.4			17.2 ± 7			6.1 ± 4.3
dAB	5	1.0	4.0	4.0		4.0	4.0		5.0	5.0		5.0	5.0	
dAB	20	1.0	5.0	5.0		7.5	7.5		14.0	14.0		9.0	18.0	
dAB	15	4.6	33.0	7.2		76.0	16.5		107.0	23.2		60.0	13.0	
dAB	14	7.8	98.0	12.5		115.0	14.8		135.0	17.3		7.0	0.9	
					7.2 ± 3.8			10.7 ± 5.9			14.8 ± 7.6			9.2 ± 7.7
NX	7	3.9	9.4	2.4		4.4	1.1		10.8	2.8		0.0	0.0	
NX	4	7.0	18.4	2.6		4.6	0.6		33.2	4.7		0.0	0.0	
NX	5	8.5	12.8	1.5		8.6	1.0		4.2	0.5		0.0	0.0	
NX	13	17.5	64.3	3.7		54.0	3.1		46.0	2.6		0.0	0.0	
NX	6	24.1	134.8	5.6		60.0	2.5		87.8	3.6		0.0	0.0	
					3.2 ± 1.6			1.7 ± 1.1			2.9 ± 1.6			0
NBX	35	6.8	37.4	5.5		10.2	1.5		65.4	9.6		<2.0	0.0	
NBX	15	7.6	23.2	3.1		8.6	1.1		57.2	7.5		0.0	0.0	
NBX	1	24.7	33.2	1.3		19.6	0.8		66.4	2.7		<2.0	0.0	
NBX	13	94.3	19.2	0.2		60.4	0.6		52.2	0.6		0.0	0.0	
					2.5 ± 2.3			1.0 ± 0.4			5.0 ± 4.2			0

[a] K18 RNA was measured by Northern blot analysis. RNA values are presented as picograms of K18 RNA per 10 μg of total RNA. Average values represent means and standard deviations. Values for the K18, K18-Nsi (Nsi), and K18-Xmn (Xmn) lines of mice have been previously reported (23). Note that the value for liver of K18-Xmn mice is higher than the value previously published; the value presented represents estimations done by the same Northern blot method as used for the remainder of the data. Vector designations are abbreviated by deleting the "K18-" designation.

gene in transgenic mice containing the whole K18 gene (24). To investigate the potential role of the *Alu* RNA Pol3 promoter in regulation of the K18 gene in transgenic mice, two mutations were constructed (Fig. 5A). In the dB mutation, the B-box element of the Pol3 promoter (30) was inactivated by substitution with a *Bgl*II site and the deletion of two nucleotides (Fig. 1 and 5A). In the second construction, both the A- and B-box elements and the region between the two elements were deleted (Fig. 1 and 5A). To confirm the effectiveness of these mutations, both constructions were tested by in vitro transcription in a Pol3 system (32). In vitro transcription of all plasmids that contained the *Alu* element located proximal to the K18 transcription start site resulted in RNAs with 5′ ends corresponding to those expected for the Pol3 promoter (Fig. 5B, lanes 1 to 3). Mutation of the B box or deletion of both the A and B boxes completely abolished Pol3 transcription of the *Alu* element (Fig. 5B, lanes 4 and 5). When replaced within the context of the whole K18 gene, these mutations did not affect expression of the K18 gene in transient transfection of L cells (Fig. 5C).

K18 transgenic mice with *Alu* Pol3 promoter mutations. To test the effect of the mutations on integrated forms of the genes, transgenic mice were generated from two fragments of the K18 gene containing the mutations. The K18-dB and K18-dAB mice contained the B-box mutation and the A- and B-box deletion, respectively, within the context of the previously tested K18-Nsi fragment (Fig. 1). Use of the *Nsi*I fragment eliminated the potential complication of a second *Alu* sequence located upstream of the targeted proximal *Alu* sequence.

The RNAs from three K18-dB and four K18-dAB transgenic mice were analyzed by Northern blot analyses and quantitated. Results are presented in Table 1 and summarized in Fig. 3A. In most respects, the two mutations of the *Alu* Pol3 promoter had little effect on K18 RNA expression. Like the wild-type K18-Nsi mice, the K18-dB and K18-dAB mice expressed the K18 RNA in liver, intestine, kidney, lung, and brain. Spleen, heart, and skeletal muscle remained negative. However, quantitatively, both the K18-dB and K18-dAB mice expressed higher levels of RNA in kidney than did the comparable K18-Nsi mice (Fig. 3A). Expression in liver, intestine, and kidney was proportional to copy number, as indicated by the variation of the expression per gene (Fig. 3B). These experiments suggest that transcriptional activity of the *Alu* Pol3 activity is not essential for

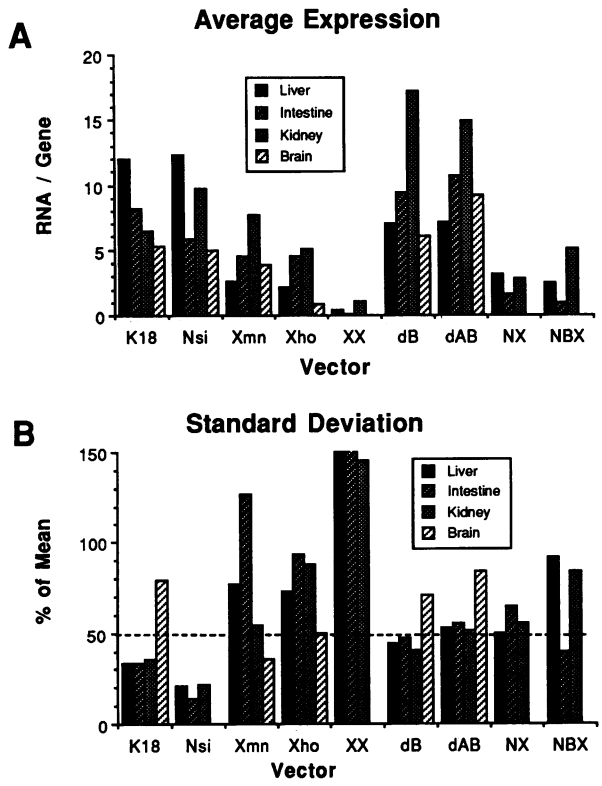

FIG. 3. (A) Average expression per transgene copy in picograms of K18 RNA per 10 μg of total RNA per gene for each vector. The vectors are designated as in Fig. 1 except for the deletion of the "K18-" prefix. Individual data are indicated in Table 1. (B) Standard deviations of the means shown in panel A. The values are represented as percentages of the mean value. Values for XX exceed 150% (see Table 1). Values of about 50% or less indicate copy number-dependent expression.

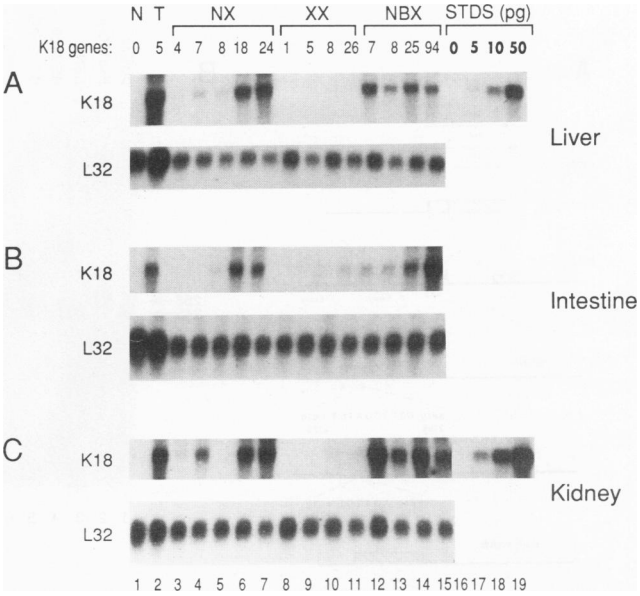

FIG. 4. Northern blot analysis of selected tissue RNAs of K18-NX, K18-XX, and K18-NBX transgenic lines. RNAs from liver, intestine, and kidney are shown in panels A, B, and C, respectively. Panels A and B represent one filter hybridized first with K18 and second with the L32 ribosomal protein probe. Standards shown in panel A are also appropriate for the K18 signals shown in panel B. Panel C represents a second filter analyzed in the same manner. At the top are indicated the transgenic lines analyzed: N, normal nontransgenic; T, K18TG1; NX, K18-NX; XX, K18-XX; and NBX, K18-NBX. STDS, synthetic K18 mRNA standards. "K18 genes" represents the nearest integral copy numbers shown in Table 1.

either efficient, tissue-specific expression or integration site-independent, copy number-dependent expression when assayed in the presence of the 3′ flanking sequences of the K18 gene. However, because the inclusion of either the 5′ or 3′ flanking sequence with the body of the K18 gene ensures expression in intestine and kidney (Fig. 3A; compare K18-Xmn and K18-Xho in intestine and kidney with K18-XX), it was possible that any effect of mutation of the *Alu* Pol3 promoter was masked by the presence of the 3′ flanking element.

To evaluate the effect of the mutations in the *Alu* Pol3 promoter in the absence of additional *Alu* elements in the 3′ flanking sequence (19), we generated additional animals which carried the B-box mutation but without the 3′ flanking sequence (Fig. 1, K18-NBX). A second set of control mice carried the same wild-type fragment (Fig. 1, K18-NX).

Representative Northern blot analyses of these animals are shown in Fig. 4. Individual animal data are presented in Table 1, and a summary of data for all animals is shown in Fig. 3. As expected, when the 3′ deletion was included, expression in liver was decreased in both sets of animals (Fig. 3). In addition, expression in intestine and kidney was also low, and no expression was detected in the brains of transgenic mice carrying either construction. While the average level of expression of the available transgenic lines reveals no difference between the K18-NX and K18-NBX mice in liver, intestine, or kidney, the copy number dependence of expression of the two sets of animals is quite different. Figure 3B and Table 1 compare the standard deviations of the RNA expression per gene copy of the K18-NX and K18-NBX mice. The efficiency of expression of the K18-NBX mice progressively decreased with increasing copy number (Fig. 6). This finding suggests that the NBX fragment did not ensure that all copies of the tandem arrays of transgenes are efficiently expressed. The standard deviations of liver and kidney RNA per gene approached 100% for K18-NBX mice, while those for the control K18-NX mice were similar to the 50% or less values found for the other copy number-dependent fragments, K18, K18-Nsi, K18-dB, and K18-dAB. The B-box mutation leads to greater variation in the efficiency of expression and a striking decrease in efficiency of expression as copy number increases (Fig. 6). However, both the K18-NX and K18-NBX transgenes are expressed in the same tissues as are the control K18 transgenes.

DISCUSSION

Tissue-specific expression of K18 in transgenic mice. K18 is expressed in a variety of simple epithelial tissues, including liver, intestine, kidney, lung, and the ependymal layer of the brain, but is not found in most mesodermally derived tissues such as skeletal muscle, heart, or lymphoid tissues such as spleen (22). The K18 gene appears to be regulated significantly by its chromatin state because the 10-kb human K18 gene is expressed promiscuously when introduced directly into cultured cell lines by transfection but is expressed appropriately in transgenic mice (1, 23). In addition, DNase

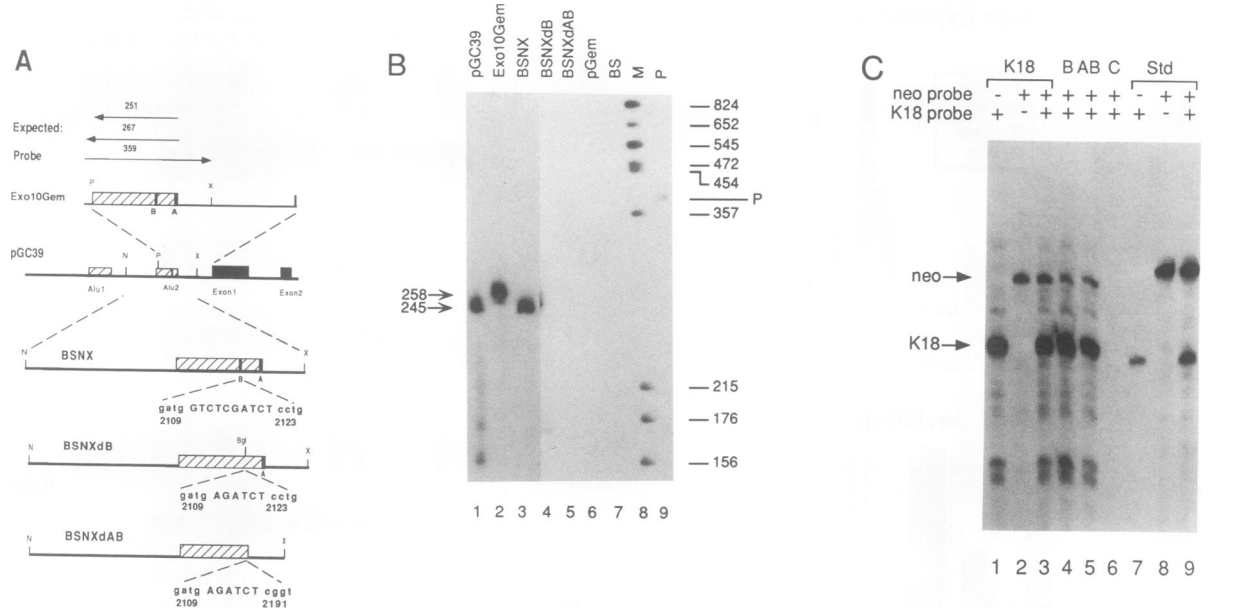

FIG. 5. (A) Schematic representations of the inserts of the plasmids indicated at the left. The plasmid pGC39 insert represents the 5′ 3.9 kb of the K18 gene. The two *Alu* sequences are indicated by hatched boxes; K18 exons are indicated by solid boxes. Restriction enzyme sites for *Nsi*I (N), *Pst*I (P), and *Xho*I (X) are shown. The BSNX series of plasmids represents the *Nsi*I-to-*Xho*I fragment of the K18 gene. Positions of the A-box (A) and B-box (B) elements of the *Alu* Pol3 promoter are indicated in the BSNX map. Alteration of the B box to a *Bgl*II site is indicated in the sequence below the map for BSNXdB, and the deletion of the region from the beginning of the B box to the end of the A box is indicated in the sequence below BSNXdAB. At the top is indicated the nonradioactive probe derived from transcription of *Xho*I-cut plasmid Exo10Gem (Probe). The radioactive fragments derived from Pol3 transcription of the *Alu* sequence after hybridization with the probe and digestion with RNase T_1 are indicated by the top two arrows, with their expected sizes in nucleotides. The protected fragments from Pol3 transcription of plasmid Exo10Gem are expected to be 16 nt longer than those from the other plasmids because of additional polylinker sequences included in the probe. (B) RNase protection analysis of *Alu* transcription in vitro. RNA derived from in vitro transcription of the plasmids indicated at the top were hybridized to a nonradioactive *Alu* probe, digested with RNase T_1, resolved by electrophoresis in an acrylamide gel containing 8 M urea, and detected by intensifer screen-enhanced autoradiography. M, size markers as indicated at the right in nucleotides; P, a synthetic radioactive transcript of the same size as the nonradioactive probe. Exposure times for lanes 1 to 3 and lanes 4 to 9 were 1 and 18 h, respectively. Numbers on the left indicate the estimated sizes in nucleotides of the protected fragments, which are in good correspondence to the sizes expected. Note that no detectable transcripts were generated from plasmids BSNXdb and BSNXdAB or from control plasmids pGEM1 and BSKSM13$^+$. (C) HR9 cells were transfected with a mixture of K18 constructions (Fig. 1) and the pMC1neopA standard plasmid by the calcium phosphate precipitate method. Ten micrograms of RNA isolated from the cells after transfection with the K18 (lanes 1 to 3), K18-dB (B; lane 4), and K18-dAB (AB; lane 5) plasmids was analyzed by RNase protection using simultaneous hybridization with probes for the *neo* gene and the K18 first exon. After electrophoresis under denaturing conditions, the protected fragments were detected by intensifier screen-enhanced autoradiographic exposures to X-ray films. Addition of K18 probe, *neo* probe, or both to the RNA is indicated above the gel. Positions of the K18 and *neo* protected fragments are indicated at the left. C, control of 10 μg of tRNA; Std, 10 pg of synthetic K18 mRNA standard. The K18 synthetic standard is slightly shorter than the authentic K18 mRNA. The relative activity of each construct is judged by the intensity of the K18 signal relative to the *neo* signal.

digestion of nuclei reveals dramatic differences in the chromatin state of the transgenic K18 gene in different tissues (24). The efficient and appropriate tissue-specific expression of the 10-kb K18 gene in transgenic mice provides the potential opportunity of identifying sequences which participate in the specification of the chromatin state of the gene in different tissues. All of the transgenic mice described here except those derived from the K18-XX vector expressed detectable levels of K18 RNA in appropriate tissues and did not show ectopic expression with the possible exception of brain. We conclude that if there are regulatory elements essential for tissue-specific expression of the K18 gene, they must reside within the 5-kb fragment bounded by the *Nsi*I site 1,075 bp upstream of the transcriptional start site and the *Xmn*I site 155 bp downstream of the last exon. Clearly, there are regulatory elements that modulate the level of expression in particular tissue, as first suggested by the much lower expression found in the livers of animals receiving the 3′-deleted K18 gene, K18-Xmn. However, even in those

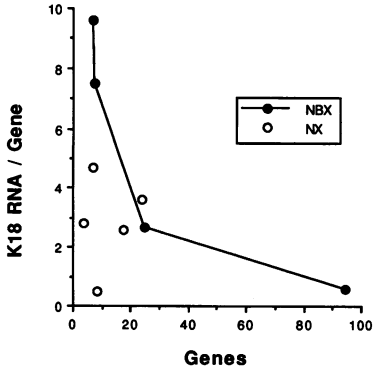

FIG. 6. Decreasing transcriptional efficiency of K18-NBX mice with increasing copy number. The efficiency of expression in kidney expressed as picograms of K18 RNA per gene copy is plotted as a function of the copy number. Each point represents a separate transgenic founder.

mice or the K18-NX and K18-NBX mice which received fragments with additional 1,459-bp 5′ deletions, every transgenic mouse expressed detectable levels of K18 RNA in appropriate tissues. It is possible that even the smallest K18 fragment tested, K18-XX, that contains only 250 bp of sequence upstream of the transcriptional start site is competent for expression, at least in some tissues, because reasonable expression was found in kidney for one animal containing this fragment (Table 1, K18-XX, line 4) and detectable expression was found in liver for two others. In addition, the K18-Xho fragment, which retained the full 3′ end, expressed nearly normal levels of RNA in intestine and kidney and detectable levels in liver and brain. However, in transgenic mice, expression of the K18-XX fragment may be subject to position effects of the sites of integration. The smallest fragment which clearly directs the same tissue specificity as does the K18 gene is the 5-kb K18-NX fragment.

Unlike expression in other tissues examined, expression of transgenic K18 in brain may deviate from the endogenous pattern. Detection of K18 protein by antibody staining had previously confirmed expression in the ependymal layer of epithelial cells lining the ventricles of K18 transgenic mice (1). However, considering that K18-positive tissue represents a very small amount of the total mass of the brain, the level of K18 RNA expression appear quite high in some transgenic lines, particularly the K18-dAB mice. Subsequent analysis of RNA from ventricle-free cerebellar tissue confirmed K18 expression that may represent ectopic expression (data not shown). Detection of K18 gene products in the brains of transgenic animals would require in situ hybridization techniques because the K18 protein is expected to be labile in the absence of a complementary type II keratin subunit (17).

Modulation of the level of K18 expression. The most complex question arising from the data for these transgenic mice concerns the level of expression in different tissues. This issue is complicated by two related considerations. The first is the copy number-dependent behavior of the gene fragments. Clearly, in the absence of position-independent and copy number-dependent expression, it is not possible to reach firm conclusions concerning tissue specific regulatory elements which modulate activity. However, both of these criteria are satisfied in comparing the K18-Nsi and K18-NX mice for tissues other than brain (Fig. 3A and B). In this case, the additional 3′ deletion found in the K18-NX fragment leads to lower activity in liver, intestine, and kidney. Thus, regulatory elements that can modulate activity in these tissues appear to reside in the 3′ flanking sequence of the K18 gene. However, this interpretation is compromised by a comparison of K18 and K18-Xmn mice, which also differ only in the 3′ end. In this case, where copy number dependence is preserved for kidney (but not liver or intestine), the level of K18 RNA in kidney is not affected by the 3′ deletion. Thus, the 3′ deletion has a greater effect when assayed in the context of the shorter 5′ flanking fragment. The simple interpretation that there exists a positive regulatory element in the 3′ flanking sequence that can contribute to kidney expression needs refinement. These complications may be due to the interaction of tissue-specific regulatory elements or, for example, tissue-specific termination activity residing in the distal 5′ flanking sequence of the gene which complements the loss of such activity in tandemly duplicated genes when the 3′ end is deleted.

Integration site-independent expression. In contrast to most transgenic experiments in which a significant proportion of animals fail to express the transgene (29), every transgenic animal from each of the active K18 gene fragments expressed the gene. The silence of many transgenes has been attributed to the *cis*-acting effects of particular sites of integration, a suggestion that has been confirmed directly (3). In transgenic mice, a few genes are expressed independently of their site of integration (1, 4, 5, 7, 13–15). In the case of the lysozyme gene, a gene fragment designated the A element that was associated with nuclear matrix attachment activity conferred both integration site-independent and copy number-dependent expression on a CAT reporter gene construct in cell lines (36). The same element conferred integration site-independent but not copy number-dependent expression on a heterologous gene in transgenic mice (21). The K18-XX fragment appears to be sensitive to its site of integration, while the K18-NX fragment is expressed in every integration site found. Thus, the region defined by the difference between these two fragments, the 825-bp region between the proximal *Nsi*I site and the *Xho*I site just upstream of the transcriptional start site, is necessary for integration site-independent expression of the K18 gene. The level of expression of the K18-NX fragment is lower than that of the K18 or K18-Nsi fragment, but expression can still be considered integration site independent because every mouse expresses the K18 RNA. The absence of nuclear matrix attachment sites within the entire 10-kb K18 gene fragment (1) distinguishes it from the A element of the lysozyme gene.

The *Alu*-type DNA element associated with a 630-bp segment of the first intron of the ADA gene was implicated in the integration site-independent and copy number dependent expression of an ADA-CAT reporter gene construction (4). The B-box mutation of the K18 *Alu* Pol3 promoter abolished the copy number-dependent behavior of the K18-NX fragment but did not destroy the integration site-independent expression of the fragment. While a relatively small number of transgenic animals were recovered for the K18-NX and K18-NBX fragments, the decreasing efficiency of expression with increasing copy number of the K18-NBX fragment was striking (Fig. 6). Thus, the B-box mutation separates the characteristics of integration site-independent expression and copy number-dependent expression. Integration site-independent expression of the K18 gene requires a 825-bp fragment including the proximal *Alu* sequence but does not require the active transcription of the repeated sequence by Pol3. DNA sequences within *Alu*-type repeats that are recognized by specific binding proteins (8, 34, 39) are among the candidate sequences which may mediate position-independent expression. However, the element(s) of the 825-bp fragment responsible for position independent expression may also reside outside the *Alu* sequence.

Copy number-dependent expression of the K18 gene. The functional separation of the K18 characteristics of integration site-independent and copy number-dependent expression is consistent with results reported for both the lysozyme gene A element (21) and the LCR of the β-globin locus (5, 10, 12, 33). A powerful tissue-specific enhancer appears to be responsible for the integration site-independent expression of β-globin LCR constructs. The only enhancer detected in the K18 gene resides within the first intron (26). Copy number-dependent expression implies that each copy of tandemly duplicated gene arrays can function independently not only of the site of integration of the array but also of each neighboring copy of the same gene. Necessary insulating elements might be expected to flank each gene copy. The different results obtained with the K18-NX and K18-NBX fragments in transgenic animals suggest that the transcrip-

tion of the proximal K18 *Alu* sequence by Pol3 is essential for providing full transcriptional insulation of the K18 gene. The minimal effect of the B-box mutation when tested in the presence of the 3′ flanking sequence (K18-dB) implicates a similar, complementary function in the 3′ flanking sequence. Perhaps the *Alu* promoter acts to alleviate transcriptional interference (31) of an upstream K18 gene on the next downstream copy of the K18 gene. Such a function for an *Alu* transcription unit has been previously described for the ε-globin gene (42). In that case, active transcription of the *Alu* sequence blocks transcriptional interference of an upstream promoter on the proximal and tissue-specific ε-globin gene promoter (42). It is possible that an *Alu* sequence implicated in the copy number-dependent behavior of the ADA-CAT fusion gene (4) acts in a similar way. This hypothesis would be consistent with a complementary function in the 3′ end of the K18 gene that may be either a separate transcriptional terminating element or similarly functioning *Alu* sequences. A separate terminating activity in the 3′ end might lead to higher expression than would alternative use of an oppositely oriented *Alu* transcription unit. It will be interesting to determine whether both characteristics of transcriptional insulation, position independence and copy number dependence, are associated with the *Alu* sequence. The concept of insulating a gene by flanking Pol3 transcription units is attractive because it provides a potential explanation of the conservation of the Pol3 elements of *Alu*-type sequences in different species without necessarily strong conservation of the transcribed element itself.

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