A Single Human Keratin 18 Gene Is Expressed in Diverse Epithelial Cells of Transgenic Mice

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Abstract. The expression of keratin 18 (K18) is restricted in humans primarily to a variety of single layered or simple epithelia. However, direct introduction of a cloned K18 gene into cultured, somatic cells by DNA transfection has been shown to result in the promiscuous expression of K18 even while the endogenous mouse form of K18 (Endo B) remains silent. To determine if the cloned K18 genomic DNA fragment contains sufficient information to be regulated appropriately when subjected to a normal developmental environment, and to determine if the cloned gene is expressed in diverse epithelia, the K18 gene, including 2.5 kb of 5′ flanking sequence and 3.5 kb of 3′ flanking sequence, has been introduced into the germ line of mice. Mice from all three resulting K18 transgenic lines express the gene in an appropriate tissue-specific pattern that includes hepatocytes, simple epithelia of the intestinal tract, ductal cells of several glands and epithelial cells of the thymus. No expression of K18 was found in muscle, heart, or in most of the brain even in mice carrying 18 copies of the K18 gene. In most tissues, the level of K18 RNA was directly proportional to copy number and was as efficiently expressed as the endogenous Endo B gene. The K18 protein was identified by both protein blotting methods and indirect immunofluorescence staining. No pathological consequences of overexpression of the K18 gene were observed. The cloned K18 gene appears to contain all *cis*-acting DNA sequences necessary for appropriate expression. In addition, diverse epithelial cell types are able to express this single human gene.

In vertebrates, the ~20 different keratin proteins represent the largest group of intermediate filament proteins. They are further subdivided into the generally smaller and more acidic type I keratins and the larger and more basic type II keratins (8, 46). Keratin intermediate filaments are formed by the polymerization of type I and type II proteins, through the initial formation of a heterotypic dimer (17) and subsequently through tetrameric complexes (16, 43). In adult tissues, the different keratins are expressed in a variety of epithelia in tissue specific patterns that generally include particular pairs of one type I and one type II keratin (8, 32). Keratin 18 (K18) appears to be the ancestral type I keratin from which the other specialized members of the group have evolved (1). Endo B, the mouse form of K18, and its complementary partner Endo A (the mouse form of keratin 8) are the first intermediate filament proteins to be expressed during mouse development. They are first detectable just before the morula stage (7, 11, 12, 36) and are found in trophectoderm at the blastocyst stage (5, 21, 36). In adults, K18 is found primarily in a variety of simple epithelial although exceptions occur (3, 29). In mice, there are five genes closely related to Endo B. Only one appears to be responsible for the expression of the protein. (20, 38, 50). However, Southern hybridization experiments under conditions that do not detect other members of the intermediate gene family, identified over 20 sequences homologous to K18 in humans (38, 50). Thus, it is possible that more than a single human K18 gene might be responsible for expression in different human tissues.

K18 appears to represent a class of genes that are more restricted in their tissue specificity than constitutively active metabolic functions but less restricted than very specialized differentiated products. In somatic mouse cell lines, expression of Endo B is transcriptionally regulated and inversely correlated with both a methylated DNA state and a condensed chromatin state (38). Somatic cell hybridization experiments have implicated *cis*-acting negative regulatory mechanisms in the control of Endo B and K18 expression (38). The observation that the characterized human K18 gene is capable of efficient expression when introduced directly into cultured fibroblastic cells by DNA transfection, even while the endogenous, homologous gene remains silent, reinforces this suggestion (26). The characteristic of inappropriate, promiscuous expression after direct transfection has been observed for other intermediate filament genes as well (22, 34, 42, 52). The results of studies using transgenic mice suggest that the flanking regions of the vimentin and keratin 14 genes may contain the regulatory signals necessary for tissue-specific expression of these intermediate filament genes (41, 52). However, recently an essential regulatory region has been identified within the first intron of the K18 gene. This enhancer element is activated by c-*fos* and

c-*jun* (39). In addition, transgenic mice that have incorporated a K18 construction that includes all available 5′ and 3′ flanking sequences as well as the first intron express a β-galactosidase reporter gene only in embryos but not in adult animals (R. Pedersen and R. G. Oshima, unpublished results). This implies that additional regulatory elements reside within the K18 gene. To determine whether the 10-kb human genomic DNA fragment containing the K18 gene has sufficient regulatory information to be appropriately expressed when subjected to a normal developmental environment, we have generated transgenic mice carrying the K18 gene. In this study, we show that the K18 gene is regulated appropriately in transgenic mice and thus appears to contain all necessary regulatory signals. In addition, diverse epithelia of these transgenic mice are capable of expressing the single cloned K18 gene.

Materials and Methods

Transgenic Mice

The genomic fragment containing the K18 gene was excised from the previously characterized plasmid pGC1853 (26, 27) by digestion with Hind III. The 10-kb fragment, at a DNA concentration of 0.5 ng/μl, was injected into the pronuclei of fertilized strain FVB/N mouse eggs and transferred to pseudopregnant strain CD-1 foster mothers. These manipulations were performed by Ms. Jacqueline Avis using standard methods (19) (Transgenic Mouse Facility of the La Jolla, California Cancer Research Foundation). RNA, protein and histological analyses were performed on tissues dissected from F1 heterozygous mice at ~6 wk. The three founder K18 transgenic mice were assigned La Jolla Cancer Research Foundation animal identification numbers 71, 56, and 59 and are referred to as K18TG1, K18TG2 and K18TG3, respectively, in the text.

Nucleic Acids Analysis

Tail DNAs from resulting progeny were screened by Southern hybridization or dot blot hybridization using the K18 cDNA (37) labeled by the random primer method (13) and nylon filters (Zetaprobe; Bio-Rad Laboratories, Richmond, CA). Final washes were performed at 65°C in 0.1 × SSC and 0.1% SDS. DNA concentrations were determined fluorimetrically (30). Gels used for Southern blot analysis were stained with ethidium bromide both before and after blotting to confirm that the same amount of DNA was loaded in each lane. For quantitative estimation of the number of K18 genes (19), multiple Southern blot and dot blot analyses were performed on multiple concentrations of transgenic animal DNAs. Hybridization to bands detected in Southern blots were compared to those of dilutions of digested plasmid DNAs by densitometry. Dot blot hybridization was quantitated by liquid scintillation counting. Estimations of copy number by the two methods were in good agreement.

Total RNA was isolated by the guanidinium isothiocyanate-cesium chloride method (6, 51). Tissues were frozen in liquid nitrogen immediately after dissection and were then homogenized directly in guanidinium isothiocyanate solutions. For Northern blots, RNAs were separated in agarose gels containing formaldehyde (31), transferred to nylon filters and hybridized at 43°C with the random-primed K18 cDNA excised from plasmid sequences or a 1,287-bp fragment of the Endo B cDNA (45, 50) derived by digestion with Eco RV and Sal I. Final washes were at 70°C for 15 min in 0.1 × SSC and 0.1% SDS. Dot blot analysis of RNA levels were quantitated by liquid scintillation counting.

S1 nuclease protection experiments were performed as previously described using a K18 probe of 471 nt that overlaps the 5′ end of the first exon by 240 nt and an Endo B probe of 658 nt that protects a 196-nt fragment of Endo B RNA (26, 38). Endo B mRNA levels were determined by densitometry of Endo B signals in comparison to standards of synthetic Endo B mRNA made by SP6 RNA polymerase transcription of the full length Endo B cDNA (45) subcloned into the SP64 plasmid.

Protein Analysis

Antibody detection of K18 proteins was performed on tissue homogenates prepared by the method used for immunoprecipitation of Endo B (35). Dissected tissues where homogenized in 0.1% SDS, 10 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mM PMSF, 1 mM *N*-ethylmaleimide, and 1:100 volume of aprotinin (Sigma Chemical Co., St. Louis, MO) solution. The homogenates were treated with 5 μg/ml of protease-free DNase I (40) for 5 min. SDS and EDTA were added to final concentration of 0.5% and 10 mM, respectively, and the samples were heated to 100°C for 2 min. NP-40 detergent was added to a final concentration of 1% and the samples were centrifuged to remove insoluble debris. Protein concentration was determined by the method of Bradford (4) (Bio-Rad Laboratories) on dilutions of the lysates. Equal amounts of protein were resolved by gel electrophoresis in the presence of SDS and transferred to nitrocellulose filters (49). Nonspecific protein binding was blocked with 1% nonfat dried milk. K18 was detected by reaction with the PKK3 monoclonal antibody (gift of Ismo Virtanen, University of Helsinki) directed against K18 (53) followed by alkaline-phosphatase-conjugated anti-mouse IgG (Sigma Chemical Co.). Alkaline phosphatase activity was visualized by reaction with 5-bromo-4-chloro-3-indoly phosphate (175 μg/ml) and nitroblue tetrazolium (340 μg/ml) in 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, and 0.1 M $MgCl_2$.

Immunofluorescence

Tissues dissected from transgenic mice or their nontransgenic siblings were embedded in OCT compound and frozen on dry ice. Frozen sections of 8 μm were fixed in methanol at −20°C for 5 min. For use with mouse monoclonal antibodies the sections were treated with 0.1% H_2SO_4 and 0.5% $KMnO_4$ for 20 s and rinsed in water for 2 min to remove endogenous mouse immunoglobulin reaction (14). To detect K18, the dried sections were treated with the PKK3 mouse monoclonal antibody and FITC conjugated sheep anti-mouse IgG (Organon Teknika-Cappel, Malvern, PA) secondary antibody. PKK3 had no reaction against Endo B in either immunofluorescence or immunoblot analysis. To detect Endo B, rabbit anti-endo B antiserum (35) and rhodamine conjugated goat anti-rabbit IgG (Organon Teknika-Cappel) was used. Endo A was detected with the use of the TROMA1 rat monoclonal antibody (5, 24) and fluorescein-coupled goat anti-rat IgG.

Results

Identification of K18 Transgenic Mice

The human K18 gene fragment used for microinjection was 10 kb containing 2.5 kb of 5′ flanking sequences and 3.5 kb of 3′ flanking sequences (Fig. 1). Dot blot and Southern blot analysis detected K18 sequences in five of 81 resulting mice. However, two of these positive mice were germ line mosaics and were not analyzed further. Southern analysis of the Bam HI digested DNA of the remaining three mice, designated K18TG1, K18TG2 and K18TG3, is shown in Fig. 1. All three mice retained the internal 1.7- and 0.9-kb Bam HI fragments characteristic of the K18 gene. With the high stringency conditions used for the experiment shown in Fig. 1, the gene for the endogenous mouse form of K18 (Endo B β-1) was not detected. Comparison of the intensity of the 1.7- and 0.9-kb fragments of the transgenic mice with plasmid standards by both additional Southern analysis and quantitative dot blot analysis (data not shown) indicated the K18TG1, K18TG2, and K18TG3 mice carried ~4, 6, and 18 copies of the K18 gene, respectively. The largest hybridizing fragments of the three mice likely represent the expected multiple arrays of the two flanking portions of the injected gene that would be expected to be 7.4 kb (3.9-kb 5′ fragment plus the 3.5-kb 3′ fragment) if the genes were integrated in the commonly found head-to-tail arrangement. The bands unique to each transgenic mice between the 6.6-kb and 2.3-kb lambda DNA markers likely represent terminal fragments of the expected tandem arrays of genes and indicate that each of the three mice has integrated the K18 gene at different positions. Additional analysis with a unique K18 gene probe located near the 5′ end of the 5′ flanking sequence of the gene confirmed that each transgenic animal had retained the distal 5′ flanking se-

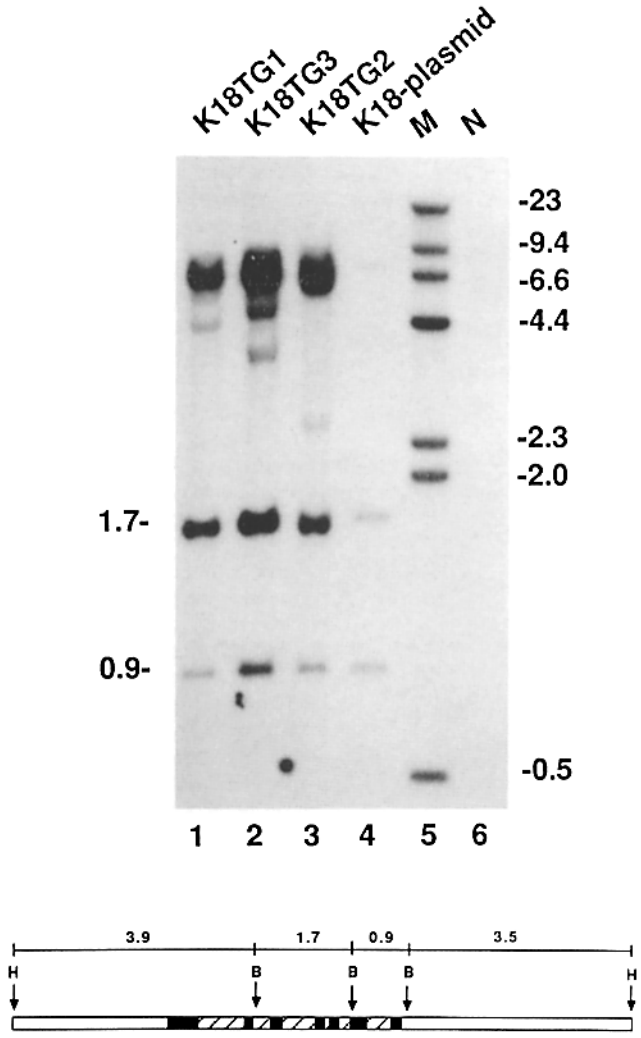


Figure 1. Southern blot analysis of K18 genes in transgenic mice. Mouse tail DNA (5 μg) was digested with Bam HI, subjected to electrophoresis in an agarose gel, transferred to nylon membranes, hybridized to the K18 cDNA probe, and exposed to x-ray film. DNAs from the transgenic mice are indicated in lanes *1–3* and nontransgenic mouse (*N*) DNA is shown in lane *6*. Lane *4* represents ~20 pg of the pGC1853 plasmid that contains the cloned K18 gene. Size markers (*M*) of Hind III digested lambda DNA are shown in lane *5* with the fragment sizes indicated at the left in kilobases. The 1.7- and 0.9-kb fragments of the K18 gene are indicated on the left. A map of the K18 gene used for microinjection is shown below. Exons are indicated by solid regions. Hatched and open regions indicate intron and flanking sequences, respectively. Bam HI (*B*) and Hind III (*H*) restriction enzyme sites are indicated. The sizes of the two internal Bam HI fragments are indicated above the map in kilobases. The cDNA probe hybridizes to all exons.

quences of the injected fragment. In K18TG3 mouse DNA (Fig. 1, lane *2*) only the two largest fragments detected with the K18 cDNA probe also hybridized with the 5′ flanking sequence probe (data not shown). These results are consistent with a single unique integration site for each of the transgenic mice.

Subsequent breeding of the founder transgenic animals have resulted in normal numbers of males and females. The frequencies of transmission ranged from 30 to 70% in individual litters, consistent with single sites of integration. To date, no abnormalities in transgenic animals have been detected. In addition, potential homozygous progeny, as determined by hybridization analysis, have been derived from both the K18TG1 (12/55 progeny) and K18TG2 (1/9 progeny) lines of mice. However, no homozygous animals were detected among 35 progeny of 4 matings from 2 different pairs of K18TG3 heterozygotes. This suggests that either the expression of 36 K18 genes, expected in homozygous K18TG3 mice, is incompatible with normal development or that the insertion of the transgenes have caused a lethal recessive mutation.

K18 mRNA Expression in Transgenic Animals

K18 RNA expression was detected in the adult tissues of all three transgenic lines. A comparison of K18 RNA expression in various tissues of the K18TG1 transgenic line with the expression of endogenous Endo B RNA is shown in Fig. 2. In nontransgenic siblings, the 1.5-kb Endo B mRNA was detected by Northern blot experiments in jejunum, liver, thymus, and cervix but not in heart, skeletal muscle, brain, or spleen (Fig. 2 *A*). Endo B transcripts detected by S1 nuclease analysis (Fig. 2 *C*) were found in similar abundance and in the same tissues of K18TG3 mice as normal nontransgenic mice. In other experiments and with longer exposures, Endo B RNA was detected in kidney tissues but always at very low levels (data not shown). Similarly, RNA from spleen occasionally provided a barely detectable signal. In contrast, Endo B RNA was not detected in muscle or heart even with very long exposures.

Under hybridization conditions that did not detect the endogenous Endo B mRNA (Fig. 2 *B*), the 1.4-kb K18 mRNA was detected easily in RNAs from jejunum, liver, and thymus of K18TG1 mice by Northern blot analysis (Fig. 2 *B*, lanes *5*, *7*, and *10*). The signal for K18 mRNA in cervix may not be visible after reproduction, but additional analysis confirmed its presence (Fig. 2 *D*, lane *10*, and Fig. 3 *A*). In contrast to Endo B RNA in normal mice, the K18 RNA was relatively abundant in kidney and the amount of K18 RNA in thymus was lower than expected from the abundance of Endo B mRNA. S1 nuclease protection experiments confirmed relatively abundant K18 transcripts in the kidney of K18TG1 mice (Fig. 2 *D*, lane *6*) and relatively low amounts in the thymus (Fig. 2 *D*, lane *7*). K18 RNA was barely detectable in RNA from the brains of K18TG3 animals but not from K18TG1 animals (data not shown). Lung tissue had barely detectable Endo B RNA (Fig. 2 *C*, lane *10*) while all K18 transgenic mice expressed detectable amounts of K18 mRNA in this tissue (Fig. 2 *D*, lane *11*, and Fig. 3 *A*). The S1 nuclease protection experiments indicated the K18 RNAs expressed in K18TG1 mice were initiated at the same position as authentic K18 RNA (Fig. 2 *D*, lane *1*) (26). A summary of the expression of K18 and Endo B RNAs, including additional data not shown, is presented in Table I.

Overall, the qualitative, tissue specific expression of K18 RNA in all three transgenic lines was very closely correlated with the expression of the endogenous Endo B RNA found in organs containing simple epithelial tissues. However, the relative level of K18 expression differed from Endo B expression in kidney, lung, and thymus. The discordance with the relative levels of Endo B RNA expression in these permissive tissues appeared not to be due to particular integration sites of the K18 genes because each of the transgenic lines had the

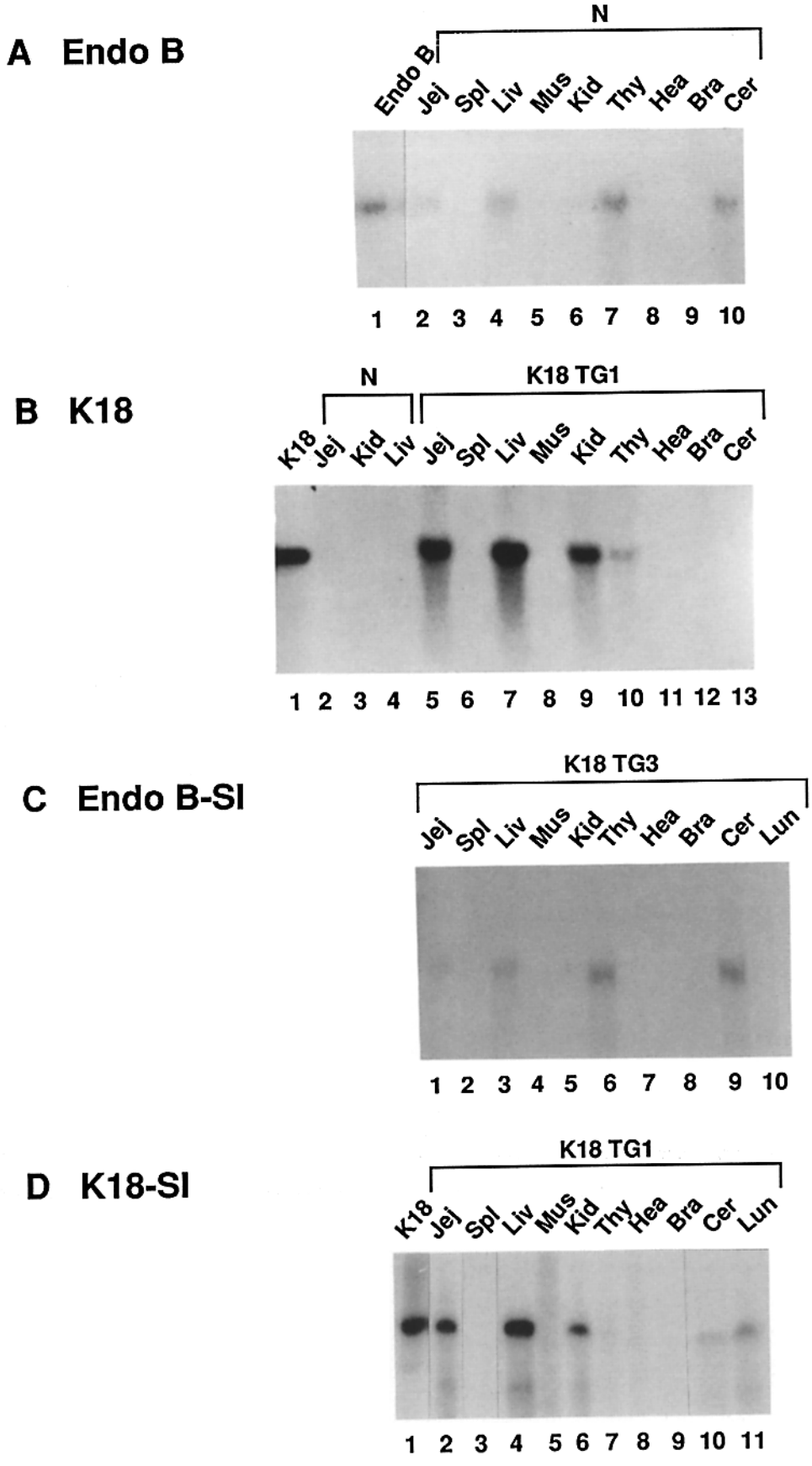


Figure 2. RNA analysis of various tissues of nontransgenic (*N*) and K18 transgenic mouse tissues. Abbreviations used for the different tissues: *Jej*, jejunum; *Spl*, spleen; *Liv*, liver; *Mus*, skeletal muscle; *Kid*, kidney; *Thy*, thymus; *Hea*, heart; *Bra*, brain; *Cer*, cervix; *Lun*, lung. (*A*) Northern blot analysis of nontransgenic tissues for Endo B RNA expression. Lane 1 contained ~25 pg of synthetic Endo B mRNA made by SP6 RNA polymerase transcription of the Endo B cDNA (45) subcloned into the SP64 plasmid. The remaining lanes received 10 μg of total RNA of the indicated tissue. A random-primed 1,287-bp fragment of the Endo B cDNA generated by digestion with Eco RV and Sal I was used as the probe. (*B*) Northern blot analysis of nontransgenic and K18TG1 transgenic tissues of K18 mRNA expression. Each lane contained 10 μg of total RNA except for lane *1* that contained 50 pg of synthetic K18 mRNA made by T7 RNA polymerase transcription of K18 cDNA in the pK187 plasmid (37). The filter was hybridized with the random-primed K18 cDNA free of plasmid sequences. (*C*) S1 nuclease protection analysis of Endo B RNA in tissues of a K18TG3 line mouse. 10 μg of RNA from the indicated tissue was hybridized with a 658 nt single-stranded DNA probe that protects 196 bp of the 5′ end of Endo B mRNA. After S1 nuclease digestion and acrylamide gel electrophoresis in the presence of 8-M urea, the protected fragments were detected by autoradiographic exposure of the dried gel in the presence of an intensified screen. The protected fragment seen in lanes *1*, *3*, *6*, *9*, and faintly in lanes *5* and *10* were the same size (~196 nt) as found for synthetic Endo B mRNA standards (not shown). (*D*) S1 nuclease protection analysis of K18 RNA in tissues of a K18TG1 line mouse. The experiment was performed as for *C* except that the K18 probe was 470 nt in length with a 240 nt overlap with authentic K18 mRNA. Lane *1* shows the migration position and relative intensity resulting from 50 pg of synthetic K18 mRNA as described for *B*.

Table I. Summary of K18 Expression in Transgenic Mice

	EndoB RNA*		K18 RNA‡			K18 prot§	EndoA protǁ
Line¶	TG3	N	TG1	TG2	TG3	TG3	TG3
Stomach	++				+++	+++	+++
Jejunum	++	++	++	++	+++	++	++
Colon	++				++++	++	++
Thymus	++	++	+	++	++	+++	+++
Liver	++	++	++	++	++++	++	++
Cervix	++		+	+	+++	++	++
Vesicular gland	++		+	+	+++	+++	++
Salivary gland	+				++	+++	+++
Kidney	±	±	++	+++	++++	+	+
Lung	±		+	++	+++	+	+
Brain	−	−	−		+	+	+
Spleen	−	−	−	+	+	−	−
Muscle	−	−	−	−	−	−	−
Heart	−	−	−	−	−	−	−
Skin	+			+	+	−	−
Tongue	+				++	−	−
Esophagus					++	−	−

* S1 nuclease protection.
‡ S1, Northern or dot blot.
§ Perceived intensity of immunofluorescent staining with PKK3 antibody.
ǁ Perceived intensity of immunofluorescent staining with TROMA1 antibody.
¶ All animals used were ~6 wk old. *N*, normal; TG1, K18TG1; TG2, K18TG2; TG3, K18TG3.

same pattern of tissue specific expression (Fig. 3 *A*). The level of expression in lung, kidney, jejunum, cervix, and liver appeared to correlate directly with copy number. Careful quantitation of the levels of K18 RNA in the livers of mice from the three transgenic lines and of Endo B in both nontransgenic and transgenic animals confirmed a linear relationship between transgene copy number and K18 RNA expression (Fig. 3 *B*). In addition, the expression of K18 RNA appears to have little or no effect on the level of Endo B RNA expression (Fig. 3 *B*, *inset*, and Fig. 2, *A* and *B*). Thus in vivo, as in cultured cells (26), there appears to be no effect on the expression of K18 RNA on the levels of Endo B mRNA. Finally, the K18 gene appears to be at least as efficiently expressed in liver as the endogenous Endo B genes. Together, the pattern of tissue specific expression, the linear dependence of expression on copy number and the efficiency of expression indicate that the 10-kb K18 genomic fragment used for microinjection appears to contain most if not all of the regulatory signals necessary for normal and position independent expression. Quantitative difference in K18 RNA levels relative to Endo B RNA in some permissive organs appears to be a characteristic of K18 genomic fragment used for microinjection.

K18 Protein Expression and Immunolocalization

To confirm the biological activity of the K18 mRNA detected in transgenic animals, whole tissue lysates of the K18TG3 line were separated by electrophoresis, transferred to nitrocellulose, and probed with a monoclonal antibody, PKK3, that binds human K18 (53) but not Endo B. Fig. 4 shows that PKK3 reactive proteins of the same mobility as authentic K18 were detected in thymus, liver, and jejunum of K18TG3 line transgenic mice but not in muscle or brain. However, the relative abundance of the protein in the three positive tissues did not correlate closely with the relative level of K18 RNA. For example, in the thymus of K18TG3 mice, relatively little K18 RNA was detected (Fig. 3 *A*). However, K18 protein was easily detectable in K18TG3 thymus (Fig. 4, lane *2*). The discordance between the level of K18 RNA and K18 protein is likely due to posttranslational mechanisms as noted in the Discussion.

The tissues that express either Endo B or K18 in transgenic animals are composed of multiple cell types. Immunofluorescent localization was used to identify those cells of complex organs that expressed stable K18 protein. Fig. 5 shows representative results of immunofluorescent staining of K18TG3 transgenic tissues with the PKK3 monoclonal antibody directed against K18. Similar results were obtained with the CK5 monoclonal antibody that recognizes K18 (48) (data not shown). All nontransgenic tissues were negative with the PKK3 antibody. For comparison, nontransgenic tissues were stained with rabbit Endo B antiserum (Fig. 5, *A*, *D*, and *G*). The patterns of K18 expression in K18TG3 jejunum, kidney, and liver were very similar to nontransgenic tissues stained for Endo B expression. K18 was localized to the epithelial cells of jejunum, kidney, colon, and vesicular gland (Fig. 5, *B*, *E*, *K*, and *L*). The pattern of kidney staining of the transgenic mice was virtually indistinguishable from that of Endo B reaction in normal mice even though the level of K18 RNA in kidney appeared relatively higher than that for Endo B (Figs. 2 and 3). The cells of kidney tubules, particularly the distal portions, were strongly reactive (Fig. 5, *E*, and data not shown). In the colon, the basement membranes and connective tissues did not react (Fig. 5, *J* and *K*). In liver, hepatocytes and bile ducts were positive for K18. In the thymus, staining was limited to the reticular cells but not thymocytes (Fig. 5, *C*) while in salivary glands, strong reaction with myoepithelial cells was evident (Fig. 5, *F*). Finally, brain tissue was negative with the exception of the ependymal cell layer that lines ventricles (Fig. 5 *I*). The detection of K18 in the ependymal cells of the brain supports an earlier report of

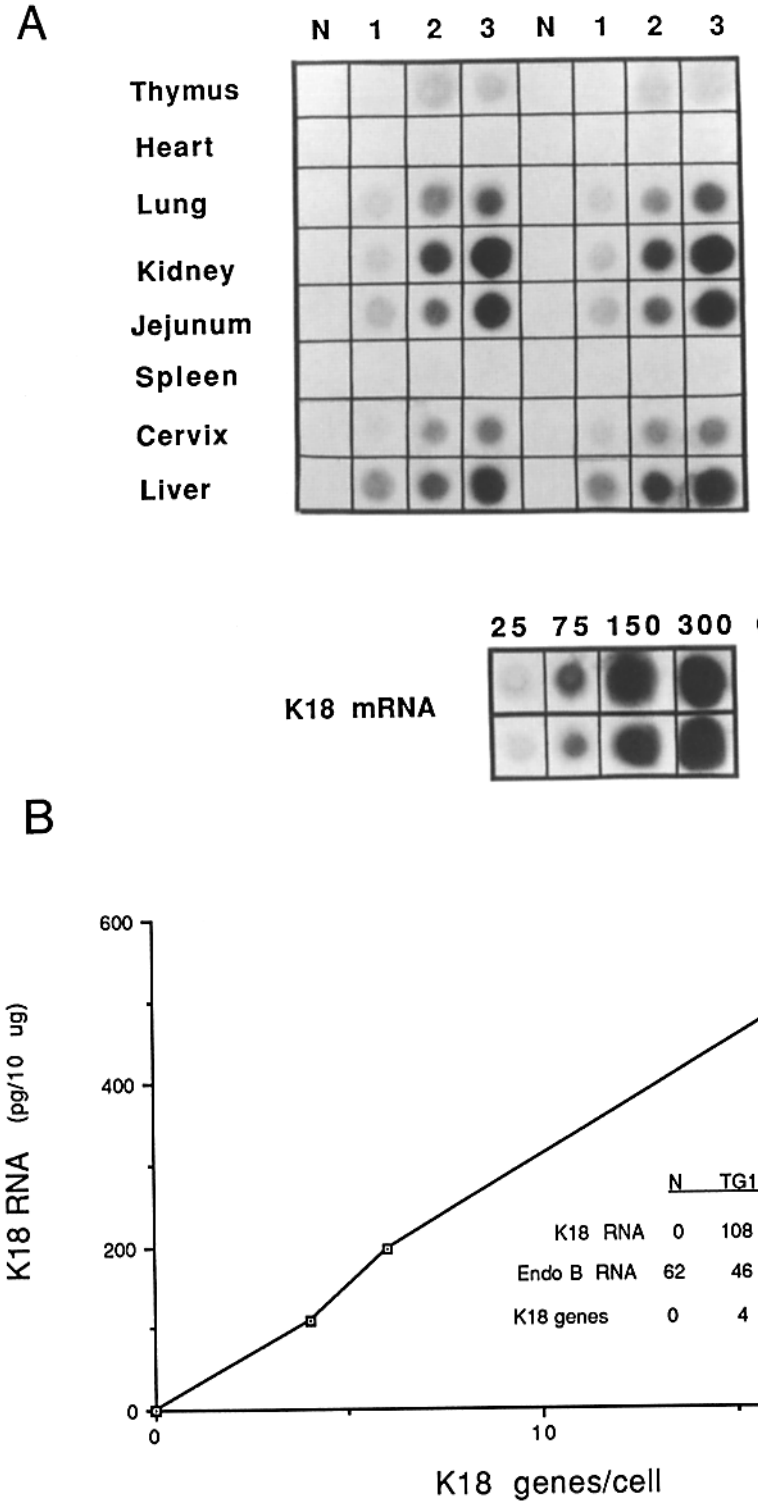


Figure 3. Comparison of the relative levels of K18 RNA in different tissues of three K18 transgenic mice. Duplicate aliquots of 10 μg of RNA from the indicated tissues of a nontransgenic (*N*) mouse, or the transgenic lines K18TG1 (*1*), K18TG2 (*2*), and K18TG3 (*3*) were denatured, immobilized on a nylon filter, hybridized to the ^{32}P-labeled K18 cDNA and washed under stringent conditions. Included on the same filter were varying amounts of synthetic K18 mRNA (small panel) diluted in the presence of tRNA carrier. After fluorographic detection of the signal shown in *A*, the spots were excised and quantitated by liquid scintillation counting. The amount of K18 RNA found in liver samples are plotted in *B* as a function of the number of K18 genes found in the different lines of mice. The inset in *B* also includes the estimates of Endo B RNA abundance in aliquots of the same RNAs used in *A* as determined by S1 nuclease protection and densitometer scanning of appropriately exposed films relative to synthetic Endo B mRNA as standards (data not shown).

keratin type epitopes expressed in these cells (15). No reaction was detectable in multiple samples of muscle and heart tissues (data not shown). At high magnification K18 staining appeared filamentous in positive tissues suggesting that K18 had complexed with an endogenous type II keratin (most likely Endo A) to form typical intermediate filament structures as was previously found in cultured cells (26). The staining patterns of the tissues of K18TG1 and K18TG2 transgenic mice were very similar or identical to those shown for K18TG3 mice. However, the degree of reaction was clearly reduced in K18TG1 mice as might be expected because of the lower gene copy number and RNA levels. Because the Endo B antiserum recognizes both Endo B and K18, it was not possible to localize Endo B independently of K18 in the transgenic mice. The distribution of K18 was compared to that of Endo A, the complementary type II keratin which is

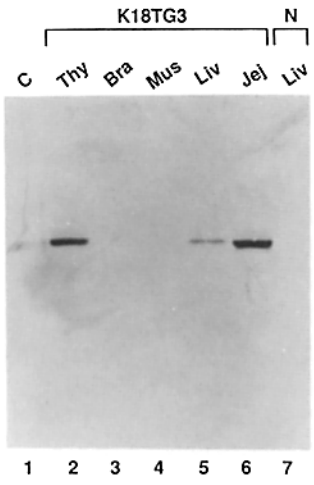


Figure 4. Immunoblot detection of K18 protein in tissue homogenates of different tissues from a line K18TG3 mouse. Proteins solubilized by SDS were separated by acrylamide gel electrophoresis in the presence of SDS, transferred to nitrocellulose and reacted with the PKK3 monoclonal antibody to K18. Antibody binding was visualized by the activity of alkaline phosphatase-coupled antimouse IgG. 20 μg of protein was loaded in each lane. Lane *1* contained a homogenate from the NT2 human embryonal carcinoma cell line as a positive control. A homogenate of liver from a nontransgenic mouse (*N*, lane *7*) did not react with the antibody.

normally coexpressed with Endo B, by use of the TROMA1 monoclonal antibody (5, 24). The distribution of Endo A matched that of K18 in all transgenic tissues examined. A summary of the expression of K18, Endo B RNA, and Endo A protein is shown in Table I. Sections of three stratified epithelial tissues of K18TG3 mice (skin, tongue, and esophagus) did not have detectable reaction with either the PKK3 or TROMA1 antibodies (except for weak reaction of hair follicles in skin) even though K18 RNA was detectable in each of these tissues.

Discussion

The tissue-specific expression of K18 provides strong evidence that the cloned K18 gene contains all regulatory signals necessary for tissue-specific expression provided the gene is exposed to a normal developmental environment. Some of the regulatory sequences of the K18 gene are located within the gene as well as in its flanking regions. For example, the first intron of the K18 gene has been shown to have transcriptional enhancer activity that is dependent upon the interaction of members of the JUN and FOS families of transcription factors (39). This provides one of several different ways of modulating the transcriptional activity of the gene and may be an important component of the restricted expression of the gene in very early embryonic cells. However, even the inclusion of the first intron and all available 5′ and 3′ flanking sequences is not sufficient to ensure the expression of a reporter gene in adult transgenic animals (R. Pedersen and R. G. Oshima, unpublished observations). This implies the existence of additional necessary regulatory sequences located within the body of the gene. It is interesting that the keratin 14 and neurofilament-L genes that are regulated relatively correctly when introduced into transgenic mice (23, 52), share with K18 the characteristic of promiscuous expression upon direct transfection into cultured cells (22, 26, 52). Other genes that are inappropriately expressed after transfection into cell lines but are regulated correctly in transgenic mice include N-*myc* (55) and alpha 1-acid glycoprotein gene (9). In the cases of K18 and N-*myc*, stably transfected cell lines that contain only a few gene copies have been examined in addition to transiently transfected cells. Expression in these permanent lines appears to rule out the possible titration of *trans*-acting negative regulatory molecules in transiently transfected cells that commonly contain many copies of the exogenous genes. Perhaps the general mechanism of restricted transcriptional accessibility that has been proposed as a component of K18 regulation (38) is also important for other intermediate filament genes.

The efficient expression of the K18 genes in the K18TG mice relative to the endogenous Endo B homologue suggests that at least for some permissive tissues, regulatory sequences necessary for appropriate quantitative expression are also included within the K18 genomic fragment. The linear dependence of K18 RNA expression in liver upon the number of integrated K18 genes reinforces this conclusion and suggests that appropriate K18 expression can be obtained independent of the integration site. However, presently this conclusion is based on only a modest number of transgenic animals and additional studies are needed for confirmation. It may be of interest that in a survey of the K18 genomic fragment used in this study, we have found no evidence for sites that preferentially bind to the nuclear scaffold (J. Bode and R. Oshima, unpublished results; reference 2). Such nuclear DNA attachment sites have been implicated in the position independent expression of the chicken lysozyme gene (47).

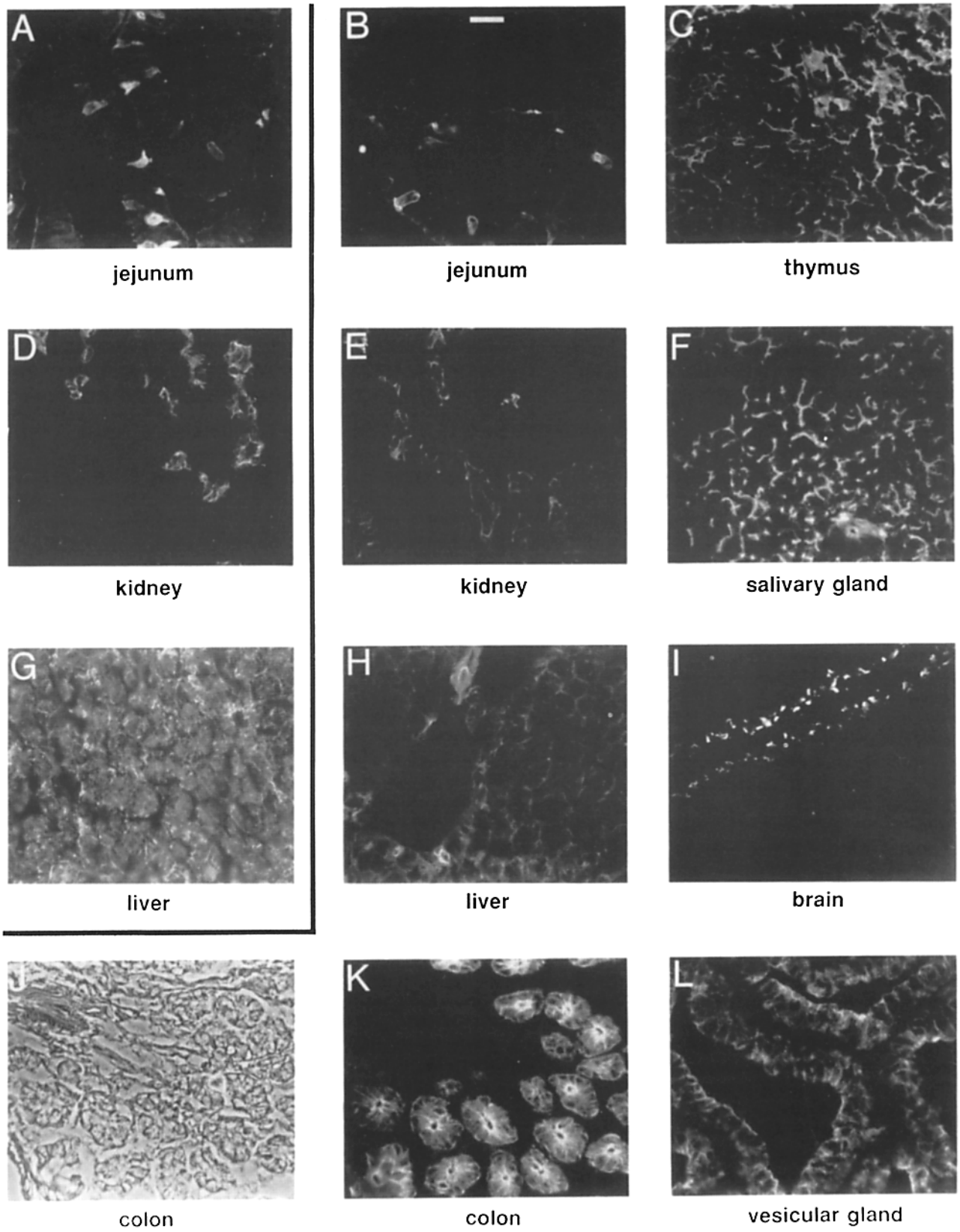
The expression of K18 in a large variety of organs of the transgenic mice described in this study is consistent with the view that only a single K18 gene may be responsible for expression of the protein in the different tissues. However, we cannot exclude the possibility that other K18 genes may be active in humans in addition to the one that has been characterized. This is a significant point because of the large number of K18 genes detectable within primate DNAs (38, 50). Many or all of the additional homologous human sequences may represent processed pseudo genes as found in the mouse (20, 38).

There is significant quantitative discordance of K18 RNA levels with Endo B RNA levels in several permissive tissues. In comparison to Endo B RNA, K18 RNA was lower than expected in thymus and higher than expected in kidney and lung in all three K18 transgenic lines. This variation may be due to species-specific differences in the effectiveness of particular regulatory sequences. Alternatively, it is possible that additional regulatory elements that function to modulate the level of K18 RNA in particular tissues may be located outside of the cloned K18 genomic fragment.

Comparison of the relative levels of K18 RNA in different organs to the level of K18 protein found in tissue homogenates of the same organs by Western blot analysis or by the intensity of immunofluorescent staining indicated that the mRNA level was not a good indicator of the level of accumulated K18 or Endo B protein. This result is in contrast to the results of transgenic mice that express the neurofilament-L gene (23). It is likely due to a combination of the stability of keratin intermediate filaments and the instability of K18 and Endo B proteins that are in excess of a complementary type II keratin. In cultured cells, K18 protein expressed either in the absence or in excess of a complementary type II keratin is degraded rapidly (10, 25, 28). In addition, overexpression of K18 does not lead to a compensatory increase in the endogenous complementary mouse keratin, Endo A.

anti B
nontransgenic

anti K18
K18TG3



The lack of apparent accumulation of K18 intermediate filaments in the K18TG mice suggests that Endo A is similarly independently regulated in vivo. Thus, the amount of K18 protein found in a particular tissue is likely the result of several processes including the rates of synthesis of Endo A, Endo B, K18, and other keratin proteins, the competition of all type I keratins for complementary type II keratin partners, the rate of degradation of excess K18, and the stability of the intermediate filaments containing K18. Such processes likely vary from tissue to tissue. These results reinforce the conclusion of studies with cultured cells that the commonly found equal amounts of Endo B and Endo A or K18 and K8 in simple epithelial cell types are not due to tight regulation of the RNA levels of the complementary keratins (28).

The posttranslational modulation of K18 protein levels may be a significant component of the explanation of why K18 RNA was found in two stratified epithelial tissues (tongue and esophagus) but no apparent antibody reaction could be detected in these tissues (Table I). Detection of K18 RNA in a third stratified epithelium, K18TG3 skin, may be due to inclusion of hair follicles that have been shown to express some K18 (18). However, the detection of Endo B RNA and K18 RNA in tongue and esophagus is consistent with the recent demonstration that both K18 and K8 RNAs are detectable in certain stratified epithelia that previously had been considered negative for K18 (3) and may be significant to the observations that carcinomas derived from stratified epithelium commonly express simple epithelial keratins (32, 33, 44, 54). The reason that K18 protein is not detected in these normal tissues may involve the particular anti-K18 monoclonal antibody because significant differences in the reaction of a few tissues with different K18 antibodies have been documented (3). However, regardless of the reason that K18 protein is not detected in these tissues, the presence of detectable K18 RNA, at least in the K18TG3 line that carries the highest number of copies of the K18 gene, suggests that three different transcriptional states of the K18 gene exist in the adult animal. In permissive tissues like liver and the epithelia of colon and jejunum, the gene is expressed efficiently. In nonpermissive tissues (heart, skeletal muscle, and the brain exclusive of the ependymal cells of the ventricles) the K18 gene appears silent even in mice containing 18 copies of the gene. In a few tissues (esophagus, tongue, and perhaps skin) the K18 genes appear to be transcriptionally active but very weakly expressed.

With the use of additional transgenic mice and pluripotent teratocarcinoma cells it should be possible to identify those regulatory elements necessary for the efficient transcriptional activity of the K18 gene, its stable repression in nonpermissive tissues, and its apparent characteristic of position independent, gene copy number dependent expression.

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Figure 5. Indirect immunofluorescent detection of K18 in K18 transgenic mouse tissues and Endo B in nontransgenic animals. Frozen sections of the indicated tissues were fixed in methanol, treated to remove reaction of the anti-mouse IgG secondary antibody with endogenous mouse antibody (see Materials and Methods), and reacted with the PKK3 mouse monoclonal antibody that recognizes K18 (anti-K18). Reaction was visualized by reaction with FITC-conjugated sheep anti-mouse IgG antibodies. Endo B (*A*, *D*, and *G*) was detected with the rabbit anti-Endo B antiserum (35) (anti-B) and rhodamine-conjugated goat anti-rabbit antibodies. *J* shows the phase-contrast appearance of the same section shown in *K*. The bar shown in *B* represents 25.6 μm. The two layers of reactive cells shown in *I* of the brain of the K18TG3 mouse represent the ependymal cells of a ventricle. Other brain tissues were negative. *A–L*, same magnification.

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