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Tissue-specific and efficient expression of the human simple epithelial keratin 8 gene in transgenic mice

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SUMMARY

Keratin 8 is a type II intermediate filament protein found in simple epithelia. We have introduced a 12 kb DNA fragment of the human K8 locus into the germ line of mice. The transgene, containing 1.1 kb of 5′ flanking sequences, 7.7 kb corresponding to the body of the gene and 3.2 kb of 3′ flanking sequences, was expressed in all six lines obtained. Immunolocalization and RNA analysis of adult tissues showed that the tissue-specific expression pattern of the transgene was almost indistinguishable from that of the endogenous gene. This pattern was found in organs containing single epithelial cell types, such as trachea, lung, stomach, intestine, liver, kidney, thymus and glands. The highest expressing line, however, also produced human K8 in tissues such as stratified epithelia, where it formed part of the pre-existing keratin cytoskeleton of basal cells. Steady state levels of human K8 RNA were proportional to the copy number of the transgene, but transgene expression was less efficient, per gene copy, than that of the endogenous gene. When in the 12 kb DNA fragment the exons and introns of the gene were replaced by the *Escherichia coli lacZ* gene, the resulting construct showed no expression in transgenic mice. This suggests that 5′ and 3′ flanking sequences, in the absence of intragenic sequences, are not sufficient for K8 expression and that important control elements are located in the body of the K8 gene.

Key words: keratin 8, simple epithelium, intermediate filament, transgenic mice, gene expression

INTRODUCTION

Keratin intermediate filaments (IF) are a major constituent of the cytoskeleton of vertebrate epithelial cells. They are encoded by two gene families corresponding to acidic (type I) and basic (type II) keratins. Keratin filaments are obligate heteropolymers of specific pairs of type I and type II polypeptides. The different epithelial cell types are characterized by specific keratin patterns determined by each of these pairs, or combination of pairs (reviewed by Coulombe, 1993; Moll et al., 1982; Quinlan et al., 1985; Steinert and Roop, 1988).

The type II keratin K8 is found associated with the type I keratin K18 and with the type I keratin K19 in single-layered (simple) epithelia (Moll et al., 1982; Sun et al., 1984). The K8-K18 pair constitutes the IF of hepatocytes, tubular epithelium of the kidney, luminal epithelium of the mammary gland and pancreatic epithelia (Franke et al., 1981a,b; Moll et al., 1982) while the predominant IF in intestinal epithelia and choroid plexus are composed of both K8-K18 and K8-K19 pairs (Franke et al., 1981b; Miettinen et al., 1986; Moll et al., 1982). The K8-K18 pair also contributes to the more complex keratin pattern of simple epithelia that comprise different cell types, such as those in trachea, or the transitional epithelium of the urinary bladder (Blobel et al., 1984; Moll et al., 1982; Summerhayes and Chen, 1982). On the contrary, K8 and K18 are not found, or only as minor components, in multilayered (stratified) epithelia (Bosch et al., 1988; Franke et al., 1981b). In addition, K8 and K18 are also found in a variety of carcinomas derived from internal epithelia, as well as in skin carcinomas (Markey et al., 1991; Moll et al., 1982; Quinlan et al., 1985).

K8, and its partner K18 (or endo A and endo B, as they are also termed in the mouse) are the first IF expressed during mouse development. They are found at the morula stage and, subsequently, in the trophectoderm of blastocysts (Chisholm and Houliston, 1987; Duprey et al., 1985; Jackson et al., 1980; Oshima et al., 1983). In the postimplantation embryo, they are present in the placenta and extraembryonic membranes, as well as in embryonic simple epithelia (Hashido et al., 1991; Thorey et al., 1993b). In fact, they are the major IF proteins in the embryo until day 10 (Franke et al., 1982). However, the recent generation of mice deficient in K8 (by gene targeting in embryonic stem cells) has shown that K8/K18 filaments are dispensable for the formation of the trophoblast and other extraembryonic membranes (Baribault et al., 1993). K8/K18 filaments, though, serve an essential function in embryogenesis, as shown by the lethality caused by the K8 null mutation at around day 12 of development (Baribault et al., 1993).

The mouse and human genomes contain single active genes for K8 and K18 among a large number of pseudogenes (Krauss

and Franke, 1990; Kulesh and Oshima, 1988; Oshima et al., 1988; Vasseur et al., 1985; Waseem et al., 1990). K8 and K18 gene expression in murine and human tissue culture cell lines is regulated, at least in part, at the transcriptional level (Knapp and Franke, 1989; Oshima et al., 1988). The K8 and K18 genes show cell-type-dependent methylation (Knapp and Franke, 1989; Oshima et al., 1988; Tamai et al., 1991). In somatic cells such as myoblasts or fibroblasts, which do not express K8 or K18, the genes are hypermethylated. In contrast, in differentiated embryonal carcinoma and endodermal cells, which express K8 and K18, the genes are hypomethylated. In undifferentiated embryonal carcinoma cells, however, the genes are also hypomethylated, although they do not express K8 or K18, perhaps due to the presence of a repressor (Crémisi and Duprey, 1987).

Recently it has been shown that a 10 kb genomic fragment containing the human K18 gene is expressed in a tissue-specific and copy-dependent manner in transgenic mice (Abe and Oshima, 1990). Both 5′ and 3′ flanking sequences are important for efficient expression of the K18 gene (Neznanov et al., 1993; Thorey et al., 1993a). In addition, an enhancer activity in the first intron of the gene has been found in tissue culture cells (Oshima et al., 1990). This *cis*-acting region contains binding sites for transcription factors such as Ets and AP-1, and it is believed to mediate the activation of K18 through the Ras signalling transduction pathway (Pankov et al., 1994).

In contrast to K18, very little is known about the sequences controlling K8 gene expression. So far, only an enhancer in the 3′ flanking region of the murine K8 gene has been found in transient transfection assays of endodermal cells (Fujimura et al., 1994; Takemoto et al., 1991). To investigate the *cis*-regulatory regions of the K8 gene, we have generated transgenic mice carrying the human K8 gene. In this study we report that a 12 kb genomic DNA fragment containing the K8 gene flanked by 1.1 and 3.2 kb of 5′ and 3′ sequences is expressed in most tissues that express the endogenous K8 gene. Moreover, we found that, in the absence of the intragenic region, the 5′ and 3′ flanking sequences are not sufficient for K8 expression.

MATERIALS AND METHODS

Plasmids

Construct HK8.12 is a *Sal*I fragment from phage λH8-101, which contains the entire human K8 gene (Krauss and Franke, 1990) and was kindly provided by Dr W. Franke. To construct K8-5′Z3′, a fragment of HK8.12 containing 1.1 kb of 5′ flanking and promoter sequences, and part of the first exon of the human K8 gene, was fused in frame to an *Escherichia coli lacZ* gene derived from pRSV*lacZ* (Edlund et al., 1985). The *Xho*I site in the first K8 exon was filled in with the Klenow fragment of DNA polymerase I and ligated to the *Kpn*I site of the *lacZ* gene previously repaired with T4 DNA polymerase. Subsequently, a 2.6 kb *Xba*I-*Sal*I fragment of HK8.12, containing 3′ flanking sequences, was cloned downstream from the SV40 polyadenlylation signals of the *lacZ* gene.

Transgenic mice and DNA analysis

DNA fragments were isolated free of vector sequences by preparative gel electrophoresis. After further purification using an Elutip column (Schleicher and Schuell), DNA was dissolved at 1-2 μg/ml in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA and microinjected in the pronuclei of fertilized oocytes from superovulated (C57BL/10 × Balb/c)F1 females mated with (C57BL/10 × Balb/c)F1 males. Surviving injected eggs were transferred to pseudopregnant recipients of either Swiss or (C57BL/10 × Balb/c)F1 strains, as described (Hogan et al., 1985). Transgenic mice were identified by Southern blot analysis of 5-10 μg of tail DNA using standard protocols (Sambrook et al., 1989). For copy number determinations, DNA obtained from animals from established transgenic lines was used in Southern blot analysis of *Pst*I-digested DNA hybridized to a *Pst*I fragment of the HK8 clones (nucleotides 4027-5974 in the HK8 sequences; Krauss and Franke, 1990). The intensity of the band corresponding to the internal *Pst*I fragment was measured by densitometric scanning of different exposures of the blots using a Personal densitometer, or using a PhosphorImager (Molecular Dynamics). Signals were compared with those of known amounts of plasmid DNA electrophoresed on the same gels. Hybridization to a mouse *Thy-1* probe was used to normalize for loading differences.

Preparation and analysis of RNA

RNA from various tissues was prepared by the acid phenol method (Chomczynski and Sacchi, 1987). Keratin transcripts were detected by northern blot analysis of total RNA with random-primed ^{32}P-labelled probes, using standard protocols (Sambrook et al., 1989). RNA from HK8.12 mice was hybridized to a 1.0 kb *Sac*I-*Xba*I genomic fragment spanning part of the last exon and 3′ flanking sequences of the human K8 gene. RNA from HK8-5′Z3′ mice was probed with a *Hin*dIII-*Bam*HI fragment from plasmid pRSV*lacZ* corresponding to the *gpt-trpS-lacZ* gene (Edlund et al., 1985). The endogenous mouse K8 was detected with a 300 bp *Bam*HI-*Eco*RI 3′ fragment of the mouse K8 cDNA (Morita et al., 1988). RNA levels were measured by densitometric scanning of different exposures of blots hybridized to K8 probes. Filters were reprobed with a rat β-actin probe to normalize for RNA loading differences.

Immunofluorescence analysis and X-gal histochemistry

Indirect immunofluorescence was used to detect human and mouse K8. Tissues were dissected out and rapidly frozen in OCT medium (Miles Inc.). Sections of 7 μm thickness were cut, mounted on poly-L-lysine-coated slides, and either used immediately or kept frozen at −70ºC. Tissue sections were air-dried and fixed with acetone at −20ºC for 10 minutes, air-dried again and rinsed three times in phosphate-buffered saline (PBS). Sections were preincubated for 20 minutes at 37ºC with a 1:100 dilution of horse serum. Then, they were incubated for 1 hour at 37ºC in a humidified chamber with either TROMA-1 (Brûlet et al., 1980), a rat monoclonal antibody that recognizes mouse and human K8, or CAM-5.2 (Becton Dickinson), a mouse monoclonal antibody that preferentially recognizes human K8. Antibodies were diluted in PBS containing 1% bovine serum albumin. Subsequently, sections were washed three times at room temperature with PBS and then incubated with 1:100 dilutions of fluorescein-conjugated donkey anti-rat or Texas Red-conjugated donkey anti-mouse secondary antibodies (ImmunoResearch Labs. Inc.) for 1 hour at 37ºC. After thorough washes with PBS, slides were mounted in Mowiol. Controls were included to asses non-specific immunofluorescent staining. For instance, antibody CAM-5.2 was tested on non-transgenic tissues, or sections were incubated only with secondary antibodies. Samples were observed with a Zeiss Axiophot fluorescence microscope and photographed with Ilford HP5 Plus ASA 400 film.

β-Galactosidase activity was assayed histochemically on sections fixed in 0.2% glutaraldehyde for 5 minutes After three washes in PBS, sections were incubated in 0.75 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside, Boehringer Mannheim Biochemicals), 10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$ and 1.2 mM $MgCl_2$ in PBS at 37ºC overnight. After incubation, sections were rinsed in PBS and counterstained with eosin.

RESULTS

HK8 RNA expression in transgenic mice

A 12 kb genomic fragment of the human K8 locus, which produces K8 protein in transiently transfected tissue cultures cells (Krauss and Franke, 1990), was used to generate transgenic mice. In this fragment, termed HK8.12 (Fig. 1A), the human K8 gene is flanked by 1.1 kb and 3.2 kb of 5′ and 3′ flanking sequences, respectively. Six founder transgenic mice were obtained, and transgenic lines were derived from all six. The analysis included only animals from established lines. The RNA expression pattern of HK8.12 transgenic mice was determined by northern blot analysis using a probe that spans the last exon of the human K8 gene (Fig. 1A). A representative blot, as well as a summary of the results, is shown in Fig. 1B,C. All lines expressed the transgene in tissues where the mouse K8 RNA was found (digestive tract, thymus, liver, lung and kidney) but not in others, such as brain or tailskin, which did not express mouse K8. An exception was the urinary bladder, an organ where human K8 RNA was detected in some but not all lines. The highest levels of human and mouse K8 RNA were found in stomach and intestine. The relative levels of mouse K8 RNA in transgenic mice did not differ from those in non-transgenic littermates (not shown), which indicates that human K8 RNA expression did not affect the expression of the endogenous gene. In some lines we also found human K8 transcripts in tissues that do not express the endogenous gene, such as spleen or striated muscle. Thus, HK8 expression was seen in the spleen of lines 1864 and 1865, whereas lines 1851, 1918 and 1865 showed transgene expression in muscle.

The levels of human K8 RNA in many tissues seemed related to the copy number of the transgene. To address this aspect of human K8 expression we studied the genomic organization of the transgenes by Southern blot. Probe 1 (Fig. 2A) hybridized to a 2.0 kb internal fragment of *Pst*I-digested DNA (Fig. 2B). The intensities of the hybridization signals for this band were used for transgene copy number determinations as described in Materials and methods. The presence of additional bands in the lane corresponding to line 1908 indicated that some of the copies were rearranged in this line. Probe 2 (Fig. 2A) was hybridized to *Sac*I-digested DNA to identify junction fragments, as well as end fragments corresponding to chromosomal DNA flanking the transgene. Four of the lines (1908, 1851, 1918 and 1865) showed the 4.5 kb band expected for the junction fragment resulting from several copies of the transgene integrated in a head to tail array (Fig. 2). The two bands in the lane with DNA from line 1910 (Fig. 2B, right panel) were not consistent with the presence of intact copies

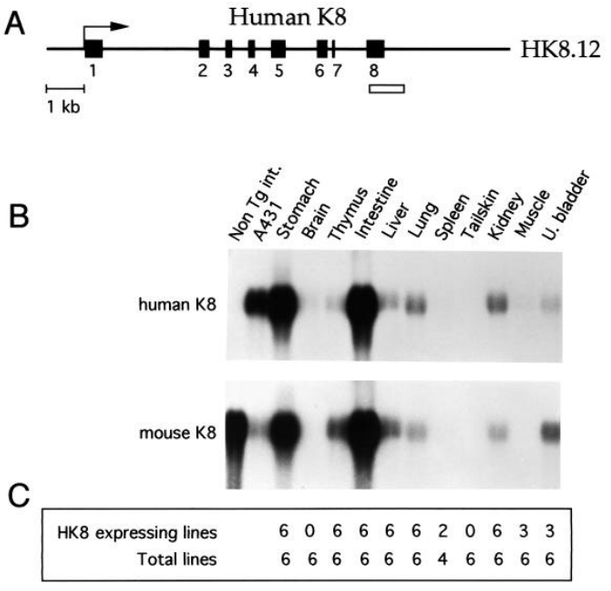

Fig. 1. Analysis of transgene (Tg) expression in tissues of HK8.12 transgenic animals. (A) Schematic representation of the HK8.12 construct. Numbered filled boxes represent exons and thin lines HK8 intronic and flanking sequences. The arrow denotes the start site of transcription. The empty box below the map indicates the probe used for northern blots. (B) Representative example of northern blot analysis using 10 μg of total RNA isolated from the indicated tissues of transgenic line 1918. RNA from the intestine of a non-transgenic mouse and RNA from the human epithelial cell line A431 were used as controls. The probe used to detect human K8 RNA was a 1.0 kb genomic fragment encompassing the last exon of the HK8 gene (indicated above). After stripping filters from the HK8 probe, they were probed with a 300 bp fragment of the 3′ end of the mouse K8 cDNA. (C) Summary of human K8 northern blot analysis from several independent transgenic lines, showing the number of lines that expressed human K8 out of the total number of lines analyzed.

of the transgene integrated in head to head or tail to tail orientations. Therefore, this mouse has at least one rearranged copy of the transgene. The unique band, larger than 4.5 kb, seen in the lane with DNA from line 1864 (Fig. 2B, right panel) agreed with copy number estimations on this line, which showed it had a single copy of the transgene. From these results, together with others from Southern blots not shown, we conclude that four of the six transgenic lines contained all (lines 1864 and 1851) or most (lines 1918 and 1865) of the copies of the transgene intact and inserted in a head to tail fashion.

Table 1. Quantitation and expression levels of HK8.12 transgenic mice

		Human K8 RNA*					
Tg line	Copies	Intestine	Kidney	Liver	Lung	Stomach	Thymus
1864	1	2207 (0.4)†	1650	452	259	1292 (0.6)	123
1851	5	2667 (0.4)	211	129	161	2663 (1.2)	420
1918	25	16080 (2.1)	1123	2103	583	9331 (4.2)	1238
1865	108	97997 (11.0)	7274	6943	3917	52743 (10.1)	8452

*HK8 RNA was quantitated by densitometric scanning of northern blots. Densitometric values within each tissue were normalized for the β-actin content of the particular tissue. Tg, transgene.

†Ratio between HK8 and mouse K8 RNA signals, after correction for differences in the specific activity of the probes.

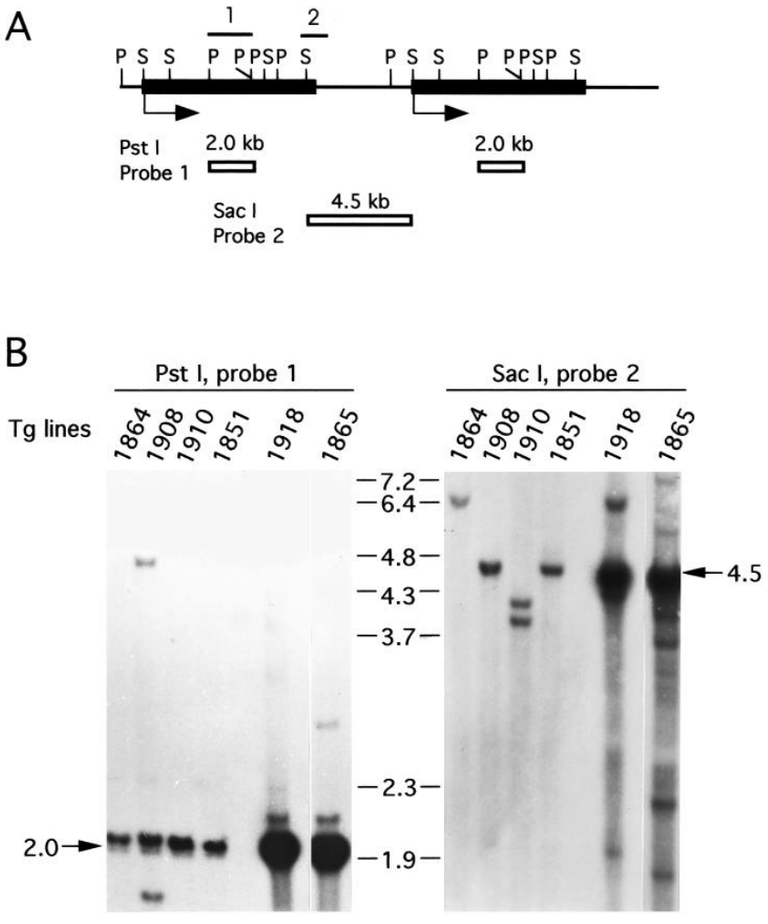

Fig. 2. Genomic organization of HK8.12 transgenes. (A) Schematic representation of the transgene as if it was in head-to-tail tandem array; the body of the gene (exons and introns) is shown as a filled box; arrows show transcription initiation sites. *Pst*I (P) and *Sac*I (S) restriction sites are indicated. Probes 1 and 2 are indicated above the map. The expected sizes of the *Pst*I and *Sac*I fragments that hybridize to probes 1 and 2 are shown as empty boxes below the map.
(B) Southern blot analysis of 10 μg of DNA isolated from the tail tip or from liver. The left panel shows a *Pst*I digest of DNA hybridized to probe 1. The right panel shows a *Sac*I digest of DNA hybridized to probe 2. The mouse lines' designations are shown above the lanes. Sizes in kbp of lambda DNA cut with *Bst*EII are shown in the center. The 2.0 kb *Pst*I internal fragment expected with probe 1 and the 4.5 kb *Sac*I junction fragment expected with probe 2 are shown by arrows.

Human K8 RNA levels in the four lines carrying intact copies of the transgene were measured by densitometric analysis of northern blots of RNA isolated from liver, kidney, stomach, lung, intestine and thymus. Results are summarized in Table 1. A direct relationship between transgenic copy number and human K8 RNA expression was found for lines 1851, 1918 and 1865, which contain 5, 25 and 108 copies, respectively. However, the single copy transgenic line 1864 showed higher human K8 RNA levels per copy than the other mice. The ratio of human to mouse K8 RNA levels (values in parenthesis in Table 1) showed that human K8 RNA expression, per gene copy, was about 10-fold less efficient than that of the endogenous gene.

Immunolocalization of human K8 in transgenic mice

The tissues that express K8 are constituted of multiple cell types. To examine the cell-type specific expression pattern of the transgene and compare it with that of mouse K8 we used immunofluorescence localization. As TROMA-1, the antibody that recognizes mouse K8, also reacts with human K8, we analyzed mouse K8 expression on tissue sections of non-transgenic littermates. CAM-5.2, the antibody that recognizes human K8, did not stain tissues from non-transgenic mice (not shown). A summary of the immunofluorescent pattern of human K8 in three transgenic lines, as well as that of mouse K8, is shown in Table 2. Fig. 3 shows representative examples of this analysis. The study of the other three lines included only some of the tissues shown in Table 2.

Overall, the expression pattern of human K8 was similar in five of the six lines, and coincided with that of mouse K8. Expression in urinary bladder, however, showed variability among lines. In these five transgenic lines (1908 and 1918 are examples) expression was restricted to simple epithelial cells. Thus, the mesothelial cells that cover many organs stained strongly with the two antibodies. In the liver, both keratins were found in hepatocytes, biliary ducts and the epithelium of

Fig. 3. Immunolocalization of human K8 in transgenic mice and of mouse K8 in non-transgenic mice. Frozen sections of transgenic tissues (A-D, I-L) were incubated with CAM-5.2, a murine monoclonal antibody that recognizes human K8. Frozen sections of non-transgenic tissues (E-H, M-P) were incubated with TROMA-1, a rat monoclonal antibody that recognizes both mouse and human K8. Staining was observed after incubation with Texas-Red-conjugated donkey anti-mouse or fluorescein-conjugated donkey anti-rat antibodies, respectively. The lack of staining of non-transgenic tissues with CAM-5.2 antibody (not shown) indicates that immunofluorescent staining of transgenic tissues is due to reaction with human K8. Abbreviations: gb, gall bladder; s, serosa; g, glandular stomach; f, forestomach. Bar: 57 μm (A,B,D,E,F,K,L,O); 90 μm (C,G,H,I,J,M,N,P).

the gall bladder (Fig. 3A,E). In the respiratory system, the antibodies reacted only with the respiratory epithelium that lines the trachea, bronchi, bronchioles and alveoli (Fig. 3B,F). Mouse and human K8 were detected throughout the thymic

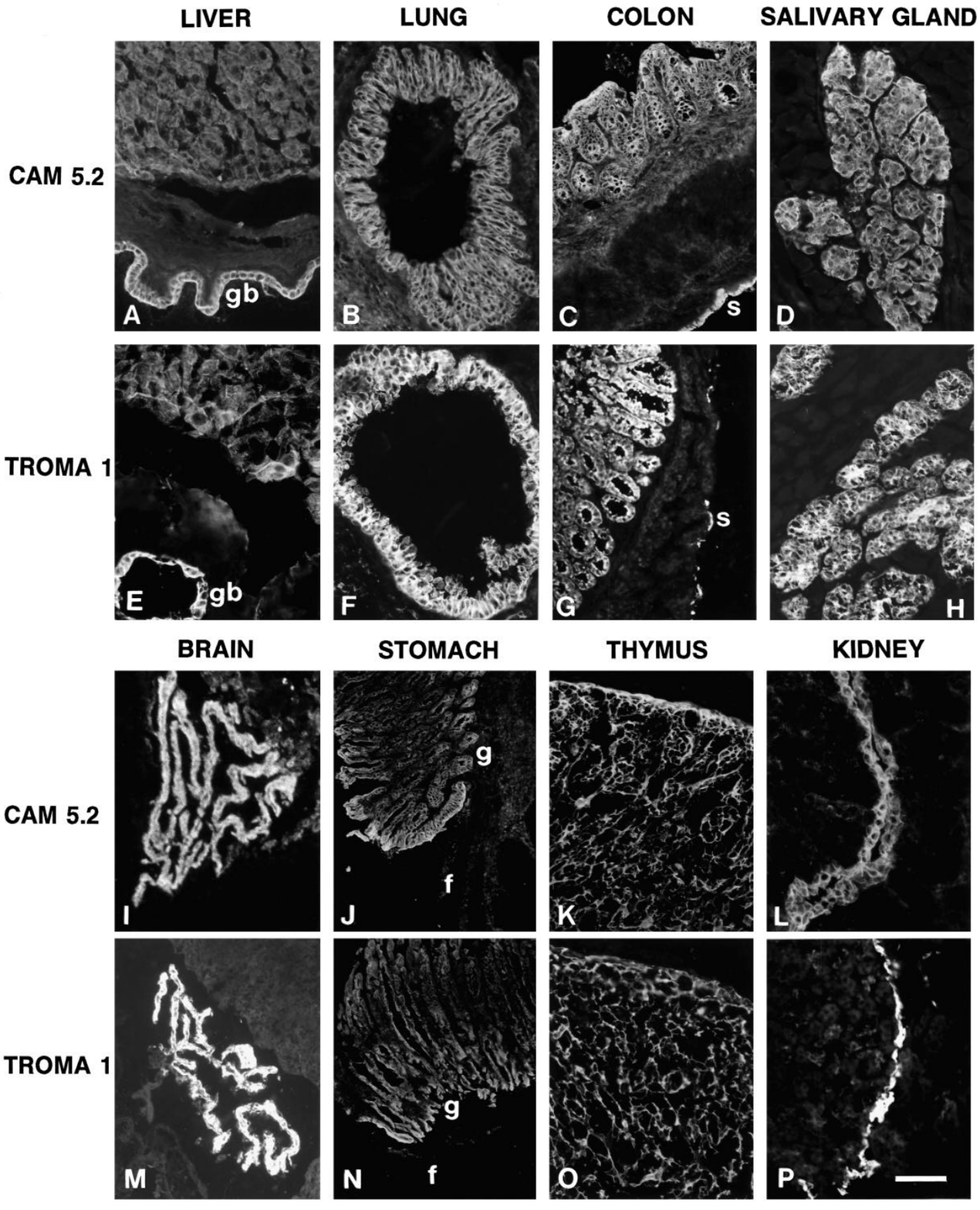

Table 2. Summary of mouse and human K8 protein expression

Tissues‡	Mouse K8* (non-Tg)	Human K8†		
		1908§	1918§	1865§
Brain				
Choroid plexus	+++	++	++	++
Thymus				
Reticular cells	+++	+	+++	+++
Spleen	–	–¶	–¶	–¶
Esophagus	–	–	–	++
Stomach				
Mucosa	++	+	++	++
Intestine				
Mucosa	++	++	+++	+++
Liver				
Hepatocytes	+	+	+	+
Gallbladder and ducts	++	++	+	++
Pancreas				
Acini and ducts	+	+	+	++
Salivary glands	+++	+	+	+++
Trachea				
Mucosa	+++	++	+++	+++
Lung				
Bronchi, bronchioles	++	+	+	+
Kidney				
Distal tubules	+	+	+	++
Renal pelvis	+++	+	++	+++
Urinary bladder	+++	–	–	++
Uterus	++	+	++	+++
Skin				
Hair follicles	+	+	+	+++
Epidermis	–	–	–	+++
Striated muscle	–	–	–	+++

*Perceived intensity of immunofluorescent staining with TROMA-1 antibody.
†Perceived intensity of immunofluorescent staining with HK8-specific CAM-5.2 antibody.
‡Mouse and human K8 staining was also seen in the mesothelial lining of the serosa in liver, spleen, kidney, lung, esophagus, stomach, intestine, thymus, pancreas and uterus.
§Transgenic line.
¶Few reticular cells stained.

reticular epithelium, but not in thymocytes (Fig. 3K,O). In the kidney, the strongest reaction was seen in the epithelium of the renal pelvis (Fig. 3L,P) and in the distal tubules. In the brain, mouse and human K8 staining was restricted to the choroid plexus (Fig. 3I,M). In the digestive tract, mouse and human K8 were found in the surface epithelium of the glandular stomach (Fig. 3J,N), as well as in the duodenal villi and crypts, and in the colon (Fig. 3C,G), but not in other cell types. Glandular epithelia such as those of salivary glands (Fig. 3D,H), sebaceous glands and others in the digestive and respiratory tracts reacted also with the two antibodies (not shown). Typically, the intensities of human and mouse K8 staining in the various tissues did not reflect the differences in steady state levels of RNA.

In the transgenic line 1865, the CAM-5.2 antibody stained tissues that expressed mouse K8, as well as others where the endogenous K8 is not detected. Thus, human K8 was found in the basal cells of stratified epithelia such as: tongue mucosa (Fig. 4C); forestomach (Fig. 4E); epidermis of backskin (but not tailskin, Fig. 4F); palate and esophagus (not shown). Staining was also seen in both skeletal (Fig. 4A) and cardiac muscle (not shown). Mouse K8 was not detected in any of these tissues, with the exception of hair follicles (not shown). Interestingly, every hemizygous mouse of this line was 70-80% of the size of non-transgenic littermates and homozygous animals died shortly (1-3 weeks) after birth (Casanova et al., unpublished observations). This suggests that either HK8 expression or insertional mutagenesis could be the cause of these phenotypes.

Lack of expression of a human K8 construct without intragenic sequences in transgenic mice

In a preliminary attempt to map *cis*-regulatory regions in the 12 kb DNA fragment of the human K8 locus, we used a construct in which the exons and introns of the gene were replaced by the *lacZ* gene of *E. coli*. In this construct, termed HK8-5′Z3′ (Fig. 5A), the *lacZ* gene was used as a reporter of the transcriptional activity of sequences flanking the human K8 gene: a 1.1 kb upstream region containing the transcription start site and 5′ flanking sequences, and a 2.6 kb region of sequences 0.5 kb downstream from the polyadenylation site. We obtained seven transgenic mice with varying transgene copy numbers, of which one contained a truncated transgene (mouse 12 in Fig. 5B). Transgene expression in three mice was studied by northern blot analysis of RNA from liver, thymus, stomach, intestine, lung and kidney, using the *lacZ* gene as a probe. However, no *lacZ* signal was obtained with any of them, although obvious signals were obtained with a β-actin probe (not shown). To determine whether the absence of *lacZ* transcripts could be due to a restricted expression of the transgene in cell subpopulations, we also assayed those tissues for β-galactosidase activity using X-gal histochemistry. However, no staining was observed in any of the above tissues, or in brain, heart, urinary bladder, tongue or spleen. A similar X-gal analysis on lines derived from the other three founders showed, again, no expression of the transgene. From these results we conclude that flanking sequences of the human K8 gene are not sufficient to confer expression on a reporter gene and that important regulatory elements are contained in intragenic regions of the K8 gene.

DISCUSSION

We show that a 12 kb DNA fragment of the human K8 locus, including the gene flanked by 1.1 kb of 5′ and 3.2 kb of 3′ sequences, contains sufficient control elements for appropriate expression in most adult tissues of transgenic mice. The expression pattern of human K8 in these tissues varied very little among the different transgenic lines, with the exception of the urinary bladder, and mimicked the cell-type specificity of the endogenous gene. As both human and murine urinary bladders express K8, the variability of transgene expression in this tissue cannot be considered a case of species-specific expression. Instead, it suggests that important *cis*-control element(s) for expression in this particular epithelium are not included in the injected DNA fragment. In some lines, human K8 RNA transcripts were also found in tissues that do not express mouse K8, such as spleen or striated muscle. These deviations from the expression pattern of the endogenous gene were rather exceptional and only the line with the highest K8 RNA levels (line 1865) showed examples of all of them. A similar result was obtained with human K18 transgenic mice:

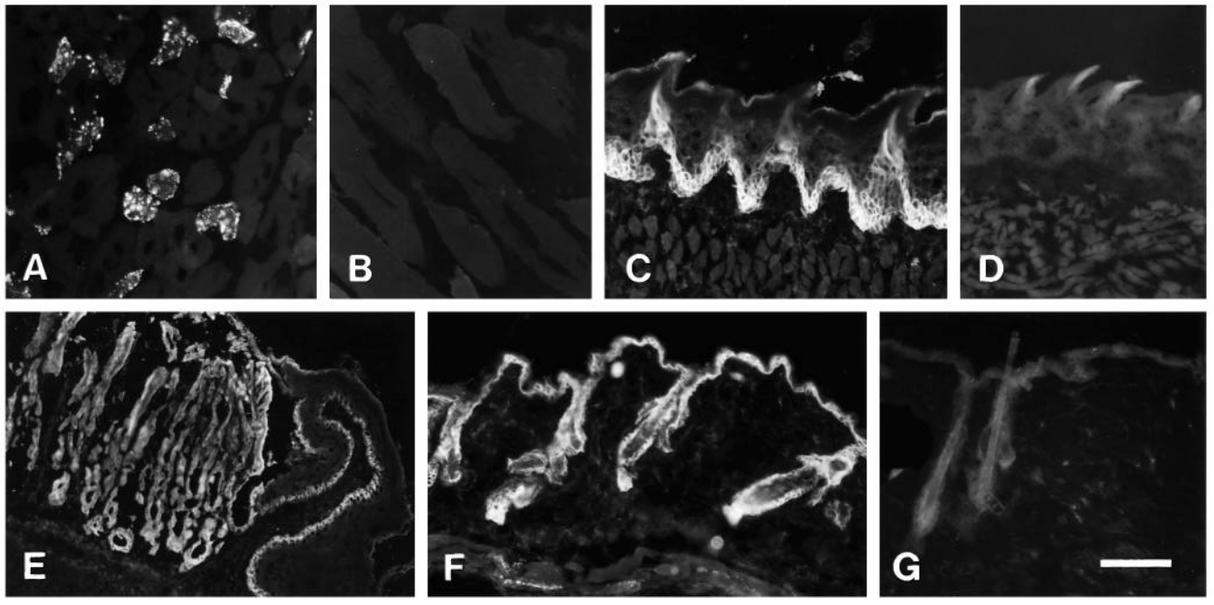

Fig. 4. Examples of ectopic expression of human K8 in transgenic mice. Indirect immunofluorescent detection of HK8 in frozen sections of striated muscle (A), tongue (C), stomach (E) and backskin (F) of transgenic line 1865 incubated with CAM-5.2 antibody. Frozen sections of non-transgenic tissues incubated with TROMA-1 antibody: striated muscle (B), tongue (D) and backskin (G); non-transgenic stomach is shown in Fig. 3N. These non-transgenic tissues did not react with CAM-5.2 antibody (not shown). Bar: 90 μm (C,E); 140 μm (A,B,D,F,G).

in an overall appropriate expression pattern, the highest expressing line produced human K18 RNA in tissues that did not express the endogenous gene (Abe and Oshima, 1990). These differences between the expression patterns of the transgene and the endogenous gene could represent genuine examples of ectopic expression. However, it is also possible that in some tissues they are a consequence of overexpression in cell types that may express undetected levels of the endogenous gene. Human vagina, esophagus or tongue are examples of tissues, previously thought to be negative for K8, that express low levels of K8 (Bosch et al., 1988).

The highest expressing HK8.12 line (1865) was characterized by the dwarf phenotype of every hemizygous mouse. Moreover, 1865 homozygous mice died shortly after birth. It is premature to speculate about the high expression level of the transgene as the cause of the physiological abnormalities in this line. However, there is already evidence of physiological alterations due to ectopic expression of keratins in transgenic mice. For example, the synthesis of epidermal keratins in pancreatic β cells of transgenic mice results in diabetes (Blessing et al., 1993). We are currently generating additional highly expressing HK8.12 transgenic lines, to rule out any effects due to insertional mutagenesis and to establish a potential relationship

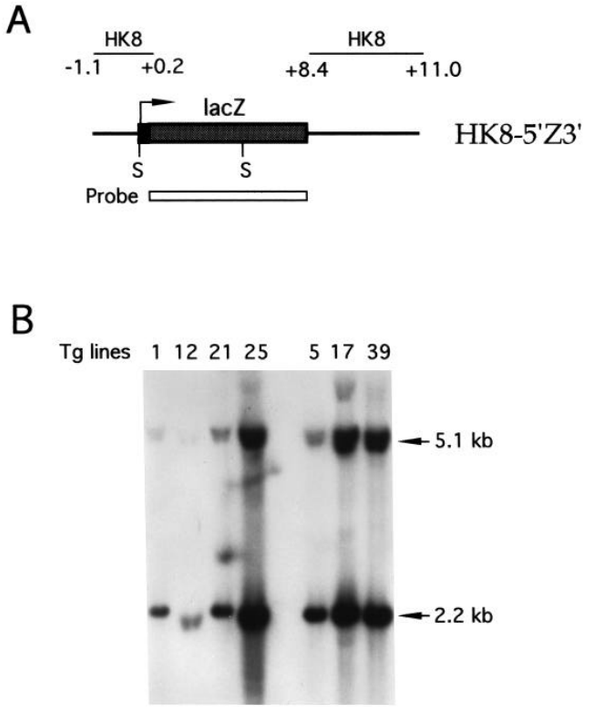

Fig. 5. Southern blot analysis of HK8-5′Z3′ transgenic mice. (A) Schematic representation of the HK8-5′Z3′ construct. Thin lines represent the HK8 flanking sequences indicated above the map. Nucleotide positions relative to the start site of transcription (+1), marked by the arrow, are indicated in kbp. The stippled box represents the protein-coding region of the *trpS-lacZ* gene and polyadenylation signals from SV40. The probe used in Southern blots is indicated by the empty box below the map. *Sac*I (S) sites are indicated. (B) Southern blot analysis of 10 μg of tail DNA digested with *Sac*I and hybridized to the *lacZ* probe indicated above. The 2.2 kb internal fragment and the 5.1 kb junction fragment expected in a head-to-tail tandem array of the transgene (Tg) are shown by arrows.

between overexpression of human K8 and the observed dwarf phenotype.

The discrepancy between human and mouse K8 staining and their RNA levels could be explained by an imbalance in the production of K8 and K18 (and K19). This post-translational regulation has been observed in K8 transfected cells, in which K8 is degraded in the absence of exogenous K18. On the contrary, when cotransfected with a K18 construct, K8 is stabilized and keratin filaments are formed (Domenjoud et al., 1988; Kulesh et al., 1989). Likewise, in mouse embryos homozygous for a null mutation in the K8 gene, K18 protein is not detected in hepatic tissues, despite the presence of K18 transcripts (Baribault et al., 1993). In the same way, we did not observe anti-human K8 staining in the few examples of ectopic expression of human K8, such as spleen or muscle. An exception, however, was the striated muscle of line 1865, which showed fluorescent aggregates, different from the dispersed, filamentous pattern seen in other tissues. A similar staining was observed for K18 in lungs of K8 deficient mice (Baribault et al., 1993). It is then possible, that due to the (presumably) high K8 expression, not all translated human K8 is degraded in striated muscle and aggregates are formed instead. In this transgenic line, human K8 was also detected in the basal layer of stratified epithelia. However, these tissues do not contain detectable amounts of K18 or K19, the known partner of K8 (Bader and Franke, 1990; Rentrop et al., 1986; Schweizer, 1993; Schweizer et al., 1988). Interestingly, the appearance of the anti-human K8 staining in stratified epithelia was similar to that seen for K5 and K14, the keratins typical of the basal layer (not shown). This results suggest that K8 could form filaments in vivo with type I keratins other than K18 or K19, such as K14.

Besides the overall correct tissue-specific expression pattern, the 12 kb DNA fragment of the human K8 locus used for microinjection showed two other properties. First, the transgene showed integration-independent expression, as indicated by the fact that all transgenic lines expressed the transgene. Second, human K8 expression in several tissues was proportional to the number of intact copies of the transgene. These two rather unusual characteristics of transgene expression (Palmiter and Brinster, 1986) are also observed in transgenic mice generated with a 10 kb DNA fragment of the human K18 locus (Abe and Oshima, 1990; Neznanov et al., 1993). However, whereas exogenous K18 RNA levels in livers of transgenic mice were similar to those of endogenous K18, our results show that the transcriptional efficiency of the human K8 transgene in stomach and intestine is lower than that of the endogenous gene. Whether the lower activity of the human K8 transgene is related to species-specific differences or to the absence of *cis*-control elements in the injected DNA fragment remains to be elucidated. Human K8 RNA levels per gene copy were higher in a single copy transgenic mouse than in multicopy transgenic mice. This indicates that although the control elements contained in the human K8 transgene allow its expression in all integration sites, they are not sufficient for absolute insulation of a single transgene from the surrounding chromatin. It has been reported that transcriptional insulation of tandemly duplicated HK18 genes in transgenic mice requires the presence of an *Alu* element proximal to the HK18 promoter (Thorey et al., 1993a). Interestingly, the HK8 locus contains an *Alu* element located 750 bp upstream of the transcription start site (Krauss and Franke, 1990), which raises the possibility that the two genes have a common mechanism of transcriptional insulation. For the HK8 gene, however, this possibility remains to be tested.

Our initial attempt to map *cis*-acting elements controlling tissue-specific expression of the human K8 gene indicated that the exons and introns of the gene contain important regulatory regions. We show that a construct containing 5′ and 3′ flanking sequences (including the transcription start site) of the human K8 gene, was unable to drive expression of a linked *lacZ* gene in transgenic mice. A similar human K18 construct, in which the exons and introns of the gene were replaced by the herpes simplex virus (HSV) thymidine kinase (TK) gene, did not exhibit the tissue-specificity of the human K18 gene, but the one corresponding to the HSVTK gene (Neznanov et al., 1993). Our HK8-5′Z3′ construct should have identified an enhancer activity in the 3′ flanking region of the gene as the one found in the mouse K8 gene (Takemoto et al., 1991). It is possible, therefore, that the localization of control elements is not conserved between the human and mouse K8 genes; or that such an enhancer (identified in parietal endoderm-like cells) is active only during early stages of development. The alternative possibility to consider is that K8 expression is regulated by the interaction of control elements dispersed throughout the gene. In any case, the mapping of the *cis*-controlling regions of the human K8 gene will require a detailed deletional analysis of intragenic sequences of the gene.

Finally, the lack of sufficient control elements in proximal flanking sequences for K8 and K18 gene expression contrasts with the activity in transgenic mice of 5′ flanking regions of stratified-epithelial keratin genes, such as K5 (Byrne and Fuchs, 1993; Ramírez et al., 1994), K6 (Ramírez et al., unpublished data), K10 (Fuchs et al., 1992) and K14 (Vassar and Fuchs, 1991). This difference in the architectural array of control elements between simple and stratified-epithelial keratin genes may be a reflection of the evolutionary history of keratin genes, which shows that K8 and K18 were the first to diverge from the ancestors of all other keratins (Blumemberg, 1988).

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