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Genetics

Silent genes in the mouse major urinary protein gene family

(submaxillary gland/gene expression/polymerase chain reaction/transgenic mice)

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ABSTRACT To date, two classes of mouse major urinary protein (MUP)-encoding genes have been described, the expressed genes and the intervening-sequence-containing pseudogenes. The data presented in this paper define a third class, the silent *Mup* genes, which are potentially functional but appear not to be expressed under normal circumstances. We describe a MUP subfamily (*Mup-1.5*) containing two genes, *Mup-1.5a* and *Mup-1.5b*, that are nearly identical, differing at only three positions (>99.9% identity) over the entire 4-kilobase (kb) transcription unit and ≈1 kb of flanking DNA. The similarity between these two genes extends over >35 kb. Using specific oligonucleotides, we have shown that the *5a* gene is expressed in BALB/cByJ mice, primarily in the submaxillary gland, whereas the *5b* gene is not expressed. However, we found that when a 9.4-kb DNA fragment containing the *Mup-1.5b* gene was introduced into the mouse germ line, mice in two of the four transgenic lines expressed this gene at a high level and with the tissue-specificity characteristic of the *Mup-1.5a* gene. These results suggest that the inactivity of the endogenous *Mup-1.5b* gene is due not to a lack of functional positive regulatory elements, but to long-range, inhibitory position effects.

Mouse major urinary proteins (MUPs) are low-molecular-weight polypeptides that were originally recognized as the major protein constituents of the urine of adult mice (1). In addition to the liver, where the urine MUPs are synthesized, MUP mRNAs are also found in the mammary, submaxillary, sublingual, parotid, and lachrymal glands (2–4). Although the function of MUPs has not been established, it has been observed that they belong to a family of proteins that function as carriers for small hydrophobic molecules, such as retinol (5), bilin (6, 7), cholesterol (8), and steroids (9). This suggests that MUPs may serve as binding proteins for as-yet-unidentified small molecules.

In most inbred strains of mice the MUPs are encoded by a family of ≈35 closely related genes (10). Most *Mup* genes belong to two large subfamilies, group 1 and group 2, each of which contains 12–15 genes (10). By structural criteria, all group 1 genes appear to be potentially functional (11, 12), although there is direct evidence for the expression of only four of the group 1 genes (4, 11, 13). All group 2 genes are pseudogenes with mutations in their exons, their introns, and their flanking sequences (14).

We have shown that in the BALB/c mice, six distinct mRNA sequences, designated MUPI–MUPVI, account for most MUP mRNAs (4, 15). MUPI and -III are found only in the liver, whereas MUPII is found in the liver and in the mammary gland; MUPIV is synthesized in the lachrymal and parotid glands, MUPV is synthesized in the submaxillary gland and at a much lower level in the sublingual gland, and MUPVI is synthesized in the parotid gland (4, 15). The genes encoding MUPI and -II belong to the group 1 subfamily, whereas the genes encoding MUPIII, -IV, -V, and -VI belong neither to the group 1 nor the group 2 subfamily (15).

In this paper we describe a subfamily containing two very closely related genes, *Mup-1.5a* and *Mup-1.5b*, which exist as a nonallelic pair in most strains of mice. We show that these two genes are nearly identical (>99.9% similar) within their transcribed and immediate flanking regions and that they have arisen through a recent duplication of a unit spanning at least 35 kilobases (kb). Nevertheless, we find that in the BALB/cByJ strain only the *Mup-1.5a* gene is expressed, whereas the *Mup-1.5b* gene is not. Studies using transgenic mice demonstrate that the cloned *Mup-1.5b* gene is potentially functional, as it is expressed at a high level and with appropriate tissue-specificity when inserted at new sites in the mouse genome. We argue that the *Mup-1.5b* gene represents a new class of *Mup* genes, the silent *Mup* genes, which are distinct from both the expressed genes and the pseudogenes, and we discuss possible mechanisms of silencing.

MATERIALS AND METHODS

Animals. BALB/cAnN mice were purchased from the Dominion Laboratories, Dublin, VA. All other strains were purchased from The Jackson Laboratory.

MUP Genomic Clones. Clones elkV.1, elkV.2, and elkV.3 were constructed using DNA from the liver of BALB/cAnN mice. DNA was partially digested with *Sau*3AI and ligated into a *Bam*HI-digested λ vector EMBL3. Clone chMUP6 was isolated from a partial *Eco*RI library constructed in Charon 4a λ using sperm DNA of BALB/c mice maintained at Stanford University (16).

DNA Sequence Analysis. Restriction fragments of the genomic clones chMUP6 and elkV.1 were subcloned into the M13mp10 and M13mp11 vectors and either sequenced directly or sequenced by generating overlapping deletions (17).

Synthetic Oligonucleotide Probes. The synthetic oligonucleotide S_{257} was prepared according to the sequence clone chMUP6 and is complementary to the region corresponding to nucleotides (nt) 3782–3798 (*Mup-1.5b* sequence) (unpublished results). The synthetic oligonucleotide S_{255} is based on the equivalent region in the MUPV cDNA clone (*Mup-1.5a* sequence) (4). Nucleotide sequences of S_{255} and S_{257} are TGTCATGG-T-TGTGTCA and TGTCATGG-G-TGT-GTCA, respectively. The oligonucleotide oSmx-G was described earlier and is complementary to both clones (4). Oligonucleotides were end-labeled as described (4).

Polymerase Chain Reactions. The polymerase chain reactions were performed according to Saiki *et al.* (19) using the DNA thermal cycler (Perkin–Elmer/Cetus). The primers correspond to nt 3634–3658 in the 3′-untranslated region (P_1), and to nt 3947–3969 in the 3′-flanking region (P_2) of the

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Abbreviations: MUP, major urinary protein; MUPI–MUPVI, six distinct MUP mRNA sequences; *Mup-1.5a* (*5a*) and *Mup-1.5b* (*5b*), nonallelic genes; nt, nucleotide(s).

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genomic clone chMUP6. The nucleotide sequences of the former and the latter are ACTCCAGCATCATCCCTTC-CCTTC and ACTCAGGTTACATTCTGGGTCA, respectively.

Preparation of Construct pE. First, the clone chMUP6 gene was modified by inserting a *Bam*HI–*Sma* I adaptor to a *Bgl* II site in the 5′-untranslated region. This modification was performed on a 1.1-kb *Bam*HI–*Eco*RI fragment, the *Eco*RI site of which is marked with an asterisk in Fig. 4. The marked pE construct was then assembled from this fragment and the flanking fragments *Hin*dIII–*Bam*HI and *Eco*RI–*Hin*dIII in the vector pUC-18. Both *Hin*dIII sites are marked with † in Fig. 4.

Production of Transgenic Mice. The *Hin*dIII fragment of plasmid pE DNA was eluted from an agarose gel and purified by centrifugation in CsCl (20). The DNA was microinjected into the pronuclei of (C57BL/6J × CBA/J)F_2 mouse zygotes at a concentration of 4 μg/ml, the eggs were transferred to foster mothers (20), and 32 offspring were obtained. DNA was isolated from the tail tips, digested with *Bam*HI, and analyzed by Southern hybridization, with use of the MUPV specific Riboprobe (unpublished results). Hybridization to the endogenous MUPV genes yielded a 6.0-kb band, whereas transgenic-mice displayed a 9.4-kb band, due to cleavage at *Bam*HI sites in adjacent tandem copies of the transgene. Four transgenic founder mice (pE1, pE9, pE14, and pE16) were obtained, carrying ≈10–20, 2–3, 6–8, and 6–8 copies of the transgene, respectively. These mice were mated to normal (C57BL/6J × CBA/J)F_1 mice to produce transgenic progeny for RNA analysis.

Preparation of RNA. RNA was isolated from fresh or frozen mouse tissues by the guanidinium thiocyanate method of Chirgwin *et al.* (21) or by the LiCl/urea method of Auffrey and Rougeon (22).

RNase Protection Analysis. P^{32}-labeled RNA was synthesized *in vitro* from a 240-bp *Hae* III–*Spe* I fragment containing the 5′ end of the marked *Mup-1.5b* gene (see Fig. 5). Five to ten nanograms of probe was hybridized with each RNA sample (see Fig. 5 legend) at 45°C in a buffer containing 80% formamide, followed by RNase digestion (23) as described (24), and analyzed by electrophoresis on an urea–polyacrylamide gel.

RESULTS

The Two Nonallelic *Mup-1.5a* and *Mup-1.5b* Genes Can Be Distinguished with Synthetic Oligonucleotide Probes. We have recently suggested that in most inbred strains of mice the gene encoding the MUPV mRNA is a member of a subfamily of two genes (unpublished results). This suggestion was based on the quantitative analysis of Southern blots of mouse DNA, by using known amounts of cloned DNA as a standard, and the finding of a substrain of BALB/c mice (BALB/cJ) in which the MUPV encoding gene is present in a lower copy-number relative to other inbred strains (unpublished results). From the initial experiments it appeared that the duplicated *Mup-1.5* genes must be highly conserved, because in a given strain a *Mup-1.5* specific probe hybridizes to a single band of digested mouse DNA irrespective of the restriction enzyme used (unpublished results).

We have previously shown that it is possible to isolate specific MUP-encoding genes by use of combinations, or panels, of oligonucleotide probes that recognize the variable portions of MUP mRNAs (4). With the MUPV-specific panel, we first isolated the clone chMUP6 (4) and, more recently, three additional genomic clones elkV.1, elkV.2, and elkV.3 (Fig. 1). The complete nucleotide sequence of clone chMUP6, including all seven exons, all six introns, ≈500 base pairs (bp) of 5′- and 500 bp of 3′-flanking sequence were determined. Clone chMUP6 differs from the MUPV mRNA by a single nucleotide substitution: at position 817 in the 3′-untranslated region of the MUPV mRNA is an adenine residue (4), whereas at the corresponding position in the chMUP6 sequence there is a cytidine residue. We suggest that these two sequences represent the two *Mup-1.5* genes and define the gene encoding MUPV mRNA as the *Mup-1.5a* gene and the gene represented by clone chMUP6 as the *Mup-1.5b* gene. To identify genomic clones of the *Mup-1.5a* gene, two oligonucleotides that include the base substitution in the 3′-untranslated region were synthesized: S_{255} corresponds to the *Mup-1.5a* sequence, and S_{257} corresponds to the *Mup-1.5b* sequence. As shown in Fig. 1, only clone elkV.1 hybridized to the S_{255} probe, whereas the other three clones hybridized to the S_{257} probe. As expected, all four clones hybridized to probe oSmx-G, which is one of the oligonucleotides defining the MUPV-specific panel (4) but does not distinguish between *Mup-1.5a* and *Mup-1.5b*. To confirm that clone elkV.1 carries the *5a* gene, the nucleotide sequence of this clone was determined (unpublished results) and was shown to agree completely with the MUPV mRNA sequence (4). This clone, thus, corresponds to the *Mup-1.5a* gene, and clones chMUP6, elkV.2, and elkV.3 correspond to the *Mup-1.5b* gene.

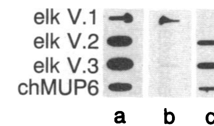

FIG. 1. Identification of genes *Mup-1.5a* and *Mup-1.5b* with synthetic oligonucleotides S_{255} and S_{257}. Genomic clones elkV.1, elkV.2, elkV.3, and chMUP6 were hybridized to one of the synthetic oligonucleotides defining the MUPV-specific panel (4), oSmx-G (lane a); to the S_{255} (lane b), and the S_{257} oligonucleotide (lane c). Hybridizations were performed at 37°C as described (15), washes were performed in 2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/0.2% NaDodSO$_4$ at 45°C (S_{255}), or at 50°C (oSmx-G and S_{257}).

To directly prove that both the *Mup-1.5a* and *Mup-1.5b* genes are present in the BALB/cByJ strain, we hybridized the S_{255} and S_{257} oligonucleotides to genomic DNA fragments amplified by the polymerase chain reaction (19). Two primers, P_1 and P_2, were synthesized based on sequences common to both *Mup-1.5* genes, but not to any other *Mup* genes or mRNAs sequenced to date. These two primers were used to amplify a 335-bp DNA fragment that includes the polymorphic nucleotide, employing either genomic or cloned DNA as the starting material, and the amplified DNA was then hybridized to the S_{255} and S_{257} oligonucleotides.

As shown in Fig. 2, the 335-bp fragment amplified from clone elkV.1 or chMUP6 hybridized only to the S_{255} or S_{257} oligonucleotide, respectively, confirming the specificity of the assay. In contrast, the 335-bp fragment amplified from the BALB/cByJ genomic DNA hybridized to both oligonucleotides, directly demonstrating the presence of two nonallelic *Mup-1.5* genes in this strain.

We also performed the same assay on genomic DNAs from BALB/cJ and C57BL/6J mice. The BALB/cJ DNA fragment hybridized only to the S_{255} oligonucleotide, and the C57BL/6 fragment DNA hybridized only to the S_{257} oligonucleotide. As BALB/cJ mice appear to carry only a single copy of the *Mup-1.5* gene (unpublished results), this result indicates that this strain carries only the *Mup-1.5a* gene. In the C57BL/6 strain, which carries two copies of the *Mup-1.5* gene (unpublished results), both copies are identical in the sequence corresponding to the S_{255} and S_{257} oligonucleotides.

Only the *Mup-1.5a* Gene Is Expressed in BALB/cByJ Mice. The MUPV mRNA is synthesized in the submaxillary gland and at a much lower level in the sublingual gland (15). If the *5a* and *5b* genes are both functional, we would expect them

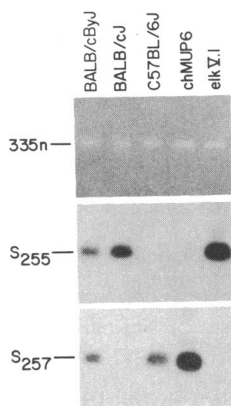

FIG. 2. Detection of the two nonallelic *Mup-1.5* genes in the genomic DNA of BALB/cByJ mice. Two micrograms of DNA isolated from livers of BALB/cByJ, BALB/cJ, and C57BL/6J mice was subjected to 50 cycles of the polymerase chain reaction (19), using the primers P_1 and P_2. This amplified a 335-bp fragment including the polymorphic nucleotide in the 3'-untranslated region of clones chMUP6 and elkV.1. As control, the reaction was also done with 1 ng of genomic clones chMUP6 and elkV.1. The amplified DNA was electrophoresed through a 2% agarose gel, stained with ethidium bromide (*Top*), transferred to a Zeta-Probe membrane (Bio-Rad Laboratories), and hybridized sequentially to the S_{255} (*Middle*) and to the S_{257} (*Bottom*) oligonucleotides. Hybridizations and washes were performed as described in the legend to Fig. 1.

to be expressed in one or both of these tissues. Recently, we constructed cDNA clones using mRNA from both the submaxillary and sublingual glands and sequenced 19 clones from the submaxillary gland and five from the sublingual gland (4). The nucleotide sequences of all these clones are identical and agree with the sequence of the genomic clone elkV.1 (4). Therefore, the *Mup-1.5a* gene is expressed in both of these tissues.

To determine whether the *5b* gene is also expressed, we first hybridized Northern (RNA) blots of submaxillary gland mRNA from BALB/cByJ mice of different ages with the S_{255} and S_{257} oligonucleotides. As shown in Fig. 3, RNA from the submaxillary gland of prepubescent, as well as older, BALB/cByJ mice hybridizes to the S_{255} probe specific for the *5a* gene but not to the S_{257} probe specific for the *5b*. This is true for all the BALB/c substrains, including BALB/cJ (unpublished results). The RNA from the C57BL/6 strain hybridizes, on the other hand, only to the S_{257} probe. This result is as expected because as shown in Fig. 2, the nucleotide sequences of both gene copies in the C57BL/6J strain are identical to the S_{257} oligonucleotide. Because of this fact it is not known whether both copies of the *Mup-1.5* gene, or only one, are expressed in this strain.

As a more stringent test for low-level expression of the *Mup-1.5b* gene in BALB/c mice, we screened a cDNA library made from the submaxillary gland of 3-week-old mice

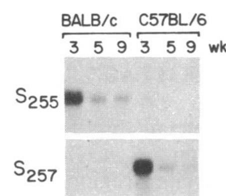

FIG. 3. Expression of the *Mup-1.5a* and *Mup-1.5b* genes. Twenty micrograms of RNA from the submaxillary gland of 3-, 5-, and 9-week old BALB/cByJ and C57BL/6 mice was electrophoresed in duplicate through a 1.4% formaldehyde gel, blotted, and hybridized to end-labeled oligonucleotides S_{255} and S_{257}, respectively. Hybridizations and washes were done as described in the legend to Fig. 1.

(4) with a MUPV-specific Riboprobe and isolated 300 MUPV clones; all 300 clones hybridized to the S_{255} oligonucleotide, and none hybridized to the S_{257} oligonucleotide. Thus, in the submaxillary gland of BALB/c mice, the *5b* gene is either silent, or its level of expression is much lower (<0.01) than that of the *5a* gene. In addition, we screened 32 MUPV cDNA clones isolated from the sublingual gland (4), and all 32 clones hybridized to the S_{255} probe, but none hybridized to the S_{257} probe. Thus, we can find no evidence for expression of the *Mup-1.5b* gene in either tissue.

Nucleotide Sequences of *Mup-1.5a* and *Mup-1.5b* Are Nearly Identical. In an attempt to localize the sequence differences responsible for the differential expression of *5a* and *5b* genes, we compared the nucleotide sequence of the *5a* gene (clone elkV.1) with that of the *5b* gene (clone chMUP6). Equivalent regions of both genes were sequenced, including ≈500 bp of 5'- and 500 bp of 3'-flanking sequences and the entire 4-kp transcription unit. In the exons, as mentioned above, the two genes differ by only a single nucleotide substitution, at position 817 of the MUPV mRNA (4) [nt 3789 in the sequence of elkV.1 (unpublished results)]. In the introns, the two genes differ at two sites: at position 1029 in intron II by a T-to-C transition and in intron III by a deletion (in elkV.1) of the sequence $(GT)_3$ within a tract of $(GT)_{15}$. In both the 5'- and 3'-flanking regions sequenced, the two genes are identical.

***Mup-1.5a* and *Mup-1.5b* Genes Are Present Within Duplicated Units of at Least 35 kb.** To define the boundaries of the duplicated units and to identify any divergent sequences that might be present within the duplicated units, we first determined the restriction maps of the cloned genes *Mup-1.5a* (elkV.1) and *Mup-1.5b* (chMUP6). As shown in Fig. 4, in the region of overlap between the two clones, which includes ≈9 kb of 5'-flanking DNA, the restriction maps for five enzymes are identical. This comparison suggests a high degree of sequence conservation and the absence of any major deletions or insertions in the 5'-flanking region. Furthermore, the conservation of restriction sites extends beyond the region of overlap shown in Fig. 4. As determined by Southern blotting of genomic DNA, the *Msp* I sites designated by asterisks in Fig. 4 are conserved in both genes (data not shown), as is an

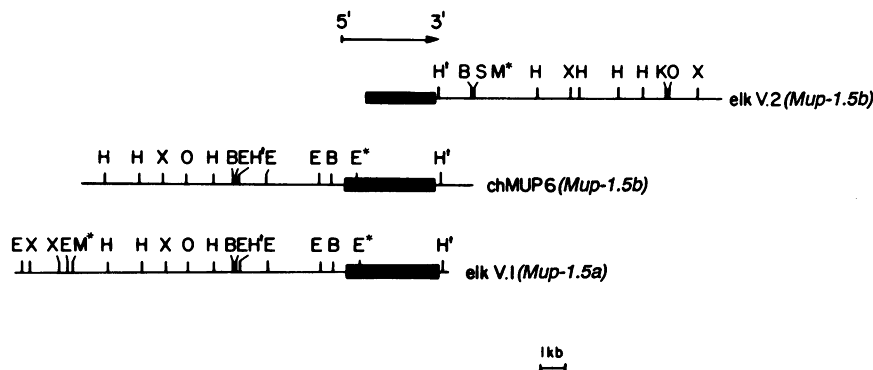

FIG. 4. Restriction maps of the *Mup-1.5a* and *Mup-1.5b* genes. Shown are restriction maps determined with enzymes *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), *Xba* I (X), *Xho* I (O), *Kpn* I (K), *Sma* I (S), and *Msp* I (M). The *Eco*RI and *Msp* I sites that are conserved in both genes, *Mup-1.5a* and *Mup-1.5b* (determined by Southern blotting of mouse DNA), are indicated by an asterisk. Not all *Msp* I sites are shown; *Msp* I sites present 5' or 3' of the two sites shown in the figure have not been mapped. Transcription units are indicated by thick lines.

*Eco*RI fragment extending from the *Eco*RI site denoted by an asterisk in Fig. 4 to an *Eco*RI site 20 kb 3′ to the polyadenylylation signal (15). Together, these data suggest that the duplicated unit is at least 35 kb in length.

The *Mup-1.5b* Gene Is Expressed in the Submaxillary Gland of Transgenic Mice. A sequence comparison of the two *Mup-1.5* genes, described above, failed to provide any obvious explanation for their differential expression, suggesting that alterations in a regulatory element outside the 5-kb region sequenced might be responsible for the observed difference. We therefore decided to use expression in transgenic mice as a functional assay to localize this putative regulatory element. As an initial experiment we produced transgenic mice carrying the silent *Mup-1.5b* gene on a 9.4-kb *Hin*dIII fragment including ≈5 kb of 5′-flanking DNA (see the two *Hin*dIII sites denoted by † in Fig. 4). To distinguish the microinjected *Mup-1.5b* gene and its mRNA from the endogenous *Mup-1.5* genes and mRNA, we marked the gene by inserting an oligonucleotide into the 5′-untranslated region. The marked gene (construct pE) was microinjected into fertilized eggs, and four transgenic founder mice were identified by Southern blot analysis (data not shown). Transgenic progeny were obtained in all four pE founder mice, and animals from each line were sacrificed for RNA analysis at 3 weeks of age, the time at which endogenous MUPV mRNA is maximally expressed (Fig. 3). RNA was isolated from the submaxillary, sublingual, lachrymal, and preputial glands, liver, and spleen and examined by RNase protection analysis with a Riboprobe that distinguishes the marked transgenic mRNA from endogenous MUPV mRNA (Fig. 5).

In two of the lines, pE1 and pE14, the transgene was expressed at a high level in the submaxillary gland and not detectably in any other tissue (Fig. 5). Although no expression of the transgene was detected in the sublingual gland, neither was endogenous MUPV mRNA observed in this tissue (Fig. 5), suggesting that both may be below detection. In the other two pE lines, no expression was detected in the submaxillary gland or in other tissues (data not shown). We were able to estimate the levels of expression of the transgene relative to the endogenous *Mup-1.5* genes, as the Riboprobe hybridized with both the transgenic and endogenous MUPV mRNAs, yielding different-sized RNase digestion products (Fig. 5). The levels of expression averaged 3.7 times the endogenous level (range 0.5–8.7) in four different mice from transgenic line pE1 and averaged 2.9 times (range 1.2–5.8) in three different mice from line pE14. We conclude that the *Mup-1.5b* gene, although normally silent, is capable of high-level, tissue-specific expression when introduced into transgenic mice as a 9.4-kb DNA fragment. Therefore, the lack of expression of the endogenous *Mup-1.5b* gene must be due to the effects of sequences outside the 9.4-kb fragment rather than to any inherent defect in the gene or its nearby flanking sequences.

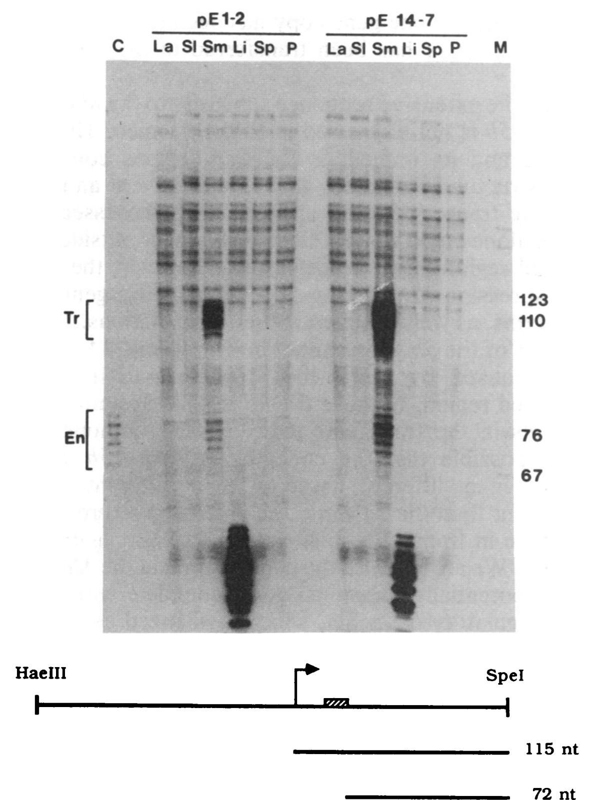

FIG. 5. Tissue-specific expression of the *Mup-1.5b* transgene. (*Upper*) Total RNA was isolated from various tissues of two male transgenic mice, one from line pE1 and one from line pE14, and 10 μg of each RNA was used for RNase protection analysis. C, control RNA from submaxillary gland of a nontransgenic mouse (0.5 μg of polyA^+ RNA). La, lacrymal gland; S1, sublingual gland; Sm, submaxillary gland; Li, liver; Sp, spleen; P, preputial gland; M, M_r markers (pBR322 DNA digested with *Hpa* II). Tr, fragments protected by transgenic mDNA; En, fragments protected by endogenous MUPV mRNA. The strong, low-M_r bands in liver lanes result from cross-hybridization to liver MUP mRNAs. (*Lower*) The 240-bp DNA fragment from the 5′ end of the transgene, used to generate the RNA probe. Arrow indicates the 5′ terminus of the mRNA, and hatched box indicates a 10-bp oligonucleotide inserted in the first exon of the gene. Hybridization to mRNA from the transgene results in a RNase-resistant fragment ≈115 nt long, whereas hybridization to endogenous MUPV mRNA, lacking the oligonucleotide, results in a shorter (≈72-nt) RNase-resistant fragment.

DISCUSSION

In this paper we describe a subfamily of mouse MUP-encoding genes, consisting of an active gene, *Mup-1.5a*, expressed primarily in the submaxillary gland and a structurally similar but functionally silent partner, *Mup-1.5b*.

The impetus for these studies stemmed from the discovery of a single nucleotide difference between the nucleotide sequence of MUPV mRNA and the genomic clone chMUP6 that was initially presumed to encode this mRNA. The experiments described here show that this difference is due to the existence of two nearly identical, nonallelic *Mup-1.5* genes. We show that the *Mup-1.5b* gene, while lacking any obvious structural defect, is apparently not expressed. Yet *Mup-1.5b* is not a pseudogene, as it is capable of efficient expression in the submaxillary gland when introduced as a transgene into the mouse germ line. The *Mup-1.5b* gene, therefore, represents a class of "silent" *Mup* genes, which are potentially functional but normally inactivated by a yet-to-be-defined mechanism.

The *Mup-1.5a* and *-1.5b* genes are conserved to a remarkable degree, exhibiting restriction-site conservation as far as 20 kb from the transcription unit and complete sequence identity in the immediate 5′- and 3′-flanking regions. Within the 4-kb transcription unit, the only differences between the two genes are two single nucleotide substitutions and the deletion of a $(GT)_3$ sequence in a *Mup-1.5b* intron. When deletion of the $(GT)_3$ sequence is scored as a single event, divergence between the two genes is 0.07% and can be attributed to neutral mutation. Assuming the neutral drift rate to be the same as the rat pre-pro-insulin gene pair (26), gene duplication can be dated to ≈85,000 years ago. The sequence homology appears to have been maintained by coordinate evolution of the two genes. Although in every inbred strain we have examined, both *Mup-1.5* genes are located within a single-sized restriction fragment, the size of this fragment may differ between strains (unpublished results). This difference implies that a mutation in a restriction-enzyme recog-

nition sequence of one gene copy has been corrected by the unmutated gene or has been transferred to the other gene copy.

Despite the extensive sequence similarity to the *Mup-1.5a*, the *Mup-1.5b* is not expressed in BALB/c mice. The *Mup-1.5b* gene and its immediate flanking regions contain no obvious structural deficiency that would prevent an mRNA from being transcribed and appropriately processed. This evidence alone could suggest that an enhancer outside of the sequenced region might be defective. However, the appropriate expression of the *Mup-1.5b* gene in transgenic mice rules out this, as well as several other, explanations. Lack of expression of the *5b* gene cannot be due to mRNA destabilization, caused by the A-to-C transversion in the 3′-untranslated region, because the *5b* mRNA accumulates to high levels in the transgenic mice. Finally, although it is formally possible that the endogenous *Mup-1.5b* gene is expressed in a different tissue or at a different time in development than the *5a* gene, the pattern of expression of the *5b* gene in transgenic mice argues strongly against this possibility. We are left with the conclusion that the *Mup-1.5b* gene is a potentially functional gene, complete with tissue-specific regulatory elements, which is silenced as a consequence of its chromosomal position.

The mechanism of silencing of the *Mup-1.5b* gene is not yet known. We have shown that both *Mup-1.5* genes are located on chromosome 4, closely linked to the rest of the *Mup* gene family (*Mup-1* locus) (unpublished results). However, the relative positions of the *5a* and *5b* genes within the cluster of ≈35 *Mup* genes are not yet known; thus it is possible that the two genes are separated by a large distance and that they are located in distinct chromosomal domains. Possibly the DNA sequences in the vicinity of the *Mup-1.5b* gene confer on it chromatin conformation incompatible with transcription. Another possibility is that when gene duplication occurred (or thereafter) the *Mup-1.5b* gene was rendered inactive by the loss of a distant positive activating element, as was the β-globin gene in Dutch γβ-thalassemia (27). However, this explanation seems unlikely because the *Mup-1.5b* gene is expressed at such a high level in transgenic mice. Even when normalized for gene copy number (see *Materials and Methods*), the *Mup-1.5b* transgene is expressed at ≈25–50% the level of the endogenous *Mup-1.5a* gene. In contrast, the β-globin transgene is expressed at only 1–2% the endogenous level per gene copy (28, 29) in the absence of the distant upstream activating element (30). It is therefore more probable that the endogenous *Mup-1.5b* gene is silenced by a negative mechanism, which can be escaped by the transgene.

Mup-1.5b is probably not the only silent gene in the *Mup* gene family. The polymorphic urinary MUPs are encoded by a gene subfamily known as group 1 (4, 13), consisting of 12–15 genes (10). However, to date only four group-1 genes are known to be expressed in BALB/c mice, MUPI and -II (4), p1057 (13), and pMUP11 (11), even though many have been shown to be expressed when introduced into cultured cells (12). Moreover, in the BALB/cJ substrain, about half of the group-1 genes are deleted, and none of the remaining six to eight group 1 genes are expressed (unpublished results). Both of these observations are consistent with the idea that many of the 12–15 group-1 genes are silent.

Silent genes of this type may be a more general phenomenon rather than peculiarities of the *Mup* family. In the human α-globin gene complex, the functional ζ gene is linked to a pseudogene (31), which carries only one identifiable (amber) mutation. In some individuals the amber mutation is corrected by interchromosomal gene conversion. The corrected gene is not expressed *in vivo* but can be transcribed when introduced into HeLa cells or *Xenopus* oocytes (24). Similarly, in the human growth hormone (hGH) gene family, the hGH-5-encoding gene differs from the hGH cDNA sequence. There is no obvious defect in the structure of this gene, and it can be expressed *in vitro*, but there is no evidence that the human *hGH-V* gene is expressed *in vivo* (18, 25). These two genes may represent additional examples of silent genes, inactivated by long-range position effects. However, neither of these genes has been shown to be expressed appropriately when moved to a new chromosomal location, and thus the possibility remains that they are inactive due to a defect in a closely linked regulatory element, such as an enhancer.

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