MOLECULAR AND CELLULAR BIOLOGY, Mar. 1992, p. 1188–1193
0270-7306/92/031188-06$02.00/0

Vol. 12, No. 3

Silent and Expressed Sister *Mup* Genes Are Located within Distinct Chromatin Domains: Analysis by Pulsed-Field Gel Electrophoresis and Polymerase Chain Reaction-Supplemented DNase I Digestion

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Received 12 June 1991/Accepted 8 December 1991

We have recently described a subfamily of two genes, *Mup-1.5a* and *Mup-1.5b*, which exist as a nonallelic pair in most inbred strains of mice. The *Mup-1.5a* and *Mup-1.5b* genes are more than 99.9% homologous, yet they are differentially expressed. While the *Mup-1.5a* gene is expressed at a high level in the submaxillary gland, the *Mup-1.5b* gene does not appear to be expressed either in this or in any other tissue. The *Mup-1.5b* gene can, however, be expressed as a transgene with the tissue specificity of its sister gene, *Mup-1.5a*. We have shown before that both the *Mup-1.5a* and *Mup-1.5b* genes are located on chromosome 4, closely linked to the *Mup-1* locus. In this report, we demonstrate the two genes are located within distinct chromosomal domains, separated by at least 150 to 200 kb of DNA. Using a novel method, detailed in this report, we show that in the submaxillary gland, the *Mup-1.5a* gene is five- to sixfold more susceptible to DNase I digestion than is the *Mup-1.5b* gene. This finding suggests that the inactivity of the *Mup-1.5b* gene is brought about by long range-acting mechanisms that establish a chromatin structure in the vicinity of this gene incompatible with transcription.

The mouse major urinary protein (*Mup*) gene family consists of ca. 35 to 40 genes, most or all of which are located in the *Mup-1* locus on chromosome 4 (2, 3). However, in the BALB/c strain, despite an extensive sequencing effort, there is evidence for the synthesis of only eight species of MUP mRNAs, MUP I through VI (13, 14), p1057 (9), and pMUP11 (5). MUP I, MUP II, MUP III, p1057, and pMUP11 are synthesized predominantly in the liver, MUP IV is synthesized in the lachrymal gland, MUP V is synthesized in the submaxillary gland, and MUP VI is synthesized in the parotid gland. We have recently suggested that the nonexpressed genes include, in addition to pseudogenes, silent genes, i.e., genes that are potentially active but are rendered nonfunctional through long-range position effects (16). In this report, we present data that support this hypothesis.

In the BALB/cByJ strain, the gene encoding the MUP V mRNA is a member of a subfamily of two highly homologous genes, *Mup-1.5a* and *Mup-1.5b* (15, 16). The *Mup-1.5a* and *Mup-1.5b* genes are nearly identical throughout their entire 4-kb transcription units. In the exons, the two genes differ only at one position, in the 3′ untranslated region, by an A-to-C substitution. In the introns, the two genes differ at two positions; there is a T-C transition in intron II and a deletion of $(GT)_3$ in a GT-rich region of intron III. In the ca. 0.6 kb of 5′ flanking and 0.3 kb of 3′ flanking sequences, the *Mup-1.5a* and the *Mup-1.5b* genes are identical (16). The two genes exhibit identical restriction patterns, indicating that the similarity between them extends over more than 20 kb into both 5′ and 3′ flanking sequences (16). However, despite their virtual identity, the *Mup-1.5a* and the *Mup-1.5b* genes are differentially expressed. Using specific oligonucleotides that encompass the A-C substitution in the 3′ untranslated region, we have shown that the *Mup-1.5a* gene is expressed at a high level, predominantly in the submaxillary gland, while the *Mup-1.5b* gene is not expressed (16).

We have shown earlier that both genes, the active *Mup-1.5a* and the silent *Mup-1.5b*, are located on chromosome 4, closely linked to the *Mup-1* locus (15). In this study, we have further examined the chromosomal locations of the *Mup-1.5a* and *Mup-1.5b* genes by using pulsed-field gel electrophoresis (PFGE). The PFGE analysis was made possible by comparing the restriction patterns of two substrains of BALB/c mice, BALB/cByJ and BALB/cJ. The BALB/cJ strain has suffered a large deletion of genes in the *Mup-1* locus which includes the silent *Mup-1.5b* gene. Our results show that in the BALB/cByJ strain, the active *Mup-1.5a* gene and the silent *Mup-1.5b* gene are separated by at least 150 to 200 kb. This finding motivated us to investigate whether different chromatin domains surround the *Mup-1.5a* and *Mup-1.5b* genes, by assessing the relative susceptibility of the two genes to DNase I digestion. To do this, we first developed a method, described here, with which it is feasible to examine chromatin structures of closely related genes. This method involves amplification via the polymerase chain reaction (PCR) of electrophoretically separated DNase I/restriction enzyme-generated DNA fragments and subsequent hybridization of the amplified DNA to gene-specific synthetic oligonucleotide probes. With this method, we have shown that the *Mup-1.5a* gene is much more sensitive to DNase I digestion than is the *Mup-1.5b* gene.

In the ca. 500 nucleotides of promoter-proximal DNA sequences, the *Mup-1.5a* and *Mup-1.5b* genes are identical (16), and they are also identical in a 1.55-kb DNA fragment which functions as a submaxillary gland-specific enhancer (17). Thus, the findings of differential chromatin structure but of identical DNA sequences suggest that the silencing of the *Mup-1.5b* gene, and perhaps of other members of the *Mup* gene family, is the consequence of their position within the *Mup-1* locus.

MATERIALS AND METHODS

PFGE. DNA was prepared either from the mouse lymphoma cell line X.63 Ag8.653 (6) or from leukocytes isolated

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from BALB/cByJ or BALB/cJ mice (Jackson Laboratory). The leukocytes were obtained from spleen after lysis of erythrocytes with 0.17 M NH_4Cl at 4°C for 5 min. After lysis, the cell pellet was washed with phosphate-buffered saline, and the number of cells was estimated by using trypan blue. Agarose plugs were prepared by resuspending about 2.5×10^6 cells per 80 μl of molten 1% low-melting-point agarose (SeaPlaque; FMC). The DNA in the agarose plugs was deproteinized and treated with restriction enzymes essentially as described by Schwartz and Cantor (11). Briefly, agarose plugs were incubated first overnight at 37°C in a buffer containing 0.5 M EDTA, 10 mM Tris-HCl (pH 7.5), 7.5% β-mercaptoethanol, and 1% sarcosyl and then for 24 h at 50°C in a buffer containing 0.5 M EDTA, 10 mM Tris-HCl (pH 9.5), 1% sarcosyl, and 2 mg of proteinase K per ml. The agarose plugs were either stored at 4°C in this solution or washed prior to digestion with restriction enzymes. Washes were performed first with 10 mM Tris-HCl (pH 7.6)–1 mM EDTA–0.1 mM phenylmethylsulfonyl fluoride (PMSF) at room temperature (16 ml per insert) and then for 24 h with two changes of 10 mM Tris-HCl (pH 7.6)–1 mM EDTA. Restriction digests were done overnight in 0.2 ml of restriction buffer supplemented with 0.02% Triton and 0.2 mg of bovine serum albumin (BSA) per ml. A 5- to 10-fold excess of restriction enzymes (Boehringer) was used. The digests were terminated by washing the agarose plugs with 1 ml of 0.5 M EDTA–10 mM Tris-HCl (pH 9.5)–1% sarcosyl. One half of each plug was inserted into a slot of a 1% agarose gel (SeaKem HGT; FMC) and electrophoresed with the pulsed electrophoresis instrument ED (12) at 15°C as detailed in the figure legends, using 0.5× Tris-borate-EDTA as the running buffer. After electrophoresis, the transfer of DNA to nitrocellulose was facilitated either by depurination or by exposing the agarose gel to UV radiation (10 min; model UVT 400M Transluminator; IBI). To visualize the *Mup-1.5a* and *Mup-1.5b* genes, the transferred DNA was hybridized to the *Mup-1.5a/5b*-specific riboprobe as described by Shi et al. (16).

Preparation of nuclei. Nuclei were prepared essentially as described by Bellard et al. (1). Finely minced tissue was first homogenized at 4°C, using a motor-driven Potter homogenizer in solution A (15 mM Tris-Cl [pH 7.4], 0.5 mM spermine, 0.15 mM spermidine, 60 mM KCl, 15 mM NaCl, 12 mM β-mercaptoethanol, 0.5 M sucrose, 2 mM EDTA, 0.2 mM EGTA, 1.0 mM PMSF), approximately 1 g of tissue per 10 ml. The homogenate was then diluted with an equal volume of solution B (15 mM Tris-Cl [pH 7.4], 0.5 mM spermine, 0.15 mM spermidine, 60 mM KCl, 15 mM NaCl, 12 mM β-mercaptoethanol, 2.1 M sucrose, 2 mM EDTA, 0.2 mM EGTA, 1 mM PMSF), layered over a cushion of solution B, and centrifuged at 20,000 rpm for 45 min in the SW27 rotor at 4°C. Pelleted nuclei were washed twice in 2 ml of solution C (25 mM Tris-Cl [pH 7.4], 0.5 mM spermine, 0.25 mM spermidine, 60 mM KCl, 25 mM NaCl, 12 mM β-mercaptoethanol, 0.34 M sucrose, 0.1% Triton X-100, 0.2 mM EDTA, 0.2 mM EGTA, 1.0 mM PMSF), and the purified nuclei were frozen in aliquots in 25% glycerol–50 mM Tris (pH 8.0) at −70°C.

DNase I digestions of nuclei. For each digestion, 3×10^6 nuclei were resuspended in 100 μl of DNase I digestion buffer (15 mM Tris-Cl [pH 7.4], 0.5 mM spermine, 0.15 mM spermidine, 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 1.0 mM PMSF). The reactions were initiated by the addition of DNase I (Worthington) to a final concentration of 5 to 30 U/ml and by the addition of $MgCl_2$ to 5 mM, in that order, on ice. The DNase I digestions were then allowed to proceed at 30°C for 20 min and were terminated by the addition of 100 μl of a solution containing 20 mM EDTA, 0.4% sodium dodecyl sulfate (SDS), 0.4 M NaCl, and proteinase K (200 μg/ml, final concentration). Following brief incubation at 37°C (15 min), the DNA was further purified by phenol-chloroform extractions and ethanol precipitation. Control nuclei were treated as described above except that no DNase I was added.

Quantitation of the *Mup-1.5a* and *Mup-1.5b* genes by PCR. From each DNase I-digested sample, 6 μg of deproteinized DNA was restricted with the restriction enzyme *Hin*dIII and electrophoresed through a 0.7% low-melting-point agarose gel. Only the lane containing DNA size markers was stained with ethidium bromide. Lanes containing submaxillary gland DNA were cut into 1.2-mm slices, which were melted at 65°C and diluted with 100 μl of a buffer containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 0.2 mM deoxynucleoside triphosphates. The DNA in each slice was subjected to amplification using the DNA thermal cycler (Perkin-Elmer/Cetus) and the *Taq* DNA polymerase (2.5 U), using oligonucleotides P1 and P2 (13 ng of each) as primers. The PCR primers P1 and P2 correspond to nucleotides 3634 to 3658 in the 3′ untranslated region (P1) and to nucleotides 3947 to 3969 in the 3′ flanking region (P2) of the genomic clone chMUP6, derived from the *Mup-1.5b* gene (16). The nucleotide sequences of P1 and P2 are ACTCCAGCATCATCCCTTCCCTTC and ACTCAGGTTA CATTCTGGGTCA, respectively. The P1 and P2 prime DNA synthesis from both the *Mup-1.5a* and *Mup-1.5b* genes. The parameters for PCR were as follows: 94°C, 1 min; 45°C, 2 min; and 72°C, 3 min. The linear range of DNA amplification was achieved when PCR was allowed to proceed for no more than 17 cycles (see Fig. 5A). To determine the amount of DNA originating in each gel slice from the *Mup-1.5a* or *Mup-1.5b* gene, the amplified DNA (a 335-bp fragment including the polymorphic nucleotide in the 3′ untranslated region) was electrophoresed through a 2% agarose gel. The gel was stained with ethidium bromide, transferred to a Zeta-Probe membrane (Bio-Rad Laboratories), and hybridized sequentially to end-labeled synthetic oligonucleotides S_{255} and S_{257}, specific for the *Mup-1.5a* gene and *Mup-1.5b* genes, respectively (16). The hybridizations were done at 37°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% SDS–10 mM EDTA–0.1% Ficoll–0.1% polyvinylpyrrolidone–0.1% BSA–0.1 mg of yeast tRNA per ml. Washes were performed in 2× SSC–0.2% SDS at 45°C (S_{255}) or 50°C (S_{257}). The nucleotide sequences of S_{255} and S_{257} are TGTCATGGTTGTGTCA and TGTCATGGGTGTGTCA, respectively.

RESULTS

The *Mup-1.5a* and *Mup-1.5b* genes are not closely linked. We have established earlier, using recombinant inbred strains of mice, that the *Mup-1.5a* and the *Mup-1.5b* genes are tightly linked to each other as well as to all other *Mup* genes (15). To estimate the distance that separates the *Mup-1.5a* and *Mup-1.5b* genes, we analyzed the restriction patterns of *Mup-1.5a* and *Mup-1.5b* genes with the aid of PFGE. High-molecular-weight DNA was prepared from the cell line X.63 Ag8.653, derived from a BALB/c strain of mice (6). The *Eco*RI restriction pattern of *Mup* genes in the X.63 Ag8.653 cells is identical to that of DNA from the liver of BALB/cByJ mice (data not shown), indicating that *Mup* genes have not undergone rearrangements in this cell line. To visualize the *Mup-1.5a* and *Mup-1.5b* genes, the re-

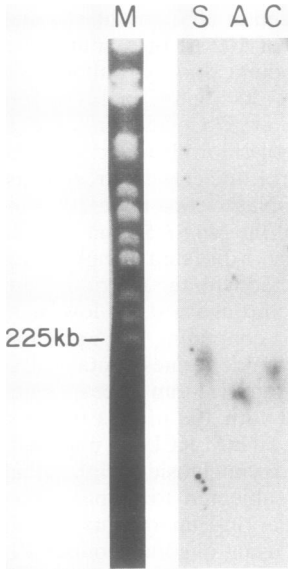

FIG. 1. PFGE restriction patterns of the *Mup-1.5a* and *Mup-1.5b* genes. DNA from about 2.5×10^6 BALB/c X.63 Ag.8653 lymphoma cells (6) was immobilized in agarose plugs and treated with restriction enzyme *Sal*I (S), *Asp* 718 (A), or *Cla*I (C). Restricted DNA was electrophoresed through a 1% agarose gel along with yeast chromosomes from *Saccharomyces cerevisiae* YNN295 (Pharmacia) as size markers (M). The electrophoresis was performed for 48 h, at 150 V with a 120-s pulse time, at 15°C. After electrophoresis, the DNA was transferred to a nitrocellulose filter and hybridized to the *Mup-1.5a/5b*-specific riboprobe (16).

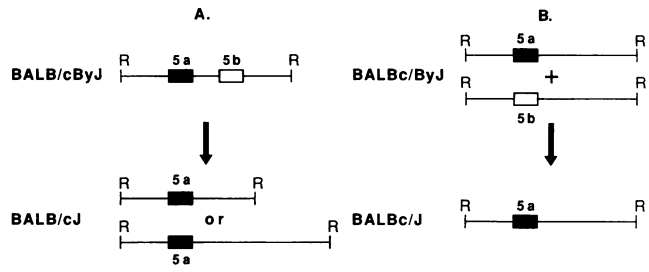

FIG. 2. Two possible arrangements of the *Mup-1.5a* and *Mup-1.5b* genes in two substrains of BALB/c mice, BALB/cByJ and BALB/cJ. The BALB/cByJ strain contains both the *Mup-1.5a* and *Mup-1.5b* genes, and the BALB/cJ strain contains only the *Mup-1.5a* gene (16). The PFGE data (Fig. 1) are consistent with two possible arrangements of the *Mup-1.5a* and *Mup-1.5b* genes in the BALB/cByJ strain. In the first one (A), the *Mup-1.5a* and *Mup-1.5b* genes are linked to each other within a region of DNA bounded by particular restriction sites R; in the second one (B), the *Mup-1.5a* and *Mup-1.5b* genes are not linked within a single restriction fragment, but they are located within restriction fragments of the same size. Diagrammed are the predicted sizes of restriction fragments of the *Mup-1.5a* gene in the BALB/cJ strain in the arrangements A and B.

stricted PFGE-separated DNA was hybridized to the *Mup-1.5a/5b*-specific riboprobe (15). This probe hybridizes to both the *Mup-1.5a* and *Mup-1.5b* genes but not to any other *Mup* genes (15). As shown in Fig. 1, in all three digests, *Asp* 718, *Sal*I, and *Cla*I, a single DNA fragment hybridizes to the *Mup-1.5a/5b*-specific probe. A single restriction fragment was also generated by *Sfi*I, *Nru*I, and *Mlu*I digestion (data not shown).

The PFGE data are consistent with two possible arrangements of *Mup-1.5* genes in BALB/cByJ strain. In the first one, the *Mup-1.5a* and *Mup-1.5b* genes are linked to each other within a region of DNA bounded by *Asp* 718 (*Sal*I, *Cla*I) restriction sites (Fig. 2A); in the second one, the *Mup-1.5a* and *Mup-1.5b* genes are not linked within the *Asp* 718 (*Sal*I, *Cla*I) restriction fragment seen in Fig. 1, but within these fragments there is only a single *Mup-1.5* gene, either *Mup-1.5a* or *Mup-1.5b*.

That only a single *Mup-1.5* gene is present within the *Asp* 718 fragment was deduced from the restriction map of the *Mup-1.5a* and *Mup-1.5b* genes. As shown in Fig. 3, the restriction map of chMUP1, a previously isolated MUP genomic clone (14), overlaps the restriction maps of genomic clones carrying the *Mup-1.5a* or *Mup-1.5b* gene. chMUP1 appears to be derived from a rearranged, nonfunctional gene, since it exhibits significant homology only to the 5′ portion of other *Mup* genes (data not shown). Establishing the linkage of chMUP1 to *Mup-1.5a* and *Mup-1.5b* allowed us to map the *Asp* 718 sites surrounding the *Mup-1.5a* and *Mup-1.5b* genes. As can be seen from Fig. 3, within a ca. 35-kb *Asp* 718 fragment there is only a single *Mup-1.5* gene, *Mup-1.5a* or *Mup-1.5b*. Within the *Asp* 718 fragment seen on the PFGE of total BALB/cByJ DNA (Fig. 1), there is thus only a single *Mup-1.5* gene, *Mup-1.5a* or *Mup-1.5b*.

That only a single *Mup-1.5* gene is present within the *Sal*I fragment was deduced from the comparison of *Sal*I restriction patterns of two substrains of BALB/c mice, BALB/cByJ and BALB/cJ. The BALB/cJ substrain has suffered a large deletion which includes, in addition to the silent *Mup-1.5b* gene (15), at least six other *Mup* genes (unpublished data). The *Mup-1.5a* gene is not deleted in BALB/cJ mice (15). As shown in Fig. 4, the *Mup-1.5a* gene is present within the same sized *Sal*I restriction fragment in the BALB/cJ strain as are the *Mup-1.5a* and *Mup-1.5b* genes in the BALB/cByJ strain. (The size of this fragment is estimated to be 150 to 200 kb, using λ concatemers as size markers [data not shown].)

As diagrammed in Fig. 2, should the *Mup-1.5a* and *Mup-1.5b* genes be linked within a particular restriction fragment, the size of this fragment would be different in the BALB/cByJ and BALB/cJ strains (Fig. 2A). On the other hand, should the *Mup-1.5a* and *Mup-1.5b* genes be not closely linked, the *Mup-1.5a* gene would reside within a restriction fragment of the same size in both strains (Fig. 2B). The fact that in both strains, BALB/cByJ and BALB/cJ, the *Mup-1.5a* gene is present within a *Sal*I restriction fragment of the same size therefore implies that the *Mup-1.5a* and *Mup-1.5b* genes are not linked within a 150- to 200-kb *Sal*I fragment in the BALB/cByJ strain.

To provide a minimum estimate of the separation of the two genes, the exact positions of the *Mup-1.5a* and *Mup-1.5b* genes within the *Sal*I fragment, and their relative orientations, would have to be known. The PFGE data, obtained with a number of different restriction enzymes (*Sal*I, *Cla*I, *Nru*I, and *Mlu*I), indicate that the *Mup-1.5a* and *Mup-1.5b* genes are located within highly homologous duplicated units of at least 150 to 200 kb. Should the *Mup-1.5a* and *Mup-1.5b* genes be centrally located within at least one of the restriction fragments (*Sal*I, *Cla*I, *Nru*I, or *Mlu*I), we would estimate that the two genes are separated by at least 150 to 200 kb of DNA.

The *Mup-1.5a* and *Mup-1.5b* genes are differentially sensitive to DNase I digestion. The observation that the *Mup-1.5a* and *Mup-1.5b* genes are separated by a large distance has led us to consider the possibility that the expressed *Mup-1.5a*

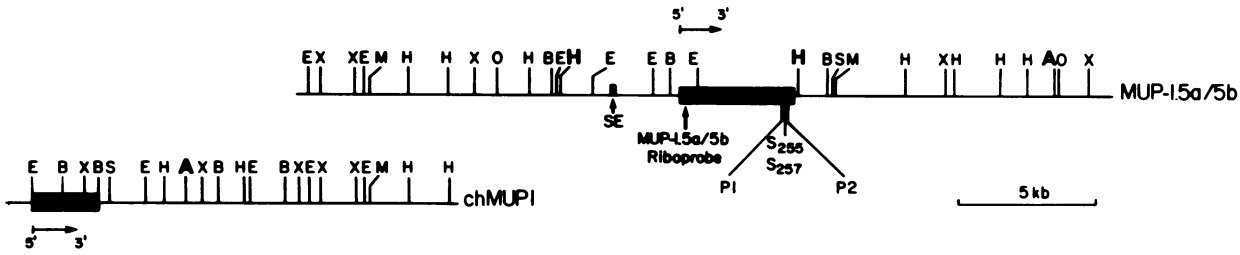

FIG. 3. Presence of a single *Mup-1.5* gene within a 35-kb *Asp* 718 restriction fragment. MUP-1.5a/5b is a composite restriction map of three genomic clones that each carries a *Mup-1.5a* or *Mup-1.5b* gene (16). chMUP1 is a randomly isolated *Mup* genomic clone (14). Shown are restriction maps determined with enzymes *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), *Xba*I (X), *Xho*I (O), *Asp* 718 (A), *Sma*I (S), and *Msp*I (M). Not all *Msp*I sites are shown. *Msp*I sites present 5′ or 3′ of the two sites shown have not been mapped. Transcription units are indicated by thick lines. Noted are also the positions of the PCR primers P1 and P2 and of the gene-specific oligonucleotide probes S_{255} and S_{257}, used in experiment shown in Fig. 5C. Arrows indicate the locations of the 1.6-kb DNA fragment that functions as a submaxillary gland specific enhancer (SE) and of the *Mup-1.5a/5b*-specific riboprobe.

gene and the silent *Mup-1.5b* gene are located within structurally distinct chromatin domains and that this may be the basis for their differential expression. As the *Mup-1.5a* and *Mup-1.5b* genes differ only by two nucleotide substitutions, they can be discriminated from each other only by synthetic oligonucleotide probes (16). While it is possible to distinguish individual *Mup* genes on Southern blots of genomic DNA with gene-specific synthetic oligonucleotide probes (13), this cannot be done with all probes or for the DNase I partial digests. We have, however, shown that in conjunction with PCR, synthetic oligonucleotides S_{255} and S_{257} can discriminate between the *Mup-1.5a* and *Mup-1.5b* genes in total mouse DNA (16). Therefore, to assess the relative susceptibilities to DNase I digestion of the *Mup-1.5a* and *Mup-1.5b* genes, the DNase I-treated chromatin was amplified prior to hybridization to the *Mup-1.5a*-specific probe S_{255} or to the *Mup-1.5b*-specific probe S_{257}.

The protocol that we developed is diagrammed in Fig. 5. Nuclei from the submaxillary gland were first incubated with increasing amounts of DNase I. Next, the DNase I-treated samples were deproteinized and incubated with a restriction enzyme (*Hin*dIII), and the restricted DNA samples were electrophoresed through a low-melting-point agarose gel. As shown in Fig. 3, both genes, *Mup-1.5a* and *Mup-1.5b*, reside within a ca. 9.4-kb *Hin*dIII fragment. To measure DNase I sensitivities of the *Mup-1.5a* and *Mup-1.5b* genes separately, we monitored selectively the amounts of each gene in the 9.4-kb *Hin*dIII restriction fragment as a function of DNase I concentration. To this end, each lane of the low-melting-point agarose gel (Fig. 5B) was first cut into 1.2-mm slices. The gel slices were then diluted with buffer, and the *Mup-1.5a* and *Mup-1.5b* genes in each gel slice were amplified, in the linear range (see below), by PCR. As PCR primers, we used oligonucleotides P1 and P2, which prime the synthesis of a 335-bp fragment, using as a template both genes, *Mup-1.5a* and *Mup-1.5b* (16). As the 335-bp fragment encompasses a polymorphic nucleotide (see the restriction map in Fig. 3), DNA amplified from the *Mup-1.5a* gene can be differentiated from DNA amplified from the *Mup-1.5b* gene by hybridization with gene-specific probes S_{255} and S_{257}. On the basis of size markers, electrophoresed in parallel, we estimated that the bulk of the full-length 9.4-kb *Hin*dIII restriction fragment has in the experiment shown in Fig. 5 electrophoresed into gel slices 2 and 3. The susceptibilities of the *Mup-1.5a* and *Mup-1.5b* genes to DNase I digestion were hence measured by monitoring the intensity of hybridization of DNA amplified from gel slices 2 and 3 to labeled S_{255} and S_{257} probes, respectively. To ensure that hybridization to amplified DNA (i.e., to the 335-bp fragment) was scored only, prior to hybridization to the S_{255} and S_{257} probes, aliquots from the PCR reaction mixtures were electrophoresed through an agarose gel and blotted to a nylon membrane.

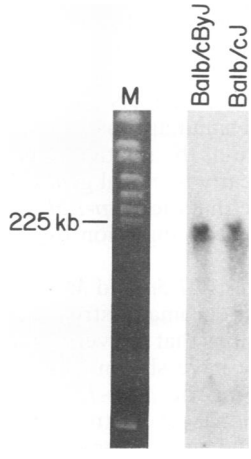

FIG. 4. PFGE patterns of the *Mup-1.5a* gene in the BALB/cByJ and BALB/cJ strains of mice. DNA immobilized in agarose plugs, prepared from leukocytes of BALB/cByJ and BALB/cJ mice as described in Materials and Methods, was treated with *Sal*I and electrophoresed through a 1% agarose gel for 48 h at 120 V, with a 120-s pulse time, at 15°C along with yeast chromosomes from *Saccharomyces cerevisiae* YNN295 (Pharmacia) as size markers (M). Following transfer to a nitrocellulose filter, restricted DNA was hybridized to the *Mup-1.5a/5b*-specific riboprobe (16).

The linear range of the PCR-aided amplification was established as follows. Aliquots of the PCR-amplified DNA obtained from gel slices from the control lane (O U of DNase I) were withdrawn after 13, 17, and 25 cycles of amplification, and each aliquot was hybridized to the S_{255} probe. As shown in Fig. 5A, after 17 cycles, the hybridization pattern is similar to that of an ethidium bromide-stained restriction digest; i.e., most of the hybridization signal is to the full-size 9.4-kb *Hin*dIII fragment (to DNA from gel slices 2 and 3). The hybridization pattern obtained after 13 cycles is qualitatively similar to that of the 17-cycle amplification but of a much lower intensity (data not shown). In contrast, after 25 cycles, the hybridization signal is no longer proportional to the amount of DNA in the different slices of the gel, i.e., the

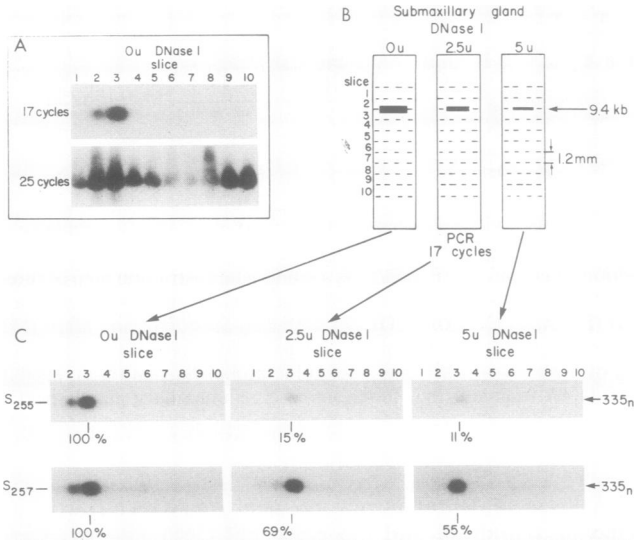

FIG. 5. Differential susceptibilities of *Mup-1.5a* and *Mup-1.5b* genes to DNase I digestion. (A and B) Isolated nuclei from the submaxillary glands of 3-week-old BALB/cByJ mice were digested with increasing amounts of DNase I (0, 2.5, and 5 U) as described in Materials and Methods. The DNase I-treated samples were deproteinized and incubated with the restriction enzyme *Hin*dIII, and the restricted DNA samples were electrophoresed through a low-melting-point 0.7% agarose gel. Each lane of the low-melting-point agarose gel was cut into 1.2-mm slices, as diagrammed. (B) As judged from size markers, electrophoresed in parallel, the bulk of the full-size 9.4-kb *Hin*dIII fragment is present within gel slices 2 and 3 (indicated by an arrow panel B). For each of the three lanes, the 1.2-mm slices were diluted with buffer, and the *Mup-1.5a* and *Mup-1.5b* genes in each gel slice were amplified by 17 cycles of PCR, using oligonucleotides P1 and P2 as primers, as described in Materials and Methods. (As shown in panel A, after 17 cycles, but not 25 cycles, the hybridization pattern is similar to that of an ethidium bromide-stained restriction digest; i.e., most of the hybridization signal is in slices 2 and 3, into which the full-size 9.4-kb *Hin*dIII fragment migrates.) (C) To determine the amount of DNA originating in each gel slice from the *Mup-1.5a* or *Mup-1.5b* gene, the amplified DNA (a 335-bp fragment) was electrophoresed through a 2% agarose gel. The DNA was transferred to a Zeta-Probe membrane (Bio-Rad) and hybridized sequentially to end-labeled synthetic oligonucleotides S_{255} and S_{257}, specific for the *Mup-1.5a* gene and *Mup-1.5b* genes, respectively (16), as described in Materials and Methods.

hybridization signal is distributed throughout the whole lane (Fig. 5A). We have therefore chosen to use 17 cycles of PCR amplification.

Using the method described above, we determined that in the submaxillary gland, the *Mup-1.5a* gene is five- to sixfold more sensitive to digestion with DNase I than is the *Mup-1.5b* gene. As shown in Fig. 5C, the amount of amplified *Mup-1.5a* DNA (scored by the S_{255} probe) originating from the full-length restriction fragment, i.e., from DNA in gel slices 2 and 3, is greatly reduced when submaxillary gland nuclei are incubated with DNase I. In contrast, the amount of amplified *Mup-1.5b* DNA (scored by the S_{257} probe) is not appreciably reduced in the same DNA samples. We determined, by densitometric tracing, that the amounts of amplified DNA in gel slices 2 and 3 are 15 and 11% for the *Mup-1.5a* gene, compared with 69 and 55% for the *Mup-1.5b* gene, in nuclei treated with 2.5 and 5 U of DNase I, respectively, compared with untreated nuclei (Fig. 5C).

DISCUSSION

The *Mup-1.5a* and *Mup-1.5b* genes comprise a *Mup* gene subfamily of two nearly identical genes (>99.99% identity). The *Mup-1.5a* gene is expressed at a high level in the submaxillary gland, but the *Mup-1.5b* gene is not expressed in this or any other tissue. The *Mup-1.5b* gene can, however, be expressed as a transgene with the tissue specificity of its sister gene, *Mup-1.5a* (16). We have recently shown that 0.53 kb of 5′ flanking sequences, together with a 1.55-kb DNA fragment located 1.85 to 3.46 kb upstream from the transcription initiation site, are sufficient for submaxillary gland-specific expression (17). The nucleotide sequences of both of these DNA regions are 100% identical in the *Mup-1.5a* and in *Mup-1.5b* genes (16, 17). We have therefore argued that the inactivity of the *Mup-1.5b* gene in vivo is due to a regulatory mechanism that has brought about a chromosomal domain in the vicinity of the *Mup-1.5b* gene in which transcription cannot take place (16, 17). In this report, we present data that support this hypothesis.

We have previously shown that both the expressed *Mup-1.5a* gene and the silent *Mup-1.5b* gene are located on chromosome 4, closely linked to the *Mup-1* locus (15). The data presented here show that within this locus, the *Mup-1.5a* and *Mup-1.5b* genes are separated by at least 200 kb of DNA and that they are located in structurally distinct chromatin domains. By measuring the sensitivity to DNase I digestion, we were able to demonstrate that the *Mup-1.5b* gene is located in a more closed chromatin domain than is the *Mup-1.5a* gene.

To examine selectively the chromatin structures of the *Mup-1.5a* and *Mup-1.5b* genes, we developed a method which employs PCR, by which genes that differ by as little as one nucleotide can be visualized on the background of similar genes. The specificity of this method is considerable. First, the PCR primers can be designed so that they prime preferentially the amplification of genes of interest, e.g., the *Mup-1.5a* and *Mup-1.5b* genes and not any other genes of this family. Second, the amplified DNA can be hybridized to gene-specific oligonucleotide probes. Third, the amplified DNA can be electrophoresed through a polyacrylamide gel, and to quantitate the amount of amplified DNA, only the hybridization to a DNA fragment defined by the PCR primers can be scored. Since the DNase I sensitivities of several genes are measured simultaneously, it is feasible to provide an accurate measure of the relative sensitivities to DNase I digestion even of nearly identical genes. Using this method, we have shown here that the *Mup-1.5b* gene is about five- to sixfold more resistant to digestion by DNase I than is the *Mup-1.5a* gene.

Even though the *Mup-1.5a* and *Mup-1.5b* genes are organized within distinct chromatin structures, they are located in chromosomal regions that are very similar to each other in DNA sequence. We have shown earlier, by examining the restriction patterns of the *Mup-1.5a* and *Mup-1.5b* genes with use of conventional gel electrophoresis, that in the 20 to 30 kb of 5′ and 3′ flanking regions, the DNA sequences of both genes are very similar, if not identical, to each other (16). In this study, we extended this analysis by examining the restriction patterns of the *Mup-1.5a* and *Mup-1.5b* genes by using PFGE. Surprisingly, we found that even when rarely cutting restriction enzymes were used to assess DNA sequence similarity, the restriction enzyme recognition sites of the two genes were found to be identical. We have therefore concluded that the *Mup-1.5a* and *Mup-1.5b* genes

are embedded within highly conserved duplication units and that the size of each unit is at least 150 to 200 kb of DNA.

Because of the scarcity of gene-specific probes, especially from the flanking sequences, it is impossible to walk in the *Mup-1* locus. By noting an overlap in restriction maps of the *Mup-1.5a* and *Mup-1.5b* gene with a previously isolated genomic clone, chMUP1, we identified the genes (*Mup* pseudogenes) that are situated immediately 5′ to the *Mup-1.5a* and *Mup-1.5b* genes. The identities of other genes that are embedded within the duplication units of *Mup-1.5a* and *Mup-1.5b* are, however, not yet known. We expect to identify these once the *Mup-1.5a* and *Mup-1.5b* genes are cloned in large continuous DNA segments, e.g., in yeast artificial chromosomes (4). It will be of particular interest to determine whether all genes located in the duplication unit carrying the *Mup-1.5b* gene are subject to gene silencing.

The characterization of the genes residing in the two duplication units with respect to whether they are functional or silent will also shed light on the mechanism of silencing of the *Mup-1.5b* gene. The present data suggest that this mechanism acts at the level of chromatin structure. It is possible that the *Mup-1.5b* gene, in contrast to the *Mup-1.5a* gene, lacks a distant positive locus-activating element (8). Alternatively, the *Mup-1.5b* gene, and perhaps all other genes that reside in the same duplication unit, are subject to negative regulation. Another possibility is that the *Mup-1.5b* gene lacks DNA elements that would insulate this gene from negative position effects (7). Whatever this mechanism may be, the fact that the *Mup-1.5a* and *Mup-1.5b* duplication units are highly conserved argues that the controlling elements are located outside of the duplication units, exerting their action over a large distance.

Long-range chromosomal position effects, negative or positive, have been shown to be dominant to regulatory mechanisms mediated by closely linked regulatory elements (see, for example, references 10 and 18). The eventual characterization of the active and silent members of the *Mup* gene family will thus provide the opportunity to investigate the molecular basis of such mechanisms.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AG07538 and by grant CD315 from the American Cancer Society to E.D.

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