VARIATION IN THE MAJOR URINARY PROTEIN MULTIGENE FAMILY IN WILD-DERIVED MICE

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ABSTRACT

The levels of expression and genomic organization of genes coding for the major urinary proteins (MUPs) were examined in several stocks of wild-derived mice. Levels of MUP mRNA in the liver varied considerably with *M. musculus* Brno and *M. castaneus* males having several-fold more MUP RNA than inbred C57BL/6 males, whereas *M. hortulanus, M. caroli* and *M. cervicolor* displayed levels much lower than C57BL/6. Analysis of RNA with MUP cDNAs specific to two different subfamilies of MUP genes revealed that *M. caroli* and *M. cervicolor* primarily expressed a MUP mRNA that was less abundant in C57BL/6, suggesting differential expression of subfamilies of genes within the MUP multigene complex. Although inbred males usually have five-fold more MUP mRNA than inbred females, male to female ratios for wild-derived stocks ranged from one to several hundred. Southern blots of genomic DNA hybridized to MUP subfamily probes revealed differences in restriction fragment sizes as well as possible variation in the number of MUP genes in some species. Analysis of urinary proteins from hybrids between C57BL/6 and *M. spretus* suggested that low MUP expression in *M. spretus* females was due to *cis*-acting genetic elements.

THE major urinary proteins (MUPs) of the mouse are a group of antigenically related, low molecular weight proteins with isoelectric points (pI) in the range of pH 4.5 to 4.9. MUPs excreted in the urine are synthesized in the liver where MUP mRNA may represent as much as 5% of all messenger (m)RNA in male mice of inbred strains (HASTIE, HELD and TOOLE 1979). mRNAs complementary to a cloned liver MUP cDNA have also been detected in lachrymal glands, salivary glands and mammary glands (SHAW, HELD and HASTIE 1983).

The MUPs are the products of a multigene complex estimated to consist of approximately 30 genes per haploid genome (HASTIE, HELD and TOOLE 1979; BISHOP *et al.* 1982). Genetic mapping studies employing recombinant inbred strains of mice as well as mouse-hamster hybrid cell lines have shown that the MUP genes are tightly linked and are located on chromosome *4* near the *Mup-1* locus (BENNETT *et al.* 1982; BISHOP *et al.* 1982; KRAUTER *et al.* 1982).

Regulation of MUP gene expression is apparently very complex. Early studies had shown that males had MUP levels in the urine up to 20 times those of females. Since castration of males reduced their output to female levels, and

testosterone treatments increased the MUP levels of castrated males and normal females, the role of androgens appeared to be clear (FINLAYSON, POTTER and RUNNER 1963). Recent studies have shown a corresponding difference in the MUP mRNA levels in the livers of such male and female mice (HASTIE, HELD and TOOLE 1979). Other hormones including growth hormone and thyroxine have also been shown to be involved in the regulation of at least some of the liver MUP genes. That these hormones act to control the activity of specific genes is suggested by the presence or absence of specific MUP mRNAs in animals in different hormonal states (KNOPF, GALLAGHER and HELD 1983; KUHN *et al.* 1984).

Two-dimensional polyacrylamide gel electrophoresis of C57BL/6 male urine reveals the presence of at least seven MUPs with slightly different pIs and molecular weights. Although posttranslational modifications may be responsible for generating some of the heterogeneity in excreted proteins, there is evidence indicating that a number of different genes are active in the liver (FINLAYSON *et al.* 1968; KNOPF, GALLAGHER and HELD 1983). Furthermore, the MUPs produced in the lachrymal glands (as deduced from *in vitro* translation of lachrymal gland MUP RNA are more basic and, hence, appear to be the products of a different set of MUP genes (SHAW, HELD and HASTIE 1983). Exactly how many of the MUP genes may be expressed in any individual is not known at this time.

Ability to distinguish among the many MUP genes and their products has been enhanced by the preparation of two cDNA MUP clones from C57BL/6 liver RNA. These clones, designated p199 and p499, can be used to identify members of two different subfamilies within the MUP multigene cluster. Evidence has recently been presented that indicates that genes within the subfamilies are active in different tissues and respond to different hormonal stimuli in the liver (KUHN *et al.* 1984).

The regulation of the MUP genes in a sex- and tissue-specific fashion, and in response to a variety of hormonal stimuli, has made them an ideal subject for studies of the relationship between gene structure and function. In connection with these studies, it seemed worthwhile to survey some wild-derived stocks of mice to determine whether they could provide additional examples of variation in MUP gene expression that could be used to analyze regulatory mechanisms. We now report on the relative levels of MUP gene activity in liver and lachrymal glands, as well as on the organization of MUP-coding sequences in the genome of several stocks of wild-derived mice. MUP cDNA clones specific to the p199 and p499 subfamilies were employed to study the relative activity and arrangement of different sets of MUP genes among the various species.

MATERIALS AND METHODS

Wild-derived mice: Mice of the following species and stocks were tested: *M. domesticus* stocks PAC and MOR; *M. domesticus praetextus* stock originating in Jerusalem; *M. musculus* stocks Brno, Belgrade and Denmark; *M. molossinus; M. castaneus; M. hortulanus; M. spretus; M. caroli* and *M. cervicolor popaeus.* The nomenclature of MARSHALL and SAGE (1981) has been used throughout this paper, since some of the stocks originated with them. Wild-derived mice for our investigations

were kindly provided by VERNE M. CHAPMAN from stocks he maintains at Roswell Park Memorial Institute. Additional information about the origin of the stocks and the degree of inbreeding (if any) of various stocks may be found in CHAPMAN, KRATZER and QUANTILLO (1983) and in DICKINSON *et al.* (1984). For information on the relationship among mice in the genus Mus refer to MARSHALL and SAGE (1981), BONHOMME *et al.* (1984) and DICKINSON *et al.* (1984).

M. cervicolor popaeus DNA was provided by KEN ABEL and was prepared as described by DICKINSON *et al.* (1984). RNA for this species was extracted from frozen liver tissue provided by M. POTTER to D. DICKINSON. Inbred mice from strains C57BL/6Jax, DBA/2Ha and BALB/c came from VERNE CHAPMAN or from the West Seneca breeding facility maintained by Roswell Park Memorial Institute. Rat DNA and RNA was obtained from a Sprague-Dawley male rat (*Rattus norvegicus*). Hybrids between the C57BL/6 inbred strain and *M. spretus* were obtained by crossing an inbred female to a wild male. Hybrid female progeny were treated with testosterone via a subcutaneous pellet (KNOPF, GALLAGHER and HELD 1983) when they were 6–8 wk old. Urine samples were collected after 2 wk of treatment.

RNA was extracted from mice between the ages of 6 and 15 wk. Tissues were either used immediately after excision or were frozen in a test tube set in dry ice/ethanol and stored at −70°.

DNA extraction: Methods similar to those of CHAPMAN *et al.* (1983) were used to extract DNA from the livers of male mice that had been deprived of food the night before. Approximately two-thirds of a mouse liver was homogenized on ice in 10 ml of 5% sucrose in TK buffer (50 mM Tris/HCl, pH 7.5, 24 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine). The homogenate was layered over 30% sucrose in TK buffer and centrifuged for 15 min at 3900 × *g* to pellet the nuclei. The nuclei were resuspended in 1× pronase buffer (50 mM Tris-HCl, pH 10, 0.15 M NaCl, 0.1 M EDTA) and SDS was added to a final concentration of 0.2%. Ribonuclease A (Sigma Chemical Company) was added to a final concentration of 100 μg/ml, and the solution was incubated at 37° for 1 hr. Then, SDS was added to bring the concentration to 0.8%, and Pronase (Calbiochem-Behring) was added to a final concentration of 100 μg/ml. This was incubated for at least 4 hr at 37°. The DNA was then extracted with phenol/chloroform/isoamyl alcohol (25:24:1) three times, followed by chloroform/isoamyl alcohol (24:1) extraction three times. Two volumes of cold ethanol were layered onto the top of the final solution, and the DNA was gently spooled onto a glass rod. The DNA was dissolved in distilled water and its concentration determined by measuring its optical density at 260 nm.

RNA extraction: Total RNA was prepared from liver by homogenizing the tissue by hand in a glass Dounce homogenizer in 8 M guanidine hydrochloride (COX 1968; CHIRGWIN *et al.* 1979; HASTIE, HELD and TOOLE 1979) and precipitating with ethanol. RNA was resuspended in 6 M guanidine and precipitated with ethanol three times. It was then suspended in RNase-free distilled water, precipitated twice more using 2% Na acetate and ethanol and suspended in distilled water. All RNAs were stored at −70°.

Total RNA was prepared from lachrymal glands by homogenizing pairs of glands from each animal in 2.5 ml of 5 M guanidine isothyocyanate (CHIRGWIN *et al.* 1979). Cesium chloride (1.1 g/2.5 ml) was dissolved in the homogenate, and this was layered over a cushion of 5.7 M CsCl prepared in NETS buffer (50 mM NaCl, 2.5 mM EDTA, 5 mM Tris-HCl, pH 7.6, 2.5% SDS). The samples were centrifuged for 18 hr at 30,000 rpm at 20° in a SW41 rotor in a Beckman ultracentrifuge. The RNA pellets were suspended in RNase-free distilled water and then reprecipitated twice with 2% Na acetate and ethanol.

cDNA clones for MUP sequences: Two cDNA clones derived from liver RNA and carrying MUP-coding sequences have been described (KUHN *et al.* 1984). The clones, designated p499 and p199, contain 519 and 406 nucleotides from the MUP-coding region, respectively, and 272 and 18 nucleotides from the 3′-untranslated portion of the message. Two subclones have been derived from each MUP cDNA sequence by cutting the cDNA insert with restriction endonuclease *Pvu*II which has a recognition site centrally located in each sequence. Nucleotide sequence analysis revealed about 94% homology between p199 and p499 at the 3′ ends, but only 80% homology at the 5′ ends (KUHN *et al.* 1984). Because the 3′ portion of these genes has apparently diverged less, the p499-3′ subclone has been used to probe for MUP sequences in general. The p499-5′ and p199-5′ subclones have been used to probe for members of what seem to be different subfamilies of MUP genes.

RNA selection using MUP cDNA clones: RNA sequences coding for MUPs were isolated from total liver RNA preparations essentially as described by SHAW, HELD and HASTIE (1983). Single-stranded DNA from cDNA clones p499, p199, p199-5′ was bound to nitrocellulose disks (Schleicher and Schuell, BA85). Disks containing complete p499 MUP sequences were used to select MUP mRNA from 40 μg of total liver RNA (200 μg of total liver RNA was used for extracts of *M. hortulanus, M. caroli, M. cervicolor* and *R. norvegicus*). For three samples (*M. caroli, M. cervicolor* and Rattus), an additional disk containing total p199 MUP sequences was included in the hybridization mixture to ensure that RNAs that might be more homologous to p199 MUP sequences would be selected. An additional sample of *M. caroli* liver RNA was selected using a disk containing cDNA from the p199-5′ MUP subclone only. As a control, two RNA samples were also selected using a disk containing only DNA from the pBR322 cloning vector but lacking MUP sequences.

Nick-translation of MUP probes: Probes for Southern, Northern and dot blots were prepared by nick translation of cDNA MUP clones (MANIATIS, JEFFREY and KLEID 1975; RIGBY *et al.* 1977). For some experiments, probes were prepared using whole cDNA plasmids, whereas for others restriction fragments containing the MUP sequences were used (KUHN *et al.* 1984). Specific activities of 10^8 to 10^9 cpm/μg were achieved by using one or two labeled nucleotides (Amersham) with specific activities of 3000 and/or 800 Ci/mmol.

Southern blotting: Genomic DNA from individual mice was digested with restriction endonuclease *Pvu*II (BioLabs) using 2 units of enzyme per microgram of DNA. Digestions were carried out under the conditions indicated by the enzyme supplier at 37° for at least 18 hr. Completeness of digestion was checked by running 1 μg of DNA on a short 0.8% agarose gel (Seakem LE) and staining with EtBr. Digested DNA was then loaded (18 μg/lane) onto an 0.8% agarose gel made up in TAE buffer (40 mM Tris-HCl, 1 mM EDTA, 0.1 mM acetic acid) and run at 35 V for 18 hr. Fragments were transferred to nitrocellulose according to the method of SOUTHERN (1975). Replicate blots were hybridized to each of the MUP probes (p499-3′, p499-5′ and p199-5′). Prehybridization of the blots was conducted for 2 hr at 63° in 10× Denhardt's solution (DENHARDT 1966), 4× SSC, 0.1% SDS, 0.1% sodium pyrophosphate and 150 μg/ml of denatured salmon sperm DNA. Hybridization was conducted at 63° for 18 hr in 5× Denhardt's, 4× SSC, 0.1% SDS, 0.1% sodium pyrophosphate, 150 μg/ml salmon sperm DNA and 2×10^6 counts/ml of MUP cDNA. Blots were then washed using 2× SSC at 60°, 1× SSC at 60° and, finally, 1× SSC at 65°. Blots were exposed using Kodak XAR film at −70° with Dupont Cronex Lightning Plus intensifying screens after each wash condition. Size markers were prepared by digesting samples of bacteriophage lambda DNA with *Eco*RI (BioLabs), *Hin*dIII (BioLabs) and a combination of the two enzymes overnight at 37°. Samples of each of the three digests were pooled and placed in a single lane of each gel. The portion of the blot corresponding to this lane was removed before hybridization and was hybridized separately using nick-translated lambda DNA.

Northern blots: Total liver RNA, 20 μg, or total lachrymal gland RNA, 15 μg, was denatured by incubating for 15 min at 60° in sample buffer containing 72% deionized formamide, 9.6% formaldehyde and 10 mM $NaPO_4$ (pH 6.5). They were electrophoresed in 1.7% agarose gel containing 5.9% formaldehyde in a 10 mM $NaPO_4$ buffer (pH 6.5). The RNA was transferred to nitrocellulose using 10× SSC. The blots were hybridized using nick-translated MUP insert in the case of the liver RNA blots and using whole p499-5′ and p199-5′ containing plasmid DNA for the lachrymal blot. Blots were hybridized overnight at 63° and were washed at the same temperature, first in 2× SSC and then in 1× SSC.

Dots blots: Five micrograms of total RNA from liver or lachrymal gland were denatured as described in WHITE and BANCROFT (1982). Samples were applied to nitrocellulose using a Schleicher and Schuell Minifold dot blot apparatus. Blots were hybridized as described for Northern blots using a total p499 MUP sequence as the probe. The hybridization temperature was 60°, whereas the blots were washed at 58° using 2× SSC, followed by 0.2× SSC. Several exposures of varying lengths were made of each blot, and the density of the spots was determined using a scanning densitometer. The relative quantity of MUP mRNA in each sample was determined by reference to a dilution series of RNA from C57BL/6 or from *M. musculus* Brno.

In vitro *translation and two-dimensional electrophoresis*: *In vitro* translation of the selected MUP mRNAs was performed in a mammalian fractionated cell-free system as previously described

(SCHREIER and STAEHELIN 1973; HELD, WEST and GALLAGHER 1977; BRUENN *et al.* 1980). Dog pancreatic membranes were added to obtain posttranslational processing of the MUPs (SZOKA, GALLAGHER and HELD 1980). Removal of the hydrophobic signal sequence is essential to achieve good isoelectric focusing because unprocessed proteins do not appear to enter the gel or they tend to smear. Control experiments indicated that the preparation of membranes used in these translations removed signal sequences, but it did not appear to be capable of glycosylating VSV G protein.

Two-dimensional polyacrylamide gel electrophoresis of translation products was carried out as described by O'FARRELL (1975) and KNOPF, GALLAGHER and HELD (1983). Isoelectric focusing gels for the narrow pH range contained 2.4% Ampholine, pH 4–6 (LKB Instruments, Inc.), whereas those for the broader pH range of pH 4–8 contained the following Ampholines (LKB): 0.8%, pH 4–6; 0.8%, pH 6–8; 0.8%, pH 3.5–10. Following electrophoresis in the second dimension, gels were impregnated with 2,5-diphenyloxozole (BONNER and LASKEY 1974) and exposed to Kodak XAR film at −70°.

Urine for two-dimensional polyacrylamide electrophoresis was collected from individual animals in an Eppendorf tube and frozen at −20°. Protein concentrations were determined using the Bio-Rad protein assay. Urine equivalent to 50 μg of protein in the case of males (and 20–30 μg for females) was applied to narrow pH (pH 4–6) isoelectric focusing gels and electrophoresed as described by KNOPF, GALLAGHER and HELD (1983). Gels were stained with Coomassie blue.

RESULTS

Quantification of MUP mRNA levels

As an initial screen for variation in levels of MUP gene expression among different stocks of wild-derived mice, dot blots of total liver and lachrymal gland RNA were hybridized against the almost full-length p499 MUP cDNA sequence (see MATERIALS AND METHODS for complete description of MUP cDNA clones used). Both liver and lachrymal gland RNA were studied because earlier experiments with C57BL/6 had suggested that different genes were active and that different modes of hormonal regulation exist in these two tissues (SHAW, HELD and HASTIE 1983; KNOPF, GALLAGHER and HELD 1983). In experiments of this type the intensity of radioactive labeling is a function of both RNA concentration and the efficiency of hybridization. Hybridization is affected by the conditions of hybridization and washing as well as by the degree of homology in nucleic acid sequence between the RNAs and the cDNA probe. Conditions of moderate stringency were employed to permit maximum possible hybridization to RNAs of more distantly related mice while not allowing an unacceptable level of nonspecific binding. As will be described, MUP cDNA from the inbred strain C57BL/6 appeared to hybridize well to RNAs from closely related strains, *i.e.*, the feral derived *M. domesticus* stocks (PAC and MOR), the commensal stocks (*M. musculus, M. molossinus* and *M. castaneus*) and the two aboriginal stocks (*M. hortulanus* and *M. spretus*) indicating that labeling intensity could be equated with mRNA concentration. Lack of homology may, however, have been a factor in the low level of hybridization observed between the p499 MUP cDNA probe and RNA sequences from more distantly related *M. caroli* and *M. cervicolor.*

Figure 1A shows a dot blot of total liver RNAs from males and females from a number of stocks. Densitometric scans of the dots allowed us to compare the signal intensity for each stock with that of C57BL/6. It was also

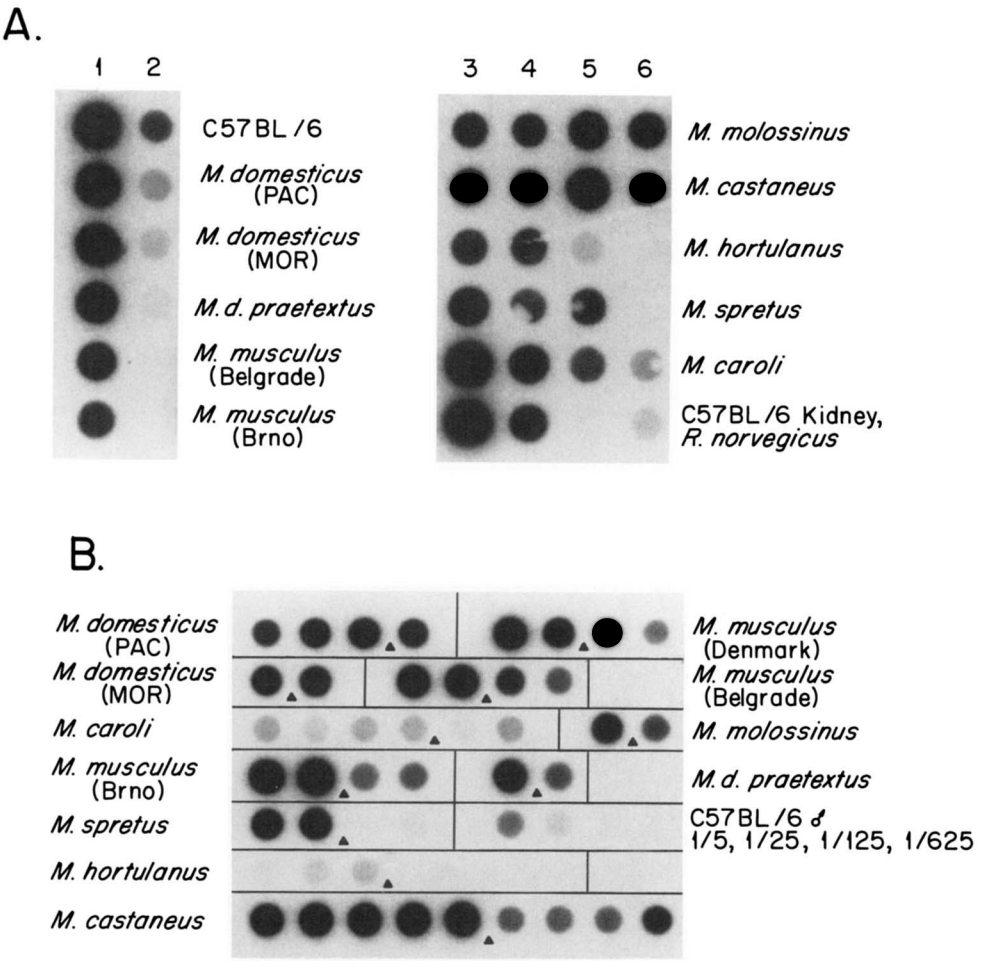

FIGURE 1.—Dot blot of total liver RNA probed with nick-translated p499 MUP cDNA. A, Columns 1 and 2 contain a reference series using RNA from *M. musculus* Brno. Quantities of RNA applied in column 1 were 3, 2, 1, 0.5, 0.2 and 0.1 μg. In column 2, dots represent 0.05, 0.02, 0.01, 0.005, 0.002 and 0.0 μg (buffer only). Other columns contained 2 μg of total liver RNA from pairs of mice of the indicated stocks (for each stock, RNA from male was to the left, female to the right). In column 5, the bottom dot contained 2 μg of total RNA from C57BL/6 kidney, a tissue that does not express MUP mRNA. The bottom dot in column 6 contained 2 μg of rat total liver RNA. [The C57BL/6 male RNA sample shown at the top of column 3 had an abnormally low level of MUP RNA; samples from other C57BL/6 males were employed in Northern blots shown in Figure 2 and gave the typical male to female ratio of about 5.] B, Total RNA equal to 5 μg was applied to each spot. Within each stock, males are to the left of the ▲, and females are to the right.

possible to determine whether the ratio of MUP mRNA in males compared to females differed from the C57BL/6 ratio of about 5. Males from several stocks appeared to have considerably more MUP mRNA than C57BL/6 males. These included the two *M. musculus* stocks, Brno and Belgrade, as well as *M. castaneus*, which were estimated to have from two- to five-fold more MUP mRNA.

By contrast, *M. hortulanus* and *M. caroli* had signal intensities that were less than half those of C57BL/6. On this blot, some hybridization was observed between the mouse MUP probe and RNA from rat liver but none between the MUP cDNA and kidney RNA, in agreement with other reports that the kidney does not express MUP RNA.

For those stocks in which the signal intensity was greater than for C57BL/6, any inefficiency of hybridization due to low homology would merely lead to an underestimate of the amount of MUP mRNA. For those strains in which low levels of labeling were observed, lack of homology could have interfered with hybridization. For example, the level of α_{2u}-globulin mRNA (the rat homologue of MUP) has been estimated at 1% of the total mRNA of the male rat liver (SIPPEL *et al.* 1976). This is a level of mRNA comparable to that observed in female inbred mice. Yet, on the dot blot (Figure 1B) the labeling intensity of the male rat sample was only about 1/10 that of the female C57BL/6, suggesting incomplete hybridization under these conditions. Although these considerations are important in comparisons of signal intensities between species, intraspecific comparisons should not be affected since hybridization efficiencies should be similar for all samples from a species.

For some of the wild-derived stocks, RNA samples from several animals were analysed, and with only a few exceptions, the concentrations of MUP mRNA seemed fairly uniform among members of the same sex for a given stock (Figure 1B). Thus, the MUP expression levels seem to be characteristic of those stocks. MUP mRNA levels of males compared to females of different stocks varied above and below 5, with *M. musculus* stocks Brno and Belgrade displaying ratios close to 10 and *M. domesticus* stocks PAC and MOR having ratios of about 1. *M. spretus* females had a very low level of MUP mRNA which led to male to female ratios on the order of 100 or more.

A dot blot of total RNA from lachrymal glands revealed far less variability in levels of MUP mRNA among the stocks (data not shown, but refer to Figure 3). For most of the wild-derived mice, signal intensities were equal to or somewhat lower than that of C57BL/6; however, a *M. hortulanus* male had a slightly higher labeling level, which was interesting in light of the very low level of hybridization observed in the liver RNA sample of this stock.

Differences in levels of MUP mRNA might reflect a general increase or decrease in the activity of all of the MUP genes transcribed in a particular tissue or might be achieved by the differential expression of a particular subset of MUP genes which could vary from stock to stock. There are two ways of addressing this issue. The first involves the use of nucleic acid probes for subfamilies within this gene cluster, and the availability of two cDNA MUP clones with divergent sequences made it possible to look for differential expression of subfamilies of MUP genes in different tissues and stocks. The second approach is to perform *in vitro* translation of mRNAs to determine which proteins are being produced and, hence, by inference which genes are active.

Since DNA sequencing of the p199 and p499 cDNA clones as well as hybridization to genomic DNA had indicated that the 3′ ends of most C57BL/6 MUP genes were fairly homologous (KUHN *et al.* 1984), we expected the

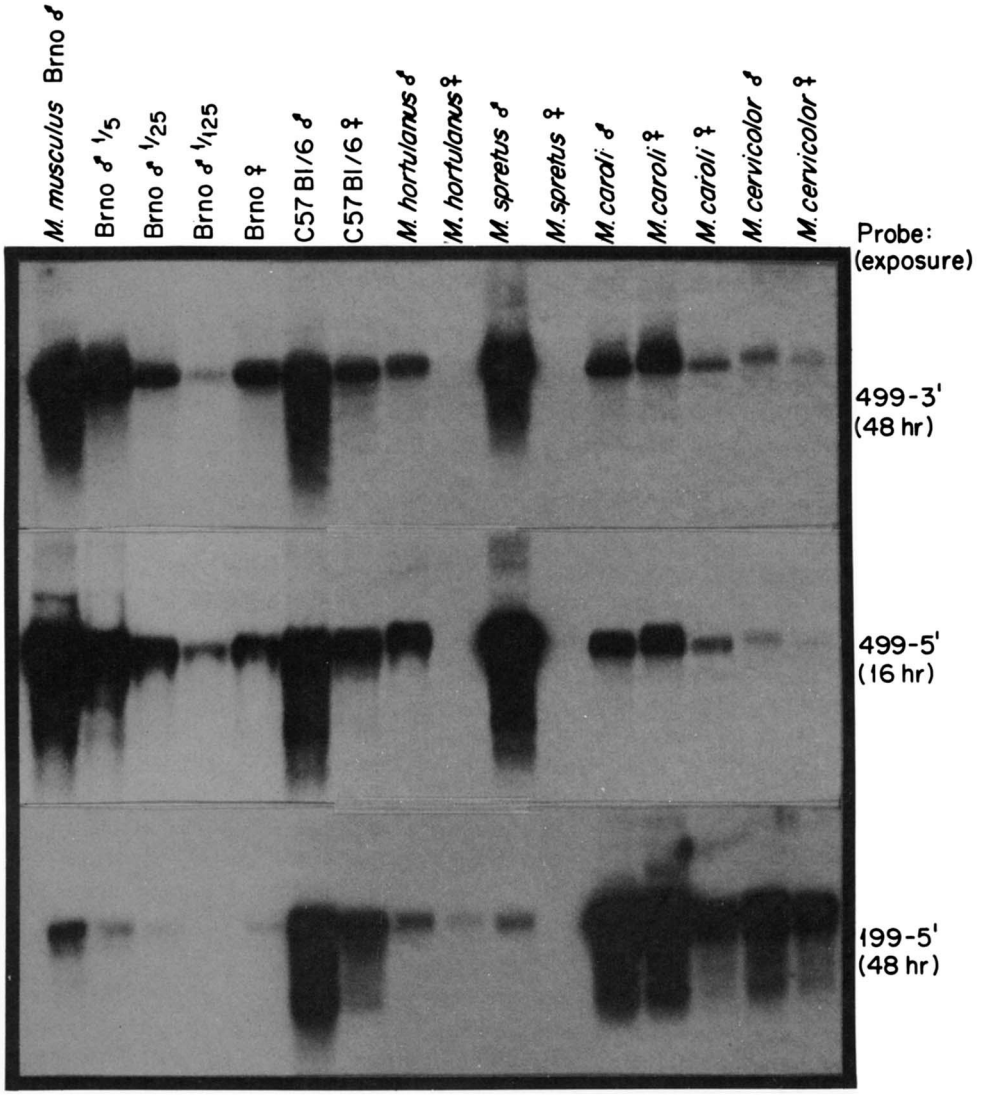

FIGURE 2.—Northern blots of total liver RNA hybridized to three different MUP cDNA sequences. Comparisons of signal intensity should be made within gels and not between gels.

p499-3′ MUP probe to hybridize with all of the MUP mRNA and provide an estimate of total MUP mRNA levels in the livers of different animals. The p199-5′ and p499-5′ MUP probes have been shown to distinguish between transcripts from different subfamilies of MUP genes (KUHN *et al.* 1984) and, thus, allowed us to determine the relative activity of each subfamily. Northern blots of total liver RNA from selected stocks that had shown MUP mRNA levels markedly different from C57BL/6 were prepared in triplicate and hybridized to each of the three subclones (Figure 2). On these blots, it can be observed that the level of MUP mRNA in the C57BL/6 male was about five

times that of the inbred female. On both p499-probed blots, the *M. musculus* Brno male had a signal intensity at least five times that of the C57BL/6 male, whereas Brno females and C57BL/6 females had very similar levels. Whereas the *M. spretus* male had a level equal to or slightly higher than the C57BL/6 male, no signal could be detected from the *M. spretus* females even on the longest exposures. For C57BL/6, *M. musculus* and *M. spretus*, the hybridization levels were quite similar for the MUP p499-3′ and p499-5′ probes, suggesting that the majority of the liver MUP RNA was similar to the p499 MUP sequence and that the p199 MUP sequence represented at most a minor proportion of the RNA.

Evidence for differential expression of a subset of the MUP genes in the liver was provided by the Northern blot hybridized with the p199-5′ MUP cDNA subclone. In C57BL/6 the p199 MUP sequence represents about 10% of the liver MUP RNA (KUHN *et al.* 1984). *M. musculus* Brno and *M. spretus* expressed very small amounts of p199-like sequences, even though they had high overall levels of MUP mRNA. By contrast, in both sexes of *M. caroli* and *M. cervicolor* this sequence occurred at a level equal to or greater than that of C57BL/6. These wild-derived stocks, however, showed relatively low levels of hybridization to the p499 probes.

In addition to the differences in relative signal intensities between stocks, there were several differences in the length of the message to which the MUP probe hybridized. Messenger RNA hybridizing to the p499 MUP sequences was slightly larger in *M. hortulanus* and *M. spretus* than in C57BL/6. Although *M. cervicolor* and *M. caroli* were similar in their higher levels of p199 MUP sequences, the sizes of the MUP mRNAs from the two species were slightly different.

Differential expression of the p499 and p199 subfamilies of MUP genes did not appear to occur in the lachrymal gland. Northern blots of total lachrymal RNA hybridized to either the p199–5′ or p499-5′ MUP cDNA gave essentially the same pattern (Figure 3). The relative amounts of p499 and p199 MUP sequences in lachrymal glands appeared to be constant in different stocks, although some individuals (*M. musculus* Brno and *M. spretus* females) had no detectable MUP mRNA, whereas their respective males had levels only slightly below C57BL/6. As previously observed on the dot blot, the *M. hortulanus* male had a MUP mRNA level somewhat higher than C57BL/6. Signal intensities of the three *M. caroli* were equivalent to about 1/5 that of C57BL/6 and with the *M. caroli* females similar in level to males. In contrast to liver, the p499 MUP sequence was more abundant than the p199 sequence in *M. caroli* lachrymal glands.

Strain-specific variation in proteins coded for by the MUP mRNAs

Strain-specific variation in proteins coded by MUP mRNAs could be due to differential expression as appears to be the case with C57BL/6 and BALB/c inbred mice (see Figure 4) or to mutations within a structural gene that alter the mobility of the resulting protein.

In vitro translation of p499 MUP cDNA-selected liver mRNA followed by

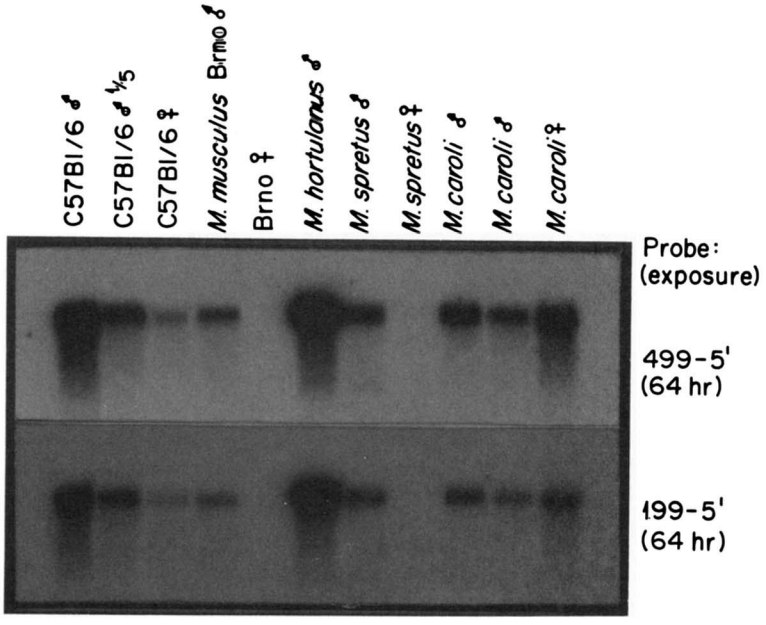

FIGURE 3.—Northern blots of total lachrymal gland RNA hybridized to either p499-5′ or p199-5′ MUP cDNA.

two-dimensional polyacrylamide gel electrophoresis of translation products has been shown to yield a pattern of MUP spots nearly identical with those seen in urine (KNOPF, GALLAGHER and HELD 1983). Rather than assume that any small urinary protein that focused in the acidic pH range was a MUP, we chose to first subject the total liver RNA sample to selection for MUP mRNA by hybridization to single-stranded MUP cDNA. The selected RNAs were then translated *in vitro*, and the translation products were electrophoresed on two-dimensional polyacrylamide gels. Translation products from all stocks were subjected to isoelectric focusing in a pH 4–6 gradient gel, and products from selected stocks were also separated on a pH 4–8 gradient gel. All samples employing the same pH range were electrophoresed at the same time so that mobilities could be compared. Fluorographs of the two-dimensional gels are shown in Figure 4. The vertical arrow on the C57BL/6 fluorograph indicates the location of the single abundant MUP spot observed in the translation products of liver MUP mRNA from females of this inbred strain (KNOPF, GALLAGHER and HELD 1983). The characteristic phenotype of males from the BALB/c inbred line (and also of DBA) differs from the C57BL/6 in having only two instead of four abundant MUP spots.

Males from the two *M. domesticus* stocks, PAC and *praetextus*, had MUP patterns that were essentially like those of C57BL/6. Two-dimensional electrophoresis of urine from the PAC and MOR males also showed the C57BL/6 pattern (B. SAMPSELL, unpublished results). We were interested in both of these *M. domesticus* stocks since MUP mRNA levels in the livers of these stocks as estimated by the dot blot method gave a male to female ratio of approximately

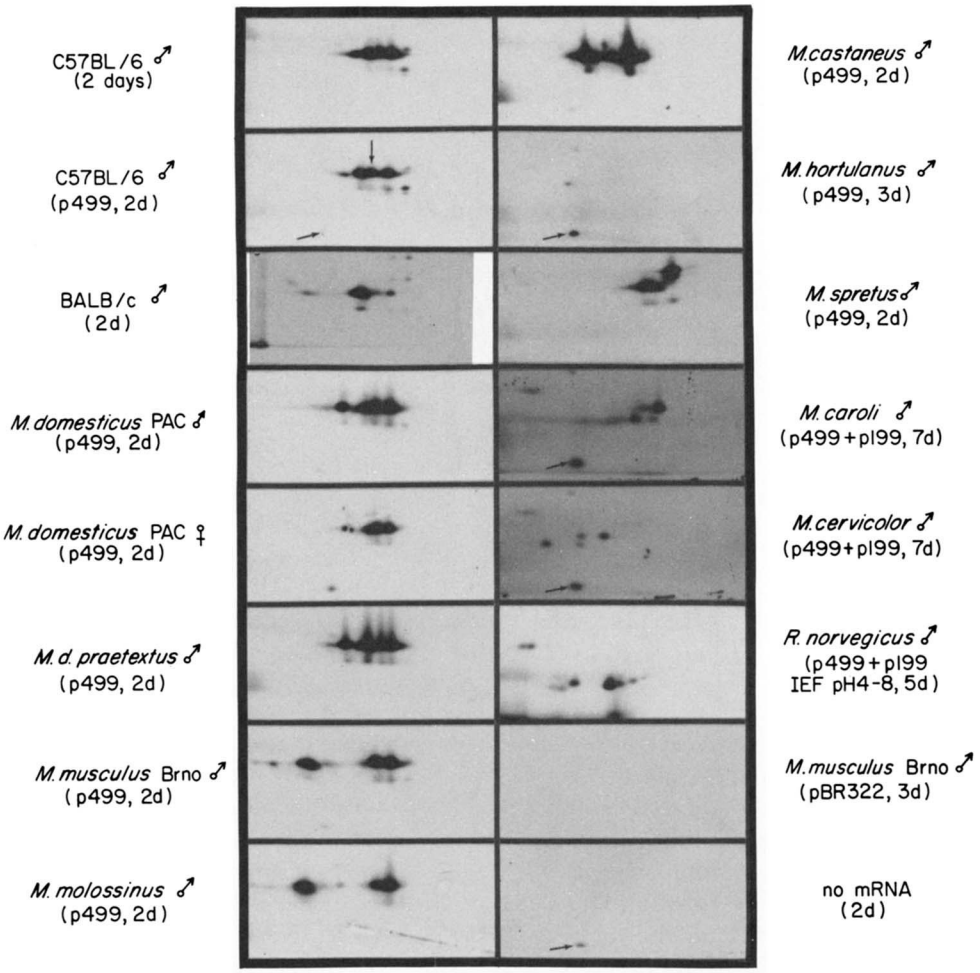

FIGURE 4.—Two-dimensional polyacrylamide gel analysis of *in vitro* translation products of MUP cDNA selected liver RNAs. Unless otherwise indicated, pH range for isoelectric focusing was pH 4–6. The acidic end of the isoelectric focusing gel was placed toward the right. The MUP cDNA clone used in message selection was p499 except where noted. Exposure times for fluorographs are shown in parentheses. The RNA from BALB/c was translated without prior message selection. The vertical arrow on the C57BL/6 gel indicates the single MUP spot that is seen in samples from a C57BL/6 female. The upward-slanting arrows on several of the gels indicate the position of a non-MUP spot which is usually seen on the gels on longer exposures and which can be used to align gels from different stocks. Photographs are taken from the corresponding section of each gel, and comigrating spots are vertically aligned.

1 (Fig. 1). Examination of the two-dimensional gels of liver RNA translation products or urine of females revealed MUP patterns that were very similar to the males and that displayed three of the four male spots in equal abundance.

Comparison of mobilities of MUP spots for stocks considered to be more genetically divergent from inbred strains revealed increasing variation at the protein level. *M. musculus* Brno and *M. molossinus* had patterns very similar to

one another. They displayed two of the four proteins that were abundant in C57BL/6 but had at least two unique proteins of more basic nature. *M. castaneus* had a number of different MUPs, some of which appeared to comigrate with those in C57BL/6. However, the relative proportions among the proteins with the same mobilities were quite different. *M. spretus* had no urinary proteins with mobilities exactly like a C57BL/6 MUP; its unique spots indicated molecules higher in molecular weight and slightly more acidic.

M. hortulanus, M. caroli and *M. cervicolor* displayed only a few MUP spots, and none of them was abundant. Samples of translation products from each of these three stocks were also subjected to isoelectric focusing using a pH range of 4 to 8 rather than the narrower pH 4–6 in case their MUPs should not have pIs in this range. For example, the homologous protein of rats, α_{2u}-globulin is more basic than the murine MUPs and will not focus well in the pH 4–6 range (see Figure 4). No additional MUP spots were observed after wide range isoelectric focusing in any of these three stocks (data not shown).

RNA from *M. caroli* was also message selected using a filter disk containing p199-5′ MUP sequences in order to determine which MUP spots were the products of p199 MUP subfamily genes. The two-dimensional gel from that message selection and *in vitro* translation was identical with the one shown in Figure 4. This is consistent with the finding that nearly all of the liver MUP mRNA from this species hybridizes to p199 rather than p499 cDNA (Figure 2). Although *M. caroli* and *M. cervicolor* both produced mRNA that appeared homologous to the p199 MUP cDNA sequence, the proteins coded for by their respective mRNAs had completely different mobilities.

To investigate the basis for the low levels of MUP expression in *M. spretus* females, individuals of this species were crossed to C57BL/6. Urine from male and female C57BL/6 and *M. spretus* as well as from hybrids between the two species was analyzed by two-dimensional polyacrylamide gels (Figure 5). As previously noted, the patterns obtained from urine samples are quite similar to those observed when liver MUP mRNAs are translated *in vitro*. The gel containing urine from the *M. spretus* female had no visible MUP spots and, hence, is not shown. Urine from the male hybrid produced a pattern of MUP spots that included proteins identical in mobility with those seen in males of each of the parental species (compare panels C and F in Figure 5). Urine from a hybrid female contained only C57BL/6-like MUPs. After the hybrid females had been treated with testosterone, urine samples were tested again and the pattern for the hybrid female was similar to that of hybrid males (data not shown) displaying both C57BL/6 and *M. spretus* spots.

Analysis of variability in gene number and arrangement within the MUP multigene family

The high and low levels of MUP mRNA observed in some wild mice might be the result of an actual increase or decrease in the number of MUP genes in the genomes of these species. This possibility was investigated by preparing Southern blots of genomic DNA digested with *Pvu*II, hybridizing with different MUP probes and examining the overall level of labeling compared to C57BL/6.

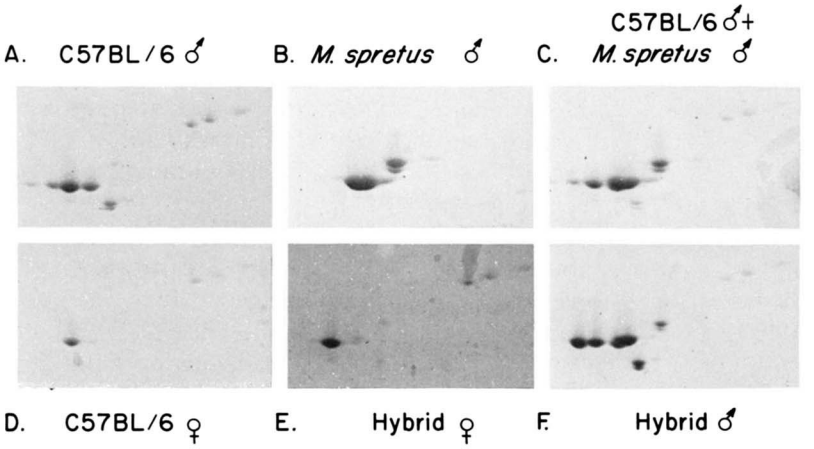

FIGURE 5.—Two-dimensional polyacrylamide gel analysis of urine from C57BL/6, *M. spretus* and interspecific hybrids. C, Equal quantities of urine from males of each of the two species were applied to the single focusing gel.

Autoradiograms of the Southern blots are shown in Figure 6. Washing of the blots was begun at fairly low stringency to detect MUP-containing fragments that may have weak homology. The autoradiograms generated after washing the blots in 2× SSC at 60° showed considerable nonspecific binding of probe. Rewashing in 1× SSC at the same temperature reduced the background level without altering the intensity of the bands (so far as could be detected). Additional washing at the highest stringency employed (1× SSC at 65°) resulted in some loss of signal, and some of the faintest bands did disappear after the final wash. The level of hybridization to *M. caroli, M. cervicolor* and rat DNA was lower than to DNA from other stocks even at the lowest stringency of washing, but these three strains also lost more labeling in the highest stringency wash.

In all three blots, the probes hybridized to a number of fragments of varying sizes. With both the p499-3′ and p499-5′ MUP probes, in the lanes containing DNA from inbred mice and their close relatives, the majority of the label appeared to hybridize to a single band, whereas the remaining radioactivity was divided among a number of different bands. This suggests that, for most of the MUP genes, the restriction recognition sequences for *Pvu*II and their spacing have been conserved during gene duplication events and the subsequent divergence. As might be expected, the sizes of labeled fragments and the band intensities were most similar to C57BL/6 in the two *M. domesticus* stocks (DBA/2Ha and *praetextus*) and in the closely related species *M. musculus* Brno and *M. castaneus. M. hortulanus*, the stock with extremely low levels of liver MUP mRNA had the same major band hybridizing to the p499-3′ probe at 2.8 kb as C57BL/6, although the labeling intensity appeared to be somewhat weaker. There also appears to have been considerably more divergence at the 5′ end of the MUP genes in this species. The darkest band was still located at 4.4 kb (just as in C57BL/6) but the labeling was less intense.

M. spretus had an unusual restriction pattern. On the p499-3′ Southern blot,

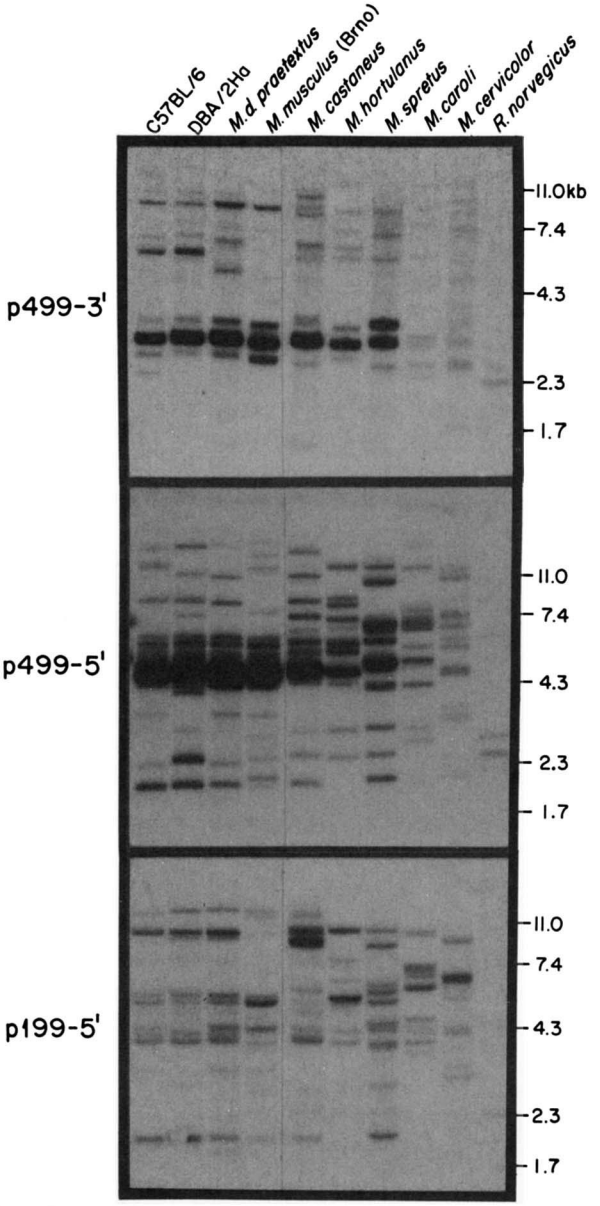

FIGURE 6.—Southern blots of genomic DNA digested with *Pvu*II restriction endonuclease and hybridized to one of three different MUP cDNA sequences indicated on the left. The sizes of lambda restriction fragment markers are shown on the right.

there were two major bands of nearly equal intensity, one of which comigrated with the major band of C57BL/6. The larger fragment (3.3 kb) may be identical in size with one that was also present as a minor component in C57BL/6. On the 5′ end *M. spretus* MUP genes have also undergone considerable divergence compared to *M. domesticus* or *M. musculus*. With the exception of

a few minor bands, none of the *M. spretus* fragments comigrated with those of C57BL/6. The *M. spretus* pattern was also quite different from that of *M. hortulanus*.

The p499-3′ and p499-5′ MUP probes hybridized poorly to the DNA from *M. caroli, M. cervicolor* and rat. In the two mouse species, there were a few faint bands that hybridized to the p499-3′ MUP probe at approximately the same position as the major C57BL/6 band. On the p499-5′ blot, slightly more hybridization was observed and, although *M. caroli* and *M. cervicolor* clearly had unique restriction patterns, a few fragments comigrated with ones present in other mouse species.

The blot hybridized with the p199-5′ MUP probe was examined to determine whether obvious differences in copy number could be observed that would correlate with the variation in level of expression of this MUP sequence. Particular attention was given to the 11-kb band which is seen in C57BL/6. Since this is the only band that remains on a C57BL/6 Southern blot washed at high stringency (0.5× SSC at 65°), it has been presumed to carry the MUP gene corresponding to the p199 cDNA (KUHN *et al.* 1984). The 11-kb band was observed in both *M. domesticus* stocks but was missing in *M. musculus* Brno, in which a 5.2-kb band was the most intensely labeled. *M. castaneus*, by contrast, which expressed somewhat higher levels of p199 MUP sequences in the liver (data not shown), had two strongly hybridizing bands: one at 11 kb and one slightly shorter. *M. hortulanus* also had two dark bands; again, one was located at 11 kb and the other was at 5.4 kb. A number of bands of various sizes showed moderate levels of hybridization in the lanes containing DNA from *M. spretus, M. caroli* and *M. cervicolor*.

DISCUSSION

We have observed variation involving the MUPs among various wild-derived stocks of mice at three different levels: genomic DNA MUP sequences, levels of MUP mRNA expression and diversity of proteins synthesized. We were interested in determining whether we could correlate any changes in the level of expression of certain MUP sequences with alterations in gene arrangement or copy number.

Throughout our study, we have assumed that the MUP sequences of different species would have retained sufficient sequence similarity to those in inbred mice that the MUP cDNAs would hybridize adequately for our purposes. In particular, we had reason to suppose that the 3′ ends of MUP genes were more highly conserved and that the p499-3′ cDNA subclone would detect most MUP-like sequences. If the sharp reduction in hybridization between the p499 cDNA and the total liver RNA or the genomic DNA from *M. caroli, M. cervicolor* and *R. norvegicus* is considered, this assumption may not be fulfilled for these more distantly related stocks. Comparison of a published sequence for a rat α_{2u}-globulin cDNA (UNTERMAN *et al.* 1981) with that of p499 MUP cDNA reveals that they differ at 20% of their sites with the substitutions distributed fairly uniformly throughout the length of the gene; the 3′ ends are only slightly more homologous (83%) than the 5′ ends (78%). Since *M.*

caroli and *M. cervicolor* are thought to have diverged from the lineage leading to inbred mice as many as 7 million yr ago compared to approximately 10 million yr since the Mus-Rattus divergence (DICKINSON *et al.* 1984), these two species may also show a large number of changes in sequences when compared to *M. domesticus*. The strong hybridization between the C57BL/6 MUP probes and the RNAs and/or genomic DNAs for the other more closely related stocks of mice supports the use of signal intensity in making estimates of RNA concentrations and gene copy number.

Among the closely related stocks, the levels of liver MUP mRNA ranged from undetectable in certain *M. spretus* females or low in the case of *M. hortulanus* up to levels in *M. musculus* stocks Brno and Belgrade and *M. castaneus* that were approximately five times as high as those found in the C57BL/6 inbred strain. Levels of MUP mRNA in the lachrymal glands showed much less variation, with C57BL/6 and one *M. hortulanus* male having the highest amounts. The *M. musculus* Brno male had relatively little MUP mRNA in its lachrymal glands. Thus, the levels of MUP gene expression appeared to be regulated independently in these two tissues.

Evidence that changes in mRNA levels reflected differences in the activity of specific genes was provided by the Northern blots using MUP cDNA probes for two different subfamilies of MUP genes. For C57BL/6, the p499 MUP sequence predominated in the liver RNA, with p199 MUP sequences representing approximately 10% of the total (KUHN *et al.* 1984). This disparity was even greater in the *M. musculus* Brno stock in which nearly all of the MUP RNA appeared to be p499-like. By contrast, sequences homologous to the p199 MUP probe were detected at relatively high levels in the livers of *M. caroli* and *M. cervicolor* of both sexes. The strong labeling of these sequences might indicate a higher degree of sequence homology for genes of this subfamily and/or a higher level of expression for this group of genes. The abundance of this sequence (which is a minor MUP component in C57BL/6) in these two species of wild mice was one of the most fascinating findings of our survey.

The relative levels of urinary protein, as judged by *in vitro* translation and two-dimensional electrophoresis of translation products as well as two-dimensional analysis of some urine samples, correlated well with relative levels of MUP mRNA in the liver. Quantification of liver MUP mRNA for the *M. domesticus* PAC and MOR stocks of mice indicated that the male to female ratio was close to 1 rather than the five-fold difference characteristic of C57BL/6. When two-dimensional gels of translation products or urine were examined, it was noted that females of these two stocks also had more protein than C57BL/6 females displaying three prominent spots instead of a single one. This means that the females of these two wild-derived stocks of *M. domesticus* appear to express MUP genes in their livers that are only active in C57BL/6 females in response to testosterone and certain other hormonal stimulation (KNOPF, GALLAGHER and HELD 1983). Whether this reflects a different physiological state in females of the wild-derived stocks or a real difference in genetic regulation will be the subject of future studies similar to those conducted with *M. spretus*.

Analysis of urine from C57BL/6, *M. spretus* and interspecific hybrids suggested that the low level of MUP expression in *M. spretus* females was the result of a *cis*-acting element. Since the MUPs produced by chromosomes derived from each parent could be distinguished by their different mobilities, it was possible to examine the expression of each species' genes in a common environment. It is clear that the hybrid female normally displayed only those MUPs coded for by the C57BL/6-derived chromosome. Treatment with testosterone resulted in the additional expression of those C57BL/6 spots seen in males, as well as the entire set of *M. spretus* proteins. These results suggest that the MUP genes in the two species may differ in the threshold levels of hormone required for induction.

The small amount of hybridizable MUP mRNA detected in total liver RNA from *M. hortulanus, M. caroli* and *M. cervicolor* produced only a small amount of protein upon *in vitro* translation. Clearly, if the homology between the MUP cDNA and the wild stock's RNA was too low to permit hybridization, very little RNA would have been selected for *in vitro* translation. Two-dimensional analysis of urine from these stocks, however, also shows less protein with MUP-like properties than from other wild stocks (W. A. HELD, unpublished results). Urine from *M. caroli* appears to contain a very heterogeneous group of proteins in low abundance that may be generated by a series of posttranslational glycosylations. Since our membrane fraction did not glycosylate newly synthesized protein, these modified forms did not appear on the two-dimensional gels shown in Figure 4. Since the gel resulting from translation of *M. caroli* mRNA selected using p199-5′ cDNA was identical with that using both full-length p199 and p499 cDNAs, the proteins observed were probably coded for by p199-like MUP sequences.

Although sequence divergence may have hampered our ability to detect some MUP sequences in some wild-derived mouse stocks, it is worth noting that hybridization can occur despite a certain amount of divergence. Thus, the C57BL/6 MUP cDNA hybridized strongly to *M. spretus* mRNA and DNA; yet, when the selected mRNAs were translated, the proteins had mobilities completely different from C57BL/6.

Southern blots of genomic DNA with the different MUP cDNA subclones did not reveal any obvious increases in the number of MUP gene copies that could explain the high levels of p499 MUP expression in *M. musculus* Brno and *M. castaneus*, although one cannot exclude the possibility that a small number of genes representing the ones active in the liver have been duplicated more than some other MUP sequences.

M. hortulanus and *M. spretus* both had restriction patterns that differed from C57BL/6, although only the former had a low level of liver MUP mRNA. Furthermore, the extent of hybridization of *M. hortulanus* genomic DNA indicates that its MUP sequences have not diverged too greatly and, thus, that homologous RNA sequences should have been detected (as they were in the lachrymal gland sample) if they had been present in the liver. On Southern blots probed with either p499 cDNA, the intensity of labeling in the band most prominent in C57BL/6 was reduced in *M. hortulanus*, which could indi-

cate a decrease in the number of p499-like MUP genes. The heterogeneity in restriction fragment size suggests there has been a great deal of alteration in MUP-coding regions within the MUP complex. The consequence of such alterations could be that certain MUP genes are no longer subject to the same regulatory control mechanisms. Another possibility is that the species is not lacking any of the relevant MUP genes but that evolution has favored the repression of the MUP genes in this species.

On the Southern blot probed with p199-5′ MUP cDNA, it was noted that *M. musculus* Brno genomic DNA was lacking the strongly labeled band at 11 kb which was present in all of the *M. domesticus* stocks. Since p199 MUP mRNA sequences were less frequent in Brno liver extracts than in C57BL/6, it is possible that the p199 MUP gene(s) of Brno have undergone some type of alteration that prevents their expression in this tissue. We plan to investigate this by isolating p199 MUP genomic sequences from Brno and C57BL/6 and comparing them by restriction mapping and DNA sequencing.

In our approach to the study of variations in gene expression, correlations can suggest but not prove cause and effect among the various genetic levels. For example, one cannot say with certainty that the absence of a particular MUP spot on a two-dimensional gel indicates the absence or divergence of the structural gene for that protein. An individual might have the gene but simply not be expressing it at the time, or in the tissue, examined. Changes in restriction patterns observed on Southern blots reflect alterations which could range from simple base substitutions within an enzyme recognition site to larger scale rearrangements, including insertions or deletions, or differences in gene arrangement resulting from the processes of gene duplication. Some of these alterations clearly have the potential to affect gene expression.

We have observed a great deal of variation at all genetic levels among the wild-derived mice with the differences compared to the inbred strains becoming greater the farther one proceeds in the taxonomic scheme. Observations of interspecific hybrids have suggested that at least some of the differences in MUP gene expression are the results of *cis*-acting elements that differ between species. This confirms the value of the wild stocks as sources of genetic variation which can be used to study the regulation and evolution of this interesting gene family.

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