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Subfamilies of the mouse major urinary protein (MUP) multi-gene family: sequence analysis of cDNA clones and differential regulation in the liver

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ABSTRACT

The mouse major urinary proteins (MUPs) are the products of a multi-gene family of 30-35 genes whose members exhibit diverse tissue specific, developmental, and hormonal controls. Three cDNA clones corresponding to liver MUP mRNAs have been sequenced. Two of the clones (p499, C57BL/6 and p1057, BALB/c) share strong homology whereas a third clone (p199, C57BL/6) has diverged considerably from the others at the nucleic acid (85% homology) and protein (68% homology) levels. The 5' regions of p499 and p199 which show the most sequence divergence were subcloned and shown to hybridize to different liver MUP mRNAs. The p499-5' sequence was expressed in all MUP expressing tissues (liver, lachrymal, submaxillary and mammary) whereas the p199-5' sequence was expressed primarily in the liver and lachrymal. Analysis of liver RNA from mice in different endocrine states indicates that the p499-5' sequence is strongly regulated by thyroxine administration whereas the p199-5' sequence is not. Both sequences appear to be regulated by growth hormone and testosterone. Southern blot analysis of mouse genomic DNA indicates that there are multiple genes homologous to each sequence.

INTRODUCTION

The multi-gene family encoding the mouse major urinary proteins (MUPs) consists of approximately 30-35 genes/haploid genome which are clustered on chromosome 4 (1-4). A similar family of genes which encode the rat urinary protein, α_{2u}-globulin, has been studied by several laboratories (5-8). Analysis of liver MUP mRNA indicates that different members of the MUP gene complement expressed in the liver are subject to differential regulation by testosterone, thyroxine and growth hormone (10,11). MUP genes are also expressed in several other secretory tissues of the mouse (lachrymal, mammary, and submaxillary glands) where they are subject to diverse developmental and hormonal controls (9). Thus, the MUP gene family appears to be somewhat unique among multi-gene families in that different members of the family are subject to very different tissue specific, developmental, and hormonal controls.

Bishop and coworkers have analyzed the structure of a number of MUP

genomic clones by restriction mapping and cross-hybridization techniques (2,12). Their results indicate that most MUP genes fall into one of two groups with some MUP genes belonging to neither group. Thus, the MUP gene family appears to be organized into two or more distinct subfamilies.

In the work described here, we have determined the nucleotide sequence of three liver MUP cDNA clones. Our intent in isolating and characterizing several liver cDNA clones was to look for regions of sequence divergence, subclone those regions, and use the subclones as probes for specific MUP genes or subsets of MUP genes. Such probes would allow us to: 1) identify genomic clones according to subfamily type; 2) determine the structure and number of genes within each subfamily; 3) determine whether each subfamily had a similar tissue distribution; and 4) determine whether the different subfamilies are subject to different hormonal controls.

Subclones of two liver cDNA clones were isolated which correspond to two different subfamilies of MUP sequences expressed in the liver. The subclones hybridize to different liver MUP mRNA species and appear to correspond to different sets of MUP genes. Furthermore, the two subfamilies of MUP mRNA sequences expressed in the liver exhibit distinct differences in hormonal control.

METHODS

Mice and Hormone Treatment

C57BL/6 mice used for the liver RNA preparation and subsequent cDNA cloning were obtained from West Seneca Laboratories, West Seneca, NY. Hypophysectomized and thyroidectomized C57BL/6 mice (8-10 weeks of age) were obtained from Charles River Breeding Labs. Hormone treatments were as previously described (10,11).

Cloning Procedures and Plasmid Preparation

The cloning procedures have been described in detail elsewhere (13). Briefly, liver mRNA from male C57BL/6 mice was prepared and fractionated on sucrose gradients (1). Fractions containing MUP mRNA were pooled and double-stranded cDNA synthesized using avian myeloblastosis virus reverse transcriptase followed by treatment with S1 nuclease. This cDNA and Pst I digested plasmid, pBR322, were tailed with dC and dG, respectively, using terminal transferase, and the annealed recombinant molecules were introduced into *E. coli* X1776. Clones identified as MUPs were subsequently transferred into *E. coli* HB101.

The 5' and 3' regions of MUP cDNA clones were subcloned as follows.

The major Pst I/Eco R1 fragment of pBR322 was isolated and reverse transcribed to fill in the Eco R1 site. This fragment was then ligated to an appropriate Pst I/Pvu II cDNA insert fragment and introduced into HB101. Subclones containing the 5' Pst I/Pvu II p499 and p199 fragments and the 3' Pvu II/Pst I p499 fragment (see Figures 1 and 2) were identified by restriction analysis and Southern blotting techniques.

DNA Sequence Strategy and Methods

Restriction enzymes (New England Biolabs) were used as recommended by the supplier. Appropriate restriction fragments were isolated and 3'-end-labeled with [α-^{32}P] cordycepin 5'-triphosphate in the presence of terminal deoxynucleotide transferase (using 3'-end-labeling kit supplied by New England Nuclear Corp.), or after removal of 5'-terminal phosphate residues with bacterial alkaline phosphatase (Worthington Diagnostics) the DNA fragments were 5'-end-labeled with [α-^{32}P]-ATP (1000-3000 Ci/mmol); New England Nuclear Corp.) in the presence of T4 polynucleotide kinase (P-L Biochemicals) as described previously (14). DNA restriction fragments from which one labeled terminus was removed by secondary restriction cleavage or strand separation were sequenced by the dimethyl sulfate-hydrazinolysis method of Maxam and Gilbert (15). Products were fractionated on 0.4 mm thin

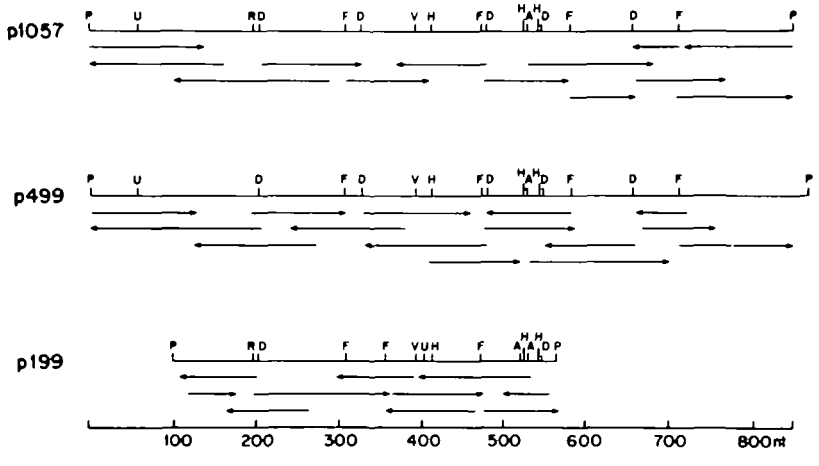
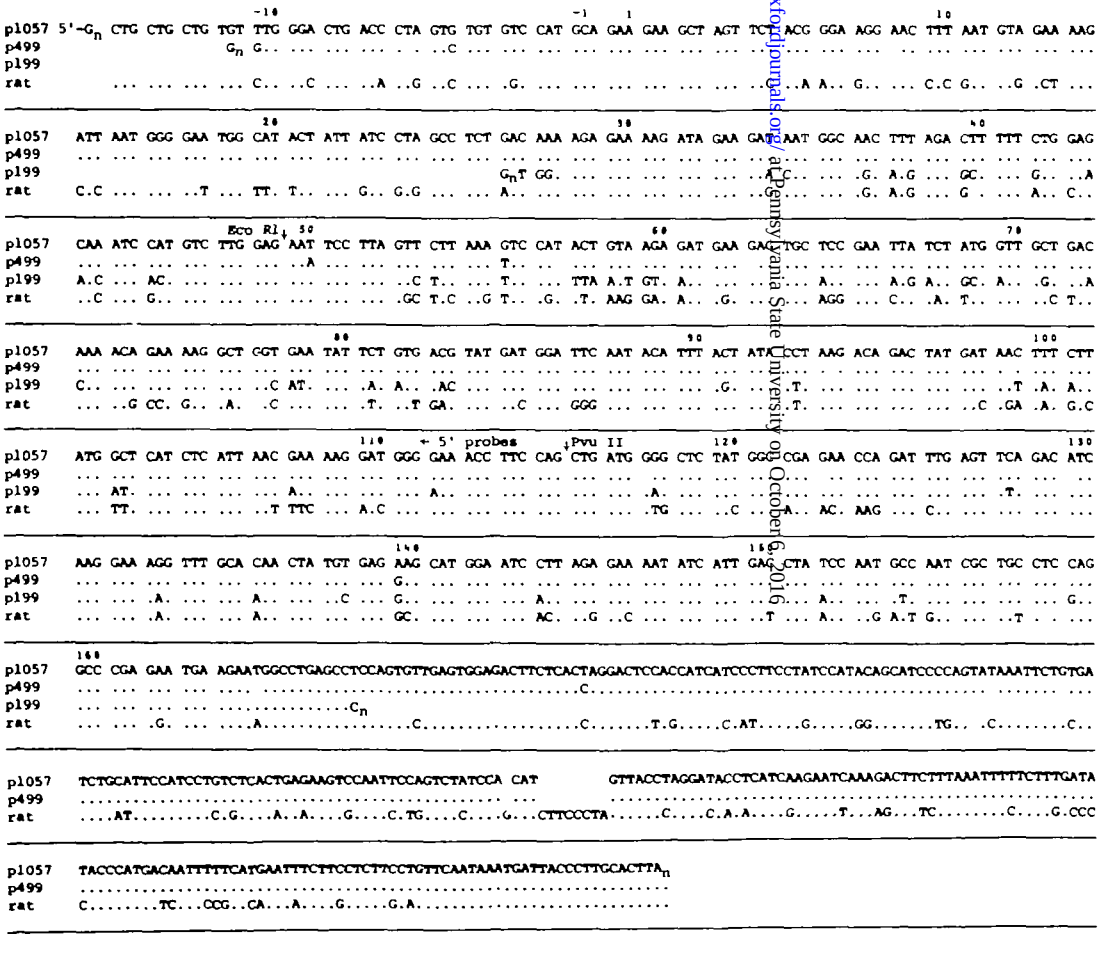


Fig. 1. Restriction endonuclease cleavage maps of the insert of three MUP cDNA clones. Vertical lines and letters refer to the location of the following restriction sites: Pst (P), Alu (U), Eco R1 (R), Dde (D), Hin f (F), Pvu II (V), Hae III (H), and Ava I (A). The nucleotide sequencing strategy is represented by horizontal arrows which indicate the extent and polarity of sequence data obtained from the various restriction fragments.



gels (16) composed of 8, 10, or 20% acrylamide-8M urea.

Hybridization Techniques

MUP mRNA selection and translation was performed as previously described (3,9). The selected mRNA was translated in a fractionated *in vitro* protein synthesizing system in the presence of dog pancreas membranes to effect processing of the pre-MUPs (17,18). The translation products were analyzed by two-dimensional polyacrylamide gel electrophoresis using a narrow range isoelectrofocusing (pH 4-6) dimension which resolves the MUP polypeptides (9-11).

Northern and Southern blotting techniques were essentially as described (9). For some experiments, plasmids were digested with appropriate restriction enzymes, the insert fragment purified by agarose gel electrophoresis, nick-translated and used as the ^{32}P-labeled probe (19).

RESULTS

DNA Sequence of MUP cDNA Clones

The DNA sequence for three different MUP cDNA clones was determined (Figures 1 and 2). The sequence of a rat α_{2u}-globulin cDNA clone previously determined (20) is shown for comparison. All three MUP sequences show extensive homology to α_{2u}-globulin confirming the relationship of the mouse and rat proteins previously inferred by cross hybridization experiments (1). The sequence of p1057 isolated from a BALB/c liver cDNA library (2) is greater than 99% homologous to the C57BL/6 p499 clone. In contrast to the strong homology between p1057 (BALB/c) and p499 (C57BL/6), p199 (C57BL/6) shows considerably more divergence in the nucleotide and deduced amino acid sequences (Figures 2 and 3). The p199 sequence differs from p1057 in 63 out of 420 base pairs and thus has diverged in this region almost as much as the rat sequence for α_{2u}-globulin (88 out of 420 base pairs) has diverged from p1057.

Thus, DNA sequence analysis of MUP cDNA clones indicates that while some MUP clones show strong sequence homology (p1027 and p499), one liver cDNA clone (p199) appears to be considerably more diverged. Current DNA

Fig.2. Comparison of nucleotide sequences of the three MUP clones p1057, p499, p199 and the α_{2u}-globulin sequence present in the rat (20). Position 1 denotes the 5'-coding triplet corresponding to the NH_2-(terminal amino) acid of the mature protein. A dot indicates homology while a letter indicates a nuleotide different from the sequence shown on the first line. Blank spaces are inserted to optimize the alignment. The termination codon (TGA) occurs at position 163.

									-10																				10
p1057 (BALB/c)				leu	leu	leu	cys	leu	gly	leu	thr	leu	val	cys	val	his	ala	glu	glu	ala	ser	ser	thr	gly	arg	asn	phe
p499 (C57BL/6)									*	*	*	*	*	*	*	*	*		*	*	*	*	*	*	*	*	*
p199 (C57BL/6)																													
α_{2u}-globulin (rat)				*	*	*	*	*	*	*	*	*	*	*	gly	*	*		*	*	*	*	*	arg	gly	*	leu
										20											30							
p1057	asn	val	glu	lys	ile	asn	gly	glu	trp	his	thr	ile	ile	leu	ala	ser	asp	lys	arg	glu	lys	ile	glu	asp	asn	gly	asn	phe
p499	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		*	*	*	*	*	*	*	*	*
p199																		gly		*	*	*	*	glu	his	*	ser	met
rat	asp	*	ala	*	leu	*	*	asp	*	phe	ser	*	val	val	*	*	asn	*		*	*	*	*	glu	*	*	ser	met
											Eco RI																		
			40								↓	50										60							
p1057	arg	leu	phe	leu	glu	gln	ile	his	val	leu	glu	asn	ser	leu	val	leu	lys	val	his	thr	val	arg	asp	glu	glu	cys	ser	glu
p499	*	*	*	*	*	*	*	*	*	*	*	lys	*	*	*	*	*	phe		*	*	*	*	*	*	*	*	*
p199	*	ala	*	val	*	asn	*	thr	*	*	*	*	*	*	*	phe	*	phe		leu	ile	val	asn	*	*	*	thr	*
rat	*	val	*	met	gln	his	*	asp	*	*	*	*	*	*	gly	phe	*	phe	arg	ile	lys	glu	asn	gly	*	*	arg	*
					70									80											90				
p1057	leu	ser	met	val	ala	asp	lys	thr	glu	lys	ala	gly	glu	tyr	ser	val	thr	tyr	asp	gly	phe	asn	thr	phe	thr	ile	pro	lys
p499	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		*	*	*	*	*	*	*	*	*
p199	met	thr	ala	ile	gly	glu	gln	*	*	*	*	*	ile	*	tyr	met	asn	*		*	*	*	*	*	ser	*	leu	*
rat	*	tyr	leu	*	*	tyr	*	*	pro	glu	asp	*	*	*	phe	*	glu	*		*	gly	*	*	*	*	*	leu	*
						100										110						Pvu II				120			
p1057	thr	asp	tyr	asp	asn	phe	leu	met	ala	his	leu	ile	asn	glu	lys	asp	gly	glu	thr	phe	gln	leu	met	gly	leu	tyr	gly	arg
p499	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		*	*	*	*	*	*	*	*	*
p199	*	*	*	*	*	tyr	ile	*	ile	*	*	*	*	lys	*	*	*	lys		*	*	*	*	glu	*	*	*	*
rat	*	*	*	*	arg	tyr	val	*	phe	*	*	*	*	phe	*	asn	*	*		*	*	*	*	val	*	*	*	*
								130										140										150	
p1057	glu	pro	asp	leu	ser	ser	asp	ile	lys	glu	arg	phe	ala	gln	leu	cys	glu	lys	his	gly	ile	leu	arg	glu	asn	ile	ile	asp
p499	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	glu		*	*	*	*	*	*	*	*	*
p199	*	*	*	*	*	leu	*	*	*	*	lys	*	*	lys	*	*	*	glu		*	*	ile	*	*	*	*	*	*
rat	thr	lys	*	*	*	*	*	*	*	*	lys	*	*	lys	*	*	*	ala		*	*	thr	*	asp	*	*	*	*
										160																		
p1057	leu	ser	asn	ala	asn	arg	cys	leu	gln	ala	arg	glu	xxx															
p499	*	*	*	*	*	*	*	*	*	*	*	*																
p199	*	thr	*	val	*	*	*	*	glu	*	*	*																
rat	*	thr	lys	thr	asp	*	*	*	*	*	*	gly																

sequence data for rat α_{2u}-globulin cDNAs has not revealed such diversity in DNA sequence (8,20).

Subcloning of Conserved and Non-conserved Regions of MUP cDNA Clones: mRNA Selection Using Subcloned Fragments

All three MUP cDNA clones have a Pvu II site corresponding to amino acid position 115 (Figures 1-3). Preliminary cross hybridization experiments indicated that the 5' Pst I-Pvu II fragment of p499 did not hybridize to the 5'-Pst I-Pvu II fragment of p199 whereas the 3'-Pvu II-Pst I fragments did cross hybridize (data not shown). Subsequent DNA sequence analysis (Figure 2) indicates that the 5' Pst-Pvu II fragment of p499 and p199 are more diverged than 3' sequences. Thus, these fragments and the 3' p499 Pvu II-Pst I fragments were subcloned in pBR322 (see Methods).

In order to determine whether the subcloned fragments recognize distinct MUP mRNA species, male C57BL/6 liver mRNA was hybridized to each of the subclones bound to nitrocellulose filters. The specifically bound MUP mRNA was eluted, translated *in vitro* in the presence of dog pancreas membranes to effect processing, and the products analyzed by 2-dimensional polyacrylamide gel electophoresis. The nomenclature for the 2D gel pattern of MUP *in vitro* translation products is the same as previously used (10). It is based on the observation that most urinary MUPs comigrate with processed *in vitro* synthesized MUPs with two exceptions. The differences between the urinary and *in vitro* synthesized pattern observed for MUPs 6 and 7 may be due to post-translational modifications (10). It should be noted, however, that we cannot be certain that each spot or group of spots represents a unique MUP protein species. The pattern of MUP polypeptides from p499 selected mRNA appears similar to total liver MUP mRNA translation products (Figure 4). The p499-3' selected mRNA translation products also appear to be similar to the total MUP translation products although some spots (MUP 6 and 7) are reduced in intensity. Thus, the p499-3' subclone appears to hybridize to all liver MUP mRNAs. In contrast, the p499-5' subclone selects mRNA coding for MUPs 1, 2, 3, 4, and 5, whereas the p199-5' subclone selected mRNA coding for MUP 6. Thus, there are at least two subfamilies of MUP mRNA sequences expressed in the liver. Since MUP 7 mRNA

Fig. 3. The amino acid sequences of MUPs deduced from the nucleotide sequences. The α_{2u}-globulin amino acid sequence (20) is included for comparison with the mouse sequences. The NH_2 terminus of the mature protein is noted as position 1; the carboxyl-terminus is indicated by three X's following amino acid 162. Potential N-linked glycosylation sites occur at position 35 in the rat and 44 in p199.

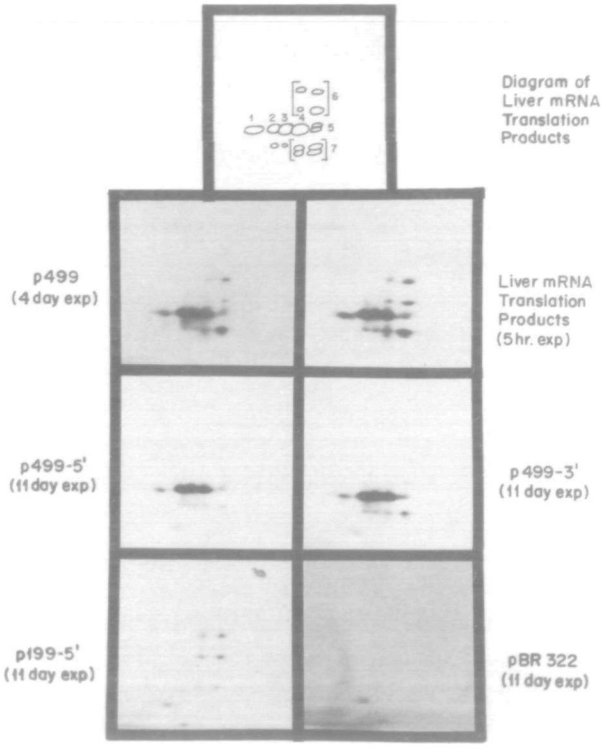


Fig. 4. Two dimemsional polyacrylamide gel analysis of MUP-plasmid-selected liver mRNA translation products. Male C57BL/6 liver mRNA (10 µg) was hybridized to MUP plasmids (p499, p499-3', p499-5', or p199-5') bound to nitrocellulose filters (10 µg of plasmid DNA per filter) as described previously (9). The eluted mRNA was translated in vitro in the presence of dog pancreas membranes and the ^{35}S-methionine labeled products analyzed by two-dimensional polyacrylamide gel electrophoresis. Most liver mRNA translation products other than the MUP polypeptides do not enter the narrow range (pH 4-6) focusing gel used in the first dimension. The plasmids used and the exposure times for the fluorographed gels (26) are indicated in the figures.

is not selected by either p499-5' or p199-5', there may be a third subfamily of MUP sequences expressed in the liver. These results indicate that the p499-5' and p199-5' subclones are specific for different MUP mRNA sequences expressed in the liver.

Tissue Specific Expression of MUP Subfamilies

MUP mRNA sequences are found in the submaxillary, mammary, and lachrymal glands as well as the liver (9). Thus, it was of interest to determine whether both the p499-5' and p199-5' liver subfamily sequences were expressed in these tissues. Northern blots of liver, lachrymal,

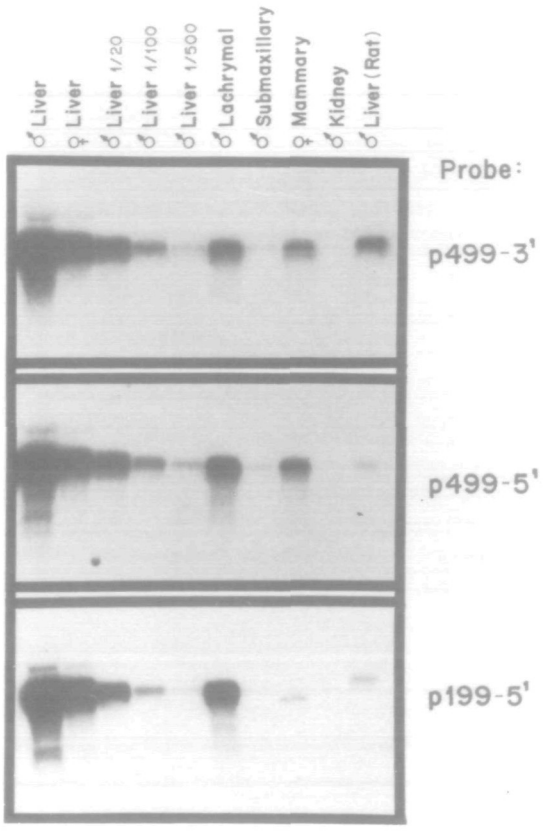


Fig. 5. Tissue distribution of MUP mRNA sequences determined by Northern blot analysis using the MUP cDNA subclones. Total RNA (20 μg) was electrophoresed on 1.7% agarose gels and blotted onto nitrocellulose as previously described (9). The plasmid DNA was digested with Pst I and Bam H1 and the fragment containing the insert plus 375 bp of pBR322 sequence was nick translated and used as a probe. The autoradiograms were exposed for 2 days (p499-3', p499-5') or 3 days (p199-5').

submaxillary and mammary RNA from C57BL/6 mice, were probed with the p499-5', p199-5', and p499-3' subclone probes (Fig. 5). Both the p499-5' and p199-5' sequences are present in approximately 5-10 fold higher abundance in male than female liver. The p199-5' sequences are most abundant in the liver and lachyrmal tissues and appear to be absent in the submaxillary gland. The p499-5' sequences are abundant in liver, lachrymal and mammary tissues. The mammary MUP mRNA which hybridizes to the p199-5' probe appears to have a somewhat lower molecular weight. The p499-3' probe

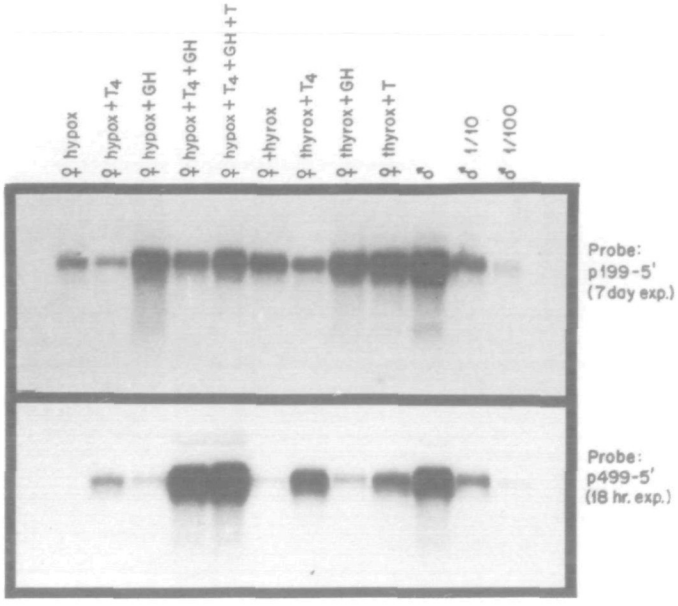


Fig. 6. Differential regulation of MUP subfamily sequences in the liver: Northern blot analysis of liver RNA from mice in various endocrine states using p199-5' and p499-5' probes. Total liver RNA (20 μg) was analyzed as previously described (9). Plasmid DNA was nicked-translated and used as probe. Exposure and source of liver RNA is given in the figure. Abbreviations are: hypox, hypophysectomized mice; thyrox, thyroidectomized; T_4, thyroxine; GH, bovine growth hormone; and T, testosterone.

detects two mRNAs in the mammary; the smaller mRNA appears to correspond to the one that hybridizes to the p199-5' whereas the larger appears to correspond to the one that hybridizes to the p499-5' probe. The somewhat stronger hybridization of heterologous male rat liver sequences to the p499-3' probe suggests the 3' sequences are more strongly conserved. As previously reported, MUP mRNA is not present in the kidney (1).

Differential Hormonal Regulation of MUP Subfamilies in the Liver

Previous work in our laboratory indicated that different members of the MUP gene complement expressed in the liver were subject to different hormonal controls (10,11). In these experiments, the differential hormonal response of different MUP genes was assessed indirectly by translation of liver RNA and analysis of MUP translation products by two-dimensional polyacrylamide gel electrophoresis. The subclones p499-5' and p199-5' allow us to directly determine whether the two subfamily sequences expressed in the liver are differentially regulated. The results shown in Figure 6

indicate that the two subfamilies have distinct differences in hormonal control. The p499-5' sequence is strongly regulated by thyroxine. This is evident from the greatly reduced level in thyroidectomized animals and the strong induction by thyroxine administration. Similarly, the p499-5' sequences are greatly reduced in hypophysectomized animals and are increased by thyroxine, especially in the presence of growth hormone. In thyroidectomized animals, the p499-5' sequence was increased by testosterone, whereas growth hormone had little, if any, effect.

In contrast, the p199-5' sequence was not significantly reduced in RNA from female thyroidectomized animals and was not increased by thyroxine administration. However, growth hormone increased the p199-5' sequence in both hypophysectomized and thyroidectomized animals and testesterone increased the p199-5' sequence in thyroidectomized animals.

The p499-5' sequences appear to represent the majority of the MUP liver mRNA sequences. Based on the MUP mRNA selection-translation experiments and the signal intensities observed in Northern blot hybridizations, we estimate that the p499-5' sequences constitute approximately 90% of the total liver MUP mRNA whereas the p199-5' sequences represent 10% or less.

Southern Blot Analysis with MUP Subfamily Probes

Mouse genomic DNA from liver was digested with Eco R1 or Pvu II, the restriction fragments separated by electrophoresis, blotted, hybridized to the different MUP subclones, and washed under low stringency (LS) and then high stringency (HS) conditions (Fig. 7). The p499-3' probe hybridized to several bands ranging in size from 2.6 to 13 kb. The relative intensity of the bands appears to be quite similar after low stringency and high stringency washes indicating the 3' regions of the MUP genes appear to be relatively homologous to the p499-3' probe.

As expected the p499-5' probe hybridizes to a different set of restriction fragments ranging in size from 1.9 to 13 kb. Clear differences in hybridization are evident after high versus low stringency washes. After the high stringency wash, several of the high molecular weight Pvu II bands (6 to 13 kb) fade and the relative intensity of some of the other bands change considerably (i.e., the band at approximately 2.3 kb, Pvu II digest). Thus, the 5' regions of MUP genes which hybridize to the p499-5' probe appear to be less homologous than the 3' regions.

One might have expected differences in the size of restriction fragments hybridizing to the p199-5' versus p499-5' probe since they

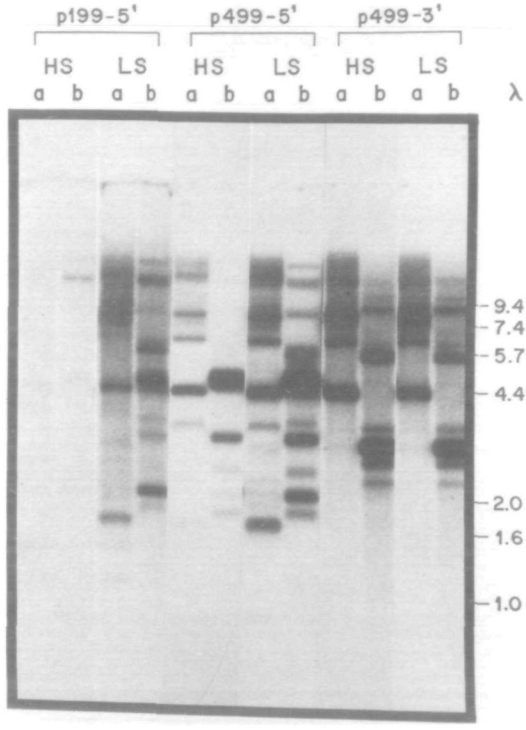


Fig. 7. Southern blot analysis of mouse genomic DNA using MUP cDNA subclones. C57BL/6 mouse liver DNA was extracted (3) and digested with Eco R1 (a) or Pvu II (b) and electrophoresed on 0.8% agarose gels. After transfer, the blots were hybridized to nicked-translated restricton fragments from the different MUP plasmid subclones at 62°. The hybridization mixture contained 4X SSC, 5X Denhardt's, 0.1% SDS, 0.1% sodium pyrophosphate, 150 ug/ml salmon sperm DNA, 1X 10^6 CPM/ml of ^{32}P-labeled, nick translated probe. The blots were washed first in 1X SSC at 60° (LS, low stringency conditions) and then autoradiographed. The blots were then washed in 0.5X SSC at 65° (HS, high stringency conditions) and autoradiographed a second time. The exposures are as follows: p499-3', LS, 4 hr, HS, 2 days; p499-5', LS, 12 hr, HS, 2 days; p199-5', LS, 12 hr, HS, 2 days. The size (kb) of λ restriction fragment markers is shown at the right of the figure.

recognize distinct MUP mRNA sequences. Using low stringency wash conditions, the p199-5' and p499-5' probes hybridize to restriction fragments of similar sizes although there are distinct differences in the relative intensities. Using higher stringency wash conditions, the p199-5' probe hybridizes strongly to a single approximately 11 kb Pvu II fragment. These results suggest that there are a small number of MUP genes, possibly

only one, which have a high degree of homology to the p199-5' probe whereas there are probably several MUP genes highly homologous to the p499-5' probe.

DISCUSSION

DNA Sequences of Liver MUP cDNA Clones

We have determined the DNA sequence of three liver MUP cDNA clones, 2 derived from C57BL/6 (p499 and p199) and 1 from BALB/c mice. The p1057 (BALB/c) and p499 (C57BL/6) sequences are nearly identical. There are only 4 base substitutions in the coding region (1 silent). Two of the amino acid substitutions are conservative and one is nonconservative. The p499 sequence would code for a MUP protein which is more acidic than the one coded by p1057. The 3' untranslated regions also exhibit extensive homology with only 1 base change out of 270 nucleotides.

In contrast, the p199 sequence shows considerably less homology to the p1057 sequence. At the amino acid level p199 (68% homology to p1057) has diverged almost as much as the rat sequence (65% homology, see Table 1). In the coding regions 43 out of 134 codons (32%) have amino acid substitutions. There is a somewhat higher number of first and second position changes (30 and 19 respectively) relative to third position changes (14). However, most of the amino acid changes are conservative (72%). Although MUP proteins have not been previously reported to be glycosylated, the p199 sequence has a potential N-linked glycosylation site at amino acid position 44. Interestingly, this is close to a potential glycosylation site in the rat α_{2u}-globulin sequence (position 35) which may suggest some functional role. It is unfortunate that the p199 cDNA clone only contains

Table 1

Comparison of MUP and Rat α_{2u}-Globulin Nucleic Acid and Amino Acid Sequence Homologies

cDNA Clones Compared	Coding Region		3' Untranslated
	% Nucleic Acid Homology	% Amino Acid Homology	% Nucleic Acid Homology
p499 and p1057	99.2 (516)[b]	98.3 (172)	99.6 (270)
p199 and p1057	84.4 (403)	67.9 (134)	-[c]
α_{2u}-globulin[a] and p1057	78.4 (528)	65.9 (176)	77.0 (270)
α_{2u}-globulin[a] and p199	77.9 (403)	64.2 (134)	-[c]

[a] Sequence data taken from Unterman et al. (20).

[b] Number in parenthesis indicates the number of nucleotide or aa residues compared.

[c] The p199 cDNA clone only contains 18 nucleotides of the 3' untranslated region.

18 bps of the 3' untranslated region. It would be interesting to determine whether this region has diverged at the same rate as the coding region.

DNA sequence analysis of several rat liver α_{2u}-globulin cDNA clones and one genomic clone indicates they all share very strong homology with each other (98%; 8). Sequence analysis of a salivary α_{2u}-globulin cDNA clone indicates a 5% nucleotide divergence from liver cDNA sequences (22). Thus, unlike the MUP genes, no strong divergence within the α_{2u}-globulin gene family has yet been reported. Both the coding and 3' untranslated regions of MUP p1057 diverged from α_{2u}-globulin at approximately the same rate (78.4 and 77.0% homology). In addition, both the p199 and p1057 sequence are about equally diverged from the rat (see Table 1). This would suggest that the duplication event leading to MUP subfamilies occurred after evolutionary separation of mice and rats. If a third subfamily of MUP sequences are expressed in the liver as implied by the mRNA selection and translation experiments, it will be interesting to determine the sequence homologies to the other MUP clones and to rat α_{2u}-globulin.

Comparison of the DNA sequence of MUP (p1057 and p199) and α_{2u}-globulin coding regions indicates that there is considerably greater homology at the nucleic acid level than at the amino acid level (see Table 1). This suggests that there has been less selective pressure to retain a precise structure compared to a protein which has a more rigid structure-function relationship. For example, the coding sequences for human β and δ globin genes are approximately 93% homologous at both the nucleic acid and amino acid levels (23), and between species the strongly conserved histone genes may have greater homology at the amino acid than at the nucleic acid level (24). Also, the extensive repetition of MUP genes to 30-35 genes per haploid genome may have allowed for greater diversity at the amino acid level. The evolutionary relationship between MUPs and α_{2u}-globulin might be less obscure if a definite functional role for these proteins was established (for a discussion of a possible functional role of MUP proteins, see ref. 9).

Subcloning and mRNA Selection

The occurrence of a conserved Pvu II site in the MUP cDNA clones allowed us to construct probes which distinguish two subfamilies of MUP mRNA sequences expressed in the liver (Figures 1 and 2). The p199-5' hybrid selected liver MUP mRNA translation products resolve into four spots by two-dimensional gel electrophoresis. The four spots may represent a single

mRNA translation product since we have frequently noted that in vitro translation in the presence of membranes results in products with both size and charge heterogenity if that protein is glycosylated in vitro (25). Indeed, we found that the set of higher molecular weight spots bind to a concanavalin A column (Held and Baumann, unpublished results), and the p199 sequence has a potential glycosylation site at a residue 44 (see Fig. 3). The p499-5' hybrid-selected liver MUP mRNA translation products are clearly distinct and probably represent the products of several different MUP mRNAs. Since MUP 7 (Fig. 4) was not selected by either 5' clone, it may represent a third subfamily of MUP mRNA sequences expressed in the liver.

Most of the other MUP expressing tissues (lachrymal, mammary, and submaxillary glands) express both p499-5' and p199-5' sequences (Fig. 5). However, due to the low level of MUP expression in the submaxillary gland, it is not clear whether there is any expression of the p199-5' sequence. The lachrymal gland contains a considerable amount of MUP mRNA homologous to the p199-5' probe. Previous work (9) indicated that the lachrymal MUP translation products differ considerably in size and charge from the liver MUP translation products. The hybridization and wash conditions used for the Northern blots (62°, 1xSSC) are sufficiently stringent to distinguish between p499-5' and p199-5' "like" sequences but probably do not distinguish between different p199-5' "like" sequences. Thus it is quite possible that the p199-5' sequences (and p499-5' sequences) expressed in the different tissues are the products of different genes.

Differential Regulation of MUP Subfamily Sequences in the Liver

Two different subfamilies of MUP sequences expressed in the liver are regulated differently (Fig. 6). The p499-5' sequences are strongly regulated by thyroxine or thyroxine in conjunction with growth hormone. Growth hormone in the absence of thyroxine has little effect on the p499-5' MUP mRNA levels. Testosterone also appears to modulate p499-5' MUP mRNA levels in thyroidectomized or normal female mice but has little or no effect in hypophysectomized animals. We previously showed that testosterone has little or no effect on total liver MUP mRNA levels in the absence of a pituitary gland (10,11). In contrast to the strong modulaton of p499-5' sequences by thyroxine, liver mRNA homologous to the p199-5' sequence shows essentially no modulation by thyroxine but is increased somewhat by either growth hormone or testosterone. The overall regulation of the p199-5' sequence is only 10-20 fold, whereas the p499-5' sequences are regulated several hundred fold (see Fig. 6).

Our previous data (10,11) indicated that administration of thyroxine to thyroidectomized mice increased MUP mRNAs corresponding to MUPs 1,2,3,4, and 5, all of which are homologous to p499-5' (Fig. 4). Administration of testosterone to thyroidectomized mice increased MUP mRNAs corresponding to MUPs 2,3,4,6, and 7. Thus, of the p499-5' MUP mRNAs, thyroxine specifically increases MUPs 1 and 5 whereas MUPs 2,3, and 4 are increased by either thyroxine or testosterone administration. This indicates that different p499-5' genes expressed in the liver are modulated by different hormones and that some p499-5' genes may be modulated by more than one hormone. These experiments, of course, do not establish whether the administered hormones are acting directly. In addition, it is quite possible that different hormones regulate the steady state levels of different MUP transcripts by different mechanisms (i.e., transcriptional, or posttranscriptional).

Genomic Organization of MUP Genes

There are 30-35 MUP genes per haploid genome which appear to be clustered in a single region of chromosome 4 (1-4). Southern blots of genomic DNA using the p499-3' probe indicate that the 3' regions of MUP genes are more conserved than the 5' regions (Fig. 7). This is also apparent from the DNA sequence data of the p499 and p199 clones (Fig. 2). The stronger hybridization of the p499-3' probe to rat liver RNA relative to the 5' probes suggests that the 3' regions have been more strongly conserved in the rat as well. The work of Bishop and co-workers indicates that most MUP genes belong to one of two groups, termed Group 1 and Group 2, which can be distinguished by restriction mapping and cross hybridization experiments. Some MUP genes, however, do not appear to correspond to either group (2). Southern blot analysis in John Bishop's lab using our p499-5' and p199-5' subclones indicates that p499-5' is homologous to group 1 genes and that p199-5' is homologous to neither group 1 or group 2 genes (John Bishop, personal communication).

Under high stringency conditions the p199-5' probe hybridizes strongly to a single 11 kb Pvu II fragment whereas the p499-5' probe hybridizes to several fragments, some of which are very intense (e.g., 4.8 kb Pvu II fragment; Fig. 7) and probably represent several genes. Thus, the p499-5' genes appear to be more homologous to one another than the p199-5' genes.

The subcloning of additional regions of sequence diversity amongst the MUP cDNA clones should facilitate their classification according to tissue expression and mode of regulation. The positions and structure of

MUP genes within the cluster on chromosome 4 can then be analyzed to determine whether the arrangement and/or structural features play a significant role in tissue specific, developmental or hormonal controls.

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