Tissue-Specific Expression of Major Urinary Protein (MUP) Genes in Mice: Characterization of MUP mRNAs by Restriction Mapping of cDNA and by In Vitro Translation

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The major urinary proteins (MUPs) in mice are coded for by a gene family which consists of ca. 30 members. The number of MUP genes that are expressed is not known. Previous studies have shown that MUP mRNAs are present in several secretory tissues in addition to the liver, in which they were originally identified. In this paper we show, through restriction analysis of MUP cDNAs, that distinct sets of MUP mRNAs are synthesized in each of the tissues studied and that these mRNAs are most likely coded for by different genes. As is shown, MUP mRNAs of different tissues are related to an extent that precludes the use of gene-specific probes in differentiating among them. The regions of homology also include the 3' untranslated regions of MUP mRNAs. The question of differential expression was thus investigated by searching for restriction polymorphisms in MUP mRNAs. We demonstrate that subtle differences in the sequences of even scarce mRNAs can be recognized by this particular approach. In addition, it is shown that MUP mRNAs of different tissues code for different, nonoverlapping sets of polypeptides, as determined by gel electrophoresis of in vitro-translated precursors to MUPs. The relevance of these results to models of evolution of tissue-specific regulation in a multigene family is discussed.

It appears that the synthesis of tissue-specific mRNAs at the appropriate stage of development is controlled through transcriptional regulation (8, 9). The mechanisms of this regulation are not known, nor is it known how this type of regulation arose during evolution. As has been suggested (see, e.g., reference 12), detailed studies of the structure of tissue-specific genes which belong to multigene families should provide clues concerning both the evolution and the molecular basis of tissue-specific regulation. In this paper we show that the genes coding for major urinary proteins (MUPs) form such a gene family.

MUPs were originally recognized because of their high concentration in the urine of adult male mice. The urinary MUPs are synthesized in the liver (13, 23) under the influence of a number of hormones, including thyroxine, growth hormone, and testosterone (16, 21). The physiological role of these proteins is not known. Because of the coincidence in timing of the appearance of MUPs in the liver and the onset of puberty, the possibility that MUPs participate in the sexual development of mice has been suggested (26). Recently it was observed that mRNAs related to liver MUP mRNA are also present in the submaxillary, sublingual, parotid, lachrymal, and mammary glands (21). In each of these tissues, MUPs are synthesized in response to different developmental and hormonal signals. For example, MUP synthesis commences in the liver at the onset of puberty and continues into adulthood (8, 21). In the submaxillary gland, the MUPs start to be synthesized 1 to 2 weeks before the onset of puberty, and MUP synthesis ceases in the postpubertal animal (unpublished data). In the lachrymal gland MUP synthesis commences at about the same time as it does in the submaxillary gland but persists into adulthood (21), and in the sublingual gland MUP synthesis occurs in postnatal animals of all ages (21).

The MUPs are coded for by ca. 30 genes that are clustered in the *Mup-a* locus of chromosome 4 (1, 5). Because of the presence of a large number of MUP genes, the possibility exists that MUP genes are differentially expressed in different tissues. This question was thus investigated with the focus on expression of MUP genes in the liver and the submaxillary, sublingual, and lachrymal glands.

MATERIALS AND METHODS

Animals. An inbred strain of Swiss white mice (NCS) raised at The Rockefeller University was used in all experiments.

Preparation of poly(A⁺) RNA. Polyadenylated [poly(A⁺)] RNA was extracted from fresh mouse tissues by the technique of Chirgwin et al. (4) as previously described (9).

RNA electrophoresis and blotting. The procedures used were those of D. Goldberg and B. Seed (unpublished) and Thomas (25). Poly(A⁺) RNA was denatured by heating for 5 min at 60°C in an electrophoresis buffer containing 50% formamide. The electrophoresis buffer consisted of 20 mM MOPS (morpholinopropanesulfonic acid; Sigma Chemical Co.), 5 mM NaOAc, 1 mM EDTA, and 1.1 M formaldehyde. Up to 10 µg of poly(A⁺) RNA per lane was electrophoresed through a 1.4% agarose gel for ca. 16 h at 40 V with circulating buffer.

After electrophoresis, the gel was soaked for 60 min in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and blotted to nitrocellulose (Schleicher & Schuell, Inc.) in 10× SSC. After overnight blotting, nitrocellulose was soaked for 15 min in 2× SSC, air dried, and baked in a vacuum oven for 4 h at 80°C.

Filters were prehybridized for 7 h in 10× Denhardt solution (7)–0.2% sodium dodecyl sulfate (SDS)–5× SSC–10 mM EDTA–100 µg of denatured salmon sperm DNA per ml at 65°C. Hybridization was performed for 16 h at 65°C in a buffer of the same composition which included 10% (wt/vol) dextran sulphate and 10⁷ cpm of nick-translated clone plivS-1

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(8, 9) with a specific activity of 1×10^8 to 2×10^8 cpm/ μ g. The nick translation was performed according to the method of Maniatis et al. (18). After hybridization, filters were washed in $2 \times$ SSC–0.2% SDS at 65°C, dried, and exposed to Kodak XAR-5 X-ray film with DuPont Lightning Plus intensifying screens.

RNA "slot" blots. Up to 5 μ g of poly(A⁺) RNA per slot was applied to nitrocellulose filters by using a commercial manifold (Schleicher & Schuell). As suggested by the manufacturer, RNA was heated for 15 min at 65°C in 4 M formaldehyde–7.5 $\times$ SSC and loaded onto the slot blotter. The filters were dried and baked in a vacuum for 4 h at 80°C. The prehybridization and hybridization were done as described above. The washes were at either 65 or 75°C in $2 \times$ SSC–0.2% SDS.

Restriction mapping of cDNA. Double-stranded cDNA was made as follows. The first-strand synthesis was done in a reaction containing 5 to 10 μ g of poly(A⁺) RNA, 50 mM Tris-hydrochloride (pH 8.3), 25 μ g of oligodeoxythymidylate (Collaborative Research) per ml, 35 mM β -mercaptoethanol, 10 mM MgCl₂, 140 mM KCl, 0.5 mM each of the deoxynucleoside triphosphates, 5 μ Ci of tracer [³H]dCTP (Amersham Corp.), and 30 to 50 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.) in a volume of 50 to 100 μ l at 42°C for 90 min. The reaction was stopped by the addition of EDTA to 25 mM. RNA was then digested with pancreatic RNase (10 μ g/ml) with T1 RNase (2 U/ml) for 30 min at 37°C and for 10 min at 65°C. cDNA was phenol-chloroform extracted, ethanol precipitated, and dissolved in water. The second strand was synthesized in a 100- μ l reaction containing 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 6.9), 50 mM KCl, 0.5 mM each of the deoxynucleoside triphosphates, 10 mM MgCl₂, 2.5 mM dithiothreitol, 20 μ Ci of tracer [³H]dCTP (Amersham), and 20 U of the Klenow fragment of *P*oI (Boehringer Mannheim Corp.) at 15°C for 20 h. The reaction was stopped by the addition of EDTA, and the double-stranded DNA was phenol-chloroform extracted and ethanol precipitated.

The DNA was then either digested directly or run through a DEAE-Sephacel column (Pharmacia Fine Chemicals) before restriction. A 100- μ l DEAE-Sephacel column was poured in a Rainin 1.0-ml pipette tip, equilibrated with low-salt buffer (0.2 M NaCl, 20 mM Tris-hydrochloride [pH 7.4], 1 mM EDTA), and loaded with cDNA diluted in low-salt buffer and eluted with high-salt buffer (1.5 M NaCl, 20 mM Tris-hydrochloride [pH 7.4], 1 mM EDTA). An amount of 0.5 to 1.0 μ g of pBU110 (a gift from J. Dubnau) was added to each digest to assess the completeness of each reaction. The restricted DNA was phenol-chloroform extracted, ethanol precipitated, and electrophoresed through 6% acrylamide gels in 89 mM Tris–89 mM boric acid–2.5 mM EDTA. The gels were denatured in 0.2 N NaOH–0.5 M NaCl for 20 min, neutralized in 40 mM Tris (pH 7.4)–20 mM NaOAc–1 mM EDTA (30 min, three changes), and electroblotted onto nylon-mesh Zeta-Probe membranes (Bio-Rad Laboratories) according to the instructions of the supplier. The filters were baked, prehybridized, hybridized to nick-translated clone plivS-1, and washed at 65°C as described above. Some blots were washed (90°C in 10 mM Tris-hydrochloride [pH 7.4], 1 mM EDTA for 5 min) and rehybridized with nick-translated probes derived from specific restriction fragments.

In vitro translation, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis. Poly(A⁺) RNA was translated in a rabbit reticulocyte lysate system (New England Nuclear Corp.) in the presence of [³⁵S]methionine according to the

instructions of the supplier, with 1.0 μ g of poly(A⁺) RNA per 25- μ l assay. Reactions were terminated by addition of 1 volume of cold water and SDS to a final concentration of 2%. The immunoprecipitation was performed essentially as described by Chang et al. (3), with antisera against urinary MUPs which were kindly provided by M. Potter (National Institutes of Health). Immunoprecipitated polypeptides were released from protein A-Sepharose Cl-4B beads (Sigma) by boiling for 5 min in sample buffer (10% SDS, 0.5 M Tris [pH 6.8], 0.5 M dithiothreitol) and electrophoresed through 12.5% SDS-polyacrylamide stacking gels (17).

Isoelectric focusing. Isoelectric focusing was performed according to the method of Singer et al. (22).

Immunoprecipitated samples were resuspended in 10% SDS–0.5 M dithiothreitol–0.5 M Tris (pH 6.8), boiled for 5 min, and run on 12.5% SDS-polyacrylamide gel (17) with dansylated (24) molecular weight (MW) markers (cytochrome *c* and carbonic anhydrase) run in adjacent lanes. Gels were run at 6 mA constant current for ca. 16 h. The next day the markers were visualized under UV light, and a molecular-mass region of 15 to 30 kilodaltons was cut out of the gel.

Isoelectric focusing was performed on slab gels containing 7% acrylamide, 0.186% bisacrylamide (Bethesda Research Laboratories), 2% Nonidet P-40 (vol/vol), 8 M deionized urea, and 2% (wt/vol) Ampholine carrier ampholytes (LKB Instruments, Inc.) with 20% pH 3.5 to 5, 60% pH 4 to 6, and 20% pH 5 to 7. Gels were polymerized by the addition of 2.5 μ l of Temed (Bethesda Research Laboratories) per ml and 0.5 mg of NH₄ persulfate per ml. 1 M H₃PO₄ was used as the anode-electrode solution, and 2% ampholines (pH 7 to 9) was used as the cathode-electrode solution. Electrophoresis was done on a LKB 2117 Multiphor flat-bed gel apparatus attached to circulating polyethylene glycol cooling bath at 5°C. Gels were prerun for 15 min at 10 W of constant power. Gel pieces cut out from SDS-polyacrylamide gel were arranged along the cathode and held in place with glass microscope slides, and the focusing was performed for 8 h at 10 W of constant power. Afterwards, 1-cm slices were removed from one edge of the gel for pH gradient determination, which ranged from pH 3.2 at the anode to 8.2 at the cathode. The isoelectric focusing gel was fixed overnight in two changes of 25% ethanol–8% glacial acetic acid and then prepared for fluorography (2).

RESULTS

Sequence homology of MUP mRNAs. mRNA homologous to MUP cDNA derived from liver RNA can be detected in several mouse tissues in addition to the liver, i.e., in the submaxillary, sublingual, parotid, lachrymal, and mammary glands. The size of predominant MUP RNA species in the liver and the submaxillary, lachrymal, and sublingual glands is ca. 900 nucleotides (Fig. 1, lanes a, b, c, and d, respectively; 21). MUP mRNAs can be detected with probes derived from any segment of plivS-1 (8), a liver MUP cDNA clone. (The restriction map of plivS-1 is shown in Fig. 2.) Hybridization of mRNA from various tissues to a probe derived from the 3' untranslated portion of a liver MUP cDNA clone is shown in Fig. 3. The concentrations of MUP mRNAs in these tissues were determined by comparison to known amounts of liver MUP mRNA (Fig. 3A). (The concentration of MUP mRNA in liver has been quantitatively determined to be about 8% [9].) MUP mRNA is estimated to be 1% in the lachrymal gland, 0.5% in the submaxillary gland of 22-day-old animals, and 0.05% in the parotid, sublingual, and mammary glands (Fig. 3B).

FIG. 1. Northern blots of MUP mRNA. Formaldehyde-denatured poly(A⁺) RNA was electrophoresed through a 1.4% formaldehyde-agarose gel, blotted, and hybridized to nick-translated plivS-1 MUP cDNA clone as described in the text. Lane a, 22-day-old-male submaxillary gland poly(A⁺) RNA (0.5 µg); lane b, male liver poly(A⁺) RNA (1 µg); lane c, male lachrymal gland poly(A⁺) RNA (5 µg); lane d, male sublingual gland poly(A⁺) RNA (5 µg). Different lanes were exposed for different times.

The probe derived from the 3' untranslated end of a liver cDNA clone did not discriminate among MUP mRNAs of different tissues (Fig. 3B). By visual examination and densitometric tracing, resulting hybrids dissociated to the same extent, irrespective of whether the probe was hybridized to liver MUP mRNA or MUP mRNA from other tissues. Similar results were obtained with probes from different coding regions of the plivS-1 clone (data not shown).

Restriction site heterogeneity in MUP cDNAs. Because of the lack of success in distinguishing among different MUP mRNAs by hybridization procedures, the question of differential expression of MUP genes was investigated by searching for tissue-specific restriction polymorphisms in MUP mRNAs. To this end, double-stranded cDNAs from the liver and the submaxillary, sublingual, and lachrymal glands were prepared. The cDNAs were then subjected to digestion with restriction enzymes that have recognition sequences in plivS-1, a cDNA clone derived from liver MUP mRNA (8) (Fig. 2). The restriction fragments generated by digestion of MUP cDNAs were visualized by hybridizing the electrophoretically separated restriction digest to labeled plivS-1 probe. The restriction patterns that were obtained by digestion of MUP cDNAs with *Hae*III and *Mbo*II indicated the existence of distinct MUP mRNA species in the submaxillary gland, the lachrymal gland, and the liver.

The *Hae*III restriction patterns of lachrymal gland and liver MUP cDNAs are indistinguishable (data not shown).

FIG. 2. Restriction map of plivS-1. Construction of plivS-1 was described previously (9). 1a, 1b, 1c, *Hae*III sites; 2a, 2b, 2c, 2d, *Mbo*II sites; 3, *Eco*RI site; 4, *Pvu*II site; 5, *Ava*I site. The position of the termination codon coincides with *Mbo*II restriction site 2c (K. Krauter, personal communication). plivS-1 is lacking ~50 nucleotides from the 5' end of liver MUP mRNA. The initiation codon is absent from the plivS-1 clone and is presumed to lie within the missing 5' end.

FIG. 3. RNA slot blots. RNA samples were applied in duplicate to a nitrocellulose membrane and hybridized to a DNA fragment corresponding to the 3' untranslated region of liver MUP mRNA. This fragment is bounded by the *Ava*I site at the 5' end and at the 3' end by the 3' end of the plivS-1 insert (Fig. 2; this paper). (A) To quantitate the amount of MUP mRNA, the intensity of the autoradiographic signals was compared with intensities of signals obtained when the same probe was hybridized to known amounts of liver mRNA. (B) After hybridization the duplicate filters were washed either at 65 or 75°C in 2× SSC–0.2% SDS. Sample a, Male liver poly(A⁺) RNA (0.2 µg); sample b, male lachrymal gland poly(A⁺) RNA (2 µg); sample c, 22-day-old-male sublingual gland poly(A⁺) RNA (2 µg); sample d, 22-day-old-male submaxillary gland poly(A⁺) RNA (2 µg); sample e, 22-day-old-male parotid gland poly(A⁺) RNA (2 µg); sample f, 2-weeks-pregnant-female mammary gland poly(A⁺) RNA (2 µg).

However, the *Hae*III restriction pattern of MUP cDNA derived from the submaxillary gland mRNA differs from the *Hae*III pattern of liver MUP cDNA (Fig. 4). The liver MUP cDNA contains three *Hae*III sites, designated 1a, 1b, and 1c (Fig. 2). In the submaxillary gland cDNA (Fig. 4, lane b) a restriction fragment of 430 nucleotides was generated which replaced a 290-base-pair (bp) fragment that was present in the digests of all other MUP cDNAs. This difference is due to the absence of *Hae*III restriction site 1b (Fig. 2) in the submaxillary gland cDNA.

The *Hae*III restriction pattern of the sublingual gland cDNA seems to be composed of two different patterns. One was generated by digestion of cDNA species that contain *Hae*III site 1b, and a second one was generated by digestion of cDNAs that lack this site. As a consequence, both restriction fragments, 430 and 290 bp, were present in the *Hae*III digest of sublingual gland MUP cDNA (Fig. 4, lane c).

The lachrymal gland MUP cDNA can be differentiated from the liver MUP cDNA by *Mbo*II digestion (Fig. 5). The liver cDNA clone contains four *Mbo*II sites, designated 2a, 2b, 2c, and 2d (Fig. 2). The *Mbo*II restriction pattern of the lachrymal gland cDNA is characterized by the absence of *Mbo*II site 2b (Fig. 2). This is indicated by the appearance of

FIG. 4. Restriction of MUP cDNAs with *Hae*III. Double-stranded cDNA was digested with *Hae*III, electrophoresed through a 6% acrylamide gel, electroblotted to a nylon-mesh membrane, and hybridized to nick-translated plivS-1 as described in the text. Lanes a and a', Liver cDNA (6 ng); lane b, submaxillary gland cDNA (200 ng); lane c, sublingual gland cDNA (300 ng). Samples a and b were run on one gel, and samples a' and c were run on another gel, both with *Hae*III fragments of ϕ X174 DNA as markers.

a 400-bp fragment in the lachrymal gland cDNA (Fig. 5, lane b) which replaced a 310-bp fragment characteristic of liver cDNA (Fig. 5, lanes a and a'). In addition, another *Mbo*II site (not mapped) was present in a subpopulation of lachrymal gland cDNA, generating a 175-bp DNA fragment (Fig. 5, lane b). The *Mbo*II restriction pattern of the sublingual gland cDNA also revealed two populations of MUP mRNAs in this tissue, one lacking *Mbo*II site 2b and one containing this site (Fig. 5, lane c). The *Mbo*II restriction patterns of the submaxillary gland and of the liver MUP cDNAs are similar (data not shown).

The different MUP cDNAs were also digested with a combination of *Ava*I-*Eco*RI and *Pvu*II-*Eco*RI restriction enzymes. Simultaneous digestion of all four MUP cDNAs with *Ava*I and *Eco*RI generated DNA fragments of ca. 310 and 290 bp, as expected (Fig. 6; see the restriction map of plivS-1, Fig. 2). Similarly, simultaneous digestion with *Eco*RI and *Pvu*II yielded the same restriction fragments in all four MUP cDNAs (data not shown).

In summary, submaxillary gland MUP mRNAs and lachrymal gland MUP mRNAs can be distinguished from each other and from the liver MUP mRNA by digestion of their respective cDNAs with *Hae*III and *Mbo*II restriction enzymes. By this analysis it also appears that at least a portion of MUP mRNAs in the sublingual gland is distinct from liver MUP mRNAs.

In addition, it appears that the variations in MUP mRNA sequences in different tissues that are detected by *Hae*III and *Mbo*II restriction enzymes are not generated by differential splicing of the same MUP mRNA precursors. If this were the case, tissue-specific restriction patterns would have been generated by *Ava*I-*Eco*RI and *Pvu*II-*Eco*RI digestion as well. These results suggest that MUP mRNAs of different tissues are the transcripts of different MUP genes.

Heterogeneity of translational products of MUP mRNAs. Because of the findings on the heterogeneity of MUP mRNAs, the nature of polypeptides coded by MUP mRNAs of different tissues was examined.

MUPs, being secretory proteins, are synthesized *in vitro* as larger-MW precursors which, in the presence of microsomal membranes, are processed into proteins of lower MW (23). Instead of studying the mature MUPs, we chose to analyze the primary translational products, the unprocessed precursors to MUPs. mRNA from the liver and the submaxillary, sublingual, and lachrymal glands was therefore translated *in vitro* in reticulocyte lysate in the absence of microsomal membrane. The translated products were immunoprecipitated with antibodies directed against urinary MUPs, and the immunoprecipitates were then electrophoresed through SDS-polyacrylamide gels.

The precursors to MUPs synthesized in the liver migrated as polypeptides with an apparent MW of 20,000 to 23,000 (Fig. 7, lane b). The translational products of mRNA extracted from unseparated submaxillary and sublingual glands are shown in lane c. The submaxillary gland mRNA codes for MUPs with an apparent MW of 22,000 (lane d). The sublingual gland mRNA codes for MUPs with an MW of 23,500 (lane e). The translational products of lachrymal gland mRNAs are MUPs with an MW of 20,000 (lane a), which are indistinguishable from the 20,000-MW precursors to MUPs in the liver (lane b) on this gel system. The observed differences in mobility of the different precursors are reproducible and are not eliminated by the addition of protease inhibitors to the reticulocyte lysate (data not shown).

The primary translational products of the liver, the submaxillary gland, and the sublingual gland MUP mRNAs can thus be distinguished from each other when they are ana-

FIG. 5. Restriction of MUP cDNAs with *Mbo*II. Double-stranded cDNA was digested with *Mbo*II, electrophoresed through a 6% acrylamide gel, electroblotted to a nylon-mesh membrane, and hybridized to nick-translated plivS-1 as described in the text. Lanes a and a', Liver cDNA (6 ng); lane b, lachrymal gland cDNA (120 ng); lane c, sublingual gland cDNA (300 ng). Samples a and b were run on one gel, and a' and c were run on another gel, both with *Hae*III fragments of ϕ X174 DNA as markers.

FIG. 6. Restriction of MUP cDNAs with *Ava*I and *Eco*RI. Double-stranded cDNA was digested with *Ava*I and *Eco*RI, electrophoresed through a 6% acrylamide gel, electroblotted to a nylon-mesh membrane, and hybridized to nick-translated plivS-1 as described in the text. Lane a, Liver cDNA (6 ng); lane b, submaxillary gland cDNA (100 ng); lane c, lachrymal gland cDNA (120 ng); lane d, sublingual gland cDNA (120 ng).

lyzed on one-dimensional SDS-polyacrylamide gels. It is shown in the next section that the lachrymal gland pre-MUPs can be distinguished from all the others by isoelectric focusing.

Isoelectric focusing of precursors to MUPs. To estimate the number of different precursors to MUPs that are synthesized in the liver and the submaxillary, sublingual, and lachrymal glands, immunoprecipitated translational products were analyzed by isoelectric focusing. On isoelectric focusing gels the liver pre-MUPs resolved to 6 to 12 components, the more basic of them being substantially reduced in the female liver (Fig. 8, lanes a and b). The submaxillary gland (lane c) and the sublingual gland (lane d) pre-MUPs both resolved to about four to six different components. All the liver, the submaxillary gland, and the sublingual gland pre-MUPs exhibited approximately the same pIs, ranging between 5.0 and 5.75. Although this gel system does not resolve the MUPs precursors of the liver, the submaxillary gland, and the sublingual gland from each other, based on the mobility differences seen on the SDS-polyacrylamide gels one can conclude that the further resolved precursors are distinct from each other. The lachrymal gland pre-MUPs (lane e) resolve into about three components that are considerably more basic, with a pI of ~6.5.

DISCUSSION

One of the major aims of our studies is to relate structural differences in MUP genes to differences in their regulation. However to do so, it is necessary to be able to differentiate among different MUP genes.

The MUP genes (5), as well as MUP mRNAs (Fig. 3; this paper), are related to an extent that has precluded the use of gene-specific probes in attempts to differentiate among mRNAs of different tissues. Such probes cannot be prepared even from the 3' untranslated region of MUP mRNAs. Moreover, it appears that this region, in contrast to what is

FIG. 7. SDS-polyacrylamide gel electrophoresis of pre-MUPs. Poly(A⁺) RNA (2 µg) was translated in vitro in 50 µl of reticulocyte lysate in the presence of [³⁵S]methionine. The translational products were immunoprecipitated as described in the text and electrophoresed through 15% (lanes a, b, and c) and 12.5% (lanes d and e) SDS-polyacrylamide gels alongside MW markers. Shown are pre-MUPs translated from lachrymal gland mRNA (lane a), male liver mRNA (lane b), mixture of 22-day-old-male submaxillary and sublingual gland mRNA (lane c), 22-day-old-male submaxillary gland mRNA (lane d), and adult male sublingual gland mRNA (lane e). kd, Kilodaltons.

the case in most other gene families (6, 14, 19), seems to be the most conserved part of all MUP genes (unpublished data).

An alternative approach by which differences in the sequence of MUP mRNAs could be recognized was thus sought. We expected that a large number of different MUP mRNAs might be synthesized in each tissue. In the liver, it was known that this number is at least 6 and perhaps as much as 20 (5, 21). A large number of MUP cDNA clones would have to be sequenced from each tissue before one could correlate any observed sequence differences with the expression of MUP genes. Therefore, instead of sequencing large number of cDNA clones, we decided to search for restriction polymorphisms that correlate with expression of MUP genes.

FIG. 8. Isoelectric focusing of pre-MUPs. Poly(A⁺) RNA was translated in vitro in the presence of [³⁵S]methionine in reticulocyte lysate and immunoprecipitated with antibodies raised against urinary MUPs. The pre-MUPs were first resolved on 12.5% SDS-polyacrylamide gels. Strips of this gel containing pre-MUPs were overlaid on a horizontal (pH 3.5 to 7) urea-Nonidet P-40-isoelectric focusing gel as described in the text. Samples were focused for 8 h at 10 W at 5°C. Shown are immunoprecipitated translational products of male liver poly(A⁺) RNA (1 µg) (lane a), female liver poly(A⁺) RNA (1 µg) (lane b), 22-day-old-male submaxillary gland poly(A⁺) RNA (2.5 µg) (lane c), adult male sublingual gland poly(A⁺) RNA (5 µg) (lane d), and male lachrymal gland poly(A⁺) RNA (5 µg) (lane e).

It is known that in large gene families distinct subfamilies can be identified by restriction sites that are not present in the rest of the family (see reference 11 for review). We argued the possibility that such sites might exist in MUP mRNAs that are expressed in the same tissue and therefore subjected double-stranded cDNAs to restriction analysis. By restriction mapping of MUP cDNAs, we were able to demonstrate that distinct sets of MUP mRNAs are synthesized in the liver, the submaxillary gland, and the lachrymal gland and that most likely these are transcribed from different genes.

We showed that differences in the sequence composition of MUP mRNAs of different tissues led to changes in the amino acid composition of proteins that these mRNAs code for. The reason this was not observed before, except in the case of the lachrymal MUPs, could be attributed to a number of differences between our studies and those of Shaw et al. (21), including differences in the strains of mice. However, the major differences are likely to be due to two factors; first, we studied precursors to MUPs rather than processed proteins, and second, the *in vitro*-synthesized polypeptides were analyzed by one-dimensional rather than two-dimensional gel electrophoresis. It has been noted before that related proteins of the same net charge can sometimes exhibit different mobilities in one-dimensional SDS-polyacrylamide gels which are not apparent when the same proteins are electrophoresed through two-dimensional gels (20).

In agreement with previous reports (5, 21), we detected a large number (6 to 12) of different MUPs coded for by liver mRNA and about 3 different MUPs coded for by lachrymal gland mRNA. However, we detected four to six different precursors to MUPs both in the submaxillary and the sublingual gland, in contrast to Shaw et al. (21), who report only one MUP in these tissues. Our results thus indicate that the number of different polypeptides coded for by MUP genes is at least 17.

In summary, the results presented here indicate that MUP genes expressed in different tissues code for different polypeptides and that different MUP genes are expressed in different tissues. The significance of these results is twofold. First, these findings suggest that it is feasible to differentiate among different MUP genes through the identification of the products of these genes: the mRNAs and the polypeptides these mRNAs code for. Second, these findings raise the possibility that the diversity in polypeptides that MUP genes code for is directly related to the expression of these genes. This suggestion is based on the observation that in multigene families base changes which lead to new protein isophorms are usually clustered in a hypervariable region of a gene, and that not all of these base substitutions lead to amino acid replacements (6, 10, 15).

MUP genes presumably arose by duplication of an ancestral gene. After gene duplication, different MUP genes acquired the potential to code for novel forms of proteins and became subject to different modes of regulation. Thus, in the MUP gene family, as seems to be the case in most other gene families, the appearance of tissue-specific regulation is paralleled by the appearance of new protein isophorms. It is therefore tempting to speculate that these two evolutionary events are interrelated and that mutations in the same, hypervariable region of a gene which led to the emergence of new protein species also led to novel modes of regulation. Experiments are in progress to define such a hypervariable region in MUP genes.

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