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Identification and Characterization of Functional Genes Encoding the Mouse Major Urinary Proteins

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Mouse Ltk$^-$ cells were stably transfected with cloned genes encoding the mouse major urinary proteins (MUPs). C57BL/6J MUP genomic clones encoding MUP 2 (BL6-25 and BL6-51), MUP 3 (BL6-11 and BL6-3), and MUP 4 (BL6-42) have been identified. In C57BL/6J mice, MUP 2 and MUP 4 are known to be synthesized in male, but not female, liver, and MUP 3 is known to be synthesized in both male and female liver and mammary gland. A BALB/c genomic clone (BJ-31) was shown to encode a MUP that is slightly more basic than MUP 2 and was previously shown to be synthesized in both male and female liver of BALB/c but not C57BL/6 mice. Comigration on two-dimensional polyacrylamide gels of the MUPs encoded by the transfecting gene provides a basis for tentative identification of the tissue specificity and mode of regulation of each gene. DNA sequence analysis of the 5′ flanking region indicates that the different MUP genes are highly homologous (0.20 to 2.40% divergence) within the 879 base pairs analyzed. The most prominent differences in sequence occur within an A-rich region just 5′ of the TATA box. This region (from −47 to −93) contains primarily A or $C(A)_N$ nucleotides and varies from 15 to 46 nucleotides in length in the different clones.

The mouse major urinary proteins (MUPs) are encoded by a multigene family of about 35 genes per haploid genome which are clustered on chromosome 4 (2, 4, 13, 16). MUP genes are expressed in several tissues (liver and lacrimal, mammary, and submaxillary glands) where they are subject to different developmental and hormonal controls (15, 17, 23, 24). In each of the tissues, MUPs are synthesized as precursors and are ultimately secreted into external body fluids. For example, MUPs synthesized in liver are secreted into the blood and subsequently excreted into the urine (10, 15, 27). MUPs have some amino acid homology to retinol-binding protein, beta-lactoglobulin, and a human urinary protein (HC protein), all of which are known or thought to bind retinoic acid or retinoic acid derivatives (28; B. Sampsell, T. Johnson, K. Manly, and W. Held, submitted). The tissue distribution, complex regulatory controls, and amino acid homology mentioned above suggest that MUPs may be involved in binding pheromones or other small molecules, thereby facilitating secretion and excretion of these molecules. Although a definitive function for MUPs has not been established, this multigene family provides an interesting model of developmental and tissue-specific multihormonal control of gene expression.

Previous studies have shown that MUP mRNA constitutes approximately 5% of total liver mRNA in male mice of most inbred strains (12, 13) and that several different MUP genes that are expressed in male liver are differentially regulated by testosterone, growth hormone, and thyroid hormone (15, 17). Two-dimensional polyacrylamide gel electrophoresis of MUP liver mRNA in vitro translation products or urinary protein from C57BL/6J mice indicates that several MUP genes expressed in male liver are not expressed in female liver (15, 17).

Based on sequence homology, MUP genes can be grouped into two major subfamilies, group 1 and group 2, each consisting of approximately 15 genes (4, 5, 9). A third group of MUP genes has diverged considerably from both group 1 and group 2, and each member of this third group has diverged from one another. One of these diverged MUP genes has been shown to be expressed in the liver (6, 17) and another in the lacrimal gland (W. A. Held, unpublished results). Sequence analysis indicates that several group 2 genes contain one or more translational termination codons within the proximal 5′ protein-coding region, suggesting that most, or all, of the group 2 genes are intervening sequence (IVS)-containing pseudogenes (11). Approximately 90% of the MUP mRNA in male liver corresponds to group 1 gene transcripts (7, 17). At least one group 1 gene(s) is also expressed in the mammary gland (W. A. Held, unpublished experiments). Thus, the majority of functional MUP genes appear to be group 1 genes.

DNA sequence analysis of group 1 genomic and cDNA clones indicates that group 1 genes are highly homologous to one another. The average divergence within exons from the group 1 consensus sequence is only 0.35% (6, 17). A high degree of homology between group 1 genes within introns and 3 to 4 kilobases (kb) of 5′ and 3′ flanking sequences has also been inferred by cross hybridization and restriction enzyme site mapping (4, 9).

The large number and high degree of homology among group 1 genes create a serious difficulty in identifying cloned MUP genes with respect to the tissue in which they are expressed and their mode of regulation. However, charge differences between MUPs indicate that five distinct group 1 C57BL/6 male liver gene products (MUPs 1 to 5) can be resolved by two-dimensional polyacrylamide gel electrophoresis (15, 17). MUPs 1, 2, and 4 are synthesized only in male liver, whereas MUP 3 is synthesized in both male and female liver and mammary gland, and MUP 5 is synthesized in both male liver and submaxillary gland (15, 24). Also, sequence differences in the protein-coding regions of seven group 1 genes predict that these seven proteins would resolve into five charge classes (6). Thus, it is possible to identify cloned

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MUP genes to some degree by analysis of the protein product.

As presented here, we have transfected a number of cloned MUP genes into mouse L cells. C57BL/6J MUP genomic clones encoding MUPs 2, 3, and 4 have been identified. In addition, a BALB/c genomic clone was shown to encode a MUP that is synthesized in BALB/c but not C57BL/6J liver.

MATERIALS AND METHODS

Isolation of MUP genomic clones. A lambda Charon 4A C57BL/6J mouse genomic library, obtained from John Seidman, was screened for MUP genomic sequences by using MUP cDNA clones (17) and the plaque hybridization procedure outline of Benton and Davis (3). A BALB/c MUP genomic clone (BJ-31) was obtained from Nicholas Hastie. Large-scale preparations of plasmid DNA and lambda bacteriophage were purified by CsCl density gradient centrifugation (19).

Growth and transfection of Ltk$^-$ cells. Ltk$^-$ cells were grown in a modified Eagle medium containing 5% calf serum as previously described (14), except the medium contained 1 μM dexamethasone. Cotransfection of MUP genomic DNA and the herpes simplex virus type 1 *tk* gene (pHSV106, provided by Steve McKnight) was essentially as described previously (30). Approximately 10^5 cells were treated with 1 ml of a calcium phosphate DNA precipitate (containing 2 μg of lambda MUP genomic DNA or 5 μg of MUP genomic plasmid DNA, 0.2 μg pHSV106, and Ltk$^-$ carrier DNA to a total of 20 μg). One to nine *tk*$^+$ colonies from each transfection were picked, purified, and analyzed for MUP gene expression. Of 24 MUP genomic clones, 6 were shown to synthesize and secrete MUPs when cotransfected into Ltk$^-$ cells.

Preparation of DNA from L-cell transformants. DNA was prepared as suggested by A. Kinniburgh (21). Briefly, cells were lysed in 2% sodium dodecyl sulfate–7.5 M urea–0.7 M NaCl–10 mM Tris (pH 8.0)–1 mM EDTA (pH 8.0) and extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and twice with chloroform-isoamyl alcohol (24:1). CsCl was added (1.1 g/2.5 ml), and the sample was layered over 3 to 4 ml of 5.8 M CsCl and centrifuged for 16 to 18 h (25°C) at 30,000 rpm (Beckman SW41). RNA pelleted to the bottom of the tube, and DNA appeared as a thin viscous band near the interface with 5.8 M CsCl. The DNA was dialyzed against 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and ethanol precipitated.

[^{35}S]methionine labeling of L-cell transformants. Initial screening of transformants for MUP gene expression was performed with 24-well plates. Approximately 4×10^5 cells were grown in HAT (hypoxanthine-aminopterin-thymidine) medium containing 1 μM dexamethasone for 24 h. After the cells were washed twice with Pucks G saline, 200 μl of modified Eagle medium minus methionine (no serum) containing 1 μM dexamethasone and 20 μCi of [^{35}S]methionine (New England Nuclear Corp.) were added, and the cells were incubated for 2 h at 37°C. One-tenth volume of complete medium (without serum) was then added, and the cells were incubated for an additional 2 or 20 h. The medium was collected and centrifuged to remove cells, and a sample was applied to a two-dimensional polyacrylamide gel. Although this was a somewhat tedious screening procedure, it was an extremely sensitive assay of exogenous MUP gene expression since untransfected Ltk$^-$ cells do not synthesize and secrete any proteins with mobilities similar to MUPs. For larger-scale preparations, approximately 10^7 cells grown in T-75 flasks were labeled with 500 μCi of [^{35}S]methionine essentially as outlined above.

Crossed immunoelectrophoresis and two-dimensional polyacrylamide gel electrophoresis. Crossed immunoelectrophoresis was performed as described previously (29). The second dimension contained 12 μl of a goat anti-MUP immunoglobulin G fraction (27) per 10 ml of gel solution. The MUP antibody was kindly provided by Paula Szoka. Approximately 5×10^6 cpm of [^{35}S]methionine-labeled cell medium was dialyzed for 24 h against 50 mM NH_4HCO_3, lyophilized, dissolved in electrophoresis buffer, and applied to the gel along with 0.16 μg of C57BL/6J MUP as a carrier. The gels were stained with 0.25% Coomassie brilliant blue, dried, and autoradiographed (18-h exposure). Only the autoradiographs are shown.

Two-dimensional polyacrylamide gel electrophoresis using a narrow-range (pH 4 to 6) focusing dimension was performed as previously described (15). Approximately 300,000 to 500,000 cpm of cell medium was applied to each gel. The mobility of the MUP that was synthesized in the transfectant relative to C57BL/6J male liver MUP mRNA in vitro translation products was determined by mixing approximately 25,000 to 50,000 cpm of male liver mRNA in vitro translation products with the cell medium. In vitro translation in the presence of dog pancreas membranes to effect removal of the MUP signal sequence was as previously described (27). The fluorographed gels were exposed to film for 2 days.

Southern blotting and restriction enzyme mapping. Restriction enzymes were purchased from New England BioLabs, Inc., or Bethesda Research Laboratories, Inc., and digestions of cloned DNA or L-cell DNA were performed as recommended by the supplier. Restriction enzyme fragments were electrophoresed in 0.8% agarose gels, transferred to nitrocellulose (26), and hybridized to nick-translated 5′ or 3′ MUP cDNA subclones (17). Restriction enzyme maps were deduced by a series of single and double restriction enzyme digests.

DNA sequence analysis. The DNA sequence of the 5′ flanking regions of the MUP genomic clones was determined by the dideoxynucleotide chain termination method (22). Double-stranded DNA from lambda MUP genomic clones (BL6-3) or plasmid MUP genomic subclones (BJ-31, BL6-42, BL6-11, and BL6-51) was used as templates for reverse transcriptase. A series of synthetic oligonucleotide primers was prepared and 5′ end labeled with T4 polynucleotide kinase. Sequencing reactions were carried out essentially as described previously (32), with 2 μg of lambda or 0.5 μg of plasmid DNA as the template and approximately 3×10^6 cpm of end-labeled primer (specific activity, 3.5×10^6 cpm/pmol). The final nucleotide concentration was 2 mM with a deoxynucleotide-to-dideoxynucleotide ratio of A = 6, C = 4, T = 12, G = 8. Initially, the sequence data of Clark et al. (8) were used to prepare synthetic oligonucleotide primers. Subsequently, primers were synthesized on the basis of our sequence data. Both strands were sequenced.

RESULTS

Isolation of MUP genomic clones and transfection into Ltk$^-$ cells. A Charon 4A C57BL/6J mouse genomic library was screened for MUP genomic clones by the plaque hybridization procedures of Benton and Davis (3). Preliminary restriction enzyme digests (*Eco*RI, *Pvu*II, and *Pst*I) of 45 purified isolates with MUP sequences indicated that there were at

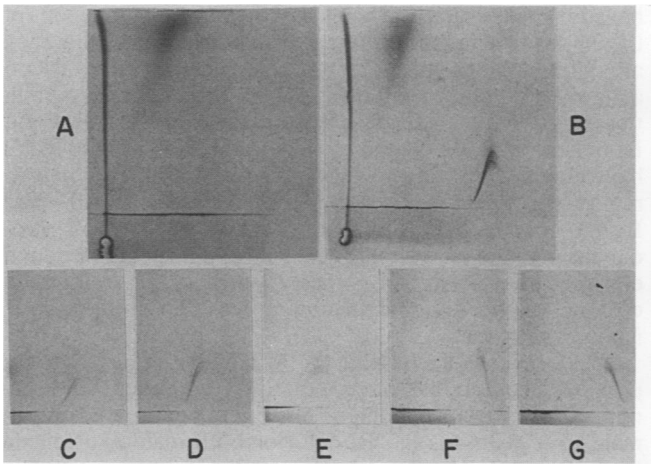

FIG. 1. Crossed immunoelectrophoresis of [^{35}S]methionine-labeled cell medium from L cells transfected with DNA from genomic MUP clones. Ltk$^-$ cells were cotransfected with HSV *tk* and genomic MUP DNAs. The transfected cell lines were labeled with [^{35}S]methionine, and the cell medium was analyzed by crossed immunoelectrophoresis as described in Materials and Methods. The control (A) was Ltk$^-$ cells transfected with HSV *tk* plasmid only. The genomic MUP clones used were BJ-31 (B), BL6-25 (C), BL6-51 (D), BL6-11 (E), BL6-3 (F), and BL6-42 (G).

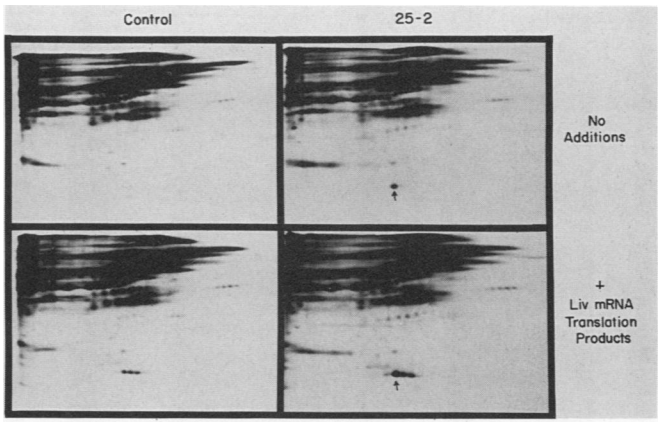

FIG. 2. Two-dimensional polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled cell medium from control and MUP BL6-25–transfected cell lines. Two-dimensional polyacrylamide gel electrophoresis was performed as described in Materials and Methods. For the isoelectrofocusing dimension, the cathode is to the left, the anode to the right. The three prominent male liver MUP mRNA in vitro translation products correspond to MUPs 2, 3, and 4, in increasing order of acidity (15, 17). The vertical arrow indicates the MUP (MUP 2) synthesized in cells transfected with BL6-25 (25-2).

least 18 different MUP genes containing the entire transcribed region plus variable amounts of 5′ and 3′ flanking regions. Twenty-four of these recombinant DNAs were transfected (with HSV *tk* DNA) into mouse Ltk$^-$ cells. The resulting *tk*$^+$ transfectants were screened for MUP gene expression by labeling the transfected cells with [^{35}S]methionine and analysis of the culture supernatant for secreted MUPs by two-dimensional polyacrylamide gel electrophoresis. Cells transfected with DNA from five C57BL/6J and one BALB/c (from Nicholas Hastie) genomic clones were found to synthesize and secrete MUP polypeptides. Transfectants of these six cloned MUP genes were analyzed in more detail.

Synthesis and secretion of MUPs by transfected Ltk$^-$ cells. [^{35}S]methionine-labeled protein secreted from transfected cell lines was analyzed for MUPs by crossed immunoelectrophoresis (Fig. 1). The Coomassie blue-stained gel (results not shown) indicated that the MUP precipitin lines formed as a broad doublet. The radioactivity tended to be localized on the basic (panels B, C, and D), middle (panel E), or acidic (panels F and G) portion of the precipitin bands, indicating that some charge separation of MUPs was occurring during the first dimension. The doublet nature of the precipitin bands (panel B) is not known. No radioactive MUP was observed in medium from Ltk$^-$ cells transfected with the HSV *tk* gene only (panel A). Thus, the MUP gene transfectants synthesized and secreted protein immunologically related to MUP (panels B to G). The precipitin bands were cut out and counted to estimate the amount of MUP synthesized by each of the transfectants. The results indicate that MUP constituted about 0.23% of the secreted protein for the BJ-31 (BALB/c) transfectant (panel B) and from 0.001 to 0.02% for the others (panels C through G).

The [^{35}S]methionine-labeled protein secreted from the L-cell transfectants was also analyzed by two-dimensional polyacrylamide gel electrophoresis to determine whether the mobility of the MUP could be correlated with MUPs previously shown to be synthesized in liver or other MUP-expressing tissues (15, 17, 24). A small amount of C57BL/6J male liver in vitro translation products was added to the cell culture medium to determine the relative mobility of the MUP synthesized by each of the L-cell transfectants (Fig. 2). The three major MUP in vitro translation products observed correspond to MUPs 2, 3, and 4 as previously described (15). The results (Fig. 2 and 3) indicate that Ltk$^-$ cells transfected with BL6-25 and BL6-51 synthesized MUP 2, BL6-11 and BL6-3 synthesized MUP 3, and BL6-42 synthesized MUP 4. Cells transfected with the BALB-c genomic clone (BJ-31) synthesized a MUP that is slightly more basic than MUP 2 from C57BL/6J. This MUP represents a strain-specific genetic variant which is synthesized in male and female BALB/c liver but not in C57BL/6J liver (B. M. Sampsell and W. A. Held, unpublished data). Analysis of several independent transfectants of each of the clones indicated that the type of MUP synthesized was a property of the cloned gene. For example, all five independent transfectants of BL6-25 synthesized MUP 2.

These results have allowed tentative identification of each transfected MUP gene with respect to the tissue in which it is expressed and its mode of regulation. MUP 2 (BL6-25 and BL6-51) and MUP 4 (BL6-42) are expressed only in male

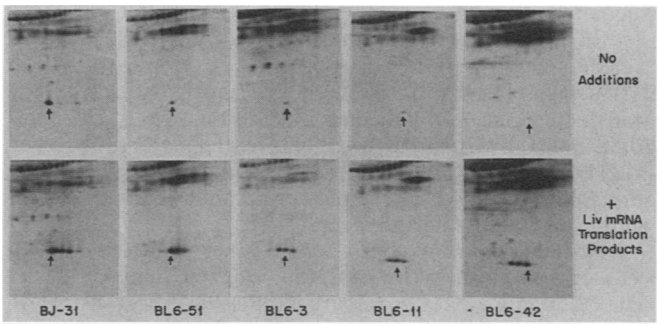

FIG. 3. Two-dimensional polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled cell medium from Ltk$^-$ cell lines transfected with DNA from MUP genomic clones. The vertical arrow indicates the position of the MUP synthesized in the transfected cell line. Only a portion of the autoradiograph is shown.

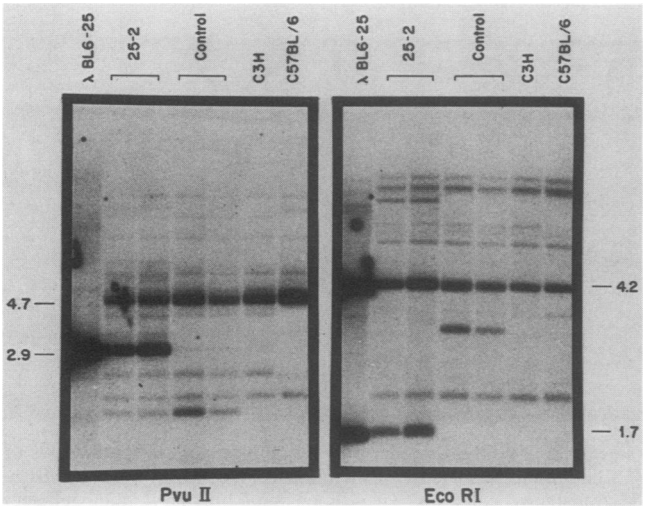

FIG. 4. Southern blot analysis of DNA from Ltk$^-$ cells transfected with BL6-25. Two different DNA preparations from cells transfected with BL6-25 (25-2) or cells transfected with HSV *tk* only (control) were digested with *Pvu*II or *Eco*RI. Digestions of genomic mouse liver DNA from C3H and C57BL/6 inbred strains and 10 ng of DNA from the MUP genomic clone BL6-25 (corresponding to approximately 30 copies of the MUP gene) is also shown. The Southern blots were hybridized to ^{32}P-labeled (nick-translated) DNA from p499-5′, a subclone of a C57BL/6 liver cDNA clone containing about 400 bp of the 5′ coding region (17). The sizes (in kilobases) of the major MUP genomic fragments are indicated.

liver (15). MUP 3 (BL6-3 and BL6-11) is expressed in male and female liver and mammary gland (15, 24). As indicated above, the MUP expressed by BJ-31 transformants is synthesized in both male and female BALB/c liver.

Southern blot analysis of MUP L-cell transfectants. DNA from L cells transfected with BL6-25 was digested with *Pvu*II or *Eco*RI, and the Southern blot was probed with a 5′ subclone (p499-5′) of a group 1 liver cDNA (17). BL6-25 contains about 500 base pairs (bp) of 5′ flanking sequence (see Fig. 6). Thus, digestion with *Pvu*II or *Eco*RI produced a unique 5′ restriction fragment which distinguishes the transfected MUP gene from the endogenous L-cell MUP genes (Fig. 4). Comparison of the signal intensity of the clone BL6-25 (10 ng, approximately 30 copies of the MUP gene) indicates that the transformant contained 10 to 20 copies of the transfected gene. Some additional fragments observed in BL6-25–transfected cells, or cells transfected with HSV *tk* only (control) are probably due to pBR322 sequences that have integrated along with the *tk* gene (see *Eco*RI digest, Fig. 4). Southern blots of DNA from L cells transfected with BL6-3, BL6-51, and BJ-31 also produced DNA fragments that were identifiable as originating from the transfected gene and indicated integration of 10 to 30 copies (data not shown). Such fragments could not be observed with DNA from BL6-11 or BL6-42 transfectants, suggesting integration of only a few copies or comigration with endogenous MUP genomic fragments.

Restriction enzyme maps of MUP genes transfected into L cells. Figure 5 shows the restriction enzyme maps of the six MUP genomic clones which are expressed after transfection into mouse L cells. A restriction map showing exon boundaries for BS-6 was taken from the published work of Clark et al. (7) and is shown for comparison. All of the genes show a high degree of restriction enzyme site homology, and all correspond to group 1 genes. BL6-25 and BL6-51 appear to have identical restriction maps within the common regions of the inserts. Preliminary sequence analysis suggests that they are different clones of the same MUP gene. L-cell transfectants of both clones synthesize MUPs with identical mobilities on two-dimensional polyacrylamide gels (MUP 2). BJ-31, the BALB/c genomic clone, and BL6-11 (C57BL/6) have almost identical restriction maps within their common region yet encode different MUPs, whereas BL6-11 and BL6-3 encode MUPs with the same mobility on two-dimensional polyacrylamide gels but have distinct restriction enzyme maps. All clones show considerable restriction enzyme site homology extending at least 2.5 kb into the 5′ flanking region.

DNA sequence analysis of the 5′ flanking regions. In the previous sections, we have tentatively identified MUP genomic clones corresponding to MUP genes expressed only in male liver (BL6-51 and BL6-42) or both male and female liver (BL6-11, BL6-3, and BJ-31). We were interested in determining whether the sex-specific differences in expression could be related to sequence differences within the 5′ flanking region of these genes, a putative regulatory region. We were also interested in the degree of sequence divergence within this region, since the coding regions are highly homologous. The DNA sequence of 879 bp of 5′ flanking region from each of the clones is shown in Fig. 6. The 5′ flanking region of BS-6 as determined by Clark et al. (8) is shown for comparison. As expected from restriction maps, all clones show a very high degree of DNA sequence homology in this region. All clones have a good consensus sequence for a TATA box clustered at about position −30 and a possible CAAT sequence at about position −110. Except for the region between the TATA box and possible CAAT sequence, differences are restricted to occasional base substitutions or additions or deletions of single bases. Sequence differences between clones tend to be limited. For example, at position −239, three of the sequences have a T and three have an A. This may reflect sequence diversion followed by duplication or gene conversion events.

The region 5′ of the TATA box shows the most striking differences in DNA sequence between the clones. The A-rich region from −47 to −93 is variable in length from 46 (BS-6) to 15 nucleotides (BL6-51).

DISCUSSION

The large number and high degree of homology of MUP genes make it exceedingly difficult to correlate a cloned MUP gene with respect to function, tissue specificity, and mode of regulation. We report here the identification of MUP genomic clones that encode MUP 2 (BL6-25 and BL6-51), MUP 3 (BL6-11 and BL6-3), MUP 4 (BL6-42), and a strain-specific variant (BJ-31). In C57BL/6J mice, MUP 2 and MUP 4 are synthesized only in male liver. Thus, we have tentatively identified MUP genes which are regulated by male-specific hormonal controls. The MUP encoded by BJ-31, which is slightly more basic than MUP 2, is synthesized in both male and female BALB/c liver and represents a protein genetic variation between BALB/c and C57BL/6J strains. MUP 3 is synthesized in both male and female liver, as well as the mammary gland in C57BL/6J mice (24). Thus, either BL6-3 or BL6-11 or both may be expressed in liver or mammary gland. It should be noted that one cannot equate a single MUP with a single MUP gene. BL6-11 and BL6-3 are clearly different genes since they have different restriction enzyme sites and differ in DNA sequence. Both genes, however, encode MUP 3 (based on two-dimensional gel

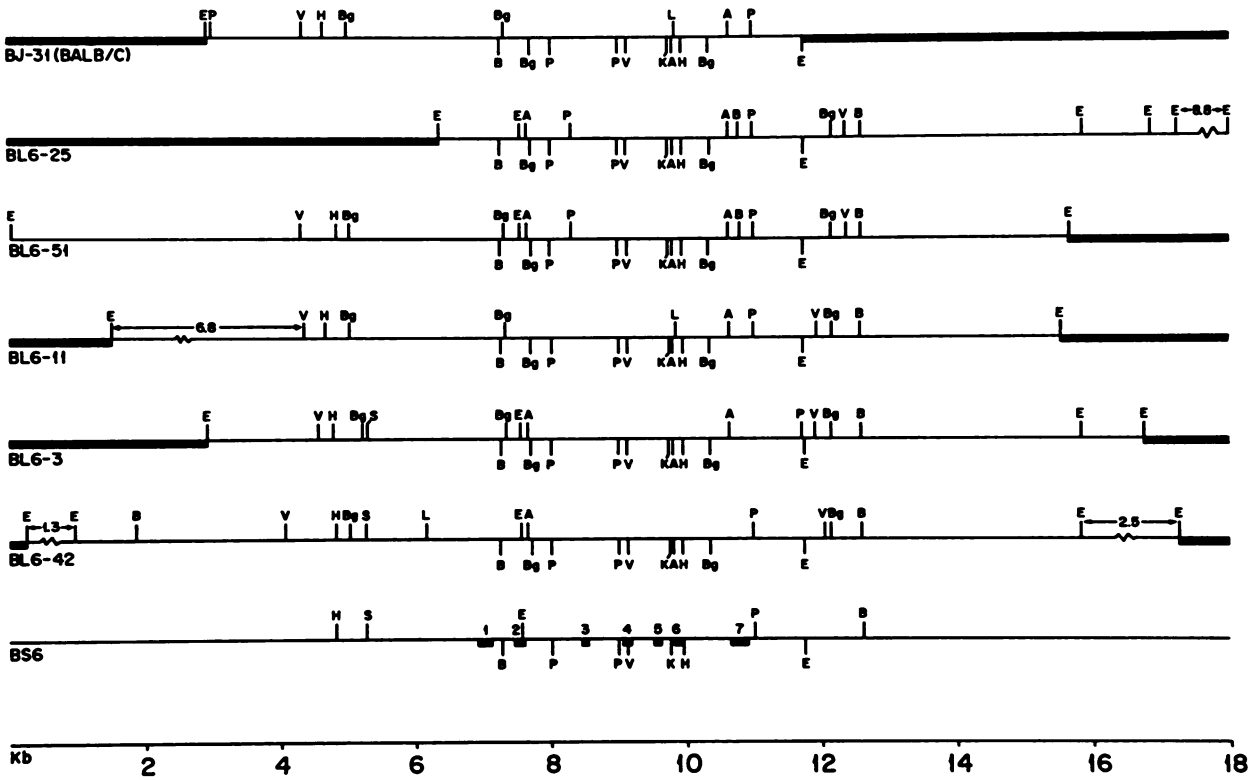

FIG. 5. Restriction enzyme maps of cloned MUP genes expressed in transfected mouse Ltk$^-$ cells. Restriction enzyme sites were deduced by a series of single and double restriction enzyme digestions and subsequent Southern blotting using 5′ and 3′ MUP liver cDNA clones as probes. Some sites were deduced through subcloning. Restriction enzyme abbreviations: A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; L, *Sal*I; P, *Pvu*II; S, *Sst*I; V, *Pvu*II. BJ-31 is a BALB/c genomic MUP sequence subcloned into the pBR322 R1 site. All other clones are from the Charon 4A C57BL/6J library. The restriction map of BS-6 showing exon positions was taken from Clark et al. (7).

mobility) but may have amino acid sequence differences which do not alter electrophoretic mobility.

Regulation of MUP gene expression has been studied in C57BL/6J mice by administration of hormones to mutant or surgically ablated mice with hormonal deficiencies (15, 17). The results indicate that MUP genes expressed in the liver are under complex, differential, multihormonal controls. Synergistic effects of hormones are indicated by the absence of testosterone-mediated MUP mRNA synthesis and the nonadditive effect of administration of both growth hormone and thyroid hormone in animals lacking pituitary function (15). Differential regulation of MUP genes expressed in the liver is indicated by differences in the pattern of MUP mRNA translation products in male versus female liver or in thyroidectomized animals after the administration of growth hormone, thyroid hormone, or testosterone (15).

Previous reports indicate that rat α_{2u}-globulin (the rat homolog to MUP) expression in the liver is regulated by glucocorticoid hormones and that cloned rat α_{2u}-globulin genes introduced into mouse L cells are regulated by dexamethasone (1, 18). Although we have not observed glucocorticoid regulation of MUPs in C57BL/6J mice, we have observed a glucocorticoid-stimulated increase of MUP mRNA in adrenalectomized and ovarectomized BALB/c mice (15; J. Knopf and W. A. Held, unpublished experiments).

The possibility that the expression of MUP genes transfected into L cells could be increased by glucocorticoid hormone was investigated by growing and labeling transfected cell lines with [^{35}S]methionine in the presence and absence of dexamethasone. Most of the transfectants showed about a twofold increase in MUP synthesis and secretion in the presence of dexamethasone. Cells transfected with BJ-31 (the BALB/c genomic clone) showed about a fivefold increase (data not shown). Currently, it is not clear whether this response is related to the general physiology of the cells or is more directly related to hormonal regulation of the transfected MUP gene. L cells are not known to have functional receptors for other hormones thought to be involved in MUP gene expression (testosterone, growth hormone, and thyroid hormone), and addition of these hormones had little or no effect (data not shown).

Considering the complex nature of the hormonal regulation of MUP gene expression, the low-level expression of transfected MUP genes in L cells is not surprising. High-level MUP gene expression may well require multiple tissue- and hormone-specific factors which are lacking in L cells. The low-level expression of the transfected MUP gene in these studies may also be due in part to the presence of inhibitory procaryotic DNA sequences. Experiments currently in progress include transfection of these genes into hepatoma cell lines and other cell lines known to have functional testosterone and thyroid hormone receptors.

All of the functional MUP genes that have been identified in this report belong to the group 1 subfamily that consists of about 15 genes. All group 1 genes exhibit high homology within protein-coding regions, 5′ and 3′ untranslated regions, and in 5′ and 3′ flanking regions (6, 8, 17), suggesting that these MUP genes were amplified relatively recently or that

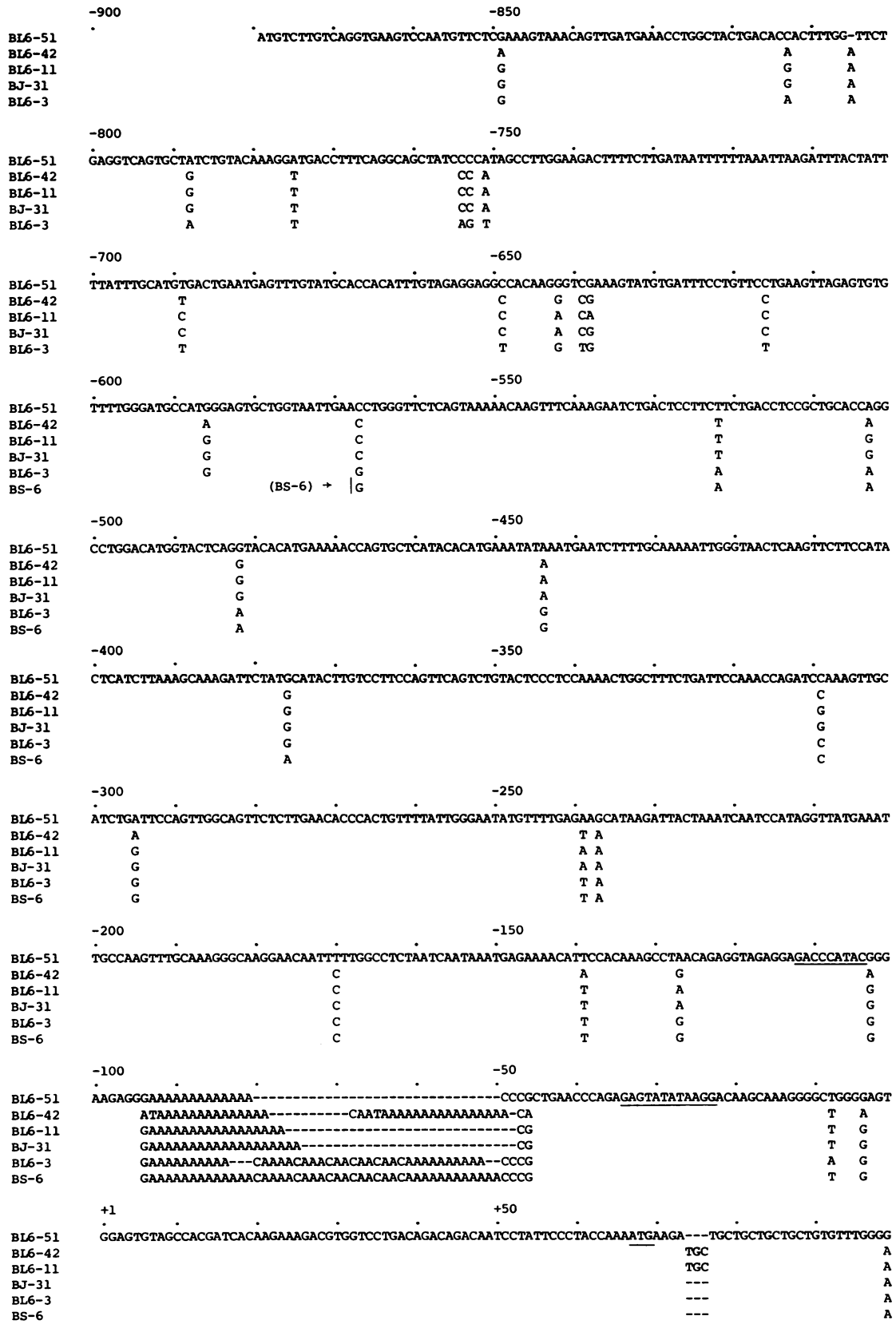

FIG. 6. DNA sequence of 5′ flanking region of five group 1 MUP genes. The entire sequence of 869 bp of the 5′ flanking region and 99 bp of the transcribed region for BL6-51 is shown. Sequence differences for the other group 1 genes are shown below. The 568-bp sequence of the 5′ flanking region of BS-6 is taken from the published work of Clark et al. (8) and is shown for comparison. The entire sequence of the A-rich region (−46 to −94) is shown for each of the clones. The translational initiation codon (ATG), the TATA box, and the possible CAAT box are underlined.

TABLE 1. Percent sequence divergence within the 5′ flanking regions of MUP genes

MUP gene region[a]	% Sequence divergence[b] with:				
	BL6-42	BL6-11	BJ-31	BL6-3	BS-6[c]
BL6-51					
A	4.09	0.91	0.91	2.27	1.82
B	0.20	1.20	1.00	2.21	1.99
C	4.39	3.51	3.51	2.63	
T	1.80	1.44	1.32	2.28	1.92
BL6-42					
A		3.18	3.18	2.73	2.27
B		1.41	1.20	2.41	1.99
C		1.75	1.75	1.75	
T		1.92	1.80	2.40	2.11
BL6-11					
A			0.00	1.36	0.91
B			0.20	3.01	2.33
C			0.00	1.75	
T			0.12	2.40	1.73
BJ-31					
A				1.36	0.91
B				2.81	2.33
C				1.75	
T				2.28	1.73
BL6-3					
A					0.45
B					0.33
C					
T					0.38

[a] Region A from position −1 to −267. Region B from position −268 to −765. Region C from position −766 to −879. Region T from position −1 to −879.

[b] The A-rich region from −47 to −93 was excluded in these calculations. Regions of unusually high homology are underlined.

[c] The sequence of 568 bp of 5′ flanking region from the MUP genomic clone BS-6 determined by Clark et al. (8) was used for comparison.

gene conversion events have maintained homology. Despite this high degree of homology, group 1 MUP genes are expressed in different tissues (liver and mammary), and in the liver, different group 1 genes are subject to different hormonal controls.

Restriction site mapping of the six group 1 genes analyzed indicates that homology extends at least 2.5 kb into the 5′ flanking sequences. Preliminary restriction mapping with four and five base-specific restriction enzymes suggests that the homology may extend as far as 6 kb (J. Lupisella and W. A. Held, unpublished data). This is consistent with previous analysis of group 1 genes (5, 9).

Since restriction site homology gives only an approximation of the extent of similarity between genes, we sequenced 879 bp of 5′ flanking regions of each of the genes to determine whether small localized regions of sequence differences occur which could reflect differences in tissue-specific or hormonal regulation of these genes. The sequence analysis indicates that all genes exhibit extensive homology throughout most of the 5′ flanking regions, with only occasional base substitutions or single-base deletions or insertions occurring. Sequence divergence within the 5′ flanking region (excluding the A-rich region from −47 to −93) is between 1.3 and 2.4% for most of the MUP genes (Table 1). Interestingly, BJ-31 (from BALB/c) and BL6-11 (from C57BL/6J) show only a 0.20% divergence. The two genes also have a very similar sequence within the A-rich region, BJ-31 having two additional A residues. Although MUPs encoded by BJ-31 and BL6-11 differ in mobility on two-dimensional polyacrylamide gels, each is synthesized in both male and female liver in their respective strains. MUP 3 (encoded by BL6-11 and BL6-3) is also synthesized in C57BL/6J mammary gland. BL6-3 (C57BL/6J) and BS-6 (BALB/c) also show extensive homology within the 5′ flanking region (0.38% divergence) and have similar sequences within the A-rich region. The MUP encoded by BS-6 is not known. BL6-51 and BL6-42, which have been tentatively identified as male liver-specific MUP genes, show an unusually high degree of homology within an approximately 500-bp segment of the 5′ flanking sequence (Table 1, region B, from −268 to −765). This may represent a gene conversion event or other type of recombination event, since the two sequences are considerably more divergent 5′ or 3′ of this region (regions A and C, Table 1). However, it is presently not clear whether the homologies noted above are related to the sex-specific differences in MUP gene expression. Differences in regulation may, of course, be determined by sequences other than within the 5′ flanking region.

The most prominent differences in sequence occur within the A-rich region just 5′ of the TATA box (from positions −47 to −93). This region contains primarily A or $C(A)_N$ nucleotides and varies in length in the different clones from 15 (BL6-51) to 46 (BS-6) nucleotides. Clark et al. (8) have also reported the presence of this variable-length A-rich tract in both group 1 and group 2 MUP genes. It is interesting that sequence analysis of the 5′ flanking region of genes encoding mouse complement C4 and the related testosterone-regulated sex-limited protein also show a high degree of overall homology (95%) (20). The sex-limited protein gene, however, lacks a 31- and a 60-nucleotide segment containing ACACCC and ACAC repeats, respectively, suggesting that simple repetitive sequences may play some role in the differential regulation of these genes. Also, $C(A)_N$ repeats or A-rich regions have also been implicated in formation of bent DNA in trypanosome kinetoplast DNA (31) and at a *Saccharomyces cerevisiae* autonomously replicating sequence (25), and may play some regulating role. However, further experiments are necessary to establish whether the sequence differences in the 5′ flanking region or other regions of MUP genes have a role in the differential regulation of MUP genes.

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ADDENDUM

In two recent reports, Shahan et al. (K. Shahan, M. Gilmartin, and E. Derman, Mol. Cell. Biol. 7:1938–1946, 1987; K. Shahan, M. Denaro, M. Gilmartin, Y. Shi, and E. Derman, Mol. Cell. Biol. 7:1947–1954, 1987) used oligonucleotide probes to identify specific MUP genes and their transcripts.

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