Proc. Natl. Acad. Sci. USA
Vol. 78, No. 9, pp. 5425–5429, September 1981
Biochemistry

Isolation of a cDNA clone for mouse urinary proteins: Age- and sex-related expression of mouse urinary protein genes is transcriptionally controlled

(*in vitro* translation/mRNA abundance/*in vitro* transcription in isolated nuclei)

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Communicated by James E. Darnell, Jr., May 29, 1981

ABSTRACT **A recombinant cDNA plasmid derived from mouse urinary protein (MUP) mRNA was isolated and used to determine the level of control of the developmentally regulated and the sex-linked expression of MUP genes by monitoring the transcription of MUP mRNA sequences in isolated liver nuclei. No transcription of MUP genes could be detected in liver nuclei of prepubescent animals whose livers do not contain measurable MUP mRNA. Transcription of MUP genes in the livers of adult male mice was 6-fold higher than in the livers of adult female mice, proportional to the difference in MUP mRNA concentrations. Transcriptional control mechanisms are therefore implicated as responsible for both the developmentally and the sex-linked changes in the expression of MUP genes.**

The mouse urinary proteins (MUPs) are tissue specific secretory proteins whose synthesis is both sex dependent and developmentally regulated. The primary site of MUP synthesis is the liver (1). The synthesis of mouse urinary proteins is higher in adult male than in female mice (2, 3) and is absent in prepubescent animals (N. Hastie, personal communication). MUPs form a family of at least four (4) closely related polypeptides that are encoded by a large number of genes in the haploid genome (1). The absolute and relative syntheses of the different MUP polypeptides have been shown to be different in different strains of mice (3, 5, 6). Study of the expression of MUP genes thus can probe different facets of regulation of eukaryotic genes—e.g., tissue specificity, commitment of cells to produce a particular protein, and the basis for hormonal regulation.

In order to initiate these studies in the MUP system, a recombinant DNA probe containing the sequences of one MUP mRNA has been obtained, and the level of regulation responsible for the age- and sex-related differences in MUP mRNA synthesis was investigated. Previous experiments (7) suggested that, for a group of 11 liver-specific mRNA molecules, transcriptional control is primarily responsible for the establishment and maintenance of liver-specific mRNA patterns. The question of the temporal relationship between the onset of transcription and the appearance of mature mRNA during tissue ontogeny, however, was not approached. The present experiments show that there is little or no transcription of MUP genes in immature liver cells which do not yet produce MUP mRNA. In addition, it appears that the male-specific increase in MUP mRNA concentration (1–3) is also accomplished by an increase in the rate of transcription of MUP genes in the liver nuclei of adult male mice compared to adult female mice.

METHODS AND MATERIALS

Animals, DNA, and RNA. An inbred strain of Swiss white mice (NCS) raised at the Rockefeller University was used in all experiments. The construction of recombinant plasmids was as described (7). Plasmid DNA was purified according to Norgard *et al.* (8). Poly(A)$^+$RNA from mouse livers was prepared as described by Chirgwin *et al.* (9).

***In Vitro* Translation and Immunoprecipitation.** Adult male liver poly(A)$^+$RNA (25 μg) was hybridized to 80 μg of plivS-1 DNA bound to a nitrocellulose filter for 4 hr in 50% formamide/0.75 M NaCl/10 mM piperazinediethanesulfonic acid, pH 7.0/10 mM EDTA/0.2% $NaDodSO_4$ at 45°C. After a washing in hybridization buffer at 45°C and then three brief washes in 50% (vol/vol) formamide/10 mM EDTA/10 mM Tris, pH 7.4/0.2% $NaDodSO_4$ at 45°C, bound RNA was eluted by boiling for 1 min in 10 mM EDTA/10 mM Tris, pH 7.4. Eluted RNA was passed through a poly(U)-Sepharose column (10), ethanol precipitated, and then translated in rabbit reticulocyte system with [^{35}S]methionine (New England Nuclear). Two-microgram samples of total poly(A)$^+$RNA from livers of 7-week-old male and female mice and from livers of 1-week-old male mice were translated in parallel. Immunoprecipitation of translational products of plasmid-selected RNA and of total poly(A)$^+$RNA from adult males was performed according to Chang *et al.* (11). The antisera raised in rabbits against MUP1 and MUP2 of BALB/c mice were kindly provided by M. Potter (National Institutes of Health). Polypeptides were fractionated on $NaDodSO_4$/13.75% polyacrylamide gels (12) and visualized by autoradiography.

Determination of RNA Size. Formaldehyde-denatured RNA was electrophoresed in 1.5% agarose/1.1 M formaldehyde gels (13) with 20 mM 3-(*N*-morpholino)propanesulfonic acid/5 mM Na acetate/1 mM EDTA, pH 7.0, for electrophoresis buffer. After transfer to nitrocellulose filters (13), the size of RNA complementary to plasmid plivS-1 (MUP) was determined by hybridization to nick-translated (14) plivS-1 DNA (7). The RNA-laden nitrocellulose filter was first incubated in 30% formamide/0.3 M NaCl/50 mM piperazinediethanesulfonic acid, pH 7.0/10 mM EDTA/0.2% $NaDodSO_4$ containing denatured salmon sperm DNA at 100 μg/ml and double-strength Denhardt's solution (15) for 10 hr at 45°C. The hybridization solution also contained 10% (wt/vol) dextran sulfate and probe DNA at 10^5 cpm/ml.

Quantitation of RNA Abundance and of Transcription Rates. These measurements were performed exactly as described (7).

RESULTS

Construction and Selection of Recombinant Plasmids Derived from MUP mRNA. Construction and selection of recombinant cDNA plasmids complementary to mRNAs present exclusively (or predominantly) in the mouse liver, compared to

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Abbreviation: MUP, mouse urinary protein.

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mouse brain or hepatoma cells, have been described (7). It was assumed that a large fraction of these plasmids was derived from MUP mRNA because this mRNA comprises a few percent (1) of total liver mRNA. The presumptive MUP recombinant plasmids were also expected to hybridize a larger proportion of adult male than adult female liver poly(A)$^+$RNA and to hybridize more liver poly(A)$^+$RNA from adult than from immature animals (1). A large number of clones satisfying these criteria were picked and one of them, plivS-1 (7), was used to prove that these plasmids were indeed derived from MUP mRNA.

Positive selection of complementary mRNA and *in vitro* translation and immunoprecipitation

Male liver poly(A)$^+$RNA (25 μg) was hybridized and eluted from plivS-1 DNA bound to a nitrocellulose filter. Eluted RNA was passed through a poly(U)-Sepharose column and bound RNA was then translated in an *in vitro* reticulocyte lysate in parallel with 2 μg of total liver poly(A)$^+$RNA from adult male and adult female mice and from 7-day-old male mice. The products of the *in vitro* translations are shown in Fig. 1. Plasmid-selected RNA was translated into two polypeptides of molecular weight 19,000 and 20,500 (Fig. 1, lane c). These two polypeptides also were the major translational products directed by total poly(A)$^+$RNA from adult female liver and adult male liver; they comprised a larger fraction of the *in vitro* synthesized polypeptides in the case of male liver poly(A)$^+$RNA. mRNA from livers of 7-day-old male mice did not direct any observable synthesis of the 19,000- and 20,500-dalton polypeptides. The molecular weights of the polypeptides translated from plivS-1-selected mRNA(s), the observed sex difference, and their apparent absence from the translational product of mRNA from immature animals all suggest that the selected mRNA(s) codes for the MUPs.

To confirm this, the translational products of plasmid selected mRNA and of the total mouse liver mRNA were immunoprecipitated with antisera raised against MUP1 and MUP2 of the BALB/c mouse (gift of M. Potter). The larger polypeptide was immunoprecipitated in both samples (Fig. 2). No precipitate was detected when a nonimmune serum was used as a control (lane not shown). The possible reasons for the lack of reactivity of the smaller polypeptide are considered in the *Discussion*.

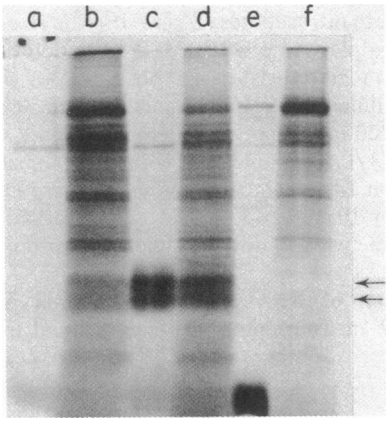

FIG. 1. *In vitro* translation of mouse liver poly(A)$^+$RNA prepared as described by Derman *et al.* (7) and translated in the presence of [^{35}S]methionine in a reticulocyte lysate (New England Nuclear). Polypeptides were electrophoresed through 13.57% NaDodSO$_4$/polyacrylamide gel and visualized by autoradiography after 3 weeks. Lanes: a, no exogenous RNA; b, 2 μg of total poly(A)$^+$RNA from from 7-week-old females; c, plivS-1 selected poly(A)$^+$RNA; d, 2 μg of total poly(A)$^+$RNA from 7-week-old males; e, globin mRNA; f, 2 μg of total poly(A)$^+$RNA from 7-day-old males.

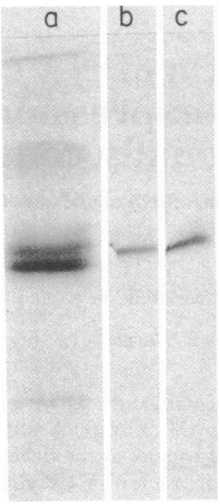

FIG. 2. Immunoprecipitations with anti-MUP antiserum. Products of *in vitro* translation were electrophoresed on a 13.75% NaDodSO$_4$/polyacrylamide gel. Lanes: a, polypeptides shown in lane c of Fig. 1; b, polypeptides shown in lane c of Fig. 1 immunoprecipitated with MUP1 and MUP2 antisera; c, polypeptides shown in lane d of Fig. 1 immunoprecipitated with MUP1 and MUP2 antisera.

RNA species complementary to plivS-1 plasmid

MUPs form a family of at least four different but related polypeptides. The translational products of adult liver mRNA(s) selected with plivS-1 DNA are at least two polypeptides (Fig. 1). To determine the size of mRNA(s) coding for these proteins,

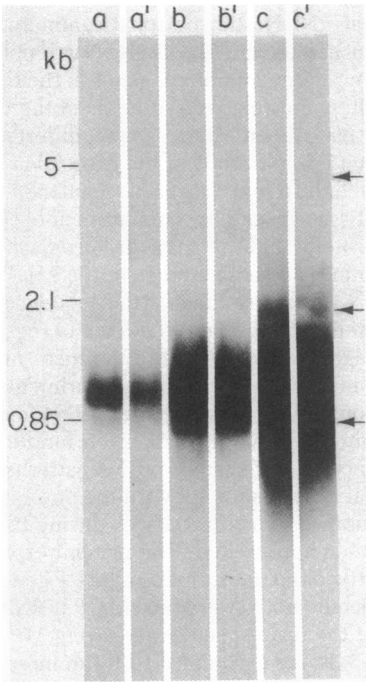

FIG. 3. Analysis of poly(A)$^+$RNA complementary to plivS-1 plasmid. Adult male liver poly(A)$^+$RNA (0.2 μg) (lanes a, b, and c) and adult female poly(A)$^+$liver RNA (1 μg) (lanes a', b', and c') were denatured, electrophoresed through a 1.5% agarose/1.1 M formaldehyde gel, transferred to nitrocellulose paper, and hybridized to nick-translated plivS-1 plasmid DNA. Autoradiographs after different exposure times are shown: a, a', 1 day; b, b', 5 days; c, c', 2 weeks. Positions of ribosomal and adenovirus marker RNAs are indicated at the left. Arrows point to the minor RNA bands hybridizing to plasmid DNA. kb, kilobases.

Table 1. Effect of age on steady-state poly(A)$^+$RNA

	Adult male, % × 10^2	Seven-day-old male, % × 10^2
plivS-1 (MUP)	8.0	<0.002
plivS-5	0.1	0.15
plivS-6	0.2	0.5

Poly(A)$^+$RNA from the livers of 7-week-old and 7-day-old male mice was prepared, ^{32}P-labeled with kinase *in vitro*, and hybridized to an excess of plasmid DNA bound to nitrocellulose filters. The fraction of total poly(A)$^+$RNA (5 × 10^6 cpm input) that remained bound to plasmid DNA after pancreatic RNase digestion is expressed as percentage of steady-state poly(A)$^+$RNA.

poly(A)$^+$RNA from male and female livers was subjected to electrophoresis, and transferred to nitrocellulose paper, and hybridized to labeled plivS-1 DNA. The predominant RNA species was 940 bases (Fig. 3). After longer exposure, RNA species of both lower (740 bases) and higher (2000 and 5000 bases) molecular weight could be detected. From the relative abundances of the different RNA species visualized, it seems probable that the 19,000- and 20,500-dalton polypeptides are translated from mRNAs that comigrate in the 940-base band.

Regulation of MUP mRNA Synthesis

Age-Related Expression of MUP Genes. Whereas MUPs are the major translational products of adult liver mRNA, these proteins are not detected in the translational product directed by mRNA from livers of 7-day-old male mice, indicating the relative absence of translatable MUP mRNA in the immature animal. It has been shown (7) that cells of the mature mouse brain, in which MUP mRNA cannot be detected, also do not transcribe MUP genes. This result implied that the tissue-specific expression of MUP genes is transcriptionally controlled and that either the transcription of MUP genes has been turned off in the lineage of cells that form the mouse brain or that transcriptional activation of MUP genes has occurred in the liver. To distinguish between these two mechanisms, and also to determine the temporal relationship between the onset of transcription and the appearance of mature mRNA, the transcription of MUP genes was measured in the liver nuclei of 7-day-old mice.

The MUP mRNA was the only mRNA of the 11 liver-specific RNAs previously studied (7) that was absent from livers of 7-day-old mice. All the others were present, although not at the same concentration as in the adult (data not shown). Two of these recently described (7) recombinant plasmids, plivS-4 and plivS-5, were chosen as controls for measuring the transcriptional activity of nuclei from 7-day-old male mice. Table 1 summarizes the concentrations of mRNAs complementary to plivS-1 (MUP) plasmid and to the two control plasmids in the livers of mature and immature males. The concentrations were obtained by measuring the fraction of *in vitro* labeled poly(A)$^+$RNA that formed a stable hybrid with excess plasmid DNA bound to a nitrocellulose filter.

Table 2 summarizes the results of two experiments in which transcription in isolated nuclei of MUP-specific sequences and RNA sequences complementary to plasmids plivS-4 and plivS-5 was measured. The nuclei were incubated in the presence of [^{32}P]UTP. RNA was then extracted and hybridized to plasmid DNA bound to nitrocellulose filters, and RNase-resistant RNA·DNA hybrids were measured. By this assay, the nuclei of the 7-day-old livers were transcriptionally active for RNA sequences that hybridized to plasmids plivS-4 and plivS-5. However, no transcription of MUP mRNA sequences could be detected in the same experiments. Thus, it appears that transcriptional activation of MUP genes in the liver underlies the tissue-specific expression of these genes.

Sex-Related Expression of MUP Genes. In the previous experiments (7), although the rate of transcription of liver-specific genes in general increased with the concentration of corresponding mRNAs, a strict proportionality of nuclear RNA transcription to mRNA concentration was not observed. Similar conclusions were also arrived at by Harpold *et al.* (16). These observations imply that, as suggested earlier (17), the concentration of a specific mRNA is determined by posttranscriptional factors in addition to the rate of transcription—that is, the efficiency of processing of the precursor or the stability of mRNA. Whether these factors are intrinsic to a given mRNA or can be varied has been the subject of a number of inquiries (18, 19). Because of this possibility, the level of regulation leading to the increase in the MUP mRNA concentrations in the male animals (ref. 4; Fig. 1) has been investigated.

The steady-state level of MUP mRNA was determined as described in Table 1; values were 8.0% for adult male liver and 1.5% for adult female liver. This sex difference is in agreement with earlier observations by Hastie *et al.* (1). Table 3 summarizes two experiments in which transcription of MUP mRNA sequences was measured in adult male and adult female liver nuclei. In both experiments the input amount of nuclear RNA was varied to demonstrate that the hybridization was performed in sufficient probe DNA excess. The results of these experiments show that, on average, MUP genes are transcribed 6-fold more frequently in male than in female nuclei. Because the observed difference in transcription is close to the 5.3-fold difference in mRNA concentration, it seems that the sex-related difference in MUP mRNA synthesis is due to the increase in transcription of MUP genes in the male.

Table 2. Effect of age on transcription in isolated nuclei

	Adult male			Seven-day-old-male		
	RNA hybridized,* cpm			RNA hybridized,* cpm		
	At 12 × 10^6 cpm input	At 15 × 10^6 cpm input	Average transcription rate × 10^6	At 15 × 10^6 cpm input	At 20 × 10^6 cpm input	Average transcription rate × 10^6
pBR322	25	30	—	24	34	—
plivS-1 (MUP)	1180	2300	135	15	34	1.4
plivS-5	300	175	18	310	ND	20
plivS-6	135	70	8	250	240	13

For preparation of nuclei, 3 livers of 7-week-old and 15 livers of 7-day-old male animals were used. Nuclei were incubated in the presence of 0.5 mCi of [^{32}P]UTP for 20 min at 25°C under conditions as described (7). Nuclear RNA was extracted, freed of DNA, and hybridized to excess plasmid DNA bound to nitrocellulose filters; the RNase-resistant RNA radioactivity was measured. The transcription rate is defined as the fraction of input RNA that is hybridized to plasmid DNA.
* Value with pBR322 subtracted. ND, not done.

Table 3. Effect of sex on transcription in isolated nuclei

Adult male			Adult female		
Input, cpm × 10^{-6}	RNA hybridized, cpm	Transcription rate × 10^6	Input, cpm × 10^{-6}	RNA hybridized, cpm	Transcription rate × 10^6
		Experiment 1			
4	380	100			
6.6	640	100	6	110	20
12	1180	100	12	180	15
		Experiment 2			
7	1165	165	5	60	12
15	2300	170	10	250	25
Mean		127			18

For preparation of nuclei, four livers were used. Nuclei were incubated in the presence of 0.5 mCi of [^{32}P]UTP for 20 min at 25°C under conditions as described (7). Nuclear DNA was extracted, freed of DNA, and hybridized to excess plasmid DNA bound to nitrocellulose filters. RNase-resistant hybridized RNA radioactivity was measured. The transcription rate is defined as the fraction of input RNA that is hybridized to plasmid DNA.

DISCUSSION

In the course of screening a collection of cDNA recombinant plasmids made from mouse liver mRNA (7), sequences common to a specific plasmid (plivS-1) were found to be present in many transformants, suggesting that this plasmid was complementary to an abundant liver mRNA. As shown in this paper, mRNA selected by plasmid plivS-1 can be translated into what on $NaDodSO_4$/polyacrylamide gels appear to be two polypeptides of molecular weights 19,000 and 20,500, in agreement with published data concerning the size of MUP precursor proteins (4). One of the two translational products was efficiently immunoprecipitated with antisera prepared against so-called MUP1 and MUP2 of BALB/c mice. The lack of crossreactivity of the second polypeptide might reflect the strain polymorphism of MUPs. The DNA sequence of the plivS-1 plasmid recently has been shown (K. Krauter and L. Leinwand, personal communication) to have the coding potential for the previously published NH_2-terminal amino acid sequence of MUP1 and MUP2 (6).

The lack (or scarcity) of mRNA complementary to plasmid plivS-1, which is here shown to be derived from MUP mRNA, in mouse brain has been shown to be due to the lack (or scarcity) of transcription of MUP genes; transcriptional control was suggested as being responsible also for the tissue-specific appearance of 10 moderately abundant liver mRNAs. Transcriptional regulation was previously demonstrated as responsible for the tissue-specific synthesis of two very abundant mRNAs, ovalbumin (20) and ovomucoid (21), and for hemoglobin switching in chicken embryos (22). The temporary relationship between the onset of transcription and the appearance of mature mRNA in the same tissue has not previously been investigated. Because it seemed possible (17) that in the immature liver the MUP genes are transcribed but no stable MUP mRNA is formed, the transcription of MUP genes in the liver of immature animals was studied.

The results presented here show that the transcription of MUP genes in the livers of 7-day-old male mice is greatly decreased relative to that in the adult, if it occurs at all. The concentration of mRNA in the livers of adult males is at least 4000-fold higher than in those of the 7-day-old males, increasing from less than 10 copies per cell to about 40,000 copies per cell. However, the measured increase in transcription of MUP mRNA sequences is only 100-fold because of the background of hybridization experiments. Any transcription rates lower than 1×10^{-6} of total RNA transcription cannot be measured by this assay. It therefore is likely that the measured decrease in the transcription rate of MUP mRNA in the immature animal relative to the adult is an underestimate of the actual difference and that the changes in expression of MUP genes during development are solely due to changes in the transcription of MUP genes. Also, as documented here, the higher concentration of MUP mRNA in the livers of adult male compared to female mice is paralled by proportional increase in transcription of MUP mRNA sequences in the adult male liver. It thus appears that both the age-dependent as well as the sex-related regulation of the expression of MUP genes is accomplished primarily at the level of transcription.

Correlations have been shown to exist between the susceptibility of chromatin to DNase I and the expression of genes (23, 24). Sites hypersensitive to DNase I whose presence can reflect the pattern of gene activity have been identified (25–27). Also, hypomethylation of DNA has been detected in the vicinity of active genes (28, 29). Which of these parallel the changes in transcription of MUP genes remains to be determined.

The author thanks F. Cross, J. Nevins, and Ch.-N. Chang for helpful conversations, M. Potter for the gift of anti-MUP antiserum, N. Hastie and W. Held for sharing their unpublished data, and J. E. Darnell, Jr., for his interest and advice. This work was supported by National Institutes of Health Grant CA 16006-07 to J. E. Darnell in whose laboratory this work was performed. The author was supported by a fellowship from the Helen Hay Whitney Foundation and by the National Institutes of Health (CA 16006-07).

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