Demonstration of a glycoprotein derived from the 24p3 gene in mouse uterine luminal fluid

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A glycoprotein in mouse uterine luminal fluid was purified to homogeneity via a series of purification steps involving Sephadex G-100 chromatography, Sephadex G-50 chromatography and HPLC on a reverse-phase C_{18} column, in that order. Automated Edman degradation was unable to determine the N-terminal residue of the glycoprotein and the partial sequences determined from its trypsin digests were found to be identical with the protein sequence deduced from 24p3 cDNA. The core protein and the total amount of carbohydrate together gave a molecular mass of 25.8 kDa. Results from the characterization of the glycopeptide bond indicated the presence of N-linked carbohydrate but no O-linked carbohydrate in the protein, which has two potential sites for N-linked carbohydrate at Asn^{81} and Asn^{85}, as deduced from analysis of the primary structure. The core protein was shown to have a molecular mass equal to that of the putative protein deduced from cDNA, suggesting that this protein may contain no signal peptide. Results of Northern-blot analysis for various tissues of adult mice revealed that the 24p3 gene was expressed in lung, spleen, uterus, vagina and epididymis.

INTRODUCTION

The uterus has complex structures composed of endometrial, epithelial, stromal and myometrial cells. It has been noted that accumulation of uterine luminal fluid (ULF) is derived from secretions of the uterine epithelial cells during the pro-oestrous phase of mature rodent reproductive cycles [1,2]. Establishing the structures of protein components in ULF is important in order to elucidate their roles in reproductive biology. Since stimulation of immature rodent by oestrogen or its analogues results in a remarkable increase in ULF, the immature murine reproductive tract provides a good experimental system for these studies. As a result, the study of lactoferrin, a major protein component of mouse ULF, has made some progress [3–6]. Less progress, however, has been made in the study of the minor protein component in mouse ULF. We report on the purification and identification of a 25.8 kDa glycoprotein, a minor protein component of mouse ULF. We have demonstrated that its core protein is derived from the 24p3 gene which is normally expressed in both sexual organs (reproductive tract, including uterus, vagina and epididymis) and non-sexual organs (such as lung and spleen), despite the fact that the gene has been screened from simian virus 40-infected kidney cells [7].

MATERIALS AND METHODS

Materials

Sephadex G-50, Sephadex G-100, markers for isoelectric focusing and thin-layer ampholine PAGE plates were obtained from Pharmacia (Uppsala, Sweden). A reverse-phase C_{18} column (Chemecopak 5 ODS-H, 7 μm pore size), a Radial-Pak C_{18} cartridge column and a Protein pak SP 5PW column were obtained from Waters (Millipore Corporation, Bedford, MA. U.S.A.). N-glycosidase F (Glycopeptidase F), O-glycosidase (endo-α-N-acetylgalactosaminidase) and N-neuraminidase were obtained from Boehringer-Mannhein G.m.b.H. All of the reagents and enzymes used in cDNA preparation, PCR, the T7 DNA polymerase sequencing system, the construction of plasmids and the preparation of the random-primed DNA probes (Prime-A-gene kit) were purchased from Promega (Madison, WI, U.S.A.). A Geneclean Kit was from BIO 101 Inc. (La Jolla, CA, U.S.A.). All chemicals were of reagent grade.

Animals

Immature (21 day-old) and sexually mature (6–8 week-old) female mice were purchased from Charles River Laboratories (Wilmington, MA, U.S.A.) and were maintained and bred in the animal centre at the College of Medicine, National Taiwan University. Animals were treated following the institutional guide-lines for the care and use of experimental animals. The immature females were injected subcutaneously with diethylstilbesterol (DES) (Sigma, St. Louis, MO, U.S.A.) in corn oil with a daily dose of 100 ng/g of body weight for three consecutive days and killed on day 24 of age by cervical dislocation. ULF was collected and mixed with 10 mM EDTA to give 1 mM EDTA in the fluid, which was stored at -70 °C before use.

Fractionation of mouse ULF

The soluble portion of mouse ULF was subjected to gel chromatography on a Sephadex G-100 column (2.5 cm × 120 cm) pre-equilibrated in PBS containing 1 mM EDTA, pH 7.4. The column was washed with the buffer at a flow rate of 8 ml/h. Fractions (2 ml) were collected and their absorbance at 280 nm was recorded. Three peaks denoted I–III in Figure 1(a) were obtained. Peak III sample was concentrated using a Centricon 10 (Amicon, Division of W. R. Grace & Co.) and rechromato-

Abbreviations used: DES, diethylstilbesterol; DMB, 1,2-diamino-4,5-methylene-dioxybenzene; NANA, *N*-acetylneuraminic acid; NGNA, *N*-glycolylneuraminic acid; ULF, uterine luminal fluid.

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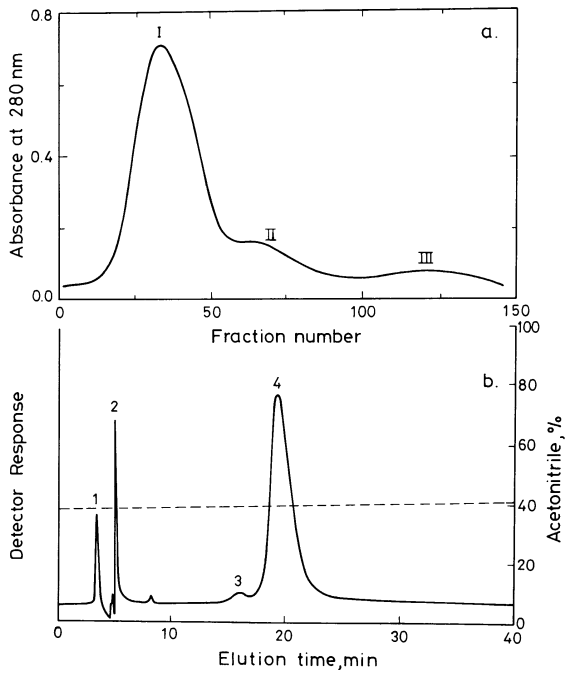

Figure 1 Separation of the protein components in mouse ULF

(**a**) Fractionation of ULF by gel chromatography on a Sephadex G-100 column (2.5 cm × 120 cm). Peak III sample was confirmed to contain a 26 kDa component by SDS/PAGE and was rechromatographed on a Sephadex G-50 column (1.5 cm × 100 cm). (**b**) The HPLC profile of the peak III sample on a reverse-phase C_{18} column (4.9 mm × 250 mm). The broken line indicates a linear gradient of acetonitrile (see text for details).

graphied on a Sephadex G-50 column (1.5 cm × 100 cm) which was washed with PBS containing 1 mM EDTA, pH 7.4. The main fraction from the rechromatography was resolved further by HPLC on a reverse-phase C_{18} column (4.9 mm × 250 mm) using a linear gradient of 35–41 % acetonitrile in 0.1 % (v/v) trifluoroacetic acid. The flow rate was 1.0 ml/min. The 24p3 protein appeared in the fractions of peak 4 (Figure 1b).

SDS/PAGE and isoelectric focusing

Proteins were resolved by SDS/PAGE on a 15 % gel slab (0.75 mm × 10 cm × 7.0 cm) according to the method of Laemmli [8]. Electrophoresis was conducted at 100 V for 2.5 h. The proteins on the gel were stained with Coomassie Brilliant Blue or periodic acid/Shiff's reagent.

Isoelectric focusing on a thin-layer ampholine/PAGE plate was performed using an LKB multiphor unit. The cathodic fluid consisted of 0.44 % Arg/0.06 % Lys, which was adjusted to pH 10 by addition of ethylenediamine. The anodic fluid contained 0.33 % Asp/0.37 % Glu at pH 3.0. Focusing was conducted for 4 h at a maximum voltage of 1.7 kV.

Fragmentation of the 24p3 protein

The protein was reduced in 6 M urea/5 mM dithioerythreitol, pH 8.5, at 45 °C for 1 h. Alkylation of the reduced group was achieved by addition of iodoacetamide to a final concentration of 40 mM at room temperature for 20 min. The alkylated derivative was digested with trypsin (enzyme:substrate = 1:50 by weight) in 0.05 % (w/v) ammonium bicarbonate, pH 6.8, at 37 °C for 6 h.

The protein was cleaved selectively with CNBr in 70 % (v/v) formic acid according to the method of Gross and Withop [9]. The CNBr-treated protein sample was reduced and alkylated as mentioned above.

The peptide fragments from either trypsin digestion or CNBr cleavage were resolved by HPLC on a C_{18} column using a linear gradient of 0–80 % acetonitrile in 0.1 % (v/v) trifluoroacetic acid. The flow rate was 1.0 ml/min and the total run time was 70 min. Fractions were collected and their absorbance at 230 nm was monitored.

Sugar analysis and amino acid sequencing

The core protein and carbohydrate moiety of the 24p3 protein were quantified by a modified Lowry method [10] and an L-cysteine sulphuric acid assay [11] respectively. The protein was deglycosylated by treatment with trifluoromethanesulphonic acid at 0 °C for 2 h [12]. N-glycosidase F digests of the glycoprotein were prepared by digestion with the enzyme at 37 °C for 24 h [13]. The N-glycosidase F-digested samples were digested further with N-neuraminidase at 37 °C for 5 h, followed by digestion with O-glycosidase at 37 °C for 24 h [14].

According to the method of Kondo et al. [15], the composition of neutral carbohydrate of the 24p3 protein was determined after it had been hydrolysed in 4 M trifluoroacetic acid at 100 °C for 4 h. The amounts of individual monosaccharides were quantified from the corresponding peak in the HPLC profile. The neuraminic acids, released by acid hydrolysis of the glycoprotein in 25 mM H_2SO_4 at 80 °C for 3 h, were converted in dilute sulphuric acid with 1,2-diamino-4,5-methylene-dioxybenzene (DMB) into highly fluorescent derivatives which could be identified by HPLC on a Radial-Pak C_{18} cartridge column [16].

The amino acid sequence was determined by automated Edman degradation with a gas-phase sequenator (477A protein sequencer with on-line 120A analyser; Applied Biosystems, Foster City, CA, U.S.A.).

RNA isolation and cDNA preparation

Total cellular RNA was isolated and single-stranded cDNAs were prepared on the polyadenylated fraction of uterus RNA by a standard procedure [17] using AMV reverse transcriptase (Promega, Madison, WI, U.S.A.).

PCR, cloning and analysis

Based on mouse 24p3 cDNA [7], we synthesized one oligonucleotide, CTGGGCCTTGCCCTGCTTGGGGTC, which represents nucleotides 44–67 of 24p3 cDNA and another oligonucleotide, GTTGTCAATGCATTGGTCGGTGGG, which is complementary to nucleotides 601–624 of 24p3 cDNA (Figure 6a). Those two oligonucleotides were employed as the primer pair for PCR, which amplified the single-stranded cDNAs of uterus with Taq polymerase for 30 cycles: 94 °C for 1.5 min; 55 °C for 2.0 min; 72 °C for 3.0 min. The reaction mixture was subjected to electrophoresis on a 2.0 % agarose gel. The amplified DNA (600 bp), which was extracted from the gel with a Geneclean Kit, was ligated to pGEM-7zf via an *Sma*I site. The constructed plasmid was introduced into *Escherichia coli* strain JM109 using a transformation technique [18]. Positive clones containing the cDNA insert were confirmed by *Xba*I–*Bam*HI digestion.

The cDNAs concerned were sequenced according to the dideoxy T7 DNA polymerase sequencing system using either oligonucleotide of the primer pair employed for PCR as the primer [19]. Each base was determined at least three times.

The random-primed DNA probe was prepared using the Prime-A-gene kit by the method of Feinberg and Vogelstein [20].

RESULTS

Stimulation of baby mice by DES caused a remarkable increase in ULF. The soluble portion of mouse ULF was divided into three peaks, denoted I–III in Figure 1(a), by gel chromatography on a Sephadex G-100 column. Peak III sample was further resolved by HPLC on a reverse-phase C_{18} column after it had been rechromatographed on a Sephadex G-50 column. As shown in Figure 1(b), four major peaks denoted 1–4 appeared on the chromatogram.

Mouse ULF was shown to contain several protein components identified by SDS/PAGE (Figure 2, lane 2). The majority of them gave a molecular mass larger than 35 kDa and the minor components a molecular mass smaller than 30 kDa. The sample giving rise to peak 4 in Figure 1(b) provided only a 26 kDa protein band on SDS/PAGE (Figure 2, lane 3). This protein appeared to be the main constituent of the minor components in the mouse ULF. Its pI was determined to be 5.8 on a thin-layer ampholine/PAGE plate. This protein band could also be stained with periodic acid/Shiff's reagent, indicating its glycoprotein nature. The ratio of carbohydrate to protein was determined to be 13:100 by weight.

The 26 kDa protein band, indicated by an arrowhead in Figure 2, was reduced to a 23 kDa band when the glycoprotein was deglycosylated by treatment with trifluoromethanesulphonic acid (Figure 2, lane 7). Exhaustive digestion of the glycoprotein with N-glycosidase F produced one protein component that appeared on SDS/PAGE at the same position as the chemically deglycosylated protein sample (cf. lanes 4 and 7 of Figure 2). On the other hand, the 26 kDa protein band did not change as it was digested with O-glycosidase (Figure 2, lane 5). Moreover, the molecular mass of the N-glycosidase F-digested sample did not decrease when it was digested further with O-glycosidase (Figure 2, lane 6). These data indicate both the lack of O-linked carbohydrate and that a certain amount of the N-linked carbohydrate is present in the protein. Apparently, the core protein of this glycoprotein has a molecular mass of around 23 kDa. An attempt was made to determine the monosaccharides in the glycoprotein. As shown in Figure 3, a certain amount of *N*-acetylneuraminic acid (NANA) and very little *N*-glycolylneuraminic acid (NGNA) could be detected in the glycoprotein.

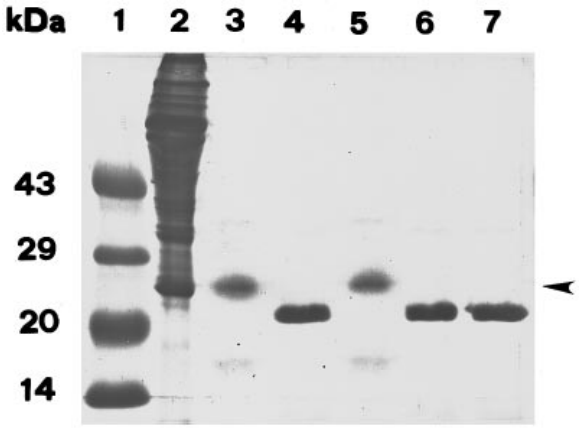

Figure 2 Protein characterization by SDS/PAGE

A suitable amount of protein was subjected to SDS/PAGE on a slab gel: lane 1, protein markers; lane 2, ULF (30 μg); lane 3, peak 4 sample of Figure 1(b) (referred to as peak 4 hereafter) (3 μg); lane 4, N-glycosidase F digest of peak 4 (4 μg); lane 5, O-glycosidase digest of peak 4 (3 μg); lane 6, O-glycosidase digest of N-glycosidase F-deglycosylated peak 4 (4 μg); lane 7, the product from the reaction of peak 4 and trifluoromethanesulphonic acid (4 μg). The arrowhead indicates the position of a 26 kDa protein band.

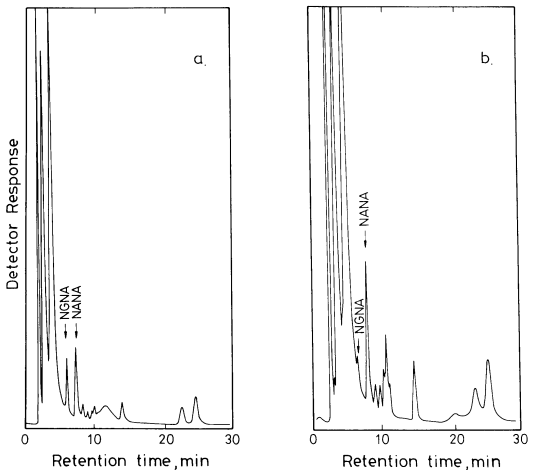

Figure 3 Identification of neuraminic acids in the glycoprotein by HPLC on a Pak C_{18} Cartridge column

(**a**) Chromatogram of the DMB derivatives of standard NANA (5 pmol) and NGNA (5 pmol). (**b**) Chromatogram of the DMB derivatives of neuraminic acids in the glycoprotein (15 pmol) which was treated as described in the text. The DMB derivatives of NANA and NGNA are indicated. The peaks other than NANA and NGNA arise from solvent, reagents and the reaction by-products.

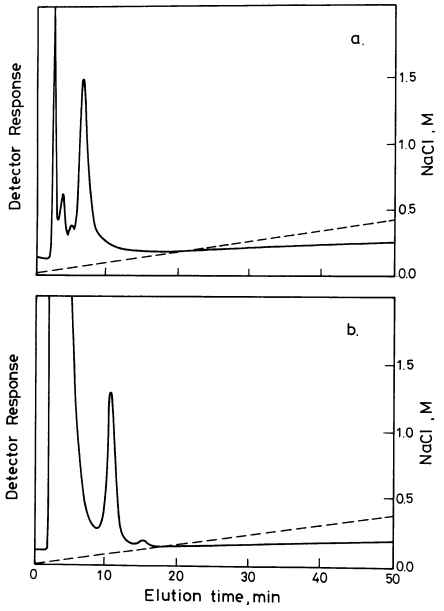

Figure 4 The sialic acid of the glycoconjugate affects the charge properties of the glycoprotein

A 15 μg amount of the parent glycoprotein (**a**) or 15 μg of the neuraminidase-digested sample (**b**) was subjected to HPLC on an SP column (7.5 mm × 75 mm), which was washed with a linear gradient of 0–0.4 M NaCl in 20 mM NaH_2PO_4/Na_2HPO_4 (pH 7.2) at a flow rate of 1.0 ml/min. The effluent was monitored at 230 nm. The broken lines indicate a linear gradient of NaCl.

Table 1 The composition of neutral carbohydrates in the glycoprotein

No correction has been made for the release of individual carbohydrates from the glycoprotein due to destruction in the course of hydrolysis.

Residue	Carbohydrate composition (mol/mol of glycoprotein)
Galactose	2.1
Mannose	1.1
Fucose	3.2
Glucose	3.0
N-Acetylglucosamine	0.8

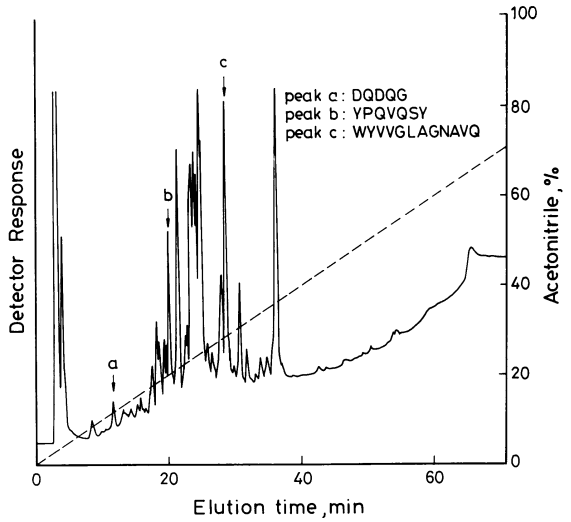

Figure 5 Resolution of trypsin digests of the glycoprotein by HPLC on a reverse-phase C_{18} column

The partial sequences determined from automated Edman degradation of peaks a–c are given in the Figure. The broken line indicates a linear gradient of acetonitrile (see text for details).

The glycoproteins were digested with neuraminidase. The HPLC chromatograms obtained with an SP column at pH 7.2, as displayed in Figure 4, indicate that the neuraminidase-digested sample associated more strongly with the cation exchanger than did the parent glycoprotein. This shows how the contribution of negative charge from the sialic acid of the glycoconjugate effects the charge properties of the glycoprotein. Analysis of the composition of neutral carbohydrates in the glycoprotein yielded galactose, mannose, fucose, glucose and *N*-acetylglucosamine (Table 1); the estimation of fucose content was suprisingly higher and that of mannose content was much lower than the value for many N-glycoproteins [21,22]. In summary, the N-glycosidase data are strongly suggestive of N-linked glycans in the glycoprotein, although further work is needed in order to elucidate the detailed structure of the carbohydrate moeity.

Automated Edman degradation of the protein, using up to 0.4 nmol, failed to reveal the N-terminal residue, suggesting a blocked amino group at this position. Partial sequence determination of this protein was then attempted on the peptide fragments obtained from trypsin digestion and CNBr cleavage of the protein. Figure 5 displays the separation of trypsin digests by HPLC on a reverse-phase C_{18} column and the reliable data from Edman degradation of three samples denoted a–c on the chromatogram. The partial sequences of the three peptide fragments were screened through the EMBL/GenBank/DDBJ protein sequence database. They could be completely aligned with Trp^{51}–Gln^{62}, Asp^{93}–Gly^{97} and Tyr^{122}–Tyr^{128} of the protein sequence deduced from 24p3 cDNA reported by Hraba-Renevey et al. [7] (Figure 6b). Although the CNBr-cleaved products were resolved only poorly under the same HPLC conditions (results not shown), N-terminal sequence analysis of the main peak gave reliable data. Two predominant amino acids could be detected in each of the first 15 cycles of Edman degradation and a single amino acid could be detected at each of the last ten cycles. The actual yield of the two sequences in an individual cycle was such that both components appeared to be present in nearly equal amounts. One of the two sequences could readily be interpreted as representing Met^{71}–Asn^{85} and the other was confirmed as Met^{142}–Ser^{167} of the reading frame of 24p3 cDNA (Figure 6b). With this information in mind, we amplified the cDNAs prepared from the uterus of DES-stimulated immature mice by PCR using the primer pair, CTGGGCCTTGCCCTGCTTGGGGTC and GTTGTCAATGCATTGGTCGGTGGG; both were designed in order to amplify nucleotides 44–624 of 24p3 cDNA (see the Materials and methods section). Ten colonies containing the amplified DNAs were identified from the transformed *E. coli* strain JM109. The ten cDNA inserts were shown to have the same nucleotide sequences, which are identical with nucleotides 44–624 of 24p3 cDNA (Figure 6a). Apparently, the 24p3 mRNA does exist in the uteri of DES-stimulated immature mice. These data together strongly support that the core protein of the 26 kDa glycoprotein band is derived from the 24p3 gene. Therefore, we named it tentatively as 24p3 protein.

The 24p3 mRNA was examined in various tissues of adult mice. The random-primed DNA probe to the amplified 24p3 cDNA was used in Northern blot hybridization to detect 24p3 mRNA in the total RNA of the tissue. 24p3 mRNA was detectable in lung, spleen, uterus, vagina and epididymis (Figure 7), but we detected no 24p3 mRNA in brain, thymus, heart, liver, kidney, pancreas, ovary, testis, seminal vesicle, coagulating gland and prostate. Apparently, 24p3 gene expression in adult mice is not tissue specific but takes place rather widely in non-sexual organs and in the reproductive tract.

DISCUSSION

We are the first to demonstrate the presence of a glycoprotein with a core protein corresponding to 24p3 cDNA in mouse uterus. The protein has a blocked N-terminus. In accordance with the amino acid sequences deduced from 24p3 cDNA (Figure 6b), the putative protein sequence of 200 amino acid residues gives a molecular mass of 22858 Da which is in agreement with the value determined by SDS/PAGE for the deglycosylated protein (Figure 2, lane 7). The core protein, together with the total amount of the conjugated carbohydrate, results in a molecular mass of 25.8 kDa, which is also in agreement with the value determined by SDS/PAGE (Figure 2, lane 3). These data together may suggest that there is no hydrophobic leader sequence in the initial translation product which is 200 amino acid in length. The results shown in Figure 2 reveal no O-linked, but only N-linked, carbohydrate in the 24p3 protein. Unlike a cytosolic glycoprotein that carries a much simpler sugar modification in which a single *N*-acetylglucosamine group is added to a serine or threonine residue of a protein, the 24p3 protein contains a rather complex glycoconjugate based on the carbohydrate composition shown in Table 1, suggesting that the 24p3 protein is secreted, but not released, as a result of cell death. Asn^{81} and Asn^{85}, being part of the consensus Asn-Xaa-(Ser/Thr)

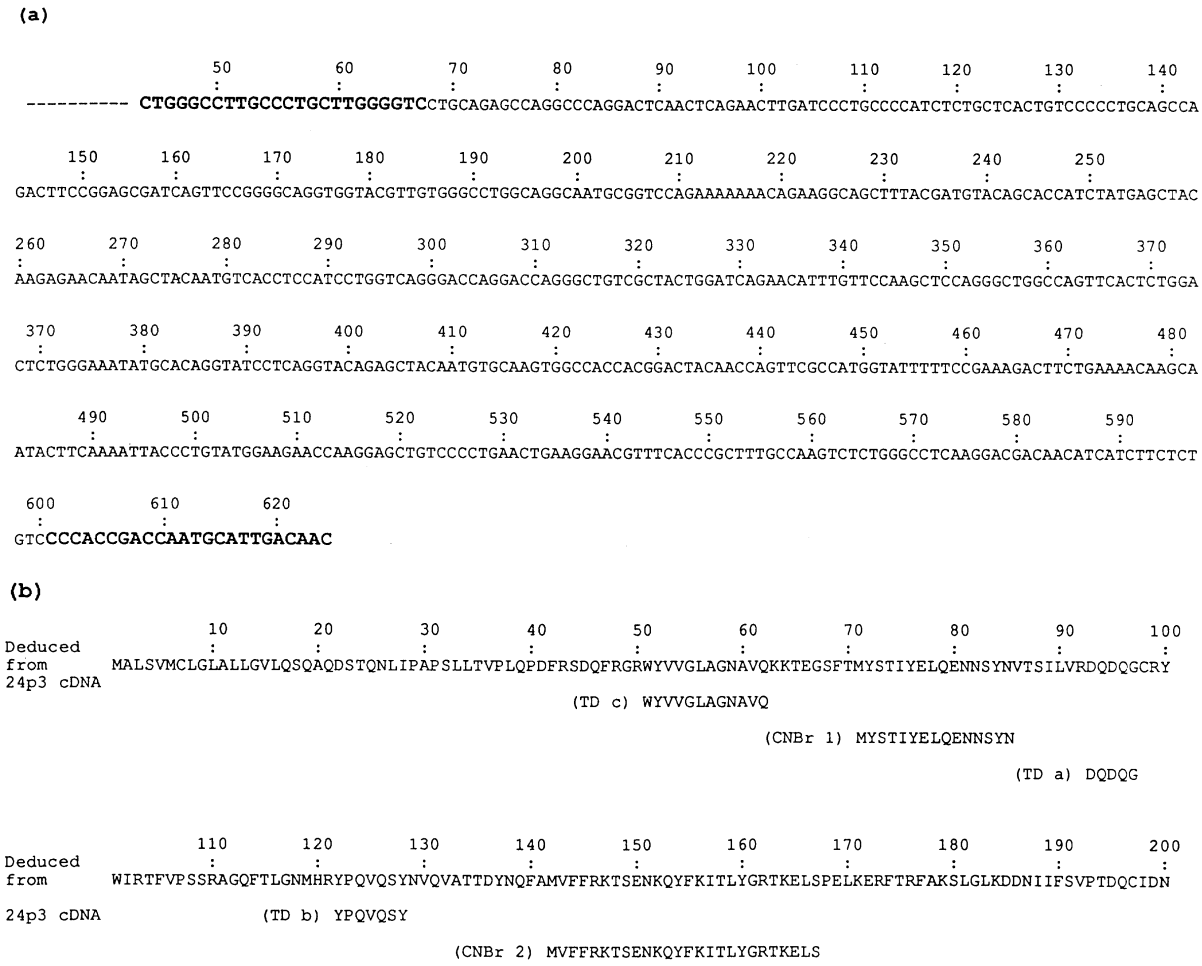

Figure 6 Comparison of the deduced protein sequence from PCR-amplified 24p3 cDNA with sequenced peptide fragments

The nucleotide sequence and the deduced protein sequence are numerated according to 24p3 cDNA reported by Hraba-Renevey et al. [7]. (**a**) The nucleotide sequences of the amplified cDNA. Bold characters indicate the primer pairs used for PCR in the present study. (**b**) A comparison of the protein sequence deduced from 24p3 cDNA with the sequence determined directly by Edman degradation. Labelling of trypsin digests (TD x) corresponds to the peaks in the HPLC profile in Figure 5. (CNBr 1) and (CNBr 2) indicate the two possible peptide sequences obtained with a peptide fragment from a CNBr digest of the protein (see text for details).

[23,24], are potentially the acceptor sites for the attachment of carbohydrate moieties. Although the hydrophobic leader sequence was previously thought to be required for cell secretion, several secretory proteins lacking a signal peptide have been found in both prokaryotes and eukaryotes [25]. In fact, Manin et al. [26] have reported a model of apocrine secretion for a mouse vas deferens protein with an N-blocked terminus and without a signal peptide. Whether secretion of the 24p3 protein follows apocrine secretion awaits future study.

The core protein has a calculated pI of 10.2 [7], but we determined the intact protein to have a pI of 5.8. This may be attributed partially to the modification of the basic core protein with the N-linked glycan which contains neuraminic acids (Figures 3 and 4). In fact, our preliminary observation from Ouchterlony analysis showed a precipitin band formed by the interaction between the 24p3 protein and lectin from horseshoe crab (Limulus polyphemus), which binds specifically to D-glucuronic acid and *N*-acetylneuraminic acid [27]. Unless there is a non-homogeneous surface-charge distribution on the protein molecule to create binding sites for either anionic or cationic ligands, independent of the final pI of the protein, the 24p3 protein may not bind to nucleic acid through ionic interaction as speculated by Hraba-Renevey et al. [7]. Instead, we found that the protein was able to bind to retinoids (S.-T. Chu, H.-L. Huang, J.-M. Chen and Y.-H. Chen, unpublished work). This supports the suggestion of Flower et al. [28] that the 24p3 protein is one member of the lipocalin family.

Hraba-Renevey et al. [7] detected very low basal levels of 24p3 mRNA in normal kidney cells. Since they found a big increase in the steady-state level of 24p3 mRNA in the cells after infection with polyoma virus or simian virus-40 in parallel with T-antigen synthesis in G_0-arrest host cells, they tended to consider the 24p3 gene as an oncogene. This might imply that the protein, though not physiologically expressed in this tissue, is synthesized in response to infection. However, we detected a certain level of 24p3 mRNA in non-sexual organs and in the reproductive tract of normal adult mice, suggesting that attendent pathways, other than the one associated with the virus infection, may be involved in the regulation of 24p3 gene expression.

This work was supported in part by National Science Council, Taipei, Taiwan, Republic of China (grants NSC84-2311-B001-069 and NSC84-2311-B001-077).

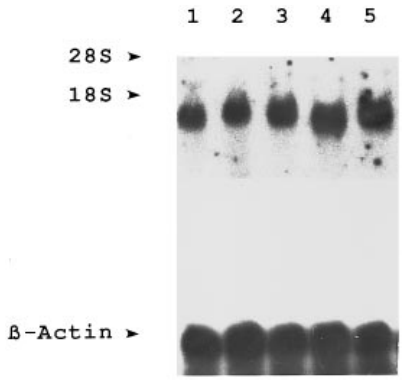

Figure 7 Northern-blot analysis for 24p3 mRNA in various tissues

Total RNA (50 μg) prepared from tissues of adult mice was run on a 1.0%-agarose/formaldehyde gel, transferred to a nylon membrane and probed with ^{32}P-labelled random-primed DNA to a cDNA of 24p3 protein (upper panel) or β-actin (lower panel): lane 1, vagina; lane 2, lung; lane 3, spleen; lane 4, uterus; lane 5, epididymis. The level of β-actin mRNA was used as an internal control. No 24p3 mRNA was detected in brain, thymus, heart, liver, kidney, pancreas, ovary, testis, seminal vesicle, coagulant gland and prostate.

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Received 24 July 1995/2 January 1996; accepted 26 January 1996