American Journal of Pathology, Vol. 144, No. 6, June 1994

An Analysis of the Expression of Cyclophilin C Reveals Tissue Restriction and an Intriguing Pattern in the Mouse Kidney

Jeff Friedman,* Irving Weissman,*
Jennifer Friedman,† and Susan Alpert*

From the Departments of Pathology and Developmental Biology,† Stanford University School of Medicine, Stanford, California*

Cyclophilin C (cyp C) is a cyclosporin A (CsA) binding protein originally isolated from a mouse bone marrow stromal cell line. We have compared the expression patterns of the mammalian cyclophilins A, B, and C in mouse tissues using* in situ *hybridization. These studies reveal that cyp C is expressed in a restricted subset of tissues including mouse ovary, testis, bone marrow, and kidney. Within the kidney, cyp C is highly expressed in a narrow zone in the outer medulla. Using monoclonal antibodies reactive against cyp C, we find that the kidney cells expressing cyp C correspond to the S3 segment of the nephron. The S3 segment has been shown to sustain histopathological damage from high dosages of CsA, raising the possibility that cyp C may be involved in mediating this damage. (Am J Pathol 1994, 144:1247–1256)

Immunophilins, the binding proteins for the immunosuppressive drugs cyclosporin A (CsA), rapamycin, and FK506, are made up of the cyclophilins (cyp) and FK binding proteins that possess *cis/trans* peptidyl-prolyl isomerase activity.[5,11,12,31] Several cyp have been isolated from mammalian tissues: cyp A,[9,11] cyp B,[14,27] cyp C,[6] and hcyp3,[3] and cyclophilin homologues have been found in a wide variety of eukaryotes including yeast,[9,17] neurospora,[39] drosophila,[29,32] as well as in *Escherichia coli*.[18] Studies using mammalian cyp C[6,19] initially identified the downstream molecular target mediating the immunosuppressive effects of CsA and FK506 as the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin. Subsequent studies demonstrated that cyp A and B could also interact with calcineurin in the presence of CsA.[20,35] However, little has been learned of the physiological function of these ubiquitous and highly expressed proteins in the absence of CsA.

Speculation concerning the function of cyp has focused on their enzymatic activity, peptidyl-prolyl isomerase activity, which is inhibited by CsA. Studies have demonstrated that cyp can act *in vitro* as catalysts of protein folding in the absence but not the presence of CsA, presumably via *cis/trans* isomerization of key proline residues in denatured or nascent polypeptide chains.[13,30] However, it has been difficult to establish the relevance of *in vitro* peptidyl-prolyl isomerase activity to *in vivo* cyp function. How the widespread expression of numerous cyp can be reconciled with the relatively tissue-specific physiological effects (immunosuppression and specific toxicities) observed with CsA treatment remains an intriguing question. We undertook a detailed analysis of the expression pattern of cyp A, cyp B, and cyp C with these issues in mind.

The expression patterns of mammalian cyp A, cyp B, and cyp C have been compared using *in situ* hybridization. Previously, the tissue distribution of mammalian cyp had been studied by labeled CsA binding to whole cell protein,[11,16] whole tissue RNA analysis of cyp A, cyp B, cyp C, and hCyP3,[3,6,9,14,27] and immunolocalization of cyp A.[16,22] Comparison of the expression patterns of cyp A, cyp B, and cyp C in a wide variety of mouse tissues reveals that cyp A and cyp B are widely expressed, whereas cyp C is tissue specific, being

This work was supported by U.S. Public Health Service grant CA 42551 and a Dean's Office Grant, Stanford Medical School. JSF was supported by U.S. Public Health Service grant NRSA 5T32 CA 09302 and SA was supported by a grant from the Irvington Institute for Medical Research.

Accepted for publication January 25, 1994.

Address correspondence and reprint requests to Dr. Susan Alpert, Department of Pathology and Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305.

Current address for Dr. Jeff Friedman, Department of Medicine, Children's Hospital, 300 Longwood Avenue Boston, MA 02115.

readily detectable in kidney, bone marrow, ovary, and testis. To characterize further cyp C expression at a cellular level, monoclonal antibodies were raised against recombinant mouse cyp C. These antibodies provide a detailed picture of the intriguing pattern of expression of cyp C within the mouse kidney. Although the mechanism of CsA-induced renal toxicity (nephropathy) remains to be elucidated, the pattern of expression observed makes cyp C a candidate mediator of the nephrotoxic actions of CsA, and a potentially interesting marker for studies of renal tubular development.

Materials and Methods

In Situ *Hybridization*

A more detailed description of the *in situ* hybridization procedure has been previously described.[8] In brief, fresh tissues dissected from 8 to 12-week-old BALB/c mice were embedded and frozen in ornithine carbanoyltransferase (OCT) (Tissue-Tek, Elkart, IN) compound on dry ice. The 6-μ frozen sections were affixed to RNAse-free, poly-L-lysine (Sigma, St. Louis, MO)-coated slides and fixed in 4% paraformaldehyde containing phosphate buffer. Slides were then washed and reacted with proteinase K (Sigma) for 30 minutes, followed by refixation in 4% paraformaldehyde buffer. Samples were then acetylated using acetic anhydride and triethanolamine.

Hybridization reactions were conducted in 40% formamide buffer at 45 to 50 C overnight. Posthybridization treatments included a 50% formamide wash in 2× standard saline citrate (SSC) followed by digestion with RNase A (Sigma) and RNAse T1 (Sigma), followed by an additional wash in 50% formamide, 2× SSC at 54 C. Images were produced by direct exposure of X-ray film (Hyperfilm M; Kodak, Rochester, NY) to tissue sections after hybridization.

Slides were dipped in 50% NTB2 nuclear track emulsion (Kodak), and 300 mM ammonium acetate and were exposed for 14 days at 4 C. The slides were then developed with Kodak D19 developer and Kodak fixer. The sections were counterstained with hematoxylin and coverslipped. Cells were considered positive for gene expression if they had 10 times as many grains as the background hybridization.

^{35}[S]-labeled RNA probes were prepared by incubating linear pBS SK- templates with either T7 or SP6 polymerase (Promega, Madison, WI), depending on the polarity desired. The 1200-bp cyp C template, comprised of the coding region (minus the first 15 amino acids) and the 3′ noncoding region of the cyp C cDNA, has been previously described.[6] Cyp A cDNA was isolated and cloned from polymerase chain reactions (PCR) between the following oligonucleotides: 5′ GCC GGA TCC ATG GTC AAC CCC A 3′ and 5′ CGG CCC GGG TAA AAT GCC CGC A 3′. The first (5′) oligonucleotide spans the start codon for the murine cyp A sequences and the second (3′) oligonucleotide spans the termination codon. Thus, the entire coding region of cyp A, approximately 550 nucleotides, was used. PCR products were digested with *Bam*HI and *Sma*I (GIBCO-BRL, Gaithersburg, MD) for cloning into pBS SK- (Stratagene, La Jolla, CA). Similarly, cyp B cDNA was isolated by PCR using the oligonucleotides 5′ GCC GGA TCC ATG AAG GTG CTC T 3′ and 5′ CGG CCC GGG ATG AGG TCC CCC A 3′. As with cyp A, the first (5′) cyp B oligonucleotide spans the start codon for cyp B and the second (3′) oligonucleotide spans the termination codon. Thus, the cyp B region included in the construct measures 650 nucleotides. PCR products were digested with *Bam*HI (GIBCO-BRL) and *Sma*I (GIBCO-BRL) for cloning into pBS SK- (Stratagene).

The cyp A and cyp B templates were sequenced and found to be identical to mouse cyp A and cyp B cDNA sequences. Templates for PCR reactions were derived from an AC.6 cDNA library,[6] because we previously found that this cell line expresses cyp A, cyp B, and cyp C (JSF, unpublished observations). Templates for RNA polymerization reactions were prepared by digesting the resulting pBS SK- constructs with *Bam*HI (GIBCO-BRL) to produce anti-sense probes.

Preparation of the probes has been previously described.[8] Specificity of the individual RNA probes was verified by Northern analysis of the AC.6 RNA. Under conditions identical to those used in the *in situ* hybridization reactions, including the RNase A and RNase T1 treatments, autoradiography revealed that each probe hybridized to a single band of the appropriate size. No cross-reactivity could be detected (data not shown). In all cases, templates were incubated with both ^{35}[S] rUTP and ^{35}[S] rCTP for probe preparation.

Production of Monoclonal Antibodies Against Cyp C

Recombinant cyp C protein was prepared using the glutathione-s-transferase (GST) fusion protein system as described.[6] Fischer rats were immunized with 100 to 200 μg of purified cyp C-GST at approximately

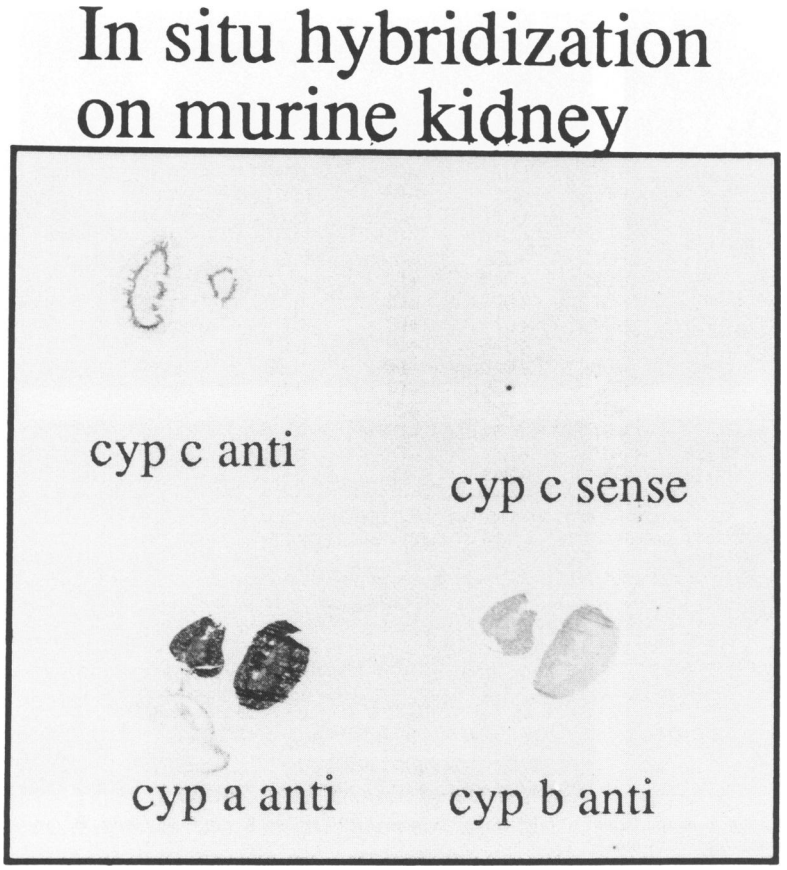

Figure 1. *Autoradiogram of* in situ *hybridizations on whole mouse kidney sections using ^{35}S-labeled RNA probes. Hybridized and washed tissue sections were placed in contact with Hyperfilm M for 7 days to produce these images.*

2-week intervals. Animals received antigen in either saline or incomplete adjuvant carrier (Ribi, Hamilton, MT). After two immunizations, a serum sample from each animal was tested for reactivity against cyp C-GST in an ELISA assay. The animal with the highest reactivity was boosted with purified cyp C (purified away from GST after cleaving cyp C-GST with thrombin; Sigma) and sacrificed 3 days later for isolation of splenic cells for use in a fusion reaction to produce hybridomas. Splenic cells were fused[28] with the partner cell line FoxNY.[36]

Individual clones growing in 96-well plates were screened by taking a sample of hybridoma conditioned medium and assaying for the presence of anti-cyp C antibodies using an ELISA reaction. In brief, cyp C-GST was immobilized on plastic wells of a 96-well plate. Hybridoma supernatants were incubated in these wells for 30 minutes, wells were then washed with phosphate-buffered saline (PBS) containing 0.2% Tween 20, and reacted with a second stage antibody (alkaline phosphatase-conjugated goat anti-rat antibody from Fisher Scientific Co., Pittsburg, PA, at a dilution of 1:1000) for 30 minutes. Wells were thoroughly washed with PBS-Tween 20 to remove unbound antibody. Wells were developed by the addition of *p*-nitrophenyl phosphate (Sigma), and plates were read by determining optical density at 405 nm. Hybridomas scoring positive in this assay were then tested against GST alone to confirm that the antigen recognized was cyp C. The hybridomas were then further tested against cyp A-GST and cyp B-GST fusion proteins using a similar ELISA protocol. Hybridomas were then expanded and subcloned until a stable cell line was obtained.

To further test the specificity of the stable hybridomas, immunoprecipitations were performed. Labeled proteins were prepared from AC.6 cells by culturing in the presence of ^{35}S methionine and ^{35}S cysteine overnight using approximately 200 μCi/ml of each amino acid per reaction. The cells were lysed by treatment with 25 mM Tris, pH 6.5, 150 mM NaCl, and 0.5% Triton X-100, and immunoprecipitations were performed. Samples were analyzed by 11.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel electrophoresis. Gels were fluorographed with 0.5 M sodium salicylate for 30 minutes before drying.

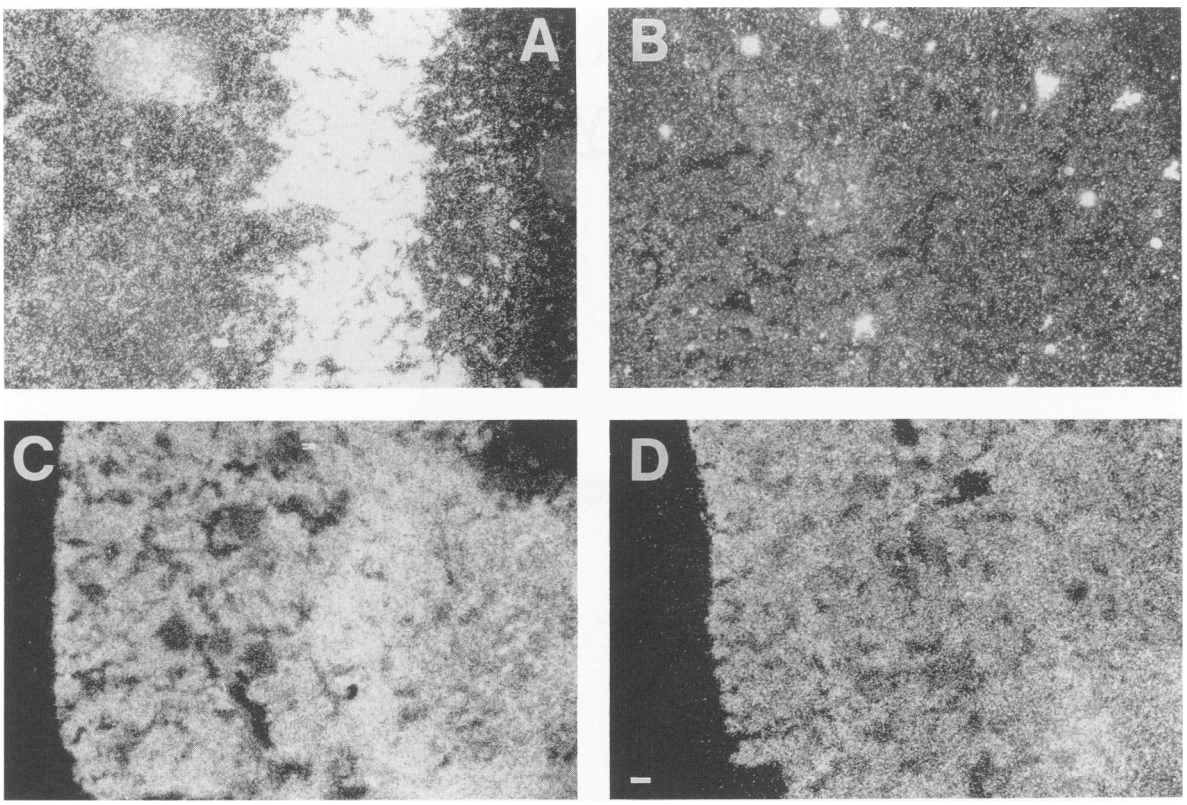

Figure 2. *Dark field micrographs of* in situ *hybridization to mouse kidney sections using* ^{35}*S-labeled RNA probes:* **A:** *cyp C anti-sense;* **B:** *cyp C sense (control);* **C:** *cyp A anti-sense;* **D:** *cyp B anti-sense. Bar = 2.5 mm.*

Immunofluorescence using Anti-Cyp C Antibodies

Freshly isolated mouse tissues were placed in OCT (Tissue-Tek) embedding medium and frozen on a block of dry ice. Frozen tissue blocks were sectioned in a Hacker cryostat to yield 6-μl sections, which were collected on glass slides and allowed to air dry. Sections were fixed for 10 minutes at room temperature in acetone and allowed to dry. A drop of antibody containing hybridoma supernatant was placed on tissue sections and allowed to incubate for 20 minutes. Sections were then washed with PBS to remove unbound antibody. A 1:100 dilution of second stage goat anti-rat antibody conjugated to fluorescein (Caltag Co., Foster City, CA) was then incubated with the washed sections for a further 20 minutes in the dark. Sections were washed with PBS and mounted with Aquamount (Lerner, New Haven, CT). Slides were analyzed using a Nikon fluorescence microscope equipped with a 35-mm camera. A titration curve showed that a signal could still be retained when the tissue culture supernatants were diluted 1:10. Samples incubated with normal rat serum as a substitute first stage antibody were negative for immunofluorescent staining under these conditions. In addition, hybridoma supernatant could be used for immunoperoxidase staining (data not shown). In these experiments, isotype-matched control antibodies were used. No staining was observed with these control antibodies (data not shown).

Results

In Situ *Hybridization of Cyp A, B, and C on Selected Mouse Tissues*

To determine the pattern of cyp A, cyp B, and cyp C expression in a variety of mouse tissues, *in situ* hybridizations were performed. Figures 2–5, present a collection of dark field micrographs of emulsion-treated slides of selected mouse tissues hybridized with mouse cyp A, cyp B, or cyp C anti-sense probes using a cyp C sense probe as a control. Because CsA has a characteristic toxic effect on the kidney, we chose to compare cyclophilin expression in this tissue. Figure 1 presents an overview of cyp expression in mouse kidney sections. The control probe used for all reactions was an identically prepared cyp C sense

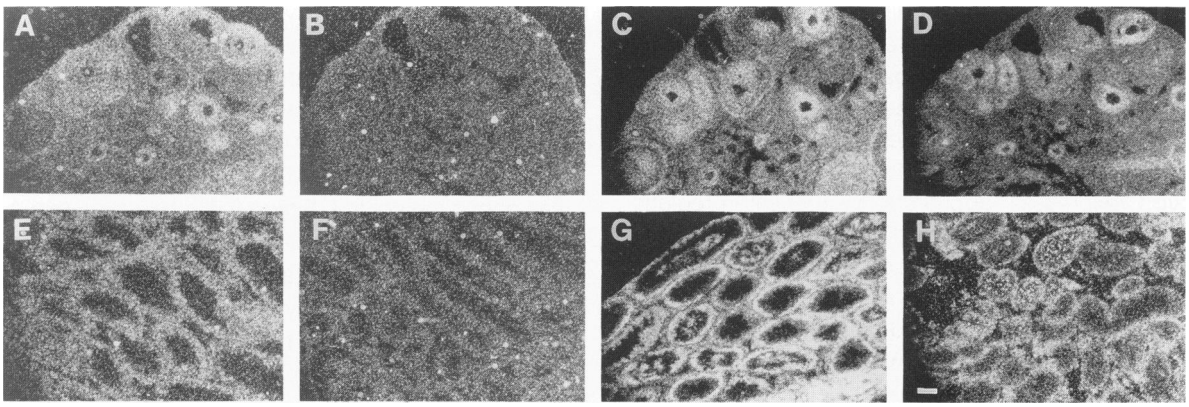

Figure 3. *Dark field micrographs of* in situ *hybridization to mouse ovary* (A-D) *and testis* (E-H) *using* ^{35}*S-labeled RNA probes:* A: *cyp C anti-sense;* B: *cyp C sense (control);* C: *cyp A anti-sense;* D: *cyp B anti-sense;* E: *cyp C anti-sense;* F: *cyp C sense (control);* G: *cyp A anti-sense;* H: *cyp B anti-sense. Bar = 5 mm.*

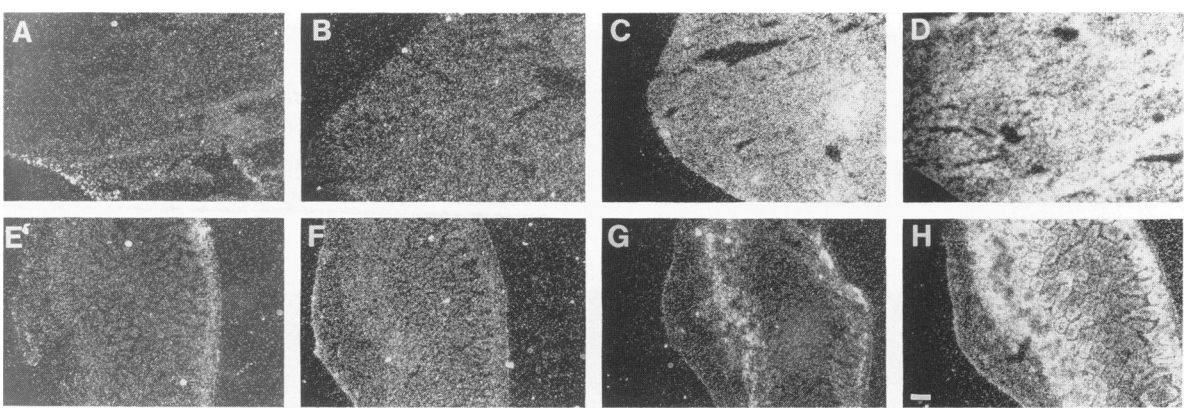

Figure 4. *Dark field micrographs of* in situ *hybridization to mouse liver* (A-D) *and intestine* (E-H) *using* ^{35}*S-labeled RNA probes:* A: *cyp C anti-sense;* B: *cyp C sense (control);* C: *cyp A anti-sense;* D: *cyp B anti-sense;* E: *cyp C anti-sense;* F: *cyp C sense (control);* G: *cyp A anti-sense;* H: *cyp B anti-sense. Bar = 5 mm.*

RNA probe. Relative signal intensities are an indicator of differences in expression levels, because probes are of equal specific activity, hybridization and wash conditions were identical, and an equal number of counts was used in each hybridization reaction. Here the restriction of cyp C expression to a band corresponding to the outer portion of the medulla is evident. Cyp A and B are expressed diffusely throughout the kidney, with higher levels of expression in cortical areas (possibly a reflection of cell density). It is also evident that cyp A mRNA is the most abundant species.

To further explore the pattern of expression in mouse kidney, hybridized sections were dipped in photographic emulsion to reveal the expression pattern on the tissue itself using silver grains. Figure 2 presents dark field micrographs of mouse kidney hybridized with anti-sense cyp C (panel A), control sense cyp C (panel B), anti-sense cyp A (panel C), and anti-sense cyp B (panel D). In each photograph, the cortex is to the left and the medulla is to the right. Here it is evident that cyp A and B are expressed relatively homogeneously throughout the kidney parenchyma, whereas the expression of cyp C is highest in the outer medulla, revealing a spiked appearance.

A battery of other tissues was examined in the same fashion. The expression pattern of the cyp in ovary and testis was particularly notable (Figure 3). All three cyp appear to be expressed throughout the ovary, with the density of expression changing in the follicular areas. Cyp A and B are highly expressed by cell layers that probably represent the zona granulosa and theca externa. Cyp C is also relatively abundant in follicular areas, particularly in the theca externa (Figure 3, A-D).

In the mouse testis expression of all three cyp is detected in a pattern that outlines the seminiferous

tubules. There are subtle differences in the expression pattern, with cyp C expression detected primarily between tubules, perhaps by interstitial cells, whereas cyps A and B appear to be expressed primarily within the tubules, demonstrating a sharp tubular boundary (Figure 3, E-H). It is interesting that cyp C expression appears to be present in regions responsible for steroid production.

The expression pattern of cyp C also diverges from the pattern of cyp A and B in the liver and small intestine (Figure 4). Cyp A and B are expressed at high levels throughout the liver, whereas cyp C is not expressed or is expressed at levels below the sensitivity of this assay (Figure 4, A-D). This agrees with previous mRNA analyses by Northern blot.[6,14,27] In the small intestine, cyp A and B expression can be easily detected, whereas cyp C message is not found (Figure 4, E-H). However, in the small intestine cyp B message appears to be far more abundant than cyp A. The linear pattern observed for cyp A message (panel G) suggests that cyp A expression is not uniform along the long axis of intestinal villi.

Cyp expression was also examined in a group of lymphoid tissues: thymus, spleen, lymph node, and bone marrow. Because cyp C was originally cloned from a cDNA library derived from a bone marrow stromal cell line,[6] we anticipated cells expressing cyp C to be detected in bone marrow sections. Indeed, the cyp C probe hybridized to small clusters cells in the bone marrow (Figure 5, M), generating a signal that was above the control (Figure 5, N). However, cyp A and B were not expressed in the bone marrow (Figure 5, O and P). The hybridization of the anti-sense cyp C probe to sections of thymus (Figure 5, A), spleen (Figure 5, E), and lymph node (Figure 5, I) was not higher than that of the control probe (thymus: Figure 5, B; spleen: Figure 5, F; lymph node: Figure 5, J). No cells could be detected that had 10 times as may grains as the appropriate control. Thus, cyp C is not expressed in these tissues, unlike cyp A and B. In the thymus, cyp A and B are expressed throughout the tissue, although there are punctate areas in which expression is not detectable (Figure 5, C and D). In the spleen, cyp A and B expression is confined to white pulp (Figure 5, G and H). In the lymph node, expression of cyp A and B is higher in follicular areas but can be detected in surrounding tissue (Figure 5, K and L).

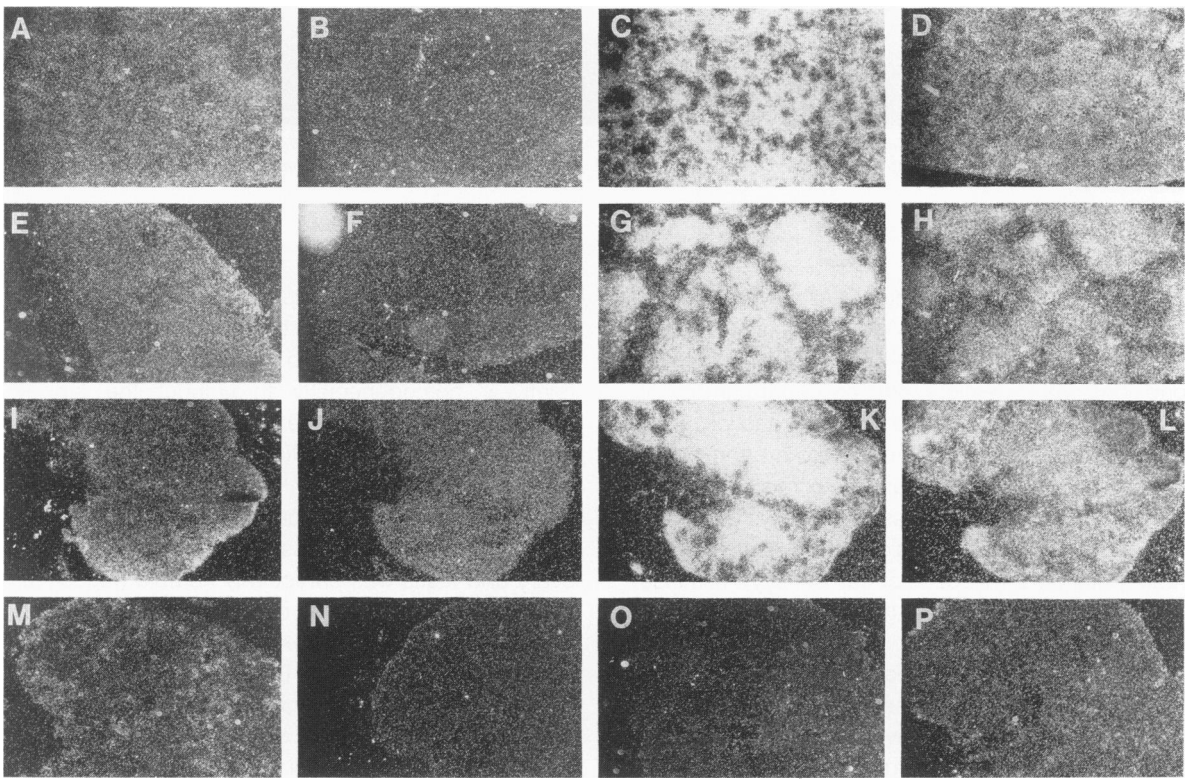

Figure 5. *Dark field micrographs of* in situ *hybridization to mouse thymus* (**A-D**), *spleen* (**E-H**), *lymph node* (**I-L**), *and bone marrow* (**M-P**) *using* ^{35}*S-labeled RNA probes:* **A**: *cyp C anti-sense;* **B**: *cyp C sense (control);* **C**: *cyp A anti-sense;* **D**: *cyp B anti-sense;* **E**: *cyp C anti-sense;* **F**: *cyp C sense (control);* **G**: *cyp A anti-sense;* **H**: *cyp B anti-sense;* **I**: *cyp C anti-sense;* **J**: *cyp C sense (control);* **K**: *cyp A anti-sense;* **L**: *cyp B anti-sense;* **M**: *cyp C anti-sense;* **N**: *cyp C sense (control);* **O**: *cyp A anti-sense;* **P**: *cyp B anti-sense. Bar = 5 mm.*

Indirect Immunofluorescence Using Anti-Cyp C Monoclonal Antibodies

In situ hybridization detects the existence of mRNA but does not address whether the RNA is translated into the protein product. Monoclonal antibodies against cyp C were prepared by immunizing rats with a cyp C:GST fusion protein (see Materials and Methods). Several independent hybridomas were obtained and each secreted a monoclonal antibody that revealed an identical staining pattern on mouse tissues. Anti-cyp C monoclonal antibodies immunoprecipitate cyp C (predicted M_r 23 kd)[7] but not cyp A (M_r 18 kd),[37] from AC.6 cells that express cyp A, B, and C (Figure 6). Because cyp B has a similar molecular weight to cyp C, ELISA assays were performed. No cross-reactivity to cyp A or B was found in these experiments (data not shown).

To examine the localization of cyp C protein, immunohistochemical studies using anti-cyp C monoclonal antibodies were conducted. Indirect immunofluorescence has confirmed the results of the *in situ* experiments detailed above, with cyp C protein being detectable in tissues that were found positive by *in situ* hybridization such as ovary, testis, kidney, and bone marrow and not being detected in tissues that were negative by *in situ* hybridization, such as brain and liver. Within the kidney and ovary, immunohistochemical studies have allowed a more precise analysis of the level and pattern of cyp C expression.

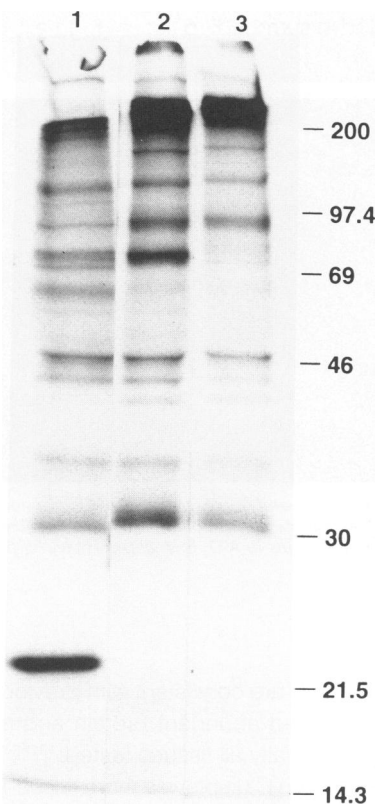

Figure 6. *Immunoprecipitation of ^{35}S-labeled protein from AC.6 cells using anti-cyp C monoclonal antibodies. Lane 1 shows AC.6 lysate immunoprecipitated with anti-cyp C monoclonal antibody D4(1)27. lane 2 shows AC.6 cell lysate reacted with an isotype-matched control antibody 9B2, lane 3 shows the AC.6 lysate without an antibody reaction. D4(1)27 immunoprecipitates a band of approximately 23 kd.*

The immunohistochemical analyses reveal that cyp C protein is concentrated in the outer medullary region. No glomeruli are observed in continuity with tubules expressing cyp C (Figure 7, A). When the staining pattern is examined at higher magnification, it is evident that the cyp C protein is expressed by renal tubular cells within this region (Figure 7, B). By morphological criteria, cyp C expression appears to be in proximal tubular cells including the S3 segment of the nephron, which occurs in the medullary rays. The outline of the cyp C-expressing cells approaches a polygonal shape, which is also characteristic of cells in the S3 segment.[38] The highest power view reveals that cyp C staining is not uniform throughout these proximal tubular cells but appears to be concentrated at their luminal aspect, perhaps as a component of their prominent brush border (Figure 7, C).

Interestingly, cyp C is also expressed by tubular structures deep within the medulla (Figure 7, D). These tubules are much narrower than the proximal tubules and a tubular lumen is not readily evident. These tubules may be the descending limb of the loop of Henle and thus be in continuity with the proximal tubular segments identified above. Within the narrow tubules of the inner medulla the expression of cyp C appears to be uniform throughout the cell without luminal concentration. It should be noted that these structures were not evident in the *in situ* hybridization experiments. The schematic drawing (Figure 7, E) demonstrates the location within the kidney of the tissue sections shown in panels A-D. Definitive identification of these tubular segments will require the use of other markers in double label immunohistochemical analysis.

To confirm the pattern detected by *in situ* hybridization, sections of mouse ovary were also processed for immunohistochemical localization of cyp C protein (Figure 7, F). In this section, it is evident that the bulk of the ovarian stroma expresses cyp C protein. The intensity of the staining varies, revealing that the concentration of cyp C protein is highest in perifollicular areas (theca externa) and lowest within the follicles (granulosa layer).

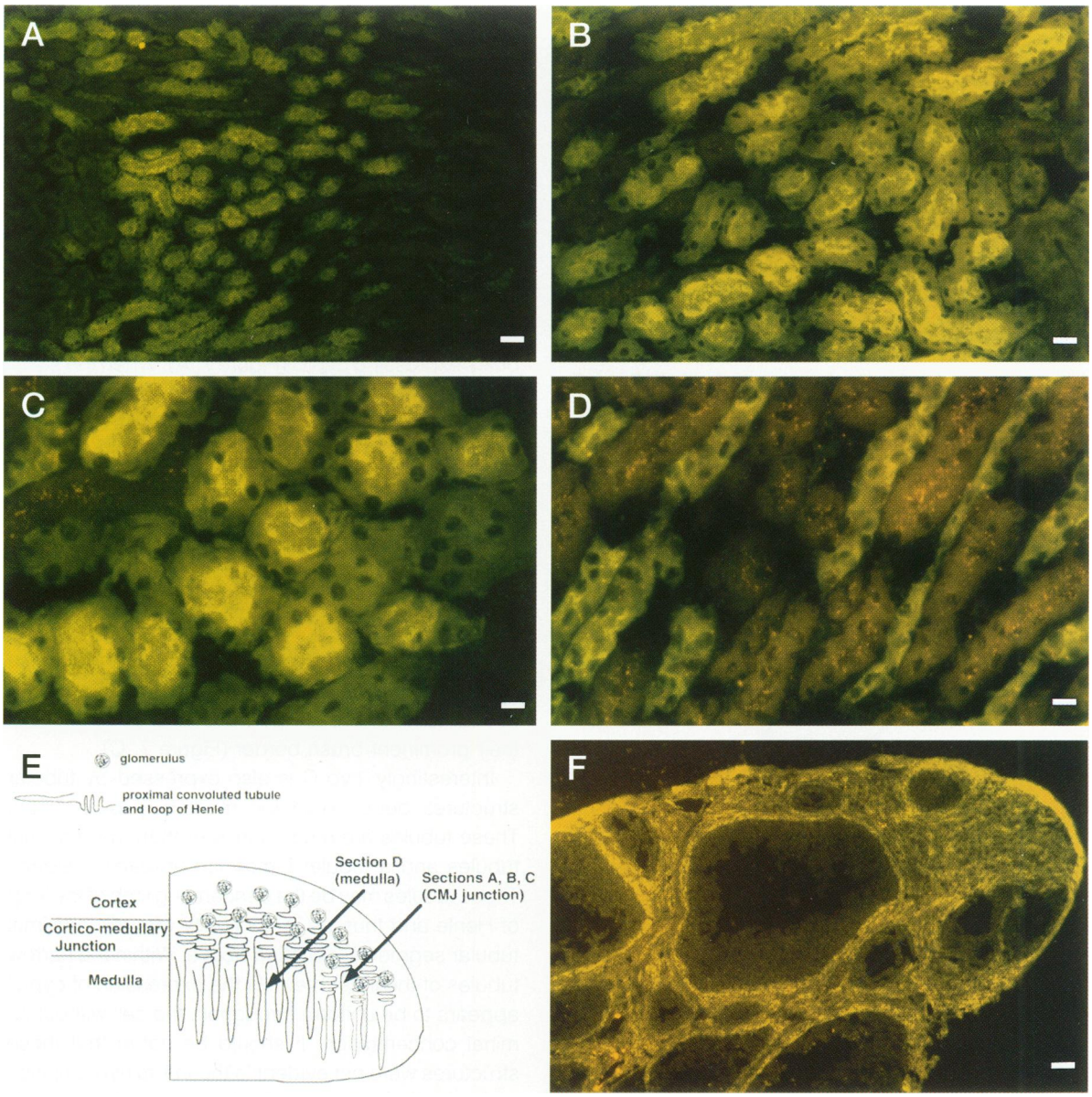

Figure 7. *Indirect immunofluorescence using anti-cyp C monoclonal antibodies on mouse kidney and ovary sections:* **A-C:** *Expression of cyp C in the region of the outer medulla.* **A,** *bar = 8 mm;* **B,** *bar = 4 mm;* **C,** *bar = 2 mm.* **D:** *High power view of tubular structures deep within the medulla that express cyp C. Bar = 2 mm.* **E:** *Schematic drawing of kidney showing location of sections in* **A-D**. **F:** *Low power view of pattern of expression of cyp C in mouse ovary. Bar = 20 mm.*

Discussion

We have compared the expression pattern of mouse cyp A, B, and C using *in situ* hybridization, which detects mRNA expression, and we have explored in detail the histological pattern of expression of cyp C in mouse kidney and ovary using indirect immunofluorescence with anti-cyp C monoclonal antibodies to detect cyp C protein. Our *in situ* hybridization results with cyp A and B are consistent with previous reports that demonstrated abundant protein and mRNA for these cyp in virtually all tissues tested.[11,14,16,21,22,27]

In contrast, cyp C displays a more tissue-restricted expression pattern, and is even further restricted to subregions of organs such as the kidney and the ovary. These results raise the possibility that there will be a number of tissue-specific cyp similar to cyp C and 'housekeeping' cyp such as cyp A and B. Pre-

cedent for such a distinction comes from studies on the Drosophila cyp homologue ninaA.[4,29,33,34] ninaA can be thought of as a highly tissue-restricted cyp, being expressed only in R1–6 photoreceptor cells of the Drosophila eye. Drosophila cells also ubiquitously express a homologue of cyp A (housekeeping) but this protein appears to be incapable of complementing ninaA (tissue specific) mutations.[34]

Clinical experience has demonstrated that the most common dose-limiting toxicity of cyclosporin therapy is deterioration of renal function.[15,23,40] Cyclosporin nephrotoxicity has been reported to occur in both acute and chronic forms.[2,15,23,24,25,26] Acute toxicity is reversible with CsA dose reduction or withdrawal, whereas chronic toxicity appears irreversible. The ultimate pathological picture encountered in chronic CsA-induced nephropathy is interstitial fibrosis[23,24] and is shared with several disease entities that result in renal failure, perhaps as a final common pathway. In acute cyclosporin toxicity, histopathological changes can be found in proximal tubular cells, predominantly in the S3 segment of the nephron.[40] The data presented here indicate that cyp C, a CsA binding protein, is expressed at high levels in proximal tubular cells, primarily in the S3 segment of the nephron. To our knowledge, no mouse model of CsA toxicity has been established, although it has been shown that cyclosporin stimulates the transcription of extracellular matrix genes in fibroblasts and proximal tubular cells.[41] However, the relationship between cyp C expression and the effects of CsA is only speculative. Nevertheless, preliminary experiments suggest that CsA treatment alters the quantity of cyp C observed in the renal tubular cells in the cortical medullary junction in mice (R. Mebius and M. Trahey, personal communication). It will be important in the future to test whether human cyp C is expressed in the same pattern in the kidney, and to examine cases of acute and chronic CsA toxicity to determine whether changes in cyp C expression occur.

In addition to the kidney, cyp C expression can be detected in the mouse ovary and testis. Because cyp C expression is associated with steroid-producing areas of the ovary, it would be interesting to study changes in the cyp C expression pattern in the ovary during the menstrual cycle and pregnancy. Direct effects of CsA therapy on testis and ovary are unknown, though anecdotal evidence suggests that patients taking CsA may remain fertile. There is evidence that high dosages of CsA can disrupt spermatogenesis and decrease testosterone levels in rats.[32] The physiological role played by cyp C in these tissues remains to be elucidated.

The results presented here demonstrate that mouse cyp A and B are expressed in a wide variety of tissues. In contrast, cyp C is expressed in a tissue-specific fashion, being detectable in mouse bone marrow, ovary, testis, and kidney. Detailed examination of the mouse kidney reveals that cyp C is expressed in a portion of the proximal tubule that is affected by CsA toxicity, and suggests that cyp C may be a mediator of the toxic effects of CsA.

Acknowledgments

We thank Lily Hu and Libuse Jerabek for excellent technical assistance in the production of antibodies against cyp C. We also thank Matt Porteus and John Rubenstein for advice on *in situ* hybridization, Meg Trahey for the immunoprecipitations and many helpful discussions, and Gladys Teitelman for helpful discussions.

References

1. Awazu M, Sugiura M, Inagami T, Ichikawa I, Kon V: Cyclosporine promotes glomerular endothelin binding in vivo. J Am Soc Nephrol 1990, 1: 1253–1258
2. Bennett W, Houghton D, Buss W: Cyclosporine-induced renal dysfunction: correlations between cellular events and whole kidney function. J Am Soc Nephrol 1991, 1: 1212–1219
3. Bergsma D, Eder C, Gross M, Kersten H, Sylvester D, Appelbaum E, Cusimano D, Livi G, McLaughlin M, Kasyan K, Porter T, Silverman C, Dunnington D, Hand A, Prichett W, Bossard M, Brandt M, Levy M: The cyclophilin multigene family of peptidyl-prolyl isomerases. J Biol Chem 1991, 34: 23204–23214
4. Colley N, Baker E, Stamnes M, Zuker C: The cyclophilin homolog ninaA is required in the secretory pathway. Cell 1991, 67: 255–263
5. Fischer G, Wittmann-Liebold B, Lang K, Kiefhaber T, and Schmid F: Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. Nature 1989, 337: 476–478
6. Friedman J, Weissman I: Two cytoplasmic candidates for immunophilin action are revealed by affinity for a new cyclophilin: one in the presence and one in the absence of CsA. Cell 1991, 66: 799–806
7. Friedman J, Trahey M, Weissman I: Cloning and characterization of cyclophilin C-associated protein: a candidate natural cellular ligand for cyclophilin C. Proc Natl Acad Sci 1993, 90:6815–6819
8. Griffiths G, Alpert S, Hershberger R, Su L, Weissman I: The granzyme A gene: a marker for cytolytic T lymphocytes in vivo. In Cytotoxic Cells. Edited by M Sitkovsky, P Henkart. Washington, DC, NIH Press, NIAID, pp 271–277

9. Haendler B, Hofer-Warbinek R, Hofer E: Complementary DNA for human T-cell cyclophilin. EMBO J 1987, 6: 947–950
10. Haendler B, Keller R, Hiestand P, Kocher H, Wegmann, G Rao Movva N: Yeast cyclophilin: isolation and characterization of the protein, cDNA and gene. Gene 1989, 83: 39–46
11. Handschumacher R, Harding M, Rice J, Drugge R, Speicher D: Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science 1984, 226: 544–547
12. Harding M, Galat A, Uehling D, Schreiber S: A receptor for the immunosuppressant FK506 is a *cis-trans* peptidyl-prolyl isomerase. Nature 1989, 341: 758–760
13. Harrison R, Stein R: Mechanistic studies of peptidyl prolyl *cis-trans* isomerase: evidence for catalysis by distortion. Biochemistry 1989, 29: 1684–1689
14. Hasel K, Glass J, Godbout M, Sutcliffe G: An endoplasmic reticulum-specific cyclophilin. Mol Cell Biol 1991, 11: 3484–3491
15. Humes H, Jackson N, O'Connor R, Hunt D, White M: Pathogenetic mechanisms of nephrotoxicity: insights into cyclosporine nephrotoxicity. Transplant Proc 1985, 17: 51–62
16. Koletsky A, Harding M, Handschumacher R: Cyclophilin: distribution and variant properties in normal and neoplastic tissues. J Immunol 1986, 137: 1054–1059
17. Koser P, Sylvester D, Livi G, Bergsma D: A second cyclophilin-related gene in Saccharomyces cerevisiae. Nucleic Acids Res 1990, 18: 43
18. Liu J, Walsh C: Peptidyl-prolyl cis-trans-isomerase from *Escherichia coli*: a periplasmic homolog of cyclophilin that is not inhibited by cyclosporin A. Proc Natl Acad Sci USA 1990, 87: 4028–4032
19. Liu J, Farmer J Jr, Lane W, Friedman J, Weissman I, Schreiber S: Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 1991, 66: 807–815
20. Liu J, Albers M, Wandless T, Luan S, Alberg D, Belshaw P, Cohen P, MacIntosh C, Klee C, Schreiber S: Inhibition of T cell signaling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity. Biochemistry 1992, 31: 3896–3901
21. Marks W, Harding M, Handschumacher R, Marks C, Lorber M: The immunochemical distribution of cyclophilin in normal mammalian tissues. Transplantation 1991, 52: 340–345
22. McDonald MT, Ardito T, Marks W, Kashgarian M, Lorber M: The effect of cyclosporine administration on the cellular distribution and content of cyclophilin. Transplantation 1992, 53: 460–466
23. Myers B, Ross J, Newton L, Leutscher J, Perlroth M: Cyclosporine-associated chronic nephropathy. N Engl J Med 1984, 311: 699–705
24. Myers B, Newton L: Cyclosporine-induced chronic nephropathy: an obliterative microvascular renal injury. J Am Soc Nephrol 1991, 2: S45–S52
25. Perico N, Dadan J, Remuzz G: Endothelin mediates the renal vasoconstriction induced by cyclosporine in the rat. J Am Soc Nephrol 1990, 1: 76–83
26. Perico N, Pasini M, Gaspari F, Abbate M, Remuzzi G: Co-participation of thromboxane A2 and leukotriene C4 and D4 in mediating cyclosporine-induced acute renal failure. Transplantation 1991, 52: 873–878
27. Price E, Zydowsky L, Jin M, Baker C, McKeon F, Walsh C: Human cyclophilin B: a second cyclophilin gene encodes a peptidyl-prolyl isomerase with a signal sequence. Proc Natl Acad Sci USA 1991, 88: 1903–1907
28. Foung SK, Sasaki D, Grumet FC, Engleman EG: Production of functional human T-T hybridomas in selection medium lacking aminopterin and thymidine. Proc Natl Acad Sci USA 1982, 79: 7484–7488
29. Schneuwly S, Shortridge R, Larrivee D, Ono T, Ozaki M, Pak W: Drosphila ninaA gene encodes an eye-specific cyclophilin. Proc Natl Acad Sci USA 1989, 86: 5390–5394
30. Schonbrunner E, Mayer S, Tropschug M, Fischer G, Takahashi N, Schmid P: Catalysis of protein folding by cyclophilins from different species. J Biol Chem 1991, 266: 3630–3635
31. Schreiber S: Chemistry and biology of the immunophilins and their immunosuppressive ligands. Science 1991, 251: 283–287
32. Seethalakshmi L, Menon M, Palllias J, Khauli R, Diamond D: Cyclosporine: its harmful effects on testicular function and male fertility. Transplant Proc 1989, 21: 928–930
33. Shieh B, Stamnes M, Seavello S, Harris G, Zuker C: The NinaA gene required for visual transduction in drosophila encodes a homologue of cyclosporin A-binding protein. Nature 1989, 338: 67–70
34. Stamnes M, Shieh B, Chuman L, Harris G, Zuker C: The cyclophilin homolog ninaA is a tissue-specific integral membrane protein required for the proper synthesis of a subset of drosophila rhodopsins. Cell 1991, 65: 219–227
35. Swanson SK, Born T, Zydowsky LD, Cho H, Chang HY, Walsh CT, Rusnak F: Cyclosporin-mediated inhibition of bovine calcineurin by cyclophilins A and B. Proc Natl Acad Sci USA 1992, 89: 3741–3745
36. Taggart RT, Samloff IM: Stable antibody-producing murine hybridomas. Science 1983, 219: 1228–1230
37. Theodore L, Pelegd D, Meyuhas O: P31, a mammalian housekeeping protein encoded by a multigene family containing a high proportion of pseudogenes. Biochem Biophys Acta 1985, 826: 137–146
38. Tisher CC, Bulger RE, Trump BF: Human renal ultrastructure. I. Proximal tubule of healthy individuals. Lab Invest 1966, 5: 1357–1391
39. Tropschug M, Nicholson D, Hartl F, Kohler H, Pfanner N, Wachter E, Neupert W: Cyclosporin A-binding protein (cyclophilin) of neurospora crassa. J Biol Chem 1988, 263: 14433–14440
40. Weinberg J: Issues in the pathophysiology of nephrotoxic renal tubular cell injury pertinent to understanding cyclosporine nephrotoxicity. Transplant Proc 1985, 17: 81–90
41. Wolf G, Killen PD, Neilson EG: Cyclosporin A stimulates transcriptional and procollagen secretion in tubulointerstitial fibroblasts and proximal tubular cells. J Am Soc Nephr 1990, 1: 918–922